Novel Insights into the Action of SHI/STY Transcriptional Regulators During Plant Development

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Abstract

One key player in plant organogenesis is the phytohormone auxin, and this thesis reveal that members of the *SHI/STY* gene family participate in the regulation of auxin homeostasis and organ development in *Arabidopsis thaliana*.

Using inducible constructs, ChIP, and EMSA, we could show that the SHI/STY members act as transcriptional activators directly binding to the promoter of the auxin biosynthesis gene *YUC4*. Additional putative downstream targets were identified encoding transcription factors, other auxin biosynthesis enzymes and enzymes involved in cell wall modulations. This work also brings novel insight into SHI/STY-dependent regulation of cotyledon and leaf vascular patterning and stamen number. Detailed expression studies of *SHI/STY* genes and several downstream targets confirm their overlapping spatial and temporal expression pattern during cotyledon, leaf, stamen and gynoecium development, clearly supporting the partially redundant function of the SHI/STY family members during the development of these organs.

In addition, the expression of *SHI/STY* members is partially mediated through a conserved motif in their promoter region, a GCC-box, which appears to be essential for *SHI/STY* expression in aerial organs. Furthermore, a group of putative upstream regulators belonging to the *AP2/ERF* family have been identified, which requires the GCC-box for their transcriptional regulation of the *SHI*/*STY* genes

Keywords: Arabidopsis thaliana, organ development, transcriptional regulation, *SHI/STY* family, *STYLISH1*

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You must do the thing which you think you cannot do.

Eleanor Roosevelt

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Eklund DM, Ståldal V, Valsecchi I, Cierlik I, Eriksson C, Hiratsu K, Ohme-Takagi M, Sundström JF, Thelander M, Ezcurra I, Sundberg E (2010). The Arabidopsis thaliana STYLISH1 protein acts as a transcriptional activator regulating auxin biosynthesis. *Plant Cell* 22(2), 349-363.

II Ståldal V, Cierlik I, Chen S, Landberg K, Baylis T, Myrenås M, Sundström JF, Eklund DM, Ljung K, Sundberg E (2012). The *Arabidopsis thaliana* transcriptional activator STYLISH1 regulates genes affecting stamen development, cell expansion and timing of flowering. *Plant Molecular Biology* 78(6), 545-559.

III Cierlik I, Landberg K, Claes A, Sundberg E. The Arabidopsis *SHORT INTERNODES/STYLISH* gene *STY1* affects the activity of, and overlaps with, gene affecting cell expansion and metabolic processes (manuscript).

IV Baylis T, Cierlik I, Sundberg E, Mattsson J (2013). *SHORT INTERNODES/STYLISH* genes, regulators of auxin homeostasis, are involved in leaf vein development in *Arabidopsis thaliana*. *New Phytologist* 197(3), 737-50.

V Eklund DM*, Cierlik I*, Ståldal V, Claes AR, Vestman D, Chandler J, Sundberg E (2011). Expression of Arabidopsis SHORT INTERNODES/STYLISH family genes in auxin biosynthesis zones of

aerial organs is dependent on a GCC box-like regulatory element. *Plant Physiology* 157(4), 2069-80.

* Indicates shared first authorship

The contribution of Izabela Cierlik to the papers included in this thesis was as follows:

I Participated in lab-work, made qRT-PCR and data analyses related to the STY1 downstream target ORA59.

II Participated in the planning of the project. Made the bioinformatic analyses and selection of the STY1 downstream targets. Participated in genotyping mutant lines and phenotypic analyses.

III Planned the project and performed the majority of the experimental work. Took active part in the result analyses and wrote the first draft of the manuscript.

IV Participated in the planning of the project and was involved in the data analyses and writing of the manuscript.

V Participated in the project planning. Made most of the work with reporter lines and performed most of the qRT-PCR studies. Took responsibility for crosses between mutant lines and the analysis of the double mutants. Participated in writing of the manuscript.

1 Introduction

Plants make up a great deal of the Earths natural environment converting sunlight into chemical energy and in doing so producing a primary source of food for life on the planet. The uses for plants are extensive from building materials to medicines and they are an essential and irreplaceable part of our everyday life. Understanding the developmental processes occurring throughout the plant life cycle and revealing their control and regulation is crucial for our understanding of healthy and profitable human-plant interactions. With each new insight we are discovering and exploring new pathways in plant development, which can be used to increase production and quality with huge social and economic impacts.

In this thesis I am presenting a novel insight into plant organ development controlled by *SHORT INTERNODES/STYLISH* family of transcription factors. Using *Arabidopsis thaliana* as a model organism I aim to understand how this gene family is being regulated and their main downstream affects.

1.1 Arabidopsis is an essential model for modern plant research

The genome sequence of *Arabidopsis thaliana* and subsequent developments in system biology changed the grounds of modern plant research. In addition there is a wealth of available online data supporting the genome at TAIR (http://www.arabidopsis.org/). With a relatively small genome size of 146 mega bases (MB) and short life cycle, Arabidopsis is one of the first and most widely used plant model organisms (Koornneef & Meinke, 2010; Van Norman & Benfey, 2009; Meyerowitz, 1987). Understanding the processes in and the regulation of the Arabidopsis life cycle is an extremely valuable reference for studies of many other plant species (Chu *et al.*, 2013; Kapazoglou *et al.*, 2012; Larsson *et al.*, 2012; Mounet *et al.*, 2012). Arabidopsis grows worldwide and a large collection of natural ecotypes

is available, complimented with an increasing number of mutants. These resources allow the study of questions relating to linking gene function with phenotype and adaptation. The great advantages of such studies have been achieved by forward genetics, the correlating phenotype to a specific gene function. However, the major impact on modern Arabidopsis research was development of the reverse genetics, where scientists could retrieve an available collection of known gene mutants and study it for the putative phenotypes (reviewed in Van Lijsebettens & Van Montagu, 2005).

Despite the huge evolutionary gap in divergence between the plants and other organisms, such as humans, a large number of cellular and biological processes are highly conserved. Details of some of these processes were first elucidated in Arabidopsis, which have led to novel insights into the pathways leading to specific human diseases such as Alzheimer and Parkinsons (Xu & Moller, 2011). Thus, although Arabidopsis will remain primarily as a plant model, it also has a value as a complementary model for more universal biological process.

1.2 Transcriptional regulation

A central aspect of every living organism is to transfer the genetic information, encoded by the DNA sequence into proteins. Generally, since the genetic information does not change during the lifetime it must be selectively transcribed under governance of various transcription factors and other co-actors, during specific time points and location. The identity and function of a cell is thus largely defined by the set of genes, apart from the housekeeping genes expressed in most cells, that it transcribes. The transcription machinery is evolutionary conserved among different eukaryotic species, but it is the differential regulation of this process that contributes to the overall organism diversity. Eukaryotes control the expression of their genetic information in several ways, and I will focus on two of the main pathways: the control via chromatin modulations and by the activity of transcription factors (TFs).

1.2.1 Chromatin modulations

The eukaryotic DNA strands are wound around histone octomeres, forming the fundamental packing unit of eukaryotic chromatin, the nucleosomes (Pfluger & Wagner, 2007). The tight coiling of the chromatin limits the access of RNA polymerase and transcription factors to the DNA. In order for transcription to take place, chromatin must “open” by a process called chromatin remodeling. Highly transcribed regions of DNA remain fairly accessible while for most of

the other genes activation requires controlled unfolding of the condensed DNA and variety of modulatory processes.

According to Pfluger and Wagner (2007) there are three main classes of epigenetic regulatory pathways controlling gene expression. These are chromatin remodeling, which is an energy dependent modulation of nucleosome position or composition; cytosine residues methylation, which affects the DNA ability to bind to TFs and other proteins and finally, histone modifications that can influence the histone-DNA interactions thus promoting or inhibiting binding of gene transcription activator proteins (Coulon *et al.*, 2013; Berger, 2007; Pfluger & Wagner, 2007; Rando & Ahmad, 2007; Klose *et al.*, 2006).

Histone modifications resulting in open chromatin permit selective binding of transcription factors (TFs) to short DNA sequences (*cis*-elements or enhancer sequences). The TFs can then recruit cofactors and RNA polymerase II to the target genes, allowing transcription to initiate (Lelli *et al.*, 2012; Spitz & Furlong, 2012; Ong & Corces, 2011; Turner & Muller, 2005). Typically, multiple TFs cooperatively bind to individual enhancer sequences (Panne, 2008) and together with co-factors regulate transcription from core promoters of nearby or distant genes through physical contacts achieved by looping of the DNA between enhancers and the core promoters (Krivega & Dean, 2012). TFs binding to core promoter cis-elements, which include sites where transcription initiation occurs, are also key components of this regulatory network (Mathelier & Wasserman, 2013; Dikstein, 2011; Goodrich & Tjian, 2010). These protein complexes also recruit histon-modifying enzymes contributing to the transcriptional control (reviewed in Lee & Young, 2013).

TFs can be classified by their characteristic DNA-binding domains (DBD), which interact with specific DNA sequences. Around 1500 TFs belonging to 30 TF families has been identified in *Arabidopsis thaliana* and about half of these have been considered plant specific (Yamasaki *et al.*, 2013; Mitsuda & Ohme-Takagi, 2009). The majority of these are involved in regulating plant specific organs or response pathways for adapting to their environment (Yamasaki *et al.*, 2013). Examples of plant specific TF families are the APETALA (AP2)/ ethylene responsive element binding factor (ERF); *CUP-SHAPE COTYLEDON 2* (NAC) and WRKY families (Swaminathan *et al.*, 2008).

The DNA binding by TFs is strictly dependent on the correct stereochemical principles of protein folding, base pair recognition and DNA structure modulations (reviewed in Yamasaki *et al.*, 2013; Luscombe *et al.*, 2000). Interestingly, a number of TFs contain multiple DBDs allowing interacting with more than one binding site in the genome, which also gives the

possibility for a TF to act as an activator or repressor of transcription. The specificity of their actions is dependent on alterations in associated protein complexes or additional signaling cues. Some TFs can exclusively act as transcriptional inhibitors. Because the *cis*- elements are usually short conserved stretches of 5-10 base pair (Riechmann, 2002), and can be positioned in the promoter region, as well as in regions further away, it has been difficult to identify which of these are actively used by the identified TFs (Klug, 2010). In addition many TFs interact with more than one binding site and are often also able to tolerate small variations within the binding sequences.

Apart from the above-described processes, the translation of the genetic code to protein sequences is affected by a multitude of additional mechanisms, including variable RNA splicing and the action of small RNAs (Ernst & Morton, 2013; Phillips & Hoopes, 2008).

1.3 The plant hormone auxin

Phytohormones are endogenous molecules, which at low concentrations affect plant growth and development, both directly and through cross talk with other hormones. Auxin is a major plant hormone involved in a variety of different developmental processes and cellular events. These include regulation and coordination of cell-specific wall modifications, elongation rates, differentiation rates, and polarity during e.g. embryogenesis, organ initiation and tissue patterning via formation of auxin maxima/minima or auxin gradients (reviewed in Sauer *et al.*, 2013). These are achieved through the spatial and temporal regulation of auxin biosynthesis, signaling, sensing, transport, degradation and compartmentalization (reviewed in Ljung, 2013).

1.3.1 Auxin biosynthesis

The most abundant endogenous auxin is indole-3-acetic acid (IAA). It is believed that the dominant route of IAA production is initiated from Tryptophan (Trp), although there is evidence of the existence of tryptophan-independent pathways of IAA synthesis (reviewed in Zhao, 2010). The four main suggested Trp-dependent biosynthesis pathways are the indol-3-pyruvic acid (IPA), the tryptamine (TAM), the indole-3-acet-amide (IAM) and the indol-3-acetaldoxime (IAOx) pathway, which are presented in Figure 1 (Mashiguchi *et al.*, 2011). The most dominant pathway in Arabidopsis appears to be the one producing IPA as an intermediate between Trp and IAA (*Figure 1, pathway enclosed in a frame*). In this pathway members of the TRYPTOPHAN AMINOTRANSFERAS OF ARABIDOPSIS 1/TRYPTOPHAN AMINOTRANSFERASE RELATED (TAA1/TAR) protein

family are responsible for conversion of Trp to IPA, while members of the flavin monooxygenase-like YUCCA (YUC) protein family are responsible for synthesizing IAA from IPA (Mashiguchi *et al.*, 2011; Stepanova *et al.*, 2011; Won *et al.*, 2011).

