Regulation of Organ Development by Auxin and the SHI/STY-family of Transcriptional Regulators

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Abstract

Transcription factors, proteins regulating the transcription of target genes, and plant hormones play important roles in the regulation of plant development. The plant hormone auxin has been assigned functions in many developmental processes and its differential distribution between cells, created mainly by local auxin biosynthesis and polar auxin transport, results in auxin maxima and gradients, which appear to be important for its role in developmental regulation.

The work in this thesis reveals that members of the *Arabidopsis thaliana* SHI/STY protein family, which redundantly regulates the development of lateral organs, act as DNA binding transcriptional activators. The SHI/STY member STY1, expressed at specific sites in developing primordia and organs, binds to the promoter of the *YUC4* auxin biosynthesis gene. Our data suggest that the control of local auxin biosynthesis, through transcriptional activation of *e.g. YUC4*, is important for SHI/STY regulated development of lateral organs. We could show that local auxin application restores *SHI/STY* mutant style defects. Furthermore, using a polar auxin transport inhibitor, auxin was found to act independently of the style-promoting factors *CRC*, *LUG*, *SEU*, *SPT* and *STY1* in style development, suggesting that auxin may act downstream or in parallel with these genes, but not in apical-basal patterning of the gynoecium.

Additional STY1 downstream targets have been identified and include genes encoding transcription factors, another auxin biosynthesis enzyme, and enzymes involved in cell expansion. Functional studies on some of these genes reveal roles in stamen and leaf development as well as in flowering time regulation.

The upstream regulation of *SHI/STY* genes has also been studied and a conserved GCC-box-like promoter element has been shown to be important for the transcriptional regulation of *SHI/STY* genes, providing a possible mechanism for the co-regulation of the SHI/STY protein accumulation.

Keywords: *Arabidopsis thaliana*, auxin, gynoecium, leaf, organ development, *SHI/STY*-family, stamen, *STYLISH1*, transcriptional activator, *YUCCA4*.

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The greatest obstacle to discovery is not ignorance – it is the illusion of knowledge.

Daniel J. Boorstin

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 Ståldal, V*., Sohlberg, J.J*., Eklund, D.M., Ljung, K. & Sundberg, E. (2008). Auxin can act independently of CRC, LUG, SEU, SPT and STY1 in style development but not apical-basal patterning of the Arabidopsis gynoecium. *New Phytologist* 180, 798-808.

II Eklund, D.M., Ståldal, V., Valsecci, I., Cierlik, I., Eriksson, C., Hiratsu, K., Ohme-Takagi, M., Sundström, J.F., 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.

III Ståldal, V., Cierlik, I., Chen, S., Landberg, K., Baylis, T., Myrenås, M., Sundström, J., Eklund, D.M., Ljung, K. & Sundberg, E. The *Arabidopsis thaliana* transcriptional activator STYLISH1 regulates genes affecting stamen development, cell expansion and timing of flowering (manuscript).

IV Eklund, D.M*., Cierlik, I*., Ståldal, V., Vestman, D., Chandler, J. & Sundberg, E. Expression of *SHORT INTERNODES/STYLISH* family genes in auxin biosynthesis zones of aerial organs is dependent on a GCC-box-like regulatory element (manuscript).

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* Indicates shared first authorship

The contribution of Veronika Ståldal to the papers included in this thesis was as follows:

I Participated in planning of the project. Made lab-work including auxin, NPA and DEX treatment, phenotypic analysis, SEM and preparations for auxin biosynthesis measurements. Participated in analyzing data and writing the manuscript. Have made around half the amount of work on this paper.

II Participated in planning of the project. Made lab-work including most q-RT-PCRs and Y1H experiment with mutated promoter. Participated in analyzing data and writing the manuscript.

III Participated in planning of the project. Made the majority of the lab-work and data analysis. Have done most of the writing, including writing the first draft of the manuscript.

IV Participated in planning of the project. Made lab-work including part of the IAA and ACC treatment experiments and part of the expression analysis of *SHI/STY* genes after DRNL-ER activation and in the *drn drnl puchi* mutant. Participated in analyzing data and writing the manuscript.

1 Introduction

Plants and other multicellular organisms are built up of different organs specialized for various tasks. The development of an organ, from a few undifferentiated cells to a multicellular structure consisting of several different tissue types and having a special form and function, is a fascinating and complicated process. Understanding the processes of organ development and how they are regulated is an important challenge for plant biologists. We are dependent on plants for food production and also use plants for many other purposes, *e.g.* as building material, for decoration, and for producing energy, clothes and pharmaceuticals. In addition to being interesting, the knowledge about how plant organ development is regulated is therefore of fundamental importance for our society.

1.1 Arabidopsis thaliana

Arabidopsis thaliana (*Arabidopsis*) is a flowering plant belonging to the mustard family (Brassicaceae). This small weed is not used for production of food, feed, fuel or other products but is today the most well studied species among all flowering plants (reviewed in Meyerowitz, 1987; Bevan and Walsh, 2005; Shindo et al, 2007; Koornneef and Meinke, 2010). The first experimental research on *Arabidopsis* was performed in the beginning of the 20th century and from the late 1970s *Arabidopsis* has become the dominating model plant species among plant molecular biologists. Several properties, such as small size, short generation time, high seed production, self-pollination and small genome size, make *Arabidopsis* suitable for use as a model organism in plant research. *Arabidopsis* has a genome size of around 146 mega bases (MB), which is small compared to most other plants, and the genome has been sequenced (The Arabidopsis Genome Initiative, 2000). Resources such as stock centres, databases and collections of insertion

mutants also exist. *Arabidopsis* can be found in many different parts of the world, in a large variety of climatic regions, allowing for studies of environmental, genetic and epigenetic adaptation. Local populations of *Arabidopsis* are called ecotypes or accessions and Columbia (Col), Landsberg *erecta* (L*er*) and Wassilewskija (Ws) are examples of accessions commonly used in research.

1.2 The plant hormone auxin

Plant hormones are small molecules that occur at low concentrations in the plant and regulate growth and development in response to different internal and external factors (reviewed in Santner and Estelle, 2009; Santner et al., 2009; Wolters and Jürgens, 2009). They can either be transported to distant tissues or act locally, in the same tissues as they are produced, and affect their target cells by binding to specific receptors. The six major classes of plant hormones are auxins, gibberellins, cytokinins, ethylene, abscisic acid and brassinosteroids, but other types of plant hormones also exist.

Auxin is a very important hormone, regulating developmental processes as well as responses to both abiotic and biotic external factors like gravity, light and pathogens (reviewed in Tanaka et al., 2006; Bari and Jones, 2009). Defects in auxin biosynthesis, transport or response have large effects on plant shape and function. During embryogenesis auxin is required for establishment of polarity, initiation of cotyledons and establishment of the root pole (reviewed in Möller and Weijers, 2009; Vanneste and Friml, 2009). During post-embryonic development auxin regulates *e.g.* root elongation, vascular differentiation, organ patterning and initiation of leaves, flower organs and lateral roots (reviewed in Alabadí et al., 2009; Overvoorde et al., 2010; Scarpella et al., 2010; Vernoux et al., 2010).

The most abundant, and most important, auxin in higher plants is indole-3-acetic acid (IAA). Several other naturally occurring and synthetic compounds have similar effects as IAA, *e.g.* 4-Cl-IAA, 1-naphtalacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D), and these compounds are also referred to as auxins (reviewed in Woodward and Bartel, 2005; Taiz and Zeiger, 2006).

Auxin has a differential distribution between cells, creating auxin concentration maxima and gradients, important for its role in developmental regulation (reviewed in Bhalerao and Bennett, 2003; Tanaka et al., 2006; Vanneste and Friml, 2009; Tromas and Perrot-Rechenmann, 2010). Auxin homeostasis and distribution are therefore strictly controlled through regulation of biosynthesis, inactivation and transport. Another level of

control is the regulation of auxin responses (reviewed in Lau et al., 2008; Kieffer et al., 2010; Tromas and Perrot-Rechenmann, 2010).

1.2.1 Biosynthesis

IAA biosynthesis in plants is complex, with several pathways contributing to the production of IAA, and there are still many gaps in our knowledge about this complicated process (reviewed in Woodward and Bartel, 2005; Chandler, 2009; Tromas and Perrot-Rechenmann, 2010; Normanly, 2010; Zhao, 2010). Plants use both tryptophan (Trp)-dependent and Trp-independent pathways for IAA synthesis, although the Trp-independent pathway remains, in large, uncharacterized. In the Trp-dependent IAA biosynthesis, four different pathways have been proposed based on experimental evidence (see fig 1): the indole-3-pyruvic acid (IPA) pathway, the indole-3-acetaldoxime (IAOx) pathway, the indole-3-acetamine (IAM) pathway and the tryptamine (TAM) pathway.

In the suggested IPA pathway, Trp is converted to IPA and then further modified to indole-3-acetaldehyde (IAAId) and IAA (reviewed in Woodward and Bartel, 2005; Tromas and Perrot-Rechenmann, 2010). In *Arabidopsis* the aminotransferase TRYPTOPHAN AMINO-TRANSFERASE of ARABIDOPSIS1 (TAA1) has been shown to convert Trp to IPA *in vitro*, and *taa1* mutants have reduced levels of free IAA (Stepanova et al., 2008; Tao et al., 2008). *TAA1* belongs to a gene family with the four additional members *TRYPTOPHAN AMINO-TRANSFERASE RELATED1* to *4* (*TAR1* to *4*). *tar1* and *tar2* mutants enhance the *taa1* phenotype and triple mutants show several auxin related phenotypes (Stepanova et al., 2008). The steps from IPA to IAA have so far been characterized in microorganisms, but not in plants (reviewed in Normanly, 2010; Zhao, 2010).

In the IAOx pathway, Trp is converted to IAOx and then via other intermediates to IAA, or to glucosinolates (reviewed in Zhao, 2010). The *Arabidopsis* enzymes CYP79B2 and CYP79B3 can convert Trp to IAOx and IAA levels are elevated in CYP79B2 overexpressors, while they are reduced in *cyp79b2 cyp79b3* double mutant plants (Hull et al., 2000; Zhao et al., 2002). Further evidence for the involvement of IAOx in IAA biosynthesis comes from studies of the glucosinolate biosynthesis genes *SUPERROOT1* (*SUR1*) and *SUR2*, where *sur1* and *sur2* mutants have elevated IAA levels, probably resulting from more IAOx available for IAA synthesis (Boerjan et al., 1995; Delarue et al., 1998; reviewed in Zhao, 2010). It is not clear how IAOx is converted to IAA but biochemical analyses have suggested both indole-3-acetonitrile (IAN) and IAM as intermediates (Sugawara et al.,

2009). Trp-converting cytochrome P450 monooxygenases have only been isolated from *Arabidopsis* and IAOx have been found in *Arabidopsis* but not in other tested species like tobacco and maize, suggesting that the IAOx pathway is not widespread among plants (Sugawara et al., 2009; reviewed in Normanly, 2010).

