A study into the regulation of root development under osmotic stress

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Abstract

The mechanisms behind the regulation of root development under a lack of water (osmotic stress) is a critical subject for plant biology and global agriculture. Previously osmotic stress has been shown to inhibit root growth via an abscisic acid (ABA)-mediated reduction in auxin transport, independent of ethylene signalling.

This thesis examines the impact of osmotic stress on critical developmental signals: auxin, ABA and reactive oxygen species (ROS) minutes after encountering osmotic stress and following a longer stress period of 24 hours. The roles of these signals in the root were analysed via loss-of-function mutants, gene expression analysis, and bioimaging in concert with exogenous chemical treatment.

Auxin, ABA and ROS (represented by level of oxidation) levels were all found to rapidly increase within 10 minutes of osmotic stress leading to downstream responses. ROS and ABA appear to have a strong positive feedback relationship that can develop with minutes. Under the longer stress period of 24 hours, auxin responses were found to decrease while both ROS and ABA responses were shown to increase.

PIN-mediated auxin transport was shown to play a key role in the reduction of auxin in the root tip following 24-hour osmotic stress treatment. PIN3 and PIN7 gene expression and protein distribution were altered under osmotic stress, associated with a reduction of the auxin maximum at the root tip.

ROP2 was found to play a central role in root development under osmotic stress root, along with the decrease of auxin and increase in ABA signalling after 24 hours. It was also shown that that several respiratory burst oxidase homologs (RBOHs) play a role in root development under standard conditions as well as under osmotic stress. PERK4 and RBOHC likely play a key role in ROS production under ABA-mediated osmotic stress response, with the loss-of-function of PERK4 significantly improving root growth under osmotic stress.

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Declaration

I certify that all of the work described in this thesis is my own original research unless otherwise acknowledged in the text or by references and has not been previously submitted for a degree in this or any other university.

Acknowledgements and Thanks

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Dedication

This thesis is dedicated to my father Mark. I guess your passion for learning and science rubbed off on me. What inspires me most was how wonderful, funny, warm, and kind you were. Every day I think about you and our times together.

"*Ipsa scientia potestas est* (Knowledge itself is power)"

Francis Bacon, 1597

Chapter 1. Introduction

1.1 Drought stress and food security

We are rapidly approaching a hotter and drier world (FAO 2019). By 2050, global human population is predicted to reach near 10 billion people (United Nations 2019), 5 billion of which are projecting to be living in water-scarce regions (UNESCO 2020). Agricultural freshwater demand is predicted to double whilst freshwater availability could halve (Gupta *et al.* 2020).

Drought is occurring more frequently, as increasing global temperature leads to more extreme weather events (Moore *et al.* 2015). This has major consequences for crop yield as water is a major limiting factor for plant productivity in the field. Lack of water leads to the failure of vital organs and cellular processes (Gupta 2020). Compared with other abiotic stresses such as flood and cold, droughts and heatwaves accounts for the largest crop losses (Lesk *et al.* 2016, Brás *et al.* 2021)

There has been widespread success for genetically modified crops with resistance to pests, pathogens, and herbicides (Raman 2017). Developing drought resistance crops is a critical target for plant breeders. However, it is a complex trait that is dependent on several mechanisms. In fact, overexpression of drought responsive genes often results in growth deficits and yield loss (Gupta *et al.* 2020).

The major focus of drought stress research has been studying plant physiology above the soil. In particular, the study of mature plants in shoot and leaf samples (Kalve *et al*. 2020). Perhaps the most studied drought responses are the role of stomata; how they rapidly open and close to limit water loss via gaseous exchange (Sarwat *et al.* 2017, Agurla *et al.* 2018, Qi *et al.* 2018).

As the primary mechanism for drought detection and the absorption of water and nutrients, root system architecture (RSA) and physiology can have a large impact on drought resistance and crop survival/yield. Depth of rooting is a critical parameter for water foraging but little consideration has been given to overall distribution of the root system and its growth patterns (Rosales *et al.* 2019, Cajero-Sanchez *et al.* 2019).

Roots exhibit remarkable plasticity to their environment (Dinneny *et al.* 2019). The root has a biphasic response to drought stress, where root growth can be promoted or hindered depending on the level of moisture in the soil (Creelman 1990, Li *et al.* 2017). Mild soil drying stimulates growth (van der Weele *et al.* 2000, Li *et al.* 2017) whilst severe soil dryness limits the growth of the root (Koevoets *et al.* 2016). Under drought we also see other responses such as the accumulation of low weight osmolytes such as proline, and an increase in suberization (Kreszies *et al.* 2019).

There remains little consensus over the mechanisms of drought (osmotic stress) response in roots and in particular, how hormonal signalling leads to changes in RSA and growth patterns. Understanding how plants respond to drought is a critical concern if we are to meet the demands of a growing population via targeted molecular breeding.

1.2 *Arabidopsis thaliana* as a model organism

Arabidopsis thaliana has been used as a model organism for over 100 years due to its diminutive size, short generation time, diploid genome, and its preference for self-pollination (Krämer *et al.* 2015). In 2000, the genome sequence was published by The Arabidopsis Genome Initiative. This project along with a number of online sources enable detailed study of the *Arabidopsis* plant system at the molecular level.

The *Arabidopsis* root system is highly organised in its cellular structure and easily studied under a microscope (Dolan *et al.* 1993). This is useful for experimental manipulation and viewing the role of hormones in developmental processes such as cell division, differentiation, and expansion.

1.2.1 *Arabidopsis* root system and structure

Early on in the development of the *A. thaliana* embryo we see the establishment on the apical/basal axis (Jürgens 2001, Robert *et al.* 2013). The apical domain develops into the cotyledons and the shoot apical meristem. The basal domain develops into the root system containing the hypocotyl, the radicle, and the stem cell niche (SCN) of the root apical meristem (RAM)

The root tip is radially organised into concentric rings of cell files: the epidermis, cortex, endodermis, pericycle, lateral root cap and stele. The stele is further subdivided into the phloem, procambium, metaxylem and protoxylem (Dolan *et al.* 1993) (Figure 1.1).

There are distinct developmental zones within the root tip, each displaying different developmental processes. The initial site of cell division and root growth is within the RAM. Here, different tissues are formed from stem cell-initials which give rise to different cell files. From the quiescent centre (QC) arise daughter stem cell initials during embryogenesis. The QC is a group of four cells with low mitotic activity which are crucial for maintaining and regulating the undifferentiated status of the initials. The group of stem cells surround the QC is called the stem cell niche (SCN) (Dolan *et al.* 1993, Petricka *et al.* 2012, Yamoune *et al.* 2021).

The RAM is protected at the tip by a layer of cells called the root cap, made up of the columella and the lateral root cap. Following repeated cell division, cells pass from the RAM through the transition

zone (TZ) to the elongation zone (EZ). At the EZ, the rate of cell division decreases, and cells expand longitudinally. Following elongation, the cells acquire their final characteristic in the area called the differentiation zone (DZ) (Dolan *et al.* 1993).

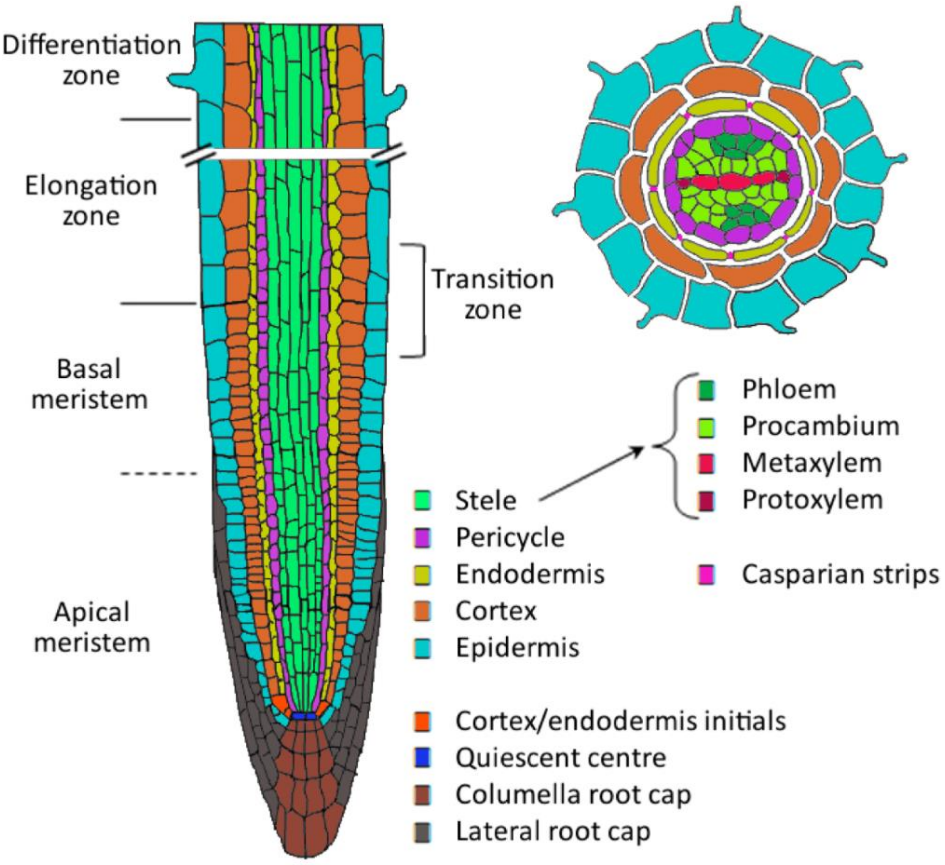

Figure 1.1. The organization of the *Arabidopsis* root: (Left) Longitudinal section of an *Arabidopsis* primary root. Each cell types are arranged in a cell file, forming concentric single-celled layers surrounding the central vascular tissue. Different developmental zones are formed as the root grows. Cell division takes place within the meristematic zone, in particular at the apical meristem. In the basal meristem, cell division begins to reduce. By the elongation zone, cell division has stopped, and cells have started to elongate. The boundary between both the meristematic and elongation zones is called the transition zone. At the differentiation zone cells have begun to differentiate. (Right) A cross section of the differentiation zone, displaying the formation of root hairs and Casparian strips (Taken from De Smet *et al.* 2015).

1.3 Plant hormones as regulators of growth

Plant hormones are responsible for controlling almost every aspect of growth and development, some more essential in specific tissues at particular times than others (Takatsuka and Umeda 2014, Moore *et al.* 2015). Hormones such as auxin, cytokinin, ethylene and abscisic acid (ABA) can be altered rapidly when subjected to abiotic and biotic stresses, resulting in coordinated developmental change (Takatsuka and Umeda 2014).

All hormones interact with each other in a mechanism of crosstalk, and it is through their interactions that root growth is maintained (Liu *et al.* 2014). Rapid hormonal crosstalk is required for integrating environmental signals into plant development (Liu *et al.* 2014, Rowe *et al.* 2016, Li *et al.* 2019, Jang *et al.* 2020). To understand osmotic stress response, we have to understand each hormone we are studying in a wider context.

1.3.1 Auxin

Almost every aspect of plant growth requires auxin (Saini *et al.* 2013). Auxin is a small organic molecule that has been well established as a regulator and coordinator of plant growth and development (Benjamins and Scheres 2008). This includes key roles in shoot and root meristem maintenance and elongation, tropic responses, leaf primordia, lateral root initiation and the development of vascular tissues (Abel and Theologis 1996, De Smet *et al.* 2007). At the cellular level, auxin alters cell division, elongation and differentiation resulting in shaping root/shoot shape and form (Naser *et al.* 2016).

High auxin concentration in the root QC and in the stem cell niche is required for the coordination and establishment of growth (Petersson *et al.* 2009, Clark *et al.* 2014). The maintenance of the auxin maximum in the root tip is achieved by a combination of shoot-derived auxin transported via auxin carriers and local auxin biosynthesis (Korver 2018, Ljung 2005). The level of auxin in a cell is crucial for determining cell fate. For example, the accumulation of auxin in pericycle cells transforms them into lateral root founder cells which further divide to form the later root primordium (Du *et al.* 2017).

Large levels of exogenous auxin application can inhibit growth, whilst lower concentrations of exogenous auxin can promote growth (Evans *et al.* 1994). Presence of auxin inhibits expansion in the elongation zone whilst promoting cell division and inhibiting differentiation in the meristematic zone (Moubayidin *et al.* 2010).

The perception of auxin and the process of gene transcription that follows is a process mediated by proteasomal degradation. Auxin is perceived by a complex of co-receptors comprising an F-box protein of the TIR1/AFB (TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX PROTEIN) family and a member of the Aux/IAA (Auxin/Indole-3-Acetic acid) family of transcriptional repressors.

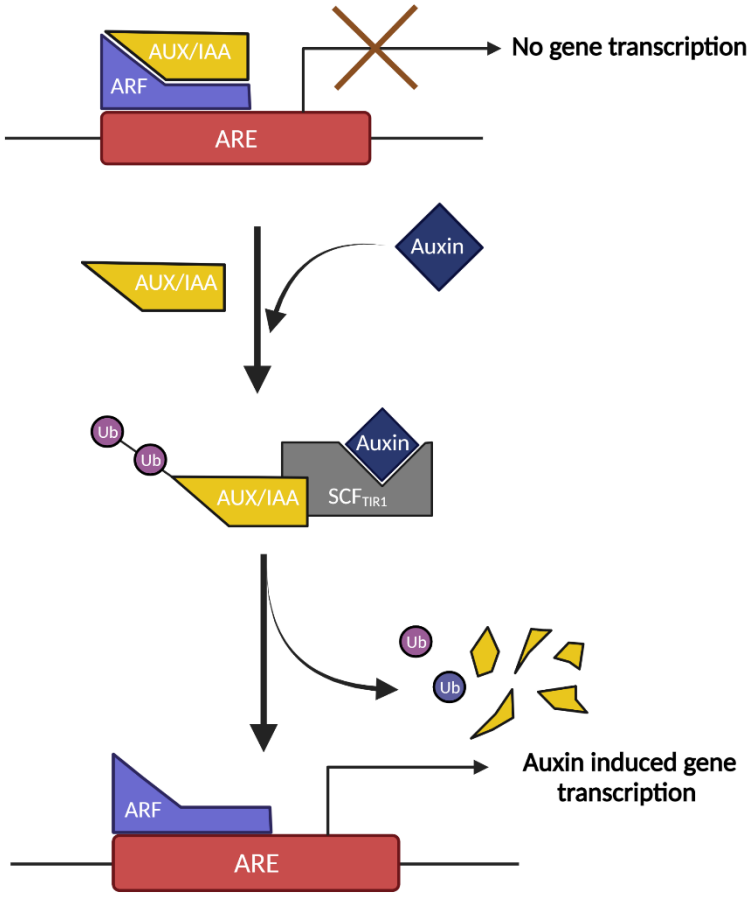

Figure 1.2. The primary auxin signalling pathway leading to the regulation of gene transcription. In the absence of auxin, AUX/IAA proteins repress the activity of ARF transcription factors which bind to ARE domains. When auxin is present, AUX/IAAs and F-box proteins of the TIR1/AFB family form an SCF-type E3 ubiquitin protein ligase complex which transfers activated ubiquitin to AUX/IAA proteins. The polyubiquitination of Aux/IAA leads to the protein's degradation. As ARFs are no longer repressed, transcription is activated, and downstream auxin responses take place (Teale *et al.* 2006, Leyser 2018).

An auxin molecule promotes interaction between TIR1/AFBs and AUX/IAAs to form an SCF-type E3 ubiquitin protein ligase complex, leading to ubiquitination and degradation of AUX/IAAs (Tan *et al* . 2007) (Figure 1.2). This degradation enables Aux/IAA-interacting Auxin Response Factor (ARF) proteins to form homodimers and bind to the Auxin Response Element (ARE) promoter domains of auxin inducible genes leading to gene expression (Bargmann and Estelle 2014, Boer *et al.* 2014, Mironova *et al.* 2014). Auxin presence ultimately triggers a large network response by increasing the affinity between TIR1/AFB and AUX/IAA co-receptor families, which in turn releases ARF-dependent outputs.

Cellular patterning in the *Arabidopsis* root requires the establishment of an auxin concentration maximum around the QC. This patterning is controlled by asymmetrically located membrane

proteins located at the plasma membrane in a process called Polar Auxin Transport (PAT). The three main groups of transport proteins are the PIN-formed (PIN) family of efflux carrier proteins, ABCB/PGP (ATP-BINDING CASSETTE SUBFAMILY B/P-GYLCOPROTEINS) efflux proteins and AUX/LAX influx proteins (Petrasek and Friml 2009, Kramer and Bennett 2006, Peer *et al.* 2011).

Recent studies have also indicated there are other auxin transporters. PILS (PIN-LIKES) are required for auxin-dependent regulation of plant growth via helping determine intracellular auxin accumulation at the endoplasmic reticulum and so the available auxin for nuclear auxin signalling (Barbez *et al.* 2012). WAT1 (WALLS ARE THIN1) is a vacuolar auxin transporter that plays a key role in auxin transport and homeostasis, and that dictates secondary cell wall thickness (Ranocha *et al.* 2013)

PIN proteins are very important for directional movement of auxin at the root tip as they help facilitate the movement of auxin out of cells (Friml *et al.* 2003, Wisniewska et al. 2006, Petrasek and Friml 2009). PINs can be quickly organised into different locations to alter the direction of auxin flow, resulting in changes in morphology (Heisler *et al.* 2005).

Strict control of PINs polar distribution on the plasma membrane is possible via clathrin-coated vesicular transport to the PM, constitutive endocytic recycling, and vacuolar degradation (Adamowski and Friml 2015). These processes are tightly regulated by ADP-ribosylation factor (ATF) GTPases. Controlled by a GTP-binding and a GTP hydrolysis cycle that activates ARF GTPases and inactivates them respectively. R-SNARE protein VAMP714 plays a key role in the insertion of PINs into the plasma membrane for polar auxin transport, root gravitropism and morphogenesis. VAMP714 likely helps the delivery of PIN-carrying vesicles to the plasma membrane and plays a role in controlling development via positive regulatory loop in which auxin activates a VAMP714-dependent PIN/auxin transport system (Gu *et al.* 2021) The actin cytoskeleton is required for vesicular trafficking of PM proteins and in the cycling of PINs (Zhu and Geisler 2015).

PIN location and polarisation is critical in determining the direction and rate of auxin flow in the root tip. The directional transport of auxin via PINs and other transport proteins forms a 'reverse fountain' of auxin leading to accumulation of auxin in specific root tip cells (Petrásek & Friml 2009). PIN1, PIN3 and PIN7 are found in the vasculature, where they transport auxin acropetally where it is then transported by PIN4 into the QC. Both PIN3 and PIN7 are also found in the columella where they transport auxin to the lateral root cap. PIN2 transports auxin basipetally (shootward) to the elongation zone (Friml *et al.* 2003) (Figure 1.3).

The temporal-spatial patterns of PIN proteins are also regulated by transcription factors. FOUR LIPS (FLP) and its paralogue MYB88 expression specifically determine the patterns of PIN3 and PIN7 during root responses to gravity (Wang *et al.* 2015)

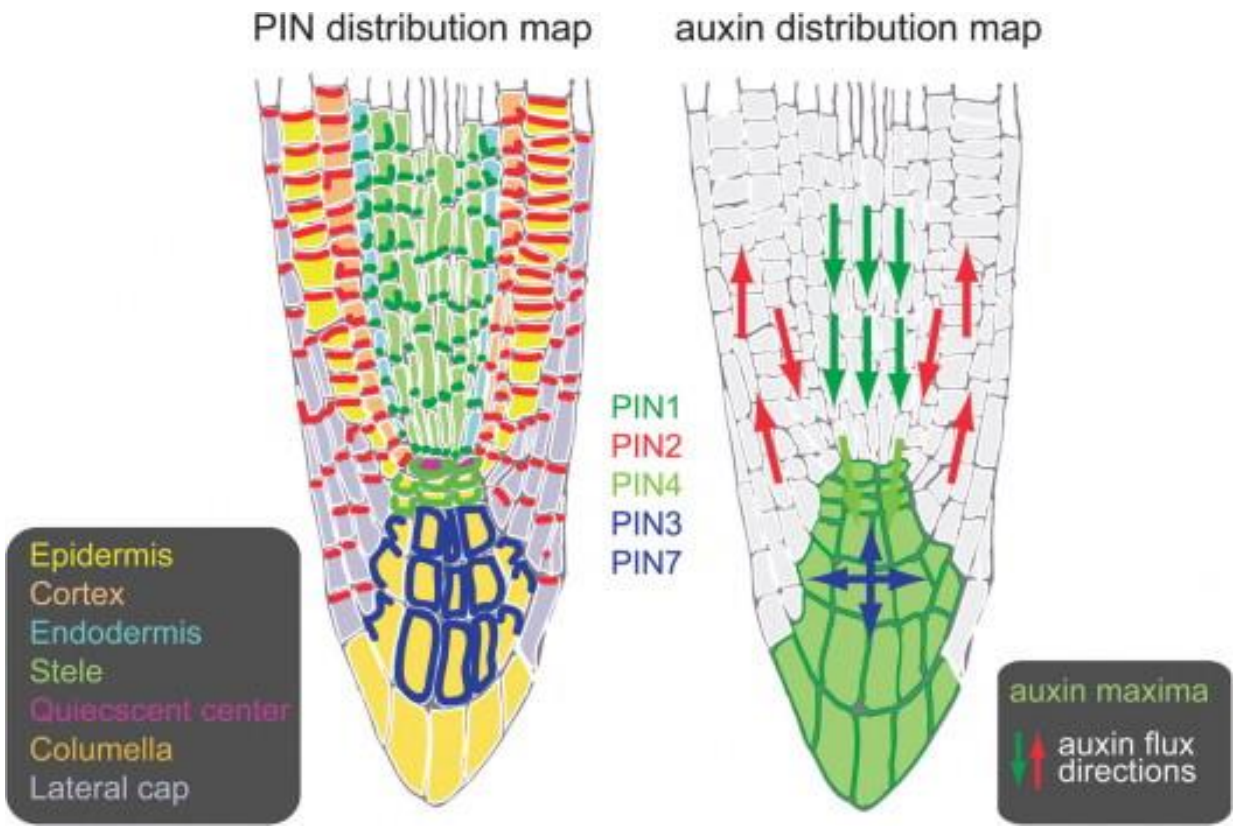

Figure 1.3. Distinct Polarization of PIN Proteins in Arabidopsis roots: PIN proteins in the stele such as PIN1 indicate a basal (root tip-orientated) polarization. In contrasts, PIN2 in the epidermal cells shows an apical (shootwards) localization. PIN3 and PIN7 are largely non-polarly localized in the gravity-sensing columella cells but undergo polarization in response to stimuli. The polar distributions of PINs determine the auxin flow direction. (Taken from Rosquete *et al.* 2012).