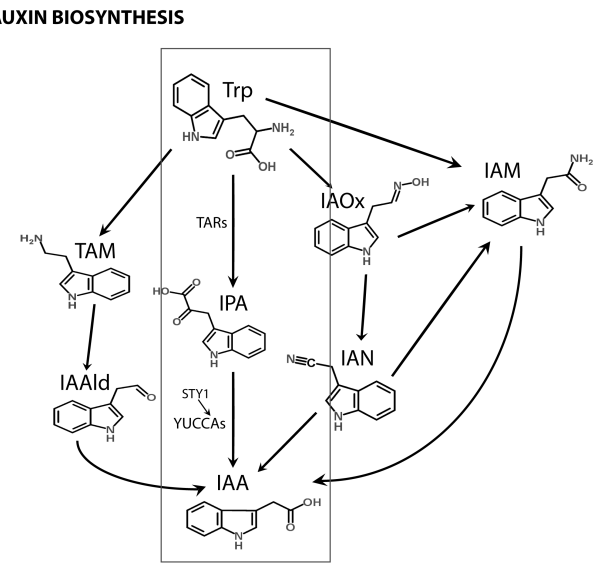

Figure 1. Overview of the proposed IAA biosynthesis pathway in Arabidopsis. Following abbreviations stand for: IAA Indole-3-acetic acid; IAAld Indole-3-acetaldehyde; IAM Indoleacetamide; IAN Indole-3-acetonitrile; IAOx Indole-3-acetaldoxime; IPA Indole-3-pyruvic acid; STY1 STYLISH1; TAM Tryptamine; TAR TAA1 RELATED; Trp Tryptophan. The IPA pathway enclosed in the box is considered to be the dominant one in Arabidopsis.

1.3.2 Auxin conjugation and oxidation

Plants produce a multitude of auxin molecules with different signaling and transport capacities, resulting in the possibility to store or transport auxin in less active forms, which can be converted to active auxin again, to trap auxin in mildly active forms or to permanently inactivate auxin. IBA is an example of a highly inactive transportable auxin that can be converted to IAA in specific competent cells (De Rybel *et al.*, 2012). Ester and amide conjugation of free auxin can temporarily inactivate auxin (Staswick *et al.*, 2005). The major fraction of auxin is conjugated to amino acids, sugars, peptides or proteins for temporary storage or for future degradation processes (Pencik *et al.*, 2013). In Arabidopsis, auxin is mostly conjugated to the amino acids alanine (Ala), leucine (Leu), asparate (Asp) and glutamine (Glu) (Kowalczyk & Sandberg, 2001; Tam *et al.*, 2000), which is facilitated by the auxin-induced GRETCHEN HAGEN3 (GH3) amido synthases (Staswick *et al.*, 2005; Hagen & Guilfoyle,

1985). Importantly, such conjugation does not lead to complete inactivation of the molecules, as they may still act in the very specific signaling pathways or may be hydrolyzed back to an unbound form. The release of conjugated IAA molecules is mediated by aminohydrolases, such as IAA-LEUCINE RESISTANT1 (ILR-1)-like (reviewed in Barbez & Kleine-Vehn, 2013). It is suggested that IAA-Asp and IAA-Glu are non-hydrolysable forms and are bound for the degradation processes. 2-oxoindole-3-acetic acid (oxIAA) and oxIAA-glucose are the main IAA degradation products (Pencik *et al.*, 2013; Novak *et al.*, 2012; Kai *et al.*, 2007; Ostin *et al.*, 1998), but the genes involved are not yet identified.

1.3.3 Auxin transport

An important aspect of auxin functioning as a developmental cue is the formation of tissue or organ specific concentration gradients or maxima. Although spatiotemporal regulation of auxin biosynthesis, degradation or phloem mediated auxin transport is important in the formation of these gradients/maxima, intercellular auxin transport plays a central role. Only protonated auxin formed at low pH conditions, as in the apoplast, can freely cross the plasma membrane whereas unprotonated IAA formed in the high pH cytosol only can leave the cell with the aid of auxin transporters (Rosquete *et al.*, 2012; Zazimalova *et al.*, 2010). There are four main groups of well-characterized auxin transporters responsible for the polar cell-to-cell auxin transport (PAT) or for auxin compartmentalization of auxin within the cell (Pencik *et al.*, 2013).

Two types of auxin efflux carriers aiding the transport of auxin out of the cell has been well characterized, and these are the PIN-FORMED (PIN1, 2, 3, 4 and 7) and the ATP BINDING CASSETTE SUBFAMILY B TRANSPORTER (ABCB) proteins. As the PIN proteins mainly have a polar localization in the PM, they play an important role in PAT leading to auxin gradient or maxima/minima formation. They are dynamically recycled within the cell and this trafficking has direct effects on their polarity. The polarity is also regulated by the antagonistic activity of the ABC protein kinase, PINOID (PID) and the serine-threonine protein phosphatase 2A (PP2A) (Ding *et al.*, 2011; Dhonukshe *et al.*, 2010; Huang *et al.*, 2010; Kleine-Vehn *et al.*, 2009; Michniewicz *et al.*, 2007; Friml *et al.*, 2004). PIN-mediated auxin efflux has been shown to be crucial for many plant developmental processes, such as embryogenesis, organ initiation, organ positioning, organogenesis, gravitropism and maintenance of the root meristem (Nakamura *et al.*, 2012; reviewed in Peer *et al.*, 2011; Titapiwatanakun & Murphy, 2009; Benkova *et al.*, 2003). At least five members of the ATP-dependent ABCB/P-glycoprotein

family have been shown to localize uniformly in the PM regardless of internal or external signals, and to mainly transport auxin out of the cell across the PM, suggesting that these proteins may act as basal auxin transporters (reviewed in Cho & Cho, 2012; Mravec *et al.*, 2008; Blakeslee *et al.*, 2007; Geisler *et al.*, 2005). Despite their apolar locations, they contribute to PAT and long-range auxin transport (Bailly *et al.*, 2008; Bouchard *et al.*, 2006; Geisler *et al.*, 2003). It has been suggested that the apolar ABCBs minimize auxin reflux from the apoplast (Bailly *et al.*, 2011), whereas polar PINs provide a vectorial auxin stream (Mravec *et al.*, 2008). However, ABCBs and PINs are also capable of interactive and coordinated transport of auxin (Blakeslee *et al.*, 2007). It has recently been suggested that PID, in addition to its function to switch PIN polarity, also has a direct and dual effect on ABCB-mediated auxin efflux activity (Henrichs *et al.*, 2012). Interestingly, one of the ABCB auxin transporting family members, ABCB4, has been shown to import auxin when the auxin level is low, and switch to an efflux carrier when the auxin concentration is high (Peer *et al.*, 2011; Kim *et al.*, 2010a).

Three PIN proteins, PIN5, PIN6 and PIN8, are not PM bound, but localize to the endoplasmic reticulum (ER), where they seem to limit nuclear auxin signaling by intracellular auxin transport (Dal Bosco *et al.*, 2012; Ding *et al.*, 2012; Mravec *et al.*, 2009). They have been shown to be important, together with intercellular auxin transport, for leaf vein formation (Sawchuk *et al.*, 2013). Apart from these three PIN proteins, a novel PIN-like family called PILS (PIN-LIKES) appears to be involved in intracellular auxin homeostasis via a similar mechanism as PIN5, PIN6 and PIN 8 (Barbez *et al.*, 2012). These transporters reduce the level of free IAA and increase the level of amino acid and glucose auxin conjugates, which suggests a link between conjugation of free IAA and auxin compartmentalization in the ER lumen (Barbez & Kleine-Vehn, 2013). Because the auxin receptor ABP1 (see section 1.3.4) mainly localizes to the ER, it has been hypothesized that this receptor may perceive ER-compartmentalized auxin or auxin conjugates (Barbez & Kleine-Vehn, 2013).

Another group of proteins, the AUXIN1/LIKE-AUX1 (AUX1/LAX) proteins, has been characterized as auxin influx carriers with mainly polar localization (reviewed in Swarup & Peret, 2012). Although IAA is readily diffused into the cell, AUX1/LAX carriers are involved in e.g. embryogenesis (Ugartechea-Chirino *et al.*, 2010), apical hook development (Vandenbussche *et al.*, 2010), root gravitropic responses (Swarup *et al.*, 2008; Bennett *et al.*, 1996), lateral root development (Swarup *et al.*, 2001), leaf phyllotaxis (Bainbridge *et al.*, 2008) and phloem loading and unloading (Marchant *et al.*, 2002). They are generally believed to pump auxin against its concentration

gradient, and mathematical modeling supports their role in local maxima formation for phyllotaxis formation in the SAM (Smith *et al.*, 2006). Recently another group of proteins, the nitrate transporter NRT1.1, has been suggested to act as an auxin influx carrier, involved in regulating lateral root formation in relation to the nitrogen status of the plant (Krouk *et al.*, 2010).

1.3.4 Auxin perception

Auxin is perceived by at least two different classes of receptors: the nuclear TRANSPORT INHIBITOR RESPONSE1/AUXIN-RELATED F-BOX (TIR1/AFB) which together with its co-receptors AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) control transcriptional responses to auxin, and the ER and extracellular space localized AUXIN-BINDING PROTEIN1 (ABP1) controlling certain aspects of growth and development (reviewed in Sauer *et al.*, 2013).

The most well studied auxin receptors are the TIR1/AFBs (reviewed in Sauer *et al.*, 2013; Calderon Villalobos *et al.*, 2012). When auxin levels are low, the AUX/IAA proteins together with TOPLESS (TPL) bind to and repress the activity of the AUXIN RESPONSE FACTOR (ARF) transcription factors, which directly regulates auxin response genes. When auxin levels increase, IAA binds TIR1/AFB in the SCF-type E3 ubiquitin ligase complex and promotes TIR1/AFB interaction to the DII domain of the AUX/IAA proteins. Auxin has been suggested to act as a glue to stabilize the TIR/AFB binding to AUX/IAAs. This binding mediates the ubiquitination of AUX1/IAAs, which leads to their degradation by the 26S proteasome and thereby also the release of ARF activity. As the II-domain of AUX/IAA proteins is responsible for the AUX/IAA-auxin-TIR/AFB binding and thus the release of auxin responses, it has been used in a reporter construct, DII-VENUS, allowing monitoring of auxin responses in plant tissues. The DII-VENUS fluorescence signal is depleted proportionally to the available amount of free auxin in auxin responsive tissues (Brunoud *et al.*, 2012).

ABP1 was identified as an auxin receptor with auxin binding activity a couple of decades ago (Jones & Venis, 1989), but it was not until an embryo lethal *abp1* mutant was identified that its biological importance was clarified (Chen *et al.*, 2001). Although ABP1 is localized to the ER, it is also secreted to the extracellular space, where it associates to the plasma membrane and acts as an auxin receptor stimulating ion fluxes at the plasma membrane, which leads to acidification of the extracellular space and activation of pH-dependent cell expansion ((Napier *et al.*, 2002); for cell expansion, see section 1.4.1). In response to auxin, ABP1 has also been shown to activate Rho-like (ROP) GTPases affecting interdigitation in leaves (Chen et al., 2012; Xu et al., 2010).

In addition, ABP1 is essential for clathrin-dependent endocytosis, whereas auxin-associated ABP1 inhibits recruitment of clathrin to the plasma membrane, which results in reduced internalization of e.g. PIN proteins (see section 1.3.3), and as a consequence, affects the rate of auxin efflux (Covanova *et al.*, 2013; Chen *et al.*, 2012; Robert *et al.*, 2010). Finally, ABP1 influences the expression of some of the early auxin-responsive genes (reviewed in Tromas *et al.*, 2010), and recent data suggest that *ABP1* genetically acts upstream of *TIR1/AFB*. Knock-down of ABP1 results in enhanced degradation of AUX/IAAs through the $SCF^{TIR1/AFB}$ E3 ubiquitin ligase pathway suggesting that the two auxin receptors collectively contribute to mediate auxin responses (Tromas *et al.*, 2013).

A third auxin receptor candidate, the F-box S-PHASE KINASE-ASSOCIATED PROTEIN 2A (SKP2A), has recently been suggested to be involved in auxin mediated cell-cycle control (Jurado *et al.*, 2010; Jurado *et al.*, 2008).