The IAM pathway is used by several plant pathogens to produce IAA from Trp in two steps with IAM as an intemediate (reviewed in Spaepen and Vanderleyden, 2010). IAM exist also in aseptically grown plants and two amidases, AMIDASE1 (AMI1) and NtAMI1, that can convert IAM to IAA have been characterized in *Arabidopsis* and tobacco, respectively, suggesting that an IAM pathway exists also in plants (Pollmann et al., 2006; Nemoto et al., 2009; Sugawara et al., 2009; reviewed in Lehmann et al., 2010). How IAM is formed in plants is not clear. IAM can be formed from IAOx in *Arabidopsis*, but since IAM is found also in plants that probably lack the IAOx pathway, this is most likely not the only pathway leading to IAM.

In the TAM pathway Trp is converted to TAM, which is converted to N-hydroxyl-TAM, and then to IAA via additional intermediates (reviewed in Woodward and Bartel, 2005; Lehmann et al., 2010; Normanly, 2010; Zhao, 2010). Trp decarboxylases catalyzing the first step from Trp to TAM have been found in several plant species, but not yet in *Arabidopsis* (Kang et al., 2007). In the second step, a flavin monooxygenase-like (FMO) enzyme, YUCCA1 (YUC1), suggested to convert TAM to N-hydroxyl-TAM, has been characterized from *Arabidopsis* (Zhao et al., 2001). Overexpression of YUC1 results in elevated IAA levels, suggesting that it catalyzes a rate-limiting step in IAA biosynthesis. *YUC1* belongs to a family of 11 members in *Arabidopsis* and overexpression of other *YUC* genes result in auxin overproduction phenotypes as well (Cheng et al., 2006; Hentrich, 2010). *YUC* family multiple mutants show developmental defects and reduced auxin response reporter expression (Cheng et al., 2006; Cheng et al., 2007). This together suggests that several of the *YUC* genes are involved in auxin biosynthesis. The role of YUC members in the conversion of TAM to N-hydroxyl-TAM has, however, been questioned and they could possibly catalyze another step in Trp-dependent auxin biosynthesis (Tivendale et al., 2010; Nonhebel et al., 2011; Philips et al., 2011). It is not clear how IAA is formed from N-hydroxyl-TAM. IAOx and IAAld have been suggested as intermediates, but biochemical analyses on wild type and *YUC* family mutant plants have not found IAOx to be a likely intermediate in this pathway (Sugawara et al., 2009).

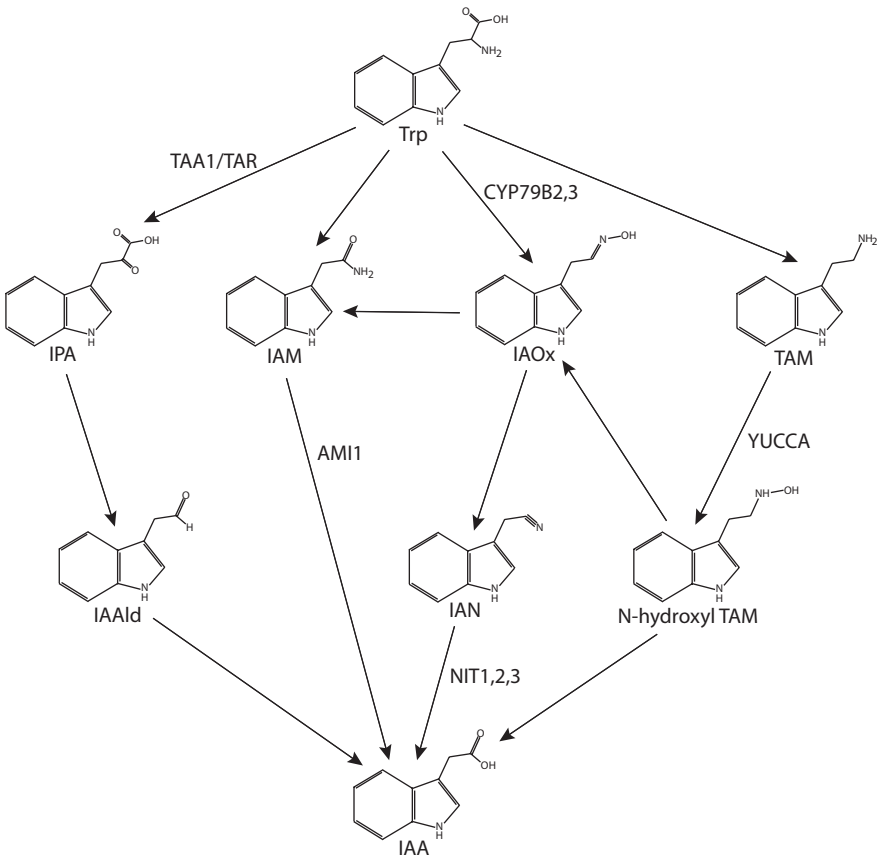

Figure 1. Proposed IAA biosynthesis pathways in *Arabidopsis*. Abbreviations: AMI, AMIDASE; CYP, CYTOCHROME P450; IAA, Indole-3-acetic acid; IAAld, Indole-3-acetaldehyde; IAM, Indoleacetamide; IAN, Indole-3-acetonitrile; IAOx, Indole-3-acetaldoxime; IPA, Indole-3-pyruvic acid; NIT, NITRILASE; TAA, TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS; TAM, Tryptamine; TAR, TAA1 RELATED; Trp, Tryptophan. Adapted from Lehmann et al., 2010; Normanly, 2010; Zhao, 2010.

Most plant tissues seem to be able to produce auxin, but the auxin synthesis rate varies between different parts of the plant (Ljung et al., 2001; Ljung et al., 2005; reviewed in Normanly, 2010; Tromas and Perrot-Rechenmann, 2010). Young leaves, cotyledons and root tips have relatively high IAA synthesis rates (Ljung et al., 2001; Ljung et al., 2005). Auxin biosynthesis genes have different expression patterns and the expression is usually restricted to a small group of cells and changes during development (Cheng et al., 2006; Tao et al., 2008; Hentrich, 2010; reviewed in Zhao, 2008; Zhao, 2010). Auxin biosynthesis thus seem to be regulated both temporally

and spatially. The expression patterns of auxin biosynthesis genes often correlate with auxin response reporter expression and inactivation of *e.g. YUC1* and *YUC4* results in a decrease in auxin response reporter expression levels specifically in the tissues where these *YUC* genes are expressed, suggesting that local auxin biosynthesis is important for the distribution of auxin and the generation of local auxin maxima/responses (Cheng et al., 2006; Cheng et al., 2007; Stepanova et al., 2008; Tao et al., 2008; reviewed in Zhao, 2008; Chandler, 2009; Zhao, 2010). Inactivation of different auxin biosynthesis genes also leads to different phenotypes and the phenotypes often correlate with the expression patterns of these genes, suggesting that local auxin biosynthesis is important for plant development (Cheng et al., 2006; Cheng et al., 2007; Stepanova et al., 2008; Tao et al., 2008). Further support for the importance of local auxin biosynthesis comes from experiments showing that the *yuc1 yuc4* phenotype can be rescued by expression of the bacterial auxin biosynthesis gene iaaM under the control of the *YUC1* promoter, but not the *YUC6* promoter, and not by exogenous auxin application (Cheng et al., 2006). Hence, loss of certain auxin biosynthesis genes with specific expression patterns cannot be compensated for by transport of auxin from other parts of the plant.

The transcription factors STYLISH1 (STY1), LEAFY COTYLEDON2 (LEC2), REVEILLE1 (RVE1), SPOROCYTELESS (SPL) and the NGATHA (NGA) family have been shown to affect the expression levels of different *YUC* genes and LEC2 also binds to the *YUC4* promoter (Sohlberg et al., 2006; Li et al., 2008; Stone et al., 2008; Rawat et al., 2009; Trigueros et al., 2009). TERMINAL FLOWER2 (TFL2) is another protein that binds to promoters and/or coding regions of *YUC* genes and affect *YUC* gene expression (Rizzardi et al., 2010). Besides this not much is known about the transcriptional regulation of auxin biosynthesis genes.

1.2.2 Storage and inactivation

IAA can be stored as indole-3-butyric acid (IBA) or as different IAA conjugates that can be converted back to IAA (reviewed in Woodward and Bartel, 2005; Tromas and Perrot-Rechenmann, 2010). Reversible conjugations include ester-linking to sugars and amide-linking to peptides or amino acids like alanine (Ala) and leucine (Leu). Conjugation to other amino acids, like glutamate (Glu) and aspartate (Asp), cannot be reversed and leads to degradation of IAA. Oxidation to OxIAA is another way to permanently inactivate IAA (reviewed in Woodward and Bartel, 2005; Tromas and Perrot-Rechenmann, 2010). The regulation of these processes plays an important role in the control of IAA levels and auxin itself

participate in the regulation, *e.g.* by inducing *GH3* family members encoding IAA conjugating enzymes.

1.2.3 Transport

Auxin is transported via at least two types of transport, passive long distance transport via the phloem and cell-to-cell polar auxin transport (PAT) (reviewed in Michniewicz et al., 2007; Petrásek and Friml, 2009; Tromas and Perrot-Rechenmann, 2010). The cell-to-cell transport contributes to the main auxin flow from the shoot towards the root apex, via transport in the vascular cambium, and also transports auxin over shorter distances within tissues. The transport of auxin between cells is highly regulated and depends on transporter proteins that can determine the direction of auxin transport via an asymmetrical cellular localization.

Auxin is a weak acid and exists in a hydrophilic anionic form (IAA^-) or a hydrophobic protonated form (IAAH) depending on the local pH. Only the IAAH form can diffuse through plasma membranes and the difference in pH between the apoplast and the cytosol favours diffusion of IAA into the cell (reviewed in Tromas and Perrot-Rechenmann., 2010). In addition to passive diffusion, auxin is also transported actively into cells via influx carriers belonging to the AUXIN RESISTANT1 (AUX1)/LIKE AUX1 (LAX) family (Bennet et al., 1996; Bainbridge et al., 2008; Swarup et al., 2008; reviewed in Petrásek and Friml, 2009). AUX1/LAX transporters are distributed uniformly around the cells in most tissues, but asymmetrical localization has been found in protophloem cells in the root (Swarup et al., 2001; reviewed in Michniewicz et al., 2007).

Efflux carriers are needed to transport auxin out of cells and proteins with auxin-exporting activity have been found in the PINFORMED (PIN) family and in subfamily B of the ATP-binding cassette (ABCB) family, also known as the multidrug resistant (MDR) or P-glycoprotein (PGP) family (reviewed in Zazimilová et al., 2010). There are eight PIN family members in *Arabidopsis* and of these PIN1-4 and 7 are localized to the plasma membrane and probably function in auxin efflux, while PIN5, 6 and 8 are localized to the endoplasmic reticulum (ER) and have been suggested to regulate intracellular auxin homeostasis (Friml et al., 2002a; Friml et al., 2002b; Petrásek et al., 2006; Mravec et al., 2009; reviewed in Petrásek and Friml, 2009). The plasma membrane localized PINs are often asymmetrically distributed around the cell as expected for PAT efflux carriers (Friml et al., 2002a; Friml et al., 2002b; Wisniewska et al., 2006; reviewed in Michniewicz et al., 2007; Feraru and Friml, 2008). PIN1, 2 and 7 have been shown to perform auxin efflux and also PIN3 and 4 seem to participate in

PAT (Friml et al., 2002a; Friml et al., 2002b; Petrásek et al., 2006; Yang and Murphy, 2009). The different *PIN* genes have different expression patterns and mutant studies have shown that they have both specific and redundant functions, with loss of some *PIN* genes leading to ectopic expression of others (Vieten et al., 2005).

