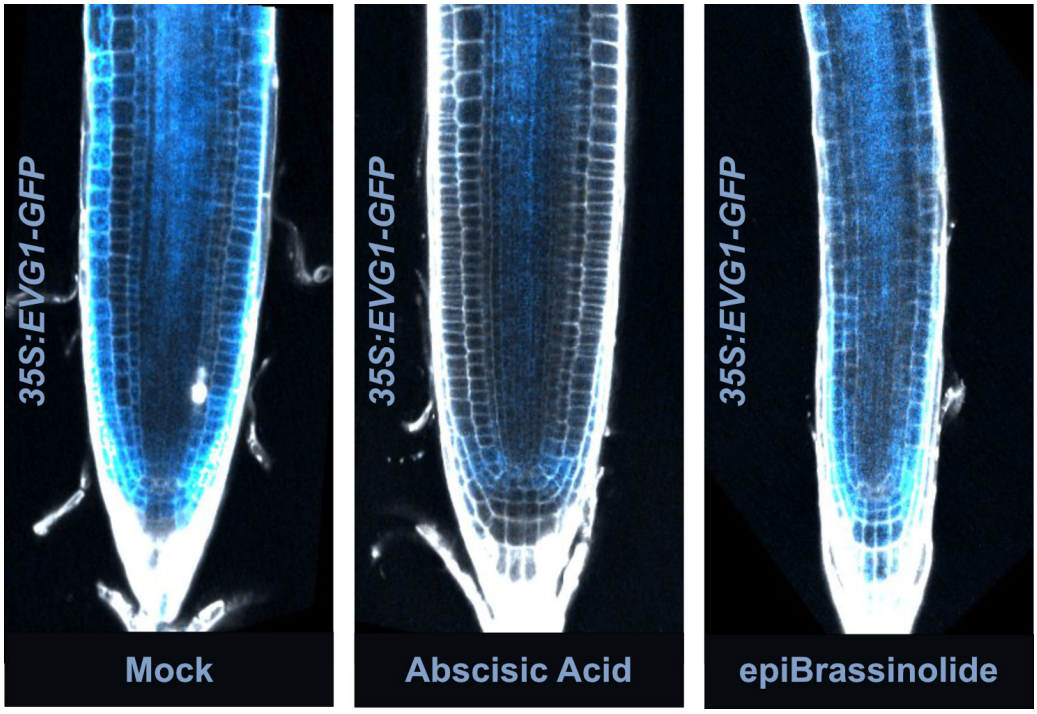
Acta Universitatis Agriculturae Sueciae

Doctoral Thesis No. 2022:52
Faculty of Natural Resources and Agricultural Scineces

The effect of stress on plant vascular development and regeneration

Shamik Mazumdar



The effect of stress on plant vascular development and regeneration

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SLU
SWEDISH UNIVERSITY
OF AGRICULTURAL
SCIENCES

DOCTORAL THESIS
Uppsala 2022

Acta Universitatis Agriculturae Sueciae
2022:52

Cover: Image of *35S:EVG1-GFP* under mock, ABA, and epiBL conditions after 24 hours (Shamik Mazumdar)

ISSN 1652-6880
ISBN (print version) 978-91-7760-979-7
ISBN (electronic version) 978-91-7760-980-3

Print: SLU Grafisk Service, Uppsala 2022

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Abstract

Plants are susceptible to stress due to their lifestyle and as such have evolved multiple adaptive strategies to ensure survival. One of the most remarkable abilities in plants is their competence to regenerate tissues. Particularly, any damage to vascular tissues is healed quickly to continue survival. This thesis aimed to identify the effect of stress on plant vascular development and regeneration using the model plant *Arabidopsis thaliana*. The thesis shows how abiotic stresses activate abscisic acid (ABA) signaling pathway, which activates VASCULAR RELATED NAC DOMAIN transcription factors to enhance xylem development to mitigate stress (Paper I). Analysis of another phytohormone signaling pathway, brassinosteroid (BR) revealed that it affects both cambium and xylem development. Additionally, both canonical BR signaling and RECEPETOR LIKE PROTEIN 44 (RLP44) associated BR signaling are required for regeneration and to maintain the balance between cambium and xylem development (Paper II). While the regenerative ability benefits plants, it is also used by biotic agents to the detriment of the plants. We identified a gene, *ENHANCER OF VISUAL AND GRAFTING 1* (*EVG1*), that was commonly induced across biotic and abiotic stresses. *EVG1* affected vascular development, regeneration, and mutation of the gene caused differential expression of cell wall related genes. The thesis demonstrates how *EVG1* is highly stress responsive and potentially acts as a stress signal and mediates developmental changes (Paper III). Overall, this thesis expands our knowledge as to how stress affects vascular development and regeneration.

Keywords: Abscisic acid, Brassinosteroid, Cambium, Cell wall, Phloem, Regeneration, Stress, Xylem

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Effekten av stress på växternas vaskulära utveckling och regeneration

Abstrakt

Växter är på grund av sin livsstil mottagliga för stress och har därför utvecklat flera adaptiva strategier för att säkerställa överlevnad. En av de mest anmärkningsvärda är växters förmåga att regenerera vävnader. Speciellt intressant och viktigt för överlevnaden är den snabba läkningen av vaskulära skador. Denna avhandling syftade till att identifiera effekten av stress på växternas vaskulära utveckling och vävnads regenereration med hjälp av modellväxten *Arabidopsis thaliana*. Avhandlingen visar hur abiotiska påfrestningar aktiverar signalering via abscisinsyra (ABA), vilket aktiverar VASKULÄRRELATERADE NAC DOMAIN transkriptionsfaktorer för att förbättra xylemutvecklingen och därmed mildra stresspåverkan (Paper I). Vi kunde också visa att både kambium- och xylemutvecklingen påverkas av signalering via ett annat fytohormon, brassinosteroid (BR). Dessutom krävs både kanonisk BR-signalering och RECEPETOR LIKE PROTEIN 44 (RLP44) associerad BR-signalering för regenerering och för att upprätthålla balansen mellan kambium- och xylemutveckling (Paper II). Även om den regenerativa förmågan gynnar växter, används den också av biotiska angripare till skada för växterna. Vi identifierade en gen, *ENHANCER OF VISUAL AND GRAFTING 1* (*EVG1*), som vanligtvis inducerades av både biotiska och abiotiska påfrestningar. *EVG1* påverkade vaskulär utveckling, regenerering och mutation av genen orsakade differentiellt uttryck av cellväggsrelaterade gener. Avhandlingen visar att *EVG1* aktiveras av stress och potentiellt fungerar som en stresssignal och förmedlar utvecklingsförändringar (Paper III). Sammantaget utökar denna avhandling vår kunskap om hur stress påverkar vaskulär utveckling och regenerering.

Nyckelord: Abscisic Acid, Brassinosteroids, Cambium, Cell wall, Floem, Regeneration, Stress, Xylem

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Contents

List of publications

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

I. **Ramachandran P, Augstein F, Mazumdar S, Nguyen TV, Minina, EA, Melnyk CW, & Carlsbecker A.** (2021). Abscisic acid signaling activates distinct VND transcription factors to promote xylem differentiation in Arabidopsis. Current biology: CB, 31(14), 3153–3161.e5

II. **Mazumdar S, Musseau C, and Melnyk CW. (**2022). The role of brassinosteroid signaling in vascular development and regeneration (manuscript)

III. **Mazumdar S, Zhang A, and Melnyk CW.** (2022). *EVG1* regulates vascular development and regeneration in response to stress (manuscript)

Paper I is reproduced with the permission of the publishers.

The following paper was written during my doctoral studies but is not part of the present dissertation:

1. **Canher B, Lanssens F, Zhang A, Bisht A, Mazumdar S, Heyman J, Wolf S, Melnyk CW, De Veylder L.** (2022). The regeneration factors ERF114 and ERF115 regulate auxin-mediated lateral root development in response to mechanical cues. *Molecular Plant*. https://doi.org/10.1016/j.molp.2022.08.008

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1. Introduction

Development of the individual is common to all organisms on this planet. It is how an organism grows and completes its life cycle. Plants have developmental trajectories as well, characterized by various growth stages. Plant development results in the different structures that comprises a plant body originating and growing. The growth is continuous and can respond adjust to external cues. Plant growth initially is dependent upon meristematic tissue found in the shoot apical and root apical meristems. Cells in the meristems divide and differentiate to give rise to primary growth. Secondary growth in plants occurs when the shoot or the root grows laterally due to the multiplication of the meristematic cells within the vasculature, a tissue termed cambium.

Plants are mostly sessile and being rooted to a certain location creates unique challenges that plants must overcome. One of the more important challenges is acquiring resources from the and the distribution of nutrients and resources within the plant. This task falls on the plant vascular system. Thus, one of the major developmental events in plants is the formation of plant vascular system which includes tissues such as xylem, cambium, and phloem. A working vascular system is at the very core of healthy plant development. The vascular system not only provides structural support to the plant, but also allows the movement of key components such as nutrients, water, sugars, RNAs, and certain signaling proteins which are important for plant survival (Lucas et al. 2013). The vascular system comprises of three different types of tissue, two of them being conductive tissues. Xylem tissues function as a hydraulic pipe, transporting water and minerals collected from the soil to the aerial parts of the plant. Phloem tissues on the other hand act as the conductive tissues that help in movement and distribution of photosynthetic

products, RNA, hormones, and proteins. A third tissue layer exists, known as procambium or cambium depending on the stage of development. Procambium or cambium acts as a meristem which divides and differentiates to form more xylem and phloem. Since the development of vasculature happens throughout the life cycle of a plant it is helpful to demarcate primary vasculature and secondary vasculature (Esau 1960; Agustí & Blázquez 2020).

1.1 Vascular development – an overview

Due to its inherent importance in the life cycle of a plant the vascular system begins development from the early growth stages of plant development. It is during formation and development of the embryo that the first steps towards developing a vascular system are initiated (Scheres et al. 1994; De Rybel et al. 2013; Yoshida et al. 2014). This thesis will focus on vascular development in the model plant *Arabidopsis thaliana*. The development of vasculature in *A. thaliana* can be divided into four sections namely, cell specification, cell identity establishment, cell identity maintenance, and finally cell differentiation(De Rybel et al. 2016).

The globular stage of embryo development sees the formation of the first cells that will act as provascular initials (Figure 1a), and this is cellular specification. This happens when the inner four cells towards the distal end of the embryo divide to generate a zone of elongated cells (Scheres et al. 1994; Caño-Delgado et al. 2010; Yoshida et al. 2014; Ruonala et al. 2017). After this, these cells undergo a series of cell divisions that are tightly controlled, and thus they establish the procambium which then differentiates into protoxylem and protophloem precursors. While the differentiation happens after embryo development, it has been found that all cell identities required for vascular formation for roots is already present at the end of embryogenesis (Bonke et al. 2003; Bauby et al. 2007; Truernit et al. 2012). Post gemination, the provascular cells in the embryo differentiate into functional vasculature for the root and the hypocotyl, but towards the shoot the vasculature is derived from the shoot apical meristem (SAM) (De Rybel et al. 2016). The development of vascular tissues and its maintenance after germination takes place in particular regions that have high rates of cell

division, regions termed as meristems. Vascular transport tissues in general have two distinct tissue types – xylem and phloem with functions as mentioned briefly before. After the cells in meristems have divided and have acquired an identity or fate, they exit the meristems to be further differentiated into specialized xylem or phloem cells (Lucas et al. 2013). Cells in both tissue types, xylem and phloem have unique cell forms that help in conducting or movement of requisite substance. Xylem possesses tracheary elements and phloem possesses sieve elements with each cell type have their own special secondary cell wall traits along with other specifications (Lucas et al. 2013).

All the vascular development discussed so far has been focused on embryo and post embryo development of the plant and is termed primary vascular development which is responsible for elongating tissues and organs. But *A. thaliana* being a dicot possess another type of vascular development as the plant continues to grow to allow radial expansion. This type of growth and development is termed as secondary growth and is characterized by the formation of a secondary vascular system (Figure 1b). As growth in plants is dependent on meristems, secondary growth is also dependent on a meristem called vascular cambium. Cambium is located on the inner side of stems, roots, and hypocotyls in a ring-like structure termed cambial ring (Agusti et al. 2011). In roots and hypocotyls, there is a massive proliferation in cell division of procambial cells (Dolan et al. 1993) which results in formation of cambial cells. Cambial cells next to primary xylem differentiate into secondary xylem (Thamm et al. 2019).This creates a chain event with more new cambial cells that are next to new secondary xylem cells differentiating into secondary xylem. This in turn increases the amount of secondary xylem cells and causes the formation of a cambial ring, which is the radial distribution of the cambium towards the outer part (Thamm et al. 2019). Cambium also differentiates to the form secondary phloem, and this results in secondary growth.

It can be observed that nature has a lot of diversity, and that holds true for the vascular system arrangement as well with the vascular system of *A. thaliana* being one of them. *A. thaliana* root has a diarch pattern of vasculature, that is a xylem axis that is in the center which is then bordered

by two phloem poles that typically has four cells each (Baum et al. 2002). Between the xylem axis and the phloem poles there are the procambial cells. A layer of cells known as pericycle surrounds this entire arrangement. *A. thaliana* roots and hypocotyl present a strong model system to understand vascular development and thus in all subsequent sections will be based on *A. thaliana* roots and hypocotyl predominantly unless mentioned otherwise.

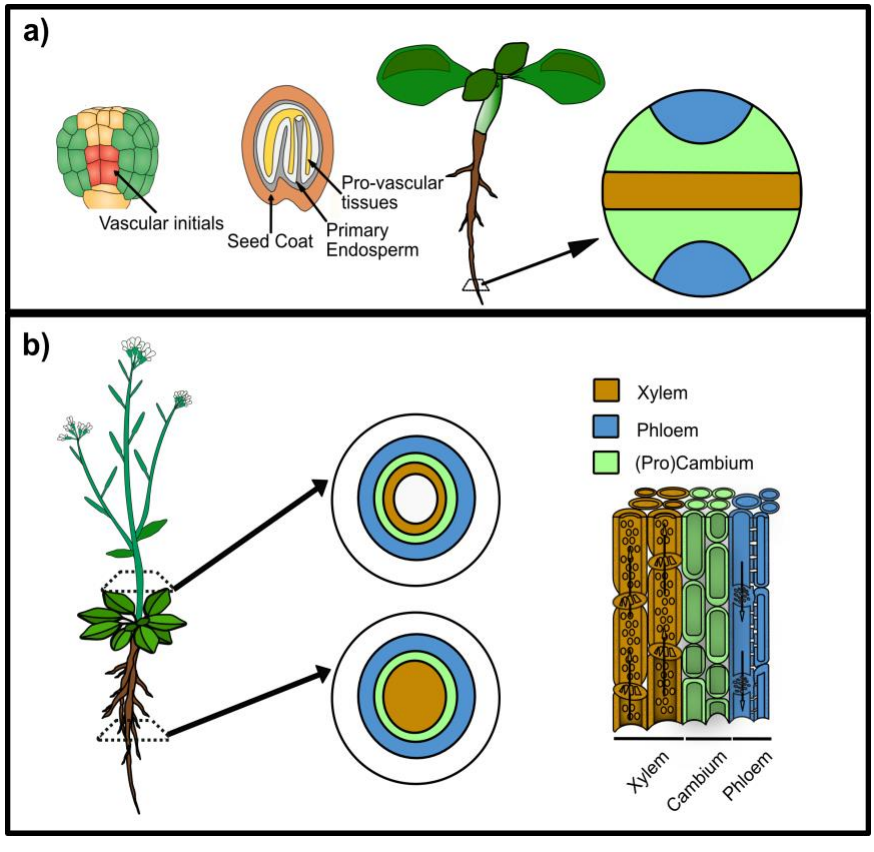

Figure 1. Development of the vascular system in plants. a) Primary development associated with tissue elongation starts from embryo with initial cells (orange). b) Secondary development associated with radial growth occurs in plants at a later stage. Adapted from Augusti and Blasquez, 2018; De Rybel et al., 2016)

1.1.1 Xylem

Xylem is the conductive tissue in the vasculature that is responsible for transport of water and nutrients from the root to all tissues above ground. Xylem is comprised of specific cell types known as tracheary elements, xylem cell fibers, and xylem parenchyma. Primary xylem in *Arabidopsis* roots occurs in two different structural forms. One is protoxylem that is characterized by the presence of spiral cell walls located at the two poles of the xylem axis. The other is metaxylem, comprising the central three cells of the xylem axis, characterized by a pitted cell wall structure (Dolan et al. 1993).). From initial xylem cell specification to the final differentiated xylem cells, there are multiple steps that occur which are governed by a highly regulated network of hormones and genetic regulators and movement of mobile signals that control patterning.

One of the major components of early xylem development is the transcription factor encoded by the gene *SHORTROOT (SHR)*. It is expressed in the procambium and moves to the endodermis where it interacts with SCARECROW (SCR), another transcription factor (Di Laurenzio et al. 1996; Carlsbecker et al. 2010). This interaction activates the transcription of MIR165A, 166A, and 166B in the endodermis (Carlsbecker et al. 2010; Miyashima et al. 2011). Movement of these miRNAs inwards causes the formation of a gradient with the highest levels on the outside of the vascular bundle and low towards the center. miRNA165/166 interact and restrict the domains of the mRNAs produced by the class III homeo-domain leucine zipper (HD-ZIP III) genes including *ARABIDOPSIS THALIANA HOMEOBOX 8 (ATHB8), CORONA (ATHB15/CNA), PHABULOSA (ATHB14/PHB), REVOLUTA (REV),* and *PHAVOLUTA (ATHB9, PHV*). High amounts of miRNA165/166 degrades HD-ZIPIII mRNAs which promotes a more protoxylem like structure and the reverse results in a metaxylem like structure (Carlsbecker et al. 2010; Miyashima et al. 2011). This results in the classical architecture of the xylem axis in young *Arabidopsis* roots (Figure 2). Apart from this classical model, recent advances have found that leucine rich receptor like kinases including BARELY ANY MERISTEM 1 (BAM1) and BARELY ANY MERISTEM 2 (BAM2) can coordinate miRNA165/166 movement into the stele, thereby regulating xylem patterning (Fan et al. 2021).

The final steps in differentiation of xylem are very dramatic. When the cells are specified and patterned, they then undergo multiple processes such as cell elongation, cell wall thickening, secondary cell wall (SCW) formation, and finally cell death (Zhong & Ye 2012; Furuta et al. 2014). Two transcription factors that are master regulators of xylem development are encoded by the genes *VASCULAR RELATED NAC DOMAIN 6 (VND6)* and *VND7*. Genes responsible for SCW formation and cell death are activated by the effects of VND6 in metaxylem and VND7 in protoxylem (Kubo et al. 2005; Ohashi-Ito et al. 2010; Taylor-Teeples et al. 2015). Alongside this, other transcription factors such as MYB83 and MYB46 also activate lignin biosynthesis and aid in the differentiation of xylem (Fisher & Turner 2007; Hirakawa et al. 2008, 2010; Etchells & Turner 2010) (Figure 2). Another gene known as *VND INTERACTING 2* (*VNI2*) hampers xylem differentiation as VNI2 interacts with VND7 (Yamaguchi et al. 2010b). Xylem differentiation is also negatively regulated by small peptides encoded by two genes known as *CLAVATA3/ESR1 LIKE 41 (CLE41)* and *CLE44* (also known as tracheary element differentiation inhibitory factor or TDIF) (Fisher & Turner 2007; Hirakawa et al. 2008, 2010).

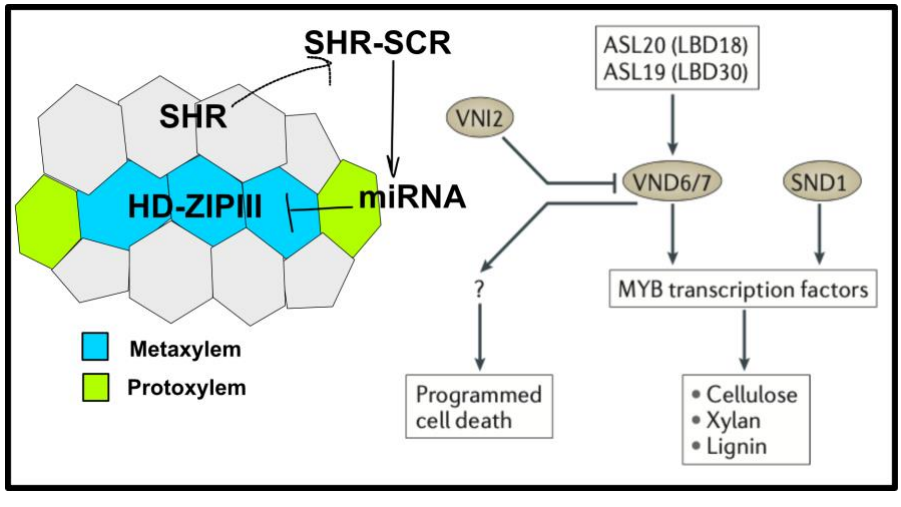

Figure 2. Xylem: Xylem carries water and minerals from roots to the shoots and is comprised of tracheary elements and xylem fibers. Adapted from Augusti and Blasquez, 2018; De Rybel et al., 2016

1.1.2 Phloem

Another conductive tissue present in the vascular system is phloem. Unlike xylem, the role of phloem is to transport photoassimilates and signaling molecules, with the general direction being from source to sink. The entire phloem tissue system comprises of sieve elements (SE), companion cells (CC), phloem fibers, and parenchyma cells. SE cells are composed of two types, protophloem and metaphloem (Figure 3). Some characteristic features of fully differentiated SE cells are that they are long and thin, and they are arranged in a straight line, to generate a sieve tube (Mullendore et al. 2010). To ensure long distance transport of photoassimilates and other compounds, the individual cells in the tube undergo cell wall thickening and nuclear breakdown. They also develop pores at the junction between two SE cells. These junctions are called sieve plates and allow the SE cells to create a continuous sieve tube. Apart from these, the SE cells also lose their organelles. The final stage in SE maturation is the loss of the nucleus known as the enucleation process (Cronshaw & Esau 1968; ESAU 1972; Eleftheriou & Tsekos 1982; Sjolund 1997; Busse & Evert 1999; Wu & Zheng 2003; Lucas et al. 2013).

ALTERED PHLOEM DEVELOPMENT or APL is the primary transcription factor that controls phloem development (Bonke et al. 2003). APL is a MYB transcription factor that controls phloem development and suppresses xylem differentiation as lack of APL results in plants with reduced phloem development but ectopic xylem formation (Bonke et al. 2003).). APL also helps in sieve element formation by controlling two the transcription factors NAC45 and NAC86 (Furuta et al. 2014), which in turn activates the *NAC45/86–DEPENDENT EXONUCLEASE DOMAIN PROTEIN 1 (NEN1*) to *NEN4* genes that control the enucleation process. NAC20 which is activated during cell specification negatively regulates APL (Kondo et al. 2016) (Figure 3). While APL regulates phloem differentiation there are other factors that control the formation and maintenance of different cell lineages present in the phloem tissue. OCTOPUS (OPS/PD4) a membrane bound protein controls protophloem specification and maintenance (Bauby et al. 2007; Truernit et al. 2012). The mutants of this gene have phloem development defect including failed differentiation of protophloem cells. Another gene, *BREVIS RADIX (BRX)*, when mutated was found to show

similar defects as that of mutant *OPS*. BRX is a target of AUXIN RESPONSE FACTOR 5 (ARF5) / MONOPTEROS (MP) and displays phenotypes associated with low penetrance MP-like embryo (Mouchel et al. 2006; Scacchi et al. 2009, 2010). BRX and MP help in maintaining sieve element identity. Recent studies have also identified markers for early phloem development. PHLOEM EARLY DOF (PEAR) genes encode transcription factors that are regulators of early protophloem sieve elements (Miyashima et al. 2019). Lastly, just like in xylem development, phloem development and differentiation are negatively regulated by another CLE peptide. CLE45 interacts with BAM3 and represses protophloem differentiation (Depuydt et al. 2013).

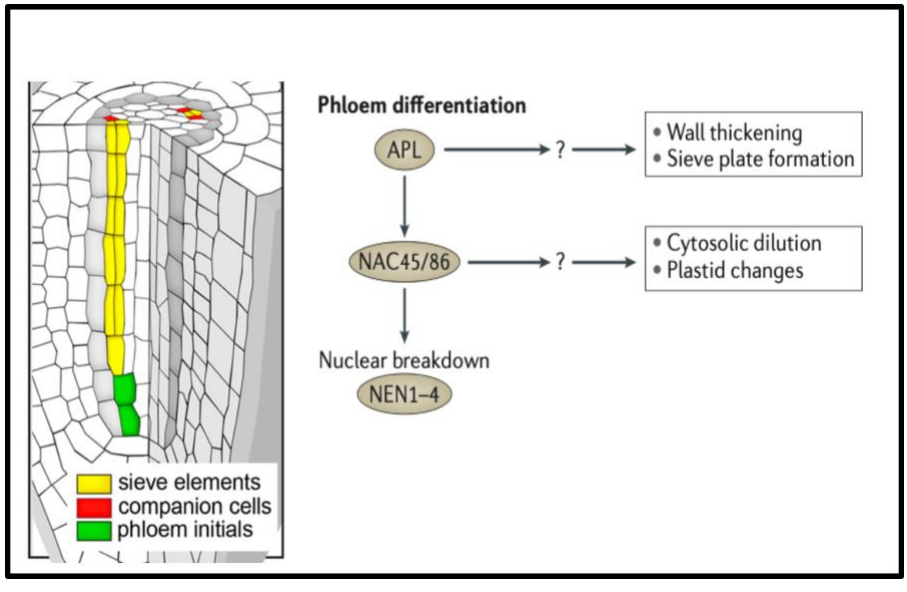

Figure 3. Phloem: Phloem functions to transport photoassimilates from shoot to root and is made up of sieve elements and companion cells. Adapted from Augusti and Blasquez, 2018; De Rybel et al., 2016

1.1.3 Cambium

The third tissue type that completes the vascular system is cambium. Cambium can be defined as the set of meristematic cells, or pluripotent cells that have the ability to form different cells of the vascular system, given the

right stimulus. Depending on the species and tissue, cambium can develop as two forms, a procambium and a secondary cambium. *Arabidopsis* as a dicot and a model plant has both procambium and the secondary cambium. Procambium can be found at the youngest regions of developing roots, hypocotyls, and leaves (Bauby et al. 2007). Procambium cells are meristematic cells that can give rise to both primary xylem and primary phloem in leaves and embryo. Development or establishment of procambium in *Arabidopsis* can be traced back to the four initial provascular cells in the embryo (Berleth et al. 2000). Post this the cells elongate and divide to generate more procambial cells (Yoshida et al. 2014). Post embryonically some procambial cells would divide to generate the precursors of both xylem and phloem, while the rest would maintain a subset of procambial cells between xylem and phloem tissues (Mähönen et al. 2000). Procambium initiation and maintenance is dependent on the interaction of many genes. It begins with MP, which activate a protein dimer formed out of two bHLH transcription factors TARGET OF MONOPTEROS 5 (TMO5) and LONESOME HIGHWAY (LHW) (De Rybel et al. 2013; Ohashi-Ito et al. 2013)(Figure 4). This then combines with hormonal signals to maintain cell divisions of procambial cells in the RAM and the whole root vasculature (Schlereth et al. 2010).

After the vascular tissues have differentiated and growth progresses in the root, secondary growth is initiated. The procambial cells close to the primary xylem undergo periclinal divisions to become cambial cells (Baum et al. 2002). Radial growth initiation in roots begins or is specified in the early protophloem (primary development stage) by the previously described PEAR genes (Miyashima et al. 2019). PEAR1 and PEAR 2, along with their closest homologs (DOF6, TMO6, HCA2, and OBP2) form a concentration gradient that is short ranged, peaking in the protophloem sieve elements, and activates expression of genes that control radial growth (Miyashima et al. 2019). PEAR proteins are antagonized by the HD-ZIPIII proteins whose expression domain is in the more internal regions of non-dividing (periclinal) procambial cells due to the restrictive action of miRNA165/166 as described previously (Carlsbecker et al. 2010; Miyashima et al. 2011, 2019). Thus, the PEAR proteins in protophloem locally antagonize HD-ZIPIII and create a negative feedback loop that generates a zone of cell division, creating in the

primary developmental stage a future basis for radial growth (De Rybel et al. 2016). Apart from the root, the mature hypocotyl also provides an interesting picture of secondary growth, where cambial cell divisions are activated and cell elongation stops (Sibout et al. 2008; Ragni et al. 2011). There are two phases of secondary or lateral growth in hypocotyls where in phase one the amount of secondary xylem and phloem produced are equal, and phase two where xylem tissue production exceeds phloem tissue production (Sibout et al. 2008; Ragni et al. 2011). As discussed previously, CLE41 and CLE44 peptides repress xylem differentiation and increase cambium formation. These peptides activate PHLOEM INTERCALATED WITH XYLEM (PXY or TDIF RECEPTOR, TDR) which is a leucine rich repeat receptor like kinase (Ito et al. 2006; Fisher & Turner 2007; Hirakawa et al. 2008, 2010). This signaling cascade activates *WUSCHEL RELATED HOMEOBOX4 (WOX4)* which regulates cambium proliferation. PXY, WOX4 and WOX14 together promote cambium activity (Etchells & Turner 2010). The CLE-PXY signaling pathway also controls cambial activity by not allowing xylem differentiation. The signaling pathway also activates *GLYCOGEN SYNTHASE KINASE 3 (GSK3)* member *BRASSINOSTEROID-INSENSITIVE 2 (BIN2)* (Kondo et al. 2014) which is a negative regulator of brassinosteroid (BR) signaling pathway and negatively regulates vascular differentiation thereby indirectly promoting cambium proliferation (Figure 4).

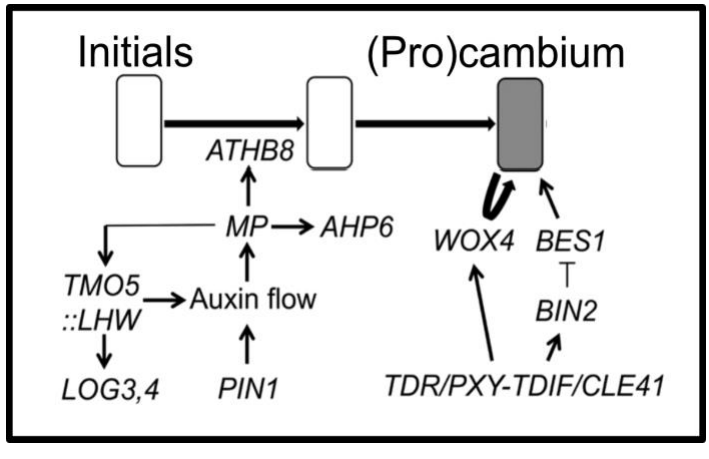

Figure 4. (Pro)cambium: (Pro)cambium acts as the meristem that can differentiate into both xylem and phloem depending on the signal. Adapted from Tan et al. 2019

Apart from the genetic control of cambium formation there are multiple hormonal interactions with these regulators that play a role in vascular development. Plant hormones including auxin, cytokinin, ethylene, abscisic acid (ABA), and brassinosteroid (BR) are thus indispensable and ubiquitous in vascular development. This will be discussed in the following sections.

1.2 Role of hormones in plant vascular development

Plant development and growth are mediated by a concerted effort and multifaceted interaction between genetic components and plant phytohormones. In fact, plant hormones or phytohormones as they are known play a role in every developmental stage and phase of a plant's life cycle, from seed, to fully-grown plant and even in their death. Thus, it is not surprising to see the involvement of phytohormones in vascular development in plants. Plant hormones interact with genetic factors and help in the initiation, specification, patterning, and differentiation of different vascular tissues during embryogenesis, primary development stage, and in secondary development. There are multiples levels of interactions and feedback loops to regulate and control the proper formation of vascular tissues at every stage and organ in the plant body

1.2.1 Auxin and Cytokinin

Auxin, the first phytohormone described and identified in plants plays a key role in vascular development and has been widely studied. Auxin plays a role from the very early stages of vascular development including in specification, tissue patterning and differentiation of cells to vascular cells. The auxin pathway is dependent on its perception by the SKP1–CUL1–F-box (SCF) ubiquitin ligase complex that contains TIR1 and AFB auxin binding proteins. These bind to and degrade Aux/IAA proteins that are negative regulators of auxin signaling. This activates AUXIN RESPONSE FACTORS or ARFs that bind to the free binding sites of downstream auxin responsive genes to change expression (Lavy & Estelle 2016; Leyser 2018). Auxin transport is mediated by various proteins including PIN-FORMED 1

(PIN1) (Scarpella et al. 2005); the hormone in turn activates genes such as *MP/ARF5*. MP also induces *PIN1*, which creates a feedback loop that increases auxin signaling which plays a further role in initiating and activating downstream genes that specify vascular cells (Scarpella et al. 2005; Wenzel et al. 2007; Donner et al. 2009). The role of auxin in promoting vascular formation can also be observed in the cases of wounding or grafting (Asahina et al. 2011; Melnyk et al. 2015; Canher et al. 2020).

Cytokinins are important in plant vascular development, cell division, growth, photosynthesis, senescence, and nutrient allocation (Kieber & Schaller 2014). Cytokinins are mobile in the plant vascular system and show movement from both root-to-shoot and shoot-to-root (Hirose et al. 2007; Matsumoto-Kitano et al. 2008). Cytokinins are synthesizedd by enzymes encoded by genes such as *LONELY GUY 3 (LOG3*) and *LONELY GUY 4 (LOG4*) (Kieber & Schaller 2014, 2018). They are percieved by resposnse regulators (RRs). There are two types of *Arabidopsis* RRs (ARRs), type B ARRs which are are needed for initial response to cyokinin and are positve regulators of cytokinin signaling (Argyros et al. 2008; Ishida et al. 2008). Type B ARRs apart from activating other cytokinin targets also stabilize and active the second type of RRs, the type A ARRs. Type A ARRs are the negative regulators of cytokinin signaling (Brandstatter & Kieber 1998; D'Agostino et al. 2000; To et al. 2008). Cytokinins are responsible for the periclinal divisions of provascular cells. Mutating a cytokinin receptor *WOODEN LEG (WOL)/ARABIDOPSIS HISTIDINE KINASE 4 (AHK4)* resulted in plants with reduced periclinal divisions in the provascular cells (Mähönen et al. 2000, 2006). Apart from the reduction in periclinal division another defect is the formation of ectopic protoxylem cells in the provasculature. The role of cytokinin in vascular development becomes clearer when observing a quadruple mutant of cytokinin biosynthesis genes ATP/ADP isopentenyltransferases (IPT), *ipt1,3,5,7*. The mutant displayed complete failure of cambium divisions and had reduced shoot and root thickness. Exogenous application of cytokinin rescued he root and shoot thickness along with cambium division (Matsumoto-Kitano et al. 2008). The root to shoot and shoot to root movement of cytokinin is important as cytokinin production either in the shoot or the root could rescue the phenotype in the whole plant (Hirose et al. 2007; Matsumoto-Kitano et al.

2008). Cytokinins also negatively regulate protoxylem formation. In fact, protoxylem formation is driven by a cytokinin signaling inhibitor ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN 6 (AHP6) (Mähönen et al. 2000). AHP6 is activated by MP, thus identifying a relationship between auxin and cytokinin signaling in vascular development. AHP6 is predominantly expressed in protoxylem cells, and thus protoxylem cells have high auxin signaling but low cytokinin signaling (Bishopp et al. 2011a; b).

Thus, a model can be visualized where the procambial cells have higher cytokinin levels and thus cause auxin to be carried out of the cell to protoxylem cells creating a tightly regulated feedback loop between auxin and cytokinin that is inhibitory and generates a vascular pattern that is bisymmetric (Figure 5). The relationship is established when *TMO5-LHW* that is activated by MP, induces the expression of *LOG3* and *LOG4*. So, auxin in a way not only has a negative regulatory effect on downstream cytokinin effects but also causes formation of cytokinin locally in the cells (De Rybel et al. 2014, 2016; Ohashi-Ito et al. 2014). Thus, we have a pattern of cells that have high levels of auxin signaling (xylem axis) placed next to the procambial cell zone that has high cytokinin signaling.

