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Mechanisms of plant root xylem developmental plasticity in response to water deficiency and salt

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Abstract
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Plants may be exposed to a variety of different environmental conditions including water deficiency and salt, both affecting the uptake of water into the plant. Water is taken up from the soil by the roots and distributed throughout the plant via the water conducting tissue, the xylem. Plants are remarkably plastic and have evolved different mechanisms to sense the environment and adjust their development accordingly. However, how xylem development may respond to water availability is not clear. In this thesis, I show how water deficiency and salt affect xylem development and how the observed phenotypic alterations are regulated on a molecular level. We found that upon water deficiency additional protoxylem strands were formed along with an early differentiation of the inner metaxylem. These phenotypes were regulated both by non-cell autonomous and cell autonomous signaling via the hormone abscisic acid (ABA). The expression of microRNA165 was induced by ABA signaling in the endodermis leading to downregulation of homeo domain leucine zipper class III (HD-ZIP III) transcription factors in the stele. This caused a shift in xylem identity from meta- to protoxylem and the formation of additional protoxylem strands. At the same time, cell autonomous ABA signaling upregulated several VASCULAR RELATED NAC DOMAIN (VND) transcription factors including VND7, which promoted the shift in xylem identity as well as VND2 and VND3, which promoted early differentiation of the inner metaxylem. In contrast, during an initial phase of salt stress, we observed the formation of protoxylem gaps specifically in response to ionic stress and distinct from ABA-signaling. We identified that protoxylem gaps were caused by lowered levels and signaling of the growth regulator gibberellin (GA). Downstream of GA-signaling, protoxylem gap formation upon salt was controlled by genes involved in secondary cell wall formation including the xylem master regulator VND6 and factors involved in cell wall modification. Salt tolerance assays suggested that protoxylem gaps may contribute to salt tolerance and the phenotypes that we observed upon water deficiency have been suggested to confer drought tolerance. We observed similar effects on xylem developmental plasticity in response to water deficiency and salt in various different dicot species indicating an evolutionary conservation. Thus, xylem development is of high relevance for breeding programs to generate plant varieties better adapted to a changing climate.

Keywords: ABA, developmental plasticity, GA, HD-ZIP III, VND, xylem

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“Auf die größten, tiefsten, zartesten Dinge in der Welt
müssen wir warten, da gehts nicht im Sturm,
sondern nach den göttlichen Gesetzten des
Keimens und Wachsens und Werdens.“

-Dietrich Bonhoeffer-

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. Ramachandran, P., Wang, G., **Augstein, F.**, de Vries, J. and Carlsbecker, A. (2018) Continuous root xylem formation and vasculature acclimation to water deficit involves endodermal ABA signaling via miR165. *Development*, 145(3), dev159202.

II. Ramachandran, P.*, **Augstein, F.***, Mazumdar, S., Nguyen T. V., Minina, E. A., Melnyk, C. W. and Carlsbecker, A. (2021) Abscisic acid signaling activates distinct VND transcription factors to promote xylem differentiation in *Arabidopsis*. *Current Biology*, 31, 3153–3161.
*equal contribution

III. **Augstein, F.** and Carlsbecker, A., DELLA-regulated root xylem developmental response to salt stress in Arabidopsis. *Manuscript*

Contribution as an Author

In Paper I, I contributed by setting up the *in vitro* water deficiency experiment in the lab following Verslues et al. (2006), measuring the water activity using the Aqualab Pre Water Activity Meter and calculating the water potential. I performed phenotyping analysis of both wild type and the *SCR:abi1-1* line under different degrees of water deficiency.

In Paper II, I contributed to the conceptualisation of the study and editing of the manuscript. I planned and performed phenotyping experiments evaluating the root xylem phenotype of different mutants upon water deficiency and ABA treatment as well as distance measurements of xylem differentiation in respect to the meristem. I designed, performed and did the downstream analysis of the presented RNA sequencing experiments.

In Paper III, I designed and carried out the experiments, interpreted the results and wrote the manuscript.

The following papers were written during the course of my doctoral studies, but are not included as part of the thesis.

I. **Augstein, F.** and Carlsbecker, A. (2018) Getting to the roots: A developmental genetic view of root anatomy and function from Arabidopsis to Lycophytes. *Frontiers in Plant Science*, 9:1410.

II. Ramachandran, P., **Augstein, F.**, Nguyen T. V. and Carlsbecker, A. (2020) Coping With Water Limitation: Hormones That Modify Plant Root Xylem Development. *Frontiers in Plant Science*, 11:570.

III. Carlsbecker, A. and **Augstein, F.** (2021) Xylem versus phloem in secondary growth: a balancing act mediated by gibberellins. *Journal of Experimental Botany*, 72(10):3489-3492.

Contents

Abbreviations

ABA	Abscisic acid
ABF	ABRE-binding factor
ABRE	ABA-responsive element
BR	brassinosteroid
DAPI	4’,6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
ER	Endoplasmatic reticulum
GA	Gibberellin
GFP	Green Fluorescent Protein
HD-ZIP III	Homeodomain leucine zipper class III
miRNA	Micro RNA
PAC	Paclobutrazol
PCD	Programmed cell death
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PI	Propidium iodide
qRT-PCR	Quantitative real time PCR
RNA	Ribonucleic acid
RNA-Seq	RNA-Sequencing
ROS	Reactive oxygen species
SCW	Secondary cell wall
TE	Tracheary element
TF	Transcription factor
WUE	Water use efficiency
YFP	Yellow Fluorescent Protein

The following nomenclature is used in this thesis:

Wild-type gene names are written in upper case italics
Wild-type proteins are written in upper case
Mutant gene names are written in lower case italics
Mutant protein names are written in lower case

Introduction

Major challenges for future agriculture

Our planet is currently being confronted with different types of extreme weather conditions: extreme heat in combination with no precipitation resulting in droughts or on the other hand, cloudbursts causing flooding. Several studies have presented that drought and heat causes major yield losses in our most important crop species. A 40% water reduction caused a decrease in yield of 21% for wheat and 39% for maize (Daryanto et al., 2016). In a different study, drought stress caused a 53-92% yield loss in a widely-grown high-yielding rice cultivar (Lafitte et al., 2007; Fahad et al., 2017). In maize, drought caused a grain yield reduction between 57 and 90% (Kamara et al., 2003) and in different wheat varieties, drought diminished yield with 57% and in combination with heat, a 75% yield loss was observed (Balla et al., 2011). In general the impact of drought on crop production is even stronger in combination with heat (Raza et al., 2019). The changed rainfall patterns and temperatures together with intensive agriculture is going to lead and is already leading to an enhanced soil degradation (Shukla et al., 2019). Major forms of soil degradation are among others erosion, loss of soil carbon acidification and salinization (Kopittke et al., 2019). Intense rains caused by climate change will further increase soil erosion that is caused by bad agricultural practices and deforestation (Borrelli et al., 2020). Another effect of a warmer climate is the rise of the sea level, which leads to salt-water intrusion causing salinization of soils in the coastal areas. At the same time, higher temperatures increase evaporation of the water from the soil leading to shallow water tables and accumulation of salt ions in the soil (Corwin, 2021). While low levels of salt may even increase yield in certain horticultural species as spinach, too high levels of salt in general lead to a reduction in yields (Shannon and Grieve, 1999). In fact, most crop species exhibit severe yield losses of around 50-80% under moderate salinity (Panta et al., 2014; Zörb et al., 2019). Together deteriorated soils and loss of area of cultivable land is threatening crop production, food security and plant biodiversity. At the same time, an increasing human population is likely to amplify the problems which are arising due to climate change (Karlova et al., 2021). One of the great challenges of our generation is therefore how to deal with the consequences of climate change to counteract the negative impact on global crop production and to secure food security worldwide (Raza et al., 2019; Karlova et al., 2021). Together with adapting

agricultural practices, breeding of crops that can withstand more dry and warm weather and that are able to re-cultivate degraded soils may be one way to tackle these problems (Raza et al., 2019).

As sessile organisms, plants need to adapt to the surrounding conditions. Plant's indeterminate and modular growth is a prerequisite for continuous adjustment of their development to changing environmental conditions. Overall plants are remarkably plastic. This means that a single genotype can give rise to a wide range of phenotypes (De Jong and Leyser, 2012). So far breeding strategies have mainly focused on optimizing plants for uniform growth and higher yield and therefor selection has not focussed on phenotypic plasticity that would help the plant to withstand changing environments and secure yield under fluctuating unpredictable conditions (De Jong and Leyser, 2012). Gaining a better understanding of how plants adjust their development to water deficiency and soil salinity, may give insights in how we can deal with the effects of climate change on crop production.

In this thesis, I have examined how environmental conditions such as water deficiency and soil salinity affect the development of the water conducting tissue, the xylem, of the plant root. I will give insights in the molecular mechanisms driving the developmental plasticity of this tissue and discuss its potential effect on plant tolerance to water deficiency and salt stress.

The path of water uptake

The root – organ of water uptake

To understand how water deficiency and salinity affect the development of the water conducting tissue, the xylem, I will first start with a description of the water flow throughout the plant and the involved organs and tissues. Roots are the plant organs that create the contact surface to the soil. From the soil, they govern the uptake of water and mineral nutrients into the plant and by this ensuring plants supply of water and nutrients for different cellular processes. At the same time, roots are also the first organ to be exposed to different conditions of the soil including low water availability or salinity. In addition to their function in uptake, they are also important to anchor plants to the ground.

In general, roots are defined by having a meristem that allows continuous growth, a root cap that protects the meristem and positive gravitropism. They have a vasculature as well as a ground tissue that almost always contains an endodermal cell layer (reviewed in Augstein and Carlsbecker, 2018). Roots

evolved at least twice during land plant evolution, once in the lycophyte lineage and once in the euphyllophyte lineage, to which all extent ferns, gymno- and angiosperms belong and consequentially evolved a vasculature (Kenrick and Strullu-Derrien, 2014). In bryophytes that lack true roots, similar functions are carried out by rhizoids, single-or multicellular filamentous outgrowths from the epidermis comparable to root hairs found in true roots (Jones and Dolan, 2012).

The continuous activity of the meristem and the development of lateral meristems give roots the ability to adjust both the pace, direction as well as the overall architecture to the surrounding conditions. Root hairs tremendously increase the surface of the root and the growing root tip ceaselessly explores the soil for new water and nutrient sources. It has therefor been suggested, that the major part of water uptake occurs close to the root tip (McCully, 1999; Taiz et al., 2015). However, also older root tissue in especially succulent species contributes to water uptake (North and Baker, 2007).

Uptake of water by the roots is dependent on differences in water potential of the surrounding soil and the plant's root cells. Water follows a gradient from regions of higher to regions of lower water potential. Uptake of water into the root occurs when the water potential of the root cells is lower than that of the surrounding soil. The cellular water potential in turn depends on the osmotic potential (Ψ_s), the turgor pressure (Ψ_p) and the matric potential (Ψ_m), which are regulated by the content of solutes, the pressure of the cell content against the cell wall and adhesion, respectively (Bray, 2007). After uptake by the root from the soil, water, minerals and nutrients are transported radially from the outer cell layers into the stele. This transport is mediated both by apoplastic transport through the cell walls as well as trans-cellular, across membranes and water channelling aquaporins, and through symplastic transport via plasmodesmata (Newman, 1976; Taiz et al., 2015). Hereby, the endodermis with its Casparian strip plays an important role as a selective barrier. Lignin and suberin deposition in the cell wall of the endodermis forces the water flow to shift from apoplastic to symplastic transport (Geldner, 2013). From the central root, water and nutrients are primarily transported passively by the transpiration flow via the xylem to the shoot.

The vasculature –tissue of transport

The plant's vasculature is the conducting tissue that connects the root with the shoot and provides a path for the transport of water, nutrients and sugars. Water and nutrients are transported from the root to the upper part of the plant by the xylem. Photosynthesis products, hormones and other substances are transported and distributed via the phloem. In this thesis, I focus on the root xylem,

as it is an intriguing question whether the development of the water conducting tissue of the plant is affected by the water availability and salt that would be transported within the water flow if not excluded from the root xylem (Møller et al., 2009).

In general, the water conducting tissue is composed of cells that have undergone programmed cell death (PCD) and form a dead hollow cylinder to minimize resistance in water transport. During the evolution of vascular plants, two types of water conducting tissue also referred to as tracheary elements (TE) have evolved: Tracheids and vessel elements. Tracheids are elongated, spindle shaped cells that are found in both angiosperms, gymnosperms, ferns and other vascular plants. Compared to tracheids, vessel elements are shorter, wider and characterized by a perforation plate at the end of each cell. The perforation plate enables stacking vessel elements on each other forming a conduit that is called the vessel. Due to their bigger radius, vessel elements are more effective in water transport, as the water flow in tubes dramatically increases with the radius. Vessel elements are commonly found in angiosperms but are lacking in most gymnosperms and ferns. They are therefore considered to be evolutionarily younger. Both tracheids and vessel elements expose pits in their lateral walls, which are characterized by the absence of a secondary cell wall (SCW) allowing the water to flow between neighbouring TEs (Esau, 1965; Taiz et al., 2015).

During the development of the plant, the first water conducting tissue is the primary xylem that is formed close to the apical meristem. The primary xylem is derived from the procambium, which is localized in the center of the stele, surrounded by the ground and dermal tissues. Depending on the pattern of SCW thickening, the primary xylem can be divided into protoxylem and metaxylem. In general, protoxylem is formed first during development (Evert and Eichhorn, 2013). Its spiral or annular SCW allows it to expand during elongation of the surrounding tissues. Later metaxylem, which displays pitted or reticulated SCWs and usually has a bigger diameter, is developed. Metaxylem contributes most to water transport in older plants (Kim et al., 2014). In general, an exarch xylem pattern with protoxylem at the periphery and metaxylem in the center is found in roots.

During secondary growth, secondary xylem is differentiated from the vascular cambium that is located between the secondary phloem in the periphery and the secondary xylem in the center (Benfey and Scheres, 2000). The secondary xylem consist of both the water conducting TEs but also xylem fibers and xylem parenchyma (Schuetz et al., 2013). Xylem fibers are dead cells that have structural functions, while xylem parenchyma cells are living cells involved in the storage of various compounds (Evert and Eichhorn, 2013).

The vasculature can be arranged in different ways. In the roots of most extant vascular plants, the vasculature is arranged in a protostele, a stele with a central xylem. In the roots of monocots, the vasculature is arranged in an eustele, with several vascular bundles surrounding a pith containing parenchyma cells (Raven and Edwards, 2001). This arrangement complicates the observation of the xylem along the longitudinal axis, which is one reason why I focussed on dicotyledonous species for this thesis.

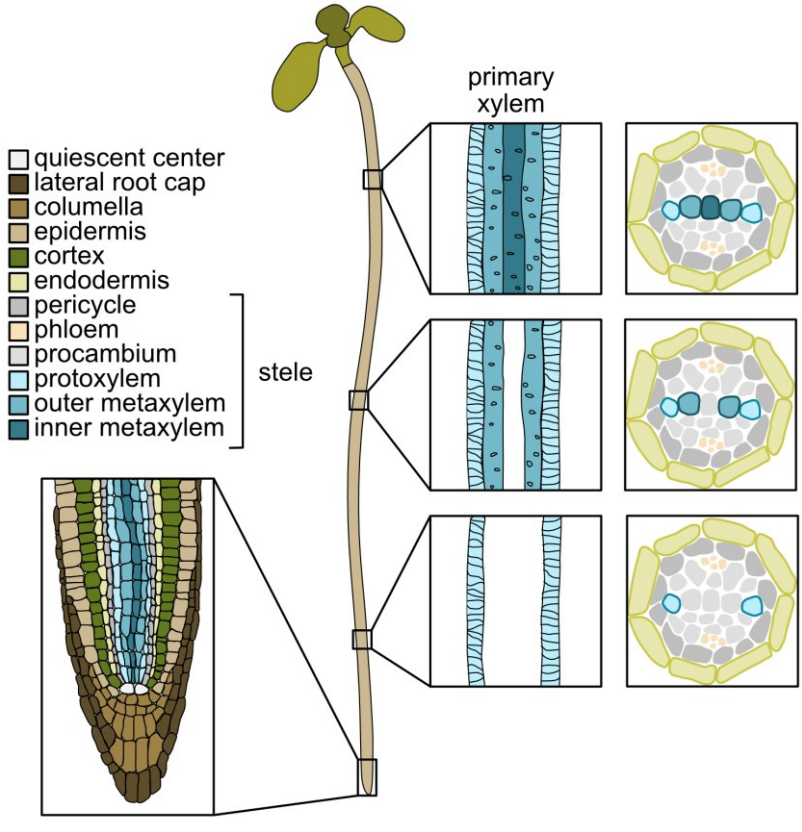

Figure 1: Xylem development in the *Arabidopsis thaliana* seedling. Illustration of the root meristem with tissue types indicated with different colours (bottom left). Xylem precursor cells differentiate into spiral-walled protoxylem and pitted metaxylem vessels. To the right cartoons showing xylem differentiation along the root both in longitudinal views and as cross sections (stele surrounded by endodermis without outer cell layers). Protoxylem vessels form close to the root tip followed by the outer metaxylem. Finally, an inner metaxylem differentiates.

The brassicaceae family member *Arabidopsis thaliana* (Arabidopsis) has been primarily used as a model plant to study root xylem development in this thesis. Its thin root as well as a high degree of pattern robustness of the root meristem

across individuals make it easy to trace cells and to analyse cell division, cell differentiation and tissue patterning (Dolan et al., 1993; Benfey and Scheres, 2000). New root cells are produced in the root meristem by the initial cells. The Arabidopsis meristem consist from the outside to the inside of epidermis, cortex, endodermis, and stele founder cells. The stele is built up by the pericycle, the xylem precursors, phloem precursors and the procambium (Figure 1). Shootward of the meristematic zone, cells undergo elongation in the elongation zone and finally reach differentiation in the differentiation zone. In Arabidopsis roots, an axis of the primary xylem transverses the stele leading to a diarch protostele arrangement, with two phloem poles that are perpendicular to the xylem, divided by the procambium and surrounded by the pericycle. Outside of the pericycle, the endodermal layer, ones fully differentiated, controls the uptake of water and nutrients into the stele with its suberization and lignified Casperian strip. The endodermis is surrounded by one layer of cortex and the epidermis on the outside. Close to the root meristem, two strands of protoxylem are formed from the xylem precursor cells at the periphery of the xylem axis. Further up, two metaxylem strands within the xylem axis and adjacent and to the protoxylem strands differentiate and even higher up, an inner metaxylem strand will differentiate to complete the primary xylem with five xylem cells in the xylem axis (Figure 1).