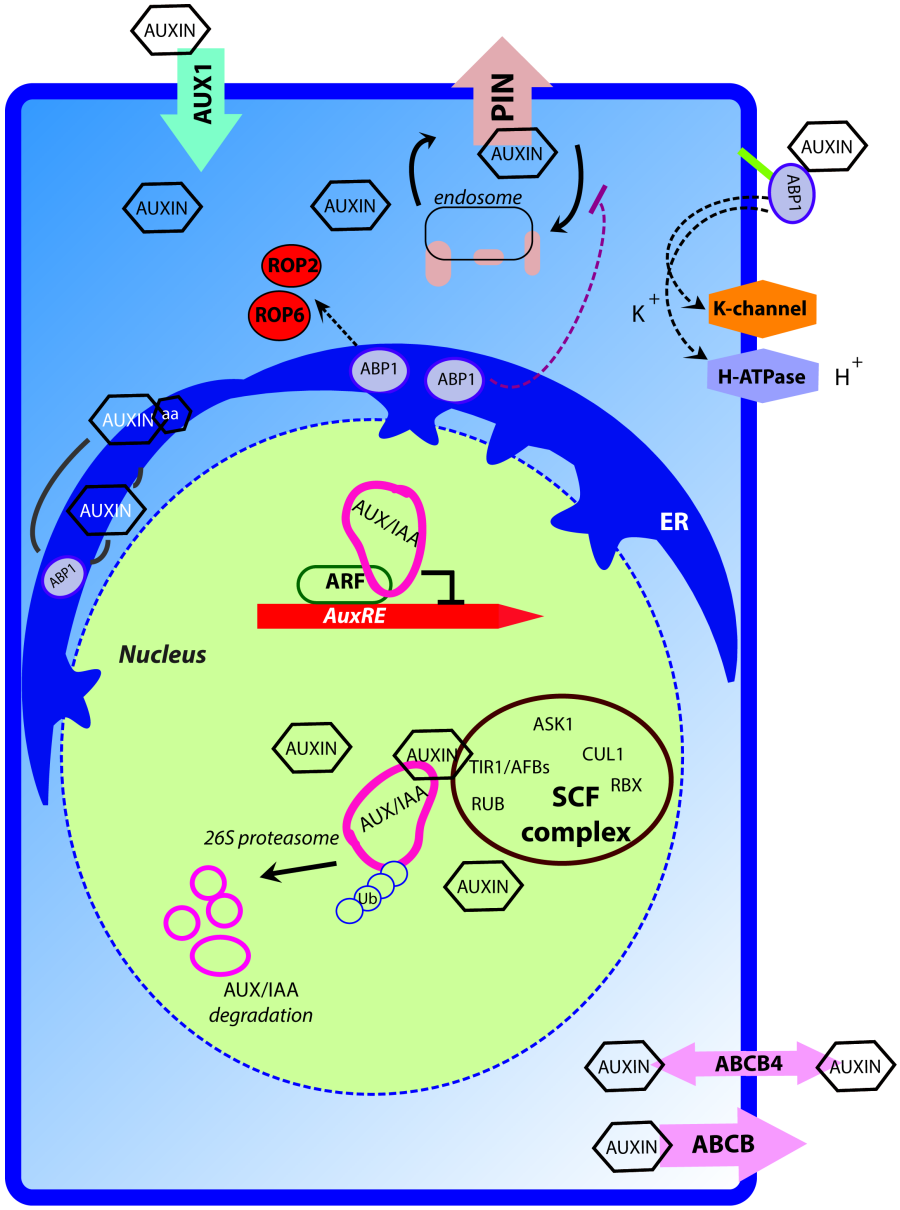

Figure 2. Model of the proposed transport and perception of auxin. Details about the processes have been described in the sections 1.3.2 and 1.3.3.

1.4 Cell expansion and plant development

Plants grow and form their shapes by controlled cell division and cell expansion. The rigidity of the plant cell wall, which contributes to mechanical support and protection of the enclosed protoplast, must be loosened in order for cell expansion to occur. The cascade of events during expansion is triggered both by internal mechanical turgor pressure and by molecular signaling pathways affecting the expression of genes encoding cell wall-modifiers and the activity of cell wall-modifying molecule.

1.4.1 Cell wall modifications regulate cell growth and expansion

Although cell wall composition is complex and varies between plant species, tissues and ages, cellulose is the key component synthesized by large cellulose synthase complexes at the plasma membrane. The inelastic cellulose microfibrils builds up the wall framework and their differential alignment determines the plasticity of the cell and the direction of the cell (Taiz & Zeiger, 2010; Wojtaszek, 2000). The cellulose microfibrils are embedded in a pectin matrix consisting of a diverse group of polysaccharides, such as galacturonans and rhamnogalacturonans, which through ionic and covalent bonds to different pectins and other polysaccharides, provide support and flexibility to the cell wall (Caffall & Mohnen, 2009). Additional cell wall polysaccharides are xylans, xyloglucans, mannans and glucomannans, usually referred to as hemicellulose. Cross-linkage of cellulose microfibrils by all these cell wall components is suggested to be a key to cell wall strength and stability (Braidwood *et al.*, 2013).

According to one of the most accepted, but still questioned theories, plant cells can expand by a controlled interaction between cell turgor pressure determined by osmolality and water uptake, and the relaxation of bonds between the existing cell wall components (for a recent review see Kutschera & Niklas, 2013; Heyn, 1981; Ray *et al.*, 1972). In addition, the composition of the cell wall components will be modified (Braidwood *et al.*, 2013). Loosening of the cell wall releases the inner turgor and allows the cell to passive uptake of water and further growth. Cellulose microfibril orientation plays a major role in determining the direction of growth and is mainly perpendicular to the main axis of expansion (Braidwood *et al.*, 2013). Many factors affect the timing, rate and orientation of expansion, such as cell type, developmental stage, and environmental ques. Plants ability to shape their cells accordingly, requires complex regulatory systems that dynamically integrate endogenous and exogenous factors to appropriately control cell growth. Integrators of signals

are often the plant hormones auxin, brassinolide, and GA. These in turn affect wall-modulating agents that can modify the physical properties of the cell wall.

Apart from affecting the activity of cell expansion genes via the nuclear TIR/AFB auxin receptor regulated auxin signaling, auxin has been suggested to have a direct effect on cell expansion via cell wall acidification, resulting in activation of pH-sensitive cell wall loosening enzymes/proteins (Hager *et al.*, 1971; Rayle & Cleland, 1970). Cell wall acidification has also been suggested to lead to K+ uptake allowing subsequent water uptake. Recently, Takahashi (Takahashi *et al.*, 2012) could support the above hypothesis by showing that auxin application to excised hypocotyl segments increased H+-ATPase proton extrusion activity, leading to acidification of the cell wall and cell expansion, by inducing penultimate threonine phosphorylation independently of TIR/AFB activity. However, as stated by Grebe (Grebe, 2006) "it remains to be revealed exactly where auxin-regulated cell wall acidification constitutes an intrinsic mechanism regulating different aspects of plant growth".

Although, key players affecting cell expansion have been discovered, the complex regulatory system of cell wall loosening is still unclear. In 1999, Cosgrove proposed that there are two types of cell wall modulating agents: the primary agents that instantly are able to act on the plasticity of the cell wall; and secondary agents that does not themselves interact with cell wall polymers, but instead leads to cell wall relaxation and increased sensitivity through the primary agents.

1.4.2 Expansins are cell wall loosening agents

One group of main primary cell wall loosening proteins is expansins. These are small extracellular proteins that disrupt non-covalent interactions between wall polysaccharides, in particular between cellulose microfibrils, resulting in enhanced extensibility (Braidwood *et al.*, 2013; Yennawar *et al.*, 2006). The expansin superfamily consists of α-, β-expansins, expansin-like A (EXLA) and expansin-like B (EXLB) (Sampedro & Cosgrove, 2005; Cosgrove, 2000a). Expansins induce cell relaxation via pH changes (McQueen-Mason & Cosgrove, 1995). The α- and β-expansins are suggested to non-enzymatically loose non-covalent bonds between xyloglucans (α), xylans (β) and cellulose and between cellulose microfibrils. The action of EXLA and EXLB are still unknown, but have been suggested to be secreted directly to the cell wall (Sampedro & Cosgrove, 2005). It is proposed that expansins most likely act via two distinct domains: the CDB domain would anchor the protein to the cellulose strands enabling the perfect positioning for cleavage of the non-covalent bonds by activation of the catalytic domain (Cosgrove, 2000b). This in turn leads to local disjoining of the polysaccharides conjunction and

immediate cell wall relaxation (Cosgrove, 2000b). Importantly, although the cell wall creep is induced, the overall covalent interactions are not affected (McQueen-Mason & Cosgrove, 1995). It has been suggested that several of the molecules integrating endogenous and environmental signals for cell expansion such as auxin (Fleming *et al.*, 1999; Rayle & Cleland, 1992), gibberellins (Vriezen *et al.*, 2000) or ethylene (Rose *et al.*, 1997) most probably regulates the expression of specific expansins.

1.4.3 A variety of enzymes affect cell growth

Apart from expansins, there are a multitude of additional enzymes affecting cell wall loosening and relaxation (see de Vries and Jaap Visser, 2001; Jamet et al., 2008; Frankova and Fry 2013 for review) (Frankova & Fry, 2013; Jamet *et al.*, 2008; de Vries & Visser, 2001). I will here only mention a few that are of relevance for my thesis work.

A large number of enzymes act on the bonds between and within cell wall polysaccharides through transglycosylation, cross-linking and hydrolysis (Cao *et al.*, 2012; Yadav *et al.*, 2009) modulating cell wall extensibility. Xyloglucan, the most abundant hemi-cellulose in many dicot plants, is modulated by xyloglucan endo-transglycosylase/hydrolase proteins, which possibly have both strengthening and loosening effects on the primary wall (Eklof & Brumer, 2010). In addition, many classes of enzymes are involved in degradation and/or modification of pectins, including the polygalacturonases (PGs), one of the pectin lyases family members. They hydrolyze the glycosidic bonds linking galacturonic acid residues together in for instance one of the most common pectins homogalacturonan. Therefore the PGs by degrading polygalacturonan polymer in the cell wall are affecting the cellular integrity, allowing cell expansion and growth events to occur.

Moreover, the FAD-dependent polyamine oxidases (*PAO*) are active in cell walls where they have been suggested to metabolize polyamines (PA) thus serving as cell wall loosening initiators (Cona *et al.*, 2005). AtPAOs are catabolizing polyamines to produce H_2O_2, 1,3-diaminopropane and aminoaldehyde (Tavladoraki *et al.*, 2006). H_2O_2 has been shown to be involved in the cell wall modifications through oxidative cross-linking of suberin and lignin, leading to strengthening effects. Additionally, H_2O_2 through formation of arabinoxylan coagula is allowing cell wall to maintain its extensibility (Angelini *et al.*, 2010; Fry *et al.*, 2000; Wisniewski *et al.*, 2000). There are five *PAO*s (*AtPAO*1-5) in *Arabidopsis thaliana* showing distinct expression patterns (Fincato *et al.*, 2012) suggesting that they may have tissue specific functions.

Metabolic reactions resulting in cell wall modifications are controlled and supported by transcriptional changes of variety of specific genes. The *Arabidopsis thaliana CYP78A* subfamily consists of 6 members (*CYP78A5-A10)* encoding for enzymes with the *cytochrome P450* (*CYP*) activity. *CYP78A* genes are differentially expressed and suggested to be involved in regulation of directional cell expansion and proliferation during organ growth, including embryogenesis (Sotelo-Silveira *et al.*, 2013; Yang *et al.*, 2013; Fang *et al.*, 2012; Lohmann *et al.*, 2010; Adamski *et al.*, 2009; Wang *et al.*, 2008; Anastasiou *et al.*, 2007; Zondlo & Irish, 1999). The overexpression of individual member results in enlarged organs and consequently the loss-of-expression leads to reduced growth as well as disrupted maintenance of SAM region. However, their exact road of actions is yet to be determined.

In summary, plants control organ growth via a tight control of cell proliferation and cell expansion. Multiple elements that are present in the cascades of these events uphold for the complexity of the multicellular organisms.

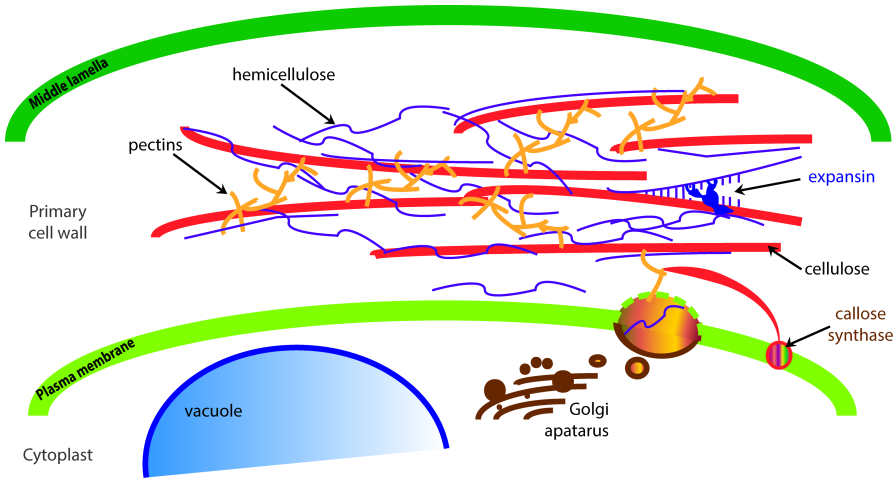

Figure 3. Schematic model of primary cell wall composition. For more details see chapter 1.4.

1.5 Aerial organ development

Plants are sessile organisms and in order to survive the environmental conditions they are exposed to they have to adapt their developmental program in accordance to this (Franks & Hoffmann, 2012). It is believed that this is one of the reasons why plant embryos, in contrast to animal embryos, do not have all organs pre-formed. Instead plants continue to form organs from the stem

cells of the meristems through out their life cycle, making it possible for the plant to make developmental decisions in accordance to the individual needs.

1.5.1 Body axis establishment and embryo development

Two axes are formed during embryo development: the apical-basal and radial (outer-inner) axes. An apical-basal axis already apparent in the egg cell is re-established in the zygote (Faure *et al.*, 2002; Laux & Jurgens, 1997; Mansfield & Briarty, 1990), and several transcription factors including WRKY2 and different WOX members have been implicated in this process (reviewed in Jeong *et al.*, 2012). The apical-basal polarity manifests itself in the first asymmetric cell division after fertilization. The apical daughter cell becomes the source of the embryonic tissue including the shoot apical meristem (SAM) and cotyledon primordia, while the basal cell will develop into the suspensor. The uppermost part of the suspensor will give rise to hypophyseal cell, incipient to the root apical meristem (RAM) (reviewed in Ueda & Laux, 2012).