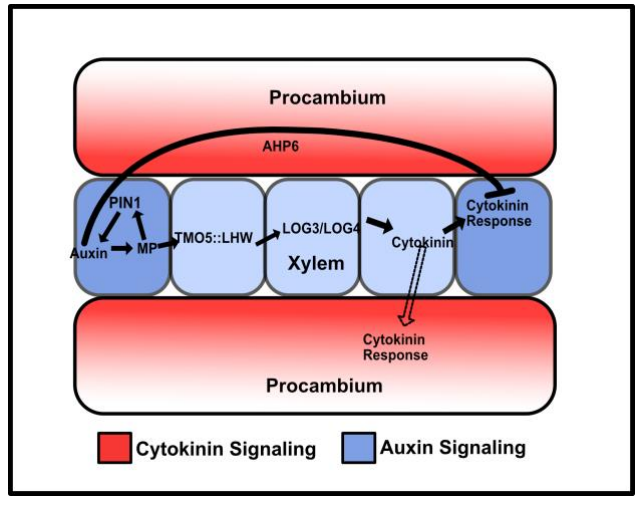

Figure 5. The role of auxin and cytokinin in patterning xylem axis and (pro)cambium cell types in young roots. Adapted from De Rybel et al., 2016

Apart from auxin and cytokinin, there are other phytohormones that play a role in vascular development as well. Brassinosteroids have been known to affect VND6 and VND7 and thus affect xylem development (Kubo et al. 2005). Moreover, ABA also activates miRNA165/166 thus affecting the patterning of the xylem axis (Ramachandran et al. 2018). These hormones also play a role in secondary development of the vasculature and cambium expansion and maintenance (Kang et al. 2017; Bloch et al. 2019; Saito & Kondo 2019). ABA also incorporates stress-based signals and has a role in regulating development in response to drought, cold, heat stress. Brassinosteroids have been reported to coordinate cell expansion, cell elongation and respond to heat stress (Albertos et al. 2022). This is why I next will focus on these two phytohormones.

1.2.2 Abscisic acid

Abscisic acid or ABA derives its name from the process of abscission as originally it was thought to play a major role in abscision. Although it was later found that ABA indirectly affects abscision by inducing ethylene, the name still persisits (Craker & Abeles 1969). Plants are remarkably adaptive, and have found ways to condition their growth so as to ensure survival under various stress conditions. Although many phytohormones interact together in complicated networks to achieve this feat, it was observed that ABA plays one of the major roles in this kind of adaptation. During drought, cold, heat, ans high salinity, plants increase the levels of endogenous ABA (Zhu 2002). ABA also controls seasonal growth by cell to cell communication. During winter or cold, plants adapt by going into a dormant state or reduced growth for survial. In peach it was found that ABA was produced in the terminal buds to protect the plant during winter months (Wang et al. 2016).. In hybrid aspen shorter days resulting short photoperiods reduce growth by suppressing FLOWERING LOCUS T2 and enhancing ABA response in the buds by enhancing ABA levels and ABA receptors (Ruttink et al. 2007; Karlberg et al. 2010). The induction of ABA causes plasmodesmata closure by enhancing levels of PDLP1 (PLASMODESMATALOCATED PROTEIN 1), thereby reducing symplastic transport (Tylewicz et al. 2018). This blocks the movement of growth promoting factors from bud such as FT1 and FT2 to meristem thereby promoting dormancy to survive the winter

months and not to grow in order to save resources (Tylewicz et al. 2018). More work identified that ABA had a role in regulating multiple developmental processes including seed dormancy, plant growth, and stomatal movement (Steuer et al. 1988; Finkelstein & Gibson 2002; Cutler et al. 2010). ABA further interacts with multiple phytohormones, often in an antagonistic manner with growth promoting hormones like gibberellins, cytokinins, and brassinosteroids (Zhang et al. 2009; O'Brien & Benková 2013; Shu et al. 2013; Du et al. 2015).

Resolving the ABA signaling pathway in detail has considerably helped in understanding the role of ABA in detail. In brief, ABA pathway relies on two groups of positive and negative regulators. PYRABACTIN RESISTANCE1/PYR1-LIKE/REGULATORY COMPONENTS OF ABA RECEPTORS (PYR/PYL/RCAR), act as the main receptors of ABA. A family of clade A Protein Phosphatase 2Cs (PP2Cs) such as ABI1, ABI2, HAB1, HAB2 and AHG3/PP2CA act both as co-receptors and as negative regulators, whereas Snf1-Related Kinase 2s (SnRK2s) act as positive regulators (Leung et al. 1994; Saez et al. 2004; Fujii et al. 2007, 2009; Ma et al. 2009; Park et al. 2009; Yoshida et al. 2010; Zhao et al. 2013; Fuchs et al. 2014). In normal conditions without the presence of ABA, the negative regulators (PP2Cs) bind to SnRK2s and impede their activity by phosphorylating them. When ABA is present, it is perceived by the PYR/PYL/RCAR which binds to the coreceptors PP2Cs. This then blocks the ability of PP2Cs to bind to SnRKs and the SnRKs are then activated which activate downstream ABA related genes by phosphorylating them (Nishimura et al. 2007; Fujii et al. 2009; Umezawa et al. 2010). The entire ABA pathway is summarized in Figure 6a.

Among various physiological and anatomical changes that are controlled by ABA, it also affects vascular development. In *Arabidopsis* root vascular development, the interaction between HD-ZIP IIIs and miRNA165/166 has been clearly identified as one of the major networks that determines the structure of the stele (Carlsbecker et al. 2010; Miyashima et al. 2011). The gradient formed by them determines the formation of protoxylem and metaxylem. Studies performed in multiple other species such as peach, wheat and barley identified that abiotic stress affect the levels of miRNA165/166

(Kantar et al. 2010; Eldem et al. 2012; Giusti et al. 2017). In populus, ABA also negatively regulates cambium development by activating miRNA169 and its target trancription factor Heme Activator Protein2 (HAP2) (Ding et al. 2016). ABA also plays a role in xylem patterning. Basal amounts of ABA are necessary for continuous xylem formation since ABA defective mutants resulted in patchy and discontinuous xylem strands including defects in secondary cell wall (SCW) formation. Moreover, simulating abiotic stresses by adding exogenous ABA or by using Polyethylene Glycol (PEG) to simulate drought increased the number of xylem cell files (Ramachandran et al. 2018). ABA also increases the transcript levels of miRNA165/166 (Ramachandran et al. 2020), non-cell autonomously affects the balance between miRNA165 and HD-ZIPIIIs causing anatomical changes in the xylem axis (Ramachandran et al. 2018). (Figure 6b and 6c). ABA is involved in secondary growth since ABA biosynthesis mutants have delayed fibre formation, although the ratio between xylem and phloem is not disrupted (Campbell et al. 2018). Addition of ABA differentiated protoxylem earlier in tomato and *Arabidopsis* root tips (Bloch et al. 2019). Although ABA generally interacts negatively with growth promoting hormones to help the plant adapt to stress conditions, overall, it causes both increases in xylem formation and earlier differentiation of xylem. This increased and early differntiation of xylem may help provide the plant root with better chances of uptaking more water during stress situations like drought and heat. Further research will help uncover whether ABA directly influences xylem differentiating genes.

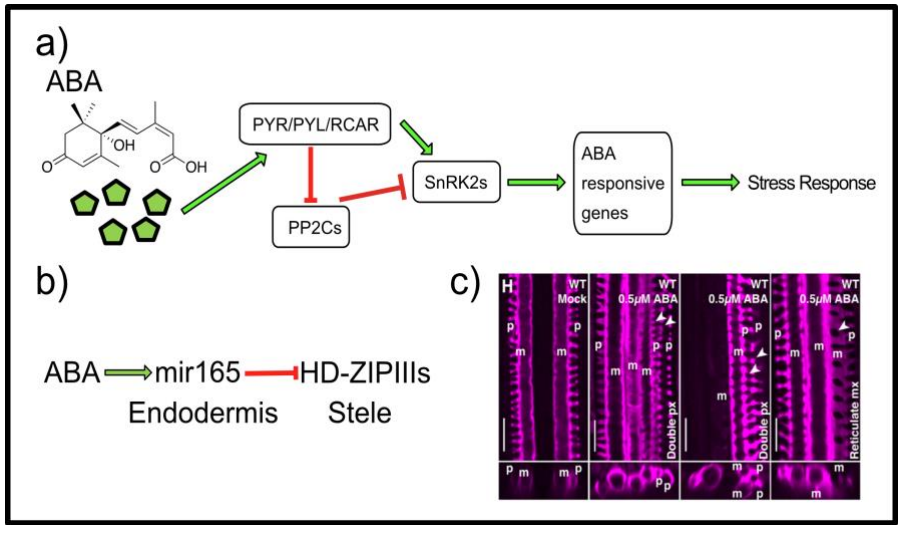

Figure 6. Abscisic acid: a) ABA signalling pathway, b) the role of ABA in maintaining the stele, c) development of extra xylem strands in ABA treated roots. Adapted from Ramachandran et al. 2018

1.2.3 Brassinosteroid

In the 1960s, a new biological compound was identified from the pollen of *Brassica napus*. This chemical was shown to promote and help growth, and thus brassinosteroids were recognized as a new class of phytohormones (Mitchell et al. 1970). Brassinosteroids (BRs) are a class of polyhydroxysteroids that have a role in plant development. The first isolated brassinosteroid was termed brassinolide and it promoted division of cells and the elongation of the stem (Grove et al. 1979). Further research since then has identified that BR is involved in and regulates various aspects of plant growth and development. Different developmental processes like vascular development, growth, cell division, cell elongation and even sex determination is controlled by BR. BR biosynthesis or signaling mutants display severe defects in growth (dwarfing) and have dark green leaves with delayed senescence (Akira & Shozo 1997; Choe et al. 1998, 1999; Klahre et al. 1998; Choe 1999; Li et al. 2001). Impairment in BR signaling also causes reduced seed yield and reduced plant fertility (Li & Chory 1997; Singh & Savaldi-Goldstein 2015). Since the discovery of BR, many new studies have

helped elucidate the multifaceted nature of BR. BR plays a role in elongation as was established in hypocotyl elongations assays (Clouse & Sasse 1998). BRs also play a role in cell division (González-García et al. 2011; Hacham et al. 2011). Since lack of BR causes severe developmental phenotypes and low concentrations of BR are present in plants all the time (Hartwig et al. 2011; Makarevitch et al. 2012) this reveals the essential nature of BR.

BR biosynthesis was first studied by labeling brassinolide precursors in periwinkle cell lines (Sakurai 1999). In absence of BR, BRASSINOSTEROID INSENSITIVE 2 (BIN2) (Figure 7a), a GLYCOGEN SYNTHASE 3 (GSK3)-like shaggy kinase downregulates the main transcription factors in the BR signaling pathway BRASSINAZOLE RESISTANT 1 (BZR1) and BRI1-EMS SUPPRESSOR 1 (BES1) by phosphorylating them. Phosphorylation of BES1 and BZR1 by BIN2 causes them to be bound to 14-3-3 proteins which causes them to be retained in the cytosol and be degraded so they cannot activate downstream genes (Li & Nam 2002; Gampala et al. 2007; Peng et al. 2008). BR is perceived at the cell membrane via membrane bound receptor, BRASSINOSTEROID INSENSITIVE 1 (BRI1) leucine rich receptor like kinase (LRR-RLK) family (Li & Chory 1997). Binding of BR to BRI1 initiates a signaling cascade where BRI1 heterodimerizes with BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED RECEPTOR KINASE (BAK1) which is also known as SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3 (SERK3). This heterodimer then causes phosphorylation level changes inside the cell and blocks the negative regulator of BR signaling BIN2 (Li & Nam 2002; Russinova et al. 2004). This causes the stabilization of the BES1 and BZR1 (Wang et al. 2002; Yin et al. 2002). These two transcription factors then control the activation or repression of multiple genes that are responsive to BR and thus control several developmental processes in plants (He et al. 2002; Sun et al. 2010; Zhao & Li 2012; Belkhadir & Jaillais 2015).

Apart from BRI1 there are two other homolog receptors known as BRI1-like 1 and BRI1-like 3 (BRL1, BRL3) that act as functional BR receptors (Caño-Delgado et al. 2004). Curiously, while BRI1 is expressed in almost every cell in the root (Friedrichsen & Chory 2001), BRL1 and BRL3 are expressed in particular tissues. BRL1 and BRL3 are more specific to the vascular stem

cell initials (Caño-Delgado et al. 2004; Fàbregas et al. 2013; Salazar-Henao et al. 2016). BRL1 and BRL3 can also heterodimerize with BAK1 and form a complex (Fàbregas et al. 2013) showing that all three receptors can form different complexes in various tissues with perhaps different signaling outputs highlighting the complicated nature of BR signaling. Moreover, with different BR receptors active in different root tissues, *Arabidopsis* roots provide an excellent model for understanding BR signaling (Figure 4e).

BR interacts with multiple hormones in both antagonistic and synergistic ways. The interaction between ABA and BR is generally antagonistic. There are different nodes of interactions, with enhanced ABA signaling stabilizing BIN2 levels and thus negatively regulating the BR pathway (Wang et al. 2018a). BIN2 also stabilizes and activates downstream ABA related genes such as *ABI5* to promote ABA signalling. The interaction between BR and auxin is more complex. BRs selectively regulate *PIN* genes where a prolonged decrease in BR levels induces *PIN4* and *PIN7* ,whereas a short increase in BR levels down regulates *PIN4* and *PIN7* (Nakamura et al. 2004). BR also induce auxin responsive genes *IAA5* and *IAA19* (Nakamura et al. 2003). BR signaling also affects the localization of PIN-LIKES (PILS) proteins by repressing the accumulation of the proteins at the endoplasmic reticulum which then increases the amount of nuclear auxin thereby causing developmental changes (Sun et al. 2020). In fact, the auxin activity required for a meristematic condition in roots relies on BR function. BR has a dual effect on auxin in the root meristem, one by increasing signal input of auxin and another by repressing signal output (Ackerman-Lavert et al. 2021; Fridman et al. 2021).

In terms of the role of BR in vascular development, BR acts on several transcription factors that control vascular differentiation. *VND6* and *VND7* that control the differentiation of xylem cells to metaxylem or protoxylem respsectively are both induced by the addition of exogeneous BRs (Kubo et al. 2005; Yamaguchi et al. 2011). Reducing BR biosynthesis resulted in reduced tracheary element differentiation in *Zinnia elegnas* cells (Iwasaki & Shibaoka 1991). BRs also function through the receptors BRL1 and BRL3 at tissues specific locations to promote xylem formation but reduce phloem formation (Caño-Delgado et al. 2004). Use of xylogenic cultures has been

instrumental in identifying the role of BR in vascular formation. Predominantly, the development of Vascluar cell Induction culture System Using *Arabidopsis* Leaves or VISUAL has helped understand the role of BR signaling in developing xylem, phloem, and cambium in the process of vascular development (Kondo et al. 2014, 2015, 2016; Kondo 2018). VISUAL uses a chemical inhibitor of BIN2, Bikinin, that competes with the binding of BIN2 and abolishes the suppressive effect of BIN2 on downstream targets BES1 and BZR1 thereby enhancing downstream BR signaling (De Rybel et al. 2009). Using VISUAL, the role of BIN2 in maintaing cambial cells was identified. BIN2 is part of the TDIF-TDR-BIN2-BES1 cascade, which acting along with the TDIF-TDR-WOX4 cascade maintains cambial cell divisions and blocks xylem development (Kondo et al. 2014), mimicking the development of xylem *in planta* (Kondo et al. 2014, 2015). Blocking of BIN2 with bikinin caused formation of more xylem cells but at the cost of cambial cell depletion (Kondo et al. 2014; Saito et al. 2018). VISUAL promoted the formation of phloem through enhancing levels of APL which is a master regulator of phloem sieve element formation and also helped identify NAC020 as the negative regulator of APL (Kondo et al. 2016; Saito et al. 2018; Saito & Kondo 2019). Lastly, reconstitutive approaches using VISUAL analyses revealed that, along with BES1 and BZR1 there are other members of the transcription factor family that control vascular development. Recently it was identified that BES1 HOMOLOG3 (BEH3) provides competitive binding sites to generate or maintain vascular stem cells instead of driving the cells towards differentiation and acts in an antagonistic manner to BES1 (Furuya et al. 2021).(Figure 4f). Thus, BRs play a role in both maintaining the vascular stem cells and stem cell niches but also promoting vascular development and affecting both cell division and cell differentiation.

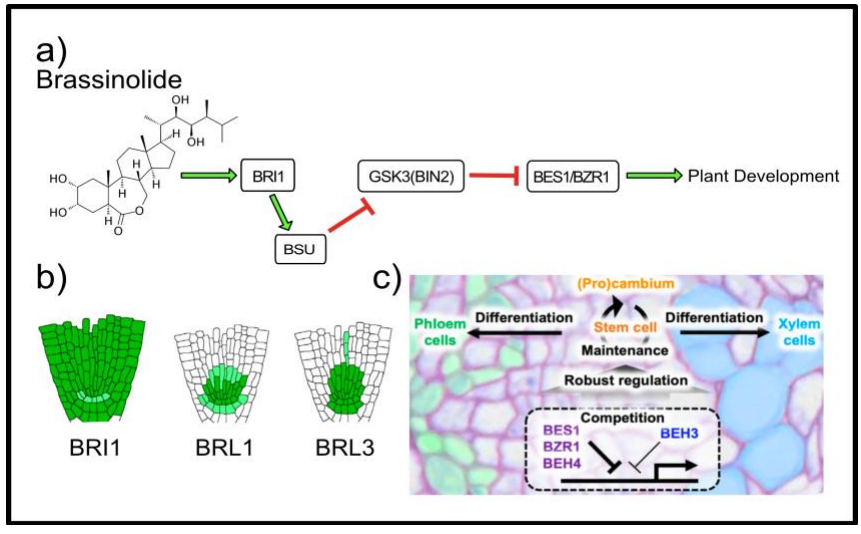

Figure 7. Brassinosteroid: a) Brassinosteroid signalling pathway, b) Expression domains of brassinosteroid receptors in different tissues of the root, c) The role of brassinosteroid signalling in maintaining cambium. Adapted from Caño-Delgado et al., 2004 and Furuya et al., 2021

1.3 Regeneration

Regeneration can be described as the ability of an organism to regrow or regenerate parts of itself that have been lost due to damage or stress. Recovery from organ loss is important to ensure survival of the organism. While most higher order organisms are capable of regeneration to an extent, the abilities differ between plants and animals. With that being said, plants are remarkable since they have amazing plastic abilities and can regenerate almost all organs or develop *de novo* organs as required (Birnbaum & Alvarado 2008; Sugimoto et al. 2011). *Arabidopsis* roots can regenerate entire apical meristems even when the root meristem is lost (Sena et al. 2009; Efroni et al. 2016). An unresolved question is why plants have such high regenerative abilities. This might be explained that since as the ability of movement is limited in plants, they are much more prone to a variety of damages and thus need efficient healing systems. Damage to plants can arise

from various biotic or abiotic stresses including grafting, wounding, and infection by pathogens and symbionts (Melnyk et al. 2015; Melnyk 2017).

Many times when plants are damaged or come across stressful situations one tissue system that is affected is that of the vasculature. Since the vascular system plays such an important role in development and function of a plant, it is imperative that any damage to it is repaired quickly. The reconnection of xylem, phloem and other important elements of plant vasculature occurs during wound healing or grafting so that the vascular strands are rejoined. It is important that during grafting the vascular connections are formed so that the graft succeeds. Rejoining of the vascular connections ensure that nutrients and signals are exchanged between both the scion and the rootstock (Melnyk et al. 2015; Melnyk & Meyerowitz 2015). The same principle can be applied to embolisms or air bubbles in xylem vessels. There often new xylem is developed around the embolism perhaps to overcome the blockage (Lucas et al. 2013; Bloch et al. 2019; Ramachandran et al. 2020; Cornelis & Hazak 2022). Although this process helps plants survive the rigors of stress that exist in the environment, this regenerative ability appears to be also used by pathogens and symbionts to generate vascular connections with plants to derive nutrients from the plants.

The vascular system also shows immense plasticity in development and reacts to environmental stresses and external cues as a form of acclimation. Countless mechanisms including genetic and hormonal interactions must act in symphony to create conditions that result in the maintenance of the vascular system and how the plant adapts. Thus, understanding how stress modulates vascular development can help elucidate the fundamentals of vascular formation and help us better understand the process. How do plants respond to stress, how is it perceived, and how regeneration is achieved as a response to stress is dependent on many genetic factors and hormonal interactions. Finally, plant cell walls act as indispensable sources of both protection from stress and also as primary indicators of approaching stress-like situations. While there are many angles to consider, this section will mainly deal with how BRs, ABA, and cell wall signals contribute to regeneration.

1.3.1 Perception of stress

When considering stress perception in plants it is imperative to define what is the source of stress. Although the effect of different kinds of stresses may end up having the same physiological and anatomical changes, defining the source of stress helps in identifying early signals and cues that can affect stress perception and downstream processes. Stress can be categorized in two forms; abiotic stresses which comprises of environmental stresses such as heat, cold, drought, salinity, freezing and wounding; and biotic stresses such as attacks by herbivores, insects, fungi, bacteria and other pathogens. Abiotic stresses cause physiological changes in plants which then leads to developmental changes, whereas biotic stresses induce defense-based genes which then causes developmental changes in plants depending on the biotic stress sources.

Stress perception requires the availability of cell membrane bound sensors or receptor proteins such as receptor like kinases (RLKs). There are more than 600 members in the RLK family in *Arabidopsis* with Lecuine Rich Repeat Receptor Like Kinases (LRR-RLKs) forming the biggest subset (Shiu & Bleecker 2003). Along with membrane bound receptors, the phytohormone ABA has been highly implicated in regulating perception and response to stress (Osakabe et al. 2005, 2013; Tanaka et al. 2012). In terms of biotic stress perception, plants relies on every individual cell to relay a cascade of response against invasion. To facilitate these, plants use cell membrane based RLKs and receptor like proteins (RLPs) to recognize foreign molecules known as microbe-asoociated molecular patterns (MAMPs) or host-derived molecules known as damage associated molecular patterns (DAMPs). For example, byproducts of cell wall damage by the pathogen invasion acts as molecular cues for the plant to either mount a defense response or to initiate physiological changes (Wan et al. 2021). Cell wall integrity (CWI) changes are also an important cue for initiating developmental changes. Pectins are complex polymers which play a large role in cell wall structure and are modified by Pectin Methylesterases (PMEs) (Mohnen 2008). PME activity is blocked by the concomitant PME inibitor (PMEI). Any changes in the PME levels, either by damage, or via genetic means results in a compensatory BR response to be intiated to control growth and development in plants (Wolf et al. 2012). BR signaling itself controls

development and expansion of cells (Wolf et al. 2012, 2014), and directly controls and activates cell wall related genes such as *ATPME41, ATPME2, ATPME3*, cellulose synthase (*CESAs*) CESA1, CESA3, CESA6, and Expansins like *EXPA1, EXPA4, EXPA8* (Sun et al. 2010; Qu et al. 2011; Xie et al. 2021). Thus BR signlaling and cell walls create a feedback loop that govern development and growth. It is also interesteing to note that BAK1 which is a co-receptor with BRI1 in BR signaling, also is an important regulator of plant immunity activating both resistance genes and cell wall changes thereby again linking cell wall integrity, stress response, and BR signaling in a network (Kørner et al. 2013; Li et al. 2019; Wang et al. 2019).

ABA signaling also affects cell wall and secondary cell wall biosynthesis. In *Medicao tranculata* direct action of ABA on cell wall related genes such as Extensins, Cellulose Synthase, and Pectinesterase resulted in reduced germination (Gimeno-Gilles et al. 2009). BRs and ABA are in most cases antagonistic to each other and interact at the levels of BIN2 and BZR1 (Cai et al. 2014; Hu & Yu 2014; Wang et al. 2018a). BIN2 increases ABA mediated stress response but negatively regulates BR signaling. Exogenous application of BR reduced ABA mediated activation of *RESPONSIVE TO DESSICATION 26 (RD26)* (Ye et al. 2017). BES1 antagonizes RD26 thereby inhibiting drought response. BR also activates WRKY transcription factors (via activation through BES1) which promotes growth while repressing drought inducible genes reducing drought tolerance (Chen & Yin 2017). BRs and ABA also interact in various other stress responses such as heat, cold and salinity. But curiously not all interactions between BR and ABA are antagonistic. During water stress conditions, BR increases Nitric oxide (NO) production which in turn enhances ABA biosynthesis (Zhang et al., 2011) thereby increasing tolerance to water stress. Thus, it can be said that the ability of the plant to perceive and tolerate stresses is reliant on its ability to swiftly shift between growth vs defense-based development dependent on what is the stimulus (Bechtold & Field 2018).

1.3.2 Regeneration in response to stress

As stress acts as a developmental cue on the plant, regeneration of cells, tissues and organs is one of the development responses of the plants.

Regeneration is an important attribute that contributes to the survival and existence of the plant. Auxin is one of the phytohormones involved in regeneration. Local auxin production as well as long distance auxin transport induces regeneration responses including development of a multiple of new organs by introducing cell division and differentiation (Asahina et al. 2011; Zhang et al. 2019; Matosevich et al. 2020). Recently it was found that damage to cell walls in the presence of auxin activates four DOF transcription factors which activate callus formation and vascular formation (Zhang et al. 2022). Organ regeneration is dependent on formation of callus at the wound site, which is a tissue comprised of organized, divided, and differentiated cells that orignate from certain subpopulations of mature stem cells (Atta et al. 2009; Sugimoto et al. 2010; Ikeuchi et al. 2013). It is also important that the cells in the callus acquire the ability of pluripotency (Kareem et al. 2015). While many phytohormones have massive roles in helping plant regenerate organs including interactions of jasmonic acid with auxin, ethylene, cytokinin; this thesis will further aim to analyze the effects of ABA and BR on regeneration.

ABA regulates stress based developmental changes in plants including changing stomatal opening and closing patterns. In terms of regeneration and developmen althought ABA is involved in somatic embryogensis. For instance, embryos of hybrid larch grew normally on ABA supplemented media but had abnormal growth in non ABA supplemented media (Gutmann et al. 1996). BRs interact with auxin downstream of BES1 and AUX/IAA proteins (Nemhauser et al. 2004). BRs have also been implicated with roles in cambium development, maintenance, and vascular development (Ohashi-Ito et al. 2002; Kubo et al. 2005; Kondo et al. 2014, 2015, 2016).). A recent study also identified that both BR and BR signaling are important for establishing a stem cell niche in roots post excision of the root tip (Takahashi & Umeda 2022). This finding was further corroborated as depletion of endogenous BR levels by a BR biosynthes inhibitor brassinazole (Nagata et al. 2001) delayed the recovery of the stem cell niche (Takahashi & Umeda 2022). Additionally, BR perception is also important for stem cell niche recovery as a quadruple BR receptor mutant *bri1,brl1,brl4,bak1* has delayed root tip regeneration compared to that of wild type control plants (Takahashi & Umeda 2022).

Lastly, the previous sections have dealt with how cell wall damage and BR signaling are part of a closed loop of interaction. Since BR controls cell elongation and cell wall related genes, the involvement of BR signaling in regeneration perhaps by cell wall damage or modifications is an interesting angle to investigate. Due to cell wall modification, PMEI overexpression lines generally display a strong BR response with wavy roots (Wolf et al. 2012). Recently, a plama membrane bound receptor was identified in a forward genetic screen of a PMEI overexpression line which resulted in a mutant that had reduced BR mediated PMEI response. The mutant identified had a premature stop codon in *RECEPTOR LIKE PROTEIN 44* (*RLP44*) (Wolf et al. 2014). RLP44 was shown to interact with BAK1 and thus modify BR signaling. RLP44 also interacts with BRI1 and activates BR signaling downstream of the receptor and thereby is not reliant on BR ligand and provides lateral input to BR signaling (Wolf et al. 2014). RLP44 incorporates cell wall damage as cues and activates BR signaling response as a result of its direct interaction with both BRI1 and BAK1 (Wolf et al. 2014; Holzwart et al. 2018). Moreover, RLP44 also associates with the receptor of the peptide hormone phytosulfokine (PSK). PSK signaling, like BR signaling, promotes cell division and growth (Sauter 2015). This interaction of RLP44 with both BR signaling and the PSK pathway allows for control of both xylem development and procambial maintenance (Holzwart et al. 2018). Additionally BRI1 itself has non canonical BR signaling properties depending on its mutant allele which can increase or decrease intereaction with RLP44 and thus modify development of both xylem and cambium (Holzwart et al. 2020a)

Thus, it can be safely said that regeneration and development in stress-based scenarios depend on incorporating and managing signals from damaged cell walls, which activates certain signals andregeneration-based genes. This thesis further aims to identify how ABA acts on development of xylem in *Arabidopsis*, how BR affects regeneration and vascular development in plants and lastly to identify factors that incorporate various stress signals and affect changes in vascular development and plant development as a whole.

2. Aims of the study

The objectives of this study can be summarized as follows:

1. To investigate the role of ABA in developing xylem and lignification in plants under stress conditions (Paper I)
2. To investigate the role of brassinosteroid in regulating vascular development and cambial maintenance (Paper II)
3. To investigate and identify novel genetic factors that can regulate vascular development and regeneration in response to stress (Paper III)

3. Results and discussion

3.1 The effect of ABA on vascular development

ABA is the major stress signaling hormone in plants (Zhu 2002, 2016), and affects both primary and lateral root development in stress conditions (Rowe et al. 2016). The role of ABA is not just limited to changing developmental aspects in relation to organ growth. Recently it was found that ABA signaling also helps plants acclimatize to stress conditions by modulating xylem development through non-cell autonomous signaling (Ramachandran et al. 2018). It does so by increasing miRNA165 quantities, which acts as the non-cell autonomous signal, blocking HD-ZIPIII transcription factors and promoting protoxylem cell differentiation in xylem (Carlsbecker et al. 2010; Miyashima et al. 2011). Although ABA controlled xylem development non-cell autonomously, involvement of ABA signaling in controlling different aspects of xylem development was not clear. In paper I we show that ABA also controls various features of xylem development via cell autonomous interactions as well. Our experiments showed that ABA controls both xylem cell fate, and xylem differentiation rates in roots (Paper I, Fig. 1). Exogenous ABA application resulted in protoxylem strands differentiating closer to the root tip when compared to control (Paper I, Fig. 1). Outer metaxylem cells (metaxylem cells next to protoxylem cells) displayed earlier differentiation when compared to mock and displayed change in morphology with a more protoxylem like structure (Paper I, Fig 1). While inner metaxylem cells also showed earlier differentiation when compared to mock, there were no morphological changes. Blocking ABA signaling using *abi1-1* a dominant negative regulator of ABA signaling, in xylem axis reduced both xylem differentiation fate and rate (Paper I, Fig. 2). Blocking ABA signaling in procambium had no effect on both fate and rate of xylem differentiation, whereas blocking ABA signaling in ground tissue led to partial reduction in xylem fate, but no affect was observed in xylem differentiation rates (Ramachandran et al. 2018) (Paper I, Fig. 2). Thus, ABA signaling in xylem cells, more than in the procambium or ground tissues is required for causing changes in the xylem development fate and rate in scenarios that result in enhanced ABA levels.

VND transcription factors are master regulators of xylem differentiation and act upstream of secondary cell wall biosynthesis genes (Kubo et al. 2005; Yamaguchi et al. 2010a, 2011; Zhou et al. 2014). Moreover, ABA directly influences the activity of *VND2, VND3,* and *VND7* (Song et al. 2016). Both RNA sequencing and independent qRT-PCR analyses of ABA treated Col-0 seedling roots showed activation of VASCULAR RELATED NAC DOMAIN (VND) transcription factor genes (Paper I, Fig. 2). ABA treatment as short as 2 hours induced genes such as *VND1*, *VND2*, *VND3*, and *VND7* and blocking ABA signaling in xylem cells reduced *VND2* and *VND3* levels (Paper I, Fig. 2). Each VND transcription factor had different domains of expression in immature xylem cells with some overlap. VND1 is expressed only in outer metaxylem, VND2 in both inner and outer metaxylem, VND3 in protoxylem and metaxylem (Paper 1, Fig. 2). VND7 is expressed in only early protoxylem cells in the meristem, but the expression extends in to differentiating protoxylem cells. Due to their expression domains being in early xylem development increased ABA levels activate VND1, VND2, VND3, and VND7 transcription factors in xylem precursor cells quite briskly. While treatment with 1μM ABA did not change the expression domains of the VNDs, increasing ABA levels does cause the lateral expansion of VND7 expression domain from protoxylem to metaxylem (Bloch et al. 2019) thereby suggesting that enhanced ABA levels can cause the expression domains of VND transcription factors to expand, thereby possibly affecting xylem development. We could also show that ABA based activation of VND transcription factors results in enhanced xylem formation by changing xylem cell fate and rate. Under mock conditions, single loss of function mutants of VND transcription factors had no defects in patterning, suggesting genetic redundancy. Higher order mutants though, like *vnd1,2,3* and *vnd2,3* had defects in metaxylem continuity, and had reduced metaxylem differentiation (Paper 1, Fig. 3). Treatment with ABA resulted in delayed inner metaxylem differentiation in mutants compared to wild type, showing VND2 and VND3 are required for early differentiation of inner metaxylem affecting cell differentiation rate (Paper 1, Fig 3). Enhanced xylem formation was also observed in water limiting conditions. Water limiting conditions enhance ABA levels in plants, and results in enhanced xylem differentiation (Ramachandran et al. 2018) and this enhancement is also driven by ABA activating VND transcription factors. While differentiation rate was reduced

in inner metaxylem, early outer metaxylem differentiation or change in morphology of the cells was not disrupted in *vnd1,2,3* and *vnd2,3* loss of function mutants. In contrast ABA treatment of *vnd7* lines while did not affect the rate of differentiation, reduced the change in cell fate in xylem differentiation with morphological changes like extra protoxylem or reticulate metaxylem showing reduction when compared to wild type (Paper 1, Fig. 3). Thus, the effect of ABA on both enhancing xylem differentiation and changing the xylem cell fate is genetically separated by virtue of ABA activating different VND transcription factors. To understand if the effects were additive when *vnd1,2,3,7* quadruple loss of function was analyzed, it reduced both xylem differentiation rate and fate (Paper 1, Fig 3).

Enhancing VND transcription factors causes the formation of ectopic tracheary elements (Kubo et al. 2005; Yamaguchi et al. 2010a). Since ABA elevated VNDs we analyzed if ABA would also induce xylem formation via transdifferentiation of cotyledon mesophyll cells to xylem cells. We used a xylogenic culture where cotyledons are treated with auxin, cytokinin, and bikinin which is a GSK3 inhibitor and activates downstream BR signaling pathway (De Rybel et al. 2009; Kondo et al. 2015). We observed that when bikinin was substituted with ABA, ectopic lignified deposits were observed on the cotyledon surface area (Paper 1, Fig 4). When we tested *abi1-1* which has suppressed ABA signaling (Leung et al. 1994), it had reduced lignification compared to that of wild type. This shows that ectopic lignification is in part reliant on ABA signaling. Like reduced xylem cell differentiation rate and xylem cell fate, the ectopic lignification was also reduced in *vnd1,2,3* and *vnd7* when compared to wild type (Paper II, Fig. S4). Thus, like in roots, ABA also activates VND transcription factors in cotyledons to generate ectopic lignified deposits. Lastly, we also observed that this effect of ABA promoting xylem differentiation is also conserved among eudicot species (Paper 1, Fig. 4). However, although ABA could form lignified deposits, we did not observe typical xylem cell with secondary cell wall (SCW) architecture. This suggests that there are other factors that contribute to formation of xylem cells in VISUAL based xylogenic cultures. Additionally, the duration of ABA treatment required for lignin deposition was also longer compared to that of bikinin based VISUAL. This suggests a possibility that the lignified deposits are not true xylem cells or have

interrupted developmental trajectories. One possibility is that ABA stabilized BIN2, thereby negatively regulating downstream BR signaling and vascular differentiation (Wang et al. 2018b). In leaves, vascular development is also reliant on procambium, and procambium development is regulated by HD-ZIPIII transcription factors (Prigge et al. 2005). Since ABA negatively regulates HD-ZIPIII levels (Ramachandran et al. 2018; Bloch et al. 2019), the lack of typical xylem cells could be a function of reduced HD-ZIPIII transcript levels. Moreover, most of the ectopic xylem formation in VISUAL is reliant on VND6 (Kondo et al. 2015). Since ABA did not affect *VND6* levels (Paper I, Fig. 2), lack of typical xylem cells may be due to this. The lignified deposits may be a function of genetic redundancy between VND transcription factors, as even loss of function of *VND6* did have spontaneous tracheary element differentiation. Thus, there might be other factors that control the formation of xylem cells and lignified deposits. MYB46 and MYB83 have been shown to act as a second layer of master regulators in xylem cell differentiation (Zhong & Ye 2012). ABA binds to the promoter of *MYB46* (Song et al. 2016).,thereby showing the role of factors other than VNDs involved in ABA based xylem development. An RNAseq analysis of ABA treated *vnd1,2,3* revealed that while xylem expressed genes had reduced up regulation in the mutant, *MYB46* and *MYB83* were not affected (Paper 1, Fig 3). Ectopic xylem differentiation was also completely lost in a *myb46myb83* double mutant (Tan et al. 2018), These results suggest that MYB46 and MYB83 transcription factors are potentially regulated by ABA independently of VNDs to govern early xylem differentiation during high ABA conditions.