The ABCB transporter family also regulates auxin efflux but is less well studied. Members of the family modulate auxin transport in a non-directional manner and do not have a high level of polar localisation. Recent research has indicated that ABCBs facilitate auxin efflux independently of PINS; however, PIN-mediated efflux is predominantly through a co-dependent efflux where PINs are co-localised with ABCBs (Mellor *et al.* 2022).

The AUX1/LAX family of carrier proteins are responsible for auxin influx into cells (Swarup *et al.* 2001). The most well studied of the family is AUX1. AUX/LAX protein carriers play a role in determining orientation and level of cell division in the root meristem

Auxin biosynthesis is a tightly regulated process contributing to control of root development, working synergistically with auxin transport to generate an auxin maximum. Local auxin production

in roots is required for maintaining functional root meristems (Brumos *et al.* 2018). Indole-3-acetic acid (IAA), the best characterized form of auxin, is predominantly produced from the aromatic amino acid L-tryptophan (Trp) via indole-3-pyruvic acid (IPyA) in a two-step pathway (Brumos *et al.* 2014). Trp is converted into IPyA (indole-3-pruvate) by the TAA1 (L-tryptophan pyruvate aminotransferase) family of amino-transferases such as WEI8, SAV3, TIR2, TAR1 and TA2 (Stepanova *et al.* 2008). IPyA is then converted to IAA by the YUCCA (YUC) family of flavin monooxygenases (Zhao *et al*. 2014). There are other biosynthesis pathways, but the Trp-dependent is the most prevalent (Wang *et al.* 2015)

The catabolism, conjugation and oxidation of auxin has a vital role in determining the actual level of active IAA, leading to control of plant development.

Auxin can be inactivated via a variety of pathways that are strictly coordinated with biosynthesis and transport. The larger proportion of IAA is stored in its conjugated form and is only made available when needed in developmental processes (Ludwig-Muller *et al.* 2011). It is often thought that the most important inactivation pathway is the oxidation of IAA, as oxIAA is the most abundant IAA metabolite in *Arabidopsis* (Zhang and Peer 2017). DIOXYGENASE FOR AUXIN OXIDATION (DAO1) is a member of the 2-oxyglutarate and Fe(II)-dependent oxygenase superfamily. DAO1 coverts IAA to oxIAA in vitro (Porco *et al.* 2016). *dao1-1* mutants display auxin-accumulation phenotypes, although overexpression of *DAO1* did not lead to obvious auxin-deficiency (Porco *et al.* 2016, Zhang *et al.* 2016).

The GH3 family of Acyl amidosynthetases are able to catalyze the conjugation between IAA and amino acids (Staswick *et al.* 2005). Six GH3 genes (GH3.1-GH3.6), induced by auxin and mainly producing IAA-Asp, likely play regulatory roles in maintaining active auxin concentrations (Staswick *et al.* 2005). GH3.9 and GH3.17 function in basal IAA inactivation, encoding for enzymes that catalyze the formation of IAA-Glu (Khan and Stone 2007). Overexpression of GH3.6 leads to severe auxin-deficient phenotypes, whereas loss-of-function mutants exhibited slightly impaired phenotypes in auxin-regulated development processes (Takubo 2020, Hayashi *et al.* 2021).

IAA-Leu-Resistant1 (IRL1) and members of the Arabidopsis IRL1-like (ILL) family convert various IAA-amino acid conjugates to IAA in vitro. IAA-Asp and IAA-Glu are reversible forms of storage for IAA which is converted back via IRL1/ILL enzymes (Hayashi *et al.* 2021).

Recent work has shown that IAA inactivation is regulated and coordinated by a GH3-IRL1-DAO pathway, where IAA is mainly inactivated by GH3 enzymes to form IAA-amino acid conjugates. IRL1/ILL enzymes can then revert IAA-Asp and IAA-Glu back to free IAA. IAA-amino acid conjugates are then irreversibly converted to oxIAA-amino acid conjugates (oxIAA-Asp & oxIAA-Glu) by DAO1.

The final form of inactive oxIAA is hydrolyzed from oxIAA-amino acids by IRL1, not from direct oxidation of IAA (Hayashi *et al.* 2021). High IAA accumulation in the *dao1-1* mutant is due to the release of IAA from IAA-Asp and IAA-Glu and that ILR1 and IAR3 primarily contribute to this conversion (Hayashi *et al.* 2021).

1.3.2 Ethylene and POLARIS

Ethylene is a gaseous hormone that plays a vital role in plant growth, fruit ripening and root development along with stress responses (Kieber *et al.* 1997). In the majority of plant tissues, ethylene causes a reduction in growth via inhibiting cell elongation, mainly though crosstalk with auxin (Vaseva *et al.* 2018, Vandenbussche *et al.* 2012). Exogenous application of ethylene leads to short, wide roots with long root hairs (Strader *et al*. 2010).

Ethylene has a strong interaction with the auxin pathway. Ethylene has been shown to increase the rate of auxin biosynthesis in *Arabidopsis* (Stepanova *et al.* 2007) whilst auxin mutants (*wei2*, *aux1*, *pin2*, *axr1* and *tir1*) show strong ACC insensitivity and ethylene mutants (*ein2*, *ein3* and *eil1*) are sensitive to exogenous auxin (Stepanova *et al.* 2007). Auxin and ethylene likely work in a reciprocal regulatory loop, with several levels of interaction (Vaseva *et al.* 2018)

The epidermis is the main site of ethylene control of plant growth in both roots and shoots and is where ethylene acts a central negative regulator of the feedback loop that controls cell elongation via restricting auxin (Vaseva *et al.* 2018).

Ethylene is perceived by five ethylene receptors, ETR1, ETR2 ERS1, ESR2 and EIN4. In absence of ethylene, ETR1 and CTR1 are activated. CTR1 phosphorylates EIN2, maintaining its ER localisation and inhibiting its function (Ju e*t al.* 2012). As EIN2's carboxyl terminal end is unable to be cleaved and stabilize EIN3, EIN3 is targeted for degradation. If ethylene is present, ETR1 and CTR1 are inactive, therefore EIN2 is not phosphorylated. As a result, EIN2 has its C-terminal end cleaved and is translocated to the nucleus where it inhibits the degradation of EIN3. EIN3 is then able to directly activate the transcription of ETHYLENE RESPONSE FACTOR (ERF1) leading to the promotion of the ethylene responsive transcription pathway and ethylene response (Solano *et al.* 1998).

POLARIS is a 36 amino acid peptide that acts as a negative regulator of ethylene responses (Casson *et al*. 2002, Chilley *et al.* 2006). It acts with both auxin and ethylene pathways with PLS transcription enhanced by auxin and repressed by ethylene (Casson *et al.* 2002). Plants with a *pls* mutation display normal levels of ethylene biosynthesis, however they display enhanced ethylene responses including short roots. The short-root phenotype *pls* mutant can be restored by inhibiting ethylene perception pharmacologically or crossing the *pls* mutant with gain-of-function ethylene resistant mutant *etr1-1,*

implying that PLS acts at the level of receptor (Chilley *et al.* 2006). PLS has been suggest to be critical in auxin-cytokinin homeostasis that modulates root growth and leaf patterning (Casson *et al.* 2002).

Ethylene receptor ETR1 and PLS interact directly with each other, co-localising to the endoplasmic reticulum (Mudge 2016). ETR1 requires a copper cofactor to bind to ethylene (Rodriguez *et al.* 1999) whilst PLS is able to bind to copper *in vitro* (Mudge 2016). PLS likely acts a negative regulator of ethylene signalling via regulating the ETR1 receptors interaction with its copper cofactor (Mudge *et al.* 2016).

There remains a number of key questions about the ethylene pathway and what role POLARIS plays. In particular how PLS interacts with other hormones that are key in development such as the cytokinin pathway.

1.3.3 Cytokinin

Cytokinin is an important regulator of meristem function and growth (Werner *et al.* 2001). Biosynthesis takes place in the root and is transported through the vasculature to other areas of the plant (Antoniadi *et al.* 2015). Plants that are deficient in cytokinin display increased root growth and larger root meristems, whereas increasing cytokinin leads to the opposite effects (Dello Ioio *et al.* 2007).

Cytokinin exerts control of root growth via altering meristematic cell differentiation (Dello Ioio *et la.* 2007, Dello Ioio *et al.* 2008) and altering cell division (Skoog *et al.* 1965, Schaller *et al*. 2014). Increasing levels of cytokinin lead to increased QC cell division (Zhang *et al.* 2013). Cytokinin triple receptor mutant displays severe phenotypes such as extreme reductions in root and shoot growth (Nishimura *et al.* 2004).

Auxin and cytokinin are thought to antagonistically interact with each other in a cell specific way, leading to control of root growth and meristem activity (Antoniadi *et al.* 2015, Schaller *et al.* 2015). In the TZ, auxin signalling repressor SHY2/IAA3 has been shown to be upregulated by various type-B ARR proteins. These are transcription factors that are produced downstream of cytokinin signalling (Dello Ioio *et al.* 2008, Moubayidin *et al.* 2010).

Cytokinin is perceived by histidine kinase receptors (AHK2, AHK3, AHK4) which auto-phosphorylate in the presence of cytokinin (Suzuki *et al.* 2001, Yamada *et al.* 2001). This leads to the transfer of the phosphoryl group to a member of the ARABIDOPSIS HIS PHOSPHOTRANSFER (AHP1-5) family, which activate type-A and B ARABIDOPSIS RESPONSE REGULATORS (ARR) by phosphorylation (Tanaka *et al*. 2004).

Type-B ARRs that are activated function as transcription factors promoting cytokinin responsive gene expression (Mason *et al.* 2005). Type-A ARRs inhibit cytokinin signalling in a negative feedback loop (To *et al.* 2007). TCSn:GFP is a synthetic cytokinin reporter for that is useful for determining cytokinin patterning in the root tip (Liu and Müller 2017).

How cytokinin interacts with other hormones remains an area that requires more research. For example, cytokinins interaction with ethylene has received little attention (Iqbal *et al. 2017*). Previously it has been shown that *pls* roots are hyperresponsive to exogenous cytokinins, along with showing increased expression of cytokinin-inducible ARR5 when compared to WT. This indicates that cytokinin increases in levels under the enhanced ethylene response seen in the *pls* mutant (Casson *et al.* 2002).

1.3.4 Abscisic Acid (ABA)

Primarily associated with stress responses in plants, abscisic acid (ABA) accumulates rapidly during unfavourable conditions such as drought (Cutler *et al.* 2010, Rowe *et al.* 2016). ABA also plays a vital role in seed dormancy, germination, stomatal response, and root growth (Finkelstein *et al.* 2008, Finkelstein 2013, Harris *et al.* 2015). Although mainly synthesised in the shoots, ABA has a crucial role in the root maintaining quiescence in the stem cell niche along with helping to regulate cell differentiation and elongation (Zhang *et al.* 2010, Osakabe *et al.*2014).

At high concentrations ABA will cause an inhibition of root growth (Sun *et al.* 2018). ABA has an effect on adaptive root development such as root gravitropism and lateral root development (Li *et al.* 2020). Higher levels of ABA inhibit lateral root development (Li *et al.* 2020). ABA exerts control of root hydraulic conductance, as an increase in ABA levels rapidly increases hydraulic conductivity (Rosales *et al.* 2019)

Exogenous application of ABA can have a complex biphasic on root growth (Li *et al.* 2017). In well-watered conditions, high levels of exogenous ABA inhibit shoot and root growth (van der Weele 2000, Rowe *et al.* 2016). Application of low concentrations of ABA in well-watered conditions can increase root growth (Mulkey *et al.* 1983). There is a 'bell-shaped' dose-dependent response to ABA application, with an increase in primary and lateral root length at low ABA concentration and inhibition at the higher concentrations of ABA application (Rosales *et al.* 2019). Root hydraulics also have a bell-shaped dependency on ABA exogenous concentrations (Rosales *et al.* 2019).

As depicted in Figure 1.4, under stressful conditions ABA levels increase and it binds with the PYRABACTIN RESISTANCE1 (PYR)/PYRABACTIN RESISTANCE1-LIKE (PYL)/REGULATORY COMPONENT OF ABA RECEPTOR (RCAR) family of ABA receptors (now abbreviated as PYL). This triggers a

conformational change in PYLs that leads to interactions with clade A protein phosphatase 2C (PP2C). This interaction leads to PP2C activity being inhibited, reducing its interaction with protein kinases such as SUCROSE NON-FERMENTING 1 (SNF1)-RELATED PROTEIN KINASEs (SnRKs), GUARD CELL HYDROGEN PEROXIDE-RESISTANT1 (GHR1), CALCIUM-DEPENDENT PROTEIN KINASEs (CIPKs). This releases them to phosphorylate a range of downstream proteins that initiate ABA responses. This includes activating or inhibiting via phosphorylation a group of transcription factors called ABRE-BINDING proteins/ABRE-BINDING FACTORs (AREB/ABFs) along with membrane proteins including SLOW ANION CHANNEL-ASSOCIATED (SLAC1) (Sheard & Zheng 2009, Fujii *et al.* 2009).

There is also an associated novel protein phosphatase 2A (PP2A) signalling pathway. Without ABA present, PYLs promote PP2A activity which counteracts the PINOID (PID)-mediated phosphorylation of PIN proteins. ABA binds to PYL, PP2A activity is inhibited thereby increasing phosphorylation of PIN proteins and inhibiting directional auxin transport (Li *et al.* 2020)

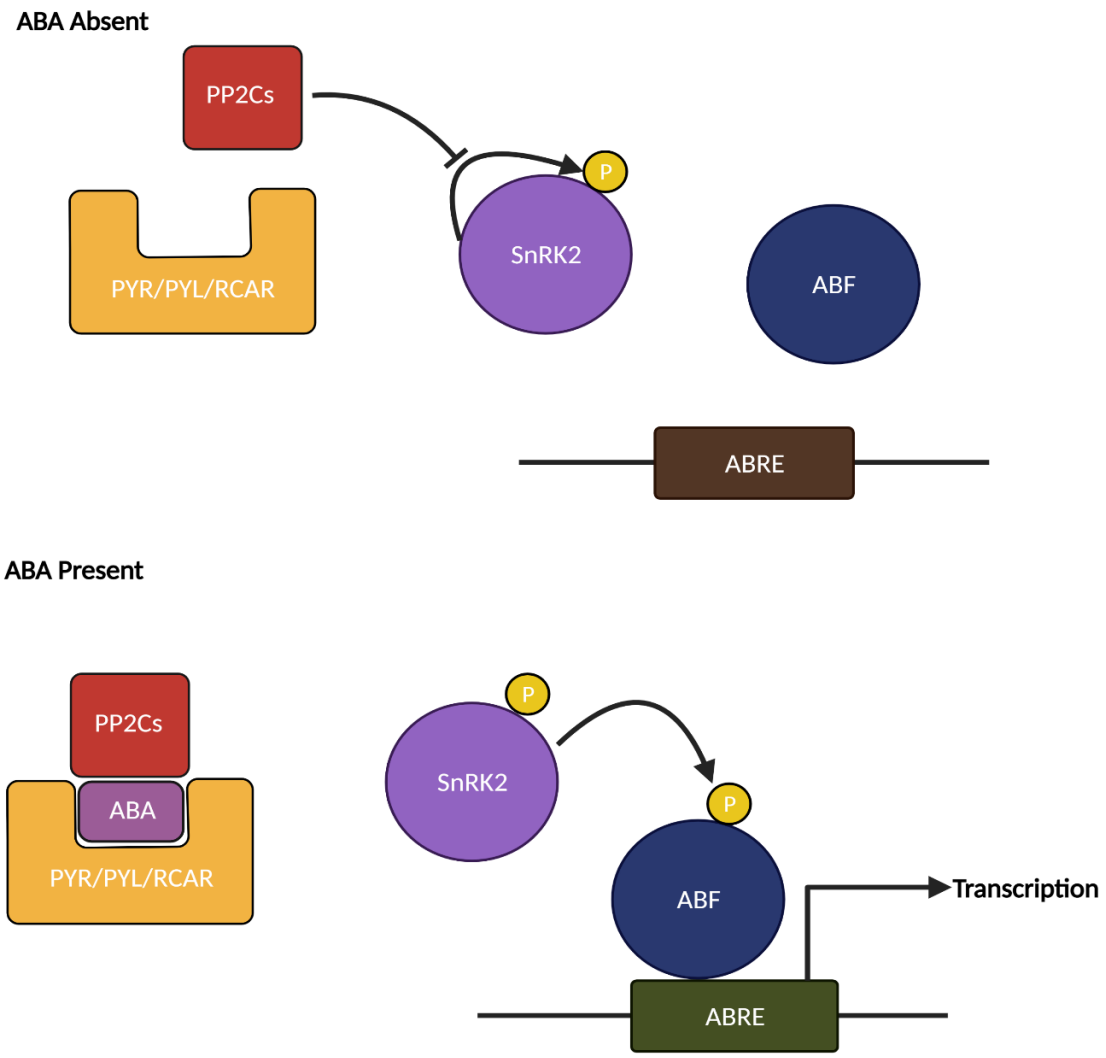

Figure 1.4. The signalling cascade of abscisic acid (ABA). In the absence of ABA, protein phosphatases (PP2Cs) inhibit the phosphorylation of SnRK2 kinases. When ABA is present, PYR/PYL/RCAR family is able to bind and sequester PP2C. As SnRK2 kinases are no longer inhibited by PP2C, they are able to phosphorylate and activate transcription factors (ABFs) which then bind to ABA-responsive promoter elements (ABREs) leading to transcription (Sheard & Zheng 2009).

ABA in higher plants is synthesized via the mevalonic acid-independent pathway. The major source of ABA is β-carotene (C_{40}) which is cleaved via an oxidative reaction in plastids. The next step in the pathway is the conversion of zeaxanthin and antheraxanthin to all-*trans*-violaxanthin, which is catalysed by zeaxanthin epoxidase (ZEP) in the plastid. All-*trans*-violaxanthin is converted to 9-*cis*-violaxanthin or 9'-*cis*-noexanthin by the 9-*cis*-epoxy carotenoid dioxygenase (NCED) which yields a C15 intermediate product called xanthoxin (Schwartz *et al.* 1997). The xanthoxin produced is then

exported into the cytosol where it is converted into ABA. In this step there are two enzymatic reactions, first xanthoxin is converted to an ABA aldehyde by an enzyme called short-chain alcohol dehydrogenase/reductase (SDR). This is encoded by the *ABA2* gene in *Arabidopsis thaliana*. The next and final step of ABA biosynthesis is oxidation of the abscisic aldehyde to ABA, catalysed by the abscisic aldehyde oxidase (AAO) (González-Guzmán *et al.* 2002, Seo *et al.* 2000, Mehrotra *et al.* 2014, Sah *et al.* 2016).

The movement of ABA between cells, tissue and organs is key to the whole plant response to stressful conditions. There are two major forms of ABA transport, passive and active. As ABA is a weak acid it is able to diffuse across plasma membranes in its protonated form (ABAH) (Kuromori *et al.* 2018). The local pH can have an impact on the levels of ABAH available therefore leading to a decline/increase in passive transport relative to pH levels. Due to the limitations of passive transport under pH, active transporter function is required for proper transportation of ABA, particularly under drought stress (Boursiac *et al.* 2013)

The ABC family of ATP-binding cassette transporter proteins are the primary transporters of ABA in *Arabidopsis* (Kang *et al.* 2010). There are eight sub-families reported, specifically the ABCG family play an important role in ABA movement. Both AtABCG25/WBC26 (half sized) and AtABCG40 (full sized) ABCG transporters play a key role in ABA movement (Kuromori *et al.* 2010). The half-sized transporter is expressed in the vascular tissues where it acts as an exporter of ABA along with a role in ABA biosynthesis (Kuromori *et al.* 2010). Full-sized transporter is needed for ABA importing and the movement of ABA into stomatal cells (Kang *et al.* 2010, Kuromori *et al.* 2010). The importance of full-sized transporter ABCG40 was shown whereby a reduced length mutant form showed reduced sensitivity to ABA and performed worse under osmotic stress conditions (Kang *et al.* 2010). Over expression of AtABCG25/WBC26 and AtABCG40 helped reduce water loss by promoting ABA accumulation in guard cells (Kuromori *et al.* 2010).

ABA-IMPORTING TRANSPORTER (AITI) is a secondary transporter of ABA. Previously identified as a nitrate transporter (NRT1.2), it belongs to the NRT1/PTR (Nitrate transporter1/Peptide transporter) family (Kanno *et al.* 2012). AITI plays an important role in ABA transport in vascular tissues of roots, stem and leaves. The loss of function of this transporter resulted in water loss during drought (Kanno *et al.* 2012).

Another secondary ABA transporter is DTX50 which belongs to DTX/MATE (Detoxification Efflux Carriers/Multidrug and Toxic Compound Extrusion) family. Expressed mainly in the vascular tissues and guard cells, it is strongly upregulated under exogenous ABA application (Zhang *et al.* 2014). *dtx50* mutant plants were found to be more sensitive to ABA in growth inhibition (Zhang *et al.* 2014).

ATDTX50 likely mediates ABA efflux from the cytosol of vascular and guard cells located in the plasma membrane (Zhang *et al.* 2014).

If stress signals have become diminished, ABA is then metabolized into inactive products. There are two major pathways in which the metabolism takes place: hydroxylation and conjugation (Nambara and Marion-Poll 2005). Hydroxylation takes place via the oxidation of three methyl groups (C-7', C-8' and C'9) on the ring of the ABA structure. C-8' is the most dominant catalytic pathway, where phaseic acid (PA) and dihydrophaseic acid (DPA) are the most abundant ABA catabolites (Okamoto *et al.* 2009, Nambara and Marion-Poll 2005).

ABA conjugation plays a key role in the regulation of ABA in both normal and stress conditions (Xu *et al.* 2012). Both ABA and its hydroxylated catabolite form can be conjugated to glucose. ABA can be synthesized into ABA glucosyl ester (ABA-GE) by glycosyltransferase within the cytosol, and then stored into vacuoles (Boursiac *et al.* 2013). Under stressful conditions, ABA glucosyl ester can then be converted back into ABA by enzyme-catalysed hydrolysis. ABA glucosyl ester (ABA-GE) can be transported by proton-driven gradient or ABC transporters (Xu *et al.* 2012, Burla *et al.* 2013).

PROLINE-RICH EXTENSIN-LIKE RECEPTOR KINASE 4 (PERK4) plays important role in ABA responses (Ma *et al.* 2019). PERK4 functions by perturbing calcium (Ca^{2+}) homeostasis in response to ABA. Acting at an early stage of ABA signalling to modulate root cell elongation, mutants of PERK4 display attenuated sensitivity to ABA. ABA-induced increase in cytosolic Ca^{2+} in root cells is lower in perk4 mutants than in wild type. Recently PERK4 has been shown to be involved in ABA-stimulated ROS production, partially mediated by RBOHC. In both *perk4*, *rbohc* and *perk4/rbohc* mutants, ABA-induced ROS production is impaired (Ma *et al.* 2019). Upon ABA treatment, it is likely that PERK4 stimulates ROS accumulation through an interaction with RBOHC which in turn inhibits primary root growth. If PERK4 is not functioning, normal ROS accumulation does not occur, and ABA signalling is blocked (Ma *et al.* 2019).