The ABCB family proteins ABCB1/PGP1 and ABCB19/PGP19 have been shown to perform auxin efflux and ABCB4 is suggested to function as either an influx or an efflux transporter depending on the auxin concentration (Yang and Murphy, 2009; reviewed in Zazimilová et al., 2010). ABCB1, 4 and 19 are localized to the plasma membrane, primarily in a nonpolar fashion (Mravec et al., 2008).

PAT is a very important process as suggested by the severe developmental defects that result from disruption of auxin transporter genes and from application of naphthylphthalamic acid (NPA) or other chemical inhibitors of PAT. This transporter-mediated process is regulated at several levels and auxin itself is an important factor in the regulation (reviewed in Petrásek and Friml, 2009). The transcription of both influx and efflux carriers is influenced by auxin as well as other factors, and degradation of PIN proteins is regulated by the MODULATOR OF PIN (MOP) proteins. Some PIN proteins are continuously internalized and recycled back to the cell surface and the internalization is inhibited by auxin, thereby increasing the amount of PIN proteins at the cell surface (Paciorek et al., 2005). The polar localization of PIN proteins affects the direction of auxin transport and can change in response to developmental and environmental signals, thereby redirecting the auxin flow (reviewed in Michniewicz et al., 2007). This localization of PIN proteins at the apical or basal side of the cell is regulated via phosphorylation/dephosphorylation by the serine/threonine protein kinase PINOID (PID) and protein phosphatase 2A (PP2A) (Benjamins et al., 2001; Friml et al., 2004; reviewed in Petrásek and Friml, 2009). In turn, *PID* expression is also regulated by auxin. Since auxin affects PAT in several ways, local auxin biosynthesis, and the resulting local auxin levels, might play an important role in regulating the direction and rate of auxin transport. A combination of both PAT and local auxin biosynthesis is also needed for many developmental processes (reviewed in Zhao, 2008; Chandler, 2009)

1.2.4 Signalling

Auxin induces expression of early auxin response genes via a short pathway involving the nuclear located auxin receptor TRANSPORT INHIBITOR RESPONSE1 (TIR1)/Auxin-Binding F box (AFB) family, the AUX/IAA

transcriptional repressors and the Auxin Response Factor (ARF) family of transcription factors (reviewed in Guilfoyle and Hagen, 2007; Vanneste and Friml, 2009; Kieffer et al., 2010; Tromas and Perrot-Rechenmann, 2010). There are 23 different ARFs in *Arabidopsis* and these interact with Auxin Response Elements (AREs) in the promoters of auxin response genes and either activate or repress transcription. The *AUX/IAAs* are themselves auxin response genes and most of the 29 members in *Arabidopsis* are upregulated by auxin. AUX/IAAs interact with activating ARFs and repress transcription of auxin response genes, *e.g.* by recruitment of the transcriptional co-repressor TOPLESS (TPL). The auxin receptor TIR1, and the related AFB proteins, are components of the Skp1-cullin-F box protein (SCF) E3 ubiquitin ligase complexes that catalyze ubiquitination of Aux/IAAs, thereby targeting them for degradation (Dharmasiri et al., 2005). When auxin binds the TIR/AFBs, the interaction between TIR/AFBs and AUX/IAAs is stabilized and the AUX/IAAs get ubiquitinated and degraded (Dharmasiri et al., 2005). Via this mechanism, an increased auxin concentration leads to degradation of AUX/IAAs, resulting in de-repression of activating ARFs and transcription of auxin response genes. The auxin-induced up-regulation of *AUX/IAA* and *GH3* transcription is important for the transient nature of auxin responses.

Different auxin response genes carry out different auxin responses and the regulation of which auxin response genes that are induced in a certain tissue at a certain time point plays an important role in auxin regulated development. Both ARFs and AUX/IAAs can form homo- and heterodimers and it has been suggested that the ARF and AUX/IAA pairs have specific target genes (reviewed in Vanneste and Friml, 2009). The response to auxin in a certain tissue is suggested to be determined by the production of a specific set of ARFs and AUX/IAAs, forming certain combinations and thereby inducing certain target genes. Different co-activators and co-repressors could also participate in the regulation of auxin responses (reviewed in Lau et al., 2008).

There seem to be other, TIR1 independent, auxin signaling pathways as well and these could be involved in *e.g.* rapid cellular auxin responses that probably are not mediated via transcription (reviewed in Vanneste and Friml, 2009; Tromas and Perrot-Rechenmann, 2010). The plasma membrane and ER located AUXIN-BINDING PROTEIN1 (ABP1) function as an auxin receptor and affects cell expansion and cell division (Braun et al., 2008). The phospatase INDOLE 3-BUTYRIC ACID RESPONSE 5 (IBR5) is another protein that promotes auxin responses in a potentially TIR1 independent pathway (Strader et al., 2008).

1.3 Plant development

Plants have a sessile lifestyle and have to adapt to the local environment where they grow. In contrast to animals that establish their adult body plan during embryogenesis, plants continue to develop new organs throughout their lifetime. During embryogenesis a plant embryo develops shoot- and root apical meristems and from these meristems new organs are formed during vegetative and reproductive development. Post germination, during the vegetative phase, leaves are formed above ground, and at a certain time point there is a switch to the reproductive phase when also flowers are formed (reviewed in Simpson et al., 1999; Taiz and Zeiger, 2006).

1.3.1 Phase transitions

The vegetative phase is divided into a juvenile phase and an adult phase, that in *Arabidopsis* and many other plants are characterized by different leaf morphologies, and it is only after the transition to the adult phase that the plant is competent to respond to signals promoting flowering (reviewed in Möller-Steinbach et al., 2010).

In *Arabidopsis*, the transition to flowering is regulated by four major pathways: autonomous, photoperiod, vernalization and gibberellin (GA) pathways (reviewed in Komeda, 2004; Taiz and Zeiger, 2006; Michaels, 2009; Möller-Steinbach et al., 2010). The input from all these pathways converge in the regulation of *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) and other floral integrators that, in turn regulate genes necessary for producing flowers.

The photoperiod pathway regulates the response to day length and *Arabidopsis*, which is a facultative long day plant, flowers earlier when grown in long day conditions (reviewed in Möller-Steinbach et al., 2010). Some *Arabidopsis* accessions also require a cold period, vernalization, to flower, but this is not the case for common laboratory accessions like Col and L*er* (reviewed in Michaels, 2009; Möller-Steinbach et al., 2010). The autonomous pathway regulates flowering in response to internal signals and in the GA pathway, required for flowering in non-inductive short day conditions, flowering is induced by the plant hormone GA (Taiz and Zeiger, 2006).

1.3.2 Organ development

The stem and aerial lateral organs, like leaves and floral organs, are formed from the shoot apical meristem (SAM) and the root system is formed from the root apical meristem (RAM). These meristems contain pluripotent stem cells and to function properly, a balance between maintaining the apical

meristem cells and forming organs and axillary meristems, must exist (reviewed in Shani et al., 2006; Wang and Li, 2008; Wolters and Jürgens, 2009; Bohn-Corseau, 2010). Several transcription factors and hormones participate and interact in regulating this balance. A negative feedback loop between CLAVATA3 (CLV3) and the transcription factor WUSHEL (WUS) is important for maintenance of the SAM and additional transcription factors, such as SHOOTMERISTEMLESS (STM), CUP-SHAPED COTYLEDONE1 to 3 (CUC1 to 3), AINTEGUMENTA (ANT) and LEAFY (LFY) participate in regulating SAM and organ identity as well. Among the hormones, cytokinins are important for maintenance of the SAM and auxin for initiation of organ primordia, but also GA and ethylene affect SAM function and the interactions between the different hormones are rather complex.

Organ initiation

The SAM is composed of several layers and zones (reviewed in Barton, 2010; Bohn-Corseau, 2010; Vernoux et al., 2010). Cells in the layers L1 to L3 generate different tissues, and lateral organs are formed from cells in the L3 layer. The three zones, central, peripheral and rib zone, also have different functions. The central zone maintains the meristem and supplies the other zones with new cells, the rib zone forms the internal tissues of the stem, and the peripheral zone initiate lateral organs. When organ initiation takes place, a group of cells in the peripheral zone is selected to increase the rate of proliferation and growth to form a primordium, followed by morphogenesis into an organ.

An important regulator of organ initiation is auxin, with auxin concentration maxima and gradients determining where new organs are initiated (reviewed in Bohn-Corseau, 2010; Krupinski and Jönsson, 2010; Vernoux et al., 2010). Several studies and computer simulations support a model where PIN1 directs auxin fluxes towards the sites of organ initiation. This produces sites of auxin maxima, leading to organ initiation, and depletion of auxin from the surrounding cells, preventing new organ initiation nearby the newly formed primordia. In addition to polar auxin transport, local auxin biosynthesis is also suggested to be important for formation of the auxin maxima at organ initiation sites. Another level of control is the competences of different cells to respond to auxin with organ initiation.

Following the formation of an auxin maximum, meristem identity factors like STM are down-regulated at the organ initiation site and instead, transcription factors involved in organ outgrowth and identity, such as ANT

and LFY, are expressed (reviewed in Bohn-Corseau, 2010; Vernoux et al., 2010).

Leaf development

Leaves are lateral organs specialized for light absorption, gas exchange and sugar synthesis. Leaves can be either simple, with a single blade, or compound, with several leaflets, and the leaf margins can be smooth, lobed or serrated (Reviewed in Efroni et al., 2010). The *Arabidopsis* leaf is a simple leaf with serrated margins and the extent of serration varies during different developmental stages and between different accessions (Tsukaya and Uchimiya, 1997; Perez-Perez et al., 2002). The rosette leaves of *Arabidopsis* have a bladeless part called the petiole that connects the leaf blade with the stem, whereas cauline leaves lack the petiole (Tsukaya, 2002). Leaves have three axes of asymmetry (see fig 2), a proximal-distal axis going from the base to the tip, a medio-lateral axis going breadthwise across the leaf blade and an adaxial-abaxial axis going from the upper to the lower surface of the leaf (Taiz and Zeiger, 2006).

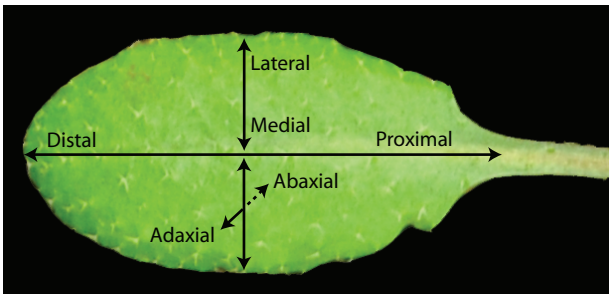

Figure 2. Arabidopsis rosette leaf with arrows showing the three asymmetry axes.