To conclude, our results show that enhanced ABA signaling in xylem precursor cells generate developmental changes in xylem differentiation and that it is through activation of VND transcription factors. Additionally, the morphological and anatomical changes that occur in the plant due to enhanced ABA levels benefit the plant. Development of protoxylem like structures not only benefits plant by reducing the chances of air bubbles and embolisms but may also benefit lateral water movement (Hwang et al. 2016). Early differentiation of xylem on the other hand increases drought resistance (Tang et al. 2018).

3.2 The effect of BR on vascular development and regeneration

BR signaling controls cellular expansion and elongation (Clouse & Sasse 1998; He et al. 2002; Sun et al. 2010). While they have been implicated in controlling tracheary element differentiation (Iwasaki & Shibaoka 1991) and activating VND transcription factors (Kubo et al. 2005), their role and mechanism in vascular development and regeneration is poorly understood. We observed that like exogenous ABA, application of exogenous BR in the form of epiBrassinolide (epiBL) could create changes in xylem morphology of primary roots (Paper 1; Paper II Fig. 3). We then proceeded to analyze the role of BR signaling in vascular development and callus formation. We analyzed expression levels of core BR signaling genes during graft formation (Melnyk et al. 2018) and observed that they were activated during graft formation (Paper II, Fig 1). We also analyzed a subset of BR inducible genes and observed they too were activated during graft formation, showing that BR related genes may be active during graft formation (Paper II, Fig 1) Using *Arabidopsi*s hypocotyl micrografting (Melnyk et al. 2015) we observed the grafting ability when BR signaling is compromised. We found that in A*rabidopsis*, a functional BR signaling is important for hypocotyl micrograft formation and phloem and xylem regeneration. Mutants of BR signaling genes *BRI1*, *BIN2, BES1, BZR1* showed reduced grafting ability as observed by reduced phloem and xylem reconnection rates at tested time points (Paper II, Fig. 1). Loss of *BES1* function also resulted in reduced grafting ability when compared to wild type, whereas a dominant version of *BZR1* also reduced grafting ability suggesting that perhaps both activation of downstream target genes and perhaps BR levels regulation by *BZR1* are important (Wang et al. 2002; He et al. 2005; Yu et al. 2011) (Paper II, Fig 1). Although BR perception is reliant on different receptors which show some degree of redundancy (Caño-Delgado et al. 2004), any perturbation in BR perception affects graft formation as we observed that even a hypomorphic mutation in *BRI1* was enough to reduce grafting ability and it could not be rescued with epiBL treatment (Paper II, Fig 5).

In terms of callus formation, BR signaling promotes callus formation in monocots (Lu et al. 2003). Our experiments in *Arabidopsis* showed that callus formation and regeneration were reduced in excised petioles of BR

signaling mutants when compared to wild type (Paper II, Fig. 2), suggesting that BR signaling perhaps influences callus formation. BES1 inversely, reduces cell divisions in rice by binding to U-type Cyclin *CYC U4;1* (Sun et al. 2015). We also observed that overexpression of *BES1* resulted in reduced callus forming abilities (Paper II, Fig. 2) suggesting that reduced callus formation may be a function of reduced cell division. With BR signaling already influencing root tip regeneration (Takahashi & Umeda 2022) and stem cell niche regeneration (Lozano-Elena et al. 2017), our results further suggest the role of BR signaling in callus regeneration. Since BR signaling controls meristematic cellular proliferation, cell expansion and elongation (Clouse & Sasse 1998; González-García et al. 2011; Hacham et al. 2011), reduced grafting ability and callus formation ability of BR signaling mutants could potentially be attributed to the inability of cell to divide or expand. Moreover, damages to cell wall activate a compensatory BR signaling cascade (Wolf et al. 2012). Mechanical injury to the cell walls is one of the first steps that occurs during grafting, which activates a compensatory BR response, thus explaining the dynamic nature of BR signaling genes during graft formation and possible involvement of BR signaling in vascular regeneration. Similarly, as cellular expansion and division are reduced in BR signaling mutants, this could potentially explain the reduced callus formation abilities.

To dissect if BR signaling affected vascular regeneration only in wounding or in normal developmental scenarios as well, we looked in non-wounded individuals. When exogenous epiBL was added, lack of BR perception and BR signaling resulted in reduced xylem formation compared to that of wild type (Paper II, Fig. 3). BR perception mutant also had supernumerary metaxylem cell file numbers. Thus, BR perception is also required for normal metaxylem architecture (Paper II, Fig. S3). When we analyzed xylem formation in cross sections at 0.5mm below shoot-root junction in 21-day old seedlings we observed that loss of function of BR signaling mutants resulted in reduced xylem area and xylem cell number compared to wild type (Paper II, Fig 4). This suggests that BR is signaling was required for normal xylem development as both *BES1* and BR receptors control xylem differentiation (Caño-Delgado et al. 2004; Kondo et al. 2015). As mentioned previously BR influences cell division and cell expansion, the reduction in xylem area or

cell number can possibly be explained as a function of reduced cell division and expansion. We also observed that when endogenous BR levels were changes by either adding epiBL or brassinazole which is a BR biosynthesis blocker (Nagata et al. 2001) we observed changes in the xylem area. Adding epiBL increased both the xylem area and xylem cell number, whereas brassinazole addition reduced xylem area in the wild type (Paper II, Fig. 5). BR signaling also has been implicated in cambial development and has contrasting roles when it comes to cambial development in roots and shoots (Ibañes et al. 2009; Fàbregas et al. 2013; Kang et al. 2017; Wang et al. 2022). Our experiments with 21-day old seedling root cross sections suggested that BR signaling mutants affected cambium and xylem area. *BES1* loss of function had more cambium area when compared to xylem area but this was a function of reduced xylem area and number of xylem cells (Paper II, Fig. 4). The resulting decrease of xylem cells and xylem area caused cambium upon xylem area ratio to be higher in *bes1-2*.This can be explained by the fact that increasing levels of *BES1* results in reduction of procambial cell layers in roots (Kondo et al. 2014; Saito et al. 2018; Furuya et al. 2021). When we added epiBL we also saw an increase in the cambium area in wild type (Paper II, Fig. 5).

Overall, our experiments suggested that while BR signaling may affect both xylem and cambium development in plants, there is a possibility of a dual output, with reduced xylem differentiation resulting in enhanced cambium and vice versa. This can be explained possibly by the node of interaction in BR signaling pathway. In VISUAL we already observe ectopic formation of xylem cells (Paper II, Fig 3) (Kondo et al. 2015), moreover enhancement of xylem differentiation in hypocotyl post bikinin addition is at the cost of cambial cell reduction (Kondo et al. 2014). . Formation of xylem cells in bikinin based VISUAL is a function of VND6 activation and down regulation of BIN2, freeing downstream BR signaling pathway. Although ABA does not activate VND6 (Paper I), it does negatively regulate HD-ZIPIII transcription factors. Lack of typical xylem cells in ABA based VISUAL may be because of the stabilization of BIN2, but lignified deposits may be a function of reduced cambium by virtue of negative regulation of HD-ZIPIII transcripts by ABA. Whatever the scenario, there may exist a

potential antagonistic relationship between cambium and xylem differentiation.

3.3 Canonical BR signaling and *RLP44* associated BR signaling

A compensatory BR signaling cascade is activated during cell wall damage (Wolf et al. 2012). Recently it was found that RLP44 associates with BAK1 at the plasma membrane level (Wolf et al. 2014) and mediates the activation of BR signaling in times of damage by associating with both BRI1 and BAK1 (Wolf et al. 2014; Holzwart et al. 2018). Additionally, association of BRI1 and RLP44 controls vascular cell fate determination in roots independent of canonical BR-BRI1 signaling cascade (Holzwart et al. 2018). We decided to investigate how *RLP44* associated BR signaling affects vascular regeneration and callus formation. We analyzed mutants of *RLP44* in the same assays as of the previously mentioned BR signaling genes. We observed that while a loss of function mutant *rlp44-3* enhanced grafting ability, an overexpression mutant *RLP44ox* (Wolf et al. 2014) reduced grafting it (Paper II, Fig 1), thus showing mutants of *RLP44* behaved in an opposite manner to that of BR signaling mutants. In VISUAL based assay, overexpression and loss of function mutants showed reduction and enhancement of ectopic xylem formation abilities respectively (Paper II, Fig 3). A possible explanation would be that RLP44 interaction with BRI1 and PSK signaling (Holzwart et al. 2018, 2020b) counteracts the depletion of cambial cells, maintaining them and thus reduces xylem differentiation and regeneration. In contrast in normal root xylem development of xylem in roots *RLP44* enhances xylem formation as *rlp44-3* had reduced xylem formation when treated with epiBL compared to wild type (Paper II, Fig. 3). Moreover, when we analyzed cross sections of *rlp44-3* roots, we saw that xylem area was reduced, although there were higher number of xylem cells per unit xylem area (Paper II, Fig. 4). This higher xylem cell per xylem area ratio also corroborated the observation of supernumerary metaxylem cell files in *rlp44-3* roots (Holzwart et al. 2018), and enhanced ectopic xylem ability (Paper II, Fig. 3). We observed that *rlp44-3* had reduced cambium area (Paper II, Fig. 4), which may be explained as RLP44 association with both BRI1 and PSK

signaling affect (pro)cambium development as. *RLP44* also influenced callus regeneration as *rlp44-3* had reduced callus formation and root tip regeneration abilities (Paper II, Fig 2; Fig S2). The amount of xylem differentiation always does not correlate with the severity of growth phenotypes related to BR-deficiency, which can possibly explain why RLP44 had stronger phenotypes. Additionally, the interaction between BRI1 and RLP44, rather than downstream BR signaling controls the xylem fate (Holzwart et al. 2018). Presence of RLP44 creates a fork in BR signaling cascade with PSK-RLP44-BRI1 forming a parallel pathway to contribute to procambial cell maintenance while at the same tine governing xylem cell fate. Moreover, different alleles of *BRI1* associate with *RLP44* with different strengths and can result in different outputs in terms of either procambial cell maintenance or xylem cell fate (Holzwart et al. 2020b). Thus, the association of different receptors at the plasma membrane does affect the developmental trajectories. Branching out of signal transduction pathways resulting in different outputs is emerging as a common feature of RECEPTOR LIKE KINASE- dependent signaling (Couto & Zipfel 2016). Overall, our results helped us propose a mechanistic model to explain how BR signaling and RLP44 associated BR signaling affects vascular development. It shows that both pathways control xylem and cambium development where canonical BR signaling controls xylem development which is most likely antagonistic to cambium development. Presence of *RLP44* associated BR signaling maintains the balance between cambium and xylem development (Paper II, Fig. 5).

3.4 Identification of *EVG1*, a cell wall linked stress responsive gene that regulates vascular development and regeneration

As seen in previous sections, plants have remarkable vascular regenerative abilities and modulate multiple signaling pathways in response to stress (Paragraph 1.3.2). But this ability of regeneration is also used by many biotic agents to generate vascular connections to plants for resources. Since vascular formation or regeneration was the common focal point in both biotic and abiotic stresses we attempted to identify if there existed a common

mechanism between them. We performed comparative analysis and identified a gene, AT3G08030 (ATHA2-1), which was highly upregulated by both biotic stress and abiotic stress (Paper III, Fig1; Fig S1). AT3G08030 has a domain of unknown function 642 (DUF642) that has been linked to cell wall development (Vázquez-Lobo et al. 2012; Salazar-Iribe et al. 2016; Cruz-Valderrama et al. 2019). Based on phylogenetic analysis of DUF642 domain containing genes in spermatophytes, AT3G08030 was placed in clade A2 (Vázquez-Lobo et al. 2012). When we analyzed its transcriptional dynamics during graft formation, AT3G08030 was highly upregulated especially at a time point that is associated with activation of cambium development related genes (Melnyk et al. 2015, 2018) (Paper III, Fig 1). Although AT3G08030 was highly induced during graft formation and in stress conditions (Paper III, Fig. 1), a loss of function T-DNA insertional line for AT3G08030, resulted in enhanced ectopic xylem formation in VISUAL (Paper III, Fig 1), and enhanced grafting ability. Due to its phenotype in VISUAL and grafting we termed the gene *ENHANCER OF VISUAL AND GRAFTING 1* (*EVG1*). The closest paralogs of *EVG1* in *Arabidopsis* have been shown to play a role in cell elongation, hypocotyl elongation and are involved in different development stages (Salazar-Iribe et al. 2016; Cruz-Valderrama et al. 2019). E*VG1* is present in lateral root and is an important molecular marker for seed ageing with *EVG1* transcripts present in all stages of *Arabidopsis* life cycle and all stages of seed development (Garza-Caligaris et al. 2012; Cruz-Valderrama et al. 2019). Additionally, *EVG1*interacts with cellulose *in vitro* (Borner et al. 2003; Vázquez-Lobo et al. 2012). Although EVG1 expression was reported to be in root cortex and hair cell (Shulse et al. 2019), we observed *EVG1* was expressed in the epidermal cells, and showed rapid response to wounding in hypocotyl, cut top, and grafted top tissues (Paper III, Fig 1, Fig 6 Fig. S1, Fig S6). Genes that activate early in grafting conditions have been associated with defense, wound response, and stress response (Melnyk et al. 2018), but *EVG1* differs from a typical defense related gene as the transcript levels of *EVG1* did not fall down rapidly at later time points.

We performed RNA sequencing of entire seedlings of the *evg1-1* loss of function mutant and found that a significant number of genes related to cell wall organization, biogenesis and cell wall loosening were differentially

expressed (Paper III Fig 4). As an example, *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 4* (*XTH4*), which controls cell expansion and secondary cell wall development (Kushwah et al. 2020) was up regulated in *evg1-1*. Moreover, a group of genes related to cell wall loosening, namely *EXPANSIN A4* (*EXPA4*), *EXPA8, EXPA16, EXPB3* were down regulated in *evg1-1*. Expansin gene family encodes a set of extracellular proteins that affect cell wall expansion and modify cell wall mechanical properties (Cosgrove 2000, 2016; Li et al. 2002). The involvement of *EVG1* in stress response was further highlighted when we observed that a subset of genes related to ABA response were differentially expressed in *evg1-1* (Paper III, Fig. 4). We observed down regulation of an ABA biosynthesis related gene *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3 (NCED3)* Moreover, we also saw upregulation of ABA receptor genes such as *PYR1* and associated paralogs such *PYL4, PYL5, PYL6* (Paper III, Fig. 4). We could show that ABA negatively regulates *EVG1* expression by treating a transcriptional *EVG1* reporter *(pEVG1:GFP*) treated with 1μM ABA (Paper III, Fig 4; Fig S4). Abiotic stress negatively regulates cellular expansion and growth in maize epidermal cells (Zörb et al. 2015). Since we observed *EVG1* expression in epidermal cells, and as cellular expansion related genes were down regulated in *evg1-1*, the negative regulation of *EVG1* by ABA is maybe an adaptive response to abiotic stress by reducing cellular expansion and growth (Liu et al. 2021). As to why loss of *EVG1* results in up regulation of ABA receptors it remains to be seen. One possible explanation could be that the differential expression of receptors is a function of generating hypersensitivity to ABA (Umezawa et al. 2010; Zhao et al. 2013). Moreover, reduction of ABA related genes like *KIN1, RD29A, ATHB-7* can also be a function of loss of *EVG1* resulting in the plant being stress primed (Liu et al. 2012). Overall, our results show that *EVG1* is stress responsive and is associated with cell walls.

As the *EVG1* expression profile during grafting peaked at time points related to activation of cambium associated genes (Melnyk et al. 2018) (Paper III, Fig. 1), we analyzed a cross sections of a region 0.5 mm below shoot-root junction of 21-day old *evg1-1*. We observed that the *evg1-1* root had smaller radial area when compared to that of Col-0 (Paper III, Fig. 2). We also noticed that *XTH19* and *XTH20* which influence hypocotyl diameter (Miedes

et al. 2013) were downregulated in *evg1-1* (Paper III, Fig 4), which could possibly explain the phenotype. Our results also showed that *evg1-1* had reduced cambium area when compared to wild type, suggesting involvement of EVG1 in cambium development (Paper III, Fig 2). Cell division and expansion are the driving forces behind cambium formation (Prislan et al. 2013). We already observed down regulation of cell wall expansion related genes in *evg1-1* (Paper III, Fig. 4), explaining one possible reason behind the reduced cambium area. In terms of xylem, we observed that although there was a reduction in the area encompassed by the xylem cells the number of xylem cells was not reduced when compared to Col-0 (Paper III, Fig. 2). This discrepancy led to the ratio of xylem cell per unit area being higher in *evg1-1* pointing towards more xylem formation (Paper III, Fig. 2). One plausible explanation is that since *EVG1* was stress responsive, enhanced xylem formation in *evg1-1* maybe an adaptive stress response. Plant cope with water loss by, for example, the formation of more xylem (Ramachandran et al. 2020) where ABA signaling also plays a role. Enhanced xylem development was also evident when we studied primary roots of *evg1-1* as they had supernumerary metaxylem cell file number compared to Col-0 (Paper III, Fig S2). Moreover, in VISUAL based ectopic xylem assays, *evg1* displayed enhanced ectopic xylem formation (Paper III, Fig 2).

Since *EVG1* was upregulated in grafting and affected cambium and xylem development we tested whether it affects vascular regeneration. Strikingly we found that *EVG1*, although being up regulated in grafting, inhibited graft formation. We found that both phloem and xylem reconnection was enhanced in the *evg1-1* mutant, whereas an overexpression *EVG1* in an overexpression line (*EVG1-OE*) reduced both phloem and xylem reconnection (Paper III, Fig. 3). This inverse relation in xylem development was also seen in ectopic xylem formation where the *EVG1-OE* line had reduced ectopic xylem, whereas the *evg1-1* line had enhanced ectopic xylem (Paper III, Fig.2). Moreover, we also found that presence of EVG1 in scion is detrimental to graft formation, as *evg1-1* in scion enhanced grafting whereas, *EVG1-OE* reduced grafting (Paper III, Fig. 3). One possible explanation could be that as a stress response gene *EVG1* acts like a signal and influences vasculature formation and reconnection as an adaptive measure. We also analyzed regeneration ability by analyzing callus

formation in excised petioles and found that the *evg1-1* line had reduced callus area compared to Col-0. Moreover, the regenerative abilities in resected root tips in the *evg1-1* line was reduced (Paper III, Fig 3, Fig. S3). Overall, we concluded that while *EVG1* positively affects cambium formation and regeneration, it negatively impacts xylem development. A possible explanation would be that since the *evg1-1* mutant affected cell wall loosening genes, there is an interference in cell expansion and division. Cambium formation relies on cellular expansion and division and we see that when there is a loss of *EVG1* it results in the reduction of genes related to cell wall loosening and increasing diameter of the hypocotyl (Cosgrove 2000, 2016; Li et al. 2002; Miedes et al. 2013). This would lead to reduced cambium formation and callus regeneration. Reduction in cambium also promotes xylem formation (Kondo et al. 2014; Saito et al. 2018; Furuya et al. 2021) and additionally, hypersensitivity to stress in the *evg1*-1 mutant may act as a trigger for forming extra xylem. Furthermore, cellular expansion is induced by pathogens such as *Agrobacterium* or nematode infection, (Deeken et al. 2007; Lee et al. 2009; Shanks et al. 2016; Olmo et al. 2020), thus up regulation of *EVG1* by biotic agents is a way to increase cell expansion. Overall, our results suggest that *EVG1* responds to both biotic and abiotic stresses and regulates cambium development, xylem formation and callus formation.

3.5 *EVG1* regulates vascular development and regeneration through *RLP44*

As *EVG1* is not a transcription factor, we questioned whether its effects on development were indirect. Moreover, the phenotypes we observed with *EVG1* mutants during graft formation, xylem development, cambium development, and callus regeneration was like what we observed with *RLP44* mutants (Paper II, Paper III, Fig 5). Like the *rlp44-3* mutant line, the *evg1-1* mutant line also had supernumerary metaxylem cell files, along with an increased xylem cell per unit xylem area (Paper II Fig 4, Paper III, Fig 2). As *RLP44* was identified to be involved in cell wall surveillance (Wolf et al. 2014; Holzwart et al. 2018) and *EVG1* showed differential expression of cell wall related genes (Paper III, Fig. 4), along with the phenocopying of the

mutants, we decided to investigate if there is relationship between the two. We performed RNA sequencing on whole seedlings of *rlp44-3* and found that there was a statistically significant overlap between the up and down regulated DEGs of *rlp44-3* and *evg1-1* including cell wall related genes (Paper III, Fig 5; Fig S5). The results of RNA sequencing along with the mutants of *EVG1* and *RLP44* phenocopied each other suggest an involvement in a common mechanism of action. Despite the similarities, there were a few key differences. The major difference between *RLP44* and *EVG1* was observed when we considered response to stress. While *EVG1* was highly responsive to stress (Paper III, Fig 1; Fig 6), *RLP44* did not show active response to stress (Paper III, Fig 6). During graft formation *EVG1* was highly induced in both grafted and cut tops as early as 6 hours after grafting, whereas *RLP44* activation was delayed with activation beyond 48 hours post grafting (Paper III, Fig 6, Fig. S6). Even in case of abiotic stress like salt stress, or biotic stress like parasitic plant infection *EVG1* was induced either earlier or stronger compared to *RLP44* (Paper III, Fig 6). Lastly, *EVG1* showed higher induction in cases of wounding-based regeneration in different tissue types (Paper III, Fig. S6). Thus, we propose a likely mechanistic model by which *EVG1* influences development and regeneration (Paper III, Fig 6). A scenario that remains to be explored is the difference in the tissue or layer of expression with *EVG1* being in the epidermal cells, whereas *RLP44* being in the cortex and vascular tissues (Wolf et al. 2014; Holzwart et al. 2018). Although the expression domains of the two genes are different, changes in *EVG1* levels affect cell walls, and *RLP44* is involved in cell wall surveillance and activating BR signaling in response to cell wall damage (Wolf et al. 2014; Holzwart et al. 2018). RLP44 also controls vascular development by virtue of its interaction with BRI1 (Holzwart et al. 2018, 2020b). Thus, it is a possibility that changes in EVG1 transcript levels in the epidermal cells due to stress generate changes in the cell walls, which is then incorporated by RLP44 affecting vascular development and regeneration.

4. Future perspectives

As plants are subjected to multiple stresses daily, the major aim of this study was to identify the effect of stress on vascular development and regeneration. In paper I we described and identified how abscisic acid (ABA) enhances xylem formation in roots. ABA activates xylem differentiation master regulators VNDs which changes both the cell fate, and the rate of differentiation of xylem cells. This is pertinent with regards to water deprivation and drought conditions which activates ABA signaling, and thus enhanced differentiation of xylem is an adaptive strategy employed by plants. Moreover, since ABA induced enhanced xylem formation was conserved across many eudicot species uncovering more aspects of this interaction will help crop production and yield stability by breeding for drought-resilience. While we identified how ABA enhanced xylem formation, the results obtained also raised some relevant questions. Primarily, while ABA activates VNDs, it also negatively regulates HD-ZIPIII transcription factors (Ramachandran et al. 2018). This poses a puzzling scenario since HD-ZIPIIIs are required for efficient xylem formation (Prigge et al. 2005; Carlsbecker et al. 2010; Miyashima et al. 2011). How is the interaction between these two factors affecting xylem differentiation? Is it time specific or tissue (space) specific? One possible way to genetically identify their interaction would be to create multi-order mutants of VNDs and HD-ZIPIIIs to see the effect on xylem development. Another viable strategy would be either block or enhance VNDs s in particular tissues in mutant backgrounds of HD-ZIPIIIs and vice versa, followed by analysis of xylem development under ABA treatments. Interestingly another relevant point was that, while ABA formed lignified deposits on cotyledon surface in a modified VISUAL assay, it could not create cells with typical xylem cell wall architecture (no secondary cell walls). This suggests that ABA might be functioning differently in terms of xylem formation in cotyledons. Development of lignified deposits instead of xylem cells, may also be an adaptive response to prevent water loss from the leaves. Additionally, the amount of ectopic lignification was much less compared to a standard bikinin based VISUAL assay. The reduced lignification may also be a function of negative regulation of HD-ZIPIII transcription factors by ABA. A way to uncover this would be to identify

differential expression of genes in terms of lignin deposition on cotyledon surface by performing a transcriptomic analysis of ABA treated cotyledons. The lack of secondary cell wall may also be dosage dependent, and thus perhaps increased ABA amounts may possibly result proper xylem cell formation, or more lignified deposits. Understanding and answering these questions these lines will further help us better fine tune xylem development.

In paper II we focused on the role of BR signaling, since it affects plant development. Although ABA is the major stress hormone, recent studies have shown that BR signaling also helps plants adapt to environmental stresses (Zhang et al. 2011; Albertos et al. 2022). We saw the BR signaling affects cellular regeneration and xylem differentiation, and callus formation. We also observed how *RLP44* associated BR signaling affected regeneration, cambium formation, and xylem differentiation. Lastly, we also observed that while BR signaling affected xylem formation, it did not have profound effects on cambium development whereas RLP44 associated BR signaling affected both cambium formation and xylem differentiation. While both canonical BR signaling and *RLP44* associated BR signaling affected vascular development, the phenotypes observed also pose some interesting questions. Why did canonical BR signaling promote vascular regeneration during grafting, whereas *RLP44* associated BR signaling inhibit the same? Similarly, why do the signaling pathways have opposite phenotypes in terms of ectopic xylem formation, while promoting xylem formation in roots? One possible explanation is the association between *BRI1* and *RLP44* which can perhaps cause the switch in fate of the development of cell to either xylem or maintain procambium cells (Holzwart et al. 2020b). This can be investigated by using alleles of *BRI1* which show a greater or lesser association with *RLP44* and observing the vascular phenotypes to confirm whether the plasma membrane level interaction between *BRI1* and *RLP44* can affect vascular development. Research along these lines can also possibly explain as to why BR signaling mutants affect xylem differentiation, but do not have profound effect on cambium development. Another interesting observation was that while BR receptor and *RLP44* loss of function mutant had supernumerary metaxylem cell file numbers in roots, addition of exogenous BR reverted the cell file numbers to wild type like. What mechanism is promoting the formation of wild type like metaxylem

architecture in roots post exogenous BR application even when BR signaling is compromised? One way to resolve this would be to study the expression of metaxylem and cambium development related genes in higher order BR receptor and *RLP44* mutants under exogenous BR treatment conditions. Lastly, multiple signaling pathways converge on BR signaling. In fact, ABA and BR have mostly antagonistic functions in physiological terms. Yet, both additions of ABA and BR led to formation of either extra xylem or changed the morphology of xylem in primary roots in our experiments. Moreover, both signaling pathways promote xylem differentiation. Why is there synergy between ABA and BR in terms of vascular development and differentiation? One possible reason is that both ABA and BR signaling pathway interact at the level of *BIN2* (Wang et al. 2018a). This could possibly modulate the responses but still needs to be explored more as *BIN2* negatively regulates vascular differentiation by blocking downstream BR signaling pathway. One way to identify this would be to generate higher order mutants comprising of elements from both ABA and BR signaling pathways and to observe their phenotypes in vascular regeneration and cellular regeneration assays. Another possible solution would be to cross treat ABA signaling mutants with exogenous BR and vice versa to see the effect on regeneration and vascular formation. Both ABA signaling and BR signaling affect vascular development. Moreover, the relationship between BR and ABA signaling may help in adaptation to environmental stresses. Further research along these lines, to identify context specific interaction between ABA and BR would help us better understand stress-based adaptation in terms of vascular development and regeneration.

While regeneration or development of vasculature as an adaptation to stress is important for plant survival, this is very often abused by biotic agents for their gain. In paper III we hypothesized that since vascular regeneration is the end step in both abiotic and biotic stresses, there might be a common mechanism between them. We identified a gene, *EVG1* which is up regulated by both biotic and abiotic stress, and it affected vascular development, vascular regeneration, and cellular regeneration. We observed that mutating *EVG1* affected cell wall related genes and mutants of *EVG1* phenocopied mutants of *RLP44*. While *EVG1* is stress responsive, our analyses show that *RLP44* is not. This could possibly point to EVG1 mediating developmental

changes through RLP44. The major question that arises is that what is the link between *EVG1-RLP44* that drives these developmental changes? Moreover, since we observed the expression domains of both EVG1 and RLP44 are different, how does EVG1 influence RLP44? Does it act like a mobile signal, or is it that the changes in *EVG1* transcript levels cause cell wall modifications and changes which act as a signal? A likely way to identify this would be to generate mutants of *EVG1* and *RLP44* and observe the resultant phenotypes in different assays such as grafting, VISUAL, and regeneration-based assays. Another method would be to use EVG1 translation reporters in *RLP44* mutant backgrounds in stress conditions and to track the signal. Lastly, performing cell wall fraction analysis on *EVG1* mutants to see if it actively affects cell wall dynamics will also help us understand if it acts as a cell wall damage-based signal. Localization studies and co-immunoprecipitation studies of *RLP44* and *EVG1* will also help identify the association between the two if it exists. Since *EVG1* affects cell walls related genes, and cell wall changes initiate a compensatory BR signaling cascade, a relationship between *EVG1* and BR signaling is not far-fetched. Moreover, loss of function of *EVG1* also had supernumerary metaxylem cell file numbers. This suggests that *EVG*1 may be a part of the BR signaling network. Treating *EVG1* mutants with BR to see how vascular development, growth, regeneration ability is affected will potentially help us identify if *EVG1* is also influenced by BR signaling. We also observed that *EVG1* was negatively regulated by ABA. Since reduction on *EVG1* levels resulted in more xylem formation in grafting (and VISUAL), this could point to another potential mode of action for ABA signaling to enhance xylem development in stress conditions. A way to identify this would be to generate mutants of *EVG1* and ABA signaling elements and to observe their vascular phenotype post ABA treatment.

In conclusion, this thesis shows how phytohormone signaling pathways like ABA signaling and BR signaling affect development of vasculature and regeneration. We also show that genetic factors contribute to stress-based development as well. The potential involvement of *EVG1* with both ABA and BR signaling also opens questions about another avenue of interaction for ABA and BR. The identification of *EVG1* further opens potential questions and exploration opportunities as to how the impact of stress on cell

wall biology can influence vascular development and regeneration. Recently, it was reported that cell wall damage activates factors that control regeneration and vascular development (Zhang et al. 2022). Further research for clear and thorough understanding of these pathways, along with a detailed analysis of *EVG1* will help us uncover mechanisms of plant adaptation to stress which can help us improve agricultural yield and generate crops that are stress resilient.