The shoot – place of transpiration

From the root, water and nutrients are transported via the xylem into the shoot and finally to the leaves. In the stem of dicotyledonous plants, the xylem is arranged in a ring of vascular bundles surrounding a central parenchymatous pit, while in monocots, scattered vascular bundles are usually observed. The stem of vascular plants normally exhibits an endarch xylem with protoxylem in the center and metaxylem in the periphery. In the vascular bundles of the Arabidopsis inflorescence stem, xylem is formed in the center and phloem periphery. In the leaves of dicotyledonous plants, xylem is formed at the upper, adaxial, side and phloem at the lower, abaxial, side of the leave.

In an adult plant, the water flow through the xylem is mainly driven by the loss of water at the leaves. A decreased water potential of the leaves creates a water potential gradient that controls the water movement from the roots to the xylem. From the xylem, water is pulled into the cell walls of the leaf mesophyll cells and evaporates in the airspace of the leaf. Water and CO_2 are needed for the production of sugars during photosynthesis. For photosynthesis plants need to open their stomata to uptake CO_2. At the same time, this leads to water loss due to transpiration. Only a small amount of the water transported up to the leaves is actually used for photosynthesis. The bigger fraction of water is needed to maintain the turgor of the plant by compensating for the

water loss due to transpiration. The amount of water that is used for photosynthesis is determined as the water use efficiency (WUE), which is defined by the amount of biomass per unit of water used by the plant (Briggs and Schantz, 1913).

Regulation of xylem development

Xylem patterning and specification

The described xylem pattern of the root is determined already in the embryo and propagated in the root meristem (De Rybel et al., 2014). Hereby, initial auxin signals from the two cotyledons may specify the diarch xylem pattern in the Arabidopsis embryo. However, mathematical modelling revealed that, the balanced action of auxin and cytokinin signalling domains in response to the stele diameter is sufficient to control xylem pole number (Mellor et al., 2019). Auxin is channelled towards the central part of the stele due to polar auxin transport via PIN-FORMED 1 (PIN1) and lateral auxin transport from the procambium via PIN3 and PIN7, forming the immature xylem axis (Bishopp et al., 2011). High levels of auxin in the xylem axis trigger the activation of *MONOPTEROS* (*MP*) (Schlereth et al., 2010). MP in turn activates *TARGET OF MONOPTEROS 5* (*TMO5*) (Schlereth et al., 2010). TMO5 together with its interaction partner LONESOME HIGHWAY (LHW) activate *LONELY GUY 3* (*LOG3*) and *LOG4* resulting in cytokinin biosynthesis within the xylem axis (Ohashi-Ito et al., 2014; De Rybel et al., 2014). Cytokinin diffuses into the procambium where high cytokinin signalling promotes cell division and the bisymmetric distribution of PIN3 and PIN7 reinforcing auxin accumulation in the xylem axis. Thus, in embryos of the *tmo5 t5l1* double mutant fewer cells are forming in the vascular tissue (De Rybel et al., 2013; Ohashi-Ito et al., 2014). In the xylem axis, MP induces the expression of *ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN 6* (*AHP6*) an inhibitor of cytokinin signalling. *AHP6* is specifically expressed in the protoxylem cells, and the neighbouring pericycle. There AHP6 suppresses cytokinin signalling, promoting the formation of distinct auxin and cytokinin domains within the stele (Bishopp et al., 2011). Consistently, both the weak mutant allele *mpS319* and the *ahp6* mutant display discontinuous protoxylem strands, supporting the importance of auxin-cytokinin balance in vascular specification (Müller et al., 2016; Figure 2).

Xylem development has been strongly connected to the activity of class III homeodomain-leucine zipper (HD-ZIP) transcription factors (Carlsbecker et al., 2010; Miyashima et al., 2011; Dello Ioio et al., 2012; Müller et al., 2016). HD-ZIP transcription factors are a plant-specific group of transcription factors that have functions in developmental and stress response processes. They got

their name because they are containing both a homeodomain (HD) for DNA binding and a leucine zipper dimerization domain (ZIP), indicating that they are acting as dimers (Sessa et al., 1998). HD-ZIP transcription factors can be classified into four subfamilies (Ruberti et al., 1991; Mattsson et al., 1992; Schena and Davis, 1992; Ariel et al., 2007). Members of subfamily class III HD-ZIPs contain a HD, LZ, steroidogenic acute regulatory protein-related lipid transfer (START), a START adjacent domain (SAD) domain and a MEKHLA domain, which is similar to the PAS domain, found in many proteins of all kingdoms and has been connected to light, oxygen and redox potential sensing (Mukherjee and Bürglin, 2006; Ariel et al., 2007). Five transcription factors are included in this family: Arabidopsis HOMEOBOX 8 (ATHB8), PHABULOSA (PHB/ ATHB14), PHAVOLUTA (PHV/ ATHB9), CORONA (CNA/ ATHB15) and REVOLUTA (REV) which is also known as INTERFASCICULAR FIBRELESS 1 (IFL1) (Sessa et al., 1998; Ariel et al., 2007). In many processes the five members of the HD-ZIP III transcription factors act in a redundant or partially redundant manner, as it is seen that single mutants often do not display a phenotypical difference (Emery et al., 2003; Prigge, 2004; Carlsbecker et al., 2010). HD-ZIP III transcription factors are involved in the control of various developmental processes, including determination of root apical meristem size, the vasculature and the leaves (McConnell et al., 2001; Ariel et al., 2007; Dello Ioio et al., 2012; Ramachandran et al., 2016).

In vascular development, HD-ZIP III transcription factors (TF) are part of a regulatory network that controls vascular specification as well as differentiation. As described in the previous chapter, auxin is essential for xylem specification. Also expression of HD-ZIP III transcription factors is induced by auxin in the xylem axis (Ursache et al., 2014). In addition to MP's above described role in the establishment of auxin and cytokinin domains in the stele, MP directly activates *ATHB8* within its expression domain (Baima et al., 1995; Donner et al., 2009). As described previously, the primary xylem of the Arabidopsis root consists of protoxylem at the periphery and metaxylem in the central part of the stele. This pattern is established by the bidirectional communication between the stele and the endodermis resulting in a post-transcriptional control of HD-ZIP III levels through micro RNAs, miR165/166 (Reinhart et al., 2002; Carlsbecker et al., 2010; Miyashima et al., 2011). The transcription factor *SHORTROOT* (*SHR*) is expressed in the stele and moves to the endodermis (Helariutta et al., 2000), where it, together with SCARECROW (SCR), activates the expression of *MIR165A*, *MIR166A* and *MIR166B*. In turn, miR165/166 moves from the endodermis into the stele, where they suppress the mRNA levels of HD-ZIP III transcription factors (Carlsbecker et al., 2010; Miyashima et al., 2011; Figure 2). High levels of HD-ZIP III promote metaxylem formation, while protoxylem is formed if the level of HD-ZIP III are lower (Carlsbecker et al., 2010). Consistently, the

quadruple HD-ZIP III loss-of function *phb phv cna athb8* mutant only has protoxylem and in the *phb-7d* mutant, in which a point-mutation in the miR165/166 targeting site renders *PHB* mRNA less susceptible to miR165/166 regulation, metaxylem is formed in place of protoxylem (Carlsbecker et al., 2010). In fact, mathematical modelling has revealed that the signaling pathways through SHR, miR165/6 and PHB are required to maintain xylem patterning (Muraro et al., 2014)

Several studies have implicated the interconnection of HD-ZIP III action and hormone signalling in xylem development, in particular auxin and cytokinin signalling. REV upregulates the expression of auxin biosynthesis genes *TRYPTOPHAN AMINO-TRANSFERASE OF ARABIDOPSIS 1* (*TAA1*) and *YUCCA 5* (*YUC5*) (Brandt et al., 2012) and direct binding of REV to the promoters of the auxin influx carriers *AUXIN RESISTANCE 1* (*AUX1*), *LIKE AUXIN RESISTANCE 2* (*LAX2*), *LIKE AUXIN RESISTANCE 3* (*LAX3*), which promote xylem differentiation, has been reported (Baima et al., 2014; Huang et al., 2014; Fàbregas et al., 2015). Additionally, PHB mediates an auxin signalling loop. After induction through auxin, PHB directly binds to the promoters of auxin signalling components *MP* and *IAA20*, activating their expression (Müller et al., 2016). Consistently, auxin signalling in the xylem axis is impaired in the PHB gain-of-function mutant *phb-7d* due to activation of the auxin signaling repressor *IAA20* (Müller et al., 2016).

Hormonal regulation of xylem differentiation

The xylem precursor cells differentiate into conducting elements by a sequence of events including proliferation, expansion, patterned SCW deposition, PCD and post-mortem differentiation that results in functional hollow capillaries with a thick secondary cell wall (Figure 2). The SCW of xylem cells consist of cellulose, hemicellulose, pectin and lignin (Mellerowicz and Sundberg, 2008). The *CELLULOSE SYNTHASE* (*CESA*) gene family is involved in cellulose biosynthesis. CESA proteins of higher plants can be divided in six different classes. While three classes represented by CESA1, CESA3 and CESA6 are involved in cellulose synthesis in the primary cell wall, CESA4, CESA7 and CESA8 are important for cellulose synthesis in the SCW (Taylor et al., 2003; Desprez et al., 2007; Persson et al., 2007). Hemicellulose biosynthesis is controlled by different *IRREGULAR XYLEM* (*IRX*) genes, *CELLULOSE SYNTHASE LIKE* (*Csl*), *GALACTURONOSYLTRANSFERASE-LIKE* (*GATL*), *GLUCURONIC ACID SUBSTITUTION OF XYLAN* (*GUX*), *XYLOGLUCAN ENDOTRNASGLUCOSYLASE/HYDROLASE* (*XTH*), *FRAGILE FIBER* (*FRA*) and more. Pectin is found in the junction between xylem and fibre cells in woody tissue. It plays an important role in both primary and SCW and its biomechanical properties are determined by the degree and pattern of methylesterification (Mohnen, 2008; Wormit and Usadel,

2018). Pectin methylesterification is controlled by *PECTIN METHYLESTERASE* (*PME*) and *PECTIN METHYLESTERSE INHIBITOR* (*PMEI*) (Wormit and Usadel, 2018). Lignin is an essential component of the xylem SCW, as it provides both strength and water impermeability. Lignin synthesis can be divided into two major steps. The first step is the biosynthesis of monolignols, which is controlled by several enzymes including phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate coenzyme A ligase (4CL), ferulate 5-hydroxylase (F5H), *p*-coumarate 3-hydroxylase (C3H), *p*-hydroxycinnamoyl-CoA:quinate/shikimate hydroxycinnamoyl transferase (HCT), caffeoyl-CoA *O*-methyltransferase (CCoAOMT), cinnamoyl-CoA reductase (CCR), caffeic acid *O*-methyltransferase (COMT), and cinnamyl alcohol dehydrogenase (CAD) (Wang et al., 2013). In the second step, lignin polymerization in the cell wall is controlled by laccases such as LACCASE 4 and 17 (LAC4 and LAC17) (Berthet et al., 2011) and xylem class III peroxidases for example PEROXIDASE 66 (PRX66) or PRX72 (Marjamaa et al., 2009; Tokunaga et al., 2009; Hoffmann et al., 2020).

Different trans-differentiation cell culture systems have illustrated the involvement of several hormones in xylem differentiation. Ectopic xylem differentiation in cotyledons can be induced in vitro by a combination of auxin, cytokinin and brassinosteroids (BR) in the KDB system (Tan et al., 2018) or auxin, cytokinin and bikinin, a chemical inhibitor of GSK3 kinases that activates BR signalling (De Rybel et al., 2009) in the Vascular Cell Induction Culture System Using Arabidopsis Leaves (VISUAL) (Kondo et al., 2014; Kondo et al., 2015; Kondo et al., 2016). In these systems, auxin and cytokinin are needed for TE differentiation (Fukuda and Komamine, 1980; Kubo et al., 2005; Kondo et al., 2015; Tan et al., 2018). However, it seems that they primarily function in the early reprogramming of mesophyll cells and by this they are likely to be involved in xylem specification rather than in the actual differentiation (Milioni et al., 2001; Bollhöner et al., 2012). In general, it is difficult to fully separate specification and differentiation processes in these systems as xylem differentiation only will occur if the cells have acquired xylem identity and xylem specification will under normal conditions lead to xylem differentiation. I will therefore in the following use the term xylem differentiation even if maybe specification is affected.

BR induces TE differentiation through induction of genes related to SCW formation and PCD (Yamamoto et al., 1997; Fukuda, 2004). BR is an early signal to induce PCD-specific genes including hydrolytic enzymes as cysteine proteases, serine proteases, RNases, S1-type nucleases, acid phosphatases and lipases. These enzymes accumulate in the vacuole. Treatment of *Zinnia elegans* cell cultures with brassinolide or uniconazole, an inhibitor of BR biosynthesis, revealed that BR is critical for expression of genes involved in the final phase of xylem differentiation (Yamamoto et al., 1997). BR may be important in

regulation of xylem specification via HD-ZIP III transcription factors but also during TE differentiation (Fukuda, 2004). Additionally, ethylene plays a role in xylem differentiation (Pesquet and Tuominen, 2011). Blocking ethylene signalling using silver thiosulphate inhibits TE differentiation and ethylene accumulates in maturing *Zinnia elegans* TEs (Pesquet and Tuominen, 2011; Bollhöner et al., 2012). However, ethylene's function in xylem development is not supported by ethylene biosynthesis or signalling mutants (Bollhöner et al., 2012). Another study in *Zinnia elegans* xylogenic culture revealed that gibberellin (GA) is important for TE differentiation and lignification. Treatment with GA slightly increased TE formation and strongly induced lignin formation by activating polymerization of lignin precursors (Tokunaga et al., 2006). In the VISUAL system, GA signalling is needed for vascular differentiation during the transversion of mesophyll to procambial cells (Yamazaki et al., 2018). The polyamine thermospermine, a structural isomer of spermine, suppresses xylem differentiation by limiting auxin signalling (Yoshimoto et al., 2012). The thermospermine synthase genes, *ACAULIS 5* (*ACL5*) is specifically expressed in the xylem vessels prior to SCW deposition (Muñiz et al., 2008). Mutation in *ACL5* results in earlier xylem differentiation due to an early initiation of PCD, while exogenous thermospermine inhibits xylem differentiation in xylogenic cell cultures of *Zinnia elegans* (Kakehi et al., 2010). ACL5 acts through a negative feedback loop via inhibition of auxin signaling and HD-ZIP III transcription factors, in particular ATHB8 (Baima et al., 2014) and a negative feedback-loop involving TMO5-LHW (Katayama et al., 2015; Vera-Sirera et al., 2015). In turn, treatment with xylemin, an inhibitor of thermospermine biosynthesis, result in excessive xylem differentiation (Yoshimoto et al., 2016).

The transcriptional hierarchy to control xylem differentiation

Because xylem cells have to undergo PCD, their differentiation is a terminal process. In the following, I will describe the transcriptional regulation of xylem differentiation including a hierarchy of different transcription factors regulating SCW synthesis and PCD. A cascade of transcription factors with a set of NAC domain transcription factors as master regulators precisely coordinate cell wall thickening. The NAC domain is localized at the N-terminal part of the protein and got its name from the *NO APICAL MERISTEM* (*NAM*) gene from *Petunia hybrida* and the Arabidopsis genes *ATAF1/ATAF2* and *CUP-SHAPED COTYLEDON2* (*CUC2*) (Aida et al., 1997). In vascular plants, several NAC domain transcription factors act as early regulators of xylem differentiation and cell wall modification. These transcription factors belong to the VND/NST/SND or VNS clade. In cell-culture trans-differentiation assays, 15 Arabidopsis VNS have been identified of which 10 belong to the VASCULAR-RELATED NAC DOMAIN (VND) and NAC SECONDARY WALL THICKENING PROMOTING FACTOR (NST) subgroups (Kubo et al.,

2005; Zhao et al., 2005). Comparative studies in the non-vascular plant *Physcomitrium patens*, show that *PpVNS* is required for death of the water conducting hydroids and cell wall thickening in supporting stereids indicating the VNS function precedes the evolution of xylem (Xu et al., 2014). *SECONDARY WALL NACs* (*SWNs*), in Arabidopsis including SND1, VND6, VND7, NST1 and NST2 bind to a SECONDARY WALL NAC BINDING ELEMENT (SNBE), an imperfect palindromic 19-bp consensus sequence, in the promoter of their targets (Zhong et al., 2010).

VND transcription factors are master regulators of TE-differentiation, while SND1 and NST control xylem fibre differentiation (Kubo et al., 2005; Mitsuda et al., 2005; Zhong et al., 2006; Mitsuda et al., 2007). VND transcription factors are upregulated during xylem vessel formation and expressed in the procambium or the xylem (Kubo et al., 2005). Recently, VND1, VND2 and VND3 have been shown to be important in xylem vessel formation in cotyledons (Tan et al., 2018). Overexpression of *VND6* and *VND7* lead to the ectopic formation of TEs, with metaxylem or protoxylem characteristics, respectively (Kubo et al., 2005). Analyses of truncated versions of VND7 revealed that the VND7 C-terminal region is important for normal development of proto- and metaxylem in roots and metaxylem in aerial organs (Yamaguchi et al., 2008). VND7 activates both downstream transcription factors but also directly a number of non-transcription factor genes that contain a SNBE site in their promoter involved in secondary wall biosynthesis, cell wall modification, and programmed cell death (Zhong et al., 2010). Among the downstream targets of VND7 are *MYB46*, *SND3*, *LATERAL BOUNDARY DOMAIN FAMILY PROTEIN 15* and *30* (*LBD15* and *LBD30*), *XND1,* and secondary wall biosynthesis and cell wall modification related genes including *I14H*, *IRX10*, *CslB01*, *CslB02* and peroxidases (Zhong et al., 2010).