During leaf development a small peg-like primordium is formed and the three axis of asymmetry are established (Reviewed in Braybrook and Kuhlemeier, 2010; Efroni et al., 2010; Scarpella et al., 2010). The adaxial and abaxial domains are determined by transcription factors that promote adaxial or abaxial identity, and small RNAs that participate in regulating the expression patterns of the adaxial- and abaxial identity factors. The class III HOMEODOMAIN-LEUCINE ZIPPER (HD-ZIP III) transcription factors PHABULOSA (PHB), REVOLUTA (REV) and PHAVOLUTA (PHV) and ASYMMETRIC LEAVES 1 and 2 (AS1 and 2) promote adaxial fate whereas KANADI (KAN) family members, auxin response factors ARF3 and ARF4, and YABBY family members promote abaxial fate (Eshed et al., 2004; Pekker at al., 2005; Fu et al., 2007). The adaxial identity

factors, together with small RNAs, repress the abaxial identity factors and vice versa. The set up of these two domains is possibly regulated by a mobile signal derived from the meristem (Reinhardt et al., 2005). The identity of this signal is unknown but lipids/sterols and small RNAs have been suggested as candidates (McConnell et al., 2001; Garcia et al., 2006). Auxin has been suggested as an abaxial signal, and an abaxial-adaxial auxin gradient across the leaf primordium has been proposed (Reinhardt et al., 2003; Pekker et al., 2005). Less is known about regulation of proximal-distal and medio-lateral asymmetry but the genes *BLADE-ON-PETIOLE1* and *2* (*BOP1* and *2*) and *AS1* and *2* have been shown to affect proximo-distal patterning, and a distal-proximal auxin gradient has been suggested to exist (Byrne et al., 2000; Benkova et al., 2003; Hepworth et al., 2005; Jun et al., 2010).

When the asymmetry axes have been established, the primordium elongates and the distal part starts expanding to form a leaf blade (Reviewed in Efroni et al., 2010). Expansion of the leaf blade first occurs through cell divisions that take place throughout the blade and gradually cease starting from the distal tip of the leaf (Donnely et al., 1999). When the cell divisions cease, expansion of the leaf blade continues through cell expansion. In most leaves maturation starts from the distal part and involves morphological changes of cells to form *e.g.* trichomes, vascular cells and guard cells.

The young developing leaves soon start to produce auxin and a peak of auxin response is first seen in the apical tip (Ljung et al., 2001, Aloni et al., 2003; reviewed in Scarpella et al., 2010). Later, high auxin response is detected in the hydathodes in the tips of the teeth that form the serrated margins and in the midvein. Formation of the serrations seem to be regulated by auxin and *CUC* genes and auxin is also regulating vein development (Hay et al., 2006; Nicovics et al., 2006).

Floral organ initiation

After the transition to flowering, the *Arabidopsis* SAM is transformed into a primary inflorescence meristem (IM) that, instead of rosette leaves, produces cauline leaves, secondary inflorescence meristems and floral meristems (reviewed in Wang and Li, 2008; Alvarez-Buylla et al., 2011; Sun and Ito, 2010). The floral meristems are initiated on the flanks of the IM and generate cells that form floral organ primordia in four concentric whorls, with sepals in the outermost whorl, followed by petals, stamens and carpels (see fig 3). The first floral organ primordia to arise are the two lateral sepal primordia and somewhat later the two faster growing medial sepal primordia. After that the four petal primordia and the four medial (long)

stamen primordia are formed. The two lateral stamen primordia appear later and somewhat outside the medial stamen primordia and the last primordia to arise are the carpel primordia.

Formation of floral meristems requires upregulation of floral meristem identity genes like *LFY* and *APETALA1* (*AP1*) and repression of IM genes like *TERMINAL FLOWER1* (*TFL1*) and *EMBRYONIC FLOWER 1* and *2* (*EMF1* and *2*) (Blazquez et al., 1997; Liljegren et al., 1999; reviewed in Alvarez-Buylla et al., 2011; Sun and Ito, 2010). Like in the SAM, the floral meristem is maintained through a *WUS-CLV* negative feedback loop, but in the floral meristems *WUS* is only expressed until all floral organ primordia are initiated and the stem cells no longer are needed. The *WUS* expression domain and the timing of repression of *WUS* expression is important for formation of the right number of floral organs and defective regulation can result in flowers with to few or to many floral organs (Laux et al., 1996; Fletcher et al., 1999). *AGAMOUS* (*AG*), induced by *WUS* and *LFY*, represses *WUS* expression via induction of *KNUCKLES* (*KNU*) (Lenhard et al., 2001; Sun et al., 2009). Other genes that participate in the regulation of floral meristems are *ULTRAPETALA1* (*ULT1*), *PERIANTHIA* (*PAN*), *SUPERMAN* (*SUP*) and *CRABS CLAW* (*CRC*).

The initiation of the right type of floral organs at the right place requires activation of different combinations of floral organ identity genes in the four whorls (reviewed in Alvarez-Buylla et al., 2011; Irish, 2010). According to the ABC model, these floral organ identity genes are the A genes *AP1* and *AP2*, the B genes *AP3* and *PISTILLATA* (*PI*), the C gene *AG*, and the four *SEPALLATA* genes (*SEP1-4*) (Bowman et al., 1991; Ditta et al., 2004; reviewed in Coen and Meyerowitz, 1991). A function specifies sepal identity, A and B function together specify petal identity, B and C function together specify stamen identity, and C function specifies carpel identity (Bowman et al., 1991). The *SEP* genes are required together with the ABC genes to specify floral organ identity (Ditta et al., 2004). The expression and/or functional domains of the floral organ identity genes are regulated by the floral meristem identity genes, other floral organ identity genes, a microRNA and genes like *WUS* and *UNUSUAL FLORAL ORGANS* (*UFO*) (Bowman et al., 1991; Lee et al., 1997; Chen, 2004; Sundström et al., 2006). The floral organ identity genes regulate expression of genes required for floral organ initiation and development, *e.g.* transcription factors and genes involved in homeostasis and response to auxin and other hormones (reviewed in Sablowski, 2010).

Within the *Arabidopsis* flower a medio-lateral asymmetry exists, with *e.g.* medial and lateral stamen primordia appearing at different time-points. One

gene involved in the medio-lateral patterning of the flower is the *WUS*-related homeobox (*WOX*) gene *PRESSED FLOWER1* (*PRS1*), that is expressed in lateral domains and affect initiation of lateral sepal and stamen primordia (Matsumoto and Okada, 2001; Shimizu et al., 2009).

The positioning of floral organs within each whorl has been suggested to depend on auxin (reviewed in Alvarez-Buylla et al., 2011). Both leaf and floral primordia are initiated at sites with auxin maxima, and auxin response maxima are found in floral organ primordia (Benková et al., 2003). Furthermore, defects in auxin transport, synthesis or response result in altered floral organ number and/or fused floral organs (Sessions et al., 1997; Tobena-Santamaria et al., 2002; Benková et al., 2003; Cheng at al., 2006). The boundaries between primordia are specified by expression of *CUC1-3* and other boundary genes, leading to repression of growth (Takada et al., 2001; Vroemen et al., 2003; Borghi et al., 2007). The boundary genes affect *e.g.* auxin transport and expression of meristem specific genes.

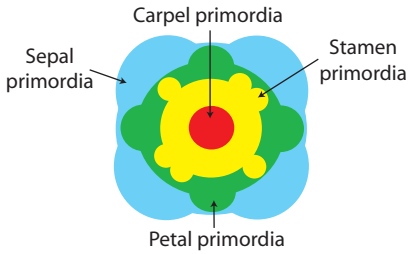

Figure 3. Schematic drawing of a floral meristem. Adapted from Alvarez-Buylla et al., 2011.

Gynoecium development

Carpels are the female reproductive organs of flowering plants and they are located in the innermost whorl of the flower. The carpels enclose the ovules and after fertilisation they form fruits that protect the developing seeds. The carpels of a flower can be either fused or unfused and together make up the gynoecium. In *Arabidopsis* the gynoecium is composed of two fused carpels (reviewed in Roeder and Yanofsky, 2005; Ferrandiz et al, 1999).

Going from the apical to the basal end the gynoecium is divided into stigma, style, ovary and gynophore (see fig 4A). The stigma consists of a layer of cells with stigmatic papillae and this is where pollen grains are captured and germinate. The style connects the stigma with the ovary and consists of a short solid cylinder surrounding transmitting tract cells. The ovary contains the ovules that after fertilization develop into seeds. Two valves (carpels) make up the walls of the ovary that is divided in the medial plane into two chambers by the internal septum and the outer replum (see

fig 4B). Between the valve and replum are the valve margins. The ovary also contains a transmitting tract, where the pollen tubes grow, and placental tissue from which the ovules form. The gynophore is a short stalk that connects the ovary with the base of the flower.

Along the medio-lateral axis the valves form the lateral domain and the placenta, septum, replum, style and stigma form the medial domain. Along the abaxial-adaxial axis the abaxial domain corresponds to the outer parts such as replum and outer valve epidermis, and the adaxial domain corresponds to placenta, ovules, septum and other inner parts.

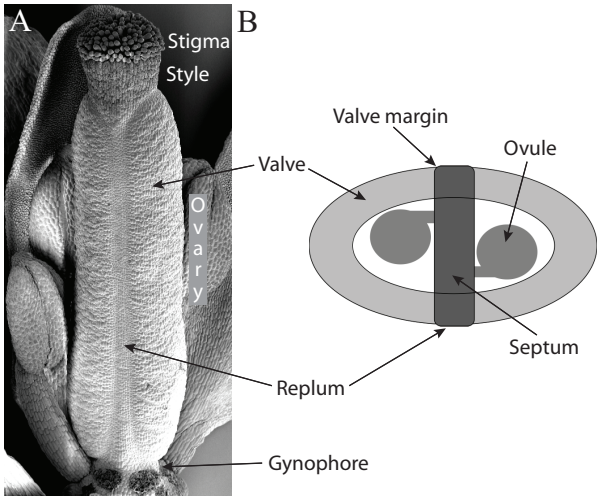

Figure 4. Arabidopsis gynoecium. (A) Scanning electron micrograph of a developing *Arabidopsis* gynoecium. (B) Schematic drawing of a cross section through the ovary.

When the gynoecial primordium first appears the two carpels are already fused and the primordium elongates to form a hollow cylinder (reviewed in Roeder and Yanofsky, 2005; Ferrandiz at al., 1999). The developing gynoecium continues to increase in size and the septum and placentae starts to form from the inner medial surfaces. The apical end of the gynoecium closes, stigma and style start to develop, and the medial ridges forming the septum meet and fuse. The gynoecium continues to grow, the cells of the valve margin and replum differentiate and the transmitting tract forms. During the development of the gynoecium, the ovules and vascular bundles also form. At anthesis the gynecium is mature and ready for fertilization of the ovules by released pollen. Subsequently the gynoecium develops into a fruit called silique.