References

Ackerman-Lavert, M., Fridman, Y., Matosevich, R., Khandal, H., Friedlander-Shani, L., Vragović, K., Ben El, R., Horev, G., Tarkowská, D., Efroni, I. & Savaldi-Goldstein, S. (2021). Auxin requirements for a meristematic state in roots depend on a dual brassinosteroid function. *Current Biology*, 31 (20), 4462-4472.e6. https://doi.org/10.1016/j.cub.2021.07.075

Agustí, J. & Blázquez, M.A. (2020). Plant vascular development: mechanisms and environmental regulation. *Cellular and Molecular Life Sciences*, 77 (19), 3711–3728. https://doi.org/10.1007/s00018-020-03496-w

Agusti, J., Herold, S., Schwarz, M., Sanchez, P., Ljung, K., Dun, E.A., Brewer, P.B., Beveridge, C.A., Sieberer, T., Sehr, E.M. & Greb, T. (2011). Strigolactone signaling is required for auxin-dependent stimulation of secondary growth in plants. *Proceedings of the National Academy of Sciences*, 108 (50), 20242–20247. https://doi.org/10.1073/pnas.1111902108

Akira, S. & Shozo, F. (1997). Studies on Biosynthesis of Brassinosteroids. *Bioscience, Biotechnology, and Biochemistry*, 61 (5), 757–762. https://doi.org/10.1271/bbb.61.757

Albertos, P., Dündar, G., Schenk, P., Carrera, S., Cavelius, P., Sieberer, T. & Poppenberger, B. (2022). Transcription factor BES1 interacts with HSFA1 to promote heat stress resistance of plants. *The EMBO Journal*, 41 (3). https://doi.org/10.15252/embj.2021108664

Argyros, R.D., Mathews, D.E., Chiang, Y.-H., Palmer, C.M., Thibault, D.M., Etheridge, N., Argyros, D.A., Mason, M.G., Kieber, J.J. & Schaller, G.E. (2008). Type B Response Regulators of *Arabidopsis* Play Key Roles in Cytokinin Signaling and Plant Development. *The Plant Cell*, 20 (8), 2102–2116. https://doi.org/10.1105/tpc.108.059584

Asahina, M., Azuma, K., Pitaksaringkarn, W., Yamazaki, T., Mitsuda, N., Ohme-Takagi, M., Yamaguchi, S., Kamiya, Y., Okada, K., Nishimura, T., Koshiba, T., Yokota, T., Kamada, H. & Satoh, S. (2011). Spatially selective hormonal control of RAP2.6L and ANAC071 transcription factors involved in tissue reunion in *Arabidopsis*. *Proceedings of the National Academy of Sciences*, 108 (38), 16128–16132. https://doi.org/10.1073/pnas.1110443108

Atta, R., Laurens, L., Boucheron-Dubuisson, E., Guivarc'h, A., Carnero, E., Giraudat-Pautot, V., Rech, P. & Chriqui, D. (2009). Pluripotency of Arabidopsis xylem pericycle underlies shoot regeneration from root and

hypocotyl explants grown *in vitro*. *The Plant Journal*, 57 (4), 626–644. https://doi.org/10.1111/j.1365-313X.2008.03715.x

Bauby, H., Divol, F., Truernit, E., Grandjean, O. & Palauqui, J.-C. (2007). Protophloem Differentiation in Early Arabidopsis thaliana Development. *Plant and Cell Physiology*, 48 (1), 97–109. https://doi.org/10.1093/pcp/pcl045

Baum, S.F., Dubrovsky, J.G. & Rost, T.L. (2002). Apical organization and maturation of the cortex and vascular cylinder inArabidopsis thaliana (Brassicaceae) roots. *American Journal of Botany*, 89 (6), 908–920. https://doi.org/10.3732/ajb.89.6.908

Bechtold, U. & Field, B. (2018). Molecular mechanisms controlling plant growth during abiotic stress. *Journal of Experimental Botany*, 69 (11), 2753–2758. https://doi.org/10.1093/jxb/ery157

Belkhadir, Y. & Jaillais, Y. (2015). The molecular circuitry of brassinosteroid signaling. *New Phytologist*, 206 (2), 522–540. https://doi.org/10.1111/nph.13269

Berleth, T., Mattsson, J. & Hardtke, C.S. (2000). Vascular continuity and auxin signals. *Trends in Plant Science*, 5 (9), 387–393. https://doi.org/10.1016/S1360-1385(00)01725-8

Birnbaum, K.D. & Alvarado, A.S. (2008). Slicing across Kingdoms: Regeneration in Plants and Animals. *Cell*, 132 (4), 697–710. https://doi.org/10.1016/j.cell.2008.01.040

Bishopp, A., Help, H., El-Showk, S., Weijers, D., Scheres, B., Friml, J., Benková, E., Mähönen, A.P. & Helariutta, Y. (2011a). A Mutually Inhibitory Interaction between Auxin and Cytokinin Specifies Vascular Pattern in Roots. *Current Biology*, 21 (11), 917–926. https://doi.org/10.1016/j.cub.2011.04.017

Bishopp, A., Lehesranta, S., Vatén, A., Help, H., El-Showk, S., Scheres, B., Helariutta, K., Mähönen, A.P., Sakakibara, H. & Helariutta, Y. (2011b). Phloem-Transported Cytokinin Regulates Polar Auxin Transport and Maintains Vascular Pattern in the Root Meristem. *Current Biology*, 21 (11), 927–932. https://doi.org/10.1016/j.cub.2011.04.049

Bloch, D., Puli, M.R., Mosquna, A. & Yalovsky, S. (2019). Abiotic stress modulates root patterning via ABA-regulated *microRNA* expression in the endodermis initials. *Development*, dev.177097. https://doi.org/10.1242/dev.177097

Bonke, M., Thitamadee, S., Mähönen, A.P., Hauser, M.-T. & Helariutta, Y. (2003). APL regulates vascular tissue identity in Arabidopsis. *Nature*, 426 (6963), 181–186. https://doi.org/10.1038/nature02100

Borner, G.H.H., Lilley, K.S., Stevens, T.J. & Dupree, P. (2003). Identification of Glycosylphosphatidylinositol-Anchored Proteins in Arabidopsis. A Proteomic and Genomic Analysis. *Plant Physiology*, 132 (2), 568–577. https://doi.org/10.1104/pp.103.021170

Brandstatter, I. & Kieber, J.J. (1998). Two Genes with Similarity to Bacterial Response Regulators Are Rapidly and Specifically Induced by Cytokinin in Arabidopsis. 11

Busse, J.S. & Evert, R.F. (1999). Vascular Differentiation and Transition in the Seedling of *Arabidopsis thaliana* (Brassicaceae). *International Journal of Plant Sciences*, 160 (2), 241–251. https://doi.org/10.1086/314117

Cai, Z., Liu, J., Wang, H., Yang, C., Chen, Y., Li, Y., Pan, S., Dong, R., Tang, G., Barajas-Lopez, J. de D., Fujii, H. & Wang, X. (2014). GSK3-like kinases positively modulate abscisic acid signaling through phosphorylating subgroup III SnRK2s in *Arabidopsis*. *Proceedings of the National Academy of Sciences*, 111 (26), 9651–9656. https://doi.org/10.1073/pnas.1316717111

Campbell, L., Etchells, J.P., Cooper, M., Kumar, M. & Turner, S.R. (2018). An essential role for Abscisic acid in the regulation of xylem fibre differentiation. *Development*, dev.161992. https://doi.org/10.1242/dev.161992

Canher, B., Heyman, J., Savina, M., Devendran, A., Eekhout, T., Vercauteren, I., Prinsen, E., Matosevich, R., Xu, J., Mironova, V. & De Veylder, L. (2020). Rocks in the auxin stream: Wound-induced auxin accumulation and *ERF115* expression synergistically drive stem cell regeneration. *Proceedings of the National Academy of Sciences*, 117 (28), 16667–16677. https://doi.org/10.1073/pnas.2006620117

Caño-Delgado, A., Lee, J.-Y. & Demura, T. (2010). Regulatory Mechanisms for Specification and Patterning of Plant Vascular Tissues. *Annual Review of Cell and Developmental Biology*, 26 (1), 605–637. https://doi.org/10.1146/annurev-cellbio-100109-104107

Caño-Delgado, A., Yin, Y., Yu, C., Vafeados, D., Mora-García, S., Cheng, J.-C., Nam, K.H., Li, J. & Chory, J. (2004). BRL1 and BRL3 are novel brassinosteroid receptors that function in vascular differentiation in *Arabidopsis*. *Development*, 131 (21), 5341–5351. https://doi.org/10.1242/dev.01403

Carlsbecker, A., Lee, J.-Y., Roberts, C.J., Dettmer, J., Lehesranta, S., Zhou, J., Lindgren, O., Moreno-Risueno, M.A., Vatén, A., Thitamadee, S., Campilho, A., Sebastian, J., Bowman, J.L., Helariutta, Y. & Benfey, P.N. (2010). Cell signalling by microRNA165/6 directs gene dose-dependent root cell fate. *Nature*, 465 (7296), 316–321. https://doi.org/10.1038/nature08977

Chen, J. & Yin, Y. (2017). WRKY transcription factors are involved in brassinosteroid signaling and mediate the crosstalk between plant growth and drought tolerance. *Plant Signaling & Behavior*, 12 (11), e1365212. https://doi.org/10.1080/15592324.2017.1365212

Choe, S. (1999). Brassinosteroid biosynthesis. *Plant Physiology and Biochemistry*, 37 (5), 351–361. https://doi.org/10.1016/S0981-9428(99)80041-2

Choe, S., Dilkes, B.P., Fujioka, S., Takatsuto, S., Sakurai, A. & Feldmann, K.A. (1998). The DWF4 Gene of Arabidopsis Encodes a Cytochrome P450 That Mediates Multiple 22␣-Hydroxylation Steps in Brassinosteroid Biosynthesis. 13

Choe, S., Dilkes, B.P., Gregory, B.D., Ross, A.S., Yuan, H., Noguchi, T., Fujioka, S., Takatsuto, S., Tanaka, A., Yoshida, S., Tax, F.E. & Feldmann, K.A. (1999). The Arabidopsis *dwarf1* Mutant Is Defective in the Conversion of 24-Methylenecholesterol to Campesterol in Brassinosteroid Biosynthesis1. *Plant Physiology*, 119 (3), 897–908. https://doi.org/10.1104/pp.119.3.897

Clouse, S.D. & Sasse, J.M. (1998). BRASSINOSTEROIDS: Essential Regulators of Plant Growth and Development. *Annual Review of Plant Physiology and Plant Molecular Biology*, 49 (1), 427–451. https://doi.org/10.1146/annurev.arplant.49.1.427

Cornelis, S. & Hazak, O. (2022). Understanding the root xylem plasticity for designing resilient crops. *Plant, Cell & Environment*, 45 (3), 664–676. https://doi.org/10.1111/pce.14245

Cosgrove, D.J. (2000). Loosening of plant cell walls by expansins. *Nature*, 407 (6802), 321–326. https://doi.org/10.1038/35030000

Cosgrove, D.J. (2016). Catalysts of plant cell wall loosening. *F1000Research*, 5, 119. https://doi.org/10.12688/f1000research.7180.1

Couto, D. & Zipfel, C. (2016). Regulation of pattern recognition receptor signalling in plants. *Nature Reviews Immunology*, 16 (9), 537–552. https://doi.org/10.1038/nri.2016.77

Craker, L.E. & Abeles, F.B. (1969). Abscission: Quantitative Measurement with a Recording Abscissor. 6

Cronshaw, J. & Esau, K. (1968). P PROTEIN IN THE PHLOEM OF CUCURBITA. 12

Cruz-Valderrama, J.E., Gómez-Maqueo, X., Salazar-Iribe, A., Zúñiga-Sánchez, E., Hernández-Barrera, A., Quezada-Rodríguez, E. & Gamboa-deBuen, A. (2019). Overview of the Role of Cell Wall DUF642 Proteins in Plant Development. *International Journal of Molecular Sciences*, 20 (13), 3333. https://doi.org/10.3390/ijms20133333

Cutler, S.R., Rodriguez, P.L., Finkelstein, R.R. & Abrams, S.R. (2010). Abscisic Acid: Emergence of a Core Signaling Network. *Annual Review of Plant Biology*, 61 (1), 651–679. https://doi.org/10.1146/annurev-arplant-042809-112122

D'Agostino, I.B., Deruère, J. & Kieber, J.J. (2000). Characterization of the Response of the Arabidopsis Response Regulator Gene Family to Cytokinin. *Plant Physiology*, 124 (4), 1706–1717. https://doi.org/10.1104/pp.124.4.1706

De Rybel, B., Adibi, M., Breda, A.S., Wendrich, J.R., Smit, M.E., Novák, O., Yamaguchi, N., Yoshida, S., Van Isterdael, G., Palovaara, J., Nijsse, B., Boekschoten, M.V., Hooiveld, G., Beeckman, T., Wagner, D., Ljung, K., Fleck, C. & Weijers, D. (2014). Integration of growth and patterning during vascular tissue formation in *Arabidopsis*. *Science*, 345 (6197), 1255215. https://doi.org/10.1126/science.1255215

De Rybel, B., Audenaert, D., Vert, G., Rozhon, W., Mayerhofer, J., Peelman, F., Coutuer, S., Denayer, T., Jansen, L., Nguyen, L., Vanhoutte, I., Beemster, G.T.S., Vleminckx, K., Jonak, C., Chory, J., Inzé, D., Russinova, E. & Beeckman, T. (2009). Chemical Inhibition of a Subset of Arabidopsis thaliana GSK3-like Kinases Activates Brassinosteroid Signaling. *Chemistry & Biology*, 16 (6), 594–604. https://doi.org/10.1016/j.chembiol.2009.04.008

De Rybel, B., Mähönen, A.P., Helariutta, Y. & Weijers, D. (2016). Plant vascular development: from early specification to differentiation. *Nature Reviews Molecular Cell Biology*, 17 (1), 30–40. https://doi.org/10.1038/nrm.2015.6

Deeken, R., Engelmann, J.C., Efetova, M., Czirjak, T., Müller, T., Kaiser, W.M., Tietz, O., Krischke, M., Mueller, M.J., Palme, K., Dandekar, T. & Hedrich, R. (2007). An Integrated View of Gene Expression and Solute Profiles of *Arabidopsis* Tumors: A Genome-Wide Approach. *The Plant Cell*, 18 (12), 3617–3634. https://doi.org/10.1105/tpc.106.044743

Depuydt, S., Rodriguez-Villalon, A., Santuari, L., Wyser-Rmili, C., Ragni, L. & Hardtke, C.S. (2013). Suppression of *Arabidopsis* protophloem differentiation and root meristem growth by CLE45 requires the receptor-like kinase BAM3. *Proceedings of the National Academy of Sciences*, 110 (17), 7074–7079. https://doi.org/10.1073/pnas.1222314110

De Rybel, B., Möller, B., Yoshida, S., Grabowicz, I., Barbier de Reuille, P., Boeren, S., Smith, R.S., Borst, J.W. & Weijers, D. (2013). A bHLH Complex Controls Embryonic Vascular Tissue Establishment and Indeterminate Growth in Arabidopsis. *Developmental Cell*, 24 (4), 426–437. https://doi.org/10.1016/j.devcel.2012.12.013

Di Laurenzio, L., Wysocka-Diller, J., Malamy, J.E., Pysh, L., Helariutta, Y., Freshour, G., Hahn, M.G., Feldmann, K.A. & Benfey, P.N. (1996). The SCARECROW Gene Regulates an Asymmetric Cell Division That Is Essential for Generating the Radial Organization of the Arabidopsis Root. *Cell*, 86 (3), 423–433. https://doi.org/10.1016/S0092-8674(00)80115-4

Ding, Q., Zeng, J. & He, X.-Q. (2016). MiR169 and its target PagHAP2-6 regulated by ABA are involved in poplar cambium dormancy. *Journal of Plant Physiology*, 198, 1–9. https://doi.org/10.1016/j.jplph.2016.03.017

Dolan, L., Janmaat, K., Willemsen, V., Linstead, P., Poethig, S., Roberts, K. & Scheres, B. (1993). Cellular organisation of the Arabidopsis thaliana root. *Development*, 119 (1), 71–84. https://doi.org/10.1242/dev.119.1.71

Donner, T.J., Sherr, I. & Scarpella, E. (2009). Regulation of preprocambial cell state acquisition by auxin signaling in *Arabidopsis* leaves. *Development*, 136 (19), 3235–3246. https://doi.org/10.1242/dev.037028

Du, Q., Avci, U., Li, S., Gallego-Giraldo, L., Pattathil, S., Qi, L., Hahn, M.G. & Wang, H. (2015). Activation of *miR165b* represses *AtHB15* expression and induces pith secondary wall development in Arabidopsis. *The Plant Journal*, 83 (3), 388–400. https://doi.org/10.1111/tpj.12897

Efroni, I., Mello, A., Nawy, T., Ip, P.-L., Rahni, R., DelRose, N., Powers, A., Satija, R. & Birnbaum, K.D. (2016). Root Regeneration Triggers an Embryo-like Sequence Guided by Hormonal Interactions. *Cell*, 165 (7), 1721–1733. https://doi.org/10.1016/j.cell.2016.04.046

Eldem, V., Çelikkol Akçay, U., Ozhuner, E., Bakır, Y., Uranbey, S. & Unver, T. (2012). Genome-Wide Identification of miRNAs Responsive to Drought in Peach (Prunus persica) by High-Throughput Deep Sequencing. Vinatzer, B.A. (ed.) (Vinatzer, B. A., ed.) *PLoS ONE*, 7 (12), e50298. https://doi.org/10.1371/journal.pone.0050298

Eleftheriou, E.P. & Tsekos, I. (1982). Development of protophloem in roots ofAegilops comosa var.thessalica. II. Sieve-element differentiation. *Protoplasma*, 113 (3), 221–233. https://doi.org/10.1007/BF01280911

ESAU, K. (1972). Changes in the Nucleus and the Endoplasmic Reticulum during Differentiation of a Sieve Element in Mimosa pudica L. *Annals of Botany*, 36 (4), 703–710. https://doi.org/10.1093/oxfordjournals.aob.a084626

Esau, K., , (1960). *Anatomy of seed plants*. https://archive.org/details/anatomyofseedpla0000unse

Etchells, J.P. & Turner, S.R. (2010). The PXY-CLE41 receptor ligand pair defines a multifunctional pathway that controls the rate and orientation of vascular cell division. *Development*, 137 (5), 767–774. https://doi.org/10.1242/dev.044941

Fàbregas, N., Li, N., Boeren, S., Nash, T.E., Goshe, M.B., Clouse, S.D., de Vries, S. & Caño-Delgado, A.I. (2013). The BRASSINOSTEROID INSENSITIVE1–LIKE3 Signalosome Complex Regulates *Arabidopsis* Root Development. *The Plant Cell*, 25 (9), 3377–3388. https://doi.org/10.1105/tpc.113.114462

Fan, P., Aguilar, E., Bradai, M., Xue, H., Wang, H., Rosas-Diaz, T., Tang, W., Wolf, S., Zhang, H., Xu, L. & Lozano-Durán, R. (2021). The receptor-like kinases BAM1 and BAM2 are required for root xylem patterning. *Proceedings of the National Academy of Sciences*, 118 (12), e2022547118. https://doi.org/10.1073/pnas.2022547118

Finkelstein, R.R. & Gibson, S.I. (2002). ABA and sugar interactions regulating development: cross-talk or voices in a crowd? *Current Opinion in Plant Biology*, 5 (1), 26–32. https://doi.org/10.1016/S1369-5266(01)00225-4

Fisher, K. & Turner, S. (2007). PXY, a Receptor-like Kinase Essential for Maintaining Polarity during Plant Vascular-Tissue Development. *Current Biology*, 17 (12), 1061–1066. https://doi.org/10.1016/j.cub.2007.05.049

Fridman, Y., Strauss, S., Horev, G., Ackerman-Lavert, M., Benaim, A.R., Lane, B., Smith, R.S. & Savaldi-Goldstein, S. (2021). *Root meristem shaping via brassinosteroid-controlled cell geometry*. Plant Biology. https://doi.org/10.1101/2021.04.01.438011

Friedrichsen, D. & Chory, J. (2001). Steroid signaling in plants: from the cell surface to the nucleus. *BioEssays*, 23 (11), 1028–1036. https://doi.org/10.1002/bies.1148

Fuchs, S., Tischer, S.V., Wunschel, C., Christmann, A. & Grill, E. (2014). Abscisic acid sensor RCAR7/PYL13, specific regulator of protein phosphatase coreceptors. *Proceedings of the National Academy of Sciences*, 111 (15), 5741–5746. https://doi.org/10.1073/pnas.1322085111

Fujii, H., Chinnusamy, V., Rodrigues, A., Rubio, S., Antoni, R., Park, S.-Y., Cutler, S.R., Sheen, J., Rodriguez, P.L. & Zhu, J.-K. (2009). In vitro reconstitution of an abscisic acid signalling pathway. *Nature*, 462 (7273), 660–664. https://doi.org/10.1038/nature08599

Fujii, H., Verslues, P.E. & Zhu, J.-K. (2007). Identification of Two Protein Kinases Required for Abscisic Acid Regulation of Seed Germination, Root Growth, and Gene Expression in *Arabidopsis*. *The Plant Cell*, 19 (2), 485–494. https://doi.org/10.1105/tpc.106.048538

Furuta, K.M., Hellmann, E. & Helariutta, Y. (2014). Molecular Control of Cell Specification and Cell Differentiation During Procambial Development. *Annual Review of Plant Biology*, 65 (1), 607–638. https://doi.org/10.1146/annurev-arplant-050213-040306

Furuya, T., Saito, M., Uchimura, H., Satake, A., Nosaki, S., Miyakawa, T., Shimadzu, S., Yamori, W., Tanokura, M., Fukuda, H. & Kondo, Y. (2021). Gene co-expression network analysis identifies BEH3 as a stabilizer of secondary vascular development in Arabidopsis. *The Plant Cell*, 33 (8), 2618–2636. https://doi.org/10.1093/plcell/koab151

Gampala, S.S., Kim, T.-W., He, J.-X., Tang, W., Deng, Z., Bai, M.-Y., Guan, S., Lalonde, S., Sun, Y., Gendron, J.M., Chen, H., Shibagaki, N., Ferl, R.J., Ehrhardt, D., Chong, K., Burlingame, A.L. & Wang, Z.-Y. (2007). An Essential Role for 14-3-3 Proteins in Brassinosteroid Signal Transduction in Arabidopsis. *Developmental Cell*, 13 (2), 177–189. https://doi.org/10.1016/j.devcel.2007.06.009

Garza-Caligaris, L.E., Avendaño-Vázquez, A.O., Alvarado-López, S., Zúñiga-Sánchez, E., Orozco-Segovia, A., Pérez-Ruíz, R.V. & Gamboa-deBuen, A. (2012). At3g08030 transcript: a molecular marker of seed ageing. *Annals of Botany*, 110 (6), 1253–1260. https://doi.org/10.1093/aob/mcs200

Gimeno-Gilles, C., Lelièvre, E., Viau, L., Malik-Ghulam, M., Ricoult, C., Niebel, A., Leduc, N. & Limami, A.M. (2009). ABA-Mediated Inhibition of Germination Is Related to the Inhibition of Genes Encoding Cell-Wall Biosynthetic and Architecture: Modifying Enzymes and Structural Proteins in Medicago truncatula Embryo Axis. *Molecular Plant*, 2 (1), 108–119. https://doi.org/10.1093/mp/ssn092

Giusti, L., Mica, E., Bertolini, E., De Leonardis, A.M., Faccioli, P., Cattivelli, L. & Crosatti, C. (2017). microRNAs differentially modulated in response to heat and drought stress in durum wheat cultivars with contrasting water use efficiency. *Functional & Integrative Genomics*, 17 (2–3), 293–309. https://doi.org/10.1007/s10142-016-0527-7

González-García, M.-P., Vilarrasa-Blasi, J., Zhiponova, M., Divol, F., Mora-García, S., Russinova, E. & Caño-Delgado, A.I. (2011). Brassinosteroids control meristem size by promoting cell cycle progression in *Arabidopsis* roots. *Development*, 138 (5), 849–859. https://doi.org/10.1242/dev.057331

Grove, M.D., Spencer, G.F., Rohwedder, W.K., Mandava, N., Worley, J.F., Warthen, J.D., Steffens, G.L., Flippen-Anderson, J.L. & Cook, J.C. (1979). Brassinolide, a plant growth-promoting steroid isolated from Brassica napus pollen. *Nature*, 281 (5728), 216–217. https://doi.org/10.1038/281216a0

Gutmann, M., von Aderkas, P., Label, P. & Lelu, M.-A. (1996). Effects of abscisic acid on somatic embryo maturation of hybrid larch. *Journal of Experimental Botany*, 47 (12), 1905–1917. https://doi.org/10.1093/jxb/47.12.1905

Hacham, Y., Holland, N., Butterfield, C., Ubeda-Tomas, S., Bennett, M.J., Chory, J. & Savaldi-Goldstein, S. (2011). Brassinosteroid perception in the epidermis controls root meristem size. *Development*, 138 (5), 839–848. https://doi.org/10.1242/dev.061804

Hartwig, T., Chuck, G.S., Fujioka, S., Klempien, A., Weizbauer, R., Potluri, D.P.V., Choe, S., Johal, G.S. & Schulz, B. (2011). Brassinosteroid control of sex determination in maize. *Proceedings of the National Academy of Sciences*, 108 (49), 19814–19819. https://doi.org/10.1073/pnas.1108359108

He, J.-X., Gendron, J.M., Sun, Y., Gampala, S.S.L., Gendron, N., Sun, C.Q. & Wang, Z.-Y. (2005). BZR1 Is a Transcriptional Repressor with Dual Roles in Brassinosteroid Homeostasis and Growth Responses. *Science*, 307 (5715), 1634–1638. https://doi.org/10.1126/science.1107580

He, J.-X., Gendron, J.M., Yang, Y., Li, J. & Wang, Z.-Y. (2002). The GSK3-like kinase BIN2 phosphorylates and destabilizes BZR1, a positive regulator of the brassinosteroid signaling pathway in *Arabidopsis*. *Proceedings of the National Academy of Sciences*, 99 (15), 10185–10190. https://doi.org/10.1073/pnas.152342599

Hirakawa, Y., Kondo, Y. & Fukuda, H. (2010). TDIF Peptide Signaling Regulates Vascular Stem Cell Proliferation via the *WOX4* Homeobox Gene in

Arabidopsis. *The Plant Cell*, 22 (8), 2618–2629. https://doi.org/10.1105/tpc.110.076083

Hirakawa, Y., Shinohara, H., Kondo, Y., Inoue, A., Nakanomyo, I., Ogawa, M., Sawa, S., Ohashi-Ito, K., Matsubayashi, Y. & Fukuda, H. (2008). Non-cell-autonomous control of vascular stem cell fate by a CLE peptide/receptor system. *Proceedings of the National Academy of Sciences*, 105 (39), 15208–15213. https://doi.org/10.1073/pnas.0808444105

Hirose, N., Takei, K., Kuroha, T., Kamada-Nobusada, T., Hayashi, H. & Sakakibara, H. (2007). Regulation of cytokinin biosynthesis, compartmentalization and translocation. *Journal of Experimental Botany*, 59 (1), 75–83. https://doi.org/10.1093/jxb/erm157

Holzwart, E., Huerta, A.I., Glöckner, N., Garnelo Gómez, B., Wanke, F., Augustin, S., Askani, J.C., Schürholz, A.-K., Harter, K. & Wolf, S. (2018). BRI1 controls vascular cell fate in the *Arabidopsis* root through RLP44 and phytosulfokine signaling. *Proceedings of the National Academy of Sciences*, 115 (46), 11838–11843. https://doi.org/10.1073/pnas.1814434115

Holzwart, E., Wanke, F., Glöckner, N., Höfte, H., Harter, K. & Wolf, S. (2020a). A Mutant Allele Uncouples the Brassinosteroid-Dependent and Independent Functions of BRASSINOSTEROID INSENSITIVE 1. *Plant Physiology*, 182 (1), 669–678. https://doi.org/10.1104/pp.19.00448

Holzwart, E., Wanke, F., Glöckner, N., Höfte, H., Harter, K. & Wolf, S. (2020b). A Mutant Allele Uncouples the Brassinosteroid-Dependent and Independent Functions of BRASSINOSTEROID INSENSITIVE 1. *Plant Physiology*, 182 (1), 669–678. https://doi.org/10.1104/pp.19.00448

Hu, Y. & Yu, D. (2014). BRASSINOSTEROID INSENSITIVE2 Interacts with ABSCISIC ACID INSENSITIVE5 to Mediate the Antagonism of Brassinosteroids to Abscisic Acid during Seed Germination in *Arabidopsis*. *The Plant Cell*, 26 (11), 4394–4408. https://doi.org/10.1105/tpc.114.130849

Hwang, B.G., Ryu, J. & Lee, S.J. (2016). Vulnerability of Protoxylem and Metaxylem Vessels to Embolisms and Radial Refilling in a Vascular Bundle of Maize Leaves. *Frontiers in Plant Science*, 7. https://doi.org/10.3389/fpls.2016.00941

Ibañes, M., Fàbregas, N., Chory, J. & Caño-Delgado, A.I. (2009). Brassinosteroid signaling and auxin transport are required to establish the periodic pattern of *Arabidopsis* shoot vascular bundles. *Proceedings of the National Academy of Sciences*, 106 (32), 13630–13635. https://doi.org/10.1073/pnas.0906416106

Ikeuchi, M., Sugimoto, K. & Iwase, A. (2013). Plant Callus: Mechanisms of Induction and Repression. *The Plant Cell*, 25 (9), 3159–3173. https://doi.org/10.1105/tpc.113.116053

Ishida, K., Yamashino, T., Yokoyama, A. & Mizuno, T. (2008). Three Type-B Response Regulators, ARR1, ARR10 and ARR12, Play Essential but

Redundant Roles in Cytokinin Signal Transduction Throughout the Life Cycle of Arabidopsis thaliana. *Plant and Cell Physiology*, 49 (1), 47–57. https://doi.org/10.1093/pcp/pcm165

Ito, Y., Nakanomyo, I., Motose, H., Iwamoto, K., Sawa, S., Dohmae, N. & Fukuda, H. (2006). Dodeca-CLE Peptides as Suppressors of Plant Stem Cell Differentiation. *Science*, 313 (5788), 842–845. https://doi.org/10.1126/science.1128436

Iwasaki, T. & Shibaoka, H. (1991). Brassinosteroids Act as Regulators of Tracheary-Element Differentiation in Isolated Zinnia Mesophyll Cells. *Plant and Cell Physiology*, 32 (7), 1007–1014. https://doi.org/10.1093/oxfordjournals.pcp.a078163

Kang, Y.H., Breda, A. & Hardtke, C.S. (2017). Brassinosteroid signaling directs formative cell divisions and protophloem differentiation in *Arabidopsis* root meristems. *Development*, 144 (2), 272–280. https://doi.org/10.1242/dev.145623

Kantar, M., Unver, T. & Budak, H. (2010). Regulation of barley miRNAs upon dehydration stress correlated with target gene expression. *Functional & Integrative Genomics*, 10 (4), 493–507. https://doi.org/10.1007/s10142-010-0181-4

Kareem, A., Durgaprasad, K., Sugimoto, K., Du, Y., Pulianmackal, A.J., Trivedi, Z.B., Abhayadev, P.V., Pinon, V., Meyerowitz, E.M., Scheres, B. & Prasad, K. (2015). PLETHORA Genes Control Regeneration by a Two-Step Mechanism. *Current Biology*, 25 (8), 1017–1030. https://doi.org/10.1016/j.cub.2015.02.022

Karlberg, A., Englund, M., Petterle, A., Molnar, G., Sjödin, A., Bako, L. & Bhalerao, R.P. (2010). Analysis of global changes in gene expression during activity-dormancy cycle in hybrid aspen apex. *Plant Biotechnology*, 27 (1), 1–16. https://doi.org/10.5511/plantbiotechnology.27.1

Kieber, J.J. & Schaller, G.E. (2014). Cytokinins. *The Arabidopsis Book*, 12, e0168. https://doi.org/10.1199/tab.0168

Kieber, J.J. & Schaller, G.E. (2018). Cytokinin signaling in plant development. *Development*, 145 (4), dev149344. https://doi.org/10.1242/dev.149344

Klahre, U., Noguchi, T., Fujioka, S., Takatsuto, S., Yokota, T., Nomura, T., Yoshida, S. & Chua, N.-H. (1998). The Arabidopsis DIMINUTO/DWARF1 Gene Encodes a Protein Involved in Steroid Synthesis. 14

Kondo, Y. (2018). Reconstitutive approach for investigating plant vascular development. *Journal of Plant Research*, 131 (1), 23–29. https://doi.org/10.1007/s10265-017-0998-1

Kondo, Y., Fujita, T., Sugiyama, M. & Fukuda, H. (2015). A Novel System for Xylem Cell Differentiation in Arabidopsis thaliana. *Molecular Plant*, 8 (4), 612–621. https://doi.org/10.1016/j.molp.2014.10.008

Kondo, Y., Ito, T., Nakagami, H., Hirakawa, Y., Saito, M., Tamaki, T., Shirasu, K. & Fukuda, H. (2014). Plant GSK3 proteins regulate xylem cell differentiation downstream of TDIF–TDR signalling. *Nature Communications*, 5 (1), 3504. https://doi.org/10.1038/ncomms4504

Kondo, Y., Nurani, A.M., Saito, C., Ichihashi, Y., Saito, M., Yamazaki, K., Mitsuda, N., Ohme-Takagi, M. & Fukuda, H. (2016). Vascular Cell Induction Culture System Using Arabidopsis Leaves (VISUAL) Reveals the Sequential Differentiation of Sieve Element-Like Cells. *The Plant Cell*, 28 (6), 1250–1262. https://doi.org/10.1105/tpc.16.00027

Kørner, C.J., Klauser, D., Niehl, A., Domínguez-Ferreras, A., Chinchilla, D., Boller, T., Heinlein, M. & Hann, D.R. (2013). The Immunity Regulator *BAK1* Contributes to Resistance Against Diverse RNA Viruses. *Molecular Plant-Microbe Interactions*®, 26 (11), 1271–1280. https://doi.org/10.1094/MPMI-06-13-0179-R

Kubo, M., Udagawa, M., Nishikubo, N., Horiguchi, G., Yamaguchi, M., Ito, J., Mimura, T., Fukuda, H. & Demura, T. (2005). Transcription switches for protoxylem and metaxylem vessel formation. *Genes & Development*, 19 (16), 1855–1860. https://doi.org/10.1101/gad.1331305

Kushwah, S., Banasiak, A., Nishikubo, N., Derba-Maceluch, M., Majda, M., Endo, S., Kumar, V., Gomez, L., Gorzsas, A., McQueen-Mason, S., Braam, J., Sundberg, B. & Mellerowicz, E.J. (2020). Arabidopsis *XTH4* and *XTH9* Contribute to Wood Cell Expansion and Secondary Wall Formation. *Plant Physiology*, 182, 1946–1965. https://doi.org/10.1104/pp.19.01529

Lavy, M. & Estelle, M. (2016). Mechanisms of auxin signaling. *Development*, 143 (18), 3226–3229. https://doi.org/10.1242/dev.131870

Lee, C.-W., Efetova, M., Engelmann, J.C., Kramell, R., Wasternack, C., Ludwig-Müller, J., Hedrich, R. & Deeken, R. (2009). *Agrobacterium tumefaciens* Promotes Tumor Induction by Modulating Pathogen Defense in *Arabidopsis thaliana*. *The Plant Cell*, 21 (9), 2948–2962. https://doi.org/10.1105/tpc.108.064576

Leung, J., Bouvier-Durand, M., Morris, P.-C., Guerrier, D., Chefdor, F. & Giraudat, J. (1994). *Arabidopsis* ABA Response Gene *ABI1* : Features of a Calcium-Modulated Protein Phosphatase. *Science*, 264 (5164), 1448–1452. https://doi.org/10.1126/science.7910981

Leyser, O. (2018). Auxin Signaling. *Plant Physiology*, 176 (1), 465–479. https://doi.org/10.1104/pp.17.00765

Li, B., Ferreira, M.A., Huang, M., Camargos, L.F., Yu, X., Teixeira, R.M., Carpinetti, P.A., Mendes, G.C., Gouveia-Mageste, B.C., Liu, C., Pontes, C.S.L., Brustolini, O.J.B., Martins, L.G.C., Melo, B.P., Duarte, C.E.M., Shan, L., He, P. & Fontes, E.P.B. (2019). The receptor-like kinase NIK1 targets FLS2/BAK1 immune complex and inversely modulates antiviral and

antibacterial immunity. *Nature Communications*, 10 (1), 4996. https://doi.org/10.1038/s41467-019-12847-6

Li, J. & Chory, J. (1997). A Putative Leucine-Rich Repeat Receptor Kinase Involved in Brassinosteroid Signal Transduction. *Cell*, 90 (5), 929–938. https://doi.org/10.1016/S0092-8674(00)80357-8

Li, J. & Nam, K.H. (2002). Regulation of Brassinosteroid Signaling by a GSK3/SHAGGY-Like Kinase. *Science*, 295 (5558), 1299–1301. https://doi.org/10.1126/science.1065769

Li, J., Nam, K.H., Vafeados, D. & Chory, J. (2001). *BIN2* , a New Brassinosteroid-Insensitive Locus in Arabidopsis. *Plant Physiology*, 127 (1), 14–22. https://doi.org/10.1104/pp.127.1.14

Li, Y., Darley, C.P., Ongaro, V., Fleming, A., Schipper, O., Baldauf, S.L. & McQueen-Mason, S.J. (2002). Plant Expansins Are a Complex Multigene Family with an Ancient Evolutionary Origin. *Plant Physiology*, 128 (3), 854–864. https://doi.org/10.1104/pp.010658

Liu, C., Yu, H., Rao, X., Li, L. & Dixon, R.A. (2021). Abscisic acid regulates secondary cell-wall formation and lignin deposition in *Arabidopsis thaliana* through phosphorylation of NST1. *Proceedings of the National Academy of Sciences*, 118 (5), e2010911118. https://doi.org/10.1073/pnas.2010911118

Liu, X., Zhu, Y., Zhai, H., Cai, H., Ji, W., Luo, X., Li, J. & Bai, X. (2012). AtPP2CG1, a protein phosphatase 2C, positively regulates salt tolerance of Arabidopsis in abscisic acid-dependent manner. *Biochemical and Biophysical Research Communications*, 422 (4), 710–715. https://doi.org/10.1016/j.bbrc.2012.05.064

Lozano-Elena, F., Planas-Riverola, A., Vilarrasa-Blasi, J., Schwab, R. & Caño-Delgado, A.I. (2017). Paracrine brassinosteroid signaling at the stem cell niche controls cellular regeneration. *Journal of Cell Science*, jcs.204065. https://doi.org/10.1242/jcs.204065

Lu, Z., Huang, M., Ge, D.-P., Yang, Y.-H., Cai, X.-N., Qin, P. & She, J.-M. (2003). Effect of brassinolide on callus growth and regeneration in Spartina patens (Poaceae). 3

Lucas, W.J., Groover, A., Lichtenberger, R., Furuta, K., Yadav, S.-R., Helariutta, Y., He, X.-Q., Fukuda, H., Kang, J., Brady, S.M., Patrick, J.W., Sperry, J., Yoshida, A., López-Millán, A.-F., Grusak, M.A. & Kachroo, P. (2013). The Plant Vascular System: Evolution, Development and Functions [F]. *Journal of Integrative Plant Biology*, 55 (4), 294–388. https://doi.org/10.1111/jipb.12041

Ma, Y., Szostkiewicz, I., Korte, A., Moes, D., Yang, Y., Christmann, A. & Grill, E. (2009). Regulators of PP2C Phosphatase Activity Function as Abscisic Acid Sensors. *Science*, 324 (5930), 1064–1068. https://doi.org/10.1126/science.1172408

Mähönen, A.P., Bishopp, A., Higuchi, M., Nieminen, K.M., Kinoshita, K., Törmäkangas, K., Ikeda, Y., Oka, A., Kakimoto, T. & Helariutta, Y. (2006). Cytokinin Signaling and Its Inhibitor AHP6 Regulate Cell Fate During Vascular Development. *Science*, 311 (5757), 94–98. https://doi.org/10.1126/science.1118875

Mähönen, A.P., Bonke, M., Kauppinen, L., Riikonen, M., Benfey, P.N. & Helariutta, Y. (2000). A novel two-component hybrid molecule regulates vascular morphogenesis of the *Arabidopsis* root. *Genes & Development*, 14 (23), 2938–2943. https://doi.org/10.1101/gad.189200