The PLETHORA (PLT) transcription factor has been suggested to act as a master regulator for root fate, whereas HDZIPIII transcription factors have been suggested to impose shoot fate in the embryo. The *PLT* gene is expressed in the basal end of the early globular embryo, and becomes confined to the vascular precursor and the incipient RAM quiescent centre in the proembryo, whereas *HDZIPIII* genes are expressed at the apex of early globular embryos (reviewed in Jeong *et al.*, 2012). In addition, dynamic auxin flow, biosynthesis and signalling are important processes during plant pattern formation, including the early embryo development, and it has been suggested that asymmetric divisions and inter-cell communication generates gene expression domains along the main apical-basal embryo axis that may serve as a basis for organizing the dynamic polar auxin flux (reviewed in Jeong *et al.*, 2012) involved in polarity and tissue identity establishment. In the two-celled zygote, PIN auxin efflux proteins mediate auxin transport from the basal to the apical cell, which induce normal apical cell division (Friml *et al.*, 2003). Later, apical-to-basal auxin transport creates a basal auxin maximum in the early embryo, required for root initiation (reviewed in Aichinger *et al.*, 2012).

Already during the late globular stage of embryo development local cell division gives rise to bilaterally symmetrical incipient cotyledons. They emerge in two apical regions of high auxin levels created by PIN-mediated transport (Moller & Weijers, 2009), and strong auxin responsiveness, visualized by activity of the synthetic auxin response reporter DR5 (Benkova *et al.*, 2003). The sites of DR5 activity correlates well with reported defects in auxin mutants (Hardtke & Berleth, 1998; Jurgens *et al.*, 1994), suggesting that auxin indeed is required for cotyledon initiation, positioning and outgrowth. A

certain set of genes appears to be specific for cotyledon development, such as e. g, *DRN* and *DRNL* (Chandler et al., 2007), whereas large sets of the genetic program are similar to the leaf developmental program (reviewed in Chandler, 2008), see below.

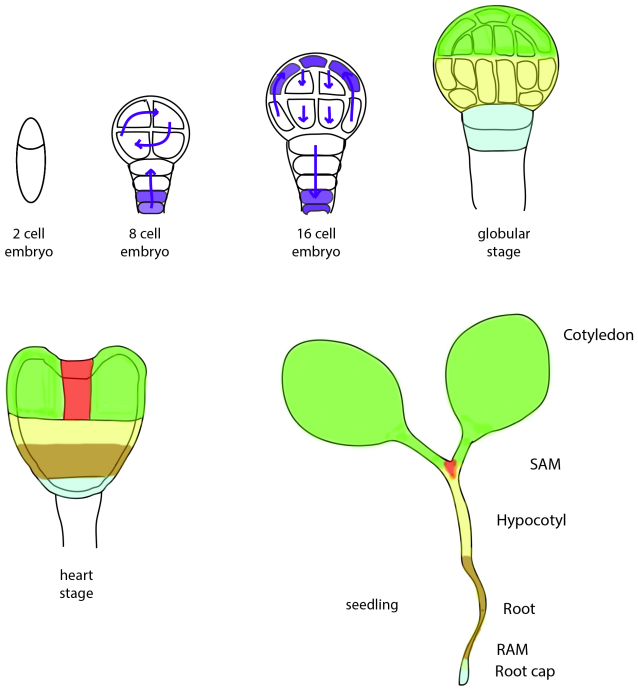

Figure 4. Overview of some of the stages of embryogenesis. Arrows indicate the direction of auxin flow and the blue-marked cells indicate high auxin sites. From globular stage onwards colors indicate pattern of organ development.

1.5.2 Post-embryonic plant development requires meristem activity

New organs form from daughter cells produced by the self-renewing population of stem cells in the SAM and RAM, established during embryo development. Their activity is responsible for the indeterminate shoot and root growth, respectively, and it has been shown that cell-cell communication and non-cell-autonomous processes play important roles in meristem function.

The shoot apical meristem

The SAM in higher plants shares a common organization. It is composed of cells forming a dome-shaped structure, with a small number of undifferentiated stem cells in the central zone (CZ), and their more rapidly dividing daughter cells, ready for recruitment into organ initiation in the more peripheral zone (PZ) (Steeves & Sussex, 1989). The maintenance of the stem cell niche is dependent on the organizing centre (OC) located directly beneath the CZ. The OC cells express the homeobox transcription factor WUSCHEL (WUS) (Mayer *et al.*, 1998), which has been indicated to move from the OC to the CZ stem cells, where it acts as a repressor of genes promoting cell differentiation in order to maintain stem cell identity (Yadav *et al.*, 2013; Yadav *et al.*, 2011; Yadav *et al.*, 2010). In addition, WUS restricts its own level by directly activating *CLAVATA3* (*CLV3*) in the CZ (Yadav *et al.*, 2011; Brand *et al.*, 2000; Fletcher *et al.*, 1999). CLV3 is a secreted peptide, which via activation of the receptor kinase CLAVATA1 pathway, restrict *WUS* transcription to a few cells only, thus forming a feedback system (Ogawa *et al.*, 2008; Fletcher *et al.*, 1999; Clark *et al.*, 1997).

Surrounding the stem cell pool, two antagonistic pathways control cell stem identity versus differentiation in the PZ. An undifferentiated cell-identity is maintained by SHOOTMERISTMLESS (STM) activity, in combination with CUP-SHAPED COTYLEDON (CUC) family members (Aida *et al.*, 1999). STM has been shown to induce cytokinin biosynthesis, and this hormone has been associated with the undifferentiated state of cells in both the CZ and PZ (Shani *et al.*, 2006; Jasinski *et al.*, 2005). *STM* and *CUC* genes are switched off by an unknown factor in the few PZ cells that is recruited to primordia formation, and remain off by the activity of the ASYMMETRIC LEAVES1 (AS1) transcription factor (Byrne *et al.*, 2000), resulting in rapid proliferation and growth rate in the developing primordium, delimited by the organ boundary region, where cell expansion is reduced.

In addition, organ primordia will only initiate in the PZ at sites of auxin maxima established by the PIN1 efflux carrier (Heisler *et al.*, 2005; Reinhardt *et al.*, 2000; Okada *et al.*, 1991). New primordia develop on the flanks of the SAM in a species-specific phyllotactic pattern (Fleming, 2006), and in Arabidopsis leaf initiation follows a spiral phyllotaxy, where primordia are initiated one at a time, at the incipient site far away from the two last formed primordia. It has been suggested that auxin itself may be able to direct its flow via PIN re-localizations to guide auxin to the next incipient site in the PZ. Two different hypotheses on how this could be achieved have been tested by computational modelling: auxin flow against the auxin gradient (Jonsson *et al.*, 2006; Smith *et al.*, 2006), and amplification of the auxin flow by induced

transport (Stoma *et al.*, 2008). Both models are sufficient to reproduce realistic PIN1 localization and phyllotactic patterns, and more detailed studies of actual auxin concentrations will help determine which fits best with reality (Bayer *et al.*, 2009). Transcription factors belonging to the auxin-signalling pathway, such as MONOPTEROS (MP) is expressed in the PZ (Vernoux *et al.*, 2011; Hardtke & Berleth, 1998) and recruits founder cells for primordia formation. The competence for organ initiation in the PZ thus also depends on the spatial modulation of auxin signal transduction. In addition, it has been suggested that mechanical forces cooperate with auxin to establish the phyllotactic pattern, for instance by reinforcing the directionality of auxin transport (reviewed in Besnard *et al.*, 2011). Cell expansion is also required for the actual outgrowth of the organ primordia (Peaucelle *et al.*, 2011).

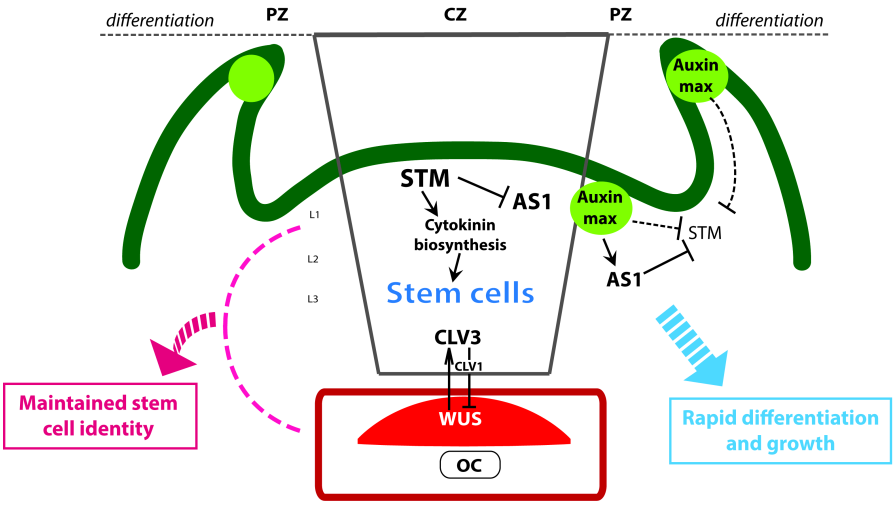

Figure 5. Proposed model for regulation of stem cell identity vs differentiation by selected genes.

Leaf initiation and development

As leaf primordia develop in the PZ on the flanks of the SAM CZ, they possess a positional relationship to the SAM. The adaxial side (which will become the photosynthetic surface of the leaf) is derived from cells adjacent to the CZ, while the abaxial side (to become the gas-exchange side of the leaf) is derived from PZ cells further away from the CZ. Already in the 1950ies, Sussex (Sussex & Steeves, 1953; Sussex, 1951) revealed by microsurgical experiments that a signal derived from the SAM is required for the establishment of the adaxial leaf primordia identity (Sussex, 1951). Without direct cell-cell communication with the SAM, leaf primordia only obtain abaxial cell identity

and fail to produce a leaf blade, suggesting that leaf lamina outgrowth is dependent on the adaxial-abaxial polarity. These observations have been supported by work with mutants failing to induce either adaxial or abaxial identity. However, the nature of the Sussex signal still remains obscure, although it has been speculated that it could be a small RNA (Chitwood *et al.*, 2007), see below.

The regulatory network controlling adaxial-abaxial polarity is based on the mutual antagonistic interactions between adaxial and abaxial determinants. The three *HD ZIPIII* genes, *PHB*, *PHV* and *REV* are, based on both loss-of-function and gain-of-function mutant phenotypes, necessary and sufficient for specification of adaxial identity in leaf primordia (Prigge *et al.*, 2005; Emery *et al.*, 2003; McConnell *et al.*, 2001; McConnell & Barton, 1998). Abaxial expression of the two *miRNAs 166* and *167* targeting *HDZIPIIIs* restricts HDZIPIII activity to the adaxial side (Emery *et al.*, 2003). The *KANADI* genes (*KAN1* and *KAN2*) and two *ARF* genes (*ETT/ARF3* and *ARF4*) specifies the abaxial domain (Pekker *et al.*, 2005; Emery *et al.*, 2003; McConnell *et al.*, 2001). Whereas KAN and HDZIPIII supresses each other, *ETT/ARF3* and *ARF4* are negatively regulated in the adaxial domain by a small RNA known as tasiR-ARF, derived from non-coding TAS3 precursor transcripts expressed in the adaxial domain (Fahlgren *et al.*, 2006; Garcia *et al.*, 2006; Allen *et al.*, 2005). The precise mechanism inducing leaf lamina outgrowth upon adaxial-abaxial juxtaposition is still not known, but members of the *YABBY* gene family have been indicated to play an important role. Although YABBY genes previously were suggested to determine abaxial identity (Sawa *et al.*, 1999; Siegfried *et al.*, 1999), recent data suggest that they are not required for initial polarity establishment, but rather polarity maintenance and lamina outgrowth (Sarojam *et al.*, 2010).

Soon after leaf primordia initiation, the primordia start to produce auxin that is transported back to the meristem to contribute to subsequent leaf primordia initiations (Heisler *et al.*, 2005; Ljung *et al.*, 2001). Initially, an apical auxin maxima in the leaf primordia, reinforced by epidermal auxin flow to this point, presumably induces distal growth (Benkova *et al.*, 2003; Reinhardt *et al.*, 2003), whereas a subsequent symmetrically distributed auxin at the leaf margins, acting downstream of the polarity genes, is thought to facilitate blade outgrowth (Wang *et al.*, 2011; Zgurski *et al.*, 2005; Aloni *et al.*, 2003; Scanlon, 2003; Mattsson *et al.*, 1999). In addition, auxin drainage flow from the apex of the very young leaf primordia through the centre of the primordium marks the positioning of the vascular leaf midvein, which will become continuous with the stem vasculature. During further development, auxin maxima at the margins of the leaf primordial correlate with the sites of lateral-vein formation

and positions of leaf serrations (see below). After primary morphogenesis, higher order leaf veins are initiated within the growing leaf lamina (reviewed in Scarpella *et al.*, 2010; Sawchuk *et al.*, 2007). All vascular cells are produced from procambial cells, differentiating from selected cells called preprocambial cells (Scarpella *et al.*, 2004; Mattsson *et al.*, 2003). In 1981, Sachs (Sachs, 1981) proposed a hypothesis suggesting that auxin flow promotes polar auxin transport, leading to the canalization of auxin flow into narrow cell files, which will become preprocambial cells. This hypothesis has been supported by many studies, and it has been shown that three components, auxin flow, PIN1, and the auxin response factor MP, form a positive feedback loop, which reinforces auxin canalization. In addition, the ATHB8 transcription factor restricts preprocambium formation to a narrow domain by spatially limiting *PIN1* expression, and by regulating MP (Ohashi-Ito & Fukuda, 2010; Wenzel et al., 2007; Scarpella et al., 2006). Interestingly, the leaf adaxial-abaxial identity tool-kit described above has been co-opted for the correct spatual positioning of the vascular components xylem and phloem in the vascular bundles (Carlsbecker & Helariutta, 2005).