Carpels and other floral organs have likely evolved from modified leaves and the development of all lateral organs is variants of a basic leaf

developmental ground plan (reviewed in Scutt et al., 2006). Carpel identity is specified by the MADS box transcription factors AG and the redundantly working SEP1-3 (reviewed in Balanzá et al., 2006; Roeder and Yanofsky, 2005). The transcription factors SHATTERPROOF1 (SHP1) and SHP2, SPATULA (SPT) and CRC are also involved in specifying carpel identity.

When carpel identity has been specified the gynoecial primordium is divided into different domains along the three asymmetry axes (reviewed in Balanzá et al., 2006; Østergaard, 2009). There are many similarities between the adaxial-abaxial patterning in leaves and gynoecia, and the leaf adaxial faxtors PHB, REV and PHV and abaxial factors KAN, ARF3 and 4 and the YABBY members FILAMENTOUS FLOWER (FIL) and YAB3 also specify adaxial and abaxial fate respectively, in the gynoecium. Additional transcription factors suggested to be involved in adaxial-abaxial patterning in the gynoecium are the potential adaxial factors JAGGED (JAG) and NUBBIN (NUB) and the potential abaxial factor, and YABBY family member, CRC.

Along the medio-lateral axis a medial domain with the meristematic medial ridge, and a lateral domain that will differentiate into valves, form (reviewed in Balanzá et al., 2006). This division is reflected by the expression of genes involved in SAM-maintenance, like the *KNOX* genes *STM* and *KNOTTED-LIKE FROM ARABIDOPSIS THALIANA1* (*KNAT1*) and *KNAT2*, and *CUC* genes, in the medial domain, and the expression of genes that repress meristem formation, like *FIL*, *YAB3*, *JAG, NUB* and *AS1* and *2*, in the lateral domain (Ori et al., 2000; Dinneny et al., 2005; Alonso-Cantabrana et al., 2007). Auxin seems to play a part in the medio-lateral patterning since disrupted auxin biosynthesis or transport result in altered distribution of medial and lateral tissues (Bennett at al., 1995; Nemhauser at el., 2000; Cheng et al., 2006; Nole-Wilson et al., 2010).

Auxin is involved also in the apical-basal patterning. Based on the phenotypes resulting from chemical or genetic disruption of PAT, or defective auxin response, an apical-basal auxin gradient has been suggested to span the gynoecium, with high levels specifying the style and stigma, medium levels specifying valves and low levels specifying the gynophore (Nemhauser et al., 2000). In this model auxin biosynthesis in apical parts together with PAT creates an auxin gradient and disrupted PAT results in a steeper gradient and a shift in the boundaries between different tissues along the apical-basal axis. ARF3 has been suggested to control the style-valve boundary by responding to medium auxin levels and repressing the activity of SPT, which promote stigma and style development, and KNUCKLES

(KNU) has been suggested to control the valve-gynophore boundary (Alvarez and Smyth, 1999; Nemhauser et al., 2000; Heisler et al., 2001; Payne et al., 2004). The expression patterns of auxin biosynthesis genes and auxin response reporters support an apical auxin maximum in the gynoecium (Aloni et al., 2003; Benkova et al., 2003; Cheng at al., 2006), but no other direct evidence of the existence of an auxin gradient has been presented. A basal cytokinin maximum together with an apical auxin maximum has also been suggested as a possible alternative model for the apical-basal patterning (reviewed in Østergaard, 2009).

Many genes required for regulation of tissue development in the gynoecium have been identified. *ANT*, *CRC*, *LEUNIG* (*LUG*), *SEUSS* (*SEU*), *SPT*, *FIL*, *SHORT INTERNODES* (*SHI*)/*STY*, *NGA*, *HECATE* (*HEC*), and *SHP* are examples of genes involved in development of style/stigma and/or other medial tissues (Alvarez and Smyth, 1999; Franks et al., 2002; Kuusk et al., 2006; Gremski et al., 2007; Alvarez et al., 2009; Trigueros et al., 2009; Colombo et al., 2010; reviewed in Balanzá et al., 2006; Roeder and Yanofsky, 2005), while *FRUITFUL* (*FUL*), *SHP*, *ALCATRAZ* (*ALC*) and *INDEHISCENT* (*IND*) are examples of genes involved in development of the lateral tissues, valves and valve margins (reviewed in Dinneny and Yanofsky, 2005).

Stamen development

In the *Arabidopsis* flower four medial (long) stamens and two lateral (short) stamens are found in the third whorl. Stamens are the male reproductive organs and consist of an apical anther and a basal filament. The anther develops four lobes, each containing a locule where pollen are produced and later released (Sanders et al., 1999). The filament is a stalk-like structure that contains vascular tissues and supplies water and nutrients to the anther (reviewed in Scott et al., 2004; Alvarez-Buylla et al., 2011). Regulated elongation of the filament is important for correct positioning of the anther.

Early in stamen development, the primordia are partitioned into two regions that develop into the anther and the filament, respectively (reviewed in Scott et al., 2004). Anther development is divided into two phases and during the first phase the connective and vascular tissues are formed and locules surrounded by tapetum layer, middle layer and endothecium, develop (Sanders et al., 1999; reviewed in Scott et al., 2004; Sundberg and Østergaard, 2009). Within the locules, pollen mother cells undergo meiosis and generate tetrads of haploid microspores enclosed by a callose wall. During the second phase the callose wall is degraded and the microspores are released from the tetrads and develop into pollen grains. The filament

elongates and pollen is released from the anther through dehiscence. After pollen release, the filaments continue to elongate, allowing the anthers of the long stamens to reach the stigma where the pollen grains germinate (Tashiro et al., 2009).

Many different transcription factors have been found to affect stamen development, *e.g.* SPL, DYSFUNCTIONAL TAPETUM1 (DYT1), JAG and NUB (Yang et al., 1999; Dinneny et al., 2006; reviewed in Scott et al., 2004; Alvarez-Buylla et al., 2011). The hormones gibberelic acid (GA), jasmonic acid (JA) and auxin have also been shown to be involved in stamen development (Sanders et al., 2000; Cheng et al., 2004; Nagpal et al., 2005; Cecchetti et al., 2008; Cheng et al., 2009; reviewed in Alvarez-Buylla et al., 2011). Both GA and JA affect pollen production and filament elongation, and JA has also been shown to be involved in anther dehiscence. The auxin biosynthesis genes *YUC2* and *YUC6* are expressed in tissues in the anthers from an early developmental stage and are later also expressed in the filaments (Cheng et al., 2006; Cecchetti et al., 2008). The *DR5* auxin response reporter has a similar expression pattern in the stamens but at a somewhat later stage (Feng et al., 2006; Cecchetti et al., 2008). Defects in auxin biosynthesis, transport or perception affect stamen development and suggest that auxin is involved in processes like pollen production, anther dehiscence and filament elongation (Feng et al., 2006; Cheng et al., 2006; Wu et al., 2006; Cecchetti et al., 2008). It has been suggested that early anther development mainly depends on local auxin biosynthesis, whereas filament elongation depends on both auxin biosynthesis and PAT (Cecchetti et al., 2008).

Root development

The root system is specialized for growing through the soil and absorbing water and minerals. In *Arabidopsis*, and other dicots, it consists of a primary root that is formed from the root apical meristem (RAM), located at the root tip, and lateral roots that are formed from primordia initiated along the primary root. An *Arabidopsis* root consists of four concentric cell layers along the radial axis: the outer epidermis, followed by cortex, endodermis, and the central stele containing the vascular tissue and pericycle (reviewed in Ueda et al., 2005). In the apical-basal direction, the root tip can be divided into four developmental zones: the root cap, the meristematic zone, the elongation zone and the maturation zone (Taiz and Zeiger, 2006). The root cap protects the RAM that is located in the meristematic zone and produces cells for root growth. In the elongation zone the newly formed cells elongate, contributing to root elongation, and in the maturation zone the

cells differentiate into different cell types, *e.g.* the root hair cells specialized for water and nutrient absorbtion. The RAM consists of the quiescent center (QC), with low mitotic activity, surrounded by mitotically active initials for the different cell types (reviewed in Scheres et al., 2002). Lateral root primordia are formed from pericycle cells and after the first stages the lateral root acquires a cellular organization similar to the primary root (reviewed in Scheres et al., 2002; Péret et al., 2009).

Auxin is important for root development and both transport from the shoot and synthesis in the root, mainly at the root tip, contributes to the pool of auxin in the root (Ljung et al., 2001; Ljung et al., 2005; reviewed in Taiz and Zeiger, 2006; Overvoorde et al., 2010; Tromas and Perrot-Rechenmann, 2010). At the root tip auxin is transported in the apical direction in the stele toward the QC and in the epidermis auxin is instead transported in the basal direction toward the end of the meristematic zone, where it is recycled to the stele. An auxin maximum is formed in the QC, important for its formation and function. Proper auxin levels is also important for root elongation, with low levels stimulating and high levels inhibiting root growth. Other processes dependent on auxin is *e.g.* lateral root formation and gravitropism.

1.4 Transcriptional regulation

Many biological processes, including organ development, are largely regulated at the transcriptional level. Together with translational regulation, as well as transport and stability of mRNA and protein, transcriptional regulation controls protein levels. Defects in this regulation often have profound effects on plant shape and function.

DNA is normally coiled around histone octamers and packed into chromatin, blocking the RNA polymerase and other members of the basic transcription machinery from binding the promoter of a specific gene and initiate transcription (reviewed in Kornberg, 1999; Riechmann, 2002). Gene transcription can be activated by transcription factors recognizing regulatory sequences in the promoter and recruiting factors that reorganize the local chromatin structure and acetylates histones. This allows additional transcription factors to bind the promoter and activate transcription by interacting with cofactors and the basic transcription machinery, leading to binding of the RNA polymerase complex to the transcription initiation site.

For proper functioning, the transcription of a gene also has to cease at the right time-point. One way to stop the transcription is targeted destruction of transcription factors (reviewed in Riechmann, 2002). Some transcription

factors also function as repressors and inhibit transcription instead of activating it, *e.g.* by recruiting histone deacetylases or competing with an activating transcription factor for binding to a regulatory sequence (reviewed in Kornberg, 1999; Liu et al., 1999).

The transcription of a gene is usually regulated by a combination of several transcription factors, each binding to a specific regulatory element in the promoter, and most transcription factors have several target genes (reviewed in Priest et al., 2009; Riechmann, 2002). There are also different cofactors and coactivators that bind to the transcription factors (reviewed in Näär et al., 2001; Riechmann, 2002). Cofactors affect the DNA-binding of the transcription factor or the interaction with the basic transcription machinery, and coactivator complexes interact with the RNA polymerase or different types of activators.

1.4.1 Transcription factors

Transcription factors can be defined as proteins that show sequence-specific DNA binding and can activate or repress transcription (reviewed in Riechmann, 2002). Most plant transcription factors contain a DNA-binding domain, an oligomerization site, a transcription regulation domain and a nuclear localization domain (reviewed in Liu et al., 1999).

DNA binding domains contain amino acids that determine the specificity of the transcription factor by binding to DNA bases at a specific cis-regulatory element and these amino acids are usually highly conserved (reviewed in Liu et al., 1999; Riechmann, 2002). Other amino acids make nonspecific contacts with DNA and enhance the binding of the transcription factor. The DNA-binding domain is often used to group a transcription factor into a transcription factor family. One example of a DNA binding domain is the zinc finger domain of transcription factors in the zinc finger family that is characterized by one or several finger motifs maintained by cysteine and/or histidine residues that are organized around a zinc ion.