Makarevitch, I., Thompson, A., Muehlbauer, G.J. & Springer, N.M. (2012). Brd1 Gene in Maize Encodes a Brassinosteroid C-6 Oxidase. Wu, S.-B. (ed.) (Wu, S.-B., ed.) *PLoS ONE*, 7 (1), e30798. https://doi.org/10.1371/journal.pone.0030798

Matosevich, R., Cohen, I., Gil-Yarom, N., Modrego, A., Friedlander-Shani, L., Verna, C., Scarpella, E. & Efroni, I. (2020). Local auxin biosynthesis is required for root regeneration after wounding. *Nature Plants*, 6 (8), 1020–1030. https://doi.org/10.1038/s41477-020-0737-9

Matsumoto-Kitano, M., Kusumoto, T., Tarkowski, P., Kinoshita-Tsujimura, K., Václavíková, K., Miyawaki, K. & Kakimoto, T. (2008). Cytokinins are central regulators of cambial activity. *Proceedings of the National Academy of Sciences*, 105 (50), 20027–20031. https://doi.org/10.1073/pnas.0805619105

Melnyk, C.W. (2017). Plant grafting: insights into tissue regeneration. *Regeneration*, 4 (1), 3–14. https://doi.org/10.1002/reg2.71

Melnyk, C.W., Gabel, A., Hardcastle, T.J., Robinson, S., Miyashima, S., Grosse, I. & Meyerowitz, E.M. (2018). Transcriptome dynamics at *Arabidopsis* graft junctions reveal an intertissue recognition mechanism that activates vascular regeneration. *Proceedings of the National Academy of Sciences*, 115 (10). https://doi.org/10.1073/pnas.1718263115

Melnyk, C.W. & Meyerowitz, E.M. (2015). Plant grafting. *Current Biology*, 25 (5), R183–R188. https://doi.org/10.1016/j.cub.2015.01.029

Melnyk, C.W., Schuster, C., Leyser, O. & Meyerowitz, E.M. (2015). A Developmental Framework for Graft Formation and Vascular Reconnection in Arabidopsis thaliana. *Current Biology*, 25 (10), 1306–1318. https://doi.org/10.1016/j.cub.2015.03.032

Miedes, E., Suslov, D., Vandenbussche, F., Kenobi, K., Ivakov, A., Van Der Straeten, D., Lorences, E.P., Mellerowicz, E.J., Verbelen, J.-P. & Vissenberg, K. (2013). Xyloglucan endotransglucosylase/hydrolase (XTH) overexpression affects growth and cell wall mechanics in etiolated Arabidopsis hypocotyls. *Journal of Experimental Botany*, 64 (8), 2481–2497. https://doi.org/10.1093/jxb/ert107

Mitchell, J.W., Mandava, N., Worley, J.F., Plimmer, J.R. & Smith, M.V. (1970). Brassins—a New Family of Plant Hormones from Rape Pollen. *Nature*, 225 (5237), 1065–1066. https://doi.org/10.1038/2251065a0

Miyashima, S., Koi, S., Hashimoto, T. & Nakajima, K. (2011). Non-cell-autonomous microRNA165 acts in a dose-dependent manner to regulate multiple differentiation status in the *Arabidopsis* root. *Development*, 138 (11), 2303–2313. https://doi.org/10.1242/dev.060491

Miyashima, S., Roszak, P., Sevilem, I., Toyokura, K., Blob, B., Heo, J., Mellor, N., Help-Rinta-Rahko, H., Otero, S., Smet, W., Boekschoten, M., Hooiveld, G., Hashimoto, K., Smetana, O., Siligato, R., Wallner, E.-S., Mähönen, A.P., Kondo, Y., Melnyk, C.W., Greb, T., Nakajima, K., Sozzani, R., Bishopp, A., De Rybel, B. & Helariutta, Y. (2019). Mobile PEAR transcription factors integrate positional cues to prime cambial growth. *Nature*, 565 (7740), 490–494. https://doi.org/10.1038/s41586-018-0839-y

Mohnen, D. (2008). Pectin structure and biosynthesis. *Current Opinion in Plant Biology*, 11 (3), 266–277. https://doi.org/10.1016/j.pbi.2008.03.006

Mouchel, C.F., Osmont, K.S. & Hardtke, C.S. (2006). BRX mediates feedback between brassinosteroid levels and auxin signalling in root growth. *Nature*, 443 (7110), 458–461. https://doi.org/10.1038/nature05130

Mullendore, D.L., Windt, C.W., Van As, H. & Knoblauch, M. (2010). Sieve Tube Geometry in Relation to Phloem Flow. *The Plant Cell*, 22 (3), 579–593. https://doi.org/10.1105/tpc.109.070094

Nagata, N., Asami, T. & Yoshida, S. (2001). Brassinazole, an Inhibitor of Brassinosteroid Biosynthesis, Inhibits Development of Secondary Xylem in Cress Plants (Lepidium sativum). *Plant and Cell Physiology*, 42 (9), 1006–1011. https://doi.org/10.1093/pcp/pce122

Nakamura, A., Goda, H., Shimada, Y. & Yoshida, S. (2004). Brassinosteroid Selectively Regulates *PIN* Gene Expression in Arabidopsis. *Bioscience, Biotechnology, and Biochemistry*, 68 (4), 952–954. https://doi.org/10.1271/bbb.68.952

Nakamura, A., Higuchi, K., Goda, H., Fujiwara, M.T., Sawa, S., Koshiba, T., Shimada, Y. & Yoshida, S. (2003). Brassinolide Induces *IAA5*, *IAA19* , and DR5, a Synthetic Auxin Response Element in Arabidopsis, Implying a Cross Talk Point of Brassinosteroid and Auxin Signaling. *Plant Physiology*, 133 (4), 1843–1853. https://doi.org/10.1104/pp.103.030031

Nemhauser, J.L., Mockler, T.C. & Chory, J. (2004). Interdependency of Brassinosteroid and Auxin Signaling in Arabidopsis. Jeffrey Dangl (ed.) (Jeffrey Dangl, ed.) *PLoS Biology*, 2 (9), e258. https://doi.org/10.1371/journal.pbio.0020258

Nishimura, N., Yoshida, T., Kitahata, N., Asami, T., Shinozaki, K. & Hirayama, T. (2007). ABA-Hypersensitive Germination1 encodes a protein phosphatase 2C, an essential component of abscisic acid signaling in Arabidopsis seed:

Arabidopsis ABA-hypersensitive mutant. *The Plant Journal*, 50 (6), 935–949. https://doi.org/10.1111/j.1365-313X.2007.03107.x

O'Brien, J.A. & Benková, E. (2013). Cytokinin cross-talking during biotic and abiotic stress responses. *Frontiers in Plant Science*, 4. https://doi.org/10.3389/fpls.2013.00451

Ohashi-Ito, K., Demura, T. & Fukuda, H. (2002). Promotion of Transcript Accumulation of Novel Zinnia Immature Xylem-Specific HD-Zip III Homeobox Genes by Brassinosteroids. *Plant and Cell Physiology*, 43 (10), 1146–1153. https://doi.org/10.1093/pcp/pcf135

Ohashi-Ito, K., Matsukawa, M. & Fukuda, H. (2013). An Atypical bHLH Transcription Factor Regulates Early Xylem Development Downstream of Auxin. *Plant and Cell Physiology*, 54 (3), 398–405. https://doi.org/10.1093/pcp/pct013

Ohashi-Ito, K., Oda, Y. & Fukuda, H. (2010). *Arabidopsis* VASCULAR-RELATED NAC-DOMAIN6 Directly Regulates the Genes That Govern Programmed Cell Death and Secondary Wall Formation during Xylem Differentiation. *The Plant Cell*, 22 (10), 3461–3473. https://doi.org/10.1105/tpc.110.075036

Ohashi-Ito, K., Saegusa, M., Iwamoto, K., Oda, Y., Katayama, H., Kojima, M., Sakakibara, H. & Fukuda, H. (2014). A bHLH Complex Activates Vascular Cell Division via Cytokinin Action in Root Apical Meristem. *Current Biology*, 24 (17), 2053–2058. https://doi.org/10.1016/j.cub.2014.07.050

Olmo, R., Cabrera, J., Díaz-Manzano, F.E., Ruiz-Ferrer, V., Barcala, M., Ishida, T., García, A., Andrés, M.F., Ruiz-Lara, S., Verdugo, I., Pernas, M., Fukaki, H., del Pozo, J.C., Moreno-Risueno, M.Á., Kyndt, T., Gheysen, G., Fenoll, C., Sawa, S. & Escobar, C. (2020). Root-knot nematodes induce gall formation by recruiting developmental pathways of post-embryonic organogenesis and regeneration to promote transient pluripotency. *New Phytologist*, 227 (1), 200–215. https://doi.org/10.1111/nph.16521

Osakabe, Y., Maruyama, K., Seki, M., Satou, M., Shinozaki, K. & Yamaguchi-Shinozaki, K. (2005). Leucine-Rich Repeat Receptor-Like Kinase1 Is a Key Membrane-Bound Regulator of Abscisic Acid Early Signaling in Arabidopsis. *The Plant Cell*, 17 (4), 1105–1119. https://doi.org/10.1105/tpc.104.027474

Osakabe, Y., Yamaguchi-Shinozaki, K., Shinozaki, K. & Tran, L.-S.P. (2013). Sensing the environment: key roles of membrane-localized kinases in plant perception and response to abiotic stress. *Journal of Experimental Botany*, 64 (2), 445–458. https://doi.org/10.1093/jxb/ers354

Park, S.-Y., Fung, P., Nishimura, N., Jensen, D.R., Fujii, H., Zhao, Y., Lumba, S., Santiago, J., Rodrigues, A., Chow, T.F., Alfred, S.E., Bonetta, D., Finkelstein, R., Provart, N.J., Desveaux, D., Rodriguez, P.L., McCourt, P., Zhu, J.-K., Schroeder, J.I., Volkman, B.F. & Cutler, S.R. (2009). Abscisic Acid Inhibits Type 2C Protein Phosphatases via the PYR/PYL Family of

START Proteins. *Science*, 324 (5930), 1068–1071. https://doi.org/10.1126/science.1173041

Peng, P., Yan, Z., Zhu, Y. & Li, J. (2008). Regulation of the Arabidopsis GSK3-like Kinase BRASSINOSTEROID-INSENSITIVE 2 through Proteasome-Mediated Protein Degradation. *Molecular Plant*, 1 (2), 338–346. https://doi.org/10.1093/mp/ssn001

Prigge, M.J., Otsuga, D., Alonso, J.M., Ecker, J.R., Drews, G.N. & Clark, S.E. (2005). Class III Homeodomain-Leucine Zipper Gene Family Members Have Overlapping, Antagonistic, and Distinct Roles in Arabidopsis Development. *The Plant Cell*, 17 (1), 61–76. https://doi.org/10.1105/tpc.104.026161

Prislan, P., Čufar, K., Koch, G., Schmitt, U. & Gričar, J. (2013). Review of cellular and subcellular changes in the cambium. *IAWA Journal*, 34 (4), 391–407. https://doi.org/10.1163/22941932-00000032

Qu, T., Liu, R., Wang, W., An, L., Chen, T., Liu, G. & Zhao, Z. (2011). Brassinosteroids regulate pectin methylesterase activity and AtPME41 expression in Arabidopsis under chilling stress. *Cryobiology*, 63 (2), 111–117. https://doi.org/10.1016/j.cryobiol.2011.07.003

Ragni, L., Nieminen, K., Pacheco-Villalobos, D., Sibout, R., Schwechheimer, C. & Hardtke, C.S. (2011). Mobile Gibberellin Directly Stimulates *Arabidopsis* Hypocotyl Xylem Expansion. *The Plant Cell*, 23 (4), 1322–1336. https://doi.org/10.1105/tpc.111.084020

Ramachandran, P., Augstein, F., Nguyen, V. & Carlsbecker, A. (2020). Coping With Water Limitation: Hormones That Modify Plant Root Xylem Development. *Frontiers in Plant Science*, 11, 570. https://doi.org/10.3389/fpls.2020.00570

Ramachandran, P., Wang, G., Augstein, F., de Vries, J. & Carlsbecker, A. (2018). Continuous root xylem formation and vascular acclimation to water deficit involves endodermal ABA signalling via miR165. *Development*, dev.159202. https://doi.org/10.1242/dev.159202

Rowe, J.H., Topping, J.F., Liu, J. & Lindsey, K. (2016). Abscisic acid regulates root growth under osmotic stress conditions via an interacting hormonal network with cytokinin, ethylene and auxin. *New Phytologist*, 211 (1), 225–239. https://doi.org/10.1111/nph.13882

Ruonala, R., Ko, D. & Helariutta, Y. (2017). Genetic Networks in Plant Vascular Development. *Annual Review of Genetics*, 51 (1), 335–359. https://doi.org/10.1146/annurev-genet-120116-024525

Russinova, E., Borst, J.-W., Kwaaitaal, M., Caño-Delgado, A., Yin, Y., Chory, J. & de Vries, S.C. (2004). Heterodimerization and Endocytosis of Arabidopsis Brassinosteroid Receptors BRI1 and AtSERK3 (BAK1). *The Plant Cell*, 16 (12), 3216–3229. https://doi.org/10.1105/tpc.104.025387

Ruttink, T., Arend, M., Morreel, K., Storme, V., Rombauts, S., Fromm, J., Bhalerao, R.P., Boerjan, W. & Rohde, A. (2007). A Molecular Timetable for Apical Bud Formation and Dormancy Induction in Poplar. *The Plant Cell*, 19 (8), 2370–2390. https://doi.org/10.1105/tpc.107.052811

Saez, A., Apostolova, N., Gonzalez-Guzman, M., Gonzalez-Garcia, M.P., Nicolas, C., Lorenzo, O. & Rodriguez, P.L. (2004). Gain-of-function and loss-of-function phenotypes of the protein phosphatase 2C *HAB1* reveal its role as a negative regulator of abscisic acid signalling. *The Plant Journal*, 37 (3), 354–369. https://doi.org/10.1046/j.1365-313X.2003.01966.x

Saito, M. & Kondo, Y. (2019). What Can Cell Culture Systems Reveal About Sieve Element Differentiation? In: Liesche, J. (ed.) *Phloem*. New York, NY: Springer New York. 459–466. https://doi.org/10.1007/978-1-4939-9562-2_36

Saito, M., Kondo, Y. & Fukuda, H. (2018). BES1 and BZR1 Redundantly Promote Phloem and Xylem Differentiation. *Plant and Cell Physiology*, 59 (3), 590–600. https://doi.org/10.1093/pcp/pcy012

Sakurai, A. (1999). Brassinosteroid biosynthesis. *Plant Physiology and Biochemistry*, 37 (5), 351–361. https://doi.org/10.1016/S0981-9428(99)80041-2

Salazar-Henao, J.E., Lehner, R., Betegón-Putze, I., Vilarrasa-Blasi, J. & Caño-Delgado, A.I. (2016). BES1 regulates the localization of the brassinosteroid receptor BRL3 within the provascular tissue of the Arabidopsis primary root. *Journal of Experimental Botany*, 67 (17), 4951–4961. https://doi.org/10.1093/jxb/erw258

Salazar-Iribe, A., Agredano-Moreno, L.T., Zúñiga-Sánchez, E., Jiménez-Garcia, L.F. & Gamboa-deBuen, A. (2016). The cell wall DUF642 At2g41800 (TEB) protein is involved in hypocotyl cell elongation. *Plant Science*, 253, 206–214. https://doi.org/10.1016/j.plantsci.2016.10.007

Sauter, M. (2015). Phytosulfokine peptide signalling. *Journal of Experimental Botany*, 66 (17), 5161–5169. https://doi.org/10.1093/jxb/erv071

Scacchi, E., Osmont, K.S., Beuchat, J., Salinas, P., Navarrete-Gómez, M., Trigueros, M., Ferrándiz, C. & Hardtke, C.S. (2009). Dynamic, auxin-responsive plasma membrane-to-nucleus movement of *Arabidopsis* BRX. *Development*, 136 (12), 2059–2067. https://doi.org/10.1242/dev.035444

Scacchi, E., Salinas, P., Gujas, B., Santuari, L., Krogan, N., Ragni, L., Berleth, T. & Hardtke, C.S. (2010). Spatio-temporal sequence of cross-regulatory events in root meristem growth. *Proceedings of the National Academy of Sciences*, 107 (52), 22734–22739. https://doi.org/10.1073/pnas.1014716108

Scarpella, E., Marcos, D., Friml, J. & Berleth, T. (2005). Control of leaf vascular patterning by polar auxin transport. 14

Scheres, B., Wolkenfelt, H., Willemsen, V., Terlouw, M., Lawson, E., Dean, C. & Weisbeek, P. (1994). Embryonic origin of the Arabidopsis primary root and root meristem initials. 13

Schlereth, A., Möller, B., Liu, W., Kientz, M., Flipse, J., Rademacher, E.H., Schmid, M., Jürgens, G. & Weijers, D. (2010). MONOPTEROS controls embryonic root initiation by regulating a mobile transcription factor. *Nature*, 464 (7290), 913–916. https://doi.org/10.1038/nature08836

Sena, G., Wang, X., Liu, H.-Y., Hofhuis, H. & Birnbaum, K.D. (2009). Organ regeneration does not require a functional stem cell niche in plants. *Nature*, 457 (7233), 1150–1153. https://doi.org/10.1038/nature07597

Shanks, C.M., Rice, J.H., Zubo, Y., Schaller, G.E., Hewezi, T. & Kieber, J.J. (2016). The Role of Cytokinin During Infection of *Arabidopsis thaliana* by the Cyst Nematode *Heterodera schachtii*. *Molecular Plant-Microbe Interactions®*, 29 (1), 57–68. https://doi.org/10.1094/MPMI-07-15-0156-R

Shiu, S.-H. & Bleecker, A.B. (2003). Expansion of the Receptor-Like Kinase/Pelle Gene Family and Receptor-Like Proteins in Arabidopsis. *Plant Physiology*, 132 (2), 530–543. https://doi.org/10.1104/pp.103.021964

Shu, K., Zhang, H., Wang, S., Chen, M., Wu, Y., Tang, S., Liu, C., Feng, Y., Cao, X. & Xie, Q. (2013). ABI4 Regulates Primary Seed Dormancy by Regulating the Biogenesis of Abscisic Acid and Gibberellins in Arabidopsis. Yu, H. (ed.) (Yu, H., ed.) *PLoS Genetics*, 9 (6), e1003577. https://doi.org/10.1371/journal.pgen.1003577

Shulse, C.N., Cole, B.J., Ciobanu, D., Lin, J., Yoshinaga, Y., Gouran, M., Turco, G.M., Zhu, Y., O'Malley, R.C., Brady, S.M. & Dickel, D.E. (2019). High-Throughput Single-Cell Transcriptome Profiling of Plant Cell Types. *Cell Reports*, 27 (7), 2241-2247.e4. https://doi.org/10.1016/j.celrep.2019.04.054

Sibout, R., Plantegenet, S. & Hardtke, C.S. (2008). Flowering as a Condition for Xylem Expansion in Arabidopsis Hypocotyl and Root. *Current Biology*, 18 (6), 458–463. https://doi.org/10.1016/j.cub.2008.02.070

Singh, A.P. & Savaldi-Goldstein, S. (2015). Growth control: brassinosteroid activity gets context. *Journal of Experimental Botany*, 66 (4), 1123–1132. https://doi.org/10.1093/jxb/erv026

Sjolund, R.D. (1997). The Phloem Sieve Element: A River Runs through It. *The Plant Cell*, 1137–1146. https://doi.org/10.1105/tpc.9.7.1137

Song, L., Huang, S.C., Wise, A., Castanon, R., Nery, J.R., Chen, H., Watanabe, M., Thomas, J., Bar-Joseph, Z. & Ecker, J.R. (2016). A transcription factor hierarchy defines an environmental stress response network. *Science*, 354 (6312), aag1550. https://doi.org/10.1126/science.aag1550

Steuer, B., Stuhlfauth, T. & Fock, H.P. (1988). The efficiency of water use in water stressed plants is increased due to ABA induced stomatal closure. *Photosynthesis Research*, 18 (3), 327–336. https://doi.org/10.1007/BF00034837

Sugimoto, K., Gordon, S.P. & Meyerowitz, E.M. (2011). Regeneration in plants and animals: dedifferentiation, transdifferentiation, or just differentiation? *Trends in Cell Biology*, 21 (4), 212–218. https://doi.org/10.1016/j.tcb.2010.12.004

Sugimoto, K., Jiao, Y. & Meyerowitz, E.M. (2010). Arabidopsis Regeneration from Multiple Tissues Occurs via a Root Development Pathway. *Developmental Cell*, 18 (3), 463–471. https://doi.org/10.1016/j.devcel.2010.02.004

Sun, L., Feraru, E., Feraru, M.I., Waidmann, S., Wang, W., Passaia, G., Wang, Z.-Y., Wabnik, K. & Kleine-Vehn, J. (2020). PIN-LIKES Coordinate Brassinosteroid Signaling with Nuclear Auxin Input in Arabidopsis thaliana. *Current Biology*, 30 (9), 1579-1588.e6. https://doi.org/10.1016/j.cub.2020.02.002

Sun, S., Chen, D., Li, X., Qiao, S., Shi, C., Li, C., Shen, H. & Wang, X. (2015). Brassinosteroid Signaling Regulates Leaf Erectness in Oryza sativa via the Control of a Specific U-Type Cyclin and Cell Proliferation. *Developmental Cell*, 34 (2), 220–228. https://doi.org/10.1016/j.devcel.2015.05.019

Sun, Y., Fan, X.-Y., Cao, D.-M., Tang, W., He, K., Zhu, J.-Y., He, J.-X., Bai, M.-Y., Zhu, S., Oh, E., Patil, S., Kim, T.-W., Ji, H., Wong, W.H., Rhee, S.Y. & Wang, Z.-Y. (2010). Integration of Brassinosteroid Signal Transduction with the Transcription Network for Plant Growth Regulation in Arabidopsis. *Developmental Cell*, 19 (5), 765–777. https://doi.org/10.1016/j.devcel.2010.10.010

Takahashi, N. & Umeda, M. (2022). Brassinosteroids are required for efficient root tip regeneration in *Arabidopsis*. *Plant Biotechnology*, 39 (1), 73–78. https://doi.org/10.5511/plantbiotechnology.21.1103a

Tan, T.T., Endo, H., Sano, R., Kurata, T., Yamaguchi, M., Ohtani, M. & Demura, T. (2018). Transcription Factors VND1-VND3 Contribute to Cotyledon Xylem Vessel Formation. *Plant Physiology*, 176 (1), 773–789. https://doi.org/10.1104/pp.17.00461

Tanaka, H., Osakabe, Y., Katsura, S., Mizuno, S., Maruyama, K., Kusakabe, K., Mizoi, J., Shinozaki, K. & Yamaguchi-Shinozaki, K. (2012). Abiotic stress-inducible receptor-like kinases negatively control ABA signaling in Arabidopsis: Receptor-like kinases in ABA signaling. *The Plant Journal*, 70 (4), 599–613. https://doi.org/10.1111/j.1365-313X.2012.04901.x

Tang, N., Shahzad, Z., Lonjon, F., Loudet, O., Vailleau, F. & Maurel, C. (2018). Natural variation at XND1 impacts root hydraulics and trade-off for stress responses in Arabidopsis. *Nature Communications*, 9 (1), 3884. https://doi.org/10.1038/s41467-018-06430-8

Taylor-Teeples, M., Lin, L., de Lucas, M., Turco, G., Toal, T.W., Gaudinier, A., Young, N.F., Trabucco, G.M., Veling, M.T., Lamothe, R., Handakumbura, P.P., Xiong, G., Wang, C., Corwin, J., Tsoukalas, A., Zhang, L., Ware, D., Pauly, M., Kliebenstein, D.J., Dehesh, K., Tagkopoulos, I., Breton, G.,

Pruneda-Paz, J.L., Ahnert, S.E., Kay, S.A., Hazen, S.P. & Brady, S.M. (2015). An Arabidopsis gene regulatory network for secondary cell wall synthesis. *Nature*, 517 (7536), 571–575. https://doi.org/10.1038/nature14099

Thamm, A., Sanegre-Sans, S., Paisley, J., Meader, S., Milhinhos, A., Contera, S. & Agusti, J. (2019). A simple mathematical model of allometric exponential growth describes the early three-dimensional growth dynamics of secondary xylem in Arabidopsis roots. *Royal Society Open Science*, 6 (3), 190126. https://doi.org/10.1098/rsos.190126

To, J.P.C., Deruère, J., Maxwell, B.B., Morris, V.F., Hutchison, C.E., Ferreira, F.J., Schaller, G.E. & Kieber, J.J. (2008). Cytokinin Regulates Type-A *Arabidopsis* Response Regulator Activity and Protein Stability via Two-Component Phosphorelay. *The Plant Cell*, 19 (12), 3901–3914. https://doi.org/10.1105/tpc.107.052662

Truernit, E., Bauby, H., Belcram, K., Barthélémy, J. & Palauqui, J.-C. (2012). OCTOPUS, a polarly localised membrane-associated protein, regulates phloem differentiation entry in *Arabidopsis thaliana*. *Development*, 139 (7), 1306–1315. https://doi.org/10.1242/dev.072629

Tylewicz, S., Petterle, A., Marttila, S., Miskolczi, P., Azeez, A., Singh, R.K., Immanen, J., Mähler, N., Hvidsten, T.R., Eklund, D.M., Bowman, J.L., Helariutta, Y. & Bhalerao, R.P. (2018). Photoperiodic control of seasonal growth is mediated by ABA acting on cell-cell communication. *Science*, 360 (6385), 212–215. https://doi.org/10.1126/science.aan8576

Umezawa, T., Nakashima, K., Miyakawa, T., Kuromori, T., Tanokura, M., Shinozaki, K. & Yamaguchi-Shinozaki, K. (2010). Molecular Basis of the Core Regulatory Network in ABA Responses: Sensing, Signaling and Transport. *Plant and Cell Physiology*, 51 (11), 1821–1839. https://doi.org/10.1093/pcp/pcq156

Vázquez-Lobo, A., Roujol, D., Zuñiga-Sánchez, E., Albenne, C., Piñero, D., Buen, A.G. de & Jamet, E. (2012). The highly conserved spermatophyte cell wall DUF642 protein family: Phylogeny and first evidence of interaction with cell wall polysaccharides in vitro. *Molecular Phylogenetics and Evolution*, 63 (2), 510–520. https://doi.org/10.1016/j.ympev.2012.02.001

Wan, J., He, M., Hou, Q., Zou, L., Yang, Y., Wei, Y. & Chen, X. (2021). Cell wall associated immunity in plants. *Stress Biology*, 1 (1), 3. https://doi.org/10.1007/s44154-021-00003-4

Wang, C., Huang, X., Li, Q., Zhang, Y., Li, J.-L. & Mou, Z. (2019). Extracellular pyridine nucleotides trigger plant systemic immunity through a lectin receptor kinase/BAK1 complex. *Nature Communications*, 10 (1), 4810. https://doi.org/10.1038/s41467-019-12781-7

Wang, C., Liu, N., Geng, Z., Ji, M., Wang, S., Zhuang, Y., Wang, D., He, G., Zhao, S., Zhou, G. & Chai, G. (2022). Integrated transcriptome and proteome

analysis reveals brassinosteroid-mediated regulation of cambium initiation and patterning in woody stem. *Horticulture Research*, 9, uhab048. https://doi.org/10.1093/hr/uhab048

Wang, D., Gao, Z., Du, P., Xiao, W., Tan, Q., Chen, X., Li, L. & Gao, D. (2016). Expression of ABA Metabolism-Related Genes Suggests Similarities and Differences Between Seed Dormancy and Bud Dormancy of Peach (Prunus persica). *Frontiers in Plant Science*, 6. https://doi.org/10.3389/fpls.2015.01248

Wang, H., Tang, J., Liu, J., Hu, J., Liu, J., Chen, Y., Cai, Z. & Wang, X. (2018a). Abscisic Acid Signaling Inhibits Brassinosteroid Signaling through Dampening the Dephosphorylation of BIN2 by ABI1 and ABI2. *Molecular Plant*, 11 (2), 315–325. https://doi.org/10.1016/j.molp.2017.12.013

Wang, H., Tang, J., Liu, J., Hu, J., Liu, J., Chen, Y., Cai, Z. & Wang, X. (2018b). Abscisic Acid Signaling Inhibits Brassinosteroid Signaling through Dampening the Dephosphorylation of BIN2 by ABI1 and ABI2. *Molecular Plant*, 11 (2), 315–325. https://doi.org/10.1016/j.molp.2017.12.013

Wang, Z.-Y., Nakano, T., Gendron, J., He, J., Chen, M., Vafeados, D., Yang, Y., Fujioka, S., Yoshida, S., Asami, T. & Chory, J. (2002). Nuclear-Localized BZR1 Mediates Brassinosteroid-Induced Growth and Feedback Suppression of Brassinosteroid Biosynthesis. *Developmental Cell*, 2 (4), 505–513. https://doi.org/10.1016/S1534-5807(02)00153-3

Wenzel, C.L., Schuetz, M., Yu, Q. & Mattsson, J. (2007). Dynamics of MONOPTEROS and PIN-FORMED1 expression during leaf vein pattern formation in Arabidopsis thaliana: MP and PIN1 expression in Arabidopsis leaves. *The Plant Journal*, 49 (3), 387–398. https://doi.org/10.1111/j.1365-313X.2006.02977.x

Wolf, S., van der Does, D., Ladwig, F., Sticht, C., Kolbeck, A., Schürholz, A.-K., Augustin, S., Keinath, N., Rausch, T., Greiner, S., Schumacher, K., Harter, K., Zipfel, C. & Höfte, H. (2014). A receptor-like protein mediates the response to pectin modification by activating brassinosteroid signaling. *Proceedings of the National Academy of Sciences*, 111 (42), 15261–15266. https://doi.org/10.1073/pnas.1322979111

Wolf, S., Mravec, J., Greiner, S., Mouille, G. & Höfte, H. (2012). Plant Cell Wall Homeostasis Is Mediated by Brassinosteroid Feedback Signaling. *Current Biology*, 22 (18), 1732–1737. https://doi.org/10.1016/j.cub.2012.07.036

Wu, H. & Zheng, X.-F. (2003). Ultrastructural Studies on the Sieve Elements in Root Protophleom of Arabidopsis thaliana.pdf

Xie, Q., Essemine, J., Pang, X., Chen, H., Jin, J. & Cai, W. (2021). Abscisic Acid Regulates the Root Growth Trajectory by Reducing Auxin Transporter PIN2 Protein Levels in Arabidopsis thaliana. *Frontiers in Plant Science*, 12, 632676. https://doi.org/10.3389/fpls.2021.632676

Yamaguchi, M., Goué, N., Igarashi, H., Ohtani, M., Nakano, Y., Mortimer, J.C., Nishikubo, N., Kubo, M., Katayama, Y., Kakegawa, K., Dupree, P. & Demura, T. (2010a). VASCULAR-RELATED NAC-DOMAIN6 and VASCULAR-RELATED NAC-DOMAIN7 Effectively Induce Transdifferentiation into Xylem Vessel Elements under Control of an Induction System. *Plant Physiology*, 153 (3), 906–914. https://doi.org/10.1104/pp.110.154013

Yamaguchi, M., Mitsuda, N., Ohtani, M., Ohme-Takagi, M., Kato, K. & Demura, T. (2011). VASCULAR-RELATED NAC-DOMAIN 7 directly regulates the expression of a broad range of genes for xylem vessel formation: Direct target genes of VND7. *The Plant Journal*, 66 (4), 579–590. https://doi.org/10.1111/j.1365-313X.2011.04514.x

Yamaguchi, M., Ohtani, M., Mitsuda, N., Kubo, M., Ohme-Takagi, M., Fukuda, H. & Demura, T. (2010b). VND-INTERACTING2, a NAC Domain Transcription Factor, Negatively Regulates Xylem Vessel Formation in *Arabidopsis*. *The Plant Cell*, 22 (4), 1249–1263. https://doi.org/10.1105/tpc.108.064048

Ye, H., Liu, S., Tang, B., Chen, J., Xie, Z., Nolan, T.M., Jiang, H., Guo, H., Lin, H.-Y., Li, L., Wang, Y., Tong, H., Zhang, M., Chu, C., Li, Z., Aluru, M., Aluru, S., Schnable, P.S. & Yin, Y. (2017). RD26 mediates crosstalk between drought and brassinosteroid signalling pathways. *Nature Communications*, 8 (1), 14573. https://doi.org/10.1038/ncomms14573

Yin, Y., Wang, Z.-Y., Mora-Garcia, S., Li, J., Yoshida, S., Asami, T. & Chory, J. (2002). BES1 Accumulates in the Nucleus in Response to Brassinosteroids to Regulate Gene Expression and Promote Stem Elongation. *Cell*, 109 (2), 181–191. https://doi.org/10.1016/S0092-8674(02)00721-3

Yoshida, S., Barbier de Reuille, P., Lane, B., Bassel, G.W., Prusinkiewicz, P., Smith, R.S. & Weijers, D. (2014). Genetic Control of Plant Development by Overriding a Geometric Division Rule. *Developmental Cell*, 29 (1), 75–87. https://doi.org/10.1016/j.devcel.2014.02.002

Yoshida, T., Fujita, Y., Sayama, H., Kidokoro, S., Maruyama, K., Mizoi, J., Shinozaki, K. & Yamaguchi-Shinozaki, K. (2010). AREB1, AREB2, and ABF3 are master transcription factors that cooperatively regulate ABRE-dependent ABA signaling involved in drought stress tolerance and require ABA for full activation. *The Plant Journal*, 61 (4), 672–685. https://doi.org/10.1111/j.1365-313X.2009.04092.x

Yu, X., Li, L., Zola, J., Aluru, M., Ye, H., Foudree, A., Guo, H., Anderson, S., Aluru, S., Liu, P., Rodermel, S. & Yin, Y. (2011). A brassinosteroid transcriptional network revealed by genome-wide identification of BESI target genes in Arabidopsis thaliana: Brassinosteroid transcriptional network. *The Plant Journal*, 65 (4), 634–646. https://doi.org/10.1111/j.1365-313X.2010.04449.x

Zhang, A., Matsuoka, K., Kareem, A., Robert, M., Roszak, P., Blob, B., Bisht, A., De Veylder, L., Voiniciuc, C., Asahina, M. & Melnyk, C.W. (2022). Cell-wall damage activates DOF transcription factors to promote wound healing and tissue regeneration in Arabidopsis thaliana. *Current Biology*, 32 (9), 1883-1894.e7. https://doi.org/10.1016/j.cub.2022.02.069

Zhang, A., Zhang, J., Zhang, J., Ye, N., Zhang, H., Tan, M. & Jiang, M. (2011). Nitric Oxide Mediates Brassinosteroid-Induced ABA Biosynthesis Involved in Oxidative Stress Tolerance in Maize Leaves. *Plant and Cell Physiology*, 52 (1), 181–192. https://doi.org/10.1093/pcp/pcq187

Zhang, G., Zhao, F., Chen, L., Pan, Y., Sun, L., Bao, N., Zhang, T., Cui, C.-X., Qiu, Z., Zhang, Y., Yang, L. & Xu, L. (2019). Jasmonate-mediated wound signalling promotes plant regeneration. *Nature Plants*, 5 (5), 491–497. https://doi.org/10.1038/s41477-019-0408-x

Zhang, S., Cai, Z. & Wang, X. (2009). The primary signaling outputs of brassinosteroids are regulated by abscisic acid signaling. *Proceedings of the National Academy of Sciences*, 106 (11), 4543–4548. https://doi.org/10.1073/pnas.0900349106

Zhao, B. & Li, J. (2012). Regulation of Brassinosteroid Biosynthesis and Inactivation [F]. *Journal of Integrative Plant Biology*, 54 (10), 746–759. https://doi.org/10.1111/j.1744-7909.2012.01168.x

Zhao, Y., Chan, Z., Xing, L., Liu, X., Hou, Y.-J., Chinnusamy, V., Wang, P., Duan, C. & Zhu, J.-K. (2013). The unique mode of action of a divergent member of the ABA-receptor protein family in ABA and stress signaling. *Cell Research*, 23 (12), 1380–1395. https://doi.org/10.1038/cr.2013.149

Zhong, R. & Ye, Z.-H. (2012). MYB46 and MYB83 Bind to the SMRE Sites and Directly Activate a Suite of Transcription Factors and Secondary Wall Biosynthetic Genes. *Plant and Cell Physiology*, 53 (2), 368–380. https://doi.org/10.1093/pcp/pcr185

Zhou, J., Zhong, R. & Ye, Z.-H. (2014). Arabidopsis NAC Domain Proteins, VND1 to VND5, Are Transcriptional Regulators of Secondary Wall Biosynthesis in Vessels. Hazen, S.P. (ed.) (Hazen, S. P., ed.) *PLoS ONE*, 9 (8), e105726. https://doi.org/10.1371/journal.pone.0105726

Zhu, J.-K. (2002). S ALT AND D ROUGHT S TRESS S IGNAL T RANSDUCTION IN P LANTS. *Annual Review of Plant Biology*, 53 (1), 247–273. https://doi.org/10.1146/annurev.arplant.53.091401.143329

Zhu, J.-K. (2016). Abiotic Stress Signaling and Responses in Plants. *Cell*, 167 (2), 313–324. https://doi.org/10.1016/j.cell.2016.08.029

Zörb, C., Mühling, K.H., Kutschera, U. & Geilfus, C.-M. (2015). Salinity Stiffens the Epidermal Cell Walls of Salt-Stressed Maize Leaves: Is the Epidermis Growth-Restricting? Bie, Z. (ed.) (Bie, Z., ed.) *PLOS ONE*, 10 (3), e0118406. https://doi.org/10.1371/journal.pone.0118406

Popular science summary

Many know the phrase that plants are sessile organisms. Being rooted results in plants being subjected to stress situations regularly. To survive, plants have developed multiple ways of adapting changing conditions. Our understanding of how plants adapt is an ever-expanding pursuit in our bid to generate crops that are stress tolerant for improved food safety and security. In this thesis I furthered our understanding of how plants adapt to stress through modifying vascular development and regeneration. I and co-authors discovered that abscisic acid (ABA) phytohormone signaling pathway, which is the major stress hormone signaling in plants directly increases levels of genes that control the formation and development of xylem. This results in xylem development being enhanced in the roots. ABA signaling also changes the identity of xylem cells. This adaptive measure may allow plants to both draw more water from the soil and provide protection from air bubbles in the roots. Plants are also susceptible to damage from wounding. Damage to the plant cell walls activates brassinosteroid (BR) phytohormone signaling pathway. BR signaling mediates growth and development. BR signaling normally affects cell growth and has antagonistic interaction with ABA signaling in normal conditions This prompted us to investigate whether BR signaling affects vascular development and regeneration. The results suggest that BR signaling is required for both cambium and xylem development. Since cells cannot expand when BR signaling is interrupted, results imply that regeneration or growth of cells to prevent injury or healing during wounding is also reduced in plants that have defects in BR signaling. I and co-authors found that when a gene called *RECEPTOR LIKE PROTEIN 44* (*RLP44*) was present, it allowed the BR signaling to maintain cambium cells and reduce xylem differentiation. These findings suggest that both general BR signaling and *RLP44* associated BR signaling is also a strategy

adopted by plants in the face of stress to regulate vascular development and regeneration. Previous studies and our results showed that plants have remarkable regenerative abilities, and this helps them adapt to stress conditions. This ability of regeneration is also used by biotic agents and pathogens to make vascular connections with the plants to extract resources and cause substantial yield losses in agriculture. In the last paper I hypothesized that there may be a common mechanism of vascular formation in both biotic and abiotic stresses. I identified a gene *ENHANCER OF VISUAL AND GRAFTING 1* (*EVG1*) which is increased by biotic and abiotic stresses. I and co-authors found that *EVG1* influences development by reducing xylem formation but enhances cellular expansion and cambium formation. Our results show that ABA reduces mRNA levels of *EVG1*. Results also pointed that *EVG1* is present in the outermost layers of the plant and mutations in the gene affected cell wall related genes suggesting that *EVG1* may act as an alarm bell in stress situations. Our results suggest that when abiotic or biotic stress activates *EVG1* in the outermost layers of plant cells, it causes changes in the cell walls, and we propose that these changes act as a signal which then is incorporated by other factors to perhaps mediate developmental changes. To conclude plants, have remarkable machineries to regenerate and modulate the development of their vasculature to adapt to various stresses. Moreover, damages to cell walls also acts as a stimulus for plant adaptation. Both ABA and BR signaling pathways affect plant cell walls and identification of *EVG1* further points to the potential role of cell walls in mediating plant adaptation, vascular development, and regeneration in stress conditions. Future research aims should investigate how cell walls can act as the primary interface in the daily battle of plant survival against stress.