1.4 Reactive Oxygen Species (ROS)

The production of reactive oxygen species (ROS) including superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH*) and singlet oxygen ($^1O_2$) is necessary for many major biological processes (Zhou *et al.* 2020, Nadarajah 2021). Basal and localized ROS production is required for processes such cell polarity and expansion and events such as root hair growth, Casparian strip formation and pollen tube elongation. Most notably, ROS act as a signalling molecule in multiple systems including abiotic and biotic stress responses (Huang *et al.* 2016, Huang *et al.* 2019). However, the production of too much ROS can cause oxidative stress which leads to damage of DNA,

lipids, and proteins. At a certain point, this can lead to the inhibition of growth and cell death (Van Breusegem & Dat 2006). As a result, plants have developed rapid detoxification and scavenging systems that tightly regulated ROS levels (Sofo *et al.* 2015). The balance of ROS production and ROS quenching is vitally important (Apel and Hirt 2004).

ROS are often produced at a low level as a toxic by-product of electron leakage during energy-generating processes such as respiration and photosynthesis (Choudhury *et al.* 2017). This low level can quickly be elevated when growth conditions are not optimal (Fu *et al.* 2021). ROS production is often a response to external cues including abiotic stress such as temperature, drought, salinity, and excess light (Hasanuzzaman *et al.* 2020). Biotic stresses such as pathogen infection leads to enhanced ROS production, often resulting in cell death of plants cells in response (Qi *et al.* 2017).

ROS production quickly responds to stimuli leading to local and systemic signal transduction (Mittler *et al.* 2011). Tightly linked with calcium signalling, ROS production in response to stress is strictly coordinated and regulated in different subcellular compartments and regions of the plant (Mazars *et al.* 2010, Niu and Liao 2016, Marcec & Tanaka 2021). The specificity of ROS signalling is achieved in some way by the production of ROS in different subcellular compartments (Kohli *et al.* 2019, Janku *et al.* 2019).

Intracellular ROS accumulation is largely due to by-products of metabolic activities in chloroplasts and mitochondria (Waszczak *et al.* 2018, Li *et al.* 2022). Plastid-produced ROS are important signals within cells. *Arabidopsis* NAC domain-containing protein 17 (ANAC017) transcription factor is located upon the endoplasmic reticulum. Upon increasing levels of H_2O_2 within the cell, it is translocated to the nucleus where it alters nuclear gene expression (Ng *et al.* 2013). A large portion of chloroplast ROS-dependent retrograde signalling involves singlet oxygen, which is primarily generated by photosystem II as a waste product of photosynthesis (Kim 2020).

Apoplastic ROS is primarily produced by plasma membrane-localized NADPH oxidases knows as respiratory burst oxidase homologues (RBOHs) along with cell wall peroxidases (Huang *et al.* 2019). *Arabidopsis* possesses 10 RBOH isoforms which play key roles in developmental processes such as root hair formation and pollen tube growth (Torres and Dangl 2005, Morales *et al.* 2016). In response to endogenous signals, RBOH enzymes are induced into ROS production. For example, RBOH proteins are involved in pathogen defence, where pattern recognition receptor activation leads to apoplastic ROS production through RBOH and peroxidase activity (Kadota *et al.* 2015, Daudi *et al.* 2012).

Of the variety of ROS species that are produced, H_2O_2 is considered the key signalling molecule. This is due to its long half-life and its similar structure to H_2O. H_2O_2 has an ability to oxidize proteins and capacity to move across plasma membranes (Mhamdi and Van Breusegem 2018). After passing through transporters such as *Arabidopsis* plasma membrane intrinsic protein 1;4 (PIP1;4) and PIP2;1, extracellularly produced ROS can react with intracellular proteins or be detoxified by scavenging systems (Tian *et al.* 2016). Interestingly, RBOH proteins produce superoxide which is thought to either act in the extracellular space or be dismutated to H_2O_2 which then can pass through aquaporins (Waszczak *et al.* 2018, Bienert *et al.* 2014). There are likely hotspots of ROS providing signalling specificity within the cytosol and chloroplasts, however it is challenging to visualise distinct ROS with high resolution.

To prevent overaccumulation of ROS, plants have a well-developed antioxidative mechanism consisting of enzymatic and non-enzymatic components (Dumanovic *et al.* 2021). These processes balance ROS synthesis and scavenging along with preventing overaccumulation and the resulting cellular damage.

Antioxidant systems such as superoxide dismutase (SOD), catalase (CAT) and peroxidases (POX) regulate ROS homeostasis within organisms (Kliebenstein *et al.* 1998, Mhamdi *et al.* 2010). These enzymes are involved in the reduction of O_2- to H_2O_2. Non-enzymatic components involved in the antioxidation mechanism are ascorbic acids, flavonoid, glutathione, carotenoids, lipids and phenolic compounds which help mitigate oxidative damage by reducing ROS activity or by working with enzymes. Glutathione peroxidases (GPXs) play an important role in scavenging ROS. The family comprising of 7 members are ubiquitously expressed and are regulated by abiotic stress through diverse signalling pathways (Hossain *et al.* 2015, Miao *et al*. 2006)

In certain cell types such lateral root primordia, root hair and xylem, ROS are produced by active mechanisms where they play a positive role in cell differentiation (Gapper and Dolan 2006, Mangano *et al.* 2016, Orman-Ligeza *et al.* 2016). ROS are required for the formation of specific cell type features such as the Casparian strip which is critical for selective nutrient uptake and pathogen defence in root systems (Fujita *et al.* 2020)

The specific temporal-spatial distribution of ROS has a significant impact on cellular process and root system development via interacting with hormone signalling processes. The QC and the RAM are associated with an oxidized environment whereas the elongation zone is more reduced (Dunand *et al.* 2007, Tsukagoshi *et al.* 2010). In *rml1* or *app1* mutants, where redox potential in the RAM is altered, the RAM is lost (Yu *et al.* 2016, del Pozo 2016, Vernoux *et al.* 2000). The elongation zone is also altered by redox status, and the *upb1* mutant has a larger RAM due to a more reduced redox

status in the elongation zone. Cell elongation is inhibited when the EZ is more oxidized, resulting in a shorter root (Tsukagoshi *et al.* 2010, Mabuchi *et al.* 2018).

ROOT MERISTEM GROWTH FACTOR1 (RGF1) plays a key role in meristem size via regulating ROS distribution along developmental zones. Changes in ROS distribution lead to altered stability of the PLETHORA (PLT) AP-2 transcription factors which function as regulators of stem cell maintenance and distal cell division (Aida *et al.* 2004, Licausi *et al.* 2013), acting independently of the auxin pathway (Yamada *et al.* 2020).

ROS signalling regulates MYB30, a key transcriptional regulator that controls root cell elongation (Mabuchi *et al.*2018). MYB30 has prominent expression induction by H_2O_2 in both meristematic and elongation zones (Mabuchi 2018), where it enhances the expression of lipid transfer proteins, LTPG1, LTPG2 and LTP5 (Mabuchi *et al.* 2018). ANAC032 is an upstream transcription factor of the MYB30 regulatory network, and plays an important role in abiotic stress response (Maki *et al.* 2019).

ROS plays a key role in the elongation zone, where cells stop proliferating and start to elongate along the longitudinal axis. This process requires cell wall loosening in which ROS is a critical player. ROS generated in the apoplast are involved in controlling cell wall rigidity (Karkonen and Kuchitsu 2015). Reactive oxygen radicals can enzyme-independently oxidize cell wall polysaccharides via electron transfer (Karkonen and Kuchitsu 2015). Apoplastic H_2O_2 and ROS scavengers can inhibit cell wall elongation (Somssich *et al.* 2016).

Numerous studies have identified the role of ROS as a rapid long-distance signal in the form of an auto-propagating ROS wave (Fichman and Mittler 2020). ROS wave can be induced by biotic and abiotic stimuli, passing a signal through the plant, and causing gene expression changes in distal tissues. ROS wave is seen as a key signal that alerts regions of the plant to stimuli along with acting as a whole-plant coordinator of abiotic and biotic signals. ROS wave works in coordination with Ca^{2+} and pH signalling as a key signal in stress response (Gilroy *et al.* 2016).

ROS wave propagation requires RBOH activity for the transmission of the signal. Apoplastic H_2O_2 leads to the activation of H_2O_2-induced Ca^{2+} increases 1 (HPCA1) leading to an influx of Ca^{2+} into the cytosol (Wu *et al.* 2020). The binding of Ca^{2+} to calcium-dependent protein kinases (CPKs) and RBOH sites leads to an increase in apoplastic ROS production, which could then result in the propagation of the initial signal. High ROS scavenging capacity in the cytosol means it is unlikely intracellular ROS contribute directly to plasmodesmata directed cell-to-cell communication (Cheval *et al.* 2018)

ROS-propagation in addition to its production is required for systemic acquired acclimatization (SAA). SAA is a system-wide response that enables plants to survive upcoming changes to their

environment. This includes metabolic and transcriptomic changes in different parts of the plant essential for plant acclimation, along with physiological responses such as changes in stomatal aperture (Devireddy *et al.* 2020). RBOHD plays key a role in SAA response to light stress. Specific expression of RBOHD in the phloem or xylem parenchyma cells of a *rbohd* mutant restores ROS signalling and SAA to local treatment of light stress (Zandalinas *et al*. 2020). It is suggested that the integration of systemic signals of ROS, calcium, electrical and hydraulic signals in plants takes place in the vascular bundles (Zandalinas *et al.* 2020)

ROS production is also required for systemic acquired resistance (SAR), although the role of ROS wave is more complex. A two-peak reaction of ROS production has been described during pathogen triggered immunity (PTI) (Yuan *et al.* 2020). First is a rapid ROS burst largely controlled by RBOH's which participates in the formation of a ROS wave (Yuan *et al.* 2020). There is a later second effector-triggered immunity (ETI)- enhanced PTI-induced ROS burst that begins several hours after pathogen perception. RBOH activity appears to be required for a second PTI ROS burst. It is less clear if there is a biphasic ROS burst in response to abiotic stress and wounding (Yuan *et al.* 2020). There is a lot of similarity between the two where the accumulation of apoplastic ROS leads to changes in cytosolic Ca^{2+} leading to stress response.

ROS signals are likely perceived via redox modifications of proteins resulting in changes in structure, activity, localization, and protein-protein interactions. This is mainly through the oxidative modification of cysteines (Waszczak *et al.* 2015). ROS can be perceived in both the extracellular apoplastic space and within the cytosol after passing through aquaporins and anion channels. Both extra and intracellular perception of ROS can occur at the same time, perhaps leading to increased sensitivity of ROS perception (Kimura *et al.* 2017).

Apoplastic alkalization can have an important impact on ROS signalling specificity as cysteine residues are readily oxidized at higher pH levels. Biotic and abiotic stimuli can trigger the transient alkalization of the apoplast from a pH of 5 to pH 6-7 via the inhibition of plasma membrane H+-ATPase activity (Geilfus *et al.* 2017). H^+-ATPase activity is altered by phosphorylation and the accumulation of cations in the apoplast (Geilfus *et al.* 2017). This increase in pH leads to increased reactivity of cysteines with ROS. The duration and magnitude of apoplastic alkalization can differ between stress signals, which suggest it may be a key component in ROS signalling specificity (Geilfus *et al.* 2017).

1.4.1 The Role of RBOH proteins

RBOHs play important roles in plant development by producing the highly reactive and unstable superoxide. Superoxide is dismutated either spontaneously or by the action of superoxide dismutase (SOD) to H_2O_2 (Waszczak 2018). The most highly expressed RBOH in *Arabidopsis* is RBOHD which is key for ROS production upon abiotic and biotic stimuli. RBOHF is also highly involved in abiotic stress responses, in particular the regulation of stomatal closure (Kadota *et al.* 2015). RBOH activity is considered to lead to H_2O_2, and as a result RBOH activity is often assessed by measuring H_2O_2 levels post dismutation. As a result, the role of extracellular superoxide as a signalling molecule is underexplored (Castro *et al.* 2021).

RBOHs appear to be spatiotemporally regulated, appearing in tissue specific patterns along with the capacity to be induced under stress stimulus (Hu *et al.* 2020, Morales *et al.* 2016). RBOH enzyme activity is regulated at the post-transcriptional level by the phosphorylation of the N-terminal region at conserved residues. The N-terminal region of RBOHs is a key hub for a number of kinases that induce ROS production. *Botrytis*-induced kinase 1 (BIK1), MAP4K serine/threonine-protein kinase 1 (SIK1) both phosphorylate N terminus leading to RBOH activation (Kadota *et al.* 2014, Li *et al.* 2014, Zhang *et al.* 2018).

The ability for multiple kinases to act on specific and convergent sites enables flexibility in the activation of RBOH enzymes to independent stimuli and developmental stages. Target protein C-terminus is also an important site for RBOH regulation, in particular limiting ROS production. Multiple kinases converge to achieve selective control of RBOH activity (Castro *et al.* 2021). The ubiquitination of RBOH enzymes is also a key factor in their regulation and control of ROS production.

1.5 Rho of plant (ROP) proteins

Unique to the plant kingdom, Rho of plant (ROP) proteins are a subfamily of Rho small GTP-binding proteins (also known as small G proteins) (Feiguelman *et al.* 2018, Eliáš and Klimeš 2012). ROPs act as molecular switches due their ability to change conformation upon GTP-binding and hydrolysis (Feiguelman *et al.* 2018).

Small G protein function has two major characteristics. As GTP hydrolysis can be inefficient, small G proteins can remain in the GTP-bound active form for long periods of time. GDP has a low dissociation coefficient therefore its release is inefficient and requires enzymatic activity (Vetter and Wittinghofer 2001). Due to these features, small G proteins cycles of GTP/GDP dependent

activation/inactivation are regulated by time and space by GDP/GTP Exchange Factors (GEFs) that facilitate the release of GDP and GTPase-Activating Proteins (GAP) that increase GTP hydrolysis (Berken and Wittinghofer 2008). Rho GDP Dissociation Inhibitors (RhoGDIs) play an important role in maintaining ROPs in distinct plasma membrane domains (Garcia-Mata *et al.* 2011)

Altering between the GTP and GDP-bound states enables ROP proteins to interact with effector and regulatory proteins that result in the periodic activation/inactivation cycles of signalling cascades. Their spatial regulation is important as ROPs can act as switches that convert intracellular and extracellular stimuli resulting in localized regulation of intracellular responses (Figure 1.5) (Feiguelman *et al.* 2018).

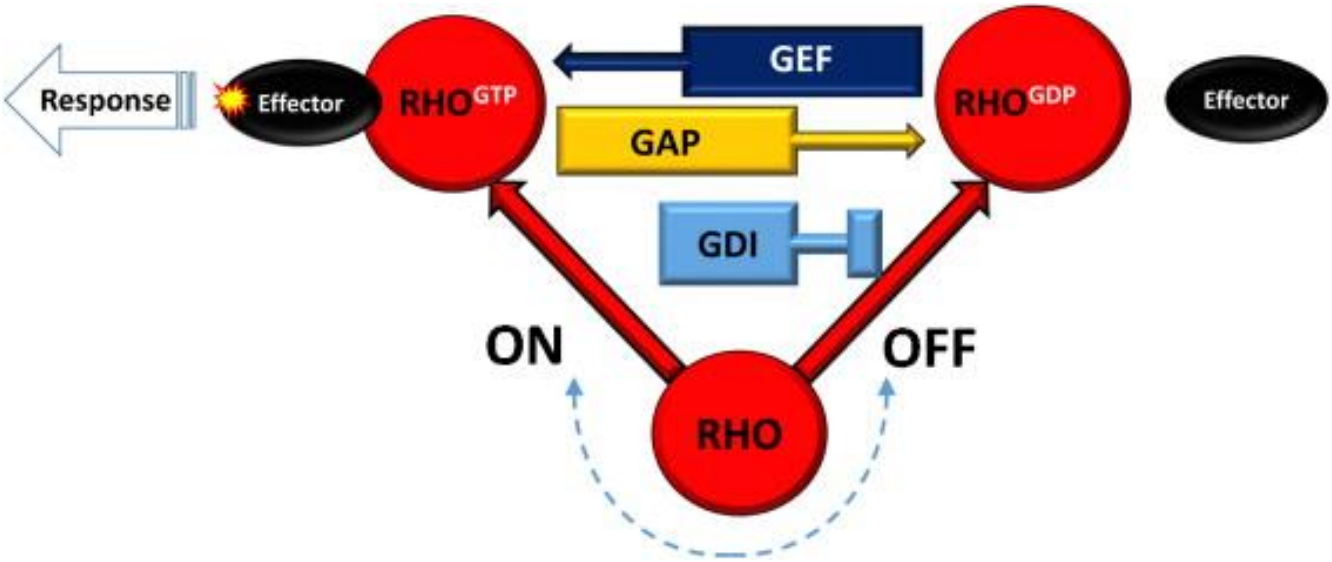

Figure 1.5. Rho of plant (ROP) proteins act as two-state molecular switches. When in GTP-bound confirmation, they are turned "ON", and are capable of binding and activating various effector molecules leading to specific cellular responses. The hydrolysis of the bound GTP results in the ROP protein turning "OFF", where effectors are released and/or inactive. The exchange of GDP to GTP can result in the protein reverting back into "ON" state. Guanine nucleiotide factors (GEFs) exchange factors (GEFs) enhance the GDP-to-GTP exchange, switching the ROP protein "ON". GTPase accelerating proteins (GAPs) promote GTP-hydrolysis that switches the protein "OFF". Guanine nucleotide dissociation inhibitors (GDIs) stabilize the "OFF" state. (Figure taken from Feher and Lajko 2015).

ROPs have a vast range of functions including regulating cell organization, growth, and shape via altering actin and the microtubule cytoskeleton (Hashimoto 2015, Takatsuka and Ito 2020). They regulate the activation of RBOHs and intracellular kinase cascades along with having a role in the regulation of ROS, ABA and auxin signalling and transport (Wu *et al.* 2011, Feiguelman *et al.* 2018).

ROPs play a key role in biotic and abiotic signalling. There is a large amount of evidence suggesting that ROPs and the ABA pathway form a negative feedback loop where ABA signalling supresses ROP

activation, and ROP signalling supresses ABA responses (Li and Liu 2012, Yu *et al* 2012). ROP10 and ROP11 inhibit ABA signalling by their physical interaction with ABA negative regulators ABI1 and ABI2 PP2C phosphatases. ROP interaction prevents ABI1/2 ABA-dependent inactivation by PYR/PIL ABA receptors (Li and Liu 2012, Yu *et al.* 2012). ABA inhibits ROP activation by promoting the degradation of several ROPGEFs. ROPGEF1 interacts with ABI1 in yeast and in vitro and is degraded in response to ABA (Li *et al.* 2016)

ROP2 is a key regulator in root hair growth and initiation (Jones *et al.* 2002) along with being involved in stress responses such as light-induced stomatal opening (Hong *et al.* 2015). ROP2 inactivation via ABA was found to critical for timely stomatal closure. The constitutively active form of ROP2 (CA-rop2) in *Arabidopsis* acts to reduce and slow stomatal closure in response to abscisic acid (ABA). Knockout of ROP2 lead to promotion of ABA-induced stomatal closure (Hwang *et al.* 2011).

ROP6 is required for auxin signalling (Platre *et al.* 2019). ROP6-mediated PIN targeting is vital for root gravitropic response (Platre *et al.* 2019). Furthermore, ROP6 has been shown to be master regulator of osmotically induced ROS accumulation, where osmotic stress stimulates ROP6 nanodomain formation within minutes. The *rop6.2* mutant has totally abolished osmotically induced ROS production (Smokvarska *et al.* 2020). These nanodomains are required for a correct spatial ROS accumulation in cells via interactions with RBOHD and RBOHF. *Rop6.2* loss of function mutant has longer primary and lateral roots in osmotic stress (Smokvarska *et al.* 2020). ROP6 nanoclusters formed after auxin or osmotic stimulation can be different in their formation, and therefore could encode signal specificity. Loss of function of ROP6 results in a reduction in osmotically induced lignin deposition in roots. The polymerisation of lignin requires cellular ROS, produced by NAPDH oxidases such as RBOHF. It is likely osmotically induced lignin to protect cells from deformation, along with mineral and water leakage is mediated by ROP6/RBOHs nandomains (Smokvaska *et al.* 2020). ROP6 likely acts immediately after cell osmotic perception, as nanoformation is only minutes after osmotic stimulation (Smokvaska *et al.* 2020).

1.6 Crosstalk between ABA, Auxin and ROS

Hormones interact with each other in a complex way, and they also interact with ROS. Each crosstalk interaction can alter the functions of each hormone. It is a challenge to summarise all hormones (such as cytokinin, ethylene and brassinosteroids (BRs)) and the interactions between them. As a result, the more hormones included the greater the complexity is. The crosstalk between ABA, auxin and ROS is the focus of my project as they play a critical role in plant development in response to abiotic stress.

1.6.1 ABA and ROS Interaction

Both critically involved in stress response, ROS and ABA have an intricate relationship with each other, regulating plant development and signalling. ROS have often been considered as amplifiers of the ABA signal, acting downstream as second messengers during process such as stomatal closure (Sierla *et al.* 2016, Rajab *et al.* 2019). This has been revealed by the study of ABA and ROS interactions in stomatal guard cell signalling (Postiglione and Muday 2020). Comparatively there has been a small amount research on how these two pathways interact in the roots and in long term development.

There are a large number of key areas where ABA and ROS interact, but they cannot all be described in detail here. There are a number of reviews which better detail their interaction (Mittler and Blumwald 2015, Postiglione and Muday 2020).

ABA induced ROS-accumulation has been repeatedly shown by a number of studies, with some reporting that takes places within minutes (Pei *et al.* 2000, Watkins *et al.* 2017). ROS has been proposed to enhance ABA biosynthesis or inhibit ABA degradation, leading to elevation in free ABA levels (Huang *et al.* 2012, Song *et al.* 2014, Mittler & Blumwalk 2015). Enhanced ROS levels could therefore result in enhanced ABA accumulation, creating a positive feedback loop resulting in stress response (Mittler and Blumwald 2015).

The roles of RBOHD and RBOHF in ABA signalling have been characterised. Gene disruption of both impairs ABA signalling, leading to reduced ABA-induced stomatal signalling and a reduction in ABA inhibition of root elongation (Kwak *et al.* 2003). Exogenous application of H_2O_2 rescues stomatal closing and Ca^{2+} channel activation. These results indicate that ROS act as rate-limiting second messengers in ABA signalling (Kwak *et al.* 2003).