Oligomerization domains are used by many transcription factors to form homo- or hetero-oligomers and this can affect DNA-binding or nuclear localization (reviewed in Liu et al., 1999).

Transcription regulation domains can either activate or inhibit the transcription of target genes and the transcription factors containing these domains are called activators or repressors, respectively (reviewed in Liu et al., 1999). Some transcription factors can act as both activators and repressors (reviewed in Finkelstein et al., 2002; Suzuki and McCarty, 2008).

Activation domains have been shown to be enriched in the amino acids proline (Pro) or glutamine (Gln).

Nuclear localization domains, important for the active translocation into the nucleus, are enriched in the basic amino acids arginine (Arg) and lysine (Lys) and can occur in one or several copies (reviewed in Liu et al., 1999).

1.4.2 Cis-regulatory elements

Transcription factors bind to specific DNA sequences that in eukaryotes often are 5-10 base pairs (bp) long, and these transcription factor binding sites are called cis-regulatory elements (reviewed in Riechmann, 2002; Priest et al., 2009). The promoter of a gene is mainly located upstream from the transcriptional start site (tss) and contains a proximal part that mediates the assembly of the RNA polymerase, and a distal part that regulates the expression (reviewed in Rombauts et al., 2003). The distal part of the promoter contains the cis-regulatory elements that guide transcription factor binding. It is not defined how far upstream from the tss the distal part ends and cis-regulatory elements can also be found downstream of the transcriptional start site, *e.g.* in introns.

Approaches for identifying cis-regulatory elements

There are several approaches for predicting cis-regulatory elements in the promoter of a gene, for testing of the predicted elements, and to analyze which element a certain transcription factor binds.

One common approach for predicting cis-regulatory elements is to look for motifs that are over-represented in promoters of co-expressed genes identified using *e.g.* microarrays (reviewed in Riechmann, 2002; Rombauts et al., 2003; Priest et al., 2009). This approach is based on the assumption that co-expressed genes are co-regulated and therefore have the same cis-regulatory elements. Another similar approach is to look for common motifs in promoters of orthologous genes, assuming that these orthologs have retained the same regulatory motifs, while non-regulatory regions in the promoters are less conserved. Different algorithms are used to search for motifs that occur more often in promoters of co-expressed or orthologous genes than in unrelated sequences. Even when the assumptions these methods are based on are true, the element prediction is complicated by the facts that regulatory elements can be found at different distances, both upstream and downstream from the tss, and that these short motifs also occur randomly in the genome.

The target genes of a transcription factor can possibly be identified by expressing the transcription factor using an inducible system, with and

without the presence of a protein synthesis inhibitor, and studying its effect on global gene expression using microarrays (reviewed in Riechmann, 2002). The identified putative target genes can then be used for prediction of cis-regulatory elements. Other ways of identifying the target genes, and possibly regulatory elements, of a transcription factor include yeast-one-hybrid (Y1H) screens and the combination of chromatin immuno-precipitation (ChIP) with microarrays or deep sequencing (reviewed in Riechmann, 2002; Priest et al., 2009). Y1H and ChIP experiments can also be used to test the interaction between a transcription factor and the predicted element. In Y1H experiments and electrophoretic mobility shift assays (EMSA) the interaction of a transcription factor with sequences containing intact and mutated versions of a predicted element can be compared.

1.5 The *SHI/STY* gene family

The *SHI/STY* gene family in *Arabidopsis* consists of nine active members, *SHI*, *STY1*, *STY2* and *SHI-RELATED SEQUENCE 3* to 7 (*SRS3* to *SRS7*) (Kuusk et al., 2006). The tenth member, *SRS8*, is probably a pseudogene (Kuusk et al., 2006). The active SHI/STY-family members in *Arabidopsis* are very similar in two highly conserved regions, a RING-like zinc finger (ZnFn) domain and an IGGH-domain that is unique to the SHI/STY-family (Fridborg et al., 2001). The SHI/STY members also contain a putative nuclear localization signal (Eklund et al., 2010). Homologues have been found in several other plant species, including the bryophyte *Physcomitrella patens*, the lycophyte *Selaginella moellandorffii* and the angiosperms rice, poplar and tomato, but there appear to be no homologues in photosynthetic algae or in non-plant species (Kuusk et al., 2006; Eklund et al., 2010).

The spatial and temporal expression patterns of *STY1*, *STY2*, *SHI*, and *SRS5* have been studied and were found to be largely overlapping, with only small variations between the different genes (Fridborg et al., 2001; Kuusk et al., 2002; Kuusk et al., 2006). As an example, *STY1* is expressed in all aerial organs of young seedlings, in the stipules, petioles and hydatodes of developing leaves, in lateral root primordia and root tips. During floral bud formation *STY1* is normally expressed already in the youngest floral primordia, in sepal and gynoecium primordia. Later during flower development the expression is found in the style and stigmatic region, ovules, sepals and receptacles.

Mutant studies have shown that at least six of the *SHI/STY*-family genes contribute to carpel fusion and marginal tissue formation in the apical part of the gynoecium (Kuusk et al., 2002; Kuusk et al., 2006). In addition, several of the *SHI/STY* genes appear to be important for proper apical–basal patterning of the gynoecium, vascular formation, as well as for leaf development and organ identity in floral whorls two and three (Kuusk et al., 2002; Kuusk et al., 2006). The SHI/STY members work redundantly and of the studied single mutants, only *sty1-1* and *sty2-1* show aberrant phenotypes (Kuusk et al., 2002; Kuusk et al., 2006). In *sty1-1* plants there is a slight depression in the style and in *sty2-1* plants the leaves are slightly more serrated than in wild type. Both these phenotypes are enhanced in SHI/STY-family multiple mutants.

Induced over-expression of STY1 results in early upregulation of the auxin biosynthesis gene *YUC4*, and the levels of IAA and IAA metabolites are affected by changes in *SHI/STY* gene expression (Sohlberg et al., 2006). *sty1-1* plants are also hypersensitive to chemical and genetic inhibition of PAT in the gynoecium (Sohlberg et al., 2006).

2 Aims of the study

The aim of this work has been to study the role of *SHI/STY*-family genes in the regulation of organ development. The main focus has been on identifying and characterising downstream targets of the SHI/STY-family and examining how these, and the plant hormone auxin, participate in SHI/STY regulated organ development. The STY1 gene was chosen for most studies and among the different plant organs a focus has been on gynoecium development. The upstream regulation of *SHI/STY*-family genes has also been studied.

3 Results and discussion

3.1 SHI/STY-family members are transcriptional activators (II, III)

Previous work shows that the predicted protein sequences of SHI/STY-family members contain a RING-like zinc finger domain, a region enriched in basic amino acids and one or two Gln-rich regions (Fridborg et al., 1999; Fridborg et al., 2001; Eklund et al., 2010). Zinc finger domains are involved in DNA or protein binding and are common in transcription factors (reviewed in Takatsuji, 1998). The region enriched in basic amino acids resembles a nuclear localization signal and the Gln-rich regions could function as transcriptional activation domains. The sequence composition thus suggests that the SHI/STY-family members could function as transcriptional activators.

3.1.1 SHI/STY-family members are localized to the nucleus

Expression of a STY1-GFP fusion protein in both onion epidermal cells and *P. patens* protoplasts showed that STY1 was localized to the nucleus (II). Also *P. patens* SHI/STY homologues have been shown to have a nuclear localization (Eklund et al., 2010). The dexametasone (DEX)-dependent ability of *35Spro:STY1-GR* to restore the *sty1-1* mutant phenotype further suggests that STY1 is active in the nucleus (Kuusk et al., 2006; Sohlberg et al., 2006). These results show that STY1, and probably also other SHI/STY-family members, are nuclear proteins and thus could function as transcriptional activators.

3.1.2 SHI/STY-family members activate transcription

Using microarray experiments we could show that DEX treatment of *35Spro:STY1-GR sty1-1 sty2-1* plants result in activation of many genes already after one hour (III). qRT-PCR studies have also shown that some

genes were significantly upregulated as early as 30 min after DEX treatment (Sohlberg et al., 2006 and unpublished data). Further experiments revealed that the STY1-GR induced upregulation of several genes was independent of protein translation (II, III). This together indicates that STY1 directly activates transcription of several genes. In agreement with this, the STY1 protein could also activate transcription in yeast assays (II).

Expression of STY1 fused to a repressor domain resulted in plants with phenotypes similar to multiple *SHI/STY*-family mutants (II). These phenotypes probably result from reduced transcription of genes normally activated by SHI/STY-family members and suggest that the function of STY1 is reversed when it is fused to a repressor domain. Taken together these results suggest that STY1 function as a transcriptional activator. The sequence similarities and functional redundancies between STY1 and other SHI/STY-family members (Kuusk et al., 2006) indicate that several of the SHI/STY proteins share this function. In acccordance with this, SHI-GR induction resulted in mostly upregulated gene expression in microarray experiments and many genes were induced by both STY1-GR and SHI-GR (unpublished data).

3.1.3 SHI/STY-family members interact with the YUC4 promoter

Using ChIP experiments, Y1H analyses and EMSA assays we have shown that STY1 interacts with the promoter of the *YUC4* gene (II). SHI was used in Y1H analyses as well, and was found to interact with the *YUC4* promoter (II). Experiments using mutated *YUC4* promoter fragments indicate that the short sequence ACTCTAC, located 173 to 167 bp upstream of a putative TATA box, is needed for efficient interactions with STY1 (II). These results suggest that STY1 activates transcription of *YUC4* by binding to the promoter and that ACTCTAC is a putative STY1 binding site. STY1, and probably also other SHI/STY-family members, thus seem to function as DNA-binding transcriptional activators.

3.2 SHI/STY-family members are positive regulators of auxin biosynthesis (I, II, III)

3.2.1 SHI/STY-family members affect auxin biosynthesis rate and auxin levels

DEX-induction of *35Spro:STY1-GR sty1-1 sty2-1* plants resulted in a significantly increased IAA synthesis rate (I). IAA levels were also significantly increased by a DEX-induced nuclear translocation of STY1-GR (Sohlberg et al., 2006), probably as a result of the increased biosynthesis. Disruption of SHI/STY-family function had the opposite effect with *sty1-1*

sty2-1 mutant plants having reduced levels of free IAA compared to wild type plants and *SHI/STY*-family quintuple mutant plants having reduced levels of both free IAA and several IAA metabolites (Sohlberg et al., 2006). SHI/STY-family members thus seem to be positive regulators of auxin biosynthesis and thereby auxin levels.