Populärvetenskaplig sammanfattning

Många känner till att växter är fastsittande organismer. Att växter är rotade vid en plats leder till att de regelbundet utsätts för stress-situationer. För att överleva har växter utvecklat flera sätt att anpassa sig till föränderliga förhållanden. Att förstå hur växter anpassar sig har blivit än viktigare i strävan att generera stresstoleranta grödor och därmed öka säkerheten och tryggheten i livsmedelsproduktionen. Med den här avhandlingen har jag bidragit med ökad kunskap om hur växter, genom att påverka sin vaskulära utveckling och regenerering av vävnader, anpassar sig till stress. Jag och mina medförfattare upptäckte att signalering via växthormonet abscisinsyra (ABA), som är den viktigaste hormonella reaktionen på stress, aktiverar gener som styr bildandet av xylem, växters vatten- och närings-vaskulatur. Som resultat förbättras utvecklingen av xylem i rötterna. ABA-signalering ändrar också xylemcellers identitet. Denna adaptiva åtgärd kan tillåta växter att ta upp mer vatten från jorden samt ge skydd mot luftbubblor i rötterna.

Växter utsätts också för sårskador. Skador på cellväggarna aktiverar signalering via brassinosteroid (BR) hormonet, vilket leder till tillväxt och utveckling. Under normala förhållanden påverkar BR-signalering cellexpansion men interagerar antagonistiskt med ABA-signalering. Detta fick oss att undersöka om BR-signalering påverkar vaskulär utveckling och regenerering av vävnad. Resultaten tyder på att BR-signalering krävs för både kambium (förstadiet till vaskulära celler) och xylemutveckling. Eftersom celler inte kan expandera när BR-signalering avbryts, tyder resultaten på att regenerering eller tillväxt av celler för att förhindra skada eller för läkning av sår, också reduceras i växter som har defekter i BR-signaleringen. Jag och mina medförfattare fann att när en gen som kallas *RECEPTOR LIKE PROTEIN 44* (*RLP44*) var funktionell, tillät den BR-

signaleringen att upprätthålla produktion av kambiumceller samt minska deras differentiering till xylem. Dessa fynd tyder på att både allmän BR-signalering och RLP44-associerad BR-signalering utgör strategier som antas av växter vid stress för att reglera vaskulär utveckling och regenerering.

Både våra och tidigare studier visade att växter har anmärkningsvärda förmågor att regenerera celler och vävnader, och att detta hjälper dem att anpassa sig till stressförhållanden. Denna förmåga används också av biotiska angripare, t ex patogener, för att skapa vaskulära förbindelser med växterna för att utvinna resurser vilket orsakar betydande förluster inom jordbruket. Mina erhållna data ledde till hypotesen att en gemensam mekanism för vaskulär bildning vid både biotiska och abiotiska påfrestningar existerar. Jag identifierade en gen, *ENHANCER OF VISUAL AND GRAFTING 1* (*EVG1*), som aktiveras av både biotiska och abiotiska påfrestningar. Jag och mina medförfattare fann att EVG1 påverkar utvecklingen genom att minska bildning av xylem, samt öka cellulär expansion och kambiumbildning. Våra resultat visar att ABA minskar *EVG1*-genens aktivitet. Resultaten pekade också på att *EVG1* är aktiv i växtens yttersta cell-lager och att mutation av *EVG1* påverkade uttrycket av cellväggsrelaterade gener, vilket tyder på att EVG1 potentiellt kan fungera som en larmklocka i stress-situationer. Våra resultat tyder på att när abiotisk eller biotisk stress aktiverar *EVG1* i växtens yttersta cell-lager, orsakar det förändringar i cellväggarna, och vi föreslår att dessa förändringar fungerar som en signal som sedan inkorporeras av andra faktorer för att aktivera utvecklingsförändringar.

För att sammanfatta, växter har anmärkningsvärda förmågor att regenerera och modulera utvecklingen av deras vaskulära system för att anpassa sig till olika påfrestningar. Dessutom fungerar skador på cellväggarna som en stimulans för anpassning. Signalering via både ABA och BR påverkar cellväggarna och identifiering av *EVG1* pekar vidare på cellväggarnas potentiella roll i att förmedla växters anpassning, vaskulära utveckling och regenerering under stressförhållanden. Framtida forskningsmål bör undersöka hur cellväggar kan fungera som det primära gränssnittet i den dagliga kampen om växternas överlevnad mot stress.

Acknowledgements

This PhD would never have been completed if not for the support and help from my supervisors, colleagues, friends, and family. I would like to take a moment to thank all of you for helping me in reaching my goal.

Thank you!

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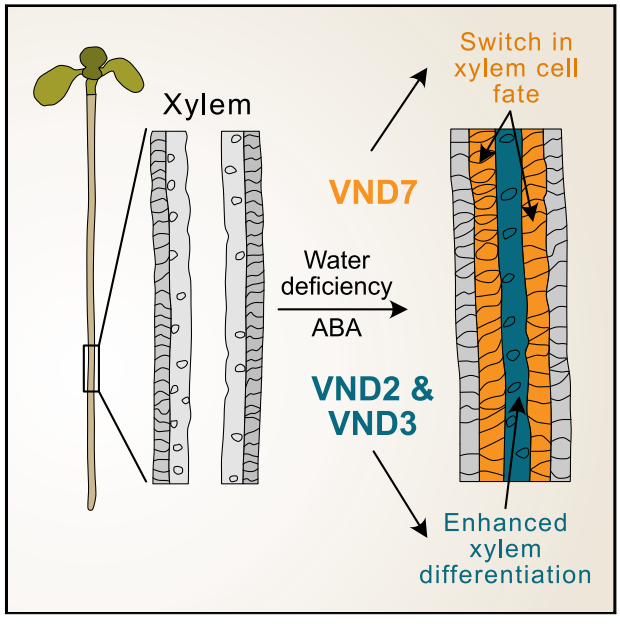
Report

Current Biology

Abscisic acid signaling activates distinct VND transcription factors to promote xylem differentiation in *Arabidopsis*

Graphical abstract



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In brief

Water limitation triggers ABA signaling to alter xylem development in roots. Ramachandran et al. show that ABA promotes both xylem cell fate change and xylem differentiation rate and that these developmental trajectories are mediated by distinct VND transcription factors. This root xylem response to ABA is conserved among eudicots.

Highlights

- Water limitation promotes cell-autonomous ABA signaling to affect xylem development
- ABA promotes both xylem cell fate change and differentiation rate
- These xylem development effects are mediated by distinct VND transcription factors
- Root xylem developmental ABA response is evolutionarily conserved among eudicots

Ramachandran et al., 2021, Current Biology *31*, 3153–3161
July 26, 2021
https://doi.org/10.1016/j.cub.2021.04.057

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Report

Abscisic acid signaling activates distinct VND transcription factors to promote xylem differentiation in *Arabidopsis*

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https://doi.org/10.1016/j.cub.2021.04.057

SUMMARY

Plants display remarkable abilities to adjust growth and development to environmental conditions, such as the amount of available water. This developmental plasticity is apparent not only in root and shoot growth rates, but also in tissue patterning and cell morphology.[1,2] We have previously shown that in response to limited water availability, *Arabidopsis thaliana* root displays changes in xylem morphology, mediated by the non-cell-autonomous action of abscisic acid, ABA.[2] Here, we show, through analyses of ABA response reporters and tissue-specific suppression of ABA signaling, that xylem cells themselves act as primary signaling centers governing both xylem cell fate and xylem differentiation rate, revealing the cell-autonomous control of multiple aspects of xylem development by ABA. ABA rapidly activates the expression of genes encoding VASCULAR-RELATED NAC DOMAIN (VND) transcription factors. Molecular and genetic analyses revealed that the two ABA-mediated xylem developmental changes are regulated by distinct members of this transcription factor family, with VND2 and VND3 promoting differentiation rate of metaxylem cells, while VND7 promotes the conversion of metaxylem toward protoxylem morphology. This phenomenon shows how different aspects of developmental plasticity can be interlinked, yet genetically separable. Moreover, similarities in phenotypic and molecular responses to ABA in diverse species indicate evolutionary conservation of the ABA-xylem development regulatory network among eudicots. Hence, this study gives molecular insights into how environmental stress modifies plant vascular anatomy and has potential relevance for water use optimization and adaptation to drought conditions.

RESULTS AND DISCUSSION

ABA affects both xylem cell fate and differentiation rate

Water-limiting conditions trigger the formation of multiple protoxylem-like cells with spiral secondary cell walls (SCWs) in place of metaxylem with pitted SCWs (Figures 1A–1C, 1E, and S1B).[2,3] This effect is partly dependent on endodermal abscisic acid (ABA) signaling resulting in enhanced levels of microRNA165 (miR165), which acts non-cell-autonomously to suppress target HOMEODOMAIN-LEUCINE ZIPPER class III (HD-ZIPIII) transcription factors within the stele, thus promoting protoxylem over metaxylem cell fate.[4,5] However, whether ABA signaling affects other aspects of xylem development and if it could act cell-autonomously is not clear. To further assess ABA's effect on xylem formation, we analyzed if it could affect xylem differentiation rate by measuring the distance from root tip to point of lignified SCWs detected in wild type (Col-0) after treatment with 1 μM ABA. Previous analyses have shown this treatment to be a good proxy for water-limiting conditions without negative root growth effects (Figure S1A).[2] A 48 h ABA treatment caused cells occupying the outer protoxylem (*px*) position of the xylem axis (Figures 1A and 1B) to differentiate slightly closer to the tip (*px* mock, 1,264 ± 139 μm [SD] versus ABA, 1,060 ± 240 μm), whereas the neighboring outer metaxylem cells (*omx*) differentiated significantly closer to the tip (*omx* mock, 2,950 ± 374 μm versus ABA, 1,912 ± 393 μm; Figures 1F and 1G). However, while *omx* cells normally have pitted SCWs characteristic of metaxylem cells, they frequently formed reticulate or spiral SCW upon ABA treatment, thus becoming protoxylem-like (Figures 1C, 1E, and S1B).[2] Because protoxylem cells normally

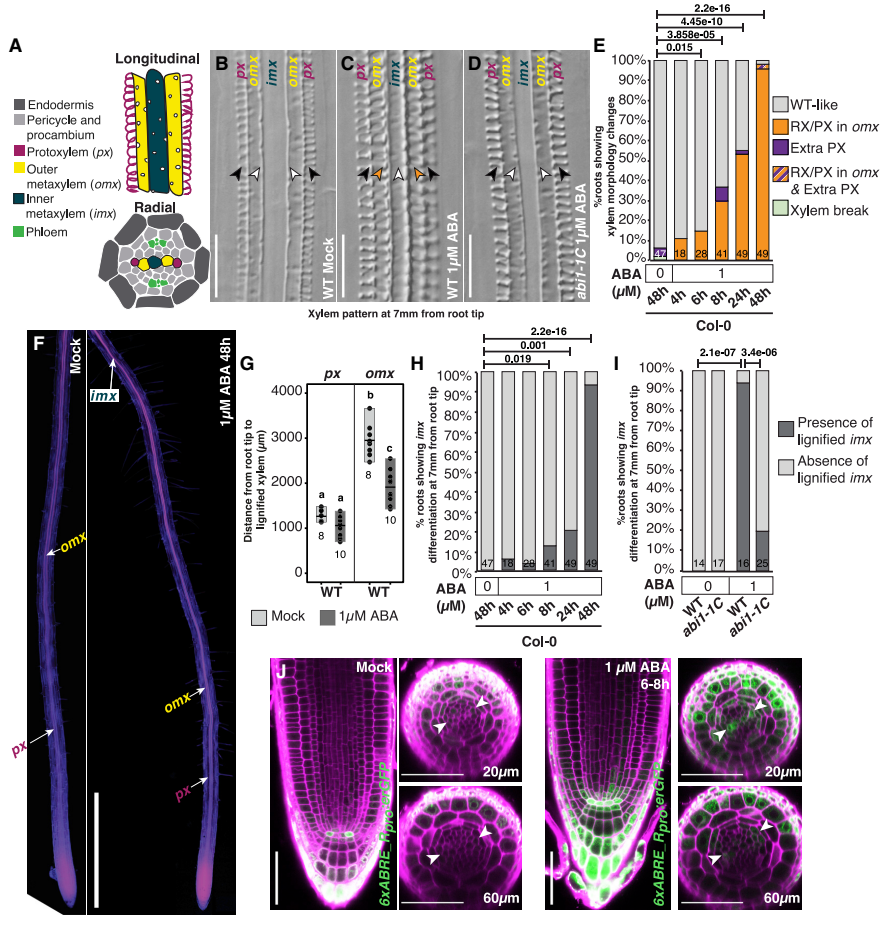

Figure 1. ABA affects both xylem differentiation fate and rate

(A) Cartoon of a longitudinal and cross-section of the *Arabidopsis* root stele (+endodermis in the cross-section) showing different cell types, highlighting the different positions in the xylem axis, *px*, *omx*, and *imx*.

(B–D) Differential interference contrast (DIC) images of the xylem pattern at 7 mm from the root tip after mock and ABA treatment in wild type (WT; B and C) and ABA treatment in *abi1-1C* (D). Differentiated protoxylem vessels are indicated by black arrowheads, metaxylem by white arrowheads, and reticulate xylem by orange arrowheads. *px*, protoxylem position; *omx*, outer metaxylem position; *imx*, inner metaxylem position. Scale bars, 50 μm.

(E) Temporal analysis of xylem morphology changes in WT roots after 1 μM ABA treatment for 4, 6, 8, 24, and 48 h. For the 4, 6, and 8 h ABA treatments, the total treatment time before root xylem analysis was 24 h. RX, reticulate xylem; PX, protoxylem.

(F) Mock- and ABA-treated WT roots double stained with basic fuchsin (magenta) and calcofluor white (blue). Pink, yellow, and blue-green text with white arrows indicate the first occurrence of a fuchsin-stained xylem vessel in the *px*, *omx*, and *imx* positions, respectively. Under mock conditions, differentiated xylem in the *imx* position was detected at a distance of 15–20 mm from the root tip (Figure S3H) and is not included within the imaged region of the root. Scale bar, 1 mm.

(G) Quantification of distances from the root tip to lignified vessel in *px* (left) and *omx* (right) positions. In the *px* position, vessels had spiral SCWs under both mock and ABA treatments, while *omx* vessels had pitted SCWs under mock and pitted, reticulate, or spiral SCWs under ABA treatment. Difference in morphology of *omx* vessels was not considered for the quantification of distances. Black filled dots represent measurements from individual roots.

(H) Quantification of early *imx* differentiation; graph shows presence/absence of lignified *imx* xylem vessel at 7 mm from the root tip, after 1 μM ABA treatment. Treatment times are as in (E).

(I) Frequency of early *imx* differentiation as in (H) in WT and *abi1-1C* after 48 h 1 μM ABA treatment.

(legend continued on next page)

differentiate closer to the root tip, the earlier *omx* differentiation observed may be coupled to cell fate changes. In contrast, the inner metaxylem cells (*imx*) (Figure 1A) never formed reticulate or spiral SCWs upon 1 μM ABA treatment (Figure 1C). In 5-day-old mock-treated seedlings, *imx* differentiated 15–20 mm from the root tip, with 0% showing differentiated *imx* at 7 mm from the root tip (Figure S3H). After 48 h 1 μM ABA treatment, 94% displayed differentiated *imx* at 7 mm from the root tip (Figure 1H), suggesting that ABA promotes metaxylem differentiation rate independent of its effect on xylem morphology. Transverse sections showed that ABA's effect was restricted to the xylem cells (Figure S1C). Furthermore, transferring back to mock conditions restored both xylem morphology and differentiation rate within 48 h (Figures S1D–S1F), further corroborating that xylem formation is highly plastic.

The dominant *ABA-INSENSITIVE1* mutant (*abi1-1*), in which ABA signaling is suppressed even in the presence of ABA,[6,7] strongly reduced the effects of ABA treatment on early *imx* differentiation (20% in *abi1-1* versus 94% in wild type; Figures 1D, 1I, and S1C), and on xylem fate change in *omx* (Figure S1G),[2] showing that canonical ABA signaling is important for xylem differentiation. However, while endodermal ABA signaling repression (using SCR_{pro}:*abi1-1*) significantly suppressed *omx* fate change (Figure S1H),[2] it had less effect on the enhanced differentiation (65% versus 100%; Figure S1I), suggesting that signaling in other cell types contributed to this response. To determine where ABA response occurs, we used synthetic ABA responsive reporters with tandem ABA RESPONSIVE ELEMENT (ABRE) repeats from two ABA responsive genes, *ABI1* and *RAB18* (*6XABRE_A*$_{pro}$*:GFPer* and *6XABRE_R*$_{pro}$*:GFPer*), respectively, which were previously described.[8] While both reporters showed strong QC and lateral root cap expression under mock conditions, optical cross-sections revealed weak expression in epidermis, cortex, endodermis, pericycle, and protoxylem precursor cells, suggesting that ABA signaling occurs in these tissues under non-stressed conditions (Figures 1J and S1J). After 6–8 h treatment with 1 μM ABA, signal intensity of both reporters increased in these tissues, and within the stele the xylem precursor cells displayed an ABA response maximum (Figures 1J and S1K). Next, we simulated water deficiency by growing plants on polyethylene glycol (PEG) overlaid media[2] (Figure S1L). This resulted in similar but stronger ABA response suggesting that exogenous ABA treatment could recapitulate cell-specific ABA responses occurring during water deprivation.

ABA signaling within the xylem activates VND transcription factors

The ABA response profile prompted us to investigate the importance of ABA signaling in different tissues for xylem differentiation. We analyzed F1 progeny of UAS_{pro}:*abi1-1*[9] crossed with enhancer trap lines J1721, expressing in the xylem axis, QC, and columella; Q0990, procambium; or J0571, ground tissue, upon ABA treatment (Figures 2A and S2A). Similar to its effect on wild type, root growth of the transactivation lines was not negatively affected by ABA treatment (Figure S2D). Strikingly, the *J1721>>abi1-1* line efficiently suppressed ABA's effects on both xylem differentiation rate and fate (Figures 2B, 2C, and S2B). Neither *Q0990>>abi1-1* nor *J0571>>abi1-1* could suppress xylem differentiation rate, but consistent with our previous observations,[2] *J0571>>abi1-1* partially suppressed xylem fate changes (Figures 2B, 2C, and S2B). Furthermore, while mock-treated *J0571>>abi1-1* occasionally displayed discontinuous metaxylem,[2] this was not detected in either of the stele-active lines (Figures S2B and S2C). These results suggest that ABA signaling in the stele is not critical for xylem formation per se, but that signaling within the xylem cells is essential to determine both xylem differentiation rate and xylem cell fate upon conditions causing elevated ABA levels.

To identify the genetic regulators involved in stress-mediated xylem developmental changes, we performed RNA sequencing (RNA-seq) of 8 h ABA-treated Col-0 roots and identified 2,368 genes upregulated by ABA ($\log_2$ FC > 0.5; $p_{adj} < 0.05$; Figure 2D; Data S1A). Of these, 114 were identified as xylem expressed by comparing with xylem-enriched genes from a single-cell RNA-seq study[10] (Figure 2D; Data S1A). ABA-responding xylem genes included *CELLULOSE SYNTHASEA4* (*CESA4*), *CESA7*, *CESA8*, *LACCASE11* (*LAC11*), *LAC17*, *XYLEM CYSTEINE PEPTIDASE1* (*XCP1*), and *XCP2*, as well as genes encoding transcription factors *MYB46*, *MYB83*, *VND2*, *VND3*, and *VND7* that act upstream of many SCW biosynthesis genes[11–15] (Data S1A; Figure 3F). In line with ABA signaling acting within the xylem cells, RNA-seq of ABA-treated *J1721>>abi1-1* revealed a reduced activation of a subset of these genes, including the *CESA*s, *LAC17*, *MYB46*, *MYB83*, and *VND3* (Figure 2D; Data S1C; $p < 0.05$).

Independent qRT-PCR analyses on ABA-treated wild-type root tips were consistent with the RNA-seq (Figure 2F), and additionally showed that 2 h treatment was sufficient to significantly upregulate not only *VND1*, *VND2*, *VND3*, and *VND7*, but also *VND4*. Longer treatment times induced *VND5*, whereas *VND6*, a regulator of metaxylem differentiation,[12] was not upregulated. A direct influence of ABA specifically on *VND1*, *2*, *3*, and *7* is supported by promoter binding of ABRE BINDING FACTORS (ABFs) and other ABA-related transcription factors[16] (Table S1). Furthermore, independent qRT-PCR testing of ABA's effect on *abi1-1* transactivation lines showed a significant suppression of *VND2* activation by *J1721>>abi1-1* with *VND3* displaying a similar trend (Figures 2E and S2E). Transcriptional reporter lines[12] revealed distinct expression patterns for *VND1*, *VND2*, and *VND3* in immature xylem cells within the meristem, with *VND1* restricted to *omx* cells, *VND2* to all metaxylem precursor cells (*omx* and *imx*), while *VND3* expression was observed in *px*, *omx*, and *imx* cells (Figure 2G). *VND3* expression extended into the differentiation zone, while *VND1* and *VND2* were restricted to the meristem (Figures 2G and S2F). *VND7*

(J) Confocal micrographs of the ABA response domains in the root apical meristem visualized using *6xABRE_R*$_{pro}$*:erGFP* after mock or 1 μM ABA treatment. Radial optical sections were captured at 20 μm and 60 μm shootward of the quiescent center (QC). Magenta, propidium iodide; green, GFP. White arrowheads indicate the xylem axis. Scale bars, 50 μm.

Statistics in (E), (H), and (I): values above the bar represent p values from Fisher's exact test, incorporating all phenotype categories. In (G), *a*,*b*,*c* represent groups with significant differences, one-way ANOVA with Tukey's post hoc testing ($p < 0.05$). Numbers at the bottom of the bars in (E) and (G)–(I) represent number of roots analyzed. See also Figure S1 and Data S2.

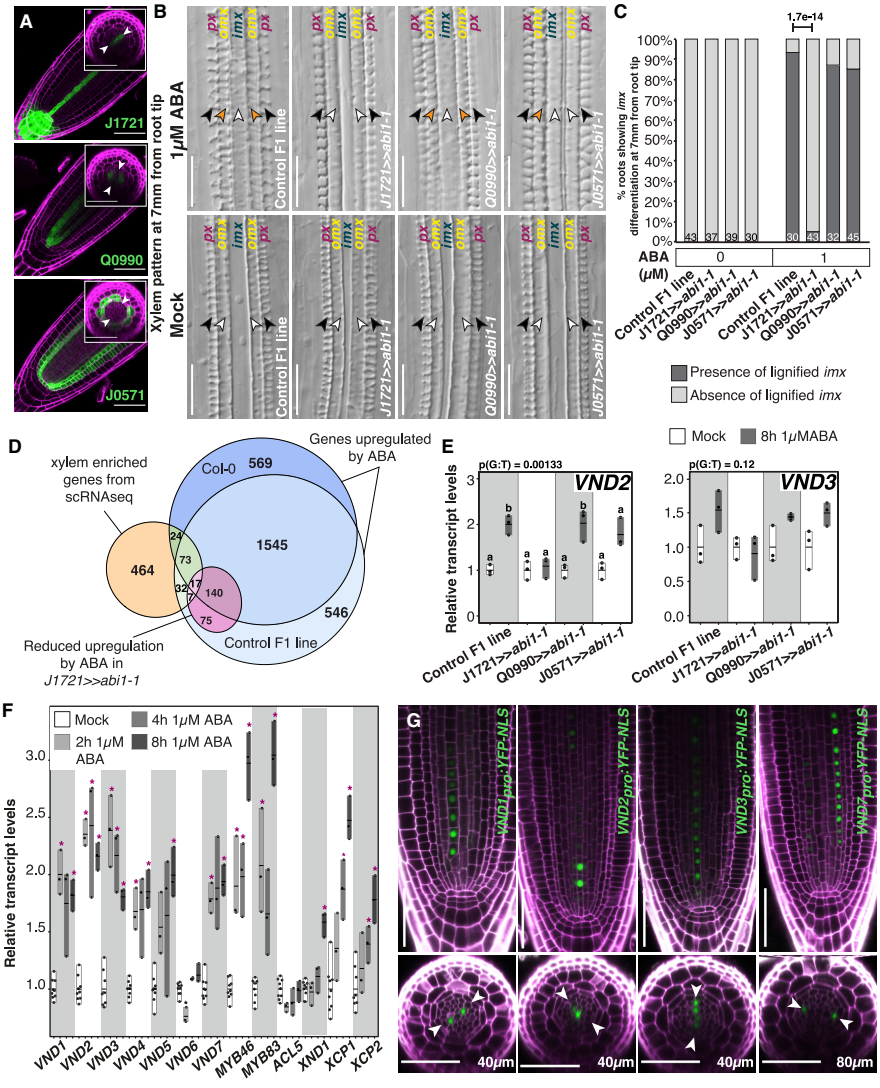

Figure 2. ABA signaling within the xylem activates VND transcription factors

(A) Confocal micrographs representing the activity domains of the J0571, Q0990, and J1721 GAL4 enhancer trap lines (green) imaged in F1 plants resulting from crosses with UAS_{pro}:*abi1-1*. Insets show radial optical sections of each enhancer trap line; arrowheads mark the xylem axis.

(B) DIC images of the xylem pattern in 48 h 1 μM ABA- and mock-treated *abi1-1* transactivation lines. Control F1 is UAS_{pro}:*abi1-1* (Col-0) X C24. Differentiated protoxylem vessels are indicated by black arrowheads, metaxylem by white arrowheads, and reticulate xylem by orange arrowheads. *px*, protoxylem position, *omx*, outer metaxylem position; *imx*, inner metaxylem position.

(C) Quantification of early *imx* differentiation after mock and 1 μM ABA treatment in different *abi1-1* transactivation lines, as measured by presence/absence of lignified xylem at 7 mm from the root tip. Values above the bar represent p values from Fisher's exact test incorporating all phenotype categories. Numbers at the bottom of the bars indicate number of roots analyzed.

(D) Venn diagram showing the overlap of genes upregulated by ABA in Col-0 and in control F1 with genes enriched in xylem expression according to single-cell RNA-seq.[10] A subset of these genes (pink) showed reduced upregulation in lines where ABA signaling was suppressed by *J1721>>abi1-1*, $p < 0.05$.

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expressed specifically in protoxylem precursors within the meristem, but extended into differentiating protoxylem cells (Figures 2G and S2J),[11] while *VND5* was only detected in differentiating protoxylem strands with the close paralog *VND4* displaying a similar pattern (Figure S2F).[12] Thus, only *VND1*, *VND2*, *VND3*, and *VND7* act in early xylem development and potentially directly downstream of ABA signaling. We, therefore, focused primarily on these factors, and analyzed the effect of 6–8 h 1 μM ABA treatment on the reporters for these genes. While expression levels increased, none of the reporters displayed obvious expression pattern changes (Figures S2G–S2J). However, after treatment with higher concentration of ABA, *VND7* has been reported to expand into the *omx* cell lineage within the meristem.[3] Taken together, these data show that *VND* expression levels rapidly and specifically increase within the xylem precursor cells upon increased ABA levels.

VNDs regulate plasticity in xylem fate and xylem differentiation rate

To test if VND transcription factors are required for the ABA-induced xylem developmental changes, we analyzed *vnd* mutants after ABA treatment and growth under water-limiting conditions. Single and most double mutants of *vnd1*, *vnd2*, *vnd3*, and *vnd7* displayed wild-type-like xylem patterns (Figure S3A). However, *vnd2vnd3* (*vnd2,3*) and *vnd1vnd2vnd3* (*vnd1,2,3*) had discontinuous metaxylem strands (Figures 3A and 3B), and in *vnd1,2,3*, metaxylem strands in *omx* and *imx* positions frequently failed to differentiate (Figures 3B and 3D). Upon ABA treatment, *vnd2,3* and *vnd1,2,3* did not show the early *imx* differentiation that occurs in wild-type plants (Figures 3A, 3C, S3B, S3C, and S3H). Importantly, the same effect was detected upon growth on water-limiting conditions, although root growth inhibition occurred in these mutants similarly to wild type (Figures 3D, 3E, S3E, and S3F). Hence, VND2 and VND3 are required to promote early *imx* differentiation upon enhanced ABA signaling and under water-limiting conditions.

Despite a role in *imx* differentiation rate, *vnd2,3* and *vnd1,2,3* displayed early *omx* differentiation and protoxylem-like or reticulate *omx* morphology upon ABA treatment (Figures 3A, 3B, and S3G). In contrast, the cell fate change was suppressed in *vnd7* (Figures 3A and 3B), while it displayed early *omx* and *imx* differentiation (Figures 3C, S3G, and S3H). Hence, ABA treatment of *vnd7* revealed a previously uncharacterized requirement for VND7 in xylem cell fate change from metaxylem toward protoxylem-like cells. Furthermore, these data show that ABA's effect on xylem differentiation rate and xylem cell fate change can be genetically separated via the activation of distinct VND genes.

To further dissect how the VNDs regulate xylem developmental plasticity, we analyzed the transcriptomic effects of ABA treatment in *vnd1,2,3* and *vnd7* (Data S1A; Figure S3I). Under mock conditions, 53 xylem-enriched genes were significantly reduced in the *vnd1,2,3* mutant ($log_2FC < 0.5$; $p_{adj} < 0.05$; Data S1B). Consistent with the wild-type-like phenotype of *vnd7*, only three xylem-enriched genes were significantly reduced in this background. Under ABA induction, *vnd1,2,3* could significantly reduce the upregulation of 21 xylem-expressed genes including the two *XCP* genes, *CESA4*, *LAC11*, and *17*, but not *MYB46* and *MYB83* (Data S1A), suggesting that these factors are regulated by ABA independently of VND1, 2, and 3. This finding is further supported by ABF binding to the promoter of *MYB46* (Table S1).[16] *vnd7* had little effect on genes induced by ABA, with only *LAC11* significantly reduced among the xylem-expressed genes (Data S1A). Taken together, these data suggest that while VND1, 2, and 3 are required for normal expression of many xylem differentiation genes, additional factors act redundantly with the VNDs to further promote xylem gene expression upon rising ABA levels.

As *vnd7* could suppress *omx* cell fate change but did not affect differentiation rate upon ABA, we reasoned that VND7 might act redundantly with VND1, 2, and 3 to regulate this trait, and we therefore generated the *vnd1vnd2vnd3vnd7* mutant. Here, both the ABA-induced *omx* fate change and the premature *imx* differentiation were suppressed (Figures 3G and S3J), showing the additivity of the two phenotypes assigned to *vnd7* and *vnd123*, respectively. However, although *omx* cells maintained metaxylem morphology, they could still respond to ABA with faster differentiation, similar to wild type (Figures 3G and 3H). Hence, factors other than VND1, VND2, VND3, and VND7 govern the early *omx* differentiation induced by high ABA levels. Our transcriptome datasets indicate that MYB46 and MYB83 are potential candidates for this role.

ABA promotes xylem differentiation in several eudicot species

Overexpression of VND transcription factors induced the formation of ectopic xylem tracheary element cells.[12,15] Since ABA had a positive effect on the expression of a number of xylem differentiation genes including the VNDs, we tested ABA's capacity to induce trans-differentiation of cotyledon mesophyll into xylem cells, as previously seen upon treatment with auxin and cytokinin along with bikinin (an inhibitor of GSK3 kinases involved in brassinosteriod signaling).[17] Strikingly, substitution of bikinin for ABA resulted in ectopic lignification, although cells did not form a typical xylem SCW pattern (Figures 4A and 4B). The ectopic lignification was nonetheless suppressed both in *abi1-1* and in *vnd1,2,3* and *vnd7* mutants (Figures S4A–S4C), suggesting that the ectopic lignification is a specific effect of the ABA treatment and that VND1, 2, 3, and 7 regulate this effect.