ABA-induced H_2O_2 production requires the activation of Ser/Thr protein kinase OST1/SnRK2.6, which plays a positive role in the ABA guard cell response. Although ABA-induced ROS production is absent

from *ost1* mutant plants, *ost1* stomata are still able to close in response to H_2O_2. This indicates that H_2O_2 is likely a signal molecule involved in the regulation of ABA-induced stomatal closure (Mustilli *et al.* 2002, Assmann 2003). OST1/SnRK2.6 phosphorylates transcription factors, anion channels and RBOHF which leads to regulation of ABA-induced stomatal closure (Sirichandra *et al.* 2009, Grondin *et al.* 2015).

PP2C phosphatases ABI1 and ABI2 are important proteins in ABA and ROS signalling (Meinhard *et al.* 2002). Plants defective in ABI1 and ABI2 are insensitive to ABA (Gosti *et al.* 1999). Treatment with ABA did not lead to the production of H_2O_2 in *abi1* mutants, whereas this response was not impaired in *abi2-1*. This suggests that ABI1 might function upstream of ABA and ROS signalling, whereas ABI2 might function downstream (Miao *et al*. 2006). ABI1 and ABI2 have been identified as negative regulators of ABA signalling, and repressors of H_2O_2 responses (Yoshida *et al.* 2006, Umezawa *et al.* 2009, Acharya *et al.* 2013). ABI1 is involved in ROS production in ABA-induced stomatal closure via sulfate treatment. ABA-triggered RBOH ROS production depends on ABI1 but not ABI2, and *ab2-1* mutants are still capable of accumulating ROS under sulphate treatment (Murata *et al.* 2001, Batool *et al.* 2018). The ABI1-OST1/SnRK2.6 phosphorylation relay is essential for the activation of RBOH ROS production, which is vital for stomatal closure under sulphate treatment (Rajab *et al.* 2019).

Glutathione peroxidases (GPXs) are key enzymes that are involved in H_2O_2 homeostasis (Miao *et al.* 2006). ATGPX3 functions both in ABA and ROS pathways. *atgpx3* mutation has high sensitivity to H_2O_2 in seedling development and enhanced production of H_2O_2 in guard cells. The *atgpx3* mutation reduces the expression of ABA and stress-responsive genes, along with disrupting ABA-induced Ca^{2+} channels. ATGPX3 interacts with ABI2, which are both regulated by H_2O_2. ABI2 phosphatase activity was reduced five-fold by the addition of oxidized ATGPX3. The reduced form of ABI2 was converted to the oxidized form by the addition of oxidized ATGPX3 in vitro, which might mediate ABA and ROS signalling (Miao *et al.* 2006). Potentially, mutation of ATGPX3 interrupts H_2O_2 and ABA signalling, thereby blocking the activation of Ca^{2+} channels in response to abiotic stress. It has been suggested that ATGPX3 senses and transduces H_2O_2 signal to downstream components via ABI2, leading to osmotic stress response (Miao *et al.* 2006).

In *Arabidopsis* it has been found that ABA triggers hydrogen sulphide (H_2S) accumulation leading to the persulphidation of RBOHD, enhancing the production of ROS. Persulphidation acts as a specific and reversible redox-based post-translational modification. If it is abolished this leads to reduced ROS production following ABA treatment (Shen *et al.* 2020).

A large amount of research on ROS and ABA interactions has been focused on stomatal response. However, there has been recent other research studying the role of both pathways in root development.

MYB30 controls root elongation through ROS production under ABA-signalling in *Arabidopsis* (Mabuchi *et al*. 2018, Sakaoka *et al.* 2018). Upregulated by ROS, MYB30 targets genes to reduce cell elongation. ABA regulates the MYB30 gene network for root elongation, as MYB30 expression is induced by ABA in the root (Sakaoka *et al.* 2018). The *myb30* mutant shows significant insensitivity to root growth inhibition following ABA treatment (Sakaoka *et al.* 2018). MYB30 protein levels are stabilized by a SUMO E3 ligase, SIZ1, and this stabilization is important in regulating expression of certain ABA response genes (Miura *et al.* 2009). ROS accumulation patterns after ABA treatment were comparable between wild type and *myb30* mutants. This indicates that insensitivity to ABA treatment in the *myb30* mutant was caused by a deficiency in the MYB30 gene regulatory network that was upregulated by the ROS produced by ABA (Sakaoka 2018). This research suggests that MYB30 is likely acting independently of auxin and cytokinin, in a role as a hub between ROS and ABA signalling to regulate root cell elongation.

ROS plays a key role in ABA-mediated inhibition of root growth. RBOHD and RBOHF, sites of apoplastic ROS production in *Arabidopsis* are markedly upregulated in expression by ABA treatment in root systems (Jiao *et al.* 2013). Mutations in *rbohd* and *rbohf* significantly reduced the impact of ABA on root elongation along with reducing the increase in H_2O_2 levels in roots following ABA treatment (Jiao *et al.* 2013). As a result, RBOHD and RBOHF have been suggested to be essential for ABA-promoted ROS production in roots. This ROS production then activates Ca^{2+} signalling and reduces auxin sensitivity in the roots, leading to ABA-inhibition of primary root growth (Jiao *et al.* 2013)

PERK4 plays an important role in ROS production under ABA response. The *perk4* mutants display attenuated sensitivity to the ABA inhibition of root growth. This is due to lack of ROS accumulation in the primary root in response to ABA treatment as PERK4 deficiency prohibits ABA-induction of RBOHC (Ma *et al*. 2019). This indicates that PERK4 and RBOHC are involved in pathway of ABA and ROS interaction influencing root growth.

The presence of ROS is required for the ABA-induced increase in hydraulic activity and aquaporin abundance. Treatment of barley plants with antioxidants reduced the impact of ABA on increasing flow rate of xylem and root hydraulic activity (Sharipova *et al.* 2021).

1.6.2 ABA and Auxin interaction

The effect of auxin and ABA on numerous growth processes has been well-documented. High levels of exogenous auxin and abscisic acid both inhibit root elongation (Fendrych *et al.* 2018, Takatsuka and Umeda 2014, Takatsuka and Umeda 2019). Depending on region/tissue, auxin tends to act downstream of ABA to regulated growth processes (Emenecker & Strader 2020).

ABA reduces growth such as hypocotyl elongation by decreasing the level of auxin (Lorrai *et al.* 2018). Exogenous ABA application reduces the expression of auxin biosynthetic genes, *YUC3*, *YUC5* and *YUC6* (Lorrai *et al.* 2018). An intact auxin signalling, and transport system is required for full responsiveness to ABA's impact on root elongation (Emenecker & Strader 2020). Disruption of auxin transport (*aux1, pin2*) and auxin signalling (*tir1, ibr5, axr1*) leads to resistance to ABA in primary root elongation (Thole *et al.* 2014). Auxin influx mutants (*aux1-7, aux1-T*) and an auxin-insensitive mutant (*iaa7/axr2-1*) were all insensitive to the inhibitory effect of high ABA concentrations (Li *et al.* 2017) Gain of function mutation that stabilizes Aux/IAA protein IAA16 lead to reduction in root responsiveness to ABA (Rinaldi *et al.* 2012).

Auxin is required for both the stimulatory effects of low concentration ABA and inhibitory effects of high ABA on root elongation (Li *et al.* 2017). It appears both auxin influx and efflux are required for the inhibitory effects of high ABA, whereas only auxin efflux is needed for stimulatory effects (Li *et al.* 2017). ABA has been found to have a key role in reducing root growth under osmotic stress via modulating PIN1 levels, leading to a reduction of auxin at the root tip (Rowe *et al.* 2016)

AUXIN RESPONSE FACTOR 2 (ARF2) expression is induced by ABA, *arf2* mutants are more sensitive to the impact of ABA on seed germination and primary root growth, whereas ARF2 overexpression leads to ABA resistance (Wang *et al.* 2011). ABA treatment was found to reduce cell division and alter auxin distribution more in the *arf2* mutant. This indicates that ARF2 is a novel regulator in the ABA pathway, which has crosstalk with auxin in the mediation of cell division in root tips (Wang *et al.* 2011).

Auxin-mediated inhibition of root elongation appears to be independent of ABA signalling. ABA signalling mutants (*abi1-1, abi2-1 and abi3-1)* display wild-type response to synthetic auxin 2,4-D (Thole *et al.* 2014). As a result, it is likely auxin acts downstream of ABA in regulation of root elongation.

The plasma membrane *Arabidopsis* H^+-ATPase 2 (AHA2) regulates apoplastic pH by pumping protons into extracellular space (Hoffmann *et al.* 2019) which is required for cell wall loosening and in turn cell elongation (Emenecker & Strader 2020). AHA2 activity is regulated by phosphoactivation, which

levels are controlled by auxin and ABA (Takahashi *et al.* 2012, Spartz *et al.* 2014). It is hypothesized ABA and auxin likely converge on AHA activity (Emenecker & Strader 2020).

1.6.3 ROS and Auxin interaction

Auxin and ROS interact in a complex way to regulate the development of plant root systems. The connection between both auxin and ROS needs to be further investigated if we are to reach a more complete understanding (Mase and Tsukagoshi 2021).

Primary growth rate of roots decreases when exposed to H_2O_2. If H_2O_2 concentration exceeds 3mM, H_2O_2 levels become toxic and leads to complete arrest of root growth (Orman- Ligeza *et al.* 2016). Upon H_2O_2 treatment, the RAM remains functional and early differentiation in the primary meristem is promoted (Orman-Ligeza *et al.* 2016).

Exogenous application of IAA increases ROS levels in the root tissues with a maximum at the root tip, indicating a possible link between ROS and auxin controlling root growth (Peer *et al.* 2013, Zwiewka *et al.* 2019). IAA induction of ROS is lowered in the *rbohd* mutant, indicating the role RBOHD has a link between auxin and ROS (Peer *et al.* 2013). In both high exogenous IAA and H_2O_2 treatment, roots adapt via accumulation ox-IAA, leading to decreasing cell division and reducing the size of the meristem. This indicates that there is interplay between ROS and auxin in the regulation of cell cycle progression during RAM adaptation to H_2O_2 (Peer *et al.*2013)

Application of H_2O_2 after three days leads to increased expression of auxin reporters DR5:GUS and R2D2, indicating increased accumulation of auxin in the root cap, columella and meristem vasculature (Zwiewka *et al.* 2019). The application of exogenous H_2O_2 leads to the rapid accumulation of auxin at the root apical meristem, along with a decrease in the abundance of PIN auxin efflux carriers (Zwiewka *et al.* 2019). Within a short period of time, H_2O_2 interrupts actin dynamics, thus modulating ARF-GEF-dependent intracellular trafficking of PIN2, leading to a decrease in PIN2 protein levels in the PM of root epidermal cells. This leads to a decrease in cell division and root meristem size by altering the auxin maxima (Zwiewka *et al.* 2019). H_2O_2 reduces the levels of PIN1, PIN2, PIN3 and PIN7 in the RAM in a dose-dependent effect (Zwiewka *et al.* 2019). Expression of YFP-tagged AUX1 protein in the root was not affected. Lower levels of PINs and the decrease of shootward auxin transport driven by PIN2 could explain the observed accumulation of auxin in the stele of stressed roots (Zwiewka *et al*. 2019).

The application of H_2O_2 restores root bending to vertical roots pre-treated with NPA. The application of ROS scavenging antioxidants also inhibits root gravitropism. These results indicate that ROS may function downstream of auxin mediated signal transduction (Hee Joo *et al.* 2001)

ROS accumulation during abiotic stress leads to depolymerization of either microtubules and actin filaments as well as oxidative modifications of tubulin and actin proteins (Livanos *et al.* 2014). Misbalance of ROS homeostasis such as that caused by osmotic stress can interfere with function of tubulin cytoskeleton therefore altering PIN distribution (Livanos *et al.* 2014)

Changes in H_2O_2 levels can also lead to alteration in peroxidases which catalyse the oxidation degradation of IAA (Kawano 2003, Tognetti *et al.* 2012). It is not only H_2O_2 that alters auxin levels, as several sources of ROS such as RBOH enzymes, apoplastic peroxidaes, acyl-CoA oxidases and mitochondrial electron transport are also involved in IAA regulatory networks (Mase and Tsukagoshi *et al.* 2021)

1.6.4 Sites of crosstalk between ABA, Auxin and ROS

ABA, auxin and ROS pathways have been detailed in key areas involving root growth, development, and stress response. It is likely there are numerous areas where all three interact to control root development. These notable regions where all three pathways connect will be listed, these could act as key centres of their regulation.

PINOID (PID) kinase, member of the ACG kinase family, regulates PIN localization on the cellular membrane, and as a result regulates polar auxin transport (PAT) (Kleine-Vehn *et al.* 2009). PID levels are upregulated by auxin and salicylic acid (SA), in pathway involving protein kinase CK2 (Armengot *et al.* 2016). Along with role is auxin transport, PIDs also act in stress signalling as a region for crosstalk between ROS, ABA and auxin (Garcia *et al.* 2012). Ectopic PID expression resulted in a disruption in hormone balance, leading to the accumulation of auxin and increased ROS production (Saini *et al.* 2017). Wide range of cellular redox processes are affected in the PID-OE lines. PID-OE has elevated ROS levels that can be explained by increased RBOH activity (Peer *et al.*2013).

Critically, PID overexpression leads to increases in ABA-induced carotenoid dioxygenase (NCED3) and ABA responsive elements-binding factors (AREB1/ABF2, AREB2/ABF4, and ABF3), and ABA insensitive (ABI1 and ABI2) along with increases in RD29B (Saini *et al.* 2017). Elevated PID levels show increased RBOH activity and an increase in antioxidants such as flavonoids. Despite the increase in auxin and antioxidants, PID overexpression did not result in drought tolerance/improvement. PID overexpression leads to increased IAA levels which already induces stress conditions. The addition of osmotic stress is likely additive, leading to higher lethality (Saini *et al.* 2017).

ABA alters auxin distribution and PLT protein stability through the production of ROS in the mitochondria of root tips (Yang *et al.*2014). The *aba-overly sensitive-8-1* (*abo8-1*) mutant has an

incomplete mitochondrial electron transport chain of complex I. As a result, it accumulates an excessive amount of ROS. *abo8-1* displays a phenotype of reduced growth and hypersensitivity to ABA. The high accumulation of ROS generated in *abo8-1* reduces the expression of PLT and activity in the root meristem, resulting in altered auxin distribution (Yang *et al.*2014). This indicates that ROS homeostasis in the mitochondria is critical for root growth and SCN maintenance via auxin distribution. It also suggests the existence of crosstalk between ROS-ABA-auxin for the regulation of root meristem size.

Jiao *et al.* (2013) found that all three pathways connected around RBOHD and RBOHF playing a role in ABA-inhibited primary root growth. Double mutants *atrbohD1/F1* and *atrbohD2/F2* are less sensitive to ABA suppression of root elongation, showing reduced ROS generation, cytosolic Ca^{2+} and activation of plasma membrane Ca^{2+} permeable channels compared with WT. Application of direct PIN transport inhibitor N-1-napthylpthalamic acid (NPA) enhanced the ABA inhibition of root growth in the mutants relative to WT. The ABA-induced decreases in auxin in the root tips were more pronounced in WT than in *atrbohD1/F1*. This study indicated that RBOHD and RBOHF are required for ABA-promoted ROS production in roots, from which the ROS activate Ca^{2+} signalling and reduce auxin in roots, therefore increasing ABA-inhibited primary root growth in Arabidopsis (Jiao *et al.* 2013)

Flavonoids, secondary metabolites induced by biotic and abiotic stresses, may influence ROS-auxin-ABA interaction (Brunetti *et al.* 2018). Produced under osmotic stress, flavonoids act as prominent ROS scavengers, leading to enhanced oxidative and drought tolerance (Nakabayashi *et al.* 2013). At lower concentrations they may act as regulators of root growth and differentiation, due to their ability to inhibit the activity of protein kinases (Brunetti *et al.* 2018). Flavonoids alter auxin transport by modifying vesicular trafficking and PIN cycling, by altering the activity of PAT regulators and by regulating PP2A (Kuhn *et al.* 2017, Saini *et al.* 2017). Flavonoids have also been found to promote auxin accumulation in a tissue-specific manner (Buer *et al.* 2013). Auxin promotes flavonoid accumulation, potentially to quench the ROS signal generated during auxin catabolism (Peer and Murphy 2007, Saini *et al.* 2017). Flavonoids may alter ABA-induced stomatal closure by the inhibition of MAPK activity or by quenching H_2O_2 which acts as a second messenger of ABA (Danquah *et al.* 2014, Brunetti *et al.* 2018). ABA has been found to promote the biosynthesis of flavonoids (Berli *et al.* 2010). The relationship between ABA-flavonoids requires more research (Brunetti *et al.* 2018)

1.7 Osmotic Stress

1.7.1 What is Osmotic Stress?

Water constitutes almost 70-80% of the fresh mass of plants (Zhou *et al.* 2021). A lack of water will greatly alter plant growth and development, as water is critical for maintaining turgor pressure, which gives plants rigidity and structure along with driving cell expansion and opening/closing of stomata (Pritchard 2001).

In the field, the availability of water is often non-uniform and can change quickly from threatening conditions to bountiful or vice versa (Ritchie 1981). The lack of water is often caused by a reduction in rainfall causing a decrease in water availability. Drought can also cause an increased soil hardness which can have a negative impact on plant growth (Whitmore and Whalley 2009)

In this thesis, osmotic stress is defined as the stress imposed by reduced water availability. Soil and plant water status is typically described by using in terms of "water potential". Water potential is derived from the free energy status of water compared to pure water at a reference state. Water potential is the sum of osmotic potential and the hydrostatic pressure, measured in MPa ($\Psi = \Psi s + \Psi p$). Water potentials of 0 to -0.3 (MPa) are typical of well-watered plants whereas water potentials of -1.5 to -2.0 MPa lead to a permanent loss of turgor and severe stress in *Arabidopsis* and most crop species (Boyer and Kramer 1995)

Water potentials that cause osmotic stress can be achieved in laboratory conditions by increasing solute concentration in the substrate on which the seedlings grow (Haswell and Verslues 2015, Osmolovskaya 2018). In order to replicate osmotic stress conditions in the lab, scientists have used NaCl or sugar derivatives such as mannitol and sorbitol (Verslues *et al.* 2006, Huan *et al.* 2017). Some of these solutes are able to enter the cytoplasm and can be toxic to plant cells (Haswell and Verslues 2015).

Mannitol and sorbitol have small molecular structures which are able to enter the area between protoplast and cell wall. This can cause plasmolysis, where the protoplast shrinks away from the cell wall. Cytorrhysis, the shrinking of both protoplast and cell wall is what occurs under a lack of water and soil drying (Haswell and Verslues 2015). Plasmolysis is not typical of what occurs to plant cells in drought field conditions and is perceived by different mechanisms and leads to different downstream responses to that of cytorrhysis. Root growth rate has been found to be much higher under cytorrhytic treatment when compared to plasmolytic treatments of the same MPa (Verslues *et al.* 2006). Cytorrhysis and plasmolysis may have different effects of plasma membrane-cell wall

connections which could elicit different downstream signals (Haswell and Verslues 2015). To achieve osmotic stress levels, we use polyethylene glycol (PEG) (molecular weight 8000) infused agar plates. PEG is a useful solute for osmotic stress as it is non-toxic and excluded by plant cells due to its large open structure (Handa *et al.* 1982, Rowe *et al.* 2016). At high concentrations a PEG-infused medium will cause cytorrhysis rather than plasmolysis (Osmolovskaya 2018).

1.7.2 The phenotypic response to osmotic stress

There are a number of potential strategies in a plant's response to drought. Some species manage to escape and complete their life cycle before the full onset of drought (Iqbal *et.* al 2020). Others attempt to avoid drought damage by modifying their root architecture and reducing water loss via stomatal conductance (Basu *et al.*2021). Plants can also attempt to tolerate drought by lowering water potentials by maintaining a level of osmolytes and molecular weight proteins (Bacelar *et al.* 2012).

When first exposed to a deficit in water plants respond by a rapid regulation of stomatal aperture together with rapid changes in root hydraulic conductivity (Bacelar *et al.* 2012, Rosales *et al.* 2019). The closing of the stomata leads to reduced transpiration which further increases the plants overall temperature. This increase in temperature can lead to denaturation of enzymes and proteins that can alter important physiological processes like photosynthesis, nitrogen metabolism and mineral uptake (Bhargava and Swant 2013). Closing stomata also limits gaseous exchange therefore increasing the levels of carbon dioxide. This accumulation of CO_2 can ultimately lead to the production of reactive oxygen species (Das and Roychoudhury 2014, Singh *et al.* 2021). Declining photosynthetic rate in plants due to drought affect photosynthetic reaction centres, oxygen-evolving complexes and xanthophyll cycle activity (Bhargava and Sawant 2013).

Over the longer term, plants alter both shoot and root growth (Koevoets *et al.* 2016). Mild water deficit can significantly promote shoot and root development, whereas severe osmotic stress results in significant reduction in both root and shoot growth (Rowe *et al.* 2016, Rosales *et al.* 2019, Smolko *et al.* 2021). The reduction in growth and development in the root and shoot is caused by the interruption of processes such as cell differentiation, division, and elongation. These processes are negatively altered by the loss of turgor pressure, the decline in enzyme activity and the loss in uptake of minerals and nutrients required for the process of photosynthesis (Ali *et al.* 2020, Singh *et al.* 2021)

Leaves and roots display different response to osmotic stress. Changes in the ratio between root and shoot length is often seen in field experiments, however sometimes there is no change (Rosales *et*

al. 2019). Above the soil we see a reduction in the number and size of leaves, total leaf area and the number of stomata. We also see a promotion in the formation of tube leaves, cell wall thickening and cutinisation (Mafakheri *et al.* 2010, Nezhadahmadi *et al.* 2015, Tenhaken 2015, Xue *et al.* 2017). There is also a reduction in photosynthetic rate by a smaller leaf surface area which increases the resistance for gaseous exchange and leaf senescence (Nezhadahmadi *et al.* 2013).

1.7.3 Root response to osmotic stress

Low levels of osmotic stress can promote primary root length and biomass and lateral root number and length (van der Weele 2000, Rosales *et al.* 2019). However, this is not always the case as some researchers have found that mild increases in osmotic stress reduce *Arabidopsis* growth (Rowe *et al.* 2016). Upon a large increase in osmotic stress levels there is a clear and sharp reduction in root growth along with a reduction in lateral root number (van der Weele *et al.* 2000, Rowe *et al.* 2016, Rosales *et al.* 2019, Yuan *et al.* 2021).