Auxin is also, together with CUC2 and its negative regulator miR164, a key regulator of leaf marginal modifications leading to leaf serration in Arabidopsis (Bilsborough *et al.*, 2011; Hay *et al.*, 2006; Nikovics *et al.*, 2006; Scarpella *et al.*, 2006). Before serration outgrowth, *CUC2* is expressed along the whole leaf margin, but eventually its expression disappears from serration initiation sites marked by high auxin activity. CUC2 is required to generate marginal epidermis PIN1 re-localizations directing auxin towards specific marginal convergence points providing local auxin maxima necessary for the localized auxin activity and serration outgrowth. In addition, auxin represses CUC2 via miR164 activation, revealing a feed-back loop critical for serration development (Bilsborough *et al.*, 2011). Auxin accumulating in the leaf serration points is then predicted to become transported into the leaf blade by basal localization of PIN 1 in subepidermal cells, defining the sites of lateral vein formation (Wenzel *et al.*, 2007; Hay *et al.*, 2006; Scarpella *et al.*, 2006).

1.5.3 Floral transition and initiation of flowering

The correct timing of initiation of flowering ensures reproductive success and therefore plants are dependent on the complex genetic and molecular signaling networks involved. Under the influence of endogenous signals, which include plant age and health, as well as environmental cues such as seasonal changes, the plant initiates the transition process from adult vegetative stage to reproductive phase. Transition from a vegetative SAM to an inflorescence meristem occurs when the required endogenous and environmental signals are

perceived. This is achieved by the stimulation of the flowering pathway integrator genes, *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), and subsequently, activation of the flowering genes *LEAFY* (*LFY*) and *AP1*. The endogenous signal network contains hormones, in particular gibberellins, sugars, and a variety of genetic factors (reviewed in Srikanth & Schmid, 2011; Amasino & Michaels, 2010; Wang & Li, 2008).

The main endogenous pathways involve the actions of miR156. Changes in expression from high levels of miR156 during seedling stage to lower levels in further developmental phases are shown to be crucial for the transition of plants to the flowering phase (reviewed in Huijser & Schmid, 2011). This age-dependent genetic pathway involves the repressive action of miR156 on the *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) mRNA. While actions of *SPL3, SPL4* and *SPL5* affect flowering time, *SPL9* and *-10* also regulate adult phase morphological traits, such as formation of trichomes on the abaxial side of the leaf (Wu *et al.*, 2009). As the levels of miR156 decrease, *SPL9* and *-10* also become more prominent in the photoperiod-induced pathway of flower transition phase (Schwarz *et al.*, 2008). Importantly, the SPL9 and SPL10 transcription factors are also involved in activation of the miR172 microRNA family. The miR172 levels increase as the plant proceeds through developmental phases and its accumulation levels are complementary to the one of miR156. The expression level of miR172 is reported to be under influence of the photoperiod (Jung *et al.*, 2007). In downstream actions, miR172 represses a number of repressors of flowering, such as *TARGET OF EAT1* (*TOE1*), *TOE2*, *SCHLAFMÜTZEN* (*SMZ*) or the *AP2* (reviewed in Huijser & Schmid, 2011). The complementary accumulation of both miR156 and miR172 in the plant tissue is critical for the timing of the transition from the juvenile to adult phase. Additionally to the endogenous signaling, a number of autonomous pathways have been reported and they all act via repression of the *FLC* either by chromatin remodeling or affecting RNA stability, thus eventually promoting flowering (reviewed in Srikanth & Schmid, 2011).

Two of the most important exogenous signals are prolonged exposure to low temperature (vernalization) and perception of day-length (photoperiod) (Turck *et al.*, 2008). Another important environmental factor is the quality of the day light that is perceived by the plants using internally oscillating system, known as the circadian clock (reviewed in Srikanth & Schmid, 2011).

Photoperiod, sensed in the leaves, is an important environmental signals for plants adapted to latitudes with varying day-length. In *Arabidopsis*, adapted to perform reproductive development in long days, the circadian clock oscillation activates transcription of the *CONSTANS* (*CO*) gene in the late afternoon/early

evening. As the CO proteins are stabilized by light and degraded in darkness, they are only able to accumulate in long day conditions, when CO transcription coincides with light. The CO transcription factor accumulating in the leaf activates transcription of the *FT* gene in the phloem companion cells and the FT proteins become actively translocated to the phloem sieve elements through which they are transported to the SAM. In the SAM, FT interacts with FLOWERING LOCUS D (FD) to induce phase transition by regulating the expression of other floral integrators such as *SOC1* and *AP1* (reviewed in Andres & Coupland, 2012; Amasino, 2010; Amasino & Michaels, 2010).

Another seasonal environmental cue used by e.g. winter annuals is temperature. In these plants, vernalization, a long period of exposure to cold temperatures, is required before the plants can enter into the reproductive phase. In Arabidopsis this process requires the activity of two genes: *FRIGIDA* (*FRI*) and *FLC* (reviewed in Amasino & Michaels, 2010). The Arabidopsis summer annuals, which do not require vernalization as an initiator of flowering, lack a functional allele of *FRI* and/or *FLC*. The *FRI*-dependent activation of *FLC* suppresses flowering (reviewed in Amasino, 2010). Similarly to FLC, another MADS-domain protein, the *MADS AFFECTING FLOWERING3* was reported to act during the repression event (Gu *et al.*, 2013). FLC activity repress flowering by blocking the transcription of the flowering pathway integrator genes (*FT* and *SOC1*). Exposure to low temperatures reduces *FLC* transcription progressively over several weeks, and by the end of the vernalization period there are low levels of *FLC* mRNA, thus releasing the repression on *FT* and *SOC1*. The cold inducted VERNALIZATION INSENSITIVE3 (VIN3) interacting with histone modification complexes like PRC2 to induce epigenetic silencing of FLC mediates the initial repression of FLC transcription. Low levels of *FLC* mRNA even when temperatures increase after the vernalization period due to activity of additional identified proteins and non-coding RNAs, is allowing the other pathways to induce flowering under right conditions (reviewed in Gu *et al.*, 2013; Kim & Sung, 2012; Smaczniak *et al.*, 2012; Amasino & Michaels, 2010).

1.5.4 Floral organ development

The inflorescence meristem (IM) is a tissue of meristematic cells, which at its PZ generates cauline leaves and axillary buds as well as floral meristems (FMs) that will develop into individual flowers (O'Maoileidigh *et al.*, 2014; Smaczniak *et al.*, 2012). As in the SAM, auxin is required for the formation of these structures, including the FMs, at the IM PZ (Cheng *et al.*, 2008; Reinhardt *et al.*, 2003; Reinhardt *et al.*, 2000; Przemeck *et al.*, 1996). The FM

stem cell fate and meristem size is initially maintained by the universal stem cell-specific WUS-CLV feedback loop (see 1.5.2.1 above; (reviewed in Smyth, 2005). Eventually, expression of *AGAMOUS* (*AG*) will inhibit the actions of WUS and thereby initiate differentiation and organ formation (Lenhard *et al.*, 2001). The phyllotaxis pattern of organ initiation is shifted in the FM from the spiral pattern used by the Arabidopsis SAM and IM, to a whorled pre-set organ arrangement (reviewed in Chandler, 2012). How this is achieved is not well understood, but it has been hypothesized that PAT and the activation of local auxin biosynthesis in the early FM results in a prolonged auxin exposure induced the switch to whorled organ initiation (van Mourik *et al.*, 2012).

Once the FM is established, AP1 and LFY activate the ABCE floral organ identity genes resulting in floral organ specification at floral stage 5 (Causier *et al.*, 2010; Smyth *et al.*, 1990; Bowman *et al.*, 1989). Class A genes (*AP1* and *AP2*) specifies the outermost whorl to become sepals, A together with B class genes *AP2* and *PISTILLATA* (*PI*) determine petal identity of second whorl organs, B together with C genes (*AG*) determine stamen fate in the third whorl, while C alone controls carpel identity in the inner-most fourth whorl. In addition, the E class genes *SEPALLATA1* (*SEP1*), *SEP2*, *SEP3* and *SEP4* co-regulate fate specification in all four whorls. Also, miR172 was suggested to suppress *AP2* expression in the inner whorls (Grigorova *et al.*, 2011; reviewed in Huijser & Schmid, 2011). Except *AP2*, all other whorl-determining genes belong to MADS-box domain transcription factor group (reviewed in O'Maoileidigh *et al.*, 2014; Huijser & Schmid, 2011). Interestingly, SOC1 (described in section 1.3.5.1) together with AGL24 and SVP has been shown to repress expression of B-, C- and E-class homeotic genes in both IM and early FM (reviewed in Smaczniak *et al.*, 2012).

1.5.5 The reproductive floral organs

The shape and number of floral organs are specific for different species. In Arabidopsis the flower consists of six stamens, the male reproductive organs hosting the pollen development events, one pistil, formed by two congenitally fused carpels, which builds up a female reproductive structure, where the pollen germination events and fruit development take place, and the sterile perianth consisting of petals and sepals (Roeder & Yanofsky, 2006).

Stamens

The male reproductive organs, the stamens, emerge from the floral meristem whorl three under control of B and C class genes expressed in the inner whorls (ABCE model described in section 1.3.5.2). Arabidopsis produces six stamens, four long medial and two short lateral stamens (Scott *et al.*, 2004). A stamen

consist of a filament and an anther, which at the point of pollination is positioned just above the stigma, allowing direct release of pollen grains onto the papillae cells, thus securing effective fertilization. Stamen filaments are thought to supply water and nutrients to anthers through the vasculature and to facilitate stamen elongation (Scott *et al.*, 2004). In Arabidopsis anthers develop four lobes that consist of epidermis, endothecium, middle layer and the adaxial tapetum. These four somatic cell layers enclose the inner reproductive tissue, the male sporocytes (or pollen mother cells), which undergo meiosis and form haploid microspores (Ma, 2005; Goldberg *et al.*, 1993). During floral stage 10 to 12 further divisions and differentiation occurs at microsporogenesis, resulting in mature pollen grains. At floral stage 13 the filaments elongate and anthers dehisce to release the mature grains during the pollination event (Song *et al.*, 2013; Ge *et al.*, 2010). The four somatic cell layers of the anthers are also essential for proper pollen formation and its release at the right time-point. For example, the layer closest to the developing pollen, the tapetum, supplies the microspores with nutrients and enzymes important for cell separation as well as cell wall synthesis.

Several phytohormones are crucial for proper stamen and pollen development (Song *et al.*, 2013). The auxin biosynthesis genes *YUC2* and *YUC6* are expressed in the tapetum and pollen mother cells from late stage 10, and the TIR/AFB receptor genes, as well as the *DR5* auxin response reporter are activated in these tissues shortly thereafter (Cecchetti *et al.*, 2013; Cecchetti *et al.*, 2008; Feng *et al.*, 2006). Although the precise role of auxin at these tissues is unknown, it has been suggested that auxin is important for the differentiation or function of e.g. the tapetum layer as a reduction of active auxin levels in the tapetum results in decreased pollen embryogenesis (Yang *et al.*, 1997). However, mutants defective in auxin biosynthesis or auxin perception are not only affected in pollen maturation, but also in filament elongation and anther dehiscence, suggesting that auxin is important for several aspects of pollen and stamen development (Cecchetti *et al.*, 2013; Cecchetti *et al.*, 2008; Cheng *et al.*, 2006; Nagpal *et al.*, 2005). Auxin appears to affect pollen grain development at least in part by controlling JA-signaling, while auxin induced stamen filament elongation is JA independent (Song *et al.*, 2013). Mutants defective in GA biosynthesis also show defects in filament elongation and anther dehiscence (reviewed in Plackett *et al.*, 2011) suggesting a complex cross-talk between plant hormones during stamen development.

The Arabidopsis *SPOROCYTELESS*/*NOZZLE* (*SPL*/*NZZ*) gene, induced by AG activity (Ito *et al.*, 2004), encodes a putative transcription factor important for early anther cell division and differentiation. The *spl*/*nzz* mutant fails to produce a pollen sac, including both sporogenous cells and nonreproductive

tissues, (Schiefthaler *et al.*, 1999; Yang *et al.*, 1999). Further work has suggested that SPL/NZZ via a regulatory feed-back loop with *BARELY ANY MERISTEM1/2* (*BAM1/2*) maintains the sporogenous activity whereas *BAM1/2* promote somatic growth, providing a balance between reproductive and somatic cells in the anther (Hord *et al.*, 2006). Interestingly, SPL/NZZ activity has also been suggested to affect auxin biosynthesis and transport (Bencivenga *et al.*, 2012; Li *et al.*, 2008). During later stages of anther development, three putative LRR-RLK proteins, EXCESS MICROSPOROCYTES1 (EMS1), SOMATIC EMBRYOGENESIS RECETOR-LIKE KINASE1 (SERK1) and SERK2, and the small peptide TAPETUM DETERMINANT1 (TDP1) are all required for tapetal cell differentiation and the repression of reproductive tissue suggesting that the microsporocyte is a default cell fate, and that cell–cell signaling triggers the differentiation of tapetal cells (Albrecht *et al.*, 2008; Zhao *et al.*, 2008; Albrecht *et al.*, 2005; Canales *et al.*, 2002; Zhao *et al.*, 2002).