(E) Relative transcript levels of *VND2* and *VND3* after 8 h ABA treatment in whole roots of F1 seedlings from crosses between UAS_{pro}:*abi1-1* and indicated GAL4 enhancer trap lines, using qRT-PCR. The significance of genotype:treatment (G:T) interaction on gene expression based on a two-way ANOVA analysis is given above the plots. Letters *a* and *b* represent groups with significant differences with Tukey's post hoc testing ($p < 0.05$).
(F) qRT-PCR quantification of xylem developmental gene transcript levels in 1 mm WT root tips after 2, 4, and 8 h of 1 μM ABA treatment. *$p < 0.05$, two-tailed Student's t test.
In (E) and (F), all values are normalized to the average of respective mock-treated samples.
(G) Confocal images of *VND1*, *VND2*, *VND3*, and *VND7* promoter activity domains in root meristem longitudinal and radial planes. Distances from QC where the radial images were captured are indicated in the images. White arrowheads indicate xylem axis.
Scale bars in (A), (B), and (G), 50 μm. See also Figure S2 and Data S1 and S2.

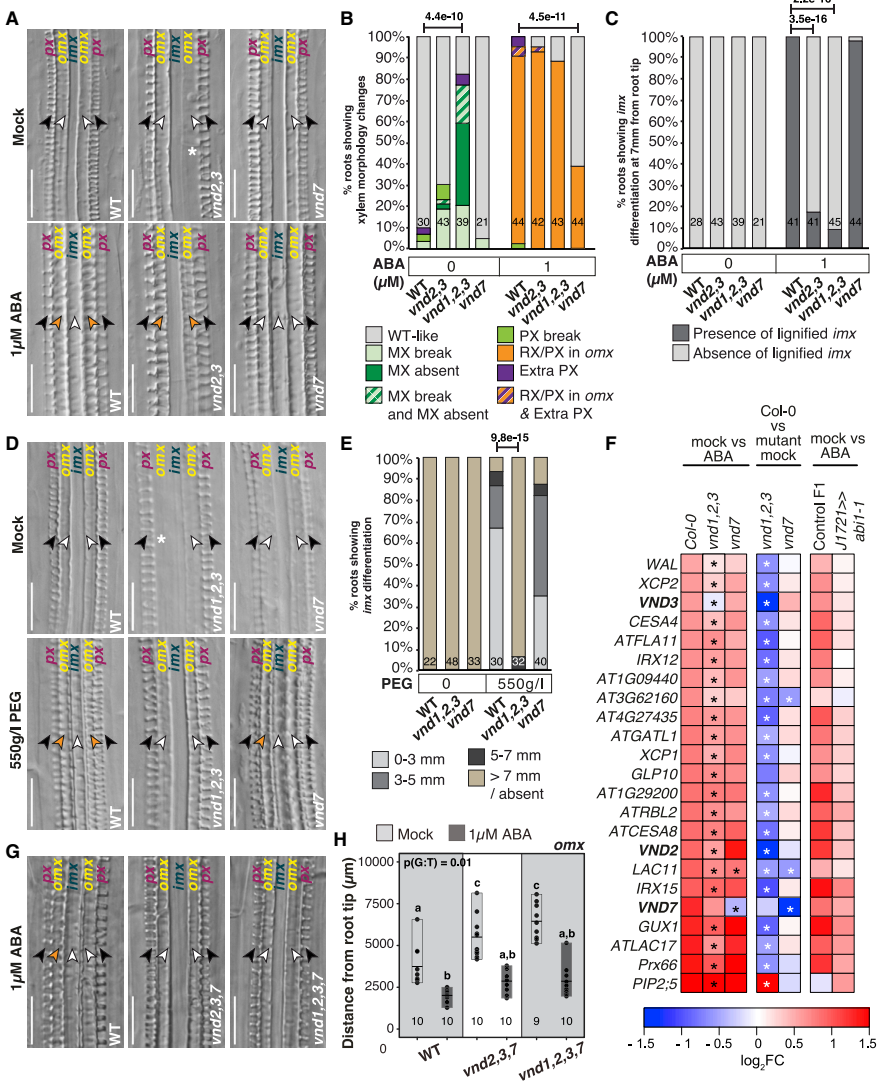

Figure 3. VNDs regulate plasticity of xylem fate and differentiation rate

(A) Representative DIC images of mock- and ABA-treated wild type (WT), *vnd2 vnd3* (*vnd2,3*), and *vnd7* roots at 7 mm from the root tip. In (A), (D), and (G), *px*, protoxylem position; *omx*, outer metaxylem position; *imx*, inner metaxylem position. Differentiated protoxylem vessels are indicated by black arrowheads, metaxylem by white arrowheads, and reticulate xylem by orange arrowheads. Asterisk (*) indicates xylem break. Scale bars, 50 μm.

(B and C) Quantification of xylem morphology (B) and *imx* differentiation at 7 mm from the root tip (C) in *vnd2,3*, *vnd1 vnd2 vnd3* (*vnd1,2,3*), and *vnd7*.

(D) Representative DIC images of mock- and polyethylene glycol (PEG)-treated WT, *vnd1,2,3*, and *vnd7* roots.

(E) Quantification of distances at which differentiated *imx* was detected in WT, *vnd1,2,3*, and *vnd7* roots subjected to mock or PEG treatments.

(F) Heatmap of xylem-enriched genes upregulated by ABA in WT ($log_2FC > 0.5$, $p_{adj} < 0.05$) and with a reduced activation by ABA in *vnd1,2,3* or *vnd7* and their pattern in *J1721>>abi1-1* lines. Black asterisks indicate significantly reduced activation upon ABA in mutants compared to WT ($p_{adj} < 0.05$); white asterisks indicate downregulation in mutants compared to WT under mock conditions.

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To examine if xylem responses upon stress are a conserved trait, we analyzed root xylem upon ABA treatment in five different eudicot species (Figures 4C–4E and S4D–S4F). This revealed that *Brassica napus* and *Brassica rapa* (Brassicales, Rosidae), *Nicotiana benthamiana* and *Solanum lycopersicum* (Solanales, Asteridae), and *Phtheirospermum japonicum* (Lamiales, Asteridae) all displayed early xylem differentiation and a higher number of xylem strands compared to mock condition, similar to *Arabidopsis* (Figures 4C–4E and S4D–S4F). Consistent with our observation, an effect of ABA on root xylem development in tomato was previously found.[3] We observed a 10-fold upregulation of the putative tomato *VND1*, *VND2*, and *VND3* ortholog (*Solyc02 g083450*) and a 4-fold upregulation of the *VND4-VND5* ortholog (*Solyco08 g079120*) after 6 h of 1 μM ABA treatment (Figure 4F). The ABA treatment had a small positive effect on one of the two *VND6* orthologs and no significant effect on tomato's two *VND7* orthologs. These results suggest at least a partial conservation in molecular and phenotypic responses to ABA among eudicots.

Taken together, here we provide insights into the molecular regulation underlying xylem developmental plasticity in *Arabidopsis*. We show that ABA signaling in the xylem precursors triggers alterations in xylem cell developmental trajectories, affecting both fate and rate of differentiation, through the activation of distinct xylem-expressed transcriptional regulators belonging to the VND gene family (Figure 4G). However, ABA also acts non-cell-autonomously via miR165 activation in the endodermis, reducing levels of HD-ZIPIII transcription factors in the stele (Figure 4G).[2,3] Intriguingly, both pathways appear important for xylem cell fate determination. While gene regulatory network studies have uncovered a complex interplay between VND and HD-ZIPIII transcription factors,[18] it remains unclear how these factors temporally interact within the pluripotent xylem precursor cells to determine xylem cell fate, under normal growth conditions and during stress.

The two distinct phenotypic changes observed under ABA treatment and water-limiting conditions may contribute two distinct advantages to the plant. A change toward more protoxylem-like cells may reduce risk of detrimental effects of air bubbles, embolisms, interrupting water transport. This is because protoxylem strands are thinner, but also may enhance lateral water movement between xylem strands for embolism repair.[19] Early metaxylem formation resulting in increased xylem area, on the other hand, may enhance hydraulic conductance and increase drought resistance.[20] Furthermore, a recent study described a maize mutant defective in a VND homolog that displayed symptoms of water stress under normal conditions due to defective protoxylem cells in adult plants.[21] This suggests that VND-dependent xylem cell acclimation to stress is a trait that evolved prior to the divergence of monocots and eudicots. Thus, ABA-VND regulation may be a potentially universal molecular toolkit for xylem cell developmental adjustments with utility for breeding of drought-resilient crop plants.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2021.04.057.

ACKNOWLEDGMENTS

We thank J.R. Dinneny, Stanford University; T. Demura, NAIST; and Nottingham *Arabidopsis* Stock Centre for materials; C. Musseau for tomato VND identifications; and M. Englund for technical assistance. We acknowledge support from the Nilsson Ehle Foundation, Lars Hiertas Minne, Lundell PO scholarship (P.R.), a Wallenberg Academy Fellowship (KAW2016.0274, C.W.M.), Vetenskapsrådet (2017-05122, C.W.M. and S.M.), and Formas (2017-00857, A.C.).

AUTHOR CONTRIBUTIONS

Conceptualization, P.R., A.C., and F.A.; Investigation, P.R., F.A., S.M., and T.V.N.; Writing – Original Draft, P.R.; Writing – Review & Editing, P.R., A.C., C.W.M., and F.A.; Funding Acquisition, P.R., A.C., and C.W.M.; Supervision, A.C., E.A.M., and C.W.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: October 5, 2020
Revised: February 3, 2021
Accepted: April 22, 2021
Published: May 26, 2021

REFERENCES

1. Finkelstein, R. (2013). Abscisic acid synthesis and response. Arabidopsis Book *11*, e0166.

(G) DIC images showing xylem pattern in WT, *vnd2,3,7*, and *vnd1 vnd2 vnd3 vnd7* (*vnd1,2,3,7*) after 1 μM ABA treatment.
(H) Quantification of distances from the root tip to *omx* for WT, *vnd2 vnd3 vnd7* (*vnd2,3,7*), and *vnd1,2,3,7*.
Statistics: in (B), (C), and (E), values above the bar represent p values from Fisher's exact test, incorporating all phenotype categories; in (H), the significance of genotype:treatment (G:T) interaction on gene expression based on two-way ANOVA analysis is given in the plot. *a,b,c* represent groups with significant differences with Tukey's post hoc testing ($p < 0.05$). Numbers at the bottom of the bars in (B), (C), (E), and (H) represent the number of roots analyzed. See also Figure S3 and Data S1 and S2.

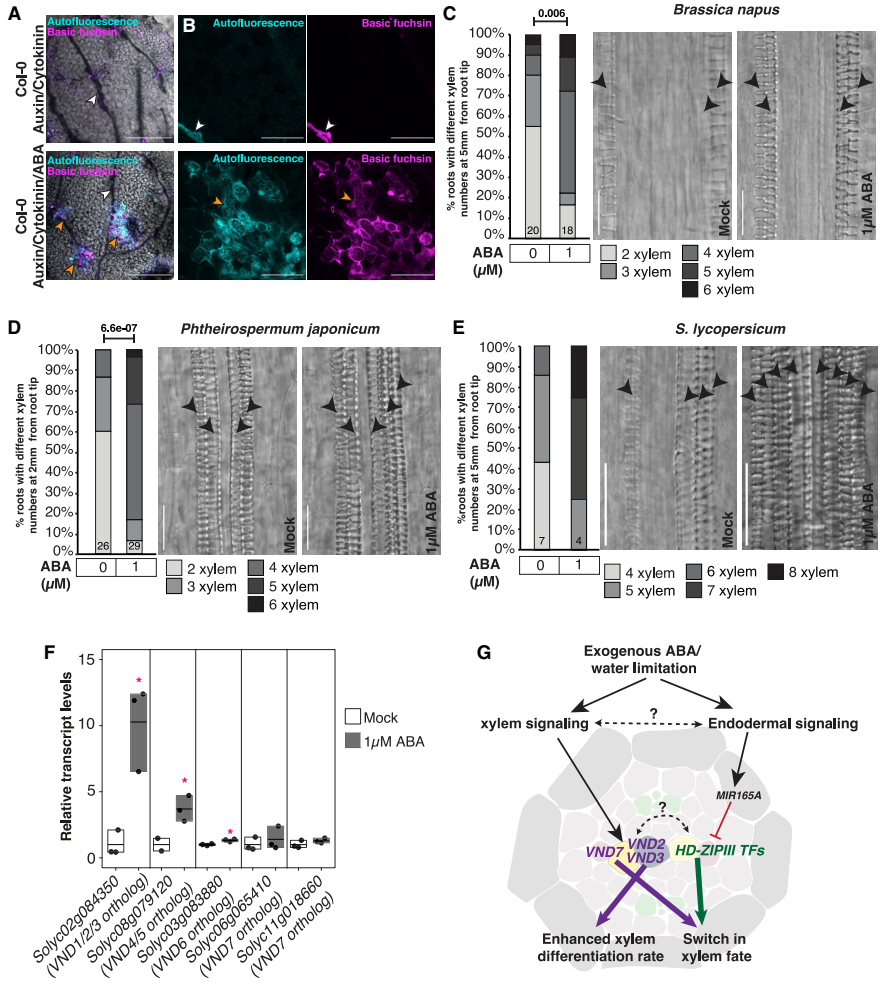

Figure 4. ABA induces ectopic lignification in *Arabidopsis* cotyledons and promotes xylem differentiation in several eudicot species

(A and B) Confocal micrographs showing the formation of ectopic lignification in wild-type (WT) *in vitro* culture in auxin-cytokinin-containing media with or without ABA. Ectopic lignification is visualized using lignin autofluorescence and basic fuchsin staining. Images in (B) show zoomed-in regions of ectopic lignification. White arrowheads indicate cotyledon venation and orange arrowheads indicate ectopic lignin deposition. Scale bars, 500 μm (A) and 100 μm (B).

(C–E) Quantification of total number of lignified xylem vessels at specific distances from the root tip in *Brassica napus* (C), *Phtheirospermum japonicum* (D), and *Solanum lycopersicum* (cv. Money Maker) (E) after mock and 1 μM ABA treatment accompanied by representative images. Black arrowheads indicate xylem strands. *$p < 0.05$ (C and D), Fisher's exact test incorporating all phenotype categories. Numbers at the bottom of the bars represent the number of individuals analyzed. Scale bars, 50 μm.

(F) qRT-PCR of *VND* homologs in tomato roots after 1 μM ABA treatment for 6 h. *$p < 0.05$, two-tailed Student's t test.

(G) Model showing genetic components regulated by ABA to mediate two different phenotypic effects. ABA signaling in the stele activates *VND2*, *VND3*, and *VND7*. While *VND2* and *VND3* are mainly involved in ABA-mediated enhancement of xylem differentiation rate, *VND7* mediates the switch in xylem morphology from pitted to a spiral or reticulate form. In the endodermis, ABA signaling activates miR165, which moves to downregulate stele-expressed HD-ZIPIII transcription factors resulting in altered xylem fate.[2,3]

See also Figure S4.

2. Ramachandran, P., Wang, G., Augstein, F., de Vries, J., and Carlsbecker, A. (2018). Continuous root xylem formation and vascular acclimation to water deficit involves endodermal ABA signalling via miR165. Development *145*, dev159202.
3. Bloch, D., Puli, M.R., Mosquna, A., and Yalovsky, S. (2019). Abiotic stress modulates root patterning via ABA-regulated microRNA expression in the endodermis initials. Development *146*, dev177097, dev29.
4. Carlsbecker, A., Lee, J.-Y., Roberts, C.J., Dettmer, J., Lehesranta, S., Zhou, J., Lindgren, O., Moreno-Risueno, M.A., Vatén, A., Thitamadee, S., et al. (2010). Cell signalling by microRNA165/6 directs gene dose-dependent root cell fate. Nature *465*, 316–321.
5. Miyashima, S., Koi, S., Hashimoto, T., and Nakajima, K. (2011). Non-cell-autonomous microRNA165 acts in a dose-dependent manner to regulate multiple differentiation status in the Arabidopsis root. Development *138*, 2303–2313.
6. Leung, J., Bouvier-Durand, M., Morris, P.C., Guerrier, D., Chefdor, F., and Giraudat, J. (1994). Arabidopsis ABA response gene ABI1: features of a calcium-modulated protein phosphatase. Science *264*, 1448–1452.
7. Meyer, K., Leube, M.P., and Grill, E. (1994). A protein phosphatase 2C involved in ABA signal transduction in Arabidopsis thaliana. Science *264*, 1452–1455.
8. Wu, R., Duan, L., Pruneda-Paz, J.L., Oh, D.-H., Pound, M., Kay, S., and Dinneny, J.R. (2018). The 6xABRE synthetic promoter enables the spatio-temporal analysis of ABA-mediated transcriptional regulation. Plant Physiol. *177*, 1650–1665.
9. Duan, L., Dietrich, D., Ng, C.H., Chan, P.M.Y., Bhalerao, R., Bennett, M.J., and Dinneny, J.R. (2013). Endodermal ABA signaling promotes lateral root quiescence during salt stress in Arabidopsis seedlings. Plant Cell *25*, 324–341.
10. Denyer, T., Ma, X., Klesen, S., Scacchi, E., Nieselt, K., and Timmermans, M.C.P. (2019). Spatiotemporal developmental trajectories in the Arabidopsis root revealed using high-throughput single-cell RNA sequencing. Dev. Cell *48*, 840–852.e5.
11. Zhong, R., and Ye, Z.-H. (2012). MYB46 and MYB83 bind to the SMRE sites and directly activate a suite of transcription factors and secondary wall biosynthetic genes. Plant Cell Physiol. *53*, 368–380.
12. Kubo, M., Udagawa, M., Nishikubo, N., Horiguchi, G., Yamaguchi, M., Ito, J., Mimura, T., Fukuda, H., and Demura, T. (2005). Transcription switches for protoxylem and metaxylem vessel formation. Genes Dev. *19*, 1855–1860.
13. Endo, H., Yamaguchi, M., Tamura, T., Nakano, Y., Nishikubo, N., Yoneda, A., Kato, K., Kubo, M., Kajita, S., Katayama, Y., et al. (2015). Multiple classes of transcription factors regulate the expression of VASCULAR-RELATED NAC-DOMAIN7, a master switch of xylem vessel differentiation. Plant Cell Physiol. *56*, 242–254.
14. Yamaguchi, M., Mitsuda, N., Ohtani, M., Ohme-Takagi, M., Kato, K., and Demura, T. (2011). VASCULAR-RELATED NAC-DOMAIN7 directly regulates the expression of a broad range of genes for xylem vessel formation. Plant J. *66*, 579–590.
15. Zhou, J., Zhong, R., and Ye, Z.-H. (2014). Arabidopsis NAC domain proteins, VND1 to VND5, are transcriptional regulators of secondary wall biosynthesis in vessels. PLoS ONE *9*, e105726.
16. Song, L., Huang, S.C., Wise, A., Castanon, R., Nery, J.R., Chen, H., Watanabe, M., Thomas, J., Bar-Joseph, Z., and Ecker, J.R. (2016). A transcription factor hierarchy defines an environmental stress response network. Science *354*, aag1550.
17. Kondo, Y., Fujita, T., Sugiyama, M., and Fukuda, H. (2015). A novel system for xylem cell differentiation in Arabidopsis thaliana. Mol. Plant *8*, 612–621.
18. Taylor-Teeples, M., Lin, L., de Lucas, M., Turco, G., Toal, T.W., Gaudinier, A., Young, N.F., Trabucco, G.M., Veling, M.T., Lamothe, R., et al. (2015). An Arabidopsis gene regulatory network for secondary cell wall synthesis. Nature *517*, 571–575.
19. Hwang, B.G., Ryu, J., and Lee, S.J. (2016). Vulnerability of protoxylem and metaxylem vessels to embolisms and radial refilling in a vascular bundle of maize leaves. Front. Plant Sci. *7*, 941.
20. Tang, N., Shahzad, Z., Lonjon, F., Loudet, O., Vailleau, F., and Maurel, C. (2018). Natural variation at XND1 impacts root hydraulics and trade-off for stress responses in Arabidopsis. Nat. Commun. *9*, 3884.
21. Dong, Z., Xu, Z., Xu, L., Galli, M., Gallavotti, A., Dooner, H.K., and Chuck, G. (2020). *Necrotic upper tips1* mimics heat and drought stress and encodes a protoxylem-specific transcription factor in maize. Proc. Natl. Acad. Sci. USA *117*, 20908–20919.
22. Regner, F., da Câmara Machado, A., da Câmara Machado, M.L., Steinkellner, H., Mattanovich, D., Hanzer, V., Weiss, H., and Katinger, H. (1992). Coat protein mediated resistance to plum pox virus in Nicotiana clevelandii and N. benthamiana. Plant Cell Rep. *11*, 30–33.
23. Ishida, J.K., Yoshida, S., Ito, M., Namba, S., and Shirasu, K. (2011). Agrobacterium rhizogenes-mediated transformation of the parasitic plant Phtheirospermum japonicum. PLoS ONE *6*, e25802.
24. Verslues, P.E., and Bray, E.A. (2006). Role of abscisic acid (ABA) and Arabidopsis thaliana ABA-insensitive loci in low water potential-induced ABA and proline accumulation. J. Exp. Bot. *57*, 201–212.
25. Ursache, R., Andersen, T.G., Marhavý, P., and Geldner, N. (2018). A protocol for combining fluorescent proteins with histological stains for diverse cell wall components. Plant J. *93*, 399–412.
26. Haseloff, J. (1999). GFP variants for multispectral imaging of living cells. Methods Cell Biol. *58*, 139–151.
27. R Development Core Team (2008). R: a language and environment for statistical computing (R Foundation for Statistical Computing).
28. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. Nat. Methods *9*, 676–682.
29. Huber, W., Carey, V.J., Gentleman, R., Anders, S., Carlson, M., Carvalho, B.S., Bravo, H.C., Davis, S., Gatto, L., Girke, T., et al. (2015). Orchestrating high-throughput genomic analysis with Bioconductor. Nat. Methods *12*, 115–121.
30. Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and biol. assays with tobacco tissue cultures. Physiol. Plant. *15*, 473–497.
31. Kondo, Y., Nurani, A.M., Saito, C., Ichihashi, Y., Saito, M., Yamazaki, K., Mitsuda, N., Ohme-Takagi, M., and Fukuda, H. (2016). Vascular cell induction culture system using Arabidopsis leaves (VISUAL) reveals the sequential differentiation of sieve element-like cells. Plant Cell *28*, 1250–1262.
32. Gutierrez, L., Mauriat, M., Guénin, S., Pelloux, J., Lefebvre, J.-F., Louvet, R., Rusterucci, C., Moritz, T., Guerineau, F., Bellini, C., and Van Wuytswinkel, O. (2008). The lack of a systematic validation of reference genes: a serious pitfall undervalued in reverse transcription-polymerase chain reaction (RT-PCR) analysis in plants. Plant Biotechnol. J. *6*, 609–618.
33. Czechowski, T., Stitt, M., Altmann, T., Udvardi, M.K., and Scheible, W.-R. (2005). Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant Physiol. *139*, 5–17.
34. Løvdal, T., and Lillo, C. (2009). Reference gene selection for quantitative real-time PCR normalization in tomato subjected to nitrogen, cold, and light stress. Anal. Biochem. *387*, 238–242.
35. Dekkers, B.J.W., Willems, L., Bassel, G.W., van Bolderen-Veldkamp, R.P., Ligterink, W., Hilhorst, H.W., and Bentsink, L. (2012). Identification of reference genes for RT-qPCR expression analysis in Arabidopsis and tomato seeds. Plant Cell Physiol. *53*, 28–37.
36. Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. *15*, 550.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Murashige and Skoog Medium (MS)	Duchefa Biochemie	Cat#M0222.0050
MES monohydrate	Duchefa Biochemie	Cat#M1503.0250
Bactoagar	Swab	Cat#B1000-1
Abscisic acid (ABA)	Sigma	Cat#14375-45-2
Polyethylene glycol 8000	Sigma	Cat#89510
Chloralhydrate	Sigma	Cat#15307
Urea	Sigma	Cat#57-13-6
Sodium deoxycholate	Sigma	Cat#1065040250
Xylitol	Sigma	Cat#X3375
Propidium iodide	Sigma	Cat#P4170
2,4-D	Sigma-Aldrich (Merck)	D70724-5G
Kinetin	Sigma-Aldrich (Merck)	K3378-1G
Critical commercial assays		
RNeasy Plant Mini Kit	QIAGEN	Cat#74904
iSCRIPT cDNA synthesis kit	Biorad	Cat#1708891
iQ SYBR Green Supermix	Biorad	Cat#17088882
Qubit BR RNA Assay	Invitrogen	Cat#Q10211
Deposited data		
Raw and processed RNaseq data files	This study	GEO: GSE169367
Experimental models: organisms/strains		
Arabidopsis thaliana: Col-0	Widely distributed	N/A
Arabidopsis thaliana: C24	Widely distributed	N/A
Nicotiana benthamiana	[22]	N/A
Ptheirospermum japonicum	[23]	N/A
Solanum lycopersicum cv. Moneymaker	Plantagen	N/A
Solanum lycopersicum cv. TinyTim	Plantagen	N/A
Brassica napus cv. Hanna	Lantmännen	N/A
Brassica rapa cv. Purple Top Milan	Impecta	N/A
Arabidopsis thaliana: *abi1-1C*	[24]	N/A
Arabidopsis thaliana: SCR_{pro}:*abi1-1* in Col-0 background	[9]	N/A
Arabidopsis thaliana: UAS_{pro}:*abi1-1* in Col-0 background	[9]	N/A
Arabidopsis thaliana: J0571 in C24 background	[25]	N/A
Arabidopsis thaliana: Q0990 in C24 background	[25]	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Arabidopsis thaliana: J1721 in C24 background	[25]	N/A
Arabidopsis thaliana: *vnd1* in Col-0 background	[26]	N/A
Arabidopsis thaliana: *vnd2* in Col-0 background	[26]	N/A
Arabidopsis thaliana: *vnd3* in Col-0 background	[26]	N/A
Arabidopsis thaliana: *vnd6* in Col-0 background	[26]	N/A
Arabidopsis thaliana: *vnd7* in Col-0 background	[26]	N/A
Arabidopsis thaliana: *vnd1 vnd2* in Col-0 background	[26]	N/A
Arabidopsis thaliana: *vnd2 vnd3* in Col-0 background	[26]	N/A
Arabidopsis thaliana: *vnd1 vnd3* in Col-0 background	[26]	N/A
Arabidopsis thaliana: *vnd1 vnd2 vnd3* in Col-0 background	[26]	N/A
Arabidopsis thaliana: *vnd1 vnd2 vnd3 vnd7* in Col-0 background	This study	N/A
Arabidopsis thaliana: *$VND1_{pro}$:NLS-YFP* in Col-0 background	[12]	N/A
Arabidopsis thaliana: *$VND2_{pro}$:NLS-YFP* in Col-0 background	[12]	N/A
Arabidopsis thaliana: *$VND3_{pro}$:NLS-YFP* in Col-0 background	[12]	N/A
Arabidopsis thaliana: *$VND5_{pro}$:NLS-YFP* in Col-0 background	[12]	N/A
Arabidopsis thaliana: *$VND7_{pro}$:NLS-YFP* in Col-0 background	[12]	N/A
Arabidopsis thaliana: *6XABRE_A_{pro}:erGFP* in Col-0 background	[8]	N/A
Arabidopsis thaliana: *6XABRE_R_{pro}:erGFP* in Col-0 background	[8]	N/A
Oligonucleotides		
Oligonucleotides are specified in Table S2	This study	N/A
Software and algorithms		
Zeiss Zen Black 2.3 SP1	Zeiss	https://www.zeiss.com/
Zeiss Zen Blue 2.3 lite and 2.5	Zeiss	https://www.zeiss.com/
R 4.02 and R studio 1.2.5019	[27]	https://www.r-project.org/; https://rstudio.com/

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Microsoft Excel 2016	Microsoft	N/A
Illustrator 2020	Adobe	N/A
Affinity Designer 1.7	Affinity	N/A
Fiji/ImageJ 2.0.0 Win64 or 2.0.0-rc-68/1.52 h	[28]	https://fiji.sc/
Bioconductor 3.11	[29]	https://bioconductor.org/
Other		
Zeiss LSM780 confocal microscope	Zeiss	https://www.zeiss.com/
Zeiss LSM800 confocal microscope	Zeiss	https://www.zeiss.com/
Zeiss Axioscope A1	Zeiss	https://www.zeiss.com/
Leica M205 FA stereo-fluorescent microscope	Leica Microsystems	https://www.leica-microsystems.com/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Annelie Carlsbecker (annelie.carlsbecker@ebc.uu.se).

Materials Availability

There are no restrictions to the availability of newly generated resources in this study.

Data and code availability

The accession number for the transcriptome data reported in this paper is GEO: GSE169367.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Arabidopsis thaliana (L.) Heynh. Columbia-0 (Col-0), *Brassica napus* cv. Hanna, *Brassica rapa* cv. Purple Top Milan, *Nicotiana benthamiana*,[22] *Ptheirospermum japonicum*[23] and *Solanum lycopersicum* cv. Moneymaker and Tiny Tim, were used in this study. All mutant and transgenic lines detailed in the Key resources table were in Col-0 background. All plant growth was carried out in growth rooms in long day conditions, 16 h light (22°C) and 8 h darkness (20°C) at light intensity of 110μmol m^{-2} s^{-1}.

METHOD DETAILS

Plant growth conditions

Seeds were surface sterilized using 70% Ethanol for 20 min and 95% Ethanol for 2-3 min, and then rinsed in sterile water four times. The seeds were imbibed and stratified for 48 h at 4°C, and plated on 0.5xMurashige and Skoog medium (MS)[30] supplemented with 1% Bactoagar and 0.05% MES monohydrate, pH 5.7-5.8. For all experiments, plants were grown vertically on 25μm pore Sefar Nitex 03-25/19 mesh, and transferred to new plates by transferring the mesh with the plants on for minimal disturbance. For experiments involving transfer from ABA back to mock conditions, seedlings were instead transferred individually to prevent effects of residual ABA on the mesh. For ABA (Sigma) treatment, stock solutions of 50mM and 5mM ABA in 95% ethanol were used to make plates with ABA concentrations as indicated. Treatment with polyethylene glycol, was done with PEG 8000, as previously described.[2,24] Briefly, 60ml of 550 g/l PEG solution in 0.5XMS was overlaid on plates containing 40ml of solid 0.5xMS media and left overnight. The excess PEG solution was discarded before transfer of plants to the plates.

For *Arabidopsis* phenotyping experiments, two-day old seedlings were transferred to 1μM ABA containing plates for treatments of times indicated. For gene expression analysis, 4-5-day old seedlings were used. For phenotyping other species, seedlings were grown until roots reached approximately 1cm in length before transfer to ABA-containing plates.

All mutants or lines used in this study are listed in the Key resources table. For generation of the *vnd1 vnd2 vnd3 vnd7* quadruple mutant, the *vnd1 vnd2 vnd3* triple mutant was crossed to the *vnd7* mutant, and segregating F2 seedlings where genotyped using the primers listed in Table S2. The ABA responsive reporters used in this study are from Wu et al.[8] and the VND transcriptional reporters are from Kubo et al.[12] For tissue specific expression of *abi1-1*, UAS_{pro}:*abi1-1*[9] were crossed to Haseloff enhancer trap lines[26] and the resulting F1 seedlings were used for further analysis.

Phenotypic analysis

Xylem morphology quantification

For analysis of xylem morphology, roots were mounted directly in chloralhydrate solution, 8:2:1 chloralhydrate:glycerol:water (w/v/v), and visualized as described previously[2] using a Zeiss Axioscope A1 microscope at 40X magnification with differential interference contrast (DIC) optics. For quantification of phenotypes, the entire primary root or part of the root grown during the treatment times were analyzed for differences from wildtype pattern, separately for the distinct xylem axis positions (*px*, *omx* and *imx*). Phenotypes were categorized and the number of plants displaying a certain phenotype was used to calculate the frequency. Presence of more than one phenotype occurring in the same root was classified into a separate category.

Quantification of xylem differentiation

For determination of the point of xylem differentiation initiation, i.e., where SCW and lignification can be detected first relative the root tip, roots were cleared and stained with ClearSee solution containing calcofluor white and basic fuchsin.[25] Briefly, seedlings were fixed in 4% paraformaldehyde solution for 1 h at room temperature and washed with 1X PBS three times. The fixed tissue was incubated in ClearSee solution overnight and stained with calcofluor white and basic fuchsin. After staining, the tissue was washed in ClearSee and tile scans of roots from the root tip were acquired using Zeiss LSM780 inverted Axio Observer with supersensitive GaAsP detectors. Distances from the root tip to xylem vessel with bright fuchsin staining (lignin) at different positions in the xylem axis was measured by drawing a line from the root tip to the point of lignification using Zeiss Zen software.

Xylem differentiation at the inner metaxylem *position*

For early *imx* differentiation phenotypes, roots were mounted in chloralhydrate solution parallel to each other with root tips aligned on glass slides. A line was draw on the glass slide at a distance of 7mm from the root tip and this 7mm section of the root from the root tip was analyzed for the presence of lignified metaxylem. Roots were scored for presence or absence of a lignified *imx* using Zeiss Axioscope A1 microscope. For *B. napus*, *B. rapa* and *S. lycopersicum*, roots were mounted similarly to *Arabidopsis* and the number of xylem vessels at 5mm from the root tip was quantified. For *P. japonicum* and *N. benthamiana*, xylem vessel number was quantified at 2mm from the root tip.

The number of primary roots analyzed in each experiment is represented in the individual figures. Most experiments were repeated at least three times with similar results.

Xylem trans-differentiation of cotyledon cells

For vascular induction in *Arabidopsis* cotyledons we followed the protocol used for xylem induction in cotyledons using bikinin with minor modifications.[17] The modifications include the following: 1. In the induction medium, all components were like in Kondo et al.[17] except that bikinin was replaced with 10μM ABA. 2. The time for induction was increased from 4 days to 6 days. At the end of the 6-day induction period, cotyledons were fixed like in Kondo et al.[31] The samples were then washed with sterile water to remove traces of fixative solution. Samples were placed in a basic fuchsin-ClearSee mixture (final basic fuchsin concentration 0.1%–0.2% in ClearSee) overnight. The following day the samples were cleared with ClearSee and mounted on slides with ClearSee for visualization of autofluorescence (UV filter) or basic fuchsin staining (dsRED filter) with a Leica M205 FA stereofluorescent microscope. The area of ectopic lignification (autofluorescence) was calculated using ImageJ[28] and normalized to the total cotyledon area. Cotyledon veins were excluded from the quantification.

Confocal analysis

Roots were mounted in 40μM propidium iodide (PI) solution between two coverslips and imaged immediately. Confocal micrographs were captured using Zeiss LSM780 inverted Axio Observer with supersensitive GaAsP detectors. For calcofluor white 405nm laser was used for excitation and emission wavelengths 410-524nm were captured in the detector. For basic fuchsin images, 561nm excitation and 571-695nm emission. For reporter lines expressing GFP and stained with PI: 561nm excitation and 650-719nm emission for PI; 488nm excitation and 500-553nm emission for GFP. For reporter lines expressing YFP, 514nm excitation for both YFP and PI, 518-562 emission for YFP and 651-688nm emission for PI was used. For ectopic lignin assays: autofluorescence 405nm excitation and 410-518nm emission; basic fuchsin 561nm excitation and 595-710nm emission. For experiments involving quantification of fluorescence intensity all imaging parameters were kept the same when imaging mock and ABA-treated roots. The Zeiss Zen software was used to quantify YFP intensity. Region of Interests (ROI) encompassing nuclei in the *Arabidopsis* root meristem were used to measure average fluorescence intensity. Nuclei from similar regions in the root was used for mock and ABA treated samples.