3.2.2 SHI/STY-family members activate transcription of auxin biosynthesis genes

YUC family members have been suggested to act as rate limiting enzymes in auxin biosynthesis and one of the *YUC* genes, *YUC4*, was shown to be induced 30 min after DEX-treatment of *35Spro:STY1-GR sty1-1 sty2-1* plants (Zhao et al., 2001; Cheng et al., 2006; Sohlberg et al., 2006). As discussed above, the STY1-GR induced upregulation of *YUC4* transcription is independent of protein translation and STY1 also binds the *YUC4* promoter. In accordance with this, the *YUC4* transcript level was reduced in *sty1-1 sty2-1* mutant plants (Sohlberg et al., 2006) as well as in *35Spro:STY1-SRDX* plants (II). One other *YUC* gene, *YUC8*, was induced by STY1-GR in the presence of a protein translation inhibitor (II). STY1 could thus be regulating auxin levels via regulation of two of the *YUC* family members. *YUC8* was however not induced to the same extent as *YUC4* and no significant reduction of *YUC8* transcript levels was found in *SHI/STY*-family quintuple mutant plants (II, III). Further, compared to *YUC4*, the *YUC8* expression pattern was not overlapping with the *STY1* expression pattern to the same extent (II). This suggests *YUC8* to play a minor role in STY1-mediated regulation of auxin biosynthesis, or to be restricted to certain specific tissues. *YUC4* was induced also by SHI-GR in microarray experiments (unpublished data), indicating that several SHI/STY-family members participate in the regulation of auxin biosynthesis genes.

3.3 SHI/STY-family members regulate gynoecium development at least partly by affecting local auxin levels (I, II, III)

Several *SHI/STY*-family genes are expressed in the apical part of developing gynoecia (Kuusk et al., 2002; Kuusk et al., 2006), suggesting a function in gynoecium development. Mutation in *STY1* resulted in plants with a slight depression in the style, and mutations in other *SHI/STY*-family members enhanced this phenotype, with quadruple and quintuple mutant gynoecia having unfused apical parts and almost completely lacking style tissue (Kuusk et al., 2002; Kuusk et al., 2006). Expression of STY1 fused to a repressor

domain also resulted in plants with a split in the style (II). Several SHI/STY-family members thus seem to redundantly promote style development and in agreement with this, ectopic expression of *STY1* and *STY2* resulted in ectopic formation of style cells on the valves (Kuusk et al., 2002). Another gynoecium phenotype seen both in multiple *SHI/STY*-family mutants (Kuusk et al., 2006) and in *35Spro:STY1-SRDX* plants (II) was an elongated gynophore. This suggests that SHI/STY-family members also participate in the regulation of the ovary/gynophore boundary. Since the SHI/STY-family members are positive regulators of auxin biosynthesis, these gynoecium phenotypes could result from reduced local auxin levels. The auxin biosynthesis gene, and STY1 downstream target, *YUC4*, has a similar expression pattern as the *SHI/STY* genes in the apical part of the gynoecium (Cheng et al., 2006), indicating that *YUC4* plays a role in SHI/STY-family regulated gynoecium development.

3.3.1 The style defects in *sty1-1 sty2-1* plants can be restored by elevated apical auxin levels

Gynoecia treated with a PAT inhibitor are suggested to accumulate auxin in the apical parts where it is produced (Nemhauser et al., 2000), and NPA treatment could restore the defective style development in *sty1-1* and *sty1-1 sty2-1* plants (Sohlberg et al., 2006). The style defects in *sty1-1 sty2-1* plants were also restored by exogenous application of auxin to the apex of young developing gynoecia, but not by spraying whole plants with NAA (I). A local elevated auxin level in the apical part of the gynoecium can thus restore normal style development in *sty1-1 sty2-1* plants. This suggests that the style defects in *SHI/STY* mutant plants result from reduced apical auxin levels. The restoration of *sty1-1 sty2-1* style defects by NPA treatment further suggests that the apical auxin synthesis is reduced but not completely abolished in this mutant line. Both *sty1-1 sty2-1* and *yuc4-1* mutant gynoecia were also hypersensitive to the effect of NPA on apical-basal patterning, indicating a defect in auxin homeostasis or signaling (I).

3.3.2 Both overexpression of STY1 and inhibition of PAT, can restore style development in diverse mutants

DEX induction of STY1-GR could restore the style phenotype in several unrelated mutants (I). A similar restoration was seen with NPA treatment (I), suggesting that the ability of STY1 to restore the style mutants is mediated by elevated auxin levels. As discussed above, the increased auxin levels resulting from STY1-GR induction is probably caused, at least partly, by an increase in *YUC* gene expression and thereby an increased auxin

biosynthesis rate. The restoration of diverse style mutants suggests that STY1 and auxin act in a parallel pathway to the studied style-promoting genes *CRC*, *LUG*, *SEU* and *SPT*, that can compensate for several of these style-promoting factors. Alternatively, STY1, and thus also auxin, act downstream of some, or all, of these factors.

3.3.3 A model for SHI/STY-family regulation of gynoecium development

Our results suggest that STY1 induces expression of *YUC4*, and perhaps also *YUC8*, in the apical part of developing gynoecia (I, II). Other SHI/STY-family members probably contribute to the induction of *YUC4* and/or other *YUC* genes. This apical expression of *YUC* genes could result in the formation of an apical auxin maximum that promotes development of style and stigma. The SHI/STY-family induced apical auxin biosynthesis could also contribute to formation of the proposed apical-basal auxin gradient. According to this model, disrupted SHI/STY function would result in decreased apical auxin biosynthesis and thereby reduced apical auxin levels. Another consequence would be that less auxin is available for the apical-basal transport, leading to a larger region of low auxin levels in the basal part of the gynoecium. These changes in auxin levels would result in reduced style and stigma formation and an apical shift of the valve/gynophore boundary, in accordance with the phenotypes seen in *SHI/STY*-family mutants and *35Spro:STY1-SRDX* plants (II).

No gynoecium phenotype was seen in *yuc4* or *yuc4 yuc8* mutant plants (III), indicating that additional SHI/STY-family downstream target genes participate in the regulation of gynoecium development. *yuc1 yuc4* and higher order *YUC* mutant plants show varying gynoecium defects, some of which are similar to those resulting from genetic or chemical inhibition of PAT in *sty1-1* or *sty1-1 sty2-1* plants (Cheng et al., 2006; Sohlberg et al., 2006). Similar to *SHI/STY*-family mutant gynoecia, *YUC* family mutant gynoecia also show defects in vascular patterning, while other gynoecium characters differ between the genotypes (Kuusk et al., 2002; Cheng et al., 2006; Kuusk et al., 2006). The SHI/STY-family regulation of gynoecium development could thus involve additional *YUC* family members, as well as other downstream genes.

3.4 SHI/STY-family members regulate organ development through different downstream target genes (II, III)

Several approached was used to identify putative downstream targets of the SHI/STY-family members. We searched for genes highly co-expressed with

SHI/STY genes in different microarray experiments, screened the annotated *Arabidopsis* genes for the putative *YUC4* STY1 binding site ACTCTAC and compared global gene expression in *35Spro:STY1-GR sty1-1 sty2-1* plants with and without DEX treatment using microarrays (III). For selected genes we also used qRT-PCR to study the effect of a protein translation inhibitor on the ability of STY1-GR to induce transcription and to analyze transcript levels in *SHI/STY*-family quintuple mutant plants (II, III). We could show that nineteen genes are induced by STY1-GR in the presence of a protein translation inhibitor and are thus possible direct downstream targets of STY1 (III). As discussed above, STY1 binds to the promoter of one of these genes, *YUC4* (II). Seven of the genes were also induced by SHI-GR in microarray experiments (unpublished data) suggesting that several SHI/STY-family members have at least some downstream targets in common.

The ectopic and elevated level of STY1 expression in the conducted experiments could possibly have resulted in induction of some genes that are not STY1 targets under wild type conditions, *i.e.* genes that are false positives. However, false positives in this experiment are not likely down-regulated in *SHI/STY* mutants. Accordingly, significantly reduced expression levels were detected for *YUC4* and an additional 11 of the 19 genes in the *SHI/STY*-family quintuple mutant (III). Additional genes may have reduced expression levels in the multiple mutant line, as our detection methods have a severe sensitivity limit for genes expressed in only a few cell types. Moreover, functional SHI/STY members might compensate for the mutated SHI/STY members in the activation of some of the downstream targets.

YUC4 and five of the other possible downstream targets were found to be highly co-expressed with several *SHI/STY*-family members in microarray experiments and/or to have a similar expression pattern as the *SHI/STY* genes in more detailed expression studies, further suggesting them to be normal SHI/STY targets (III). The other genes, so far not revealed as strongly co-regulated with *SHI/STY* genes, could still be normal SHI/STY targets at specific sites, and may in addition require other factors only overlapping with SHI/STY proteins at specific time-points/sites.

Ten of the 19 genes have the putative STY1 binding site, ACTCTAC, in their promoter (1000 bp upstream of the tss) (III). The other genes could possibly be regulated by STY1 through ACTCTAC sites located further upstream, or downstream of the genes, or through similar sequences. A shorter sequence ACTCTA is found in the promoters of four additional of the 19 genes (our unpublished data). The ACTCTAC sequence is also found in the promoters of many genes that are not induced by STY1,

indicating that additional factors are required for STY1-regulated transcriptional activation.

STY1 and the other SHI/STY-family members most likely have additional direct downstream targets that were not found among the 19 genes identified so far.

3.4.1 Two of the downstream targets affect auxin levels

As already mentioned, two of the *YUC* family members, *YUC4* and *YUC8*, are among the 19 putative direct downstream targets of STY1 and the *YUC4* promoter has been shown to interact directly with STY1 (II, III). YUC4 acts redundantly with YUC1 in flower development and YUC4 overexpression has been shown to cause an auxin overproduction phenotype, suggesting that YUC4 has a similar function as YUC1 in auxin biosynthesis (Zhao et al., 2001; Cheng et al., 2006). Overexpression of YUC8 result in increased auxin levels and an auxin overproduction phenotype (Hentrich, 2010), suggesting that it plays a similar role in auxin biosynthesis as other characterized YUC members, including YUC1. Auxin has an important role in many developmental processes and one pathway through which the SHI/STY members regulate plant development appears to be the direct regulation of auxin biosynthesis genes, and thereby the regulation of local auxin levels.

We could show that mutation in *YUC4* results in plants lacking one or both of the lateral stamens in at least half of the flowers, and a similar phenotype was detected in the *sty1* mutant, suggesting that SHI/STY members affect the initiation or development of lateral stamens through the regulation of *YUC4* (III). As discussed in chapter 3.3, *YUC4* is probably also involved in SHI/STY regulated gynoecium development. In addition to floral meristems and gynoecia, *YUC4* expression is found in other floral organs and in leaves (Cheng et al., 2006). *YUC4* could thus, redundantly with other genes, have a role in SHI/STY regulated development of other organs as well.

YUC8 is expressed in roots, sepals, petals, ovules, nectaries, anthers and stamen filaments and disruption of the *YUC8* gene results in reduced filament elongation and seed production (III). This indicates that YUC8 is required for auxin-regulated filament elongation, and possibly also other processes affecting fertility. The fertilization defect, but not the filament elongation defect, is enhanced in *yuc4 yuc8* double mutants (III). Both genes are expressed in anthers and gynoecia (III; Cheng et al., 2006) but no obvious visible pollen developmental defect was found in the *yuc4 yuc8* double mutant (III), suggesting that YUC4 and YUC8 together may affect

female fertility. *SHI/STY* quintuple mutant and *35Spro:STY1-SRDX* plants have shorter stamens and produce fewer seeds compared with wild type as well, although in these genotypes, the gynoecia are also shorter and defective (III). This suggests that SHI/STY members could regulate processes affecting filament elongation and male/female fertility through the downstream targets *YUC8* and *YUC4*.