Behind this reduction in growth is a decline in RAM size due to a decrease in the number of cells divisions and cell size. (Rowe *et al.* 2016, Cajero Sanchez 2019, Yuan *et al.* 2021). Despite this reduction in root growth, the morphology of the SCN is unaltered by osmotic stress. This maintained functionality may allow plants to restore growth once returned to favourable conditions (Cajero Sanchez *et al.* 2019) (Figure 1.6).

Direct exposure of roots to water stress results in the inhibition of hydraulic activity and aquaporin activity at the root system and cellular level. (Sutka *et al.* 2011, Hachez *et al.* 2013). Turgor pressure, which is critical for rigidity and cell structure in plants, is reduced under osmotic stress (Pritchard *et al.* 2001)

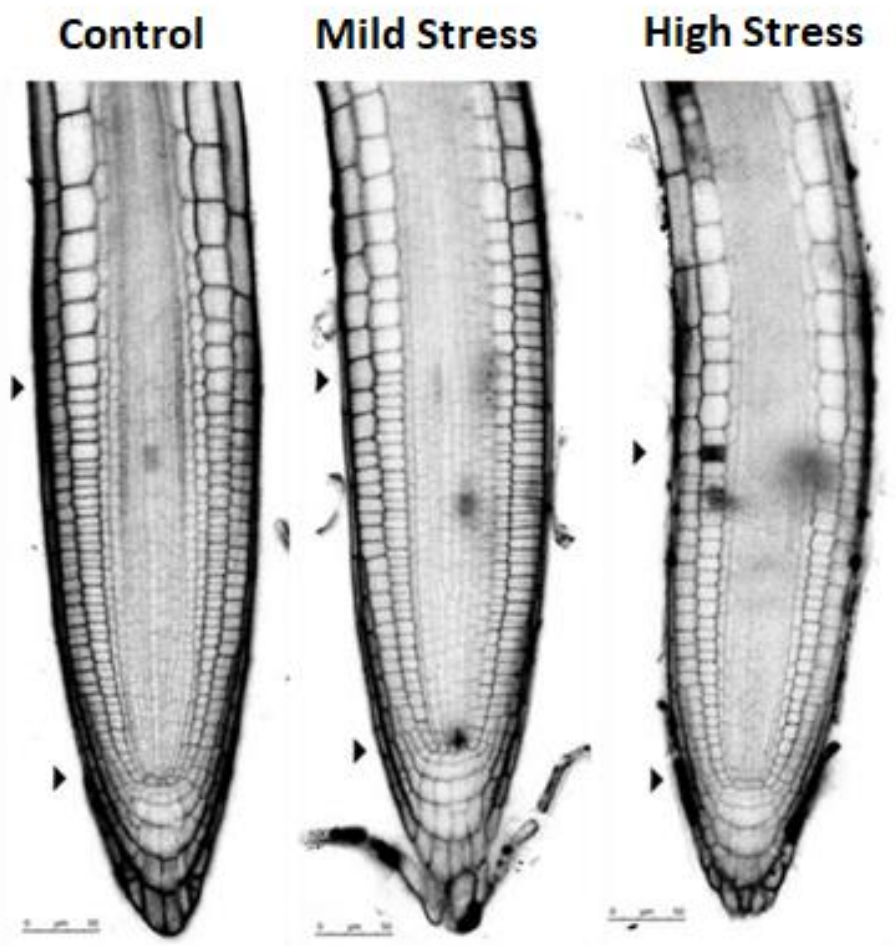

Figure 1.6. Reduction in meristem size under osmotic stress. Primary root tips stained with propidium iodide after 24hr osmotic stress treatment. Arrowheads indicate quiescent centre and approximate end of the meristematic zone. Taken from Rowe *et al.* (2016).

Under osmotic stress we see the accumulation of low molecular weight osmolytes such as proline in the root tip (Chun *et al.* 2018). Proline confers tolerance to osmotic stress by aiding cellular osmotic adjustment along with stabilizing proteins, membranes, and subcellular structures (Kishor *et al.* 2005). Proline accumulation also helps in the scavenging of ROS that can damage the cell when at high concentrations. As discussed above, proline may act as a signalling molecule (Zhang and Becker 2015, Kovacs *et al.* 2019).

There is also an increase in suberization of the root under osmotic stress. Increased suberin levels in the cell walls of the root, leading to a decrease in the hydraulic conductivity. Sealing the apoplast with suberin results in a reduced backflow of water from the root to the soil medium (Kreszies *et al.* 2019)

1.7.4 Primary sensing mechanisms

Despite a high level of interest, uncovering the mechanisms of how osmotic stress is perceived by plants is challenge. It remains an area of little understanding with few clear candidates for perception. Osmotic stress likely causes an osmotic imbalance across the plasma membrane and/or causes changes in membrane tension and integrity. This could then trigger downstream signalling leading to osmotic stress response (Haswell and Verslues 2015).

One of the first major potential osmotic sensors was identified as REDUCED HYPEROSMOLALITY-INDUCED [Ca^{2+}] INCREASE1, OSCA1 (Yuan *et al.* 2014). The mutant *osca1* resulted in a deficiency in Ca^{2+} accumulation when exposed to sorbitol but not in response to H_2O_2 or ABA. There also was a visible increased sensitivity to osmotic stress, represented by a reduction in primary root length when exposed to osmotic stress (Yuan *et al.* 2014).

OSCA1 is a hyperosmolality-gated calcium channel which is located at the plasma membrane (Yuan *et al.* 2014). High osmotic potential or plasma membrane tension caused by a water deficit potentially triggers the opening of the pore and allows Ca^{2+} influx within seconds after the stress condition is perceived (Liu *et al.* 2018). The differences between growth conditions under osmotic stress was subtle between *osca1* mutants. This indicates that it is likely there are multiple redundant osmosensors present in plants. Arabidopsis has 15 homologs of OSCA1 indicating that osmotic stress sensing could be mediated by a family of redundant calcium channels (Liu *et al.* 2018).

CALCIUM PERMEABLE STRESS-GATED CATION CHANNEL1.2 (AtOSCA1.2) has been identified as a calcium channel that is induced by osmotic stress (Hou *et al*. 2014). With a high sequence similarity to OSCA1, both may have a similar function. Despite its interesting structure, the function and subcellular location of OSCA1.2 in plants is currently unknown (Lamers *et al.* 2020).

AHK1 has been identified as a two-component phosphorelay system (TCS), similar to those acting as an osmotic pressure sensing system in bacteria (Yuan *et al.* 2017). AHK1 does appear to play a role in the transcriptional regulation response to drought, and the *ahk1* mutant displays reduced survival under drought stress (Tran *et al.* 2007). However, no direct phosphorylation activity of AHK1 has been found in response to osmotic stress, as well as no reduction in ABA levels or stomatal closure were observed in the *ahk1* mutant (Yuan *et al.*2017, Sussmilch *et al.* 2017). This suggests that AHK1 might have some role in osmotic stress, but it is unlikely that AHK1 acts as a direct osmosensor (Lamers *et al.*2020).

Several receptor-like kinases (RLKs) that are found on the plasma membrane measure the integrity of the cell wall. Dissociation of the plasma membrane from the cell wall during osmotic stress could

lead to increased phosphorylation of downstream target proteins (Feng *et al*. 2016). FERONIA (FER), a RLK, sees its activity increased under salt stress and the plasmolysis that follows (Feng *et al.* 2018). FER could act as an osmotic sensor, but it is more likely it is a sensor of cell wall integrity via measuring distorted pectin filament organization rather than a specific osmotic sensor (Lamers *et al.* 2020).

The action of osmotic stress sensing is still not well understood despite some key breakthroughs with OSCA1/OSCA1.2. Potentially there is some strong overlap in the mechanisms of perception of other abiotic stresses such as salt, temperature, cold etc. (Lamers *et al.* 2020).

1.7.5 Short-term signalling

Once osmotic stress has been detected, there is a flurry of short-term (within minutes) downstream signalling that leads to a developmental response. Downstream signalling has received a great deal more attention than the initial perception of osmotic stress (Haswell and Verslues 2015). In a process sometimes called Systemic Acquired Acclimation (SAA), a signal can quickly propagate through the whole plant leading to an acclimation where tissues are able to withstand stress even if they did not sense or experience it (Zandalinas *et al.* 2019)

Systemic signals such as ROS, calcium, electrical and hydraulic waves are, within minutes, able to propagate from a local tissue to the entire plant (Kollist *et al.* 2019). There are also seen rapid changes in hormones such as abscisic acid, auxin, ethylene and different metabolites (Choudhury *et al.* 2018). These efficient and coordinated signals lead to whole-plant system changes that enables tissues to withstand stresses and developmental changes (Suzuki *et al.* 2013, Devireddy *et al.* 2018). For example, stomata are able to close or open in response to light stress or pathogen infection within minutes (Kollist *et al.* 2019).

It is not known how hydraulic waves are linked to electric, Ca^{2+} or ROS waves. It is suggested that the members of the MECHANOSENSITIVE CHANNELS OF SMALL-CONDUCTANCE-LIKE (MSL) family, which are permeable to Ca^{2+} and/or other cations and anions, could perceive systemic hydraulic waves at tissues distant from the stress locations and translate them into Ca^{2+} signals (Fichman and Mittler 2021).

Ca^{2+} signals are able to alter ROS through the action of many specific Ca^{2+} binding proteins and Ca^{2+} dependent kinase/phosphatase switches. For example, Ca^{2+} can directly bind to the EF-binding domains of the RBOHD protein (Dubiella *et al.* 2013). OSCA1 and OSCA1.2 are likely involved in the calcium signalling that takes place immediately following osmotic stress perception (Yuan *et al.* 2014, Liu *et al.* 2018). Apoplastic alkalization occurs in tandem with calcium influx in the first

minutes after exposure of roots to salt (Choi *et al.* 2014). This likely triggers a number of responses including ROS production.

ROS accumulates within minutes of osmotic stress treatment contributed by the production of RBOHD and RBOHF, as well as independently of RBOH enzymes (Martiniere *et al.* 2019). Apoplastic ROS generated via RBOHD and other enzymes can be quickly transported via aquaporins such as PM-intrinsic protein channels (PIPs). These can transport H_2O_2 across the PM from the apoplast to the cytosol (Rodrigues *et al.* 2017, Qiu *et al.* 2020). This translocation of H_2O_2 into the cytosol can leads to changes in redox-dependent reactions, kinase and phosphatase molecular switches along with Ca^{2+} permeable channels. Further driving local and SAA pathways (Miller *et al.* 2009, Rodrigues *et al.* 2017, Fichman and Mittler 2021).

ROS signalling molecules such as H_2O_2 can travel though the continuous apoplastic space. Once produced in one space, ROS can enter neighbouring plant cells and trigger reactions within them. This leads to the activation of ROS production within the neighbouring cells and a chain of cell-to-cell transmission known as ROS wave developing (Suzuki *et al.* 2013, Fichman *et al.* 2021). RBOHD is required for ROS generation and ROS wave propagation under high light stress, along with proteins localized to plasmodesmata (Fichman *et al.* 2021). The RBOH pathway is key for PIP2;1 internalization which occurs rapidly within the minutes of osmotic stress treatment (Martiniere 2019). The function of RBOHD is also required for the propagation of electric signals in response to stress (Suzuki *et al.* 2013).

It has been suggested that the specificity of the signal created by ROS is due to the localized production (by RBOHs) and the ROS microenvironment they create in their vicinity, not due to the species of ROS produced (Martiniere *et al.* 2019).

ROP6 shows rapid changes in localisation within minutes of osmotic stress stimulation that subsequently generate ROS and lead to signalling changes. Due to the speed of this change, it is likely ROP6 is acting right after cell osmotic perception to trigger downstream responses (Smokvarska *et al.* 2020)

These rapid signals can trigger changes in gene expression, leading to changes in protein and/or metabolite levels (Kollist *et al.* 2019). Pre-existing mRNA with polysomes could be altered, resulting in the rapid synthesis of new proteins (Sablok *et al.* 2017). The ubiquitination of proteins and other post-translational modification could also easily be affected by rapid stress signalling (de Vega *et al.* 2018) Large changes in protein concentrations could be taking place rapidly, for example under 5 minutes of salt treatment PIN2 protein concentrations were found to reduce (Smolko *et al.* 2021).

1.7.6 Long-Term Osmotic Signalling

Following the rapid change in signalling under osmotic stress perception, we see an acclimatization to the stressful conditions. After this process takes place, we witness a longer-term change in hormonal signalling where clear patterns emerge over 24-hours and beyond leading to developmental changes in the plant. There has been more focus on this area of osmotic stress signalling (Rowe *et al.* 2016), however there still remain large areas that required study. In this section I will focus on longer term (24 hours +) changes that have been described.

This thesis will focus on auxin, ABA and ROS pathways. There are a number of other hormones that have a critical role in osmotic stress response including gibberellins etc (Colebrook *et al.* 2014, Khan *et al.* 2015). Epigenetic regulation, including DNA methylation, histone modifications and nucleosome assembly by chaperons, also play critical roles in osmotic-stress response and are summarized in a number of reviews (Ueda *et al.* 2019, Bulgakov *et al.* 2019).

1.7.6.1 The role of auxin during osmotic stress

In addition to regulating root growth under optimal conditions, it is clear that auxin plays a direct role in mediating osmotic stress response in the root (Naser & Shani 2016). Auxin response is down-regulated in wild-type roots under osmotic stress leading to auxin-responsive genes being expressed differently (Yuan *et al.* 2021, Huang *et al.* 2008, Ha *et al.* 2013).

Application of low concentration of auxin (1 nM IAA) was found to rescue root growth under mild osmotic stress, and partially rescue root growth under severe stress (Rowe *et al.* 2016). The *axr3-1* mutant, highly sensitive to auxin, displays an exaggerated reduction in root growth and meristem size under osmotic stress (Rowe *et al.* 2016)

There is a significant reduction in levels of endogenous IAA in leaves and roots under water deficit conditions (Liu *et al.* 2015). In both osmotic and salt stress there is a reduction in levels of DR5:GFP at the root tip indicating a reduction in auxin levels (Smolko *et al.* 2021). This is created via an alteration of polar auxin transport, biosynthesis, and homeostasis under osmotic stress (Smolko *et al.* 2021, Rowe *et al.* 2016). Interestingly in longer term (13 days) osmotic stress study we see a tendency for IAA levels to increase (Smolko *et al.* 2021).

Osmotic stress alters auxin transporter levels and localization, leading to reduction in root auxin concentrations (Rowe *et al.* 2016). PIN1 levels are reduced via an ABA-dependent manner, overriding the effects of ethylene (Rowe *et al.* 2016). Interestingly, PIN2 expression levels increase under osmotic stress. It appears there are tissue-specific effects of osmotic stress leading to

differential expression of auxin transporters (Rowe *et al.* 2016). Osmotic treatment of root meristem cells with mannitol resulted in the immediate internalization of PIN1 and PIN2, as well as the decrease recycling of internalized PINs to the plasma membrane (Zwiewka *et al.* 2015). Changes in PIN1 and PIN2 expression and localization work together to reduce auxin concentrations at the root tip (Rowe *et al.* 2016).

PIN3 and PIN4 transcriptional levels decrease under osmotic stress (Rowe *et al.* 2016, Yuan *et al.* 2021, Smolko *et al.* 2021). HOMEODOMAIN ARABIDOPSIS THALIANA2 (HAT2), HAT2 belongs to the Class II HD-ZIP transcription factor family which plays an important role in plant development and environmental response. Members of this family can act as repressors which bind to their target gene promoters. HAT2 regulates auxin activity by directly repressing PIN3 transcription (Yuan *et al.* 2021). Under osmotic stress we see an increase in HAT2 repression of PIN3. In the *hat2* loss-of-function mutant under the same osmotic stress conditions, we see the alleviation of PIN repression, an increase in auxin and a reduction in osmotic stress inhibition of root growth. HAT2 expression was not significantly increased after ABA, BRs or ethylene treatment (Yuan *et al.* 2021).

AUX1 expression is reduced under osmotic stress along with decreased protein fusion fluorescence. It is implied that ABA biosynthesis plays a key role in *AUX1* expression (Rowe *et al.* 2016). ARF-GEF cycle, responsible for polar-recycling of PINS, is likely altered by abiotic stress which leads to changes in intracellular trafficking in the RAM (Tognetti *et al.* 2017). Despite numerous studies, the role of polar auxin transport in response to osmotic stress at the molecular level is poorly understood (Yuan *et al.* 2021). More work is needed in order to understand how auxin patterning is formed.

The inhibition/reduction of auxin biosynthesis is likely to play a role in osmotic stress. IAA content in leaves and roots much lower under salt stress and water deficit conditions (Du *et al.* 2013, Shi *et al.* 2014, Liu *et al.* 2015,), although is some studies that has not proven to be the case (Smolko *et al.* 2021). Changes in transcripts of *YUC*, *GH3* and *UGT* under salt and osmotic stress suggest the disruption of auxin biosynthesis and the processes of amide and ester conjugation (Smolko *et al.* 2021). IAA biosynthesis genes (YUCCAs) were found to be significantly downregulated under osmotic stress in both *Arabidopsis* and rice, along with an increase in IAA conjugation genes (Du *et al.* 2013, Shi *et al.* 2014). Overexpression of YUCCA7, a gene involved in the auxin biosynthesis pathway, leads to increase resistance to drought stress in *Arabidopsis* (Lee *et al.* 2012).

Plants with auxin over-production (iaaM-OX) exhibited enhanced drought resistance (Shi *et al.* 2014), whilst those with reduced production *yuc1/yuc2/yuc6* triple mutants showed decreased drought resistance (Shi *et al.* 2014). Exogenous and endogenous auxin positively modulated abiotic stress

genes (RD29B, DREB2A etc) and positively affected ROS metabolism and antioxidant activities (Shi *et al.* 2014).

IAA hydrolysis is a rapid process releasing free IAA from IAA sugar or amino acid conjugates. IAR3 hydrolyses IAA-alanine and releases bioactive IAA. It has been shown that high osmotic stress reduces miR167a levels, thereby increasing the mRNA levels of the direct target IAR3. *iar3* mutants has a reduced level of free IAA and have shown reduced osmotic stress-induced root architecture (Kinoshita *et al.* 2012).

There appears to be some fluctuation in auxin response depending on the time period studied (Naser & Shani 2016). Smolko *et al.* (2021) has indicated in long-term stress (13-day assay) that there was an increase in the stability of the auxin metabolome and an increase in IAA, particularly during salt stress, whereas under short term stress (3-hour assay) we see a decrease in IAA levels. Further study is required to understand auxin homeostasis during osmotic stress (Naser and Shani 2016).

1.7.6.2 The role of ABA during osmotic stress

ABA plays a key role in osmotic stress. (Nakashima *et al*. 2014, Rosales *et al.* 2019). Under osmotic stress we see a rapid accumulation of ABA, inducing stomatal closure to reduce water loss (Wu *et al.* 2018, Wilkinson *et al.* 2012) and a subsequent inhibition of plant growth (Rowe *et al*. 2016, Rosales *et al.* 2019). ABA was found to override the positive effects of ethylene on *PIN1* expression, leading to a decrease in auxin transport and a reduction in root tip growth (Rowe *et al.* 2016). This is characterised by an increase in *RD29B* expression, a highly ABA-responsive gene (Rowe *et al.* 2016).

Recently several independent studies have identified different families of Raf-like protein kinases, operating upstream of ABA-dependent and independent signalling pathways in Arabidopsis under osmotic stress (Takahashi *et al.* 2020, Lin *et al.* 2020, Soma *et al.* 2020, Katsuta *et al.* 2020). All show that Raf-like MAPKK-kinases (M3Ks) are activated in response to osmotic stress and function upstream of SnRK2 kinases in *Arabidopsis* (Fabregas *et al.* 2020). These MAPK signalling cascades play important roles in plant growth, development, and stress response (Fabregas *et al.* 2020).

The B2/B3 family of Raf-like M3Ks have been found to regulate the osmotic stress ABA-signalling pathway by phosphorylation of the ABA-responsive SnRK2s from subclass III, whereas B4 Raf-like kinases regulate the ABA-independent signalling pathway by phosphorylating subclass I SnRK2s (Takahashi *et al.* 2020, Soma *et al.* 2020, Lin *et al.* 2020). These subclass III SnRK2s require both dissociation from PP2Cs in response to ABA and activation by B2/B3 Raf-like kinases for full activation. Interestingly, subclass I SnRK2s may be fully activated by B4 Raf-like kinases before the accumulation of ABA (Fabregas 2020).

B2, B3 and B4 RAF Kinases (RAFs) are critical for early osmotic stress and ABA signalling. This group is rapidly activated by osmotic stress and are required for the phosphorylation and activation of SnRK2 leading to downstream ABA response (Lin *et al.* 2020). B3 Raf-like M3Ks, RAF3, RAF4, RAF5 and RAF6 were shown to be the upstream kinases that phosphorylate and activate ABA-responsive SnRK2 in response to ABA and osmotic stress (Takahashi 2020). B4-Raf-like M3Ks, RAF18, RAF20 and RAF24 were shown to regulate SnRK2-VARICOSE signalling pathway under osmotic stress but not ABA-responsive SnRK2s (Soma 2020). The component that detects osmotic stress and leads to the activation of RAFs and SnRK2s has not yet been identified. Importantly, it has been shown to be independent of OSCA-mediated Ca^{2+} signalling (Lin *et al.* 2020, Fabregas *et al.* 2020).

In the absence of stress, PYLs promote PP2A activity, resulting in a reduction in PINOID (PID)-mediated phosphorylation of PIN-FORMED (PIN) proteins. Under osmotic stress conditions, ABA binds to PYLs thus inhibiting PP2A activity, leading to increased PIN phosphorylation and consequently modulating auxin transport and root architecture, particularly root gravitropic response and lateral root development (Rosales *et al.* 2019).

NINE-CIS-EXPOXYCAROTENOID DIOXYGENASE 3 (NCED3) is a vital enzyme in the accumulation of ABA under drought stress. Highly induced in the vascular tissues, NCED3 mutants displayed drought stress phenotypes and revealed decreased ABA accumulation under drought stress (Endo *et al*. 2008, Sato *et al.* 2018). NGTHA1 (NGA1) positively regulates ABA accumulation under osmotic stress by transcriptionally activating NCED3 (Sato 2018). NGA1 was found to be degraded under non-stress conditions, whereas under osmotic stress NGA accumulation was enhanced even in ABA-deficient mutants, indicating the role of ABA-independent pathway (Sato *et al.* 2018). Small peptide CLAVATA3/EMRYO-SURROUNDING REGION-RELATED 25 (CLE25) has been shown to induce NCED3 expression during drought stress (Takahashi *et al.* 2018), although how this activates NCED3 has not yet been elucidated.