Expression analysis by quantitative RT-PCR

RNA samples were extracted using the RNeasy Plant Mini Kit (QIAGEN), cDNA was synthesized using iScript reverse transcriptase enzyme. qRT-PCR analysis was performed as previously described using iQ SYBR Green Supermix in an iCycler iQ Real-Time PCR (Bio-Rad) instrument,[2] for primers see Table S2. The following program was used for the qRT-PCR analysis: initial denaturation 95°C for 3mins, 40 cycles of 95°C for 15sec, 60°C for 1min and was followed by melt curve analysis to confirm the absence of off target amplification. For *Arabidopsis*, either 1mm root tips or whole roots were used, as indicated in text. For *S. lycopersicum* (cv Tiny Tim), whole roots were collected after mock or 1μM ABA treatments for 6 h. Putative VND orthologs in tomato were annotated according to TAIR (https://www.arabidopsis.org/) and gene sequences obtained from Sol Genomics Network (https://www.solgenomics.net/). Primers used in this study are listed in Table S2. Three biological replicates were used for all samples and individual data points are represented in graphs. APT1 and GAPDH for *Arabidopsis*[32,33] and ACTIN and TIP41 for tomato[34,35] was used as reference genes, respectively.

RNAseq analysis

Five-day old *Arabidopsis* seedlings of Col-0, *vnd1 vnd2 vnd3* and *vnd7* were treated for 8 h with 1 μM ABA or mock. Three biological replicates, each consisting of 50-100 seedlings, were collected for each treatment-genotype combination. The lower part of the root (1 cm) was collected directly in RLT buffer (QIAGEN) and frozen in liquid nitrogen. In an independent experiment, samples from *Arabidopsis* Col-0(UAS_{pro}:*abi1-1*)xC24, J1721>>abi1-1, J0571>>abi1-1 and Q0990>>abi1-1, mock and ABA treated, were similarly collected. RNA was extracted using the RNeasy Plant Mini Kit (QIAGEN). RNA concentration was measured with Qubit BR RNA Assay and quality and integrity of the RNA was checked with the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). A total amount of 500ng RNA per sample was used for library preparation. Sequencing was performed by Novogene (UK) on their Illumina sequencing platform with paired-end read length of 150 and 250-300bp cDNA library resulting in 5.4 to 10.5 G raw data per sample. Initial processing of the reads as well as mapping was done by Novogene. Briefly, mapping to the *Arabidopsis thaliana* reference genome was done using Hisat2. Count files were generated using HTSeq. 96%–98% of the total reads were mapped to the *Arabidopsis* genome, whereby 94%–95% of the total reads were uniquely mapped.

Differential expression analysis was done independently for both experiments using DESeq2 in Bioconductor.[29,36] For statistical analysis of ABA effects on the different genotypes compared to wildtype, a DESeq2 model including a combinatorial effect was used (~genotype+genotype:condition). Log2fold changes were extracted from the pairwise comparison mock versus treatment for each genotype, while p values and adjusted p values were extracted from the comparison between the mutants/transactivation lines and wildtype. The effect of the different genotypes under mock condition was analyzed in an additional differential expression analysis and all values were extracted from the pairwise comparison of wildtype versus mutant. A cut-off > 0.5 was applied to the $\log_2$FC of Col-0 mock versus ABA comparison as well as to the $\log_2$FC of Col-0(UAS_{pro}:*abi1-1*)xC24 mock versus ABA. A list of xylem enriched genes from a single cell RNaseq[10] was generated by subtracting genes from the endodermis, cortex, trichoblast, atrichoblast and QC-columella cluster from the xylem cluster and used to identify xylem expressed genes influenced by ABA.

QUANTIFICATION AND STATISTICAL ANALYSIS

For categorical data, Fisher's exact test using the fisher.test function in R[27] was performed for greater than 2x2 matrices (i.e., considering all phenotype categories in a sample) and p values less than 0.05 were considered significant. For other data, Two-way ANOVA, One-way ANOVA or Student's t test was used. Statistical tests and significance threshold used are mentioned in figure legends, and summary of the ANOVA statistics in figures is presented in Data S2. The number of roots analyzed in all experiments are mentioned in the corresponding figures.

Current Biology, Volume *31*

Supplemental Information

Abscisic acid signaling activates distinct VND transcription factors to promote xylem differentiation in *Arabidopsis*

Prashanth Ramachandran, Frauke Augstein, Shamik Mazumdar, Thanh Van Nguyen, Elena A. Minina, Charles W. Melnyk, and Annelie Carlsbecker

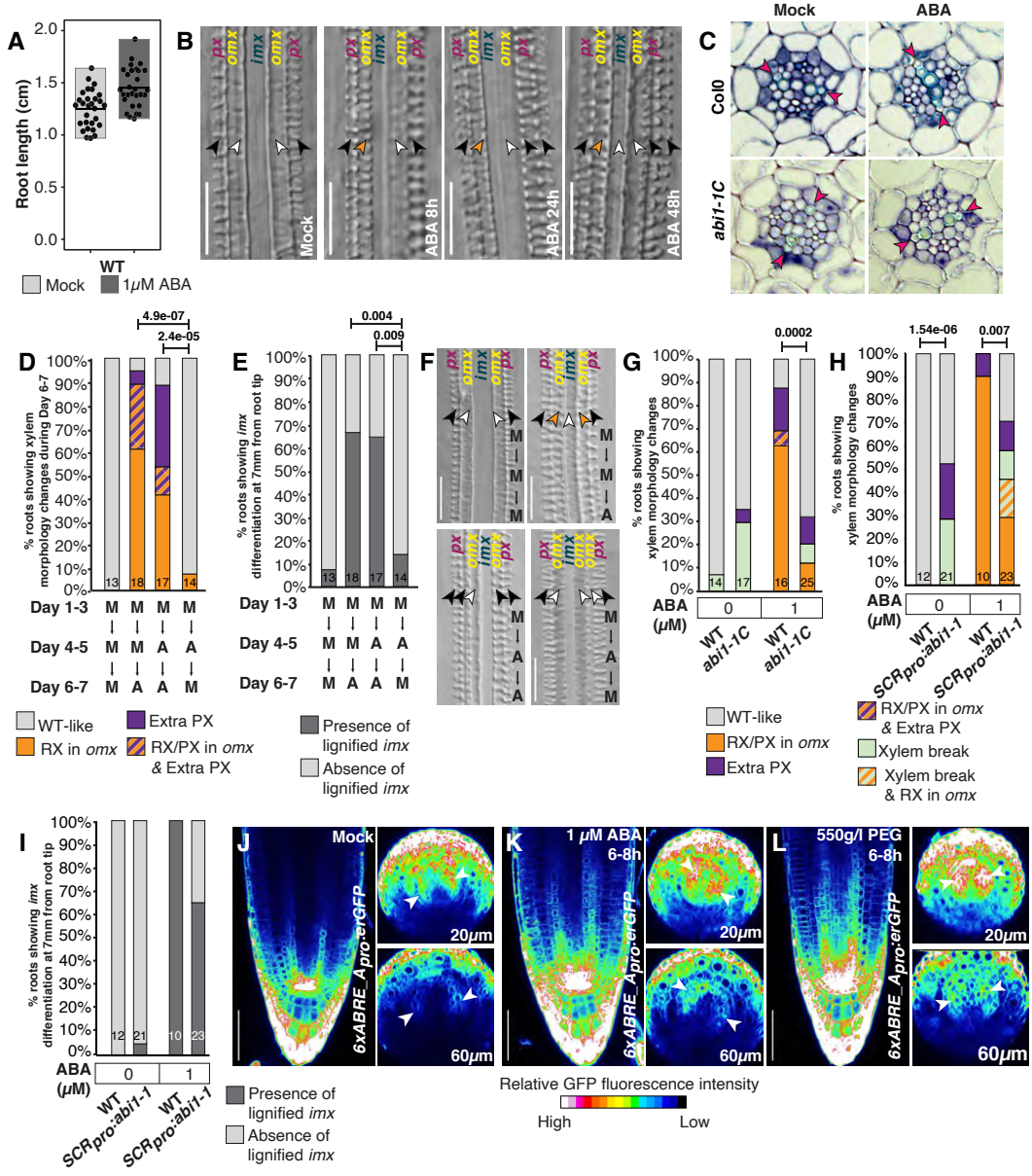
A
Root length (cm)
2.0
1.5
1.0
0.5
0.0
WT
Mock
1μM ABA
B
px
omx
imx
Mock
ABA 8h
ABA 24h
ABA 48h
C
Mock
ABA
Col0
abi1-1C
D
% roots showing xylem morphology changes during Day 6-7
4.9e-07
2.4e-05
13
18
17
14
Day 1-3
Day 4-5
Day 6-7
WT-like
RX in omx
Extra PX
RX/PX in omx & Extra PX
E
% roots showing imx differentiation at 7mm from root tip
0.004
0.009
Presence of lignified imx
Absence of lignified imx
F
G
% roots showing xylem morphology changes
0.0002
14
17
16
25
ABA (μM)
WT
abi1-1C
H
1.54e-06
0.007
12
21
10
23
SCRpro:abi1-1
Xylem break
RX/PX in omx & Extra PX & Xylem break
I
J
Mock
K
1 μM ABA 6-8h
L
550g/l PEG 6-8h
6xABRE_Apro:erGFP
20μm
60μm
Relative GFP fluorescence intensity
High
Low

Figure S1: ABA affects both xylem differentiation fate and rate in Arabidopsis roots. Related to Figure 1.

(A) Quantification of root lengths in mock and ABA treated wildtype (WT) roots. **(B)** Representative DIC images of xylem morphological changes in 1µM ABA treated WT roots quantified in Figure 1E. In B and F: *px*, protoxylem position; *omx*, outer metaxylem position; *imx*, inner metaxylem position. Differentiated protoxylem vessels are indicated by black arrow heads; metaxylem by white arrow heads; reticulate xylem by orange arrow heads. **(C)** Cross sections of control and 1µM ABA treated WT and *abi1-1C* roots. Red arrows indicate position of the xylem axis. **(D-E)** Quantification of xylem morphology (C) and *imx* differentiation (D) in ABA treated roots after transfer and growth for two days in mock, M, or ABA, A, conditions and further transfer for growth for another two days under mock or ABA conditions. In D, G and H: RX, reticulate xylem; PX, protoxylem. **(F)** Representative DIC images showing the xylem pattern after transfer of ABA treated roots to ABA or mock plates. **(G)** Quantification of xylem morphology changes in WT and *abi1-1 C* after 48h 1µM ABA treatment. **(H-I)** Quantification of xylem morphology (H) and *imx* differentiation (I) changes in SCR_{pro}*:abi1-1* lines after 48h 1µM ABA treatment. **(J-L)** Confocal micrograph showing ABA response domain after ABA treatment visualized using the *6xABRE_*A_{pro}*:erGFP* reporter after control (J), ABA (K) and 550g/l polyethylene (PEG) treatment (L), generating a negative water potential of -1.2 MPa[2]. Radial optical sections obtained at 20 and 60µm from the QC in J, K and L. GFP expression intensities are color coded, scale shown below the images. White arrow heads indicate xylem axis. Scale bars: 50µm in B, E, I and J. Statistics: In D, E, G, H, values above the bar represent p-values from Fisher's exact test, incorporating all phenotype categories. Numbers at the bottom of the bars in D, E, G, H and I represent the number of roots analyzed.

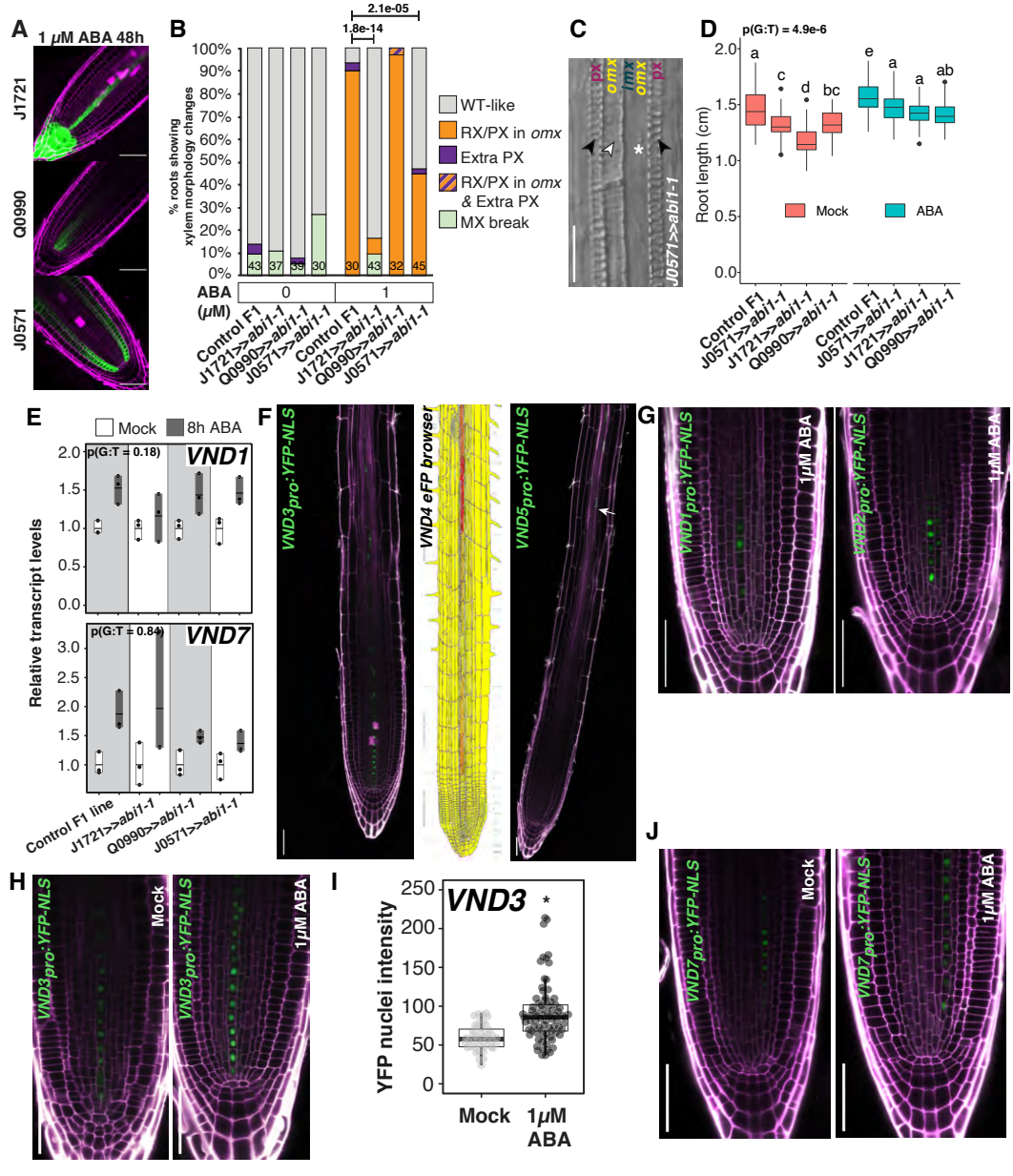
A
1 μM ABA 48h
J1721
Q0990
J0571
B
2.1e-05
1.8e-14
% roots showing xylem morphology changes
WT-like
RX/PX in omx
Extra PX
RX/PX in omx & Extra PX
MX break
43 37 39 30 30 43 32 45
ABA (μM)
0 1
Control F1
J1721>>abi1-1
Q0990>>abi1-1
J0571>>abi1-1
C
px
omx
J0571>>abi1-1
D
p(G:T) = 4.9e-6
Root length (cm)
Mock
ABA
a c d bc e a a ab
E
Mock
8h ABA
VND1
p(G:T = 0.18)
VND7
p(G:T = 0.84)
Relative transcript levels
Control F1 line
F
VND3pro:YFP-NLS
VND4 eFP browser
VND5pro:YFP-NLS
G
VND1pro:YFP-NLS
VND2pro:YFP-NLS
1μM ABA
H
VND3pro:YFP-NLS
Mock
1μM ABA
I
VND3
YFP nuclei intensity
Mock
1μM ABA
J
VND7pro:YFP-NLS
Mock
1μM ABA

Figure S2: ABA signalling within the xylem activates VND transcription factors. Related to Figure 2 and Data S2.

(A) Activity domains of enhancer trap lines after ABA treatment. **(B)** Quantification of xylem morphology changes observed in *abi1-1* transactivation lines. p-values from Fisher's Exact test incorporating all phenotype categories is mentioned above the bars; numbers at the bottom of the bars in B represent the number of roots analyzed. **(C)** DIC image showing xylem breaks in the *J0571>>abi1-1* lines, indicated with *; protoxylem vessels are indicated by black arrow heads and metaxylem by white arrow head. *px*, protoxylem position; *omx*, outer metaxylem position; *imx*, inner metaxylem position. **(D)** Quantification of root lengths in the different driver lines after mock and ABA treatment. The significance of genotype:treatment (G:T) interaction on root length based on two-way ANOVA analysis is mentioned above the plots. Letters *a,b,c,d,e* identify groups with significant differences with Tukey's post-hoc testing ($p<0.05$). **(E)** qRT-PCR quantification of relative *VND1* and *VND7* transcript levels after 8h ABA treatment in whole roots of F1 seedlings from a cross between UAS_{pro}*:abi1-1* and indicated GAL4 enhancer trap line. All values are normalized to the average of mock treated samples; black dots represent biological replicates. The significance of genotype:treatment (G:T) interaction on gene expression based on two-way ANOVA analysis is given above the plots. **(F)** Confocal micrographs of $VND3_{pro}$*:YFP-NLS* and $VND5_{pro}$*:YFP-NLS* promoter activity domains and Arabidopsis eFP browser (http://bar.utoronto.ca/) cartoon showing expression domain of *VND4*. **(G)** Confocal images of $VND1_{pro}$*:YFP-NLS* and $VND2_{pro}$*:YFP-NLS* after ABA treatment. **(H)** Confocal images of $VND3_{pro}$*::YFP-NL*S after 1µM ABA treatment for 6-8h. **(I)** Quantification of YFP nuclear intensity in mock and ABA treated $VND3_{pro}$*::YFP-NL*S. Grey dots represent each quantified nucleus. * represent $p<0.05$, two tailed Student's t-test. **(J)** Confocal images of $VND7_{pro}$*:YFP-NLS* after mock and ABA treatment. Scale bars: 50µm in A, C, F, G, H and J.

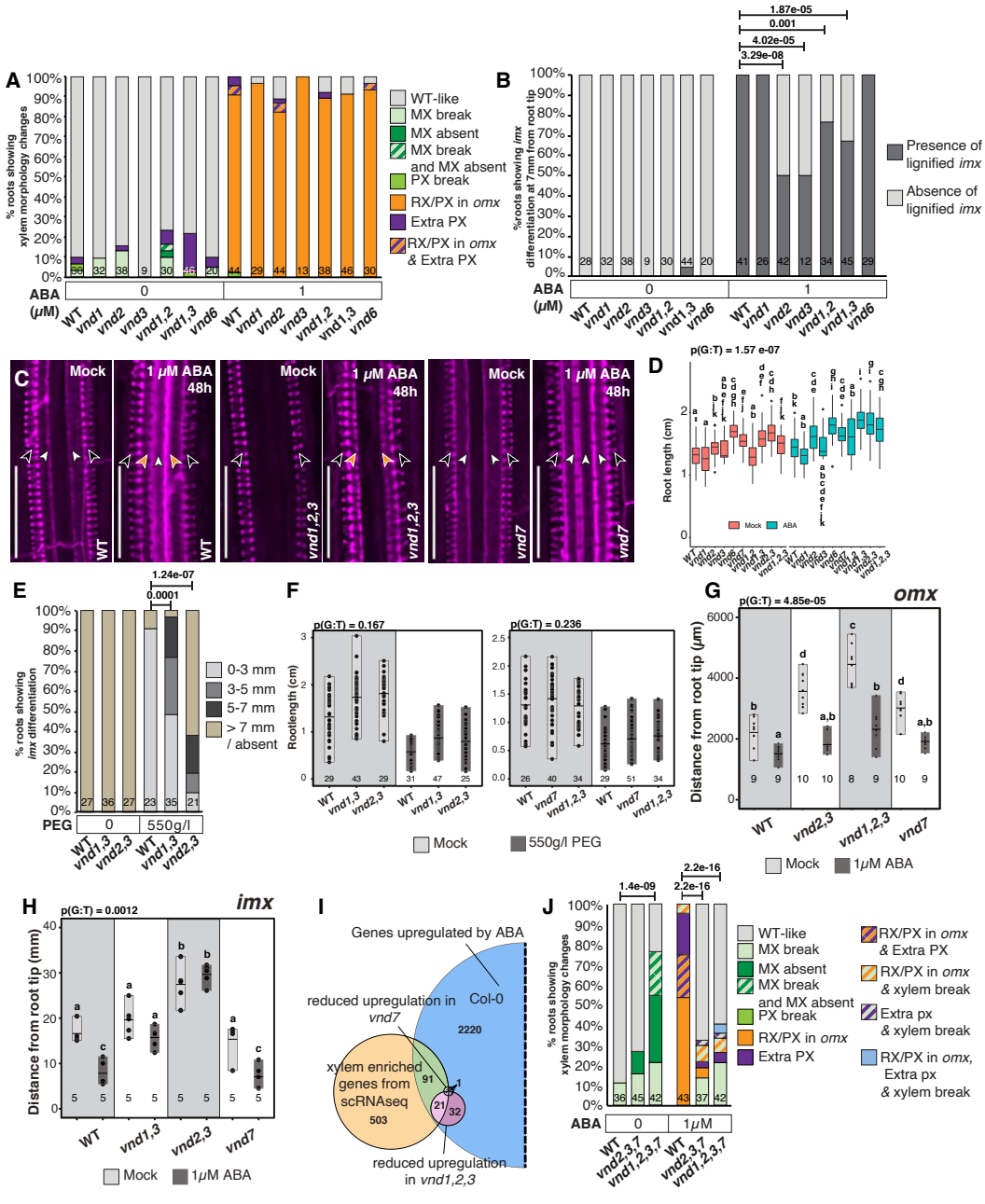
A
% roots showing xylem morphology changes
WT-like
MX break
MX absent
MX break and MX absent PX break
RX/PX in omx
Extra PX
RX/PX in omx & Extra PX
ABA (μM)
WT vnd1 vnd2 vnd3 vnd1,2 vnd1,3 vnd6
B
%roots showing imx differentiation at 7mm from root tip
Presence of lignified imx
Absence of lignified imx
1.87e-05
0.001
4.02e-05
3.29e-08
C
Mock
1 μM ABA 48h
WT
vnd1,2,3
vnd7
D
p(G:T) = 1.57 e-07
Root length (cm)
Mock
ABA
E
1.24e-07
0.0001
% roots showing imx differentiation
0-3 mm
3-5 mm
5-7 mm
> 7 mm / absent
PEG
0
550g/l
F
p(G:T) = 0.167
p(G:T) = 0.236
Rootlength (cm)
Mock
550g/l PEG
G
p(G:T) = 4.85e-05
omx
Distance from root tip (μm)
Mock
1μM ABA
H
p(G:T) = 0.0012
imx
Distance from root tip (mm)
Mock
1μM ABA
I
Genes upregulated by ABA
Col-0
2220
reduced upregulation in vnd7
xylem enriched genes from scRNAseq
503
91
1
21
32
reduced upregulation in vnd1,2,3
J
1.4e-09
2.2e-16
2.2e-16
% roots showing xylem morphology changes
WT-like
MX break
MX absent
MX break and MX absent
PX break
RX/PX in omx
Extra PX
RX/PX in omx & Extra PX
RX/PX in omx & xylem break
Extra px & xylem break
RX/PX in omx, Extra px & xylem break
ABA
0
1μM
WT vnd2,3,7 vnd1,2,3,7

Figure S3: VNDs regulate plasticity of xylem fate and differentiation rate. Related to Figure 3, Data S1 and Data S2.

(A-B). Quantification of xylem morphology (A) and differentiation in inner metaxylem position (*imx*) at 7mm from the root tip (B) after mock and 1µM ABA treatment (48h) in wildtype (WT), single and double *vnd* mutants. **(C)** Basic fuchsin stained confocal micrographs of WT, *vnd1,2,3* and *vnd7* showing xylem morphology after mock and ABA treatment. Scalebars: 50µm. **(D)** Quantification of root lengths in *vnd* mutants after mock and 1µM ABA treatment (48h). **(E)** Quantification of *imx* differentiation after polyethylene glycol (PEG) treatment in WT, *vnd1 vnd3* (*vnd1,3*) and *vnd2 vnd3* (*vnd2,3*). Plants were categorized depending on the distances from root tip to lignified *imx* xylem vessels. **(F)** Quantification of root lengths in *vnd1,3, vnd2,3, vnd7* and *vnd1 vnd2 vnd3* (*vnd1,2,3*) after PEG treatment. **(G)** Quantification of distance from root tip to a lignified xylem vessel in the outer metaxylem position (*omx*) in WT, *vnd2,3*, *vnd1,2,3* and *vnd7*. **(H)** Quantification of distance from root tip to a lignified xylem vessel in *imx* in WT, *vnd1,3*, *vnd2,3* and *vnd7*. **(I)** Venn diagram illustrating the reduced activation of several xylem enriched genes [10] by ABA in *vnd1,2,3* compared to WT. Only half of the blue circle is shown. **(J)** Quantification of xylem morphology in *vnd2 vnd3 vnd7* (*vnd2,3,7*) and *vnd1 vnd2 vnd3 vnd7* (*vnd1,2,3,7*) upon mock and ABA treatments. Statistics: In A, B, E, J, values above the bar represent p-values from Fisher's exact test, incorporating all phenotype categories. In D, F-H, the significance of genotype:treatment (G:T) interaction based on two-way ANOVA analysis is mentioned above the plots. Letters *a-k* identify groups with significant differences with Tukey's post-hoc testing ($p<0.05$). Black dots in F-H represent biological replicates. Numbers at the bottom of the bars in A-B and F-J represent the number of roots analyzed.

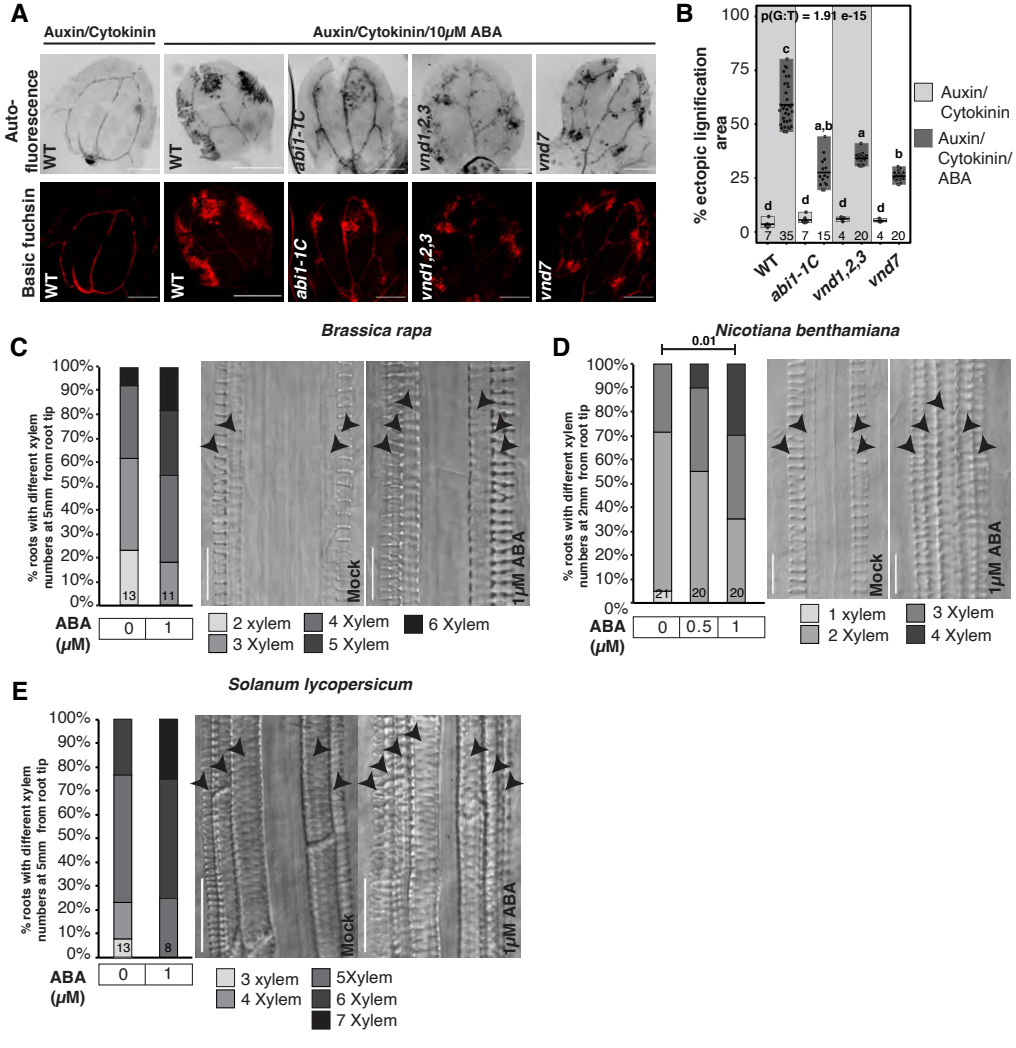
A
Auxin/Cytokinin
Auxin/Cytokinin/10μM ABA
Auto-fluorescence
Basic fuchsin
WT
abi1-1C
vnd1,2,3
vnd7
B
p(G:T) = 1.91 e-15
% ectopic lignification area
Auxin/ Cytokinin
Auxin/ Cytokinin/ ABA
C
Brassica rapa
% roots with different xylem numbers at 5mm from root tip
ABA (μM)
2 xylem
3 Xylem
4 Xylem
5 Xylem
6 Xylem
Mock
1μM ABA
D
Nicotiana benthamiana
% roots with different xylem numbers at 2mm from root tip
1 xylem
2 Xylem
3 Xylem
4 Xylem
E
Solanum lycopersicum
% roots with different xylem numbers at 5mm from root tip
3 xylem
4 Xylem
5Xylem
6 Xylem
7 Xylem

Figure S4: ABA induces ectopic lignification in Arabidopsis cotyledons and promotes xylem differentiation in several eudicot species. Related to Figure 4 and Data S2.

(A) Fluorescent micrographs showing autofluorescence (top) and basic fuchsin staining (bottom) of lignified cells in the cotyledons subjected to auxin/cytokinin/ABA or auxin/cytokinin (control) treatment. Scale bars are 1mm. **(B)** Quantification of ectopic lignification area from an independent experiment in *in vitro* cultured WT, *abi1-1C, vnd1,2,3* and *vnd7* cotyledons. The significance of genotype:treatment (G:T) interaction based on two-way ANOVA analysis is mentioned above the plots. *a,b,c,d* represent groups with significant differences with Tukey's post-hoc testing ($p<0.05$). **(C-E)** Quantification of total number of lignified xylem vessels at specified distances from the root tip in *B. rapa* (C), *N. benthamiana* (D) and *S. lycopersicum* (cult. Tiny Tim) (E) after mock and 1µM ABA treatment accompanied by representative images showing an increase in xylem number. Scale bars: 50µm. In D, values above the bar represent p-values from Fisher's exact test, incorporating all phenotype categories; Numbers at the bottom of the bars in C-E represent the number of roots analyzed.

qRT-PCR primers	
VND1_F	GGAAAACACTTGTGTTCTACAAAGG
VND1_R	AACCACCCATCCTTCTTCCT
VND2_F	CGAACCATGGGATTTACAAGA
VND2_R	TGTTTGTTCTTGTTCCTGTTGG
VND3_F	ACCGGAAAGCTTCCCTCTTGCC
VND3_R	TCCCGGGAAGGTCCCAAGGAT
VND4_F	GGTCAACGACGGATTCTCC
VND4_R	TGGCATATGTTGTTGGTGCT
VND5_F	TGAATCAGCCTGTTTTTGAGTTT
VND5_R	TCTTCATCAGTAGGATGAAATCTGA
VND6_F	ACCCTATGGAAGATGGAGGGACCA
VND6_R	CGCCACACTCCCGCACACTT
VND7_F	TTCGAAACGCAGTCGTATAATCC
VND7_R	ATTAGCTTCGACCTCATTATAGCTTTG
MYB46_F	TCTTCGTCCTGACCTCAAGC
MYB46_R	CTGCAATCTGAGACCACCTG
MYB83_F	AACGTGGATCCTTCTCTCCTC
MYB83_R	AGCCGAGTAGCTATTTGAGACC
LBD15_F	GCATGCCTGAATGTCAAGAG
LBD15_R	GGACCCGACATTGGTCTTC
LBD30_F	AGCGAGCAACGTCTCCAA
LBD30_R	ACGGCGTCTGGTCGTTTAT
ACL5_F	CGCTCCTTCTTTCGTCTCTG
ACL5_R	TCTAGCGCGAGAGAGATGGT
XND1_F	GCTCCTCGGCAACAGATGGTCG
XND1_R	GCATGCGGCTTTGACGGCAG
XCP1_F	TCCAGTTCTACAAAGGGGGAGTGTT
XCP1_R	CTGCCACACCGTGGTCTAGGT
XCP2_F	TAGCGGCGGCGTGTTTGATGG
XCP2_R	CGCAGCCACACCGTGGTCAA
APt1_F	GTTGCAGGTGTTGAAGCTAGAGGT
APT1_R	TGGCACCAATAGCCAACGCAATAG
GAPDH_F	ATCAAGAAGGCCATCAAGGA
GAPDH_R	CCTCAGTGTATCCCAAAATTCC
Solyc02g084350_F	CCGATTTGGGAATGTTGCTA
Solyc02g084350_R	CGAACTCAAGATCTCATTCAGC
Solyc08g079120_F	CCCTCAATTAGAGTCACCCAAG
Solyc08g079120_R	CATTGAGTTGTTGTTGATTGATCC
Solyc03g083880_F	CATGGGATCTTCAAGAATTATGC
Solyc03g083880_R	TTGTGGCTAAAGAAGTACCATTCA
Solyc11g018660_F	TTATATGAGAGGAACCAACACAAGTTA
Solyc11g018660_R	AATAACTTGGCATTTGTGAAAGG
Solyc06g065410_F	ATGCATGAATACAGGCTCCA
Solyc06g065410_R	CCCTACATACCACCCATCCTT

TomatoActin_F	GCTCCACGAGCTGTATTTCC
TomatoActin_R	TTTTGACCCATACCCACCAT
TomatoTIP41_F	TTGGGGAGCATAACAAATCC
TomatoTIP41_R	GACAAGGCCTGAAATGTGGT
Genotyping primers	
VND1_LP	CTCGTTTTAAGCGGATGTTTG
VND1_RP	ATGACGGGAAATTGGAGAGAG
VND1_BP	ATTTTGCCGATTTCGGAAC
VND2_LP	TCTACAGAATCGAACCATGGG
VND2_RP	AGTATGCCAAACCTTTAGGCC
VND2_BP	ATTTTGCCGATTTCGGAAC
VND3_LP	GCGTCGTCGTAGAATAAGCAG
VND3_RP	CACATGTCATCGTTCAAGTGG
VND3_BP	GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC
VND7_LP	CATGTGTGTGGTCCTGTTGAG
VND7_RP	CCATGGCTCCATTTTGTAGAG
VND7_BP	ATTTTGCCGATTTCGGAAC

Table S2. List of primers used in the study. Related to STAR Methods.

Acta Universitatis Agriculturae Sueciae

Doctoral Thesis No. 2022:52

Plants possess remarkable regenerative abilities but are susceptible to stress due to their sessile lifestyle. Here, we demonstrate how stress affects vascular development and regeneration in *Arabidopsis thaliana*. We investigate the role of abscisic acid in xylem development under stress conditions. We indicate how brassinosteroid affects vascular development. Lastly, we describe a cell wall associated gene which is induced by stress and mediates vascular development and regeneration. This thesis contributes to our understanding of stress-based plant vascular development and regeneration.

Shamik Mazumdar received his graduate education at the Department of Plant Biology, SLU, Uppsala. He obtained M.Sc. degree from Maharshi Dayanand University, Rohtak, India and his B.Sc. degree from Savitribai Phule University of Pune, India.

Acta Universitatis Agriculturae Sueciae presents doctoral theses from the Swedish University of Agricultural Sciences (SLU).

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ISSN 1652-6880
ISBN (print version) 978-91-7760-979-7
ISBN (electronic version) 978-91-7760-980-3