Xylem development is regulated by a highly interconnected hierarchy of developmental switches including a series of feed-forward loops (Taylor-Teeples et al., 2015). HD-ZIP III transcription factors REV and PHV bind the *VND7* promoter regulating xylem differentiation (Endo et al., 2015). At the same time, VND7 suppresses *REV* in a negative feedback loop (Taylor-Teeples et al., 2015). Besides being important for vascular specification and patterning, HD-ZIP III transcription factors have also been implicated in controlling vascular differentiation. REV is a positive regulator of xylem cell wall depositions especially in xylem fibers (Zhong and Ye, 1999), as shown by a decrease of xylem fibers in the *rev* mutant and increased SCW thickening when *REV* is upregulated (Zhong and Ye, 1999; Liu et al., 2014).

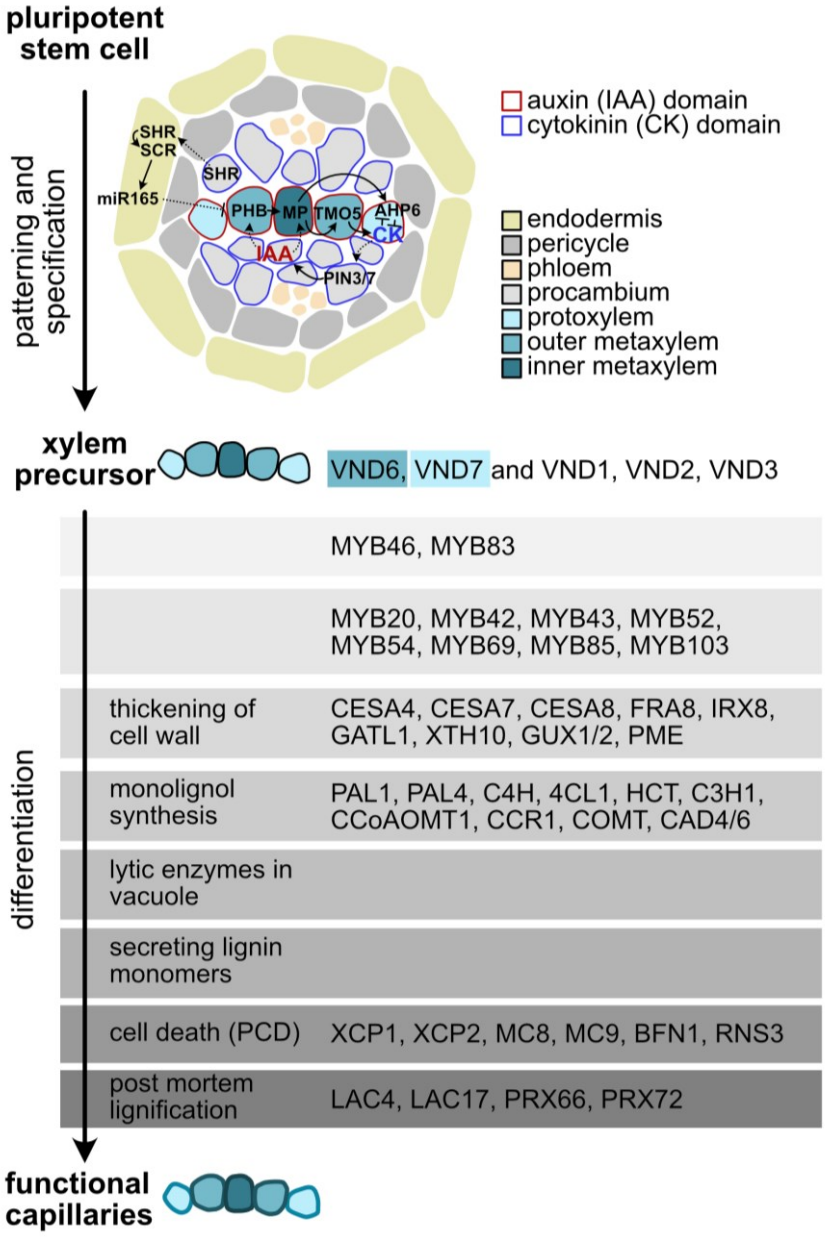
pluripotent stem cell
patterning and specification
SHR
SCR
SHR
miR165
PHB
MP
TMO5
AHP6
CK
IAA
PIN3/7
auxin (IAA) domain
cytokinin (CK) domain
endodermis
pericycle
phloem
procambium
protoxylem
outer metaxylem
inner metaxylem
xylem precursor
VND6, VND7 and VND1, VND2, VND3
differentiation
MYB46, MYB83
MYB20, MYB42, MYB43, MYB52, MYB54, MYB69, MYB85, MYB103
thickening of cell wall
CESA4, CESA7, CESA8, FRA8, IRX8, GATL1, XTH10, GUX1/2, PME
monolignol synthesis
PAL1, PAL4, C4H, 4CL1, HCT, C3H1, CCoAOMT1, CCR1, COMT, CAD4/6
lytic enzymes in vacuole
secreting lignin monomers
cell death (PCD)
XCP1, XCP2, MC8, MC9, BFN1, RNS3
post mortem lignification
LAC4, LAC17, PRX66, PRX72
functional capillaries

Figure 2: Regulation of xylem specification and differentiation. Pluripotent stem cells specify into xylem precursor cells that differentiate into functional capillaries. Signalling circuits establishing vascular patterning and controlling xylem specification are shown on top of a cross section of the stele and the endodermis. Hormones are in bold red (auxin, IAA) and blue (cytokinin, CK) letters. Arrows indicate activation, bars inhibition. Dashed arrows indicate movement. Xylem patterning is controlled by the balanced action of IAA and CK together with the action of miR165 and HD-ZIP III, exemplified by PHB (Muraro et al., 2014; Mellor et al., 2019). VND transcription factors are master regulators of xylem differentiation, with VND6 inducing metaxylem and VND7 protoxylem formation (Kubo et al., 2005) and VND1, 2 and 3 involved in xylem vessel formation in cotyledons (Tan et al., 2018). Xylem precursor cells differentiate into functional xylem vessels under the control of different transcription factors and enzymes by a sequence of events including thickening of cell wall, monolignol synthesis and secretion, programmed cell death (PCD) and postmortem differentiation. Examples of factors involved in the different steps of xylem differentiation are shown (Hussey et al., 2013; Yu et al., 2021).

VND6 and VND7 act upstream of many genes that control xylem differentiation and SCW deposition. Downstream of VND7 and VND6, MYB46 and MYB83 are central second level regulators of xylem differentiation (Zhong et al., 2008). MYB46 and MYB83 are thought to be functional redundant. Overexpression of *MYB46* or *MYB83* activates biosynthesis of cellulose, and lignin, while simultaneous knockout of *MYB46* and *MYB83* result in no formation of secondary walls (Zhong et al., 2010). However, it has been suggested that VND7 initiates xylem cell identity with a sharp switch. Only a small subset of genes is involved in generating the switch, while other downstream targets as MYB46 are essential for SCW synthesis but not for the identity switch (Turco et al., 2019). MYB46 and MYB83 in turn regulate the expression of other MYB transcription factors such as *MYB20*, *MYB42*, *MYB43*, *MYB52*, *MYB54*, *MYB69*, *MYB85*, *MYB103* and other transcription factors such as *SND2* and *SND3*. These on the other hand regulate the biosynthesis of cellulose, hemicellulose and lignin. Thus xylem differentiation is regulated by a complex network of transcription factors acting on different levels (Hussey et al., 2013; Yu et al., 2021).

While most of the identified NAC domain transcription factors are positive regulators of xylem development, there are also examples of negative regulators. The *XYLEM NAC DOMAIN 1* (*XND1*) is highly expressed in the xylem (Kubo et al., 2005; Zhao et al., 2005) most often associated with differentiating TEs (Zhao et al., 2008). *XND1* and its homologs are specific to angiosperms (Zhao et al., 2017). Overexpression of XND1 lead to dwarf growth and severely affected xylem formation, with gaps in xylem or totally absence of xylem due to failure in SCW depositions and PCD (Zhao et al., 2008). Thus, XND1 influences TE size and xylem development by negatively regulating secondary wall synthesis and PCD (Zhao et al., 2008). XND1 action on xylem differentiation depends on a protein-protein interaction with the cell cycle and

differentiation regulator RETINOBLASTOMA-RELATED (RBR) due to a highly conserved C-terminal region. Also *XND1* is a direct target of VND7 (Zhong et al., 2010). *VND-INTERACTING 2* (*VNI2*) is another NAC transcription factor that negatively regulate TE differentiation. This negative regulation depends on its interaction with VND7 and VND6 and repression of downstream targets (Yamaguchi et al., 2010; Zhong et al., 2021), indicating a complex network of negative-feedback loops.

To form functioning xylem vessels the conducting element has to undergo PCD to form a hollow cylinder for water transport. Most pharmacological agents that block xylem cell death inhibit at the same time SCW formation indicating a co-regulation of these two steps of xylem differentiation (Bollhöner et al., 2012). Xylem PCD occurs autonomously without input from other cells and is controlled by several genes involved in autolysis. During PCD lytic enzymes and monolignols are secreted. Consequently, primary cell walls are degraded by specific expression of cell-wall-degrading enzymes in the developing TE (Milioni et al., 2001; Demura et al., 2002; Fukuda, 2004). *XYLEM CYSTEINE PEPTIDASE 1* and *2* (*XCP1* and *XCP2*) are specifically expressed in xylem vessels (Funk et al., 2002). Both enzymes are located in the vacuole where they redundantly control autolysis (Avci et al., 2008). Also *METACASPASE 9* (*MC9*) is specifically expressed in differentiating xylem elements and it functions in the degradation of vessel cell contents after rupture of the vacuole (Bollhöner et al., 2013). The *BIFUNCTIONAL NUCLEASE 1* (*BFN1*) is generally expressed in tissues that undergo senescence and developmental cell death and together with *RIBONUCLEASE 3* (*RNS3*) it is involved in the degradation of nuclear DNA and RNA after TE cell death (Perez-Amador et al., 2000; Ohashi-Ito and Fukuda, 2010). Interestingly, many of these PCD related genes are under the control of VND6 or VND7, indicating that VND6 and VND7 are not only master regulators for SCW biosynthesis but also control cell death during xylem differentiation (Ohashi-Ito and Fukuda, 2010; Bollhöner et al., 2012).

As the last step of xylem differentiation, after the TE have undergone programmed cell death, they undergo a post-mortem lignification with the help of neighbouring parenchyma cells that contribute with lignin monomers as well as needed enzymes (Pesquet et al., 2013; Figure 2).

Secondary xylem specification and differentiation

Even though I am focussing on the primary xylem development in this thesis, I will briefly discuss the action of different hormones during secondary xylem development. During secondary development, phloem on the outside and xylem on the inside are proliferated due to periclinal cell divisions that originate from the vascular cambium (Shi et al., 2019). Auxin maxima in cells direct

adjacent to the vascular cambium on the xylem side, promote xylem identity as well as an organizer function of these cells to specify xylem and phloem cells during secondary growth. Auxin induces *MP*, *AUXIN RESPONSE FACTOR 7* (*ARF7*) and *ARF19*, that in turn induce the expression of HD-ZIP III transcription factors, in particular *ATHB8* defining xylem identity (Smetana et al., 2019). Furthermore, cytokinin has been shown to be important for cambial cell proliferation and by this controlling secondary development (Matsumoto-Kitano et al., 2008). Loss-of-function of BR-receptors cause abnormal phloem:xylem differentiation ratios in the secondary xylem (Caño-Delgado et al., 2004). Abscisic acid (ABA) signalling controls xylem lignification in the inflorescence stem (Liu et al., 2021). Additionally, mutants defective in ABA biosynthesis have delayed fibre production, indicating an essential role for ABA in the regulation of fibre formation (Campbell et al., 2018). Even GA enhances fibre formation. GA induces increased lignin content and SCW thickening as well as greater number of fiber-like cells but not TEs in *Nicotiana tabacum* plants (Falcioni et al., 2018). Enhanced GA biosynthesis in hybrid aspen and Arabidopsis inflorescence stems induces the formation of higher amounts and longer xylem fibers (Eriksson et al., 2000; Ragni et al., 2011). Additionally, GA increases the relative amount of xylem compared to phloem during secondary development (Mauriat and Moritz, 2009; Ragni et al., 2011; Felipo-Benavent et al., 2018). In the Arabidopsis hypocotyl the xylem expansion was shown to be regulated via a cross-talk between auxin and GA during secondary growth (Ben-Targem et al., 2021). GA plays a role in vascular lignification in several different species (Eriksson et al., 2000; Mauriat and Moritz, 2009; Gou et al., 2011; Ragni et al., 2011; Guo et al., 2015; Wang et al., 2017; Singh et al., 2019). In birch, cell wall biosynthesis related genes including *CESA* and *PAL* were induced by GA3 and decreased by treatment with the GA-biosynthesis inhibitor paclobutrazol (PAC) (Guo et al., 2015). Similarly, xylem lignification was increased by GA in sweet potato and carrot roots (Wang et al., 2017; Singh et al., 2019). Furthermore, GA signalling is involved in processes related to cell wall synthesis and cell wall remodelling (Locascio et al., 2013). Concluding, secondary xylem development is tightly regulated by an interplay of different hormones that may have similar function during primary xylem development.

Symptoms and responses of plant roots to water deficiency and salt

Symptoms of drought and salt stress

In the previous chapters, I describe how plants take up water from the soil and how it is transported via the xylem to the shoot where it is needed to compensate for the water loss during transpiration. Furthermore, I have described the

details of xylem development regulation under well-watered conditions. However, during their life plants may be subjected to periods of low water availability due to drought or osmotic changes that prevent water uptake for example caused by salt. In the following chapter, I will discuss how these conditions affect the plant.

Drought is a weather event usually defined as a period without rainfall leading to a shortage of water that occurs in almost all parts of the world and is especially common in arid and semiarid regions (Hossain et al., 2016). This reduced water availability may induce water deficiency stress to the plants when the transpiration rate from the leaf surface is higher than the water uptake by the roots (Hossain et al., 2016). Lowered water levels in the soil decrease the soil water potential and by this disturb the water flow from soil to root (Bray, 2007). However, drought and water deficiency may not be used as synonymous terms as certain plants have adapted to low water availability by physiological or anatomical measures. Due to that they avoid a water deficit and by this a stressful condition during drought (Bray, 2007; Hossain et al., 2016).

Plants depend on water for driving photosynthesis and maintaining their turgor pressure. A reduced possibility of water uptake therefor has strong effects on plants and may pose a variety of physiological and morphological changes. It is not always obvious whether observed morphological changes are symptoms of water deficiency stress or a developmental adjustment to acclimatize to a stressful condition and they may differ a lot depending on the plant species, developmental stages, growth condition and other environmental factors (Hossain et al., 2016). Common symptoms of water deficiency are wilting related to a drop in leaf turgor pressure, drooping, etiolation, yellowing and premature leaf downfall. Furthermore, plant growth, development and yield can be reduced substantially (Hossain et al., 2016). Plants often react to decreased water availability with stomata closure to decrease the water loss due to transpiration. However, this reaction also prevents the uptake of CO_2, which in turn leads to a lowered photosynthesis rate and by this to a decrease in energy supply (Zargar et al., 2017). The deficiency in CO_2 may also trigger the initiation of secondary stress due to the production of reactive oxygen (ROS) species (Raza et al., 2019). Under prolonged drought stress, water deficiency may lead to gas bubble expansion in the xylem, a process called cavitation. Cavitation may result in embolism, gas-filled voids, that cuts off water transport within the xylem and may impact water supply, even if water would be available again (Tyree and Sperry, 1989).

Water uptake by the plants from the soil is both affected by the water availability, but also when the water potential of the soil is lowered due to high soil salinity (Munns, 2002). Due to this fact, soil salinity may induce similar effect

in the plant as water deficiency. However, apart from osmotic stress, soil salinity exposes plants to ionic toxicity (Munns and Tester, 2008). Together with the water, sodium and chloride ions are taken up by the roots and transported throughout the plant. The accumulation of salt ions, mainly sodium, but also chloride, causes cytotoxic effects impairing photosynthesis, respiration, ion uptake and membrane integrity. For instance, chloride induces chlorosis, chlorophyll deficiency, and by this impairs photosynthesis (Tavakkoli et al., 2011). Furthermore, salt negatively affects cell cycle progression (West et al., 2004), leaf temperature, growth rate and photosynthetic efficiency (Awlia et al., 2021). Additionally, root hair length and density are decreased upon salt exposure (Wang et al., 2008). Similar to drought stress, salt stress also induces the accumulation of ROS that is linked to PCD in the meristem (West et al., 2004). Additionally, soil salinity lowers the solubility of many micronutrients and consequently affects the uptake of micronutrients that are needed in photosynthesis and protein biosynthesis (Talei et al., 2012; Zhao et al., 2021a).

In the following chapters, I will discuss the plant's ability to morphological change upon stress conditions to avoid or tolerate stress. Hereby, it is important to distinguish between genetically programmed developmental processes that confer stress tolerance and negative effects of the stress on plant growth (Abley et al., 2016).

Strategies to overcome stressful conditions

Plants react to changing environmental conditions on various levels with the changes being more or less fast and more or less permanent. Physiological and developmental responses may give the plant the possibility to overcome unfavourable conditions. Physiological responses can for example include stomata closure upon water limitation or changes in the plant's metabolism upon oxygen deprivation. These changes are relatively short term and do not necessarily lead to a morphological change of the plant. Developmental acclimation is another strategy to overcome stressful conditions. On the other hand, persistent environmental conditions may drive evolution of the species to adapt to new conditions to conquer a new habitat. The term developmental adaptation is used to describe when morphology of a plant species is changed to fit specific conditions and will be inherited between generations (Booth and Biro, 2008). By this, adaptation of a species describes an evolutionary concept. Many examples of plant species adaptation to drought and salinity can be observed in nature. Among these are the submersion of stomata in succulent and xerophyte plants, or salt tolerance and salt avoidance mechanisms of halophytes.