The gynoecium

The purpose of the female reproductive organ, the gynoecium, developing from the centre of the FM, is to host the ovules and guide fertilization events. After fertilization the gynoecium differentiates into the fruit tissue, which harbors the developing seeds and aids in seed dispersal. At stage 5 of floral development the FM expands laterally and forms a platform of cells that will give rise to the gynoecium. Shortly thereafter, AG will terminate the FM activity, as no further organ initiation is required (Sun & Ito, 2010; Lenhard *et al.*, 2001). In Arabidopsis, the gynoecium primordium emerges as an open cylinder consisting of two carpels congenitally fused at their margins, and these margins correspond to the medial domain (Sattler, 1973). Further development in the apical-basal, medial (fused carpel margins)-lateral as well as adaxial-abaxial results in the mature gynoecium, which is the most complex floral organ (Larsson *et al.*, 2013; Roeder & Yanofsky, 2006). The medial marginal tissues of the carpels are meristematic and express the meristem-specifying gene *STM* (Scofield et al., 2007). Internally (adaxial side) the marginal tissue gives rise to placentae and ovules, as well as a septum that divides the Arabidopsis ovary into two separate chambers and also harbors the transmitting tract guiding the pollen tubes to the ovules (reviewed in Larsson *et al.*, 2013). The medial marginal tissue is also suggested to give rise to the apical stigma and style after postgenital apical fusion of the carpels. The stigma, consisting of papillar cells, where pollen will adhere and germinate, is localized on a cylindrical style that guides the pollen tubes via the transmitting tract to the ovary and ovules. On the adaxial side (outside) of the gynoecium,

medial cells form a replum, to which the carpel margins and the carpels are attached. The ovary is connected to the base of the flower through a short stalk of cells, referred to as the gynophore (Larsson *et al.*, 2013; Staldal & Sundberg, 2009; Roeder & Yanofsky, 2006). At floral stage 13, the flower open and is ready for the pollination (Roeder & Yanofsky, 2006; Smyth *et al.*, 1990).

The marginal tissues are dependent on a number of genes, including *AINTEGUMENTA* (*ANT*), *AINTEGUMENTA-LIKE6* (*AIL6*), *LEUNIG* (*LUG*), *SEUSS* (*SEU*) and *FIL*, and loss of more than one of these results in a more or less complete loss of the marginal derived medial tissues. It has been suggested that ANT, SEU, LUG and FIL form a transcriptional co-regulator complex important for medial tissue development (Azhakanandam *et al.*, 2008; Nole-Wilson & Krizek, 2006).

Auxin plays an important role during gynoecium tissue differentiation and proliferation. Reduced auxin signaling, biosynthesis or transport results in reduced ovary (carpel) development and an enhanced style and gynophore proliferation (Stepanova *et al.*, 2008; Cheng *et al.*, 2006; Sohlberg *et al.*, 2006; Christensen *et al.*, 2000; Nemhauser *et al.*, 2000; Hardtke & Berleth, 1998; Przemeck *et al.*, 1996; Bennett *et al.*, 1995; Sessions & Zambryski, 1995). Based on these findings, Nemhauser *et al.* (2000) suggested that an apical-basal auxin gradient with highest concentration in the apical part and lowest in the base determine the apical-basal tissue border positioning (Nemhauser *et al.*, 2000). However recent studies propose that auxin gradients or auxin signaling pathways acting in abaxial-adaxial or medial-lateral axes affects the apical-basal patterning of the gynoecium (reviewed in Larsson *et al.*, 2013).

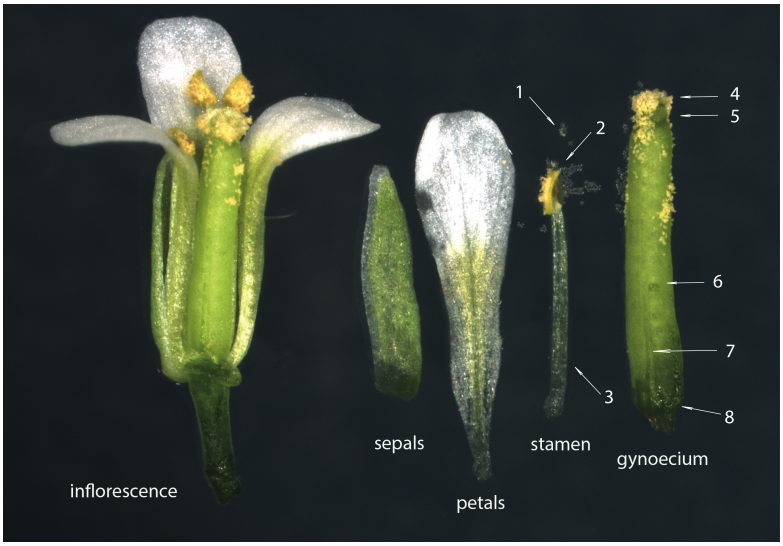

Figure 6. Picture of the Arabidopsis flower. Numbers indicate: 1- pollen grains; 2- anther; 3- stamen filament; 4- stigma; 5- style; 6- valve; 7- replum; 8- gynophore.

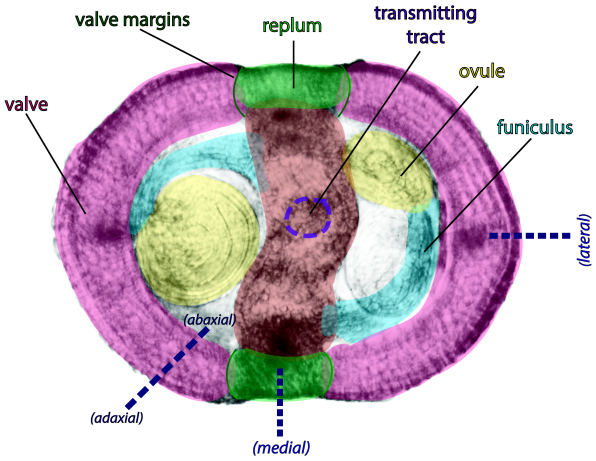

Figure 7. Cross-section of gynoecium with indicated structures and axis.

1.6 The *SHORT INTERNODES/STYLISH* gene family

The Arabidopsis *SHORT INTERNODES* (*SHI*) gene was discovered in an activation-tagging screen for genes affecting plant development (Fridborg *et al.*, 1999). Further studies revealed that the *SHI* gene belongs to a plant specific gene family, *SHI*/*STYLISH* (*STY*), important for organ development in a variety of plants, such as Arabidopsis, barley, *Populus*, and the moss *Physcomitrella patens* (Yuo *et al.*, 2012; Zawaski *et al.*, 2011; Eklund *et al.*, 2010; Kuusk *et*

al., 2006; Kuusk *et al.*, 2002). The Arabidopsis genome encodes nine functional and highly redundant members, *SHI*, *STY1*, *STY2* and *SHI-RELATED SEQUENCE 3* to *7* (*SRS3*-*SRS7*) as well as a putative pseudogene, *SRS8* (Kuusk et al., 2006; Fridborg et al., 2001). The Arabidopsis *sty1* mutant is characterized by a split style of the gynoecium. This phenotype is drastically enhanced by reduction in expression of additional *SHI/STY* family members and the *shi/sty* multiple mutant plants produce gynoecia with dramatically reduced proliferation of apical and medial tissues (Kuusk *et al.*, 2006). The *SHI/STY* are expressed in an overlapping pattern in the apical part of gynoecia from the earliest stage (floral stage 5, see section 1.5.2.3.1.2 above) to gynoecium maturity, as well as in ovules. In addition, SHI/STY proteins are required for proper leaf development. The *sty2* mutant show increased leaf serration and this phenotype become enhanced in the *SHI/STY* family multiple mutant lines, which also develop some radialized leaves (Kuusk *et al.*, 2006; Kuusk *et al.*, 2002), and as expected from these phenotypes, members of this gene family are expressed in leaf primordia, leaf tips and hydathodes/leaf serration sites. In addition they are active in cotyledon initiation sites, floral organ initiation sites, floral organ primordia, stamens, root tips, and lateral root initiation sites, and lateral root primordia (Kuusk *et al.*, 2006; Kuusk *et al.*, 2002), suggesting that they are of general importance for organ initiation and development.

The SHI/STY proteins carry two unique and conserved regions: a C-terminal domain with an IGGH motif and an N-terminal RING-like zinc finger (ZnFn) domain with a C_3HC_3H cysteine/histidine consensus arrangement (Kuusk *et al.*, 2006; Fridborg *et al.*, 2001; Fridborg *et al.*, 1999). Using yeast two-hybrid system, it was shown that SHI/STY proteins interact with each other in vivo, thereby forming homo- and heterodimers (Kuusk *et al.*, 2006). This is a strong indication that these proteins may form molecular complexes and act cooperatively in common pathways. In addition, the proteins carry a nuclear localization signal (NLS) and one or two glutamine amino acid rich region, suggesting that they act as transcription factors. This was further confirmed by using a fusion protein consisting of the full-length STY1 coding sequence fused to the rat glucocorticoid receptor (GR) domain, driven by the cauliflower mosaic virus 35S promoter in the *$35S_{pro}$:STY1-GR* construct (Kuusk *et al.*, 2006). The GR domain makes the protein cytoplasmic, but it is shuttled to the nucleus upon treatment with the synthetic ligand dexamethasone (DEX) (Lloyd *et al.*, 1994; Schena *et al.*, 1991).

By comparing the transcriptome of DEX-treated with untreated *$35S_{pro}$:STY1-GR* lines the *YUCCA4* (*YUC4*) auxin biosynthesis gene was identified as a potential direct or indirect downstream target, activated by

nuclear STY1-GR (Sohlberg *et al.*, 2006; Zhao *et al.*, 2001). In addition, nuclear translocation of STY1-GR results in a significant increase in free IAA levels, whereas loss of function of *SHI/STY* genes leads to reduction of auxin biosynthesis rates, auxin catabolism, as well as mRNA levels of *YUC4*, *IAA1* and *GH3* genes (Sohlberg *et al.*, 2006). In agreement with these data the *sty1-1* mutant is hypersensitive to alteration of polar auxin transport (PAT) (Sohlberg *et al.*, 2006), and the style defects in *sty1-1 sty2-1* plants can be restored by application of exogenous auxin directly onto the apical end of very young gynoecia, before style differentiation had occurred (Staldal *et al.*, 2008). Together these results indicate that SHI/STY members, at least in part, affect organ initiation and development via direct or indirect regulation of auxin homeostasis.

2 Aims of the present study

The goal of this work was to investigate the role of SHI/STY proteins in organ development. We aimed to detect if the SHI/STY proteins act as DNA-binding transcriptional regulators and identify downstream target genes of SHI/STY proteins in order to identify putative pathways co-regulated by the SHI/STY activity. In order to do so, we also aimed to characterize several of the downstream targets. With the goal to place the SHI/STY regulated processes in a wider network, we also aimed to identify regulatory factors controlling *SHI/STY* genes expression.

3 Results and discussion

3.1 *SHI/STY* family members act as DNA-binding transcriptional activators (I, IV)

3.1.1 The SHI/STY family proteins are localized in the nucleus (I)

As previous sequence analysis of the nine functional Arabidopsis SHI/STY family members revealed that they carry a putative nuclear localization signal (NLS) in addition to the conserved IGGH- and RING-like zinc finger domains (Fridborg *et al,* 2001; Kuusk *at al.,* 2002), we studied the intracellular localization of STY1-GFP fusion protein in transiently transformed onion epidermal cells and in *Physcomitrella patents* protoplasts (Paper I). We could show that the STY1 protein indeed is localized in the nucleus, which fits well with previous data, showing that when STY1-GR is trans-located to the nucleus by DEX treatment the *sty1-1* mutant style phenotype is rescued (Sohlberg *et al.,* 2006).

3.1.2 *SHI/STY* members are transcriptional activators (I, II)

We could show that STY1-GR, upon DEX induced nuclear translocation, within one hour activates the transcription of a large number of genes (II), including *YUC4* (Sohlberg *et al.,* 2006). To further verify the role of STY1 as a transcriptional activator, we used a *35Spro:STY1-SRDX* construct (I). SRDX is a 12-amino acid long peptide acting as a strong repressor domain and we could show that it changes STY1 into a transcriptional repressor, which strongly down-regulated *YUC4* transcription. In addition, fusion to the repressor domain caused strong phenotypic effects resembling those of *shi/sty* multiple loss-of-function mutants. This suggests that STY1-SRDX represses the transcription of genes normally activated by members of the SHI/STY protein family. It has been shown that at least SHI-GR activates the same set of genes as STY1-GR, supporting the hypothesis that STY1-SRDX affects the activity of several

common targets (unpublished data). We could also reveal that STY1 has several functional transcriptional activation domains by testing the transcriptional activation capacity of STY1 deletions fused to the GAL4 DNA binding domain in a yeas-two-hybrid (Y2H) assay, and that these act synergistically (I). To test if the SHI/STY proteins activate transcription in Arabidopsis without *de novo* protein synthesis, we induced STY1-GR in the presence of the translational inhibitor cycloheximide (CHX), and found that STY1 mediated transcriptional activation of *YUC4* and a large number of other genes is indeed independent on protein translation (I, II), suggesting that the SHI/STY proteins may act as DNA-associated transcriptional activators.