Mutants in ABA biosynthesis (*aba2-1*) and signalling (*snrk2.2*, *snrk2.3*, *hab1-1*, *abi1-2*) display an altered response to osmotic stress. All mutant lines lose the stimulatory effects of mild osmotic stress. Under severe stress, *hab1-1* and *abi1-2* show a higher reduction in growth up to 50% in comparison with wild-type. Both *aba2-1* and *snrk2.2 snrk2.3* were not significantly altered in comparison to wild type. In both *hab1-1* and *abi1-2* mutants, lateral root elongation was dramatically repressed under severe osmotic stress when compared with wild-type (Rosales *et al.* 2019). The application of ABA biosynthesis inhibitor fluridone resulted in rescuing osmotic stress phenotype under mild osmotic stress (Rowe *et al.* 2016).

ABA is involved in the stimulation of root growth and hydraulic conductivity by mild osmotic stress. The mild osmotic stress stimulation is thought to be controlled by ABA stimulatory effect on aquaporin function (Rosales *et al.* 2019). Interestingly, the same paper suggests that under severely high osmotic stress, reduction in root growth and hydraulic conductivity is largely ABA independent and instead governed by local signals (Rosales *et al.* 2019). The molecular basis of these ABA-independent root growth reduction is yet to be identified.

Under drought, ABA regulates a large number of transcription factors that are thought to play crucial roles in stress response. ABA regulates most of the target genes through ABA-responsive elements (ABRE) binding protein/ABRE binding factor (AREB/ABF) transcription factors. Drought-responsive genes are also regulated by ABA-independent mechanisms. The network of the transcriptional regulation of drought response is complex and too large to be summarised in this section, there are many reviews that cover the subject in more detail (Singh and Laxmi 2015, Yao *et al.* 2021).

ABA-independent stress responses through DREB2B were shown to increase under osmotic stress (Rowe *et al.* 2016). DREB2B encodes for a transcription factor that bind to Drought Responsive Elements (DRE) in promoters to enhance expression of dehydration responsive genes (Nakashima *et al.* 2000). Transcriptional analysis has shown that ABA-independent differentially expressed genes were enriched in response to jasmonic acid (JA), salicylic acid (SA) and gibberellin (GA) stimuli (Liu *et al.* 2018). There is significant overlap and crosstalk between ABA-dependent and ABA-independent signalling. For example, DELLA, a key component in GA signalling likely mediates the interaction between ABA and GA pathways. Both ABA-dependent and ABA-independent signalling converge at the SnRK2s, as well as in their upstream and downstream factors (Fabregas *et al.* 2020).

1.7.6.3 The role of ROS during osmotic stress

In unstressed conditions, ROS production within cells is at low levels. Once subjected to abiotic stresses, ROS levels are elevated, which then activates stress pathways within plant cells (Mittler *et al.* 2004, Shabala *et al.* 2015, Baxter *et al.* 2014).

ROS is accumulated within minutes under osmotic stress (Martiniere *et al.* 2019). Performing a number of functions including acting as secondary messengers, regulating cell endocytosis and root water conductivity and the intracellular accumulation of osmolytes such as proline (Boursiac *et al* 2008, Ben Rejeb *et al.* 2015). ROS produced under osmotic stress, likely acts in a ROS wave, triggering stomatal closing. H_2O_2 has been shown to mediate stomatal closure by activating plasma membrane Ca^{2+} channels (Murata *et al*. 2001).

There appear to be two major processes generating ROS under osmotic stress signalling. Plasma membrane localized NADPH oxidases, RBOHD and RBOHF catalyse the production of superoxide free radicals by transferring one electron to oxygen from the cytoplasmic NADPH. RBOHD and RBOHF both contribute to osmotically induced ROS accumulation in a nonadditive manner (Martiniere *et al.* 2019). There is non-enzymatic action via the Haber-Weiss reaction where reduced transition metals transfer one electron to dioxygen. Limiting the level of free apoplastic Fe^{2+} results in a significant reduction of ROS accumulation upon sorbitol treatment (Martiniere *et al.* 2019). These pathways are RBOH and Fe/ASc are activated under a wide range of mild to high osmotic stresses (Martiniere *et al.* 2019).

Increased ROS levels can have a negative impact on the plant system due to the toxic nature of oxidative stress. In response to elevated ROS levels, antioxidative systems such as ROS-scavenging catalase, glutathione etc. are upregulated to achieve homeostasis (Gill & Tuteja 2010). Previous research has shown that increasing levels of glutathione (GSH) led to improved *Arabidopsis* root systems and tolerance to drought and salt stresses (Chen *et al.* 2012). Wildtype plants treated with GSH demonstrated more tolerance to drought and salt stresses (Chen *et al.* 2012).

Despite a large amount of evidence of the involvement of ROS under osmotic stress, there is a lack of research into how ROS signalling is affected by osmotic stress in the root tip, and what role ROS plays in osmotic stress induced root growth inhibition.

1.7.6.4 Crosstalk under osmotic stress

ABA, ROS and auxin all interact with each other in a complex way under osmotic stress. There are likely numerous areas where potential pathway interactions take place both in rapid and long-term signalling. We shall attempt to detail a few key areas that these interactions could be taking place.

Auxin and ROS have both been shown to interact with each other under osmotic stress conditions. Both *iaaM-OX* transgenic lines and exogenous IAA pre-treated WT plants displayed lower levels of H_2O_2 and O_2^- along with performing better under osmotic stress conditions. The *yuc1/yuc2/yuc6* triple mutants displayed higher levels of H_2O_2 and O_2^- in comparison to wild-type plants and performed worse under osmotic stress conditions (Shi *et al.* 2014). *iaaM-OX* transgenic lines and plants pre-treated with exogenous IAA exhibited higher activities of antioxidant enzymes under drought stress conditions, whereas *yuc1/yuc2/yuc6* mutants displayed lower activities of these enzymes compared to wild-type (Shi *et al.* 2014) This experimentation suggests that auxin can promote drought stress resistance by modulating root architecture, ABA-responsive gene expression and ROS metabolism (Shi *et al.* 2014).

YUC6 appears to be a play an interesting role, where is its overexpression is found to improve drought stress tolerance. Although involved in auxin biosynthesis pathway, the improvement to osmotic stress is not due to IAA overproduction but due to the novel thiol-reductase activity of YUC6 (Cha *et al.* 2016). YUC6 has both FAD and NADPH-dependent sulfide oxidoreductase activity and is tightly regulated as it can result in H_2O_2 production (Dai *et al.* 2013). YUC6 is endowed with NADPH-dependent TR activity which requires Cys-85. The TR activity of YUC6 conveys ROS production and stress tolerance independently of its activity in auxin biosynthesis (Cha *et al.* 2016). YUC6 could act as a crosstalk point between auxin and ROS pathways under osmotic stress. TR activity of YUC6 could function in activating redox systems to scavenge ROS produced under water deficit. It may be that other YUC proteins have TR activity as well (Cha *et al.* 2016).

Glutathione peroxidase, encoded by *ATGPX3*, likely plays a key role in H_2O_2 and ABA interaction, but also how they relate under osmotic stress conditions. *atgpx3* mutation leads to high sensitivity to osmotic stress and increased water loss (Miao *et al.* 2006, Miao *et al.* 2007) ATGPX3 expression is upregulated under osmotic stress where it is thought to sense and transduce H_2O_2 signal, leading to ABA and drought stress response (Miao *et al.* 2007). Other members of the GPX family may also have key roles in osmotic stress response due to their role in regulating abiotic plant responses (Bela *et al.* 2018).

Other proteins involved in ROS-ABA stomatal closure have been identified. CHYR1, a ubiquitin E3 ligase play a role in ABA-induced stomatal closure, along with ROS production and plant drought tolerance. Regulated by OTS1/SnRK2.6 which is strongly activated by osmotic stress, CHYR1 promotes ABA-induced stomatal closure in response to drought (Ding *et al.* 2015).

Heat shock transcription factors HSFA6a and HSFA6b may regulate ROS homeostasis leading to ABA and osmotic stress insensitivity (Wenjing *et al.* 2020) ABA-responsive element binding factor (ABRE), a key regulator in ABA-signalling pathway has been shown to be involved in the transcriptional regulation of HSFA6a (Hwang *et al.* 2014). Plants overexpressing HSFA6a exhibit hypersensitivity toward ABA and tolerance to drought stress (Hwang *et al.* 2014). HSFA6a is known to be involved in ABA-mediated stress responses (Hwang *et al*. 2014) HSFA6a has been indicated to be a transcriptional activator of stress-responsive genes via the ABA-dependent pathway, HSFA6b acts as a downstream regulator of the ABA-mediated stress response (Hwang *et al.* 2014, Huang *et al.* 2016). Acting in functional redundancy with each other, the *hsfa6a/hsfa6b* double mutant is resistant to exogenous ABA and osmotic stress compared to WT. HSFA6a and HSFA6b act as positive regulators of ABA and drought stress response. *Hsfa6a/hsfa6b* double mutant has reduced ROS

accumulation upon ABA treatment (Wenjing *et al.* 2020), indicating HSFA6a and HSFA6b may act as a link between ABA and ROS signalling under osmotic stress response.

PERK4 could a key area in ABA and ROS regulation of osmotic stress response. PERK4 plays a key role in ABA-regulated inhibition of root growth, stimulating ROS accumulation via RBOHC. *perk4* and *rbohc* display attenuated sensitivity to ABA root inhibition along with reduced ROS accumulation. PERK4 deficiency prohibits the ABA-induced expression of RBOH genes. (Ma *et al.* 2019). With ABA playing a key role in osmotic stress response, potentially the PERK4/RBOHC pathway is an area in which ABA exerts control over root growth under osmotic stress.

ROPs could also be a site of ROS, ABA and auxin interaction under osmotic stress. ROP6 has recently been shown to be a master regulator of osmotic stress induced ROS production, where osmotic stress stimulates ROP6 nanodomain formation within minutes (Smokvaska *et al.* 2020). ROP6 has also been shown to be required for auxin signalling, in particular gravitropic response (Lin *et al.* 2012). ROP2 has been identified to have a role in stress responses, especially in the role of ABA-induced stomatal closure (Hwang *et al*. 2011). How ROP2 and ROP6 are involved in osmotic stress, in particular if they contribute to ROS production leading to ABA and auxin signalling has not been elucidated and requires study.

The crosstalk between hormones is very complex, with each pathway containing multiple sites where they interact with other pathways. One change in one signalling component can lead to changes in other signalling components. In order to make sense of this complexity, using systems approach to understand the patterning of hormones is a viable strategy (Liu *et al.* 2010, Rowe *et al.* 2016).

To quantitatively link a mutant gene with root development/size, all changes in the relevant hormones must be quantitatively analysed. Previously a network detailing the interactions between auxin, ethylene, cytokinin and PLS was developed revealing a circuit of crosstalk that regulates root growth (Liu *et al.* 2010).

Rowe *et al.* (2016) were able to successfully include the action of osmotic stress along with the action of ABA and auxin transport (PIN1, PIN2, PIN4, AUX1) into the network. The network revealed that ABA regulates root growth under osmotic stress conditions via its interactions with auxin, cytokinin and ethylene. ABA overrides ethylene and cytokinin regulation of osmotic stress. ABA effect is tissue specific, PIN1 in stele decreases whilst PIN2 in epidermis/cortex increases. These changes in PIN levels reduces auxin concentrations in the root tip. PLS expression is reduced as auxin

promotes and ethylene inhibits expression of the peptide (Figure 1.7). It appears in that cytokinin signalling is downstream of ABA regulation of root growth under osmotic stress (Rowe *et al.* 2016).

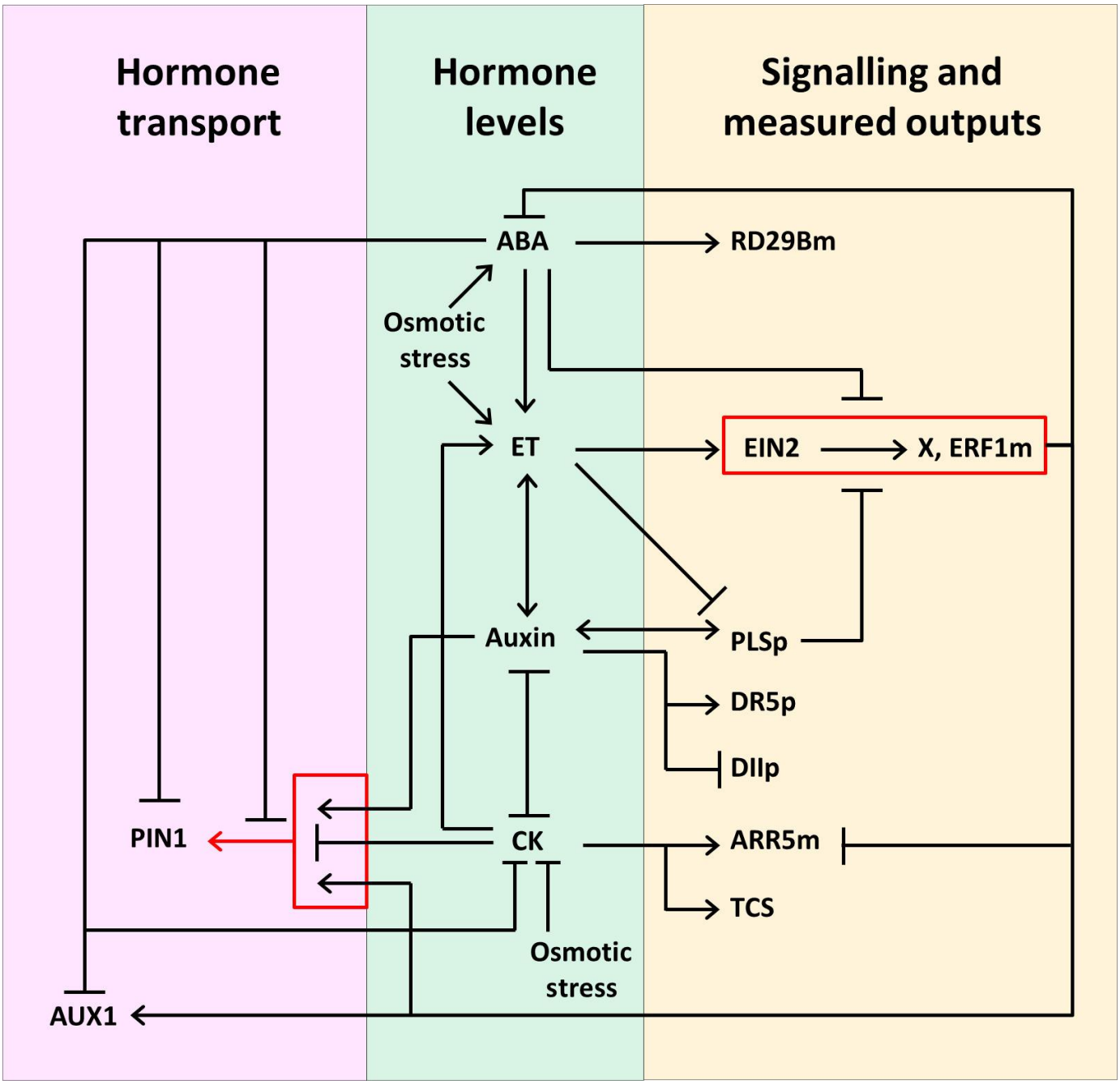

Figure 1.7. A simplified hormonal crosstalk network for the regulation of root growth under osmotic stress conditions. This network takes place in a vascular cell expressing PIN1. The network demonstrates that the responses of auxin transporters, hormones and signalling components to osmotic stress are nonlinear and complex. Abbreviations: DR5p, *DR5* regulated yellow fluorescent protein; DIIp, DII-VENUS protein; PLSp, POLARIS peptide; PIN1, PIN1 auxin efflux transporter protein; AUX1, AUX1 auxin influx transporter protein; ET, ethylene; X, the unknown factor that regulates auxin transport from the aerial tissues; EIN2, EIN2 ethylene signalling protein; ERF1m, ERF1 mRNA transcript; ABA, abscisic acid; RD29Bm, *RD29B* mRNA transcript; CK, active cytokinin; ARR5m, *ARR5* mRNA transcript; TCS, cytokinin response reporter; osmotic stress, the osmotic stress imposed by the growth medium. Red boxes group related activities. Taken from Rowe *et al.* (2016).

This previous work did not factor ROS into the study of osmotic stress in root development, nor did it look at the speed at which key signals take place. As indicated previously, ROS have strong interactions with both auxin and ABA pathways. In order to understand osmotic stress response further, we need to study ROS response to osmotic stress and how this interacts with the auxin and ABA in both short and long-term. In order to better understand how changes in auxin levels are achieved, both PIN3 and PIN7 require studying under osmotic stress. These transporters play key roles in response to other stimuli such as gravitropism, therefore they could play a key role in osmotic stress response.

1.8 Project Aims and Objectives

It is critically important to understand how plants respond to osmotic stress as this can help us improve crop development under drought conditions. How quickly plants are able to respond to osmotic stress and develop signal crosstalk could have significant ramifications to the performance of crops under stress conditions. It is particularly important to see how key signals such as auxin, ABA and ROS interact with each other within a short time frame, leading to osmotic stress response, and how critical are these signals for each other's development.

My research focused on signalling in a much shorter time period (within minutes) in combination with a longer time period (24hrs). Previous work has focused on longer time periods such as 12 or 24 hrs, and if shorter time periods were studied this was looking at above-ground regions such as leaf tissue and stomata (Qi *et al.* 2018, Yuan *et al.* 2021). Studying how rapidly stress signals appear in root systems is critical if we are to understand this aspect of plant development. It might be expected that ABA, ROS and auxin all show rapid signalling changes within minutes of osmotic stress stimuli.

Previous work has elucidated the important role PLS has in plant development as an auxin-responsive negative regulator of ethylene responses (Casson *et al.* 2002, Rowe *et al.* 2016). There remain gaps in our understanding of PLS's role in plant development, in particular it has not been fully studied how PLS affects cytokinin distribution. The first section of my thesis tests the role of POLARIS (PLS) in plant development via loss-of-function mutants, and then determines how cytokinin levels are affected, using a TCSn:GFP cytokinin reporter.

The second section of my thesis describes a further study of the role of auxin under osmotic stress. Auxin has been shown to play a key role in plant development under osmotic stress in combination with other hormones. However there has not been a detailed study on how quickly auxin patterns develop and influence plant development during osmotic stress. Short-term auxin response to osmotic stress has yet to be studied. This chapter focuses on studies using loss-of-function mutants (such as *pin3* and *pin7*) and fluorescent reporters to reveal the requirement for auxin during osmotic stress response.

The third section of my thesis focuses on the speed and temporal distribution of response of the critical stress hormone ABA under osmotic stress, in particular using the ABAleonSD1-3L21 reporter. How quickly ABA appears and how it could alter other hormones in this stress response is critical if we are to understand plant response to stress. I also study how ABA interacts with other signals via studying ABA response (using RT-qPCR) in ROS production mutants.

The fourth section of my thesis investigates the role of ROS in signalling and plant development under osmotic stress. ROS response in roots to osmotic stress is not well understood when compared to other signals, despite its key roles in stress signalling in stomata (Divreddy *et al.* 2020). Using loss-of-function mutants along with fluorescent reporters, key areas such as ROS production via RBOHs were analysed for their role osmotic stress response and plant development.

ROPs play a key role in plant development acting as switches that can convert stimuli into intracellular responses. There are number of questions remaining around how ROPs influence root development and hormonal crosstalk under osmotic stress. This could be a critical area which could reveal important new findings in abiotic stress response in plants. In the fifth section of my thesis, I study the role of ROPs in osmotic stress response using loss-of-function mutants and qRT-PCR to reveal how ROPs alter plant development and stress response.

Chapter 2. Methods and Materials

2.1 Materials

2.1.1 Chemical Suppliers

All chemicals were supplied by Sigma Aldrich or Fisher Scientific unless otherwise stated.

2.1.2 Plant material

Seed stocks were obtained from the Keith Lindsey laboratory or from the Nottingham Arabidopsis Stock Centre (NASC) (Table 2.1). All mutant and reporter lines are in the Col-0 background unless otherwise stated. T-DNA insertional mutants (SALK mutants) were checked for T-DNA insertion via PCR using primers including T-DNA Left-border (LB) and the target gene sequence (right primer, RP, and left primer, LP), only mutants with homozygous T-DNA insert were used.

Seed Line	**Notes**	**Location**
rbohd - SALK_074825C	*rbohd* mutant in Col background	NASC
rbohf - SALK_129025C	*rbohf* mutant in Col background	NASC
rbohd/rbohf (atrbohD/F)	rbohd/rbohf double mutant in Col background	Courtesy of Alistair Hetherington (University of Bristol)
rbohc SALK_071801C	*rbohc* mutant in Col background	NASC
rbohi SALK_031831	*rbohi* mutant in Col background	NASC
perk4-1 SALK_034666C	*perk4* mutant in Col background	NASC
rop6 SALK_091737C	*rop6* mutant in Col background	NASC
rop2 SALK_055328C	*rop2* mutant in Col background	NASC
upb1-2 SALK_133978C	*upb1-2* mutant in Col background	NASC
upb1-1 SALK_115536	*upb1-1* mutant in Col background	NASC
pin3	*pin3* mutant in Col background	Keith Lindsey Lab
pin7	*pin7* mutant in Col background	Keith Lindsey lab
pin3/pin7	*pin3/pin7* mutant in Col background	Keith Lindsey lab
ABAleonSD1-3L21	ABA reporter in Col background	Courtesy of Karin Schumacher (Heidelberg University)
roGFP2-Orp1 cytosolic	Monitor of Oxidation in Col background	Courtesy of Marcus Schwarzländer (University of Münster)
proPIN3:PIN3:GFP	Reporter of PIN3 in Col background	Keith Lindsey Lab
proPIN7:PIN7:GFP	Reporter of PIN3 in Col background	Keith Lindsey Lab
R2D2	Ratiometric auxin reporter in Col background	NASC
TCSn:GFP	Synthetic Cytokinin Reporter in Col background	NASC

Table 2.1 Seed stocks used in thesis

2.2 Plant tissue culture

2.2.1 Seed sterilisation

To ensure contamination-free experiments, seeds were surface sterilised. Seeds were placed in 1.5ml Eppendorf tube and washed with 70% v/v ethanol for 1 minute. Ethanol solution was replaced with bleach solution (20% v/v) and left for 15 minutes. Bleach solution was removed, and seeds were washed with sterile distilled water (sdH_2O) four times. Following washing, seed were stratified in 1ml of sdH_2O for 4-7 days in the dark at 4C^o. This was to ensure synchronous germination.

2.2.2 Growth conditions

Seedlings were grown on 10cm square plates of agar media containing 1/2 MS Salts (Sigma, 2.2 g/l), MES buffer (Sigma, 6mM, 1.2 g/l) and high gel strength agar (Melford 5g/l) sealed with micropore tape. Seedlings were grown in SANYO growth cabinets (22C°, 18hr photoperiod).