It is important to note that developmental acclimation and adaption are two different processes even though they may lead to similar phenotypic characteristics making it not always easy to differentiate between them (Demmig-Adams et al., 2008). In general, acclimation occurs only upon exposure to the stress, while morphological features due to adaptation will also be developed without the stressor. While these stable traits can give a certain species advantages in a specific environment, plastic acclimation responses can give a particular plant the ability to react to changes in the environment to survive environmental changes. In my thesis, I will focus on developmental acclimation of the xylem, but I will also briefly mention adaptive traits as they can give us some insights in which kind of traits may be favourable under specific environmental conditions.

Phenotypic plasticity as a premise for developmental acclimation

While more extreme environments may select for a constrained adaption of the trait, changing environments may favour the development of phenotypic plasticity (De Jong and Leyser, 2012). In this thesis, I am focusing on the plant's phenotypic plasticity as a response to environmental conditions. The term phenotypic plasticity describes the observation that genetically identical plants can differ substantially in morphology and anatomy in response to their environment. By this, plasticity enables plants to acclimate their development to the current conditions (Demmig-Adams et al., 2008). Even though morphological changes caused by a plastic response will not be inherited to the next generation, the ability of plants to modulate their development in response to the environment would be transferred to the next generation (De Jong and Leyser, 2012). This makes it possible to identify the genetic regulation of developmental plasticity.

In general, plants are incredibly plastic. Abley et al. (2016) proposed a model in which they divided plant phenotypes into invariant, plastic and variant depending on their ability to change with or without environmental cues. While an invariant phenotype is very consistent between different plants of the same genotype, variable phenotypes differ a lot independent of the environmental condition. In contrast, plastic phenotypes are controlled by the environment due to environmental conditions feeding into gene-regulatory networks that control the development of the phenotype (Abley et al., 2016). By this, developmental plasticity depends on the plant's ability to sense the environmental condition and to predict whether the unfavourable condition will persist long enough to make it worth to adjust the developmental program (De Jong and Leyser, 2012). Furthermore, the continuous nature of plant development by the action of meristems at the shoot and root apical part and lateral meristems, giving rise to root and shoot branching, facilitates plasticity. Phenotypic plas-

ticity may rely on general developmental pathways that adjust the development under stress conditions. Usually, the change is permanent for the part of the plant that is formed under the specific conditions but new parts can have other morphological and anatomical features. The irreversibility of the developmental plasticity constitutes a higher risk than biochemical or physiological adjustments where changes are reversible (Pigliucci et al., 2006).

In general, roots exhibit a high developmental plasticity. Perhaps the most visible sign of their adjustment to the environment are changes in root system architecture (RSA) upon different environmental conditions (Karlova et al., 2021; Zou et al., 2021). Upon drought stress, the RSA is altered depending on the severity of the drought, with a deeper primary root and fewer lateral roots forming (Dinneny, 2019). This phenotype promote the uptake of water from deeper soil and can be therefore considered a stress avoidance response (Dinneny, 2019). Post emergence lateral root growth is tightly controlled by environmental stimuli, such as nutrient and water availability. Exposure to salinity causes an arrest of primary and lateral root growth which is characterized by a quiescent phase and a recovery phase (Geng et al., 2013). Under salinity conditions, salt sensitive species bend away from salt to avoid salinity stress. This negative halotropism is an example of phenotypic plasticity and depends on the redistribution of auxin (Galvan-Ampudia et al., 2013). Additionally, tissue specific acclimation are observed, for example the enhanced deposition of suberin in the endodermis upon both drought and salt stress (Barberon et al., 2016).

Even though we have insights in how root developmental plasticity is regulated in response to water limiting conditions and salt, little is known about the phenotypic plasticity of the water conducting xylem and its regulation in response to environmental conditions.

Hormonal regulation of root development in response to water deficiency and salt stress

Tissue-specific hormone signallinsg regulates root phenotypic plasticity

Plant hormones are important factors in controlling plant development, as I have illustrated in the section about xylem development. Upon environmental perturbation, intrinsic hormone levels are changed. This in turn regulates developmental alteration and by this the plastic response.

The well-known stress hormone ABA plays an important role in regulating many root plastic responses upon water deficiency and salt stress. ABA is induced by both water deficiency and salt and affects the RSA. Low levels of ABA promote root growth and hydrotropism (Rosales et al., 2019; Miao et al., 2021). However, during severe water limitation, ABA inhibits both primary root growth via a GA-dependent pathway (Rowe et al., 2016) and lateral root emergence due to inhibition of auxin signalling (Orman-Ligeza et al., 2018; Orosa-Puente et al., 2018). Auxin also controls root growth by modulating cellular responses to GA (Fu and Harberd, 2003). Furthermore, ABA inhibits lateral root development at the post-emergence stage (Signora et al., 2001; De Smet et al., 2003). ABA, JA, BR and GA signalling have been suggested to be the main actors in salt stress signalling (Geng et al., 2013). High levels of ABA during early salt stress promote primary root quiescence, while lower levels during a later phase of salt stress promote root growth. BR and GA, which are reduced during an early phase of salt stress but induced during a later phase, promote growth and also JA is induced during a later phase of salt stress (Geng et al., 2013). Salinity has an even more severe effect on lateral root growth, which show an extended quiescence (Duan et al., 2013).

Plastic responses of the RSA to water deficiency and salt depend on hormone signalling in specific tissue types. Tissue-specific ABA-signalling in the cortical cells of the elongation zone controls root hydrotropism (Dietrich et al., 2017). In the context of primary root quiescence upon salt, ABA signalling in the endodermis has been identified to be specifically important (Geng et al., 2013). Additionally, the extended quiescence of the lateral roots is also dependent on endodermal ABA signalling and a cross-talk with GA that promotes lateral root growth (Duan et al., 2013). At the same time, spatial expression of GA downstream targets indicates that GA mainly acts in the cortex and epidermis during primary root quiescence in response to salt (Geng et al., 2013). Another study indicates that primary root growth under control conditions is mediated by GA-signalling specifically in the endodermis, regulating the cell expansion in the elongation zone of growing roots (Ubeda-tomás et al., 2008).

Both ABA and GA have been involved in the plastic response of root development to drought and salt stress. However, whether they are involved in plastic responses of the xylem is not clear and will be investigated in this thesis. Here, I will summarize the current knowledge about biosynthesis and signalling mechanisms of these hormones. This knowledge can give hints on how to disturb the function of these hormones and to study their effect on xylem development.

Abscisic acid – the stress hormone

ABA is a plant hormone that is probably most commonly mentioned in the context of stress response. Its important role in integrating various stress signals and controlling downstream stress responses has given it the connotation "stress hormone" (Tuteja, 2007). ABA is involved in the response to both biotic stresses such as pathogens as well as abiotic stresses such as drought, salinity, cold and flooding. Apart from its role in stress response, ABA is also involved in a variety of developmental processes such as seed maturation, seed dormancy and seedling growth where it usually acts antagonistic to GA (Hauser et al., 2011; Shu et al., 2018).

ABA is de novo synthesized from carotenoids involving the action of a cascade of different enzymes including the ZEANTHIN EPOXIDASE ABA DEFICIENT 1 (ZEP/ABA1), 9-CIS-EPOXYCAROTENOID DIOXYGENASE 3 (NCED3), SHORT-CHAIN DEHYDROGENASE REDUCTASE/ ABA DEFICIENT 2 (SDR1/ABA2), ABA ALDEHYDE OXIDASE (AAO3) and the molybdenum cofactor sulphurase ABA DEFICIENT 3 (ABA3) (Xiong and Zhu, 2003; Tuteja, 2007). Several mutants have been identified to be deficient in ABA biosynthesis including *aba1, aba2,* and *aba3* (Marin et al., 1996; Schwartz et al., 1997; Koornneef et al., 1998). In parallel to ABA biosynthesis, ABA homeostasis is controlled by activation of an inactive form of ABA to fine tune ABA levels. The BETA-GLUCOSIDASE 1 and 2 (BG1 and BG2) proteins catalyse the hydrolysis of glucose-conjugated inactive ABA in the vacuoles and the endoplasmatic reticulum (ER) to rapidly increase the levels of active ABA (Lee et al., 2006; Xu et al., 2012). In Arabidopsis, ABA inactivation occurs due to two types of reactions, hydroxylation and conjugation. Hydroxylation of the C-8' position is thought to be the predominant ABA catabolic pathway and leads to the generation of 8'-hydroxy-ABA, phaseic acid (PA), dihydrophaseic acid (DPA) and epi-DPA. The first step of this pathway is catalysed by the CYTOCHROME P450 FAMILY 707 SUBFAMILY A (CYP707A) ABA 8'hydroxylase (Kushiro et al., 2004). The products of the ABA hydroxylation can be conjugated to glucose and ABA glucosyl ester (ABA-GE) is the most widespread ABA-conjugate. In this form ABA is physiological inactive (Nambara and Marion-Poll, 2005).

Vascular tissue is probably the main site of ABA biosynthesis in unstressed plants (Nambara and Marion-Poll, 2005). AAO3, the enzyme that catalyses the last step of the ABA biosynthesis, is localized in the root tip, vascular bundles of the root, hypocotyl and inflorescence stem and the leaf veins as well as in the guard cells of dehydrated leaves (Koiwai et al., 2004). From the site of biosynthesis ABA is presumably transported throughout the plant via the vascular tissue (Nambara and Marion-Poll, 2005). In the root tip, ABA predominantly localizes in the endodermis (Ondzighi-Assoume et al., 2016).

However, monitoring ABA response using transcriptional reporters including the ABA-responsive element (ABRE) elements from *ABI1* (6xABRE_A:erGFP) or *RAB18* (6xABRE_R:erGFP) revealed that ABA response in the root tip under non-stress conditions is enriched at the quiescent center (QC), the stem cell niche and the lateral root cap and weakly in the epidermis, cortex, endodermis, pericycle and protoxylem precursor cells (Wu et al., 2018).

The canonical ABA signalling pathway as defined by Cutler et al. (2010) includes a cascade of dephosphorylation and phosphorylation events. ABA is sensed by receptors of the PYRABACTIN RESISTANCE 1 (PYR1)/ PYR1-like (PYL)/ REGULATORY COMPONENT OF ABA RECEPTORS (RCAR) START domain protein superfamily. Upon binding of ABA, these proteins build a complex with PROTEIN PHOSPHATASE 2C (PP2C) leading to an inhibition of their function (Umezawa et al., 2010). ABA INSENSITIVE 1 and 2 (ABI1 and ABI2) are PP2Cs and in the *abi1-1* and *abi2-1* mutants, ABA signalling is partially inhibited rendering the mutants insensitive to ABA (Leung et al., 1994; Meyer et al., 1994; Leung et al., 1997). Without ABA, PP2C phosphatases dephosphorylate and thereby inhibit SUCROSE NON-FERMENTING RELATED KINASE 2 (SnRK2) family proteins. When this inhibition is released upon ABA signalling, active SnRK2 phosphorylates and thereby activates ABRE and ABRE-binding factors (ABF)-type basic leucine zipper (bZIP) transcription factors (Umezawa et al., 2010). Among these bZIP transcription factors there are ABA INSENSITIVE 3 (ABI3) (Giraudat et al., 1992) and ABA INSENSITIVE 5 (ABI5) (Finkelstein, 1994). ABF transcription factors induce expression of genes that exhibit an ABA-responsive cis-regulatory elements in their promoter (ABRE) activating downstream responses.

Gibberellin – hub between plant growth and stress response

GA is a growth-stimulating hormone that is commonly associated with the "green revolution" (Silverstone and Sun, 2000). At the same time, GA is known as an antagonist to ABA. It is involved in many developmental processes, such as seed germination, organ elongation and floral transition (Shu et al., 2018). However, reduced GA levels and signalling restrict plant growth upon exposure to stresses as cold, drought, salt and flooding (Colebrook et al., 2014).

The first steps in GA-biosynthesis take place in the plastids with the action of terpene cyclases. Geranylgenaryl di-phosphate (GGDP) is catalysed by COPALYL DIPHOSPHATE SYNTHASE (CPS)/ GA REQUIRING 1 (GA1) into copalyl diphosphate (CDP) that is converted by ENT-KAURENE SYNTHASE (KS)/ GA REQUIRING 2 (GA2) into ent-kaurene. In the ER P450

monooxygenases catalyse the conversion of ent-Kaurene to GA12 aldehyde in several steps that are catalysed by the ent-kaurene oxidase GA REQUIRING 3 (GA3). In the cytoplasm, 2-oxoglutarate dependent dioxygenases catalyse in several steps the formation of the bioactive GAs in plants including GA7-oxidase, GA20-oxidase GA REQUIRING 5 (GA5) and GA-3-beta-hydroxylase GA REQUIRING 4 (GA3OX1/ GA4) (Yamaguchi and Kamiya, 2000). In total, more than 130 GAs have been identified, but only GA1, GA3, GA4 and GA7 are thought to function as bioactive hormones (Hedden and Sponsel, 2015; Binenbaum et al., 2018). GA2 oxidases (GA2OX) catalyse the reversion into bio-inactive forms of GA (Yamaguchi and Kamiya, 2000).

Tolerance to drought stress can be enhanced by treating plants with PAC, an inhibitor of the P450 monooxygenase step of GA biosynthesis, indicating that decreased GA levels confer stress tolerance (Gilley and Fletcher, 1998; Vettakkorumakankav et al., 1999). Consistently, while reduced GA-biosynthesis in the *ga20ox1/2*, *ga3ox1/2* and *ga20ox1/2/3* mutants leads to enhanced drought tolerance, the *35S:GA20ox* or *ga2ox*, possessing elevated GA levels, are less tolerant to drought (Colebrook et al., 2014). Similarly, reduced GA biosynthesis in the *ga1-3* mutant leads to enhanced survival upon severe salt stress (Achard et al., 2006). Furthermore, salt stress induces the expression of *GA2OX7*, a gibberellin-deactivating gene (Magome et al., 2008).

GA signalling involves a mechanism of degradation of the negative regulator DELLA. DELLA proteins form a subgroup of the GRAS family proteins (Hirsch and Oldroyd, 2009). This subgroup exhibits the unique conserved DELLA domain within the N terminus that is necessary for GA-dependent degradation (Fu et al., 2002). Even though DELLAs have been shown to act both as transcriptional repressors and activators, they lack a DNA-binding domain suggesting its function as a transcriptional coactivator that is in need of another transcription factor to induce or repress gene expression (Yoshida et al., 2014). In absence of GA, DELLA interacts with GA-dependent transcription factors and prevent them from DNA-binding and thus induction of gene expression. However, upon perception of GA through GA INSENSITIVE DWARF 1 (GID1) GA-receptor, a complex between GID1 and DELLA is formed and the SCF E3 ligase in a complex with SLEEPY and GA INSENSITIVE DWARF 2 ($SCF^{SLY1/GID2}$) is recruited to induce ubiquitination of DELLA and by this degradation in the 26S proteasome (Ueguchi-Tanaka et al., 2005; Griffiths et al., 2006). At the same time, GA-responsive transcription factors are released and bind to promotors of GA-responsive genes to induce gene expression.

Several plant species, such as rice, barley and tomato, encode a single DELLA gene: in rice, SLENDER (SLR) (Ikeda et al., 2001); in barley SLENDER PROTEIN 1 (SLN1) (Chandler et al., 2002); and in tomato, PROCERA

(Bassel et al., 2008). In Arabidopsis, there are five DELLA proteins, REPRESSOR OF GA (RGA), RGA-like 1 (RGL1), RGA-like 2 (RGL2), RGA-like 3 (RGL3) and GIBBERELLIC ACID INSENSITIVE (GAI) (Peng et al., 1997; Silverstone et al., 1998; Lee et al., 2002; Wen and Chang, 2002; Piskurewicz and Lopez-Molina, 2009). A high degree of redundancy has been observed and domain-switch studies have revealed that differences in function of the different DELLA proteins is caused by specific spatiotemporal expression rather than by a distinct functionality (Gallego-Bartolomé et al., 2010).

Salinity leads to a reduction of bioactive GAs and consequently to DELLA accumulation (Achard et al., 2006). Salt stress tolerance is impaired in the *della* quadruple mutant potentially through DELLA's effect on restraining the accumulation of ROS (Achard et al., 2006; Achard et al., 2008). *RGL3* expression is induced by high salinity and drought (Magome et al., 2008; Colebrook et al., 2014) and RGL3 stabilization upon salt stress in connection to IAA17 and salt-induced NO production confers salt tolerance (Shi et al., 2017). Altogether, GA levels and signalling do not only control plant growth but are also involved in response to stress.

Xylem phenotypes related to drought and salt stress

Xylem traits connected to adaption to drought and salt stress

The adaptation of plant species to drought and salt stress as well as the role of different xylem traits in stress tolerance have been analysed in various species, from monocots, herbaceous dicots to woody trees. For example, large xylem cells have been associated with high conductivity together with deeper rooting in drought-adapted bean species compared to drought-sensitive beans (Strock et al., 2021). However, several studies indicate that bigger vessels lead to a higher risk of embolism under drought stress (Lovisolo and Schubert, 1998; Lucas et al., 2013; Scoffoni et al., 2017; Levionnois et al., 2021) and that the resulting xylem cavitation is the main factor causing death because of drought in trees (Barigah et al., 2013). Studies in maize leaves have revealed that the smaller protoxylem vessels are less vulnerable to embolism (Hwang et al., 2016). Consistently, plants that produce smaller but more vessels show better performance under drought stress. In wheat, plants with a smaller xylem diameter show a higher yield during drought (Richards and Passioura, 1989). In bean (*Phaseolus vulgaris* and *Phaseolus acutifolius*) and soybean (*Glycine max.*) an increased root xylem area enhances hydraulic conductance and lead to increased drought tolerance and yields in the field (Prince et al., 2017; Strock et al., 2021). Summarizing, decreased vessel size accompanied with increased vessel number provide high hydraulic conductance while reducing the risk of hydraulic failure (Karlova et al., 2021; Li et al., 2021)

A study looking at natural variation of hydraulic conductivity identified the negative regulator of xylem development XND1 as important (Tang et al., 2018). Loss-of-function mutants of XND1 can deal better with water limitation compared to wild type, while overexpression of XND1 leads to impaired drought stress tolerance. This is connected to the enhanced formation and early differentiation of xylem cells in the root of *xnd1* loss-of-function mutants (Tang et al., 2018) indicating that enhanced xylem formation positively influences root hydraulics and xylem water conductivity and thereby improving the plant's ability to handle drought stress. The natural variation of the XND1 locus among Arabidopsis accessions indicates that root hydraulics might be an important feature that leads to adaption of plants to the environmental conditions of their habitat (Tang et al., 2018).