3.1.3 STY1 interacts with promoter elements of the *YUC4* promoter (I)

As STY1 activation of *YUC4* transcription is independent of protein synthesis, we tested if STY1 directly acts on the *YUC4* promoter using chromatin immunoprecipitation (ChIP), electromobility shift assay (EMSA) and yeast-1-hybrid (Y1H) experiments. In the ChIP experiments using STY1-GR and anti-GR antibodies, *YUC4* promoter sequences were amplified at a significantly higher level after DEX-treatment, and in Y1H experiments this region could be narrowed down to a 124 bp fragment binding both STY1 and SHI. By comparing this fragment with promoter sequences used in *in vitro* and Y1H binding studies of a SHI/STY ortholog in *Catharanthus roseus* and the promoter of a downstream *AP2/ERF* target gene*, ORCA3* (Vom Endt et al., 2007), we could identity a putative binding site, ACTCTAC. Mutational analysis of this fragment in Y1H revealed that it is important for STY1 binding. In addition, EMSA studies comparing binding of STY1 produced by *in vitro* transcription/translation to a 30 bp fragment including the putative binding site, and fragments with a mutated binding site, revealed a strong competition for binding to the functional ACTCTAC site.

3.2 SHI/STY members are positive regulators of several groups of genes

3.2.1 Auxin biosynthesis genes (I, II)

To investigate if STY1 potentially could interact with the promoters of additional members of the *YUC* gene family, we searched for ACTCTAC sequences in all members, but found that it is specific for the *YUC4* promoter. However, a similar sequence, ACTCTAA, was found in *YUC1, 5, 8* and *9* promoters, and in *YUC8,* just as in the *YUC4* promoter, this putative binding site was located in close proximity to the TATA box. qRT-PCR studies confirmed that STY1 can induce expression of *YUC8* independently of protein

translation, but not *YUC1, 5* nor *9* gene expression, suggesting that the presence of the TATA box in near proximity to the ACTCTAC binding site is required for transcriptional activation by STY1. Additionally, *YUC4* expression was also induced by 35Spro:SHI-GR in microarray studies (unpublished data), strongly suggesting that other *SHI/STY* members are involved in regulation of auxin biosynthesis as well.

Interestingly, the *AP2/ERF* gene *ORCA3*, a downstream target of the *C. roseus* SHI/STY ortholog, appears to activate transcription of a gene (*TDC*) encoding Trp decarboxylase (van der Fits & Memelink, 2000), which mediates synthesis of the auxin biosynthesis substrate tryptamine. We have searched for closely related *AP2/ERFs* in Arabidopsis carrying the SHI/STY binding element less than 500 bp away from the transcriptional start site, and identified the *OCTADECANOID- RESPONSIVE ARABIDOPSIS AP2/ERF59* (*ORA59*) gene (Pré et al., 2008). Using qRT-PCR, we could show that *ORA59* transcription is activated by STY1-GR in the presence of DEX and CHX.

ORA59 is affecting auxin responsiveness (II)

van der Fits and Memelink (2000) suggest that *ORA59* is involved in synthesis of tryptamine. Therefore, we analyzed the IAA content in lines with modulated levels of *ORA59*. No significant change was found in the *ora59-1* null mutant or in the *XVEpro:*ORA59 inducible line. However, *DR5pro:*GUS activity was increased in leaf margins and sometimes in leaf vasculature after induction of *XVEpro*:ORA59, indicating that *ORA59* may not function in a rate limiting step of auxin biosynthesis but rather in auxin signaling or other steps in auxin homeostasis regulation.

In summary these data suggest that STY1 and other SHI/STY members are capable of activating IAA biosynthesis and possibly also auxin responses, through transcriptional regulation.

3.2.2 Transcription factor genes (I, II)

Microarray data suggests that *SHI/STY* family members are not only involved in regulation of auxin biosynthesis, but can also induce expression of genes involved in other processes. Based on at least three criteria: microarrays studies, the presence of STY1 binding site and co-expression analysis, a number of putative downstream targets were selected for analysis. The ability of STY1-GR to activate these genes in the presence of CHX was tested, and their expression levels in quintuple *shi/sty* mutants were analyzed (II).

All seven selected genes belonging to transcription factor families were up-regulated by STY1-GR in the presence of DEX and CHX, suggesting that they are direct STY1 targets. Four of the selected genes, *ORA59*, *ETHYLENE-*

RESPONSIVE ELEMENT BINDING FACTOR 15 (ERF15), *ERF38* and *ERF43,* belong to the *AP2/ERF* family and except *ERF38* they all carry putative STY1 binding sites (I,II). *ERF38pro:GUS* is active at all major *SHI/STY* activity sites throughout development, and becomes ectopically expressed when STY1-GR is activated (II). *NGATHA2 (NGA2)*, belonging to a B3 transcription factor superfamily, is co-expressed with *STY1* and carries the *YUC4* STY1 binding site in its promoter sequence. Although *35Spro:STY1-GR* activates *NGA2* transcription, it could not induce ectopic *NGA2pro*:*NGA2:GUS* activity outside the normal *NGA2* expression domain. Instead, ectopic *NGA2pro*:*NGA2:GUS* activity was detected in the *35S:STY1-SRDX* background, suggesting that STY1-SRDX is interfering with *NGA2*-bound repressors (II). Two other downstream targets, *BEL1-LIKE HOMEODOMAIN 11 (BLH11)* and *REPRODUCTIVE MERISTEM 1 (REM1)* are also co-expressed with *SHI/STY* members and with *YUC4* at various developmental stages. The expression of these genes is also significantly reduced in the quintuple *SHI/STY* loss of function mutant (II). Interestingly, the spatial and temporal expression pattern of *REM1* in floral meristem resembles that of *STY1* (Kuusk *et al.,* 2002).

To summarize, the STY1 downstream targets encodes a diverse group of TFs, suggesting that the SHI/STY family members affect plant development via several different pathways.

3.2.3 Genes encoding enzymes involved in metabolic processes or cell wall modifications (II, III)

In the experiment presented in section 3.2.2, we also identified a group of STY1 target genes encoding enzymes, of which a couple may be involved in cell wall modulations. We studied the expression patterns of the selected genes *POLYAMINE OXIDASE 5* (*PAO5*), *PECTIN LYASE-LIKE 1* (*PGL1)*, *L-ASCORBATE OXIDASE 1* (*LAO1*), *EXPANSIN LIKE A2* (*EXPL2*), and *CYP78A6* in detail and revealed that the activity of most of them show some overlap with a number of domains characteristic for *SHI/STY* gene expression, such as in apical tip of cotyledons, leaves and floral organs, in stipules, hydathodes and in internal tissues of gynoecia and anthers.

PGL1 belongs to a large group of pectin lyases carrying a secretory peptide, suggesting that it may modulate cell wall polymers. Apart from the *PGL1pro:GUS* activity connected to vascular development, *PGL1* expression completely overlaps with known *SHI*/*STY* expression sites (e.g. organ apices, and hydathodes), suggesting that PGL1 mediates SHI/STY regulated cell wall modifications in these sites. In addition, *PGL1*, just as *STY1*, is expressed in maturing pollen before they become released. The pollen cell wall is complex

and dynamic with successive synthesis and degradation of cell wall components during pollen development (Jiang et al., 2013; Blackmore et al., 2007; Hasegawa et al., 2000; Heslop-Harrison, 1968), implicating that PGL1 may play an important role in cell wall-related pollen maturation processes. EXPL2 is an uncharacterized member of a large group of different enzymes classified as expansins (see section 1.4.2), also involved in cell wall modifications, however as we only could detect expression of *EXPL2* close to the vascular fan of the style, its function may be specific to reproductive development.

The CYP78A sub-family members are heme-dependent enzymes that through oxidation of different molecules interplay in many biosynthetic pathways (Nelson, 1999). *CYP78A6*, also known as *EOD3,* is together with two of its closest paralogs*, CYP78A8* and *CYP78A9*, involved in maternal control of seed size (Fang *et al.*, 2012). Our detailed expression analysis shows that *CYP78A6proGUS* is mainly active outside the *SHI*/*STY* expression domain, in connection to the vasculature in many different plant organs. In addition, both *CYP78A6* and *CYP78A9*, as well as *PAO5,* encoding a putative oxidase, overlap with *SHI*/*STY* expression in three anther cell layers surrounding the male germ-line. Modifications of these cell layers, such as degradation of the tapetum and middle layers and lignification of the endothecium layer, are important for proper pollen maturation and anther dehiscence (Sanders et al., 1999). Overexpression of *SHI/STY* genes results in reduced anther dehiscence and incomplete tapetum degeneration, and subsequently, reduced pollen maturation and release (Kim et al., 2010b). Based on these data, we hypothesise that correct expression levels of *SHI*/STY genes and their downstream targets in the three anther cell layers is necessary for proper tapetum development and anther dehiscence.

3.3 SHI/STY members affect flowering time, floral organ morphology and floral organ number (I, II)

The role of SHI/STY family members in Arabidopsis organ development has previously been reported (Fridborg *et al.* 1999; Fridborg *et al.* 2001; Kuusk *et al.,* 2002; Kuusk *et al.,* 2006; Sohlberg *et al.,* 2006; Staldal *et al.,* 2008), and here we reveal additional SHI/STY functions during plant development.

3.3.1 STY1-SRDX has severe effects on plant development (I; II)

Constitutive expression of the STY1-SRDX repressor of SHI/STY family targets results in dramatic developmental defects. The majority of individual *35Spro:STY1-SRDX* transgenic lines are seedling lethal lacking a shoot apical

meristem. It is yet to be determined if loss of a functional meristem is an effect of reduced activity of SHI/STY targets, or if it is an effect of STY1-SRDX interfering with other processes in the meristematic region when ectopically expressed. A very small percentage of transgenic lines produce individuals that survive the seedling stage, showing a phenotype similar to *shi/sty* multiple mutants (I), including the characteristic split of the style and reduced fertility (I, II).

3.3.2 *STY1* as well as its downstream targets *YUC4*, *ORA59*, *ERF38* and *NGA2* are affecting lateral stamen number, and the length of stamen filaments (II)

In order to investigate the function of several of the downstream targets, we identified new insertion mutants (*ora59-1*, *erf15-1, erf38-1, blh11-1, rem1-2* and *rem1-3*) as well as studied the previously published mutant lines *yuc4-1* and *nga2-2* (Trigueros *et al.*, 2009; Zhao *et al.*, 2001). None of these single mutants showed defects in gynoecia morphology, which may not be surprising as they belong to gene families with high functional redundancy. Interestingly, *NGA2* belongs to a small gene family important for gynoecium development, and it has been suggested that SHI/STY and NGA proteins act cooperatively during style development (Trigueros *et al.,* 2009). Nevertheless, we were able to identify a new role of STY1 and some of its downstream targets during floral development. We could observe that *yuc4-1, ora59-1, erf38-1,* and *nga2-2* as well as the *sty1-1* mutant had a statistically significant reduction in lateral stamen number, and that *35Spro:STY1-SRDX* and *yuc8-1* stamen filaments are reduced in length. It is known that auxin (described in section 1.5.5 *Stamens*) is required for filament elongation, and thus it is not surprising that *YUC8*, being expressed in the filaments, is involved in the elongation process.

3.3.3 *STY1* and its putative downstream target *REM1* affect flowering time (I, II)

The *REM1* gene is highly co-expressed with *SHI/STY* family members in the floral meristematic region and in gynoecia (Franco-Zorrilla *et al.*, 2002). We could show that *rem1* mutants, as well as *sty1-1 sty2-1* and *35Spro*:*STY1-SRDX* lines are flowering later than wild type plants. Interestingly, some of the original *35Spro*:*STY1-SRDX* lines never bolted or flowered very late (I). The transcript level of *FLC*, a known flowering time transcriptional regulator (see section 1.5.3), showed no altered expression in the aerial parts of the studied lines. These findings indicate that *STY/SHI* family members affect flowering time through *REM1* activity independently of the *FLC* pathway (II).

3.4 SHI/STY members affect leaf morphology and vascularization (I, IV)

The *SHI*/*STY* genes are expressed already in incipient cotyledon and leaf primordia, and as mentioned in section 1.6, *sty2-1* and *shi/sty* multiple mutants show increased leaf serration and leaf polarity defects (Kuusk *et al.,* 2002; 2006). In addition, the *35Spro:STY1-SRDX* plants also develop severe leaf serration, and narrow cotyledons (I). As auxin homeostasis is important for vascular cell fate and differentiation (see leaf sub-section in 1.5.2), we hypothesized that SHI/STY mediated auxin biosynthesis may be important also for vascular development in cotyledon and leaves. We therefor studied in detail the expression pattern of several *SHI*/*STY* genes during early venation establishment in cotyledon and leaf primordia, as well as the cotyledon- and leaf venation pattern of the *shi/sty* mutants, see below (V).