Seven days after germination (DAG) seedlings were transferred to polyethylene glycol (PEG) infused ½ MS agar plates with water potential of (ψw) -0.14, -0.37 or -1.2 MPa, adapted from Rowe (2016) and Verslues *et al.* (2006). Plates were sealed with micropore tape and placed in growth room for 24hrs or 1 week depending on the form of experimentation (22^{o}C, 18 hr photoperiod).

Rank and age play an important role in root growth response to water deficit conditions and ABA response (Rosales *et al.* 2019); as a result, seedling age was kept the same at 7 days.

2.3 Preparation of osmotic treatment

Polyethylene glycol (PEG) breaks down under high temperatures so cannot be autoclaved. As a result, ½ MS agar media (5g/L) was poured and left to dry. Once dried, an overlay solution containing PEG was poured over the media. 24 hours was then given for PEG to diffuse into the agar media.

Both agar media and overlay solution contained ½ MS salts and MES buffer and were adjusted to 5.7 pH. Once autoclaved, PEG-8000 was added to overlay solution.

40 ml of agar solution was poured into 10 cm square plates and allowed to dry. Following this, 60 ml of PEG overlay solution was poured on top. Plates were sealed with parafilm and given 24 hours to equilibrate. After 24 hours, overlay solution was removed, and seedlings were transferred to the PEG-infused media plates.

Desired osmotic pressure of the plates was achieved by adding certain level of PEG to overlay solution following protocol from Rowe *et al*. (2016) (Table 2.2).

Predicted final media water potential (ψ_w) of agar media (MPa)	PEG added to overlay solution (g/l)
-0.15 to -0.25 (Control)	0
-0.3 to -0.5 (Mild Stress)	250
-1.2 to -1.5 (High Stress)	550

Table 2.2 The mass of PEG required in 1 litre of overlay solution to achieve desired osmotic pressure (Verslues *et al.* 2006)

For confocal microscopy, 10% (w/v) PEG solution was created via dissolving PEG-8000 into imaging buffer (1g to 10ml). All other hormone treatment was created via adding stock solution to imaging buffer solution. Previous papers have used 5-20% (w/v) PEG solution to cause a decrease in water potential (Osmolovskaya *et al.* 2018). 10% (w/v) PEG solution was chosen as it is a comparable level of osmotic stress and MPa to that of the High Stress PEG agar plate (-1.2 to 1-5 MPa) (Michel 1983).

2.4 Hormone & chemical treatments

Chemical	**Notes**	**Method of Preparation**
Diphenylene iodonium (DPI)	An inhibitor of NADPH oxidase and other flavin-containing enzymes	Dissolve 31.5 mg DPI in 10 ml DMSO
N-1-naphthylphthalamic acid (NPA)	An inhibitor of PIN-mediated auxin transport	Dissolve 27.3 mg NPA in 10 ml DMSO
Fluridone	An inhibitor of carotenoid biosynthesis, a key ABA biosynthesis pathway	32.9 mg of fluridone was dissolved in 10 ml of methanol and filter sterilised
Hydrogen Peroxide (H_2O_2)	A key reactive oxygen species	4% (w/w) Hydrogen Peroxide diluted in water
Sorbitol	A sugar alcohol used for osmotic stress treatment	Dissolved into water at desired concentration

Table 2.3. Hormone and chemical treatments at 10mM stocks

Hormones were made as 10 mM stocks and stored for a maximum of 6 weeks at 4^{o}C.

For preparation of chemical treatment agar plates, media was melted and allowed to cool to around 40^{o}C. Under a laminar flow hood, the cooled media was poured into 50ml falcon tubes where stock solutions were added to the required concentration. Falcon tube was gently inverted to allow for even distribution of chemical within media.

For chemical treatment in laser scanning microscopy, stocks solutions were added to imaging buffer solutions at room temperature.

2.5 DNA Extraction and Preparation

For genotyping and sequencing, crude genomic DNA extraction was used. A 1.5ml Eppendorf tube was used to pinch out a disc into the tube from a young leaf. Using a small, sterilized pestle, leaf material was ground in the bottom of the tube. Following this, 400ul of Extraction buffer (200 mM Tris-HCl (pH 7.5), 250 mM NaCl, 25 mM EDTA, and 0.5% SDS) was added. After debris was pelleted by centrifuge, 300ul of the supernatant was transferred into a fresh 1.5ml Eppendorf tube. 300µl of isopropanol was added to the supernatant and the solution was left for two minutes. The sample was then spun in a centrifuge for five minutes to pellet the DNA. The supernatant was removed, and the pellet was left to gently dry. 100µl of TE Buffer (10 mM Tris-HCl (pH 8) and 1 mM EDTA) was added and the DNA pellet was dissolved. gDNA sample was then stored at -20C° until required.

2.6 RNA Extraction & cDNA Synthesis

20 mg of plant tissue was placed in a 1.5 ml Eppendorf tube and flash frozen in liquid nitrogen. These samples are stored at -80C° until required. Plant tissue was ground using an autoclaved pestle.

mRNA was extracted from ground plant tissue using Dynabeads mRNA Purification Kit (Thermofisher). Kit specifically captures and purifies mRNA molecules from total RNA preparations. Ribosomal RNA and small RNA molecules do not bind to the beads and are discarded. Extracted RNA was analysed using a Nanodrop 1000 spectrophotometer.

cDNA is synthesised from mRNA directly off the Dynabeads using the SuperScript IV First Stand Synthesis System with Reverse Transcriptase (RT). cDNA samples were tested for genomic DNA contamination via PCR amplification using ACT2 primers designed over an intron (see Primer sequence elsewhere). cDNA samples were then diluted by 5 times with autoclaved MilliQ water to be used for qPCR.

2.7 Polymerase Chain Reaction (PCR)

2.7.1 Primers

Primers were designed using Primer-BLAST or taken from previous papers (Wang *et al.* 2015, Rowe *et al.* 2016) (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Full list of primers is found in Table S1 in the appendix.

2.7.2 Standard PCR reaction

The following reaction used 2x PCRBIO Taq Mix Red to a 20 µl Reaction (Table 2.4). The 2x mix contains PCRBIO Taq DNA Polymerase, 6 mM $MgCl_2$, 2 mM dNTPs, enhances, stabilizers and a red dye for tracking during agarose electrophoresis.

2x PCRBIO Taq Mix Red	10 µl
Forward primer (10 µM)	1 µl
Reverse primer (10 µM)	1 µl
Template DNA Sample	2 µl
PCR grade dH_2O	5 µl

Table 2.4. Reaction mixes for 1x PCR Reaction

A Pro-Flex PCR Applied Bio Systems (Thermofisher Scientific) machine was used with the following program (Table 2.5). Specific annealing temperature was chosen for each primer.

Step	**Temperature (C°)**	**Time**	**Number of Cycles**
Initial Denaturation	95	1 min	1
Denaturation	95	15 s	40
Anneal	55 to 65 depending on primer pair	15 s	
Extension	72	1 to 90 s (15 s per kb)	

Table 2.5. Programme used for PCR, run using a Pro-Flex PCR Machine

2.7.3 Gel electrophoresis

Following completion of the PCR reaction, DNA samples were visualised by size using gel electrophoresis. 1% w/v gels were created via dissolving Agarose into 1xTAE buffer (diluted 1 in 50 from 50X TAE Buffer: 242g Tris (50 mM), 37.2g, $Na_2EDTA.2H_2O$ (2 M), 57.1 ml glacial acetic acid (1 M), in 1 L).

Ethidium bromide was added to a concentration of 0.5 µg/ml. 2xPCR Bio contains mixed in loading buffer. Hyperladder of appropriate length was loaded into gel to help with visualisation. Gels were run for around 40 mins then imaged using a BioRad Gel-Doc 1000 (BioRad).

2.7.4 Quantitative real-time polymerase chain reaction (qRT-PCR)

qPCRBIO SyGreen Blue Mix Lo-ROX (PCR Biosystems) was used with a Qiagen Rotor-Gene Q for all qRT-PCR analyses.

Reaction mix for one reaction (20µl total) (Table 2.6):

2x qPCRBIO SyGreen Blue Mix	10 µl
Forward primer (10 µM)	0.8 µl
Reverse primer (10 µM)	0.8 µl
cDNA Sample	5 µl
PCR grade dH_2O	3.4 µl

Table 2.6. Reaction mix for 1x qPCR reaction

Step	Temperature (C°)	Time	Number of Cycles
Polymerase activation	95	2 mins	1
Denaturation	95	5s	40 Data captured on FAM channel
Anneal/Extension	60-65 (depending on primer pair)	20-30s	
Melt analysis	50-90	3 mins at 50°C, then increase 10C° every 5 s	

Table 2.7. Programme used for qRT-PCR, run using a Rotor-Gene Q Machine

Samples from three or four biological replicates were amplified, with each reaction having three technical replicate reactions. Amplification specificity was checked using the analysis of melt curves in the Rotor-Gene Q series software v1.7. Using a method outlined by Taylor *et al.* (2019), normalized relative expression was calculated from the three technical repeats relative to the amplification of a reference gene in Microsoft Excel. Outliers were removed.

AT5G15710 was selected as a reference gene. This is due to the stability of its expression patterns under osmotic stress, hormone application and at various developmental stages (Czechowski *et al.* 2005).

2.8 Primary seedling growth length analysis

Seedling were plated on sterilized nylon mesh material. Seedlings were transferred to treatment plates after one week post germination via mesh material. On the bottom of each plate, the site of the primary root tip was marked with a permanent marker on every seedling. From this mark the distance of primary root growth was measured. Scans of plates were taken following one week's growth on treatment plates. These images were then analysed via ImageJ. Once an accurate scale was set, length was measured via segmented line. Number of lateral roots on each seedling was also counted.

2.9 Microscopy

2.9.1 Laser Confocal Scanning Microscopy (LCSM)

Seedlings were examined using a Zeiss LCSM 800 or LCSM 880 (https://www.zeiss.com/-microscopy/int/home.html). Roots were imaged using either a x10 or x20 air objective lens. Z-stacks were taken of each seedling to gain the maximum information possible. Settings such as gain, line, Z-step, averaging etc. were altered between each fluorescent reporter to optimise image quality and consistency. These settings were kept the same between individual seedlings of a reporter line to ensure fair comparison.

2.9.2 Steady state imaging

After 7 days growth on agar medium, seedlings were either transferred with forceps to a 10-minute solution treatment or a 24-hour agar plate treatment. For the 10-minute treatment, seedlings were immersed to either a control imaging solution (10 mM MES–Tris base (pH 5.8), 10 mM $CaCl_2$, 5 mM KCl) or an imaging solution containing PEG/Sorbitol etc. Following 10 minutes of immersion in control/treatment, seedlings were transferred to a slide. The longer 24-hour treatment, seedlings were transferred to a control agar plate or a plate with a treatment. Following 24-hours of treatment, seedlings were transferred to a slide and imaged immediately.

On the slide, seedlings were suspended in imaging solution before the placement of a cover slip (22x22, 0.13-0.16mm thick). To prevent damage of the seedling by compression of the lens on the coverslip, two additional thinner cover slips (22x22, 0.13-0.16mm thick) were placed either side of the root before placement of the top cover slip. Cover slips were sealed and secured with nail polish.

Excitation of fluorophores was performed as follows

Fluorophore/Dye	**Excitation/Laser Line (nm)**
Calcofluor White	405
YFP/Venus	488 or 514
GFP	488
tdTomato	543
Propidium Iodide	543
mTurqouoise	458
cpVenus173	458
roGFP2-Orp1	405/458

Table 2.8. Excitation of fluorophores and dyes for LCSM

2.9.3 Preparation of fixed samples using ClearSee

Following 24hr treatment, instead of immediately imaging on that day; TCSn:GFP, *pls-3/*TCSn:GFP, R2D2, proPIN3:PIN3:GFP and proPIN7:PIN7:GFP seedlings were fixed using the ClearSee method previously described by Kurihara *et al.* (2015). The ClearSee protocol enables rapid fixing and clearing of plant tissues whilst retaining the activity of fluorescent proteins and is compatible with various fluorescent dyes (Ursache *et al.* 2018)

To prepare 4% (w/v) paraformaldehyde (PFA) solution for the fixing procedure. In a fume hood, 4g of PFA powder is added to 1L of 1x phosphate-buffered saline (PBS) solution on a magnetic stirrer and heated to around 6OC^{o}. To ensure the PFA powder is dissolved, the pH is raised using a 1M KOH solution until the solution is clear. The pH is then adjusted to 6.9 with 1M HCL solution. The solution is cooled and filtered before use. The PFA solution was used fresh or kept a 4C^{o} and used within a week.

Seedlings were transferred with forceps to the 4% (w/v) PFA solution where they were fixed under vacuum for 30 mins. Following fixation, seedlings were washed in 1X PBS solution twice before the addition of ClearSee solution, where they were again placed under vacuum for 30 mins.

ClearSee solution was prepared via mixing together xyltiol (10% w/v), sodium deoxycholate (15% w/v) and urea (25% w/v) and H_2O in a solution for 30 mins. Fixed seedlings were left in ClearSee solution at room temperature for at least a week with the ClearSee replaced every few days. Following clearing, seedlings were stained and imaged.

To visualise cell structure and organisation under LCSM, cleared seedlings were submerged in 0.1% (w/v) Calcoflour White in ClearSee solution for 30 minutes. Following staining, Calcolfuor White solution was replaced by ClearSee and seedlings were washed for another 30 minutes. For LCSM, fixed seedlings were mounted on sides in ClearSee solution using coverslip placement previously described.

2.10 Analysis of confocal images

Images were opened and analysed in FIJI (FIJI is Just Image J). Analysis was conducted as follows. Ratiometric reporters were calculated using Image Calculator (https://imagej.nih.gov/ij/docs/menus/process.html#calculator) which using arithmetic and logical operations is able to create a ratio between two channels. The polygon tool was used to select regions on the root for analysis. Mean grey value was used as a measurement of the fluorescent ratio.

2.10.1 Analysis of R2D2 reporter lines

R2D2 (Ratio-metric version of 2 D2s) is a combination of RPS5A-driven DII fused to n3xVenus and RPS5A-driven mDII fused to ntdTomato on a single transgene (Liao *et al.* 2015). Auxin is measured as the as the change in ratio of fluorescence between the two channels. An auxin increase is measured by a reduction of the Venus signal relative to the steady dTomato signal.

The ratio between Venus and dTomato signals was calculated via the Image Calculator function of ImageJ. The dTomato channel SUM-STACK image was divided by the Venus SUM-STACK image, resulting in an image that was analysed to calculate a ratio.

2.10.2 Analysis of proPIN3::PIN3:GFP, proPIN7::PIN7:GFP, TCSn:GFP and *pls-3/*TCSn:GFP

The polygon tool in ImageJ was used to select regions and fluorescence (mean grey area) was measured. To calculate internalisation, mean green channel intensity was measured at the cell membrane and in the intracellular region of the cell to find a ratio of fluorescence between cell membrane and cytoplasm.

2.10.3 Analysis of ABAleonSD1-3L21 and roGFP2-Orp1

ABAleon uses PYR1 fused to a truncated ABI1 as a sensory domain linked to mTurquoise (FRET donor) and circularly permutated Venus (cpVenus, FRET acceptor), these are modulated upon ABA binding, triggering changes in fluorescence emission (Isoda *et al.* 2021).

Without ABA, ABAleon is able to have FRET to occur between mTurquoise to cpVenus. ABA triggers an increase in the distance between probes, resulting reduced FRET efficiency (Waadt *et al.* 2014). Increase in ABA increases mTurquoise and decreases cpVenus173 emission, resulting in a decrease in emission ratios. Low ratios indicate high ABA concentrations and high ratios indicate low ABA concentrations (Waadt *et al.* 2014). ABAleonSD1-3L21 is an improved version of ABAleon, with a faster response to ABA than that of ABACUS1-2u (Isoda *et al.*2021), thought its presence might alter ABA signalling to some effect (Waadt *et al.* 2020). ABAleonSD1-3L21 was excited at 458nm, mTurquoise emission was recorded at 465-505nm and cpVenus173 emission was recorded at 520-550nm. Emission ratio was then calculated by cpVenus173 divided by mTurquoise emission.

ROS sensors such as Hyper are pH sensitive and therefore unreliable in certain situations (Schwarzlander *et al.* 2008). roGFP2-Orp1 was used as it displays insensitivity to pH changes and displays rapid intracellular oxidation changes. Cytosolic roGFP2-Orp1 was excited at 405 and 488nm and emission was recorded at 510-530nm, with the pinhole set to 0.7AU. Cytosolic oxidation state was measured by calculating the ratio between 405/488 laser emission in ImageJ. Autofluorescence was collected at 430-470 nm and removed via ImageJ. An increase in the 405/488 ratio represented an increase in oxidation. Dynamic monitoring of specifically of H_2O_2 is only possible under the assumption that reduction rate remains constant. Potentially changes in oxidation may not be H_2O_2 induced but rather the changes in cytosolic antioxidant capacity. As a result, roGFP2-Orp1 cannot be used as a direct H_2O_2 sensor in cytosol but will give us a representation of oxidation levels (Nietzel *et al.* 2019). roGFP2-Orp1 was tested via treatment of 20mM DTT (reducing) and 100mM H_2O_2 (oxidising) to check if the reporter was working.

2.11 CRISPR/Cas9 gene editing of POLARIS (*gPLS*; At4g39403)

This work was performed in collaboration with Dr Johan T.M. Kroon, Department of Biosciences, Durham University.

2.11.1 Molecular construction of CRISPR/Cas9 plant vector with 2 *POLARIS (PLS)* guide RNAs

For CRISPR/Cas9-mediated generation of *pls* mutants, two gRNA spacer sequences on the - strand were chosen as Cas9 targets: for *pls*; target 1 (5'- CCATTGAtcaccctatcatttt -3') and target 2 (5'- CCATTTCTCCATGTTATATATCA -3') were chosen.

Target specificities were evaluated with CAS-OFFINDER, using an algorithm for potential off target sites of Cas9 RNA-guided endonucleases (Bae *et al.* 2014). The *PLS* expression module, was constructed via PCR using pCBC-DT1T2 as a template according to Xing *et al.* (2014) and Golden Gate assembled into the custom vector pJK PMCEE401E (*to be published*) allowing egg cell-specific EC1.2en:EC1.1 promoter-controlled expression of 3× FLAG-NLS573 zCas9-NLS46.

PCR primers incorporating the target sequences and a Type IIS restriction enzyme sequence (*Bsa*I) were combined with the plasmid pCBC-DT1T2-PCR0 as a template to generate a 626 bp PCR product producing a gene expression module for two pls gRNAs decorated with a U6-26tail for the target 1 gRNA and a U6-29promoter for target 2 gRNA (Figure 2.1).

Gtatcgcatttgtttcaagttttttttctataatgtttcttcgaaatccatgatcatatagtatataagaag
catgtatttataatgttccacttaatatattagtattggagactaaagcgaacatataaaacccaaataaacc
tttctttaagttttattaaaagtctaaacacttgatttgtgttttagtttgggtagtagtgagaaaagaaaaa
taaataatcaaaaagattaaagaagaaagaatttgaaagcaaggaacacgaaatccgaagagcgagggGAGCG
AAGACAGTCCACGTAGCTGcagagagaaagaGAAGAGCACGTGAGGCACACGttccttgtgtaagacttgttg
tggtgatgttggcgcagtgtctcactgaaacatgaATGAAACCCAGACTTTGTTTTAATTTCAGGCGAAGGTC
CATTTCTCCATGTTATATATCAATCTCTTATTTATTAGTAGCAAAATTGTTTAAACTTTTTAAAATCCATTGA
tcacCCTATCATTTTCAATATCTACATACaatcttatgtctcgataaaggtttatctttatcttattatgcaa
tacatatccctcccatttctatattgcaaattatgacatcaaaaaaccattcttttgattctacttgggccaa
taacaaaatcaatagtaatggaaaaaataacgtagatggatataaatatagtccaacGGTTCAATTTCATCAG
TAATAAGTATATAGcaaaaaaaaaaacgtttaactcagcatagttcaaaagggaccacattggtcatgtaaat
ataacatccataaaaacaaatttaagtctcatctattctcactttcccattcccgtgcagtgtgaattcccca
aattaattccgacaataatgaat

Figure 2.1A

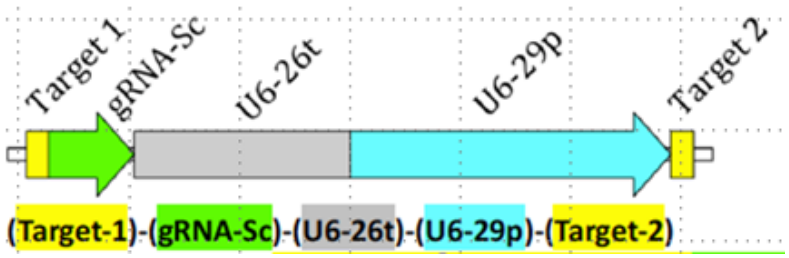

Figure 2.1B

Figure 2.1A Genomic DNA sequence of the POLARIS (PLS), At4g39403**.** Translational Start/Stop domain highlighted in Blue. Exon in green CAPITAL letters. UTR in red lowercase letters. The PAM domains are highlighted green, and the two chosen gRNA spacer sequences are highlighted in yellow. **Figure 2.1B**. Sequence of gene expression module two *pls* gRNAs decorated with a U6-26tail for the target 1 gRNA and a U6-29promoter for target 2 gRNA in *Arabidopsis*

This product was then purified and in turn incorporated into pJK_PMCEE401E vector (provided by Dr. Johan Kroon, Durham University) using a Golden Gate reaction. The pJK PMCEE401E vector will allow selection of positively transformed plants via fluorescence microscopy screening for red fluorescent seeds (dried) instead of using more time consuming and expensive antibiotic screening of seedlings in tissue culture.

E.coli cells were transformed with the ligation product, and recombinants were selected colony PCR and validated through DNA sequencing.

The *PLS*-CRISPR/Cas9 construct was transformed into *Agrobacterium tumefaciens* strain GV3101/pMP90 via electroporation and used in floral dip plant transformation of *Arabidopsis thaliana* Col-0 WT plants, synthetic cytokinin reporter line plants, (TCSn:GFP) and ratiometric auxin-reporter line (R2D2) plants in order to generate single *pls* -CRISPR knock-out mutants.