Drought stress may confront plants with the trade-off of the need to enhance hydraulic conductivity and on the other hand the risk of embolism. However, salt stress potentially adds another level of complexity with affecting the plant not only due to osmotic stress but also through ion toxicity (Munns and Tester, 2008). Decreasing xylem size and by this hydraulic conductivity may not only be a precaution to prevent embolism caused by salt-induced osmotic stress, but also in itself lead to higher salt tolerance. It has been reported that lowered hydraulic conductance upon salt stress due to reduced abundance of aquaporins is a mechanism of adaptation of different Arabidopsis accessions to salt (Sutka et al., 2011). Furthermore, the thermospermine-deficient mutant *acl5* is salt hypersensitive because of excessive xylem development (Shinohara et al., 2019).

Xylem plasticity in response to drought and salt stress

As summarized above, adaptive vascular traits that are connected to drought tolerance are the formation of many, but smaller vessels. Several observations indicate that plants have developed mechanisms to control vessel size and the amount of vessels in response to drought stress. Poplar (*Populus*) reacts to drought stress with the formation of more vessels with a decreased diameter (Arend and Fromm, 2007; Awad et al., 2010; Yu et al., 2021) also referred to as “stress-wood” (Yu et al., 2021), indicating a plastic response of the xylem formation to water availability. In maize (*Zea mays*), more but narrower metaxylem vessels are observed under drought (Klein et al., 2020) and in rice (*Oryza sativa*), plants exhibit smaller xylem diameter (Henry et al., 2012). These observations are consistent with findings from studies with mutants affected in xylem development. Overexpressing the *MdMYB88* and *MdMYB124* xylem regulators in apple lead to higher vessel density and confers higher drought stress tolerance (Geng et al., 2018). Furthermore, the increase in vessel number is often accompanied by an increase in vessel wall thickness

(Arend and Fromm, 2007; Awad et al., 2010; Geng et al., 2018; Yu et al., 2021).

Similar to what is observed upon drought stress, reduced xylem cell size and vessel diameter has been reported as a reaction to salt both in tree species as well as in herbaceous species that are relatively tolerant to salt (Junghans et al., 2006; Sellami et al., 2019a). On the other hand, in olive roots, a decreased stele area together with large diameter xylem vessels has been reported, indicating that different species may react differently to salt (Tan et al., 2020). Apart from vessel size, salt has also an effect on SCW composition. Enhanced lignification of root xylem cells upon salt stress has been reported in tomato (Sánchez-Aguayo et al., 2004), bean (*Phaseolus vulgaris*) (Cachorro et al., 1993) and soybean (*Glycine max.*) (Dolatabadian et al., 2011). In Arabidopsis both increased lignification (Taylor-Teeples et al., 2015) and decreased lignification (Sellami et al., 2019b) have been observed. In general, the composition of the xylem SCW is altered upon salt stress in Arabidopsis, with an increase of cellulose and hemicellulose such as xylans and less lignin (Sellami et al., 2019b). This changed composition potentially leads to collapsed or deformed irregular xylem vessels in the stem to the vessels in *irx* mutants (Sellami et al., 2019b). Moreover, it appears that plant cell walls undergo extensive remodelling upon salt stress, with proline-rich extension-like family proteins and proline-rich proteins being downregulated, while glycine-rich proteins, arabinogalactan proteins, invertases and expansins being upregulated (Shen et al., 2014; Zhang et al., 2021). Furthermore, overexpression of rice expansin *OsEXPA7* not only changed the structure of the vasculature, but also led to a reduced accumulation of sodium ions in the plant (Jadamba et al., 2020).

In summary, plants have evolved many developmental adaptations and acclimation mechanisms, which help them to withstand drought or salt stress. Several studies indicate acclimation or adaptation of the water conducting tissue to low water availability and salt stress and its potential importance for plant survival. However, surprisingly little is known about the underlying molecular regulation of xylem acclimation. Combining our knowledge about stress signalling and developmental pathways will help us to further understand how plants can adjust their development to environmental conditions in the long-term perspective leading to new breeding strategies. In this thesis, I focus specifically on plastic responses of the root xylem development to changing environmental conditions including water deficiency (**Paper I** and **II**) and salt stress (**Paper III**).

Research Aims

The overall aim of this thesis is to decipher the genetic and molecular mechanisms that drive the developmental plasticity of the Arabidopsis root xylem in response to water deficiency and salt. To accomplish this, I have investigated the following questions:

- How do water deficiency (**Paper I** and **Paper II**) and salt (**Paper III**) affect xylem development?
- What molecular and genetic mechanisms control the observed xylem developmental plasticity upon water deficiency (**Paper I** and **Paper II**) and salt (**Paper III**)?
- Are observed xylem developmental changes resulting in enhanced stress tolerance (**Paper III**)?

Results and Discussion

Mechanisms of xylem developmental plasticity under water deficiency

Water deficiency and ABA affect xylem development

As I have described in the previous chapters, xylem is the water conducting tissue of vascular plants. As such, it is critical for water transport throughout the plant and the idea that its development may be adjusted to water availability is intriguing. We therefore decided to study the effect of water deficiency on xylem development using the primary xylem of Arabidopsis seedlings as a model.

Water deficiency can be induced in a reproducible and specific way by lowering the water potential of agar plates either by polyethylene glycol (PEG) infusion (**Paper I** and **Paper II**; Van Der Weele et al., 2000; Verslues and Bray, 2004) or mannitol (**Paper III**). In contrast to mannitol, PEG is not taken up by the plant and has therefore been suggested to be more suitable to adjust the water potential of the medium (Hohl and Schopfer, 1991). However, on the xylem phenotype, PEG and mannitol had the same effect. PEG prevents hot agar from solidifying, therefore we followed Van Der Weele et al. (2000) protocol to prepare PEG infused agar plates overlaying ½ MS plates with liquid ½ MS containing PEG and allowing the PEG to diffuse into the plate overnight. On the next day, the remaining liquid solution was removed and the plates were used for experiments. Preparation of plates with 550 g/l PEG altered the water potential of the medium to -1.6 MPa in our conditions. Growth of two to three-day old Arabidopsis seedlings for 72 h under these conditions induced the formation of double protoxylem strands that we also referred to as extra protoxylem strands or additional protoxylem and a shift of the SCW pattern of the outer metaxylem from pitted to more protoxylem-like reticulate pattern in the roots (**Paper I** and **Paper II**). These results indicate that water availability indeed alters the development of the primary xylem suggesting phenotypic plasticity of the xylem towards environmental conditions.

As we saw that water deficiency was changing the development of the xylem, we wanted to understand how these developmental changes are regulated. Water deficiency lead to elevated endogenous ABA levels and ABA's function as

a stress hormone under drought stress is well established (Xiong and Zhu, 2003). We therefore hypothesized that altered levels of ABA mediated the morphological changes in the xylem. To test this hypothesis, we treated Arabidopsis seedlings with 0.5 µM or 1 µM of ABA. These concentrations did not compromise root growth in our studies in alignment with what had been observed before (Ghassemian et al., 2000). Treatment with ABA induced the formation of double or extra protoxylem strands as well as a shift of the outer metaxylem into reticulate metaxylem or protoxylem, similar to water deficiency caused by PEG treatment (**Paper I** and **Paper II**). Hereby, the extra protoxylem strands were forming aligned to the xylem axis or next to it. Moreover, expression of transcriptional reporters of the protoxylem marker genes *AHP6* (Mähönen et al., 2006) and *VND7* (Kubo et al., 2005) indicated that additional protoxylem cells and protoxylem characteristics in the outer metaxylem are caused by a cell identity shift towards protoxylem identity (**Paper I**).

To test if ABA not only causes similar effects as water deficiency, but that the effects of water deficiency on xylem development were mediated through ABA signalling, we used the *abi1-1* mutant. In the *abi1-1* mutant ABA signalling is partially inhibited rendering the mutant insensitive to ABA (Leung et al., 1994; Meyer et al., 1994). Upon treatment with ABA (**Paper I**) or reduced water availability caused by mannitol (**Paper III**), the phenotypic adjustments observed in wild type, were strongly reduced in the *abi1-1* mutant. In a similar study by Bloch et al. (2019) using the ABA receptor mutant *pyr1 pyl1,2,4* the adjustments towards more protoxylem upon ABA treatment or reduced water availability was not observed. These findings indicate that xylem morphology changes induced by water deficiency are mediated via canonical ABA signalling (Cutler et al., 2010). Together, these results suggest that water deficiency indeed affects xylem development through ABA and consequentially, ABA treatment can be used as a proxy for water deficiency.

In addition to the above-described phenotype, we observed an earlier xylem differentiation when growing the plants on plates infused with 550 g/l PEG (**Paper II**). Yet, plants grown under this condition exhibited inhibited root growth imputing that this phenotype might be caused by effects on the root meristem (**Paper I**). However, even though treatment with 1 µM ABA did not compromise root growth, the xylem was differentiating closer to the root tip compared to control conditions. While there was only a slight effect on protoxylem differentiation, the outer and inner metaxylem were differentiating significantly closer to the root tip upon 48h ABA treatment (**Paper II**). The earlier differentiation of the outer metaxylem potentially could be related to the identity shift to protoxylem or protoxylem-like identity. In contrast to the identity shift we observed in the outer metaxylem, the inner metaxylem retained in all cases its metaxylem identity indicating that ABA promoted its earlier differentiation independently of its effect on xylem morphology (**Paper II**).

The early differentiation of the inner metaxylem upon ABA treatment or in plates with reduced water potential by mannitol was suppressed in *abi1-1* mutant, indicating that the canonical ABA signalling pathway mediates both observed xylem phenotypes (**Paper II** and **Paper III**).

In summary, we showed that water deficiency alters the development of the water conducting tissue, the xylem, and that this process is mediated by ABA in Arabidopsis. We confirm that ABA treatment can be used as a proxy for water deficiency in this process, facilitating the specific evaluation on xylem developmental changes without disturbing root growth.

Cell autonomous and non-cell autonomous signals control xylem development upon water deficiency

As we observed that xylem developmental adjustment was controlled by ABA-signalling, we wanted to further understand how ABA affects xylem developmental plasticity. In the root, ABA is predominantly localized in the endodermis (Ondzighi-Assoume et al., 2016) and the effect of non-cell autonomous endodermal ABA signalling on primary root quiescence and lateral root plastic development under saline conditions has been described (Duan et al., 2013; Geng et al., 2013). We wanted to know whether ABA signalling is restricted to the endodermis. Synthetic ABA responsive reporters with tandem ABRE repeats from the two ABA responsive genes *ABI1* and *RAB18* (*6xABRE_A:GFPer* and *6xABRE_R:GFPer*) have been generated (Wu et al., 2018). Upon 6-8h treatment with 550 g/l PEG, we observed that ABA signalling was induced within the stele in the xylem precursor cells in addition to an enhanced signal in other tissue types, where weak expression was also observed under control conditions. The same response was seen upon treatment with 1 μM ABA, however less strong (**Paper II**).

The observation that ABA signalling upon water deficiency takes place in various tissue types including the endodermis and the xylem axis, prompted us to ask the question, in which tissues ABA signalling upon water deficiency contributes to the observed changes in xylem development. We used the previously published *UAS:abi1-1* GAL4-enhancer trap line system to answer this question (Duan et al., 2013). GAL4 binds to and activate the upstream activating sequence (UAS) that was fused to *abi1-1*, encoding a dominant negative form of a PP2C phosphatase that blocks ABA signalling. Tissue-specificity of *abi1-1* was accomplished by expressing GAL4 under different tissue specific promoters. Reducing ABA-signalling in the ground tissue, including cortex and endodermis, using the *J0571>>abi1-1* line reduced significantly the effect of ABA on the formation of extra protoxylem or the cell fate switch of the outer metaxylem to protoxylem or reticulate metaxylem upon ABA

treatment (**Paper I** and **Paper II**). Furthermore, we saw a reduction of xylem morphology changes upon treatment with 400g/l PEG or ABA when reducing ABA signalling in the endodermis using the *pSCR:abi1-1* line (Duan et al., 2013; **Paper I** and **Paper II**), indicating that xylem morphology switch upon water deficiency and ABA signalling is mediated by a non-autonomous endodermal signal (**Paper I**). The effect of endodermal ABA signalling on xylem development was even highlighted in an independent study (Bloch et al., 2019).

However, *J0571>>abi1-1* and *SCR::abi1-1* were not able to suppress the enhanced xylem differentiation rate upon ABA treatment, suggesting that ABA signalling in other cell types contributed to this phenotype (**Paper II**). In contrast, suppression of ABA in the xylem axis using *J1721>>abi1-1* had an effect on both xylem fate change and early inner metaxylem differentiation. Reduced ABA-signalling within the procambium cell files of the stele using the stele-specific *Q0990>>abi1-1* did not prevent neither the xylem morphology switch nor the enhanced xylem differentiation rate (**Paper I** and **Paper II**), indicating that the action of ABA within the stele to control xylem development is highly specific to the xylem axis. The results suggested that distinct cell autonomous ABA-signalling is controlling both phenotypical changes of the xylem upon ABA treatment (**Paper II**).

Together, these results indicated that xylem developmental plasticity is controlled by at least two distinct ABA-dependent pathways, one cell autonomous pathway in the xylem and one non-cell autonomous pathway in the endodermis.

miR165 and HD-ZIP III control non-cell autonomously xylem cell fate upon water deficiency

Now that we knew, the observed xylem developmental phenotypes were controlled by two independent ABA-signals, one coming from the endodermis and the other one occurring directly in the xylem, we wanted to further understand downstream processes. The miR165 is a well-known endodermal signal that by its restriction of HD-ZIP III transcription factor activity to the central part of the stele controls vascular development (Carlsbecker et al., 2010; Miyashima et al., 2011). Its involvement in xylem development as well as its movement from the endodermis into the stele made miR165 an interesting candidate to be involved in the non-cell autonomous endodermal regulation of xylem development upon water deficiency. We tested whether *MIR165A* was induced upon water deficiency and ABA treatment both using a transcriptional reporter and evaluating miR165 levels using qRT-PCR. Treatment with 550 g/l PEG for 6h significantly induced *MIR165A::GFP* expression in the

endodermis and we observed a similar effect upon 4h treatment with 50µM ABA (**Paper I**). Furthermore, mature miR165 levels were enhanced upon treatment with 50 µM ABA (**Paper I**). Consistent with our findings, that miR165 levels were elevated, the levels of transcripts encoding the HD-ZIP III transcription factors *PHB* and *ATHB8* upon treatment with ABA were reduced significantly and growth on plates infused with 550 g/l PEG reduced *PHB*, *ATHB8*, *REV* and *CNA* expression (**Paper I**). Similar effects of ABA on HD-ZIP III transcription have been observed in an independent study (Bloch et al., 2019). Even though the effect on miR165 and HD-ZIP III levels were marginal, previous studies had shown that a similar suppression of HD-ZIP III in a weak miR165 induction line causes protoxylem formation in metaxylem position (Müller et al., 2016), indicating that the ectopic protoxylem formation that we observed upon water deficiency can be caused by enhanced miR165 levels and a consequent downregulation of HD-ZIP III.

The hypothesis that reduced levels of HD-ZIP III causes the xylem cell fate switch upon water deficiency and ABA signalling was further supported by several *hd-zip III* mutants being more sensitive to 72 h of 0.5 µM ABA treatment with showing higher fractions of extra protoxylem. This was especially seen in *athb8* and *athb8 phb* consistent with the previous finding that ATHB8 and PHB are the main actors in double protoxylem formation (Carlsbecker et al., 2010; **Paper I**). This further supports that ABA is negatively affecting multiple HD-ZIP III transcription factors. On the other hand, PHB has been shown to directly control the expression of the ABA responsive gene *ABI4* and at the same time PHB upregulates the expression of *BG1* and thereby influences ABA homeostasis (Yan et al., 2016).

All together, these results suggested that the observed xylem fate changes upon water deficiency and ABA treatment are caused by a non-cell autonomous endodermal pathway due to the suppression of *HD-ZIP III* through miR165 (**Paper I**).

Transcriptome analysis reveals ABA's effect on many xylem developmental genes

Since the non-cell autonomous regulation of xylem development upon water deficiency involving miR165 and HD-ZIP III could not explain the earlier differentiation of the inner metaxylem, we performed several RNA-Seq experiments to get an overview about transcriptomic changes related to xylem development that occur upon ABA signalling. We generated transcriptomes using roots of five-day old Arabidopsis wild type plants treated 8h with 1 µM ABA. Transcriptome analysis revealed that 2368 genes were induced by ABA (**Paper II**).

We wanted to know whether among these genes, genes relevant for xylem development could be found. Comparing the 2368 ABA induced genes with genes from a single cell RNA-Seq study (Denyer et al., 2019) showed that 114 were xylem-enriched. Present amongst these genes were master regulators including *VND7*, *VND2*, *VND3* and *MYB46* and *MYB83* and genes more involved in downstream pathways such as *CESA4*, *CESA7*, *CESA8*, *LAC11*, *LAC17*, *XCP1* and *XCP2* (**Paper II**). These results indicated that ABA affects, apart from *HD-ZIP III,* several other master regulators of xylem development that are potentially involved in the regulation of extra protoxylem formation or early inner metaxylem differentiation upon water deficiency.