3.4.1 *SHI/STY* spatiotemporal expression partially overlaps with auxin biosynthesis during embryogenesis and leaf development (IV)

Using *SHI*, *STY1*, *STY2* and *SRS5* promoter:GUS reporter lines (Fridborg e*t al.,* 2001; Kuusk *et al.,* 2002, Kuusk *et al.,* 2006) we could observe that whereas *SHI* is expressed already in early globular embryos, *STY1* is only active in later globular stages. Both genes are expressed in the apical and central tiers, with the strongest expression in the site of the incipient cotyledons. At the triangular stage *STY2* expression appears, and from this stage *SHI*, *STY1* and *STY2* activity is more or less confined to the sites of cotyledon initiation, and at later stages, mainly in the apices of cotyledon primordia. Additionally, *STY1* expression is visible in the margins of the cotyledon primordia. *SRS5* expression could only be detected in the apical tip of cotyledons in the torpedo stage. This suggests that the five genes have partly overlapping functions, but differ in on-set during embryo development. At the globular stage, their expression overlaps in the apical tiers with that of auxin biosynthesis genes, including *YUC4* (Robert et al., 2013; Cheng et al., 2007), suggesting that the SHI/STY proteins may play a role in the induction of the apical auxin biosynthesis. Interestingly, it has been suggested that apical auxin biosynthesis at this stage is required for PIN1 polarization in the pro-embryo (Robert *et al.*, 2013), adding to the importance of spatiotemporal regulation of auxin biosynthesis. In addition, *YUC4* expression also overlaps with *SHI*/*STY* activity in apices of cotyledon primordia (Cheng *et al.,* 2007).

Although the expression of *SHI*/*STY* genes also partially overlaps in apices of very young leaf primordia, and in hydathodes, preceding *YUC4* expression (Wang *et al.,* 2011), they appear to have more diverged functions during the differentiation process. Only *STY1* is active in the margin and base of the leaf

primordia, sites where *YUC1* also is active, whereas *STY2* is expressed in apical areas of incipient midvein formation, again preceding the expression of *YUC4* (Wang *et al.,* 2011).

3.4.2 *SHI/STY* members act redundantly during vascular patterning and vein development (IV)

Indeed, cotyledons and leaves of the *shi/sty* mutants show defective venation pattern and also defective continuity of the venation system. The number of secondary veins was significantly reduced and the frequency of free vein ends was increased, suggesting that SHI/STYs are necessary both for vein formation and differentiation. These venation phenotypes resemble those of multiple *yuc* mutants (Cheng *et al.*, 2007; Cheng *et al.*, 2006), suggesting that the SHI/STYs affect venation via their regulation of auxin biosynthesis at cotyledon and leaf marginal tissues. Importantly, gaps in the vessels were observed in *shi*/*sty* mutants, which were either lacking any types of the vascular tissue, suggesting that procambial cells were not selected at these sites, or only xylem, implicating that differentiation was affected. Additionally, we could note an ectopic vascular fragment at the apex, further referred to as a "distal peg" phenomenon. The frequency of all of the detected defects increases with the number of mutated *SHI/STY* family members, implying that they act partially redundantly in controlling procambium formation, vein patterning and differentiation, which is consistent with their partially overlapping expression pattern during cotyledon and leaf development.

3.4.3 The expression pattern of *SHI/STY* genes is altered when auxin accumulation sites and levels are modulated (IV)

Positive feedback mechanisms in auxin homeostasis and signaling are important in regulating plant development (Leyser, 2006), and in an attempt to study if the *SHI*/*STY* genes are activated by auxin during cotyledon and leaf development we studied their expression patterns when auxin transport or auxin levels were altered. Generally, treatment with the auxin transport inhibitor NPA resulted in an ectopic expansion of the *SHI*/*STY* expression domains from marginal foci towards larger parts of marginal area in both cotyledons and leaves. A similar expansion of the *SHI*/*STY* expression domain was also observed upon exogenous auxin treatment of leaf primordia, although *STY1* activity was also partially induced in the leaf blade, implying that the *SHI/STY* genes are able to respond to altered auxin distribution and auxin levels only in certain domains. As expected, ectopic auxin responses detected by *DR5* activity was also induced in overlapping marginal sites in NPA treated leaves (IV; Mattsson *et al.,* 1999), whereas exogenous auxin treatment resulted

in elevated auxin responses in the central domain of leaf primordia, suggesting that auxin regulates *DR5* and *SHI*/*STY* genes through at least partially different pathways. The results suggest that a positive feed-back loop could potentially be in action in the *SHI/STY* activity domains, which ensures a stable and high auxin biosynthesis rate at these sites.

3.5 Expression of *SHI/STY* members is regulated via a GCC box element (V)

3.5.1 A conserved GCC-box regulates *SHI/STY* expression in aerial plant tissues (V)

The overlap in expression domains and function of the *SHI*/*STY* genes suggests that they may be partially co-regulated by common transcription factors and regulatory elements. By comparing the promoter sequences of the *SHI*/*STY* genes we identified a gene-family specific GGCGGC element, defined as an inverted GCC-box (GCCGCC). Mutation of this motif in the *STY1* promoter resulted in entire loss of *STY1* expression in the distal part of the cotyledon tip, leaf primordia, apical end of the young gynoecia, style, stigma, ovule and receptacle, whereas *STY1* expression in hypocotyl, petiole, proximal part of the cotyledon, and lateral root primordia appears to be GCC-box independent. In addition, loss of the GCC-box in the *lrp1* mutant results in restriction of the *LRP1* activity to lateral root primordia (Smith & Fedoroff, 1995). Although GCC-boxes originally were implicated in AP2/ERF mediated ethylene responses, we could not observe any activation of *STY1* after ACC-treatment, suggesting that the *SHI*/*STY* GCC box is not related to ethylene signaling.

3.5.2 DRNL and its homologues regulate the *SHI/STY* genes expression (V)

In a search for AP2/ERF proteins potentially involved in transcriptional regulation of *SHI*/*STY* genes via the GCC-box, we found the work by Ikeda (Ikeda *et al.*, 2006) and Marsch-Martinez (Marsch-Martinez *et al.*, 2006) revealing that the activity of at least four *SHI/STY* genes was induced by constitutive expression of the AP2/ERF protein ESR2 (ENHANCER OF SHOOT REGENERATION2)/ DRNL (DORNRÖSCHEN-LIKE), and we could show that this activation do not require any intermediate protein synthesis. Interestingly, the close homologue of DRNL, DRN, can interact with GCC-sequences both in vitro and in vivo (Matsuo & Banno, 2008; Banno *et al.*, 2001) and when the *STY1* GCC-box was mutated the ability of DRNL to activate *STY1* was abolished. In addition, *DRN/DRNL* and *SHI*/*STY* expression overlaps in embryo, cotyledon primordia, leaf primordia, tip of young leaves, stipules, ovules and carpels (Cole *et al.*, 2009; Kirch *et al.*, 2003) and ectopic

expression of members of both gene families results in similar phenotypic defects, such as short internodes and hypocotyls (Nag *et al.*, 2007; Ikeda *et al.*, 2006; Marsch-Martinez *et al.*, 2006; Kirch *et al.*, 2003; Kuusk *et al.*, 2002; Fridborg *et al.*, 1999). However, these defects were completely restored when DRNL was ectopically expressed in the *shi*/*sty* multiple mutant background, suggesting that the *SHI*/*STY* members are mediating the phenotypic effects of ectopic DNR/DRNL activity.

In order to test if DRNL and its homologues regulate *SHI*/*STY* members in its native expression domains, we generated a triple mutant carrying mutations in *DRN*, *DRNL* and a third member of the same *AP2*/*ERF* subfamily, *PUCHI* (Nakano et al., 2006). The *STY1* transcript level was not affected in the *drnl-1* or the *drnl-1 drn-1* mutants, but significantly reduced in the strong *drnl-2* mutant and the *drnl-1 drn-1 puchi-1* triple mutants, suggesting that DRN/DRNL family members indeed regulate *STY1* activity during plant development. Furthermore, the *STY1pro:GUS* expression in *drnl-2* was specifically reduced in tissues requiring the GCC-box for *STY1*transcription.

Apart from cotyledon defects, *drn-1 drnl-1* plants produce gynoecia with a reduced valve-length, a phenotype typical also of *SHI*/*STY* multiple mutants, and the *35Spro:STY1-SRDX* line (I; Chandler *et al.*, 2011; Kuusk *et al.*, 2006). In the *puchi-1 drn-1 drnl-1* triple mutant, this phenotype is dramatically enhanced and a high frequency of valveless gynoecia with a ring of meristem-like tissue around stigma is produced. This is a phenotype also resembling multiple *YUC*-family mutants (I; Cheng *et al.,* 2006; Kuusk *et al.,* 2006). Since *YUC4* is a direct downstream target of STY1 (I), this creates a strong link between *DRNL* and other VIII *AP2/ERF* genes, *STY1* activation, and *YUC4* in the regulation of e.g. gynoecia development.

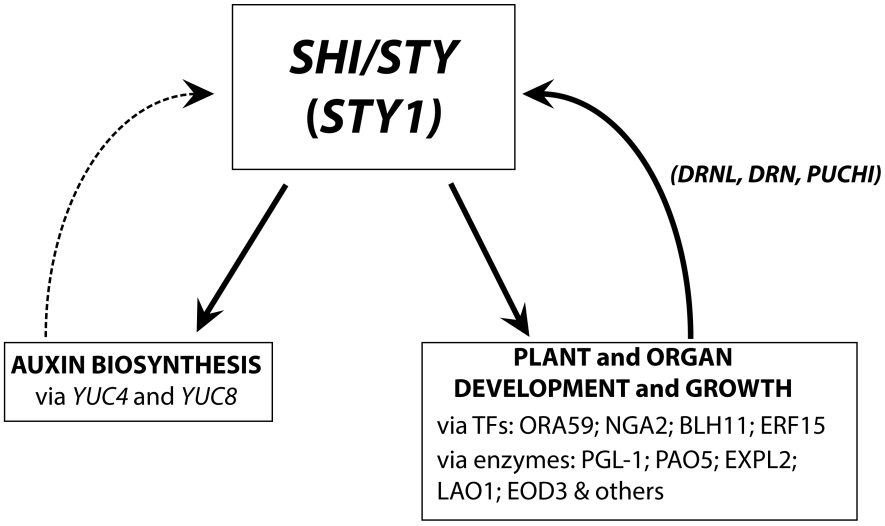

Figure 8. Model summarizing presented data

4 Conclusions

This work presents some of the new insights into regulation of plant organ development regulated by the *SHORT INTERNODES/STYLISH* gene family.

The main conclusions are:

The *SHI/STY* genes encode DNA-binding transcriptional activators acting highly redundant in various developmental processes. They are activating the expression of genes encoding other transcription factors and also specific enzymes involved in auxin biosynthesis or processes of cell wall modulations.

In addition to their role in gynoecium and leaf development, we can conclude that the redundant actions of SHI/STY family members also are important for proper cotyledon and leaf venation patterning, stamen number, and flowering time.

Expression of *SHI/STY* members is partially co-regulated through a conserved promoter element, the GCC-box-like regulatory motif, and members of the *AP2/ERF* gene family are potential upstream regulators.

5 Future perspectives

Decades of Arabidopsis research have brought us tremendous knowledge of plant biology. It is of great interest to try to verify these findings and interconnect them into a "bigger picture". Therefore, I believe that studies of this model organism have not yet reached the highest peak. Research work investigating the role of *SHI/STY* gene family members is also far from completed.

Future research include following possibilities and goals:

- To investigate which developmental processes the *SHI/STY* genes affects via the regulation of auxin homeostasis, constructs modulating the endogenous auxin levels in the *STY1* expression domain could be used. Can elevated auxin degradation in this domain mimic all or just a subset the *shi/sty* multiple mutant phenotypes? And can elevated auxin biosynthesis in the *STY1* expression domain rescue all, or only a subset of the *shi/sty* multiple mutant phenotypes?

- Which additional roles do the SHI/STY members play during plant development? This could be approached by making even higher order multiple mutants, and to characterize the expression domains of all the members of the gene family. In addition, constructs repressing common downstream targets in the STY1 expression domain could be made (e.g. *STY1pro*:STY1-SRDX). It would be specifically interesting to understand their role in tissues where *SHI/STY* gene expression overlaps, but where no phenotypic defects so far has been detected in the existing multiple mutant lines (such as e.g. lateral root primordia).

- To identify additional downstream targets using e.g *STYpro*:STY1-GFP for ChIP-sequencing. This may, in addition to revealing new

downstream pathways, also reveal if additional genes belonging the same gene family as already identified SHI/STY downstream targets are regulated by SHI/STY members.

- To further characterize the function of identified downstream targets by e.g. crossing lines carrying mutations in the target genes with mutants in closely related genes.

- To further characterize *SHI/STY* promoter elements and to identify additional upstream regulators. As only a subset of the *STY1* expression domain appears to be dependent on the identified GCC-box, the *SHI/STY* genes must be regulated through as yet unidentified promoter elements. In addition, additional AP2/ERF proteins might also be involved in regulation of *SHI/STY* gene expression via the GCC-box.

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