T1 seed was obtained and screened for positive independent transformants via fluorescence microscopy screening of dried seeds for red fluorescence

Genomic DNA was extracted from T1, T2 and T3 transgenic plants grown in soil. To analyse CRISPR/Cas9 generated genomic editing events, fragments surrounding the target sites were amplified by PCR using gene-specific primers *gPLS CRISPR F* and *gPLS CRISPR R.* Products were submitted for amplicon sequencing using nested primers (*gPLS CRISPR seq F* or *gPLS CRISPR seq R*) to identify the nature and genetic status of mutations. The sequences of the primers used are provided in Table 2.9**.** DNA sequencing was performed by Durham University Biosciences Genomics

As one *pls* mutant is already isolated (Casson *et al.* 2002), the new CRISPR/Cas9 mutants are called *pls-2* and *pls-3/*TCSn:GFP, representing independent CRISPR/Cas9 editing events.

Primer Name	Sequence	Description
gPLS DT1 F	ATATATGGTCTCGATTGaaaatgatag ggtgaTCAAGTTTTAGAGCTAGAAA TAGC	Golden gate cloning of gPLS-DT1DT2 gRNA expression module into pJK_PMCEE401E
gPLS DT2 R	ATTATTGGTCTCGAAACTTTCTCCA TGTTATATATCCAATCTCTTAGTCG ACTCTAC	Golden gate cloning of gPLS-DT1DT2 gRNA expression module into pJK_PMCEE401E
gPLS CRISPR F	gttccacttaatatattagtattgg	Genotyping of gPLS CRISPR-Cas9 targets 1 and 2
gPLS CRISPR R	ctatatacttattactgatgaaattgaacc	Genotyping of gPLS CRISPR-Cas9 targets 1 and 2
gPLS CRISPR seq F	gaagagcacgtgaggcacacg	Sequencing of gPLS CRISPR-Cas9 targets 1 and 2
gPLS CRISPR seq R	gaaaatgatagggtgaTCAATGG	Sequencing of gPLS CRISPR-Cas9 targets 1 and 2

Table 2.9. Sequences of Primers used in CRISPR/Cas9 gene editing of PLS

2.12 Statistical Analysis

All statistical analysis and plotting were carried out in R Studio. The statistical tests used are detailed on the figures. 0.05 level of significance was used.

Chapter 3. The role of PLS in root development

3.1 Introduction

POLARIS (PLS) is a small peptide that acts as a negative regulator of ethylene responses. PLS likely regulates the interaction of ethylene receptor ETR1 and its copper cofactor. Previously a *pls* mutant was developed and found to display normal level of ethylene biosynthesis but have a short root enhanced ethylene phenotype. PLS has been shown to act as a molecular site of crosstalk between auxin, cytokinin and ethylene responses (Chilley *et al.* 2006, Liu *et al.* 2014).

PLS expression was shown to decrease under osmotic stress conditions (Rowe *et al.* 2016), thought to be caused by lowering auxin and increased ethylene levels. Lowered PLS expression likely decreases promotion of auxin in the root tip and enhances the ethylene pathway (Rowe *et al.* 2016, Chilley *et al.* 2006). This previous study uses a *pls-1* mutant that was developed in the C24 *Arabidopsis* background, which has been shown to display tolerance to abiotic stresses (Bechtold *et al.* 2018). As a result, a new *pls* mutant was developed in the Col-0 background via targeting using CRISPR-Cas9 genome targeting and was called *pls-2*. This was developed in order to analyse the role of PLS in the Col-0 background under osmotic stress. Unfortunately, not enough time was available to study the how *pls-2* performs under osmotic stress conditions.

There remain large gaps of knowledge of how ethylene and cytokinin interact with each other (Iqbal *et al.* 2017). It is important to study how cytokinin is altered by the absence of PLS, a key negative regulator of ethylene response. As a result, the *pls-3* mutant was developed in the synthetic cytokinin reporter TCSn:GFP (Liu and Muller 2017) via CRISPR Cas9 genome targeting (*pls-3/*TCSn:GFP). This enables study of cytokinin levels within the *pls* mutant.

In order to understand the role of PLS in root development, I aimed to analyse the primary root growth in *pls-2* mutant seedlings in the dark. To reveal the role PLS plays in cytokinin distribution, TCSn:GFP distribution in *pls-3* mutant will be studied via LCSM.

3.2 Results

3.2.1 Loss of PLS alters seedling growth in the dark

Figure (3.1) shows that the *pls-2* mutant has significantly shorter shoots and roots when compared to Col-0 after 1 week's growth in the dark following germination ($p<0.05$). When studying shoot length and root length against each other, the *pls-2* mutant has significantly larger shoot:root ratio when compared to Col-0 after growth in the dark ($p<0.05$). These observations are consistent with an enhanced ethylene response expected for the *pls* mutant (Chilley *et al.* 2006).

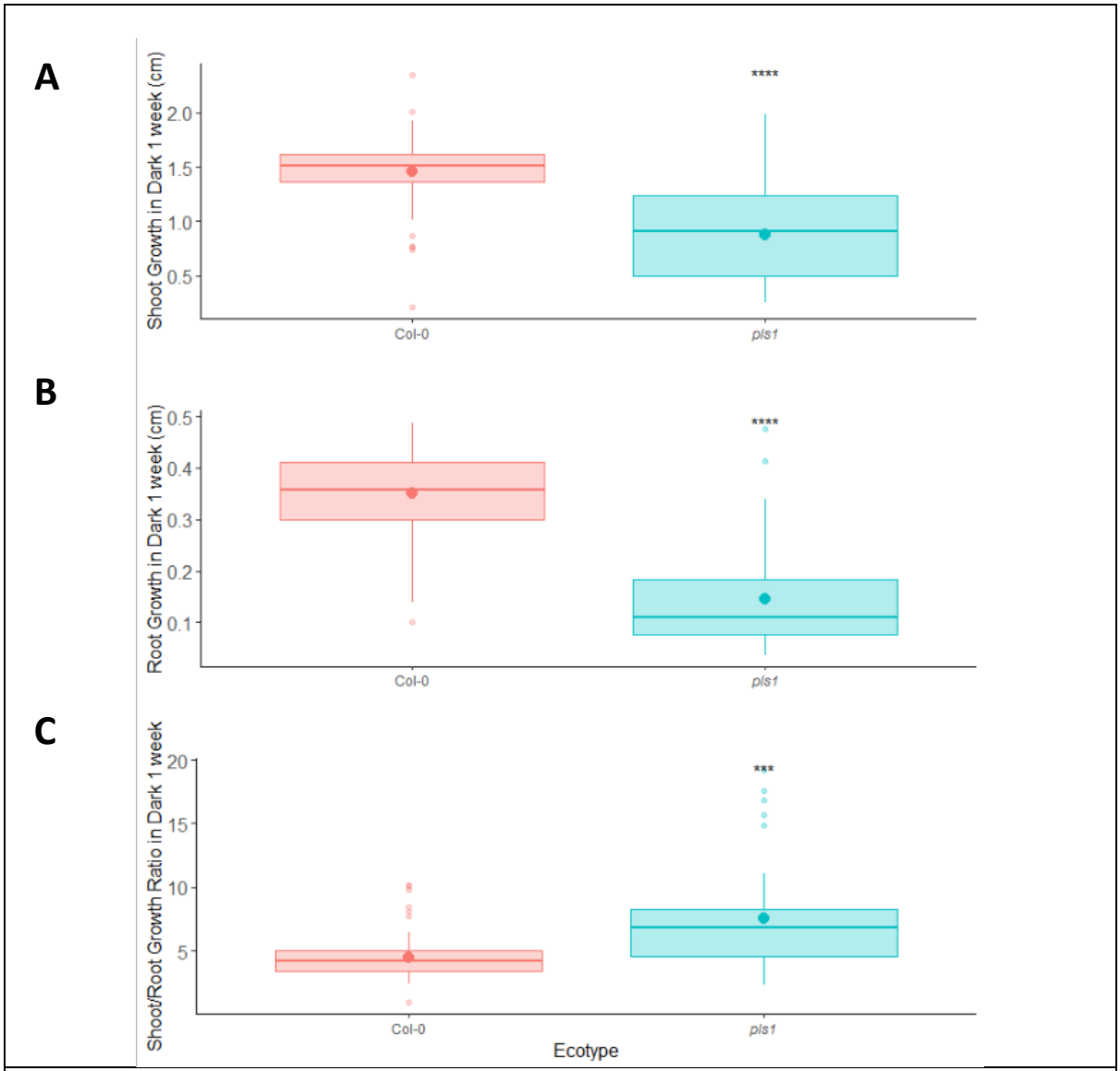

Figure 3.1. **(A)** Shoot growth of Col-0 and *pls-2* in the dark for 1 week post germination. **(B)** Primary root growth of Col-0 and *pls-2* in the dark for 1 week post germination. **(C)**. Ratio between shoot/root growth in Col-0 and *pls-2* after 1 week post germination. For boxplots, upper and lower boundaries of the box indicate the interquartile range (IQE), dark circle within the box represents the mean, a line within the box represents the median, and the whiskers represent the min and max excluding outliers. Small circles represent outliers. Stars and brackets indicate significance from t-test of treatment compared to Col-0 (NS = not significant, *<0.05, **<0.01, ***<0.001).

3.2.2 PLS influences TCSn:GFP distribution

TCSn:GFP is a synthetic cytokinin reporter that allows the visualization of cytokinin in plant roots without the need for tissue processing steps (Liu and Muller 2017). By removing PLS function via

CRISPR-Cas9 genome targeting in TCSn:GFP, it was possible to visualise how the presence/absence of PLS alters cytokinin distribution in TCSn:GFP. Results show that *pls-3*/TCSn:GFP has an increased fluorescence in both the entire root (non-significant) and in the QC (t-test, near p=0.05) indicating that cytokinin levels rise if the *pls* mutant is absent (Figure 3.2, 3.3).

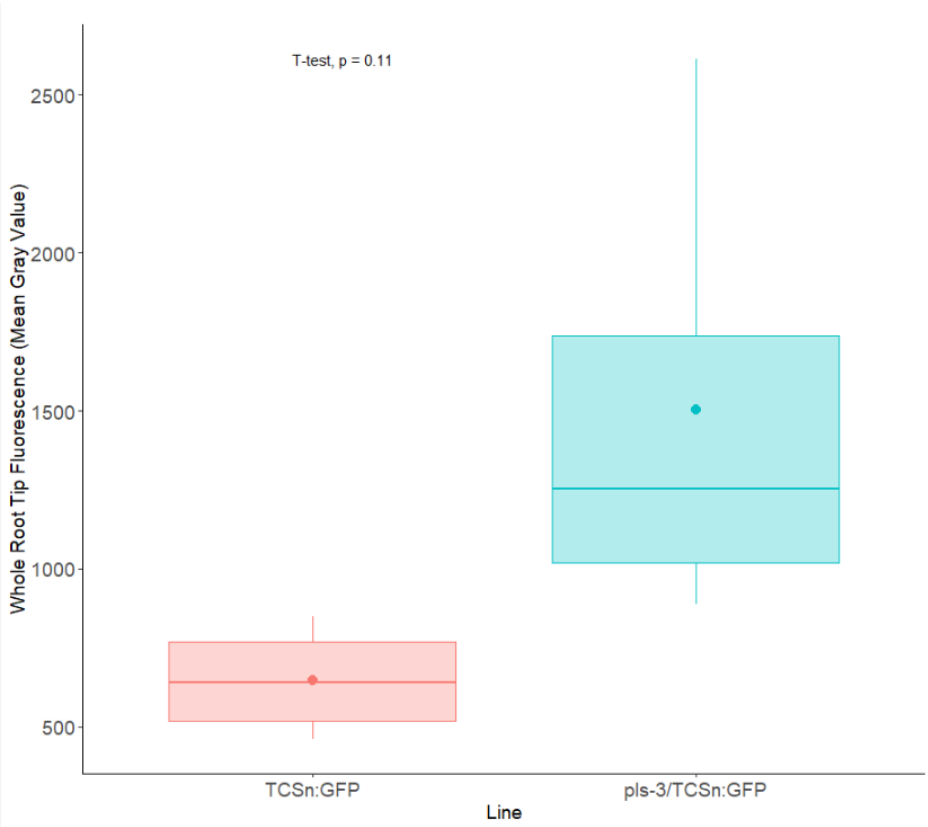

Figure 3.2

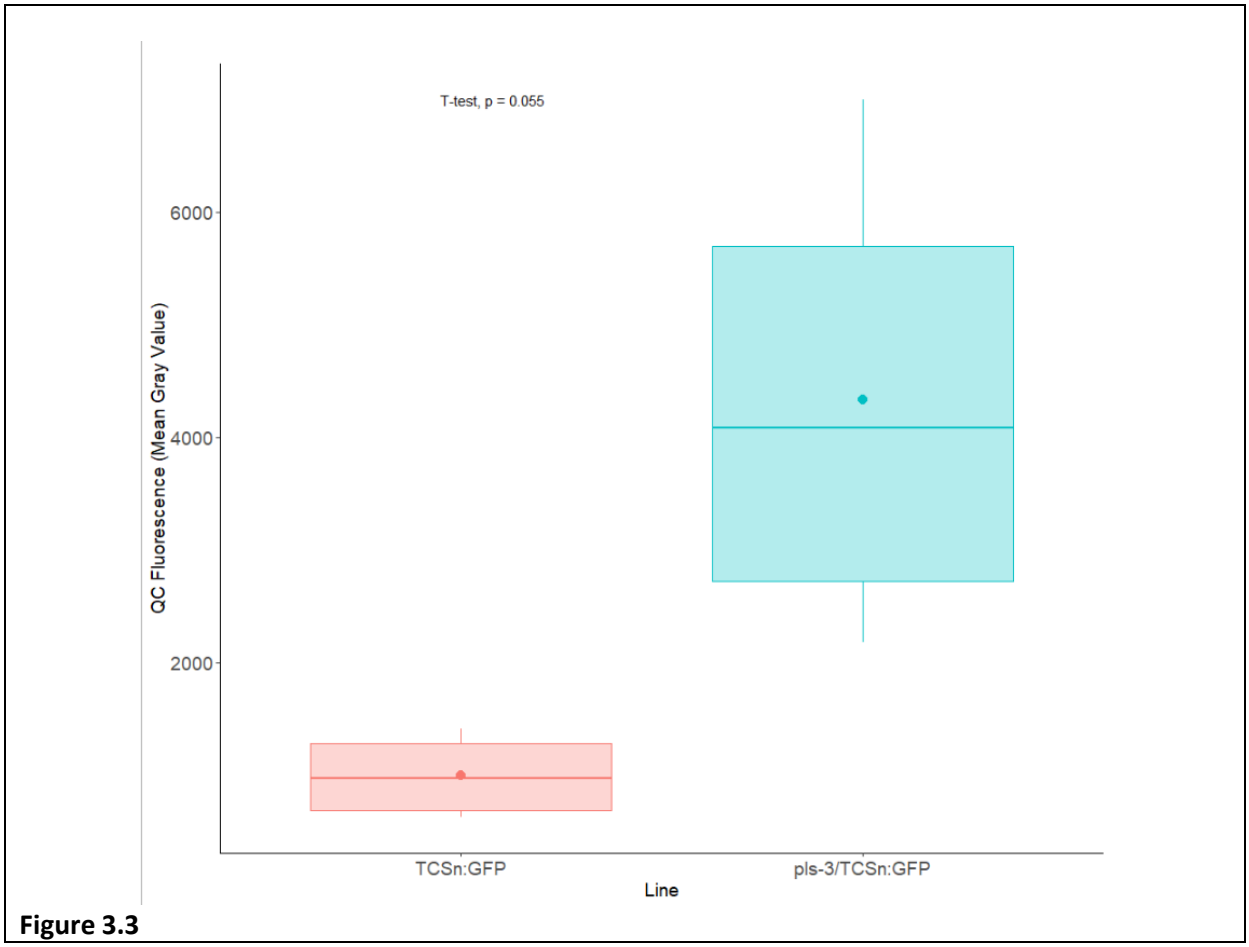
T-test, p = 0.055
6000
4000
2000
QC Fluorescence (Mean Gray Value)
TCSn:GFP
pls-3/TCSn:GFP
Line

Figure 3.3

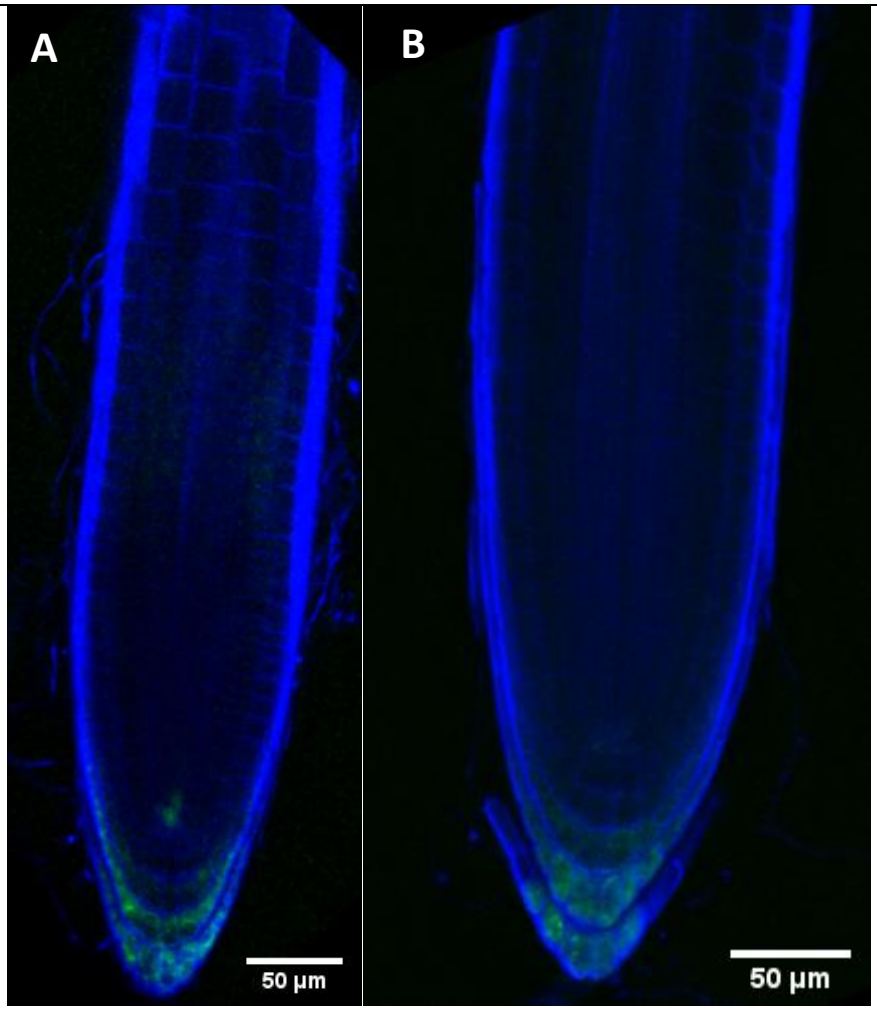

Figure 3.4

Figure (3.2) The whole root fluorescence (Mean Gray Value) of TCSn:GFP and *pls-3*/TCSn:GFP 7 DAG. **Figure (3.3)** The level of fluorescence (Mean Gray Value) in the quiescent centre (QC) in both TCSn:GFP and *pls-3*/TCSn:GFP 7 DAG **Figure (3.4) (A)** 20x confocal image of TCSn:GFP and **(B)** *pls-3/*TCSn:GFP. For boxplots, upper and lower boundaries of the box indicate the interquartile range (IQE), dark circle within the box represents the mean, a line within the box represents the median, and the whiskers represent the min and max excluding outliers. Small circles represent outliers. Stars and brackets indicate significance from t-test of treatment compared to control (NS = not significant, *<0.05, **<0.01, ***<0.001). Anova performed with significance detailed.

3.3 Summary

PLS appears to play a key role in root development, with the *pls-2* mutant demonstrating significantly smaller roots and shoots when compared with Col-0.

PLS function has a significant impact on cytokinin distribution as the *pls-3* mutant displays higher levels of TCSn:GFP distribution when compared to the control.

Chapter 4. The role of auxin and its distribution under osmotic stress

4.1 Introduction

The role of auxin is critical in root development. Maintaining high auxin concentration in the root QC and stem cell nice is critical for the coordination and establishment of growth (Clark *et al.* 2014). Previous work has identified that auxin levels reduce under osmotic stress condition, likely due to the modulation of auxin transporter levels/distribution, which contributes to a reduction in root growth. PIN1 levels were found to reduce under osmotic stress in an ABA-dependent manner (Rowe *et al.* 2016).

There remain a number of questions of how auxin levels change under osmotic stress, in particular the response within minutes to osmotic stress. Using auxin reporter R2D2 it was possible to study the response of auxin to osmotic stress in detail. The roles of PIN3 and PIN7 have yet to be analysed in their role in root development under osmotic stress. How the process of auxin oxidation alters osmotic stress response has also not been studied in detail.

How ROS alter auxin response to osmotic stress has not received much attention. ROS have previously been shown to have an important impact on auxin, as result auxin response was studied in the *rbohc* mutant. It is also important to see whether key cellular signalling components ROP2 plays a role in the decrease in auxin under osmotic stress.

In order to understand how roots are altered by osmotic stress, seven days after germination (DAG) seedlings were transferred to ½ MS agar containing high molecular weight polyethylene glycol (PEG). This gives us the opportunity to study the impact of osmotic stress independently of other stresses that salt/mechanical etc that can result from dry soil. A similar protocol of Rowe *et al.* (2016) was followed, with two osmotic stress treatments – a moderate stress (-0.37 MPa) and a severe stress (-1.2 MPa). Control plates with no PEG were found to have an osmotic pressure of -0.14MPA.

Using osmotic stress media, primary seedling root length and number of lateral roots were analysed in Col-0 seedlings to determine my own study and reinforce previous results (Rowe *et al.* 2016)

In order to understand auxin level response to osmotic stress in both short term (10 mins) and after 24hrs were assessed using fluorescent reporter R2D2 via LCSM. The PIN-transporter inhibitor NPA was used to assess the role of PIN transport under osmotic stress, and to determine whether its inhibition has an impact on auxin levels.

To understand how auxin transport via PIN3 and PIN7 distribution was altered by osmotic stress, proPIN3:PIN3:GFP and proPIN7:PIN7:GFP were studied under osmotic stress. *PIN3* and *PIN7* expression was studied under osmotic stress conditions via qRT-PCR.

The primary root growth of auxin mutants *pin3, pin7, pin3/pin7* and *dao1* mutants was assessed under osmotic stress conditions, to determine how critical auxin transport and oxidation is for osmotic stress response.

Both the role of RBOHC and ROP2 in auxin response to osmotic stress was assessed via qRT-PCR of auxin-responsive gene *IAA2* in *rbohc* and *rop2* mutants. This aimed to determine whether the presence of these proteins is critical for the decrease in *IAA2* levels that has previously been observed (Rowe *et al.* 2016).

4.2 Results

4.2.1 Osmotic stress results in a reduction of primary root growth and the number of lateral roots