As we had observed that driving *abi1-1* in the xylem axis using *J1721>>abi1-1* repressed both observed xylem phenotypes, we wondered whether the upregulation of certain xylem regulators upon ABA treatment would be repressed in this line. We performed a second RNA-Seq experiment using the *J171>>abi1-1*, *J0571>>abi1-1* and *Q0990>>abi1-1* lines. Transcriptome analyses of *J1721>>abi1-1* revealed that a subset of the xylem-specific genes that were induced by ABA, exhibited a reduced activation including *CESA*s, *LAC17*, *MYB46*, *MYB83* and *VND3*. In contrast only a few xylem-specific genes that were induced by ABA, were reduced in *J0571>>abi1-1* and *Q0990>>abi1-1* reflecting the less severe changes of the xylem phenotype when inhibiting ABA signalling in the endodermis or the procambium (**Paper II**). qRT-PCR revealed that while *VND2* and *VND3* were activated by ABA, this activation was significantly suppressed when ABA signalling was specifically blocked in the xylem axis of the stele using *J1721>>abi1-1*, indicating that *VND2* and *VND3* are induced by cell autonomous ABA signalling (**Paper II**).

Overall, transcriptome analysis indicated that genes controlling xylem development respond to ABA and that this response is mediated by ABA-signalling in distinct tissue types.

VND7 controls the switch in xylem fate cell autonomously

Since xylem cell fate was controlled both non-cell autonomously, via *miR165* and HD-ZIP III, but also via cell autonomous ABA signalling, additional factors are presumably involved in this process. Among other xylem regulatory genes, we found that *VND2*, *VND3* and *VND7* were upregulated in the root transcriptome of ABA treated plants (**Paper II**). VND transcription factors are master regulators of xylem development and in cell culture, VND7 is specifically controlling protoxylem differentiation (Kubo et al., 2005). To get an idea about whether VND7 could be an interesting candidate for xylem developmental regulation upon water deficiency, we observed *VND7* expression

upon ABA treatment. *VND7* expression was induced by ABA as early as 2h upon treatment (**Paper II**).

To further support VND7's role in xylem developmental plasticity response to water deficiency, we used the *vnd7* loss-of-function mutant. This mutant does not exhibit an apparent root xylem phenotype under normal conditions. Interestingly, the xylem cell fate change that we observed in wild type was suppressed in *vnd7* upon ABA and growth on PEG-infused plates, suggesting that VND7 is required for xylem morphology change upon ABA treatment (**Paper II**). It has previously been suggested that ABA causes protoxylem formation by affecting VND7 levels in the provascular cells and that VND7 controls the expression of SCW related genes by suppressing HD-ZIP III transcription factors (Taylor-Teeples et al., 2015; Bloch et al., 2019). We generated a trancriptome of Col-0 and *vnd7* seedling roots treated with ABA or control conditions. However, among the ABA-induced xylem-expressed genes, only *LAC11* was significantly reduced in our data set (**Paper II**). Upon ABA treatment *VND7* expression had been observed closer to the meristem (Bloch et al., 2019), so we wondered whether VND7 also is involved in the earlier differentiation of the inner metaxylem. However, the early inner metaxylem differentiation upon ABA treatment and growth on PEG-infused plates was not affected in *vnd7*, indicating that other xylem regulators control this process (**Paper II**).

Our results indicate that together a non-cell autonomous mechanism involving *miR165* and HD-ZIP III and a cell autonomous mechanism involving VND7 control xylem cell fate switch upon water deficiency.

VND2/3 transcription factors control enhanced xylem differentiation

After we identified HD-ZIP III transcription factors and VND7 as regulators of xylem cell fate switch upon water deficiency, we wanted to understand how the enhanced inner metaxylem differentiation was regulated. Our transcriptome analysis had revealed that also several other *VND* genes were upregulated by ABA, with only *VND6* being unaffected (**Paper II**). A qRT-PCR confirmed that treatment with 2h of ABA induced *VND1*, *VND2*, *VND3*, *VND4* and *VND7*, while longer ABA treatment led to *VND5* induction (**Paper II**), indicating that VND transcription factor expression is regulated during an early phase of ABA signalling. We got a further support for our hypothesis, that ABA directly regulates VND transcription factor expression, by searching for ABREs in the *VND* promoter sequences extracted from a data set generated by Chip sequencing after ABA-treatment (Song et al., 2016). ABFs were binding to the promoters of *VND1*, *VND2* and *VND3* as well as to a couple of other

xylem regulators (**Paper II**), indicating that VND1, VND2 and VND3 may regulate xylem development upon ABA signalling.

It had been reported that VND1, VND2 and VND3 are involved in xylem formation in the cotyledons (Tan et al., 2018). We wanted to know whether they could be involved in xylem differentiation in the root as well and checked therefor their expression patterns. *VND1*, *VND2* and *VND3* had distinct expression patterns in the immature xylem cells within the meristem. *pVND1:YFP* expression was restricted to the outer metaxylem cells within the meristem, while *pVND2:YFP* was expressed in both outer and inner metaxylem precursors within the meristem. In contrast, *pVND3:YFP* expression was detected in both protoxylem, outer metaxylem and inner metaxylem precursor within the meristem and extended into the differentiation zone (**Paper II**). Together, these results indicated that *VND1*, *VND2* and *VND3* are regulated by ABA and specifically expressed in the xylem, where we observed developmental alteration upon water deficiency and ABA treatment rendering those interesting candidates.

Under ABA induction, the *vnd1 vnd2 vnd3* loss-of-function mutant significantly reduced the upregulation of 21 xylem-enriched genes, including *XCP* genes, *CESA4*, *LAC11* and *LAC17*. XCP are cysteine peptidases that are involved in autolysis processes during PCD of the developing TE (Avci et al., 2008). *CESA4* is important for cellulose synthesis during SCW formation and *LAC11* and *LAC17* encode for laccases that control lignification (Berthet et al., 2011). Thus, VND1, VND2 and VND3 control genes that are involved in different steps of xylem differentiation in response to ABA treatment (**Paper II**). VND2 and VND3 requirement for xylem development upon ABA treatment was further supported by the phenotype of the knock-out mutants. The *vnd2 vnd3* double and *vnd1 vnd2 vnd3* triple mutant did not show early inner metaxylem formation upon ABA treatment as well as under water limiting conditions. This is especially interesting, because root length was reduced upon PEG treatment, uncoupling root length and inner metaxylem differentiation, indicating that VND2 and VND3 control inner metaxylem differentiation independently of the root meristem activity (**Paper II**).

The ABA-dependent effect of water deficiency caused both changes along with the protoxylem cell file and the inner and outer metaxylem (**Paper I** and **Paper II**). While ABA-dependent effects on the protoxylem cell identity were mediated by both HD-ZIP III transcription factors and VND7 (**Paper I** and **Paper II**), the early differentiation of the inner metaxylem was depending on VND2 and VND3 function (**Paper II**). However, upon ABA treatment, also an early differentiation of the outer metaxylem was observed and even though changes in the xylem fate were inhibited in the *vnd1237*, the outer metaxylem was differentiating closer to the root tip (**Paper II**). The involvement of VND2

and VND3 in the early differentiation of the inner metaxylem upon ABA treatment but not in the outer metaxylem indicate that even though their similar morphological characteristics, the inner and outer metaxylem are different cell types and that other factors are responsible of the response of the outer metaxylem to ABA treatment and osmotic stress. Interesting candidates could be MYB46 and MYB83 as they are well-known xylem development regulators and their upregulation upon ABA was not reduced in the *vnd1,2,3* mutant (**Paper II**).

Together, the results suggest that upon water deficiency VND2 and VND3 are induced by cell autonomous ABA signalling and promote early inner metaxylem differentiation (**Paper II**).

Mechanisms of xylem plasticity under salinity

Salt induces protoxylem gaps

Together with the water stream, nutrients and salts are transported throughout the xylem. At the same time soil salinity is a cause of both osmotic and ionic stress for plants (Munns and Tester, 2008) and due to this salt lowers the water uptake. We therefore wanted to know how salinity affects xylem development. Treatment of three-day old Arabidopsis seedlings with 140mM NaCl for five days induced the formation of additional protoxylem and early differentiation of the inner metaxylem in an ABA-dependent manner, similar as observed upon water limitation and ABA treatment (**Paper I, II** and **III**). However, NaCl treatment for three days induced the formation of discontinuous protoxylem strands that I will refer to as protoxylem gaps with one to four gaps per root and an average of 1.4 gaps per root distributed over both protoxylem strands or on the same strand (**Paper III**). Experiments including different concentrations of salt indicated that protoxylem gap formation occurs in a concentration dependent manner. On the contrary, treatment with isosmotic concentration of mannitol did not induce protoxylem gap formation (**Paper III**). These results indicate that salt treatment, even though it induced similar phenotypes as other osmotic treatments, induced the distinct formation of protoxylem gaps during the initial phase of stress (**Paper III**).

We wanted to understand whether the observed protoxylem gaps were caused by cells collapsing due to changed osmotic conditions under salinity or whether salt has an effect on xylem differentiation. Further analyses of protoxylem gaps using stains for different cell wall components revealed that gaps observed in the light microscope are likely to be caused by a range of effects from cells that did not have lignified SCW to cells that did not produce a SCW at all (**Paper III**). Moreover, some of the cells within the xylem axis did not

undergo PCD and exhibited expression of the procambium and immature xylem identity marker *pANT:his-YFP* (**Paper III**, Randall et al., 2015). PCD is a critical step during xylem differentiation (Schuetz et al., 2013) and precedes lignification (Pesquet et al., 2013). Transferring salt treated Arabidopsis seedlings back to mock conditions led to the formation of a normal xylem phenotype in the newly grown part of the root, indicating that protoxylem gap formation upon salt stress is, similar to extra protoxylem formation upon ABA, a plastic response (**Paper II** and **Paper III**). However, already established protoxylem gaps were not closed by transferring to mock treatment, indicating that the differentiation or specification of these cells was permanently altered (**Paper III**).

Summarizing, we observed the formation of distinct protoxylem gaps upon salt stress. Protoxylem gap formation is likely caused by altered or stunted differentiation and this can be considered a plastic response to salt.

GA-signalling controls protoxylem gap formation

We wanted to understand the molecular regulation that controls protoxylem gap formation upon growth on salt. In **Paper I** and **Paper II,** we had identified the important role of ABA in xylem development upon water deficiency. However, treatment with ABA did not induced protoxylem gap formation and reducing ABA signalling using the *abi1-1* mutant did not affect protoxylem gap formation upon salt stress, indicating that other factors than ABA are involved in the developmental regulation of protoxylem gap formation upon salt stress (**Paper III**).

As described in the introduction, GA is involved in the salt tolerance mechanisms and salt stress induces the upregulation of *GA2OX* genes that convert bio-active GA into a bio-inactive form (Magome et al., 2008; Colebrook et al., 2014). Consistently, salt treatment reduces the levels of bioactive GAs (Achard et al., 2006). We therefore wanted to test whether we could induce protoxylem gaps by lowering GA levels. Parallel treatment with salt and PAC, an inhibitor of GA-biosynthesis (Lee et al., 1985), led to increased protoxylem gap formation and even PAC treatment alone induced protoxylem gaps under control conditions. Furthermore, PACs effect was compensated by additional GA3 treatment, indicating that the phenotype was caused by PACs effect on GA biosynthesis (**Paper III**). GA-biosynthesis occurs through the sequential action of different enzymes. GA1 is a terpene cyclase that catalyses an early step during GA biosynthesis, while *GA4* encodes for a GA 3-beta-hydroxylase that controls the last step during the synthesis of bioactive GA. Two different alleles of the loss-of-function mutant *ga1* (*ga1-3* and *ga1-5*) as well as the *ga4* mutant had similar effects as PAC treatment upon salt stress, with *ga4* having the strongest effect. In *ga4,* protoxylem gaps were formed even under control

conditions, while this was not the case for *ga1-3* and *ga1-5*. Furthermore, the effect of GA-biosynthesis mutants on xylem development was restored with additional treatment with GA3 (**Paper III**).

We investigated whether altering GA signalling had a similar effect of protoxylem gap formation as manipulating GA levels. Reduced GA signalling in the GA-receptor triple mutant *gid1a-2 gid1b-3 gid1c-1* similarly led to increased protoxylem gap formation upon salt stress (**Paper III**). Stabilization of DELLA protein RGL3 that is a negative regulator in GA signalling has been shown to confer to salt stress resistance (Shi et al., 2017). RGA accumulation has been reported upon salt stress (Achard et al., 2006) and we detected that its accumulation also occurs in the stele (**Paper III**). However, in general, DELLA proteins have quite redundant functions (Gallego-Bartolomé et al., 2010). It is therefore not surprising that single mutants *gai-td1*, *rgl3-5* and *rga-28* did not show an effect on protoxylem gap formation upon growth on salt (**Paper III**). However, *rga-28 rgl3-5 della* double mutant, *gai-t6 rga-24 rgl1-1 rgl2-1* (*della* quadruple mutant) as well as *gai-t6 rga-t2 rgl1-1 rgl2-1 rgl3-4* (*della* quintuple mutant) exhibited reduced protoxylem gap formation when grown on salt (**Paper III**).

These results suggested that reduced GA levels and signalling promote the formation of protoxylem gaps under saline conditions.

DELLAs are required to activate factors related to xylem differentiation

GA signalling controls xylem expansion during secondary xylem development through distinct repression of DELLA in the phloem and cambium (Ragni et al., 2011; Ben-Targem et al., 2021). However, an effect of GA signalling on primary xylem development has not been reported before. We therefore wanted to understand how decreased GA signalling in response to salt controls the formation of protoxylem gaps. Since we had observed a strong reduction in protoxylem gap formation in salt grown *della* quintuple mutant seedlings, we generated transcriptomes of seedling roots grown under control conditions and upon 1h or 8h of salt exposure. Furthermore, we had observed an increase in protoxylem gap formation in the *ga4* and *gai* mutant upon salt stress and in the *ga4* mutant even under control conditions. Therefore, we included these two genotypes in the experiment to get an idea about transcriptomic changes that are related to protoxylem gap formation.

Genes upregulated upon 1h of salt exposure that were expressed in a DELLA-dependent manner in the immature xylem, according to two different single cell transcriptomes (Denyer et al., 2019; Wendrich et al., 2020), were mostly

belonging to GO-terms related to stress response, while among the downregulated genes we found enzymes involved hemicellulose synthesis (**Paper III**). Upon 8h of salt exposure, we found that genes that were expressed in a GA-dependent manner and filtered for xylem expression were enriched in GO-terms related to 'response to stress' but also 'cell wall organization and biosynthesis' (**Paper III**). Among the genes related to cell wall organization and biosynthesis, we found genes involved in cellulose and hemicellulose biosynthesis as well as genes of the alpha expansin family. The effect of salt treatment on the expression of genes involved in cell wall remodelling has been reported before (Shen et al., 2014; Zhang et al., 2021) and SCW composition may confer to a higher elasticity of the vessels and by this protect vessels from collapsing due to osmotic stress (Sellami et al., 2019b). It further links to the observed modified cell wall composition of xylem gaps observed in wild type upon salt treatment (**Paper III**).

With this approach we also identified the xylem master regulator *VND6* that has been described as a positive regulator of metaxylem differentiation (Kubo et al., 2005) along with its downstream target *MYB83* (McCarthy et al., 2009) as being upregulated in response to 8h of salt in a GA-dependent manner. Interestingly, *VND6* is the only VND transcription factor gene that was not regulated by ABA and both *VND6* and *MYB83* promoters lack recognition sites for ABFs (**Paper II**). This is consistent with our findings that protoxylem gap formation in response to salt is not controlled by ABA. Previously, VND7 and MYB46 were hypothesised to be important regulators during salt stress (Taylor-Teeples et al., 2015). However, in our conditions, *VND7* was not significantly upregulated upon salt and the upregulation of *MYB46* upon 8h of salt was independent from DELLA, indicating that they may not be involved in the formation of protoxylem gaps upon salt stress (**Paper III**).

It has been shown that overexpression of VND6 lead to ectopic metaxylem formation and that overexpression of VND6 fused to the SRDX strong repression domain lead to repression of inner metaxylem formation, while no apparent phenotype has been reported for the *vnd6* mutant (Kubo et al., 2005). However, we found that protoxylem gap formation upon salt was reduced in the *vnd6* mutant, suggesting that *VND6* activation upon salt is involved in inhibiting protoxylem cell differentiation resulting in protoxylem gaps (**Paper III**). Thus, our results indicate that VND6 activation upon salt may have a different role as described before for normal conditions even though the details of this function need further investigation. However, most likely additional factors contribute to xylem gap formation, since *vnd6* mutants were able to form protoxylem gaps to some extent (**Paper III**).

These results indicate that xylem plasticity in response to salt stress is regulated by GA signalling resulting in altered expression of xylem master regulator *VND6* and several classes of genes related to cell wall formation.

Intrinsic xylem regulatory pathways are co-opted for plastic responses during stress

The plant specific occurrence of HD-ZIP proteins led to the speculation that HD-ZIP proteins might mediate the coupling of development to environmental factors, since adjusting its developmental program to external conditions is an important trait for sessile organisms (Schena and Davis, 1992). Co-opting a developmental regulatory network is likely a key mechanism to facilitate adaption in response to stress (Taylor-Teeples et al., 2015).

Discontinuous xylem or xylem gaps is not a phenotype specific to salt response. We have found that ABA is required for proper xylem development and reduced ABA biosynthesis or signalling resulted in discontinuous xylem (**Paper I**). Reducing ABA biosynthesis with fluridone (Bartels and Watson, 1978) caused the formation of discontinuous metaxylem (**Paper I**). ABA biosynthesis mutants *aba2-1* and *aba3-1* are involved in the final steps of ABA synthesis and exhibit substantial reduced ABA levels (Léon-Kloosterziel et al., 1996). Observing the root xylem it was apparent that 40-60% of the *aba2-1* and *aba3-1* roots had discontinuous or absent protoxylem or metaxylem strands (**Paper I**). Both the effect of fluridone and the ABA biosynthesis mutants on xylem development was restored by treatment with 250nM and 5nM ABA respectively for 72h (**Paper I**). Furthermore, the *abi1-1* mutant that is partially insensitive to ABA (Leung et al., 1994; Meyer et al., 1994) exhibited discontinuous metaxylem strands (**Paper I**).