3.4.2 Other downstream targets probably directly affect cell expansion

Among the 19 putative direct downstream targets are four genes encoding enzymes potentially involved in cell expansion; a polygalacturonase family gene, an L-ascorbate oxidase encoding gene, an expansin family gene and *POLYAMINE OXIDASE 5* (*PAO5*) (III). This suggests that in parallel of affecting organ growth and development via auxin mediated cell expansion, SHI/STY-family members directly regulate cell expansion through activation of genes encoding enzymes involved in this process.

The polygalacturonase family gene is highly expressed in gynoecia (III) and could thus be involved in SHI/STY regulated gynoecium development.

3.4.3 Many of the downstream targets are transcription factors and can affect organ development via target genes

Seven of the 19 genes encode putative transcription factors that, in turn, regulate downstream target genes, probably involved in SHI/STY regulated organ development. These seven transcription factors are OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF 59 (ORA59), ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR 15 (ERF15), ERF38, ERF43, BEL1-LIKE HOMEODOMAIN 11 (BLH11), REPRODUCTIVE MERISTEM1 (REM1) and NGA2 (III).

ORA59 and ERF15 are two closely related AP2/ERF family transcription factors and ORA59 has been shown to be involved in plant defense and to induce expression of genes involved in tryptophan biosynthesis (Pré et al., 2008). In plant development ORA59 affect the number of lateral stamens (III) and could thus, together with YUC4, be involved in SHI/STY regulated initiation or development of lateral stamens. Interestingly, the three SHI/STY targets ORA59, ERF15 and YUC4 together appear to regulate the leaf shape, size and pigmentation (III). If this common effect on leaf development is related to auxin mediated processes is still unknown, although our data indicates that ORA59 could be involved in regulating auxin transport, synthesis or response, as both overexpression and disruption of *ORA59* result in altered auxin response reporter expression (III). SHI/STY members could thus potentially affect auxin-

regulated development also through indirect regulation of auxin transport or signaling via regulation of *ORA59*.

The expression pattern of *ERF38* as well as *NGA2* strongly overlaps with that of *SHI/STY* genes, for example in the apical part of gynoecia (III), suggesting that they could be involved in SHI/STY regulated development of the gynoecium and/or other organs. *NGA* genes have been shown to redundantly regulate gynoecium development and it has been suggested that there is a positive feedback loop between the *NGA* and *SHI/STY* genes during this process (Alvarez et al., 2009; Trigueros et al., 2009). *ERF38* and *NGA2* also affect the number of lateral stamens and could function together with other SHI/STY downstream targets in the regulation of stamen number (III).

BLH11 is co-expressed with *SHI/STY* genes and is highly active in gynoecia (III). However, no discernible gynoecium defects were found (III), suggesting that *BLH11* may act redundantly with other *BEL1*-like family members in gynoecium development. Interestingly, disruption of *BLH11* results in longer roots, indicating that it is involved in regulating root elongation (III). No root developmental deviations have so far been found in *SHI/STY*-family mutants, but since *SHI/STY* genes are expressed in roots, it is possible that they redundantly affect this process.

Also *REM1* is highly co-expressed with *SHI/STY* genes, with expression *e.g.* in floral meristems and gynoecia (III). REM1 seems to be involved in flowering time regulation, since *rem1-3* mutants flower somewhat later than wild type in both long and short days (III). Disruption of *STY1* alone does not affect the flowering time, but one trait found among the *35Spro:STY1-SRDX* primary transformants was plants that never bolted (II), and other *35Spro:STY1-SRDX* transformants flower later than wild type (III). It is thus possible that several *SHI/STY* members redundantly regulate the transition to flowering via *REM1* and other downstream targets. *REM1* could also work redundantly with other *REM* family genes in *SHI/STY* regulated gynoecium development.

3.5 SHI/STY-family promoters contain a GCC-box-like regulatory element that could be important for regulation of STY1-mediated auxin biosynthesis (IV)

The similar expression patterns and redundant functions of the *SHI/STY*-family members suggest that they are co-regulated. We performed promoter analyses to identify putative transcription factor binding sites and a short conserved promoter element was found in the promoter or 5' UTR of all

SHI/STY members except *SRS3* (IV). The element is 12-15 bp long with the consensus GGCGGC and is similar to an inverted ethylene responsive element called a GCC-box (IV). This GCC-box-like element is only found in promoters of a few other *Arabidopsis* genes, but is present also in promoters of rice *SHI/STY* homologues (IV).

Mutation of the GCC-box in the *STY1pro:GUS* construct results in a complete loss of the *STY1* promoter regulated expression in the distal parts of the cotyledons, leaf primordia, style, ovules and receptacle (IV). This suggests that the GCC-box is important for regulating *STY1* activity, and probably also the expression of other *SHI/STY* members. The *STY1pro:GUS* expression in lateral root primordia, hypocotyls, petiole and proximal part of the cotyledons remained after mutation of the GCC-box (IV), indicating that the expression in these tissues is regulated via other promoter elements. The GCC-box dependent expression domains overlap with those of *YUC4*. The GCC-box could thus be important for the regulation of *YUC4* expression via *SHI/STY* genes and thereby for SHI/STY-mediated auxin biosynthesis.

Initital studies did not reveal any significantly altered *STY1* expression after treatment with an ethylene precursor, suggesting that the *SHI/STY* GCC-box is not ethylene responsive (IV). Similarly, IAA-treatment did not significantly alter the *STY1*-expression level (IV). This indicates that *STY1* expression is not regulated by either of the hormones ethylene or auxin.

Several members of the AP2/ERF transcription factor family have been shown to regulate GCC-box-containing genes and four of the *SHI/STY* genes were upregulated by overexpression of the AP2/ERF protein DORNRÖSHEN-LIKE (DRNL) in microarray experiments (Ikeda et al., 2006; Marsch-Martinez et al., 2006). Using an inducible *DRNL-ER* construct and a protein translation inhibitor we could show that DRNL can activate transcription of *SHI/STY* genes independently of protein translation (IV). *SHI/STY* genes could thus be direct downstream targets of DRNL. In addition, DRNL induced ectopic activation of *STY1pro:GUS* is dependent on a functional GCC-box (IV). This suggests that DRNL activates transcription of SHI/STY genes through interaction with the GCC-box.

4 Conclusions

These are the main conclusions from the work presented in this thesis:

SHI/STY-family members function as DNA-binding transcriptional activators.

SHI/STY-family members regulate plant development through activation of downstream target genes encoding *e.g.* auxin biosynthesis enzymes, transcription factors and enzymes involved in cell expansion.

SHI/STY-family members regulate development of lateral organs like the gynoecium through positive regulation of local auxin biosynthesis.

Auxin can compensate for loss of function of several transcription factors, such as SPT, CRC, LUG and SEU, in style development.

A conserved GCC-box-like promoter element is important for the co-regulation of *SHI/STY*-family gene expression.

5 Future perspectives

Downstream targets of SHI/STY-family members

We have shown that STY1 binds to the promoter of *YUC4* and have identified 18 additional putative direct downstream targets. It would be interesting to test the binding of STY1 to the promoters of these additional 18 genes, using *e.g.* YIH and/or ChIP experiments.

We have made functional studies on several of the downstream target genes and have found some interesting phenotypes, but further investigations of the function of these genes and their connections to SHI/STY function could be made. What role do *YUC4* and other STY1 downstream target genes play in the regulation of lateral stamen development? Do they affect the initiation or outgrowth of the organs and why are only lateral stamens affected? PRS1/WOX3 have been shown to affect the initiation of lateral stamens and sepals (Matsumoto and Okada, 2001; Shimizu et al., 2009) and a potential connection between PRS1 and *e.g.* YUC4 could be studied. Disruption of *YUC8* results in a fertilization defect that is enhanced in the *yuc4 yuc8* double mutant and it would be of interest to study if these two *YUC* genes regulate other processes affecting fertility in addition to stamen filament elongation. The switch to flowering is delayed in the *rem1-3* mutant, but this defect is less severe in the *rem1-2* mutant. This flowering trait could be dependent on the ecotype, something that could be resolved by crossing *rem1-2* to Col or by studying additional insertion lines. It would also be interesting to further investigate what role REM1 plays in the complicated process of flowering time regulation. Disruption or overexpression of *ORA59* results in altered auxin response reporter expression but further studies are needed to establish if ORA59 affects auxin transport, synthesis or response. One thing to investigate could be a potential effect of ORA59 on the expression or localization of PIN members. *BLH11* is expressed in flowers but no aberrant flowers were

found in the mutant. Studies of higher order *BLH* family mutants could possibly identify a potential redundant role in flower development.

It is still not clear if *SHI/STY* genes are involved in the regulation of all processes found to be affected by STY1 downstream target genes and this could be investigated further. Are the *SHI/STY* genes involved *e.g.* in the regulation of root development? Knockout of additional members of this redundantly working gene family could perhaps reveal additional functions of SHI/STY members.

It would also be interesting to study the function of the other downstream target genes. Some of the genes belong to families with a suggested function in cell expansion and the involvement of these genes in cell expansion processes during development of different organs could be studied through characterization of mutant lines.

Additional downstream target genes of STY1 could possibly be identified using ChIP experiments with STY1-GR combined with sequencing of STY1-bound DNA fragments, or microarray experiments with STY1-GR induced in the presence of a protein synthesis inhibitor. The *SHI-GR* construct could also be used to identify downstream targets of SHI. This could give a better understanding of how the SHI/STY members regulate plant development.

Gynoecium development

We have shown that both overexpression of STY1 and inhibition of PAT could restore style development in several unrelated mutants. It would be interesting to further investigate the relation of these style-promoting factors to STY1 and auxin. Do STY1 and auxin act downstream of these factors, or in a parallel pathway that can compensate for the loss of several different style-promoting factors?

Which of the STY1 downstream targets participate in SHI/STY regulated gynoecium development? Several of the downstream target genes have a relatively high expression in the gynoecium and mutants in these, perhaps combined with mutants in related genes, could reveal potential involvement in gynoecium development.

Upstream regulation of SHI/STY genes

A conserved GCC-box-like element is important for the regulation of STY1 expression in *e.g.* leaves and flowers, but part of the STY1 expression domain seems to be independent of the GCC-box. It would be interesting to investigate if other promoter elements regulate the GCC-box-independent expression.

DRNL induces expression of *SHI/STY* genes but *STY1pro:GUS* or *SHIpro:GUS* expression was not altered in a *drn drnl* double mutant. Other AP2/ERF proteins could act redundantly with DRN and DRNL in the regulation of *SHI/STY* gene expression and a slight, but not statistically significant, down-regulation of *STY1* expression was detected in the *drn drnl puchi* triple mutant. This potential redundant regulation of *STY1* expression could be further tested by introducing *STY1pro:GUS* into the triple mutant, and by creating even higher order AP2/ERF mutants.

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