ABA's effect on xylem development upon water deficiency was mediated through both a non-cell autonomous endodermal signal and a cell autonomous stele signal (**Paper I** and **Paper II**). However, under control condition, discontinuous metaxylem was observed in *Q2500>>abi1-1* reducing ABA signalling in the pericycle, endodermis and weakly in the cortex and in *J0571>>abi1-1* reducing ABA signalling in the ground tissue (**Paper I** and **Paper II**). Also reducing ABA signalling in the epidermis and lateral root cap using *J0951>>abi1-1* induced discontinuous xylem (**Paper I**). Reducing ABA signalling in the xylem-pole pericycle (*J0121>>abi1-1*), columella and lateral root cap (*J3411>>abi1-1*), procambium (*Q0990>>abi1-1*) and xylem axis (*J1721>>abi1-1*) did not affected xylem morphology (**Paper I** and **Paper II**). The *pSCR::abi1-1* with reduced ABA signalling in the endodermis displayed metaxylem gaps as well (**Paper I**). Together these results indicate

that for normal xylem development endodermal ABA-signalling is critical while ABA signalling in the stele is less important. These results accord with the finding that ABA signalling in the xylem axis is specifically induced upon treatment with ABA or water deficiency and may be more connected to stress response (**Paper II**).

We wanted to know whether similar mechanisms that control xylem developmental plasticity upon water deficiency utilize the same pathways that control xylem development under control conditions. The regulation of xylem development by miR165/6 and HD-ZIP III transcription factors is a well described process (Carlsbecker et al., 2010; Miyashima et al., 2011). However, it had not been studied before how ABA affect primary xylem development. We found that treatment with 10μM Fluridone for 24h reduced *pMIR165:GFP* intensity and mature *miR165* levels were reduced in *pSCR::abi1-1*, while HD-ZIP III levels were elevated. Moreover, *hd-zip* III loss-of-function mutants displayed reduced sensitivity to fluridone treatment. These results suggested that the non-cell autonomous ABA signalling pathway that was identified under stress condition is controlling ABA-dependent xylem development even under control conditions (**Paper I**).

As we had observed xylem gap formation under different conditions, we wondered whether the expression of a common set of genes is changed under all these conditions potentially indicating their involvement in xylem differentiation. We compared the list of genes that were differentially expressed in a DELLA dependent manner upon growth on salt for 1h and 8h, genes that are changed in the *ga4* mutant compared to wild type and genes that were differentially expressed after inhibiting ABA signalling in the endodermis using the *J0571>>abi1-1* under control conditions. We identified three genes that were differentially expressed in all four datasets, *EXPANSIN 1* (*EXP1*), *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE (XTH20)* and a gene belonging to the peroxidase superfamily (**Paper III**). *EXP1* is a member of the alpha expansin family and is involved in cell wall remodelling processes (Ramakrishna et al., 2019). As mentioned before, in extensive cell wall remodelling may occur under salt acclimation in Arabidopsis (Shen et al., 2014) and rice expansin OsEXP7 is involved in salt stress tolerance and vascular development (Jadamba et al., 2020). Interestingly, the loss of function mutant *expa1-1* exhibited reduced protoxylem gap formation upon salt stress suggesting a role of alpha expansins in xylem gap formation (**Paper III**).

All in all, these results indicate that both proper ABA and GA-signalling are needed for primary xylem differentiation and that these pathways may be co-opted for plastic responses during stress regulating some common genes involved in cell wall remodelling.

Plastic response of xylem development in other species

Arabidopsis is a great model to study xylem development and how xylem development is affected by environmental conditions. We found that both water deficiency (**Paper I** and **II**) as well as salinity (**Paper III**) causes alterations in xylem development through distinct molecular mechanism including ABA and GA signalling respectively. However, we wanted to understand whether these plastic responses are specific to Arabidopsis or whether they are also found in other eudicots.

To see whether ABA's function in controlling enhanced xylem differentiation is evolutionary conserved, we tested the effect of ABA on xylem development in five eudicot species. *Brassica napus*, *Brassica rapa*, *Nicotiana benthamiana*, *Solanum lycopersicum*, *Phtheirospermum japonicum* displayed all early xylem development and higher number of xylem strands, indicating that this is an evolutionary conserved process (**Paper II**). This is also supported by another study were an effect of ABA on root xylem development in tomato had been described (Bloch et al., 2019). Moreover, in tomato *VND1*, *VND2* and *VND3* orthologues were upregulated upon 6h of ABA treatment, suggesting an evolutionary conservation of the underlying gene regulatory network (**Paper II**). Xylem morphology changes upon water deficiency have been reported for different species ranging from grasses to trees (Lovisolo and Schubert, 1998; Arend and Fromm, 2007; Awad et al., 2010; Henry et al., 2012; Prince et al., 2017; Klein et al., 2020; Zhang et al., 2020; Strock et al., 2021; Yu et al., 2021). It would be interesting to know whether similar molecular mechanisms also underlie these changes.

Similarly, we wondered whether the xylem developmental alteration that we observed upon growth on salt in Arabidopsis are also evolutionary conserved. Different species have different abilities to tolerate salt stress due to different morphological or physiological adaptations. Arabidopsis generally is considered as a salt sensitive species. Also tomato is salt sensitive (Sun et al., 2010), while sugar beet (*Beta vulgaris*) is more salt tolerant (Skorupa et al., 2019) and the brassicaceae *Eutrema salsugineum* is a halophyte (Yang et al., 2013). However, all four species developed protoxylem gaps upon treatment with salt, even though for the salt tolerant species higher concentrations of salt were needed to induce protoxylem gap formation (**Paper III**). This indicates that protoxylem gaps are not restricted to salt sensitive species, but may be a general reaction to salt stress. Whether a species appear to be salt sensitive or salt tolerant may be more related to other morphological or physiological features.

Overall, xylem developmental plasticity upon both water deficiency and salt appeared at least to some degree to be evolutionary conserved, gathering these processes as interesting aspects for crop improvement.

Physiological relevance of xylem plasticity

To breed plants that can better react to changing environments it is important to select for plastic traits that specifically improve plants fitness. As presented in the introduction, changes in the plant's morphology might be a plastic response of the plant to increase the plant's performance under the stressful conditions, but they may also be a result of stress that is not favourable to the plant. Phenotypic variation between plants grown in different environments may not affect fitness at all, or it may even have a negative impact (De Jong and Leyser, 2012). Identifying whether the morphological changes in the xylem that we observe upon water limitation and salt are part of a stress avoidance or tolerance mechanism is challenging as most likely other processes are triggered by stress potentially conferring to tolerance due to a pathway non-related to the observed xylem phenotype. However, previous studies as well as our results give us some insights in how xylem plasticity potentially confers to drought and salt tolerance.

Upon water deficiency, we observed enhanced xylem formation with earlier inner metaxylem differentiation and the formation of extra protoxylem strands. Furthermore, we observed an identity shift to smaller protoxylem cells in the outer metaxylem cell files (**Paper I** and **Paper II**). These results are in line with xylem traits that have been connected to be advantageous under drought in other species with the formation of more xylem strands (Arend and Fromm, 2007; Prince et al., 2017; Geng et al., 2018; Tang et al., 2018; Strock et al., 2021; Yu et al., 2021) as well as the decrease of vessel diameter (Lovisolo and Schubert, 1998; Arend and Fromm, 2007; Awad et al., 2010; Henry et al., 2012; Zhang et al., 2020; Yu et al., 2021). Moreover, GWAS studies had identified that a loss-of-function mutation of the negative regulator of xylem development *XND1* lead to enhanced inner metaxylem differentiation and enhanced drought tolerance by affecting hydraulic conductance, while a gain-of function mutation had the opposite effect (Tang et al., 2018) further connecting our observed xylem traits to drought tolerance. In future studies, it would be interesting to analyse the effect of ABA treatment on hydraulic conductance to get a better insight in how xylem morphology changes influence plant's tolerance to low water availability.

During the initial phase of salt stress, we observed the formation of protoxylem gaps (**Paper III**). Also in a different study, no additional protoxylem formation was observed early upon growth on salt (Bloch et al., 2019). The specific adjustment of xylem development to water deficiency and salt has been suggested before, but the difference has not been characterized in depth (Agustí and Blázquez, 2020; Karlova et al., 2021). We wanted to know whether protoxylem gap formation upon growth on salt has a physiological

function. Young seedlings of the *della* quintuple mutant exhibited less protoxylem gap formation compared to wild type. Interestingly, they appeared to be less tolerant when grown for 4 days on 200mM salt (**Paper III**), in line with the previously reported decreased salt tolerance of the *della* quadruple mutant and that reduced GA-levels lead to enhanced salt tolerance (Achard et al., 2006; Achard et al., 2008). However, the decreased tolerance of the *della* quadruple mutant has been associated to increased ROS levels during an initial phase of salt stress (Achard et al., 2008) and it is difficult to prove that it is related to the observed protoxylem gap phenotype. We therefore investigated the salt tolerance of mutants that exhibit an increased protoxylem gap formation. The *ahp6* and *vnd1237* mutant exhibit protoxylem gap under control conditions (Mähönen et al., 2006; Ramachandran et al., 2021) and we observed an increase in protoxylem gap formation upon salt (**Paper III**). Even though root growth was affected in all genotypes, *ahp6* and *vnd1237* displayed less bleaching of the leaves than wild type plants, indicating a higher salt tolerance (**Paper III**). These results support the hypothesis that protoxylem gap formation may be a measure to help plants to withstand salinity. The idea that the extent of xylem differentiation is connected to a decrease in salt tolerance has also been suggested before (Shinohara et al., 2019). Excessive xylem differentiation in the thermospermine biosynthesis mutant *acl5* led to salt hypersensitivity. This is in line with our hypothesis that on the contrary, decreased xylem differentiation due to the formation of xylem gaps may have a positive effect on salt tolerance.

The xylem is the main tissue for water transport. Together with the water stream, also nutrients and ions are transported from the root to the shoot. It is therefore likely that the observed plastic response of the xylem to water deficiency and salt are measures to affect water transport. The formation of an increased amount of xylem vessels may lead to higher hydraulic conductance and the protoxylem morphology may protect them from embolism, which would be of advantage during drought and osmotic stress. However, during an initial phase of salt stress, these traits might be a disadvantage because they would along with increased water transport accelerate the transport of ions to the shoot were unprepared cells would be harmed (Tavakkoli et al., 2011). Transport of salt ions via the xylem is an important factor for salt accumulation in the shoot and exclusion of salt from the stele is important for regulating salt transport (Møller et al., 2009). Even though toxic effects of salt mainly appear during a later phase of salt stress (Munns and Tester, 2008), Na^+ ions are detected rapidly during the stress (Zhao et al., 2021b). We therefore hypothesized that protoxylem gap formation may be a measure of lowering hydraulic conductance to decrease the transport of salt ions to the shoot in an initial phase of salt stress. This hypothesis find some support in the literature. Salt tolerant Arabidopsis accessions have a lower proportion of xylem relative to phloem and smaller vessels upon salt treatment (Sellami et al., 2019a), even

though the *irx* phenotype observed in the stem of salt treated Arabidopsis plants was not correlated to salt tolerance (Sellami et al., 2019a). Furthermore, GA3 treatment of wheat under salt stress caused the accumulation of salt ions in both the shoot and the roots, even though the photosynthetic capacity was not impaired by this (Ashraf et al., 2002).

Together, our results as well as findings described in the literature indicate that the xylem developmental plastic responses that we found during the work in this thesis may have beneficial effects on plant's survival under water deficiency and salt stress.

Potential cross-talk between ABA and GA to regulate xylem development in response to the environment

As an osmotic component, salt diminish the plant's ability of water uptake, inducing a situation of water deficiency, even though there may be enough water present. It is therefore not surprising that the plant after an initial formation of protoxylem gaps in response to salt, produces additional protoxylem strands as well as an enhanced differentiation of the inner metaxylem (**Paper III**). This indicates that the xylem may be specifically adjusted to the needs of the plants. Transferring ABA treated plants back to mock conditions restored both xylem differentiation rate and xylem morphology within 48h (**Paper II**) and protoxylem gaps are specifically formed when the plant is exposed to salt, indicating that the regulation of xylem development is a highly plastic response (**Paper III**). Prioritization of the phenotype that addresses the most limiting environmental factor has been proposed as a regulatory mechanism of developmental plasticity (De Jong and Leyser, 2012).

We have identified ABA and GA as two important hormones that regulate xylem developmental plasticity in response to environmental factors. Several connections between GA- and ABA signalling under different conditions have been reported (Shu et al., 2018) and the case of drought and salt stress emphasises the importance of the right timing of different developmental acclimations. ABA's effect on DELLA stabilization in the root meristem upon stress has been shown (Rowe et al., 2016) indicating a possible cross talk that regulates the formation of different xylem phenotypes upon stress.

We identified the endodermis and the xylem as the main tissues that are involved in the ABA-depend regulation of xylem plasticity. Root growth is primarily regulated by GA-signalling in the endodermis (Ubeda-tomás et al., 2008). However, it is not clear whether this is also the case for GA's and DELLAs effect on xylem development upon salt stress. GA-signalling has been

shown to increase along the root growth axis with higher levels in the elongation zone, but not restricted to the endodermis (Rizza et al., 2017), and an interplay of the cortex and the vasculature has been shown in the case of *PHB*, *GA2OX2* and *GA1* action during cortex development (Bertolotti et al., 2021). Downstream targets of GA signalling that overlap with the salt stress response have been mainly identified in the epidermis and cortex (Geng et al., 2013). It would be of great interest to get an insight on in which tissue reduced GA signalling regulates protoxylem gap formation upon salt stress.

Understanding the interplay of GA and ABA under these stress conditions may reveal important knowledge on how different environmental stresses fine tune vascular development.

How to apply knowledge about xylem plasticity

In this thesis, I have described that xylem development is altered by water deficiency and salt stress in a plastic fashion. Furthermore, we have identified molecular mechanisms that drive this xylem developmental plasticity including hormones and genetic regulation and that these are at least to some degree conserved between different dicotyledonous species. Finally, we could give indications that these plastic changes confer a higher tolerance against water deficiency and salt, respectively.

This thesis also indicates that the developmental adjustment of the xylem to environmental conditions is a complex process that need further investigation both to understand the developmental regulation as well as the physiological relevance of it. It is likely that improving plant fitness to one condition will not be enough, as plants are exposed to many different stresses during their life. The developmental changes of the xylem of young seedlings exposed to salt stress from protoxylem gap formation in an initial phase to the development of additional protoxylem strands and early differentiation of the inner metaxylem can be an example for such delicate adjustments. However, natural conditions are much more complex than the controlled laboratory conditions that have been used to study xylem development in this thesis. For example, a combination of abiotic and biotic stress may challenge the plant in an again different way. The fungal plant pathogen *Verticillium longisporum* that proliferates in the plant vascular system induces de novo xylem formation through activation of *VND7* which in turn improves the plants tolerance to drought stress (Reusche et al., 2012). Bacterial pathogens as *Pseudomonas syringae* pv. *phaseolicola* induce the expression of the negative regulator of xylem formation *XND1* and pathogen-associated molecular patterns such as flg22 reduce xylem area in a XND1 dependent manner to limit bacterial pro-

liferation in the plant and subsequently protect the plant against bacterial infection (Tang et al., 2018), illustrating a delicate interaction of different environmental factors.

To transfer knowledge gathered by molecular biology to generating new crops and improve plants fitness is in general a great challenge. Nevertheless, molecular biology can reveal connections of traits and genetic regulation that can be useful for future breeding or generation of GMO crops. This thesis work is at the intersection of both physiology and developmental biology. Looking at a process or phenotype from both a physiologist and developmental biologist perspective can reveal new insights that might have been overlooked otherwise. The findings from **Paper I** indicate that developmental processes as the specification of xylem through a non-cell autonomous pathway are employed during reaction to stress. On the contrary, the previous observed stress tolerance of the higher order *della* mutants, could potentially be connected to a previously unknown developmental process (**Paper III**). All in all, observing and questioning biological phenomena from different angles will give us a better understanding of the sophisticated processes that fine tune plant's development to and under stress conditions.

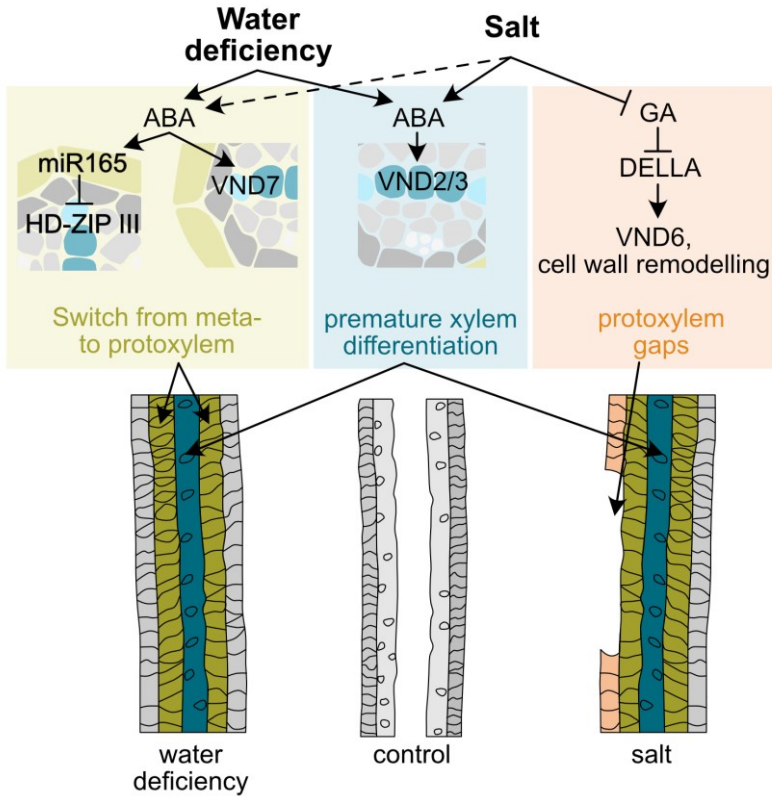

Figure 3: Mechanisms regulating xylem plasticity in response to water deficiency and salt. The bottom row illustrates the observed xylem phenotypes upon water deficiency and salt in comparison to the control conditions. Spiral pattern indicates protoxylem, pitted pattern indicates metaxylem. The upper row depicts pathways identified in this thesis that control xylem development in response to water deficiency and salt. Water deficiency induces a switch from metaxylem to protoxylem (green) via non-cell autonomous endodermal abscisic acid (ABA)-signalling enhancing miR165 leading to reduced HD-ZIP III levels (**Paper I**) and cell-autonomous xylem ABA-signalling inducing VND7 (**Paper II**). Additionally, water deficiency induces premature xylem differentiation (petrol blue) via cell-autonomous xylem ABA-signalling inducing VND2 and VND3 (**Paper II**). Salt predominantly induces protoxylem gaps (orange) via reduced gibberellin (GA) levels and signalling, leading to stabilisation of DELLA that result in activation of VND6 and cell wall synthesis and remodelling genes (**Paper III**).

Summary

During their life, plants may be exposed to a variety of different environmental conditions including times of water deficiency and high soil salinity, both affecting the uptake of water into the plant. Plants take up water from the soil by the roots and distribute it throughout the plant via its water conducting tissue, the xylem. Primary xylem of the model plant *Arabidopsis thaliana* is composed of three metaxylem strands in the centrum of the stele surrounded by two protoxylem strands at the outside. In general, plants are quite plastic and different developmental adjustments of the root to water deficiency and salt stress have been reported. The idea that water availability is affecting the development of plant's water conducting tissue is intriguingly as xylem structure seem to affect plant's ability to transport water. In this thesis, I have studied how water deficiency and salt affect xylem development and how the observed phenotypic plasticity is regulated on a molecular level.

Upon water deficiency, we have observed the formation of additional protoxylem strands and an early differentiation of the inner metaxylem. Inhibiting ABA-signaling in different tissue types, we found that these phenotypes were regulated by a non-autonomous and a cell autonomous ABA-signaling pathway. The expression of the well-known xylem regulatory miRNA miR165 was induced by ABA signaling in the endodermis and led to the consequent downregulation of HD-ZIP III transcription factors in the stele causing a shift to protoxylem identity and the formation of additional protoxylem strands. At the same time, cell autonomous ABA signaling caused the upregulation of *VND7,* which also conferred a shift to protoxylem identity. The stele autonomous ABA signal also induced *VND2/3* expression causing the early differentiation of the inner metaxylem. We further found a set of xylem differentiation related genes differentially expressed in the *vnd123* mutant upon ABA treatment.

While water deficiency through ABA signaling increased xylem differentiation and additional formation of protoxylem, we observed the formation of protoxylem gaps during an initial phase upon growth on salt. This phenotype was not observed when exposing the plants to other kinds of osmotic stress that would induce water deficiency indicating that this response is specific to ionic stress. We further could identify that protoxylem gaps were caused by

lowered GA levels and signaling. Furthermore, an increase in GA signaling in a mutant lacking the negative regulator of GA signaling DELLA, led to a reduction of protoxylem gap formation upon growth on salt. Moreover, we could identify genes involved in cell wall formation and remodeling, as well as the xylem master regulator *VND6* to be differentially expressed in the *della* quintuple mutant in response to salt. Consistently, *vnd6* mutants exhibited decreased protoxylem gap formation upon growth on salt.

We observed xylem gaps upon growth on salt in a GA dependent manner, but also when inhibiting ABA signaling in the endodermis indicating that altering both GA and ABA signaling may cause a changed xylem differentiation. ABA signaling in the endodermis was needed to induce the miR165-HD-ZIP III pathway, illustrating that a developmental pathway is employed upon stress. Common genes that were differentially expressed in these processes included genes involved in cell wall remodeling and peroxidases.

We saw similar effects on xylem developmental plasticity in response to water deficiency and salt in various different dicot species indicating an evolutionary conservation. Salt tolerance assays on different plants that show alterations in protoxylem gap formation indicate that the observed plastic response upon salt confer higher tolerance. Furthermore, the phenotypes that we observed upon water deficiency have been suggested to confer drought tolerance rendering xylem developmental plasticity an interesting trait for future breeding programs to generate plants better prepared for a changing climate.

Sammanfattning

De senaste åren har vi haft många dagar av torka eller extremt mycket regn. Dessutom har översvämningar i kustnära områden lett till att odlingsmarker i många delar av världen blivit saltare. Alla dessa faktorer uppfattas av växten som stress, minskar skörden och riskera därmed vår matförsörjning. Men, växternas kontinuerliga tillväxt ger förutsättningar för att de ska kunna anpassa sig till olika förhållanden och man har många gånger observerat att rotsystemets struktur och tillväxt anpassas till torka eller salt. In den här avhandlingen har jag undersökt om och hur växternas vattenledningsvärvnads, xylemets, utveckling kan anpassas till torka och salt och vilka molekylära mekanismer som reglerar den processen.

Växternas ledningsvävnad består av xylem och floem. Genom xylemet transporteras vatten och näringsämnen från roten upp till skottet, medan socker från bladens fotosyntes transporteras via floemet till andra växtdelar. I modellväxten backtrav, *Arabidopsis thaliana*, finns det en xylemaxel i roten med protoxylem ytterst och metaxylem i mitten. Protoxylem har spiralformiga cellväggsmönster och utvecklas nära rotens spets. Metaxylem har poriga cellväggar och utvecklas högre upp i roten. Vi undrade om xylemets utveckling är påverkat av torka och salt då xylemets struktur, antal xylemsträngar och hur nära rotspetsen det utvecklas skulle kunna på verkar växtens förmåga att ta upp och transportera vatten och andra ämnen. För att undersöka den här frågan fokuserade vi på roten hos Arabidopsis eftersom den har en relativt enkel och under normala förhållanden oföränderlig uppbyggnad som gör att förändringar i xylemets utseende enkelt kan observeras här.

Vi såg att när vi begränsade växternas tillgång till vatten fick växten fler xylemsträngar och fler med protoxylemstruktur. Dessutom utvecklades xylemet närmare rotspetsen än när växten hade god tillgång till vatten. Båda reaktionerna var plastiska, vilket betyder att xylemutvecklingen återgick till den normala när stressen upphörde. Växternas reaktion på omvärldsfaktorer styrs av växthormoner. Särskilt växthormonet abskissinsyra (ABA) är viktigt för att signalera abiotisk stress som torka och hög saltkoncentration i miljön. Genom att begränsa växtens ABA-signalering i olika vävnadstyper såg vi att xylemutvecklingens svar på torka styrs av signaler både i endodermis, som omger

ledningsvävnaden, och i omogna xylemceller. Medan xylemidentiteten regleras från båda dessa vävnader, så är xylemdifferentieringen närmare rotspetsen bara reglerat av ABAs signalering i xylemet.

Vi ville förstå de underliggande mekanismer som kontrollerar xylemets utveckling när växtens tillgång till vatten begränsats. Xylemidentiteten kontrolleras även i vanliga fall av ett mikroRNA (miR165) som vandrar från endodermis och in till ledningsvävnaden där det nedreglerar nivåerna av HD-ZIP III transkriptionsfaktorer. Låga nivåer av HD-ZIP III leder till protoxylemutveckling, medan höga nivåer leder till utveckling av metaxylem. Vi fann att *MIR165A* var uppreglerat i endodermis under torka och efter ABA-behandling och därmed ledde till en nedreglering av HD-ZIP III som ledde till protoxylem identitet. Protoxylemidentitet är också kopplat till ABA-signalering i xylemet. Genom transkriptomanalys identifierade vi faktorer som kontrollerar xylemets utveckling och är annorlunda uttryckta efter ABA-behandling. Bland dessa var VND7, som är en viktig faktor sedan tidigare känd för att kontrollera protoxylemets utveckling. I en mutant av VND7 begränsades förmågan att bilda mer protoxylemsträngar när växten behandlades med ABA. Det betyder att VND7 är en viktig komponent för det här svaret på stress. Tidig xylemdifferentiering däremot visade sig vara kontrollerat av några andra VND transkriptionsfaktorer, VND2 och VND3.

Salt utsätter växter både för minskad möjlighet att ta upp vatten på grund av osmotiska förhållanden, men natrium och kloridjoner kan också vara toxiska för växten. Vi undrade därför om salt hade en liknande effekt på xylemets utveckling som torka, men vi fann att det bildades protoxylemsträngar där vissa celler inte var differentierat, och därför bildat luckor i strängarna, så kallade ”xylem-gaps”, när växten vuxit på salt. Det här var en fenotyp som vi inte hade sett under torka och som inte kontrollerades av ABA signalering. Vi såg att xylem-gaps i stället verkade orsakas av en lägre gibberellinnivå och -signalering. Vi identifierade VND6, vilken normalt reglerar metaxylem differentiering, som en faktor som under salt-stress bidrar till utvecklingen av xylem-gaps. För att identifiera fler faktorer som inblandade i utvecklingen av xylem-gaps jämförde vi olika transkriptomdataset av olika genotyper och behandlingar som alla ger xylem-gaps. På så vis identifierade vi expansiner och andra cellväggsmodifierande enzymer som potentiellt viktiga faktorer.

Även om Arabidopsis är en bra modell för att studera molekylära mekanismer, så är det viktig att veta om processerna är likartade, och därför evolutionärt bevarade, i andra arter med relevans för jordbruket. Vi såg att både tidig xylemutveckling under torka och utvecklingen av xylem-gaps under salt sker i andra tvåhjärtbladiga arter, som tomat och sockerbeta. De här fenotyperna verkar ge växterna en större möjlighet att överleva salt och torka, särskilt under deras etableringsfas.

Vi har alltså funnit att utvecklingen av växternas vattenledningsvävnad, xylemet, anpassas till olika förhållanden, förmodligen för att hjälpa växten att överleva perioder av stress. Xylemets plastiska svar på torka och salt är reglerad av olika molekylära mekanismer som troligen interagerar. Detta indikerar komplexitet men ger också kunskap som potentiellt kan vara viktig för förädling av växter som bättre kan anpassa sin utveckling till olika omvärldsfaktorer i ett föränderligt klimat.

Zusammenfassung

In den letzten Jahren sind extreme Wetterereignisse, wie Trockenheit oder ungewöhnlich starke Niederschläge, häufiger geworden. Zudem haben Überschwemmungen, durch einen erhöhten Meeresspiegel, in Küstengebieten die Versalzung von Ackerflächen beschleunigt. All diese Faktoren werden von Pflanzen als Stress wahrgenommen, verringern Ernteerträge und bringen dadurch die Nahrungsmittelversorgung in Gefahr. Pflanzen haben jedoch durch ihr kontinuierliches Wachstum gute Voraussetzungen, sich an unterschiedliche Umweltbedingungen anzupassen und Veränderungen in der Struktur des Wurzelsystemes und des Wurzelwachstum als Antwort auf Trockenheit und Salzstress sind bekannt. In meiner Doktorarbeit habe ich untersucht, ob und wie sich die Entwicklung des Wasserleitgewebes der Pflanze, des Xylems, an Trockenheit und Salz anpasst und welche molekularen Mechanismen diesen Prozess regulieren.

Das Leitgewebe der Pflanzen setzt sich aus Xylem und Phloem zusammen. Durch das Xylem transportiert die Pflanze Wasser und Nahrung von der Wurzel in die Sprossachse und die Blätter, während sie durch das Phloem Zucker, der in der Photosynthese in den Blättern gebildet wurde, in andere Teile der Pflanze transportiert. In der Modellpflanze Acker-Schmalwand, *Arabidopsis thaliana*, besteht das Xylem der Wurzel aus zwei Protoxylempolen außen und Metaxylem in der Mitte. Protoxylem hat spiralförmig verdickte Zellwände und entwickelt sich in der Nähe der Wurzelspitze. Metaxylem hat löchrige Zellwände und entwickelt sich weiter oben in der Wurzel.

Wir wollten wissen, ob die Entwicklung des Xylems durch Trockenheit und Salz beeinflusst wird, weil sowohl die Xylemstruktur, die Anzahl der Xylemstränge, als auch der Umstand, wie nah an der Wurzelspitze sich das Xylem entwickelt, die Fähigkeit der Pflanze, Wasser und andere Substanzen aufzunehmen und zu transportieren beeinflussen könnte. Um diese Fragestellung zu untersuchen, verwendeten wir die Arabidopsis-Wurzel, weil diese einen relativ einfachen und unter normalen Bedingungen stabilen Aufbau hat, der es vereinfacht, Veränderungen in der Xylemstruktur zu beobachten.

Es stellte sich heraus, dass der begrenzte Zugang zu Wasser, die Entwicklung von Xylem mit Protoxylemeigenschaften und die Ausbildung zusätzlicher

Protoxylemstränge begünstigte. Zudem entwickelte sich das Xylem näher an der Wurzelspitze im Vergleich zu Pflanzen, die Zugang zu ausreichend Wasser hatten. Beide Reaktionen waren plastisch, das bedeutet, dass die Xylementwicklung wieder normal ablief, nachdem der Stress aufgehört hatte. Die Reaktion von Pflanzen auf Umweltbedingungen wird durch Phytohormone kontrolliert. Besonders das Phytohormon Abscisinsäure (ABA) ist wichtig, um abiotischen Stress wie z. B. Trockenheit und hohe Salzkonzentrationen zu signalisieren. Die Begrenzung des ABA-Signalweges in verschiedenen Gewebetypen der Pflanze zeigte, dass die Antwort der Xylementwicklung auf Trockenheit durch ABA-Signale sowohl in der Endodermis, eine Zellschicht, die das Leitgewebe umgibt, als auch in undifferenzierten Xylemzellen kontrolliert wird. Während die Xylemidentität durch Signale in beiden Geweben kontrolliert wird, wird die Xylemdifferenzierung in der Nähe der Wurzelspitze nur durch ein ABA-Signal im Xylem reguliert.

Daraufhin untersuchten wir die zugrundeliegenden Mechanismen, die die Xylementwicklung kontrollieren, wenn der Zugang der Pflanze zu Wasser begrenzt ist. Die Xylemidentität wird normalerweise durch eine microRNA (miR165), die von der Endodermis in das Leitgewebe migriert, wo sie das Niveau an HD-ZIP III Transkriptionsfaktoren senkt, reguliert. Ein niedriges HD-ZIP III Niveau veranlasst Protoxylementwicklung, während ein hohes Niveau Metaxylementwicklung begünstigt. Die MIR165A ist während Trockenheit und nach ABA-Behandlung in der Endodermis hochreguliert. Dieses führt zu einer Herunterregulierung von HD-ZIP III , was die Ausbildung von Protoxylemidentität zur Folge hat. Protoxylemidentität ist auch an ein ABA-Signal im Xylem gekoppelt. Durch eine Transkriptionsanalyse identifizierten wir Faktoren, die die Xylementwicklung kontrollieren und deren Expression nach ABA-Behandlung verändert war. Unter diesen war der Transkriptionsfaktor VND7, welcher als ein wichtiger Faktor für die Protoxylementwicklung bekannt ist. In einer Mutante von VND7 war die Fähigkeit, verstärkt Protoxylem zu bilden, wenn die Pflanze mit ABA behandelt wurde, verringert. Das bedeutet, dass VND7 eine wichtige Aufgabe in dieser Stressreaktion hat. Desweiteren stellte sich heraus, dass die verfrühte Xylemdifferenzierung von anderen VND Transkriptionsfaktoren, VND2 und 3, kontrolliert wird.

Salz beeinflusst Pflanzen negativ, sowohl durch eine verringerte Möglichkeit der Wasseraufnahme auf Grund von osmotischen Umständen, als auch durch Ionentoxizität. Wir untersuchten, ob Salz einen ähnlichen Effekt auf die Xylementwicklung hatte wie Trockenheit. Wenn die Pflanze auf Salz wuchs, wurden jedoch Protoxylemstränge gebildet, in denen bestimmte Zellen nicht differenziert waren und die Stränge deswegen Lücken enthielten. Dieser Phenotyp entstand nicht während Trockenheit und er wurde nicht durch ein ABA-Signal kontrolliert. Stattdessen wurden Xylemlücken durch eine Abnahme des Niveaus des Phytohormons Giberellin (GA) und der GA-Signalübertragung

verursacht. Wir identifizierten VND6, welches im Normalfall die Metaxylemdifferenzierung reguliert, als einen Faktor, der bei Salzstress zur Entwicklung von Xylemlücken beiträgt. Um weitere Faktoren, die an der Entwicklung von Xylemlücken beteiligt sind, zu indentifizieren, wurden verschiedene Transkriptome von unterschiedlichen Genotypen und Behandlungen verglichen, die Xylemlücken hervorrufen. Auf diese Weise wurden Expansine und andere zellwandsmodifizierenden Enzyme als potentiell wichtige Faktoren gefunden.

Auch wenn Arabidopsis eine gute Modellpflanze ist, um molekulare Mechanismen zu verstehen, ist es wichtig zu wissen, ob ähnliche Prozesse auch in anderen Pflanzenarten mit Relevanz für die Landwirtschaft ablaufen und damit evolutionär konserviert sind. Sowohl verfrühte Xylementwicklung bei Trockenheit, als auch die Entwicklung von Xylemlücken bei Salzstress kamen in anderen zweikeimblättrigen Arten, wie Tomaten und Zuckerrüben, vor. Diese Phenotypen scheinen Pflanzen eine verbesserte Möglichkeit zum Überleben unter Salz und Trockenheit zu geben, besonders während der Etablierungsphase junger Keimlinge.

Wir haben also herausgefunden, dass die Entwicklung des Wasserleitgewebes der Pflanze, des Xylems, sich an unterschiedliche Bedingungen anpasst, vermutlich um der Pflanze zu helfen, Zeiten von Stress zu überleben. Die plastische Antwort des Xylems auf Trockenheit und Salz ist durch unterschiedliche molekulare Mechanismen, die vermutlich interagieren, reguliert. Dies weist auf Komplexität hin, aber hilft uns auch dabei zu verstehen, wie neue Pflanzen gezüchtet werden können, die ihre Entwicklung besser an verschiedene Umweltbedingungen in einem sich verändernden Klima anpassen können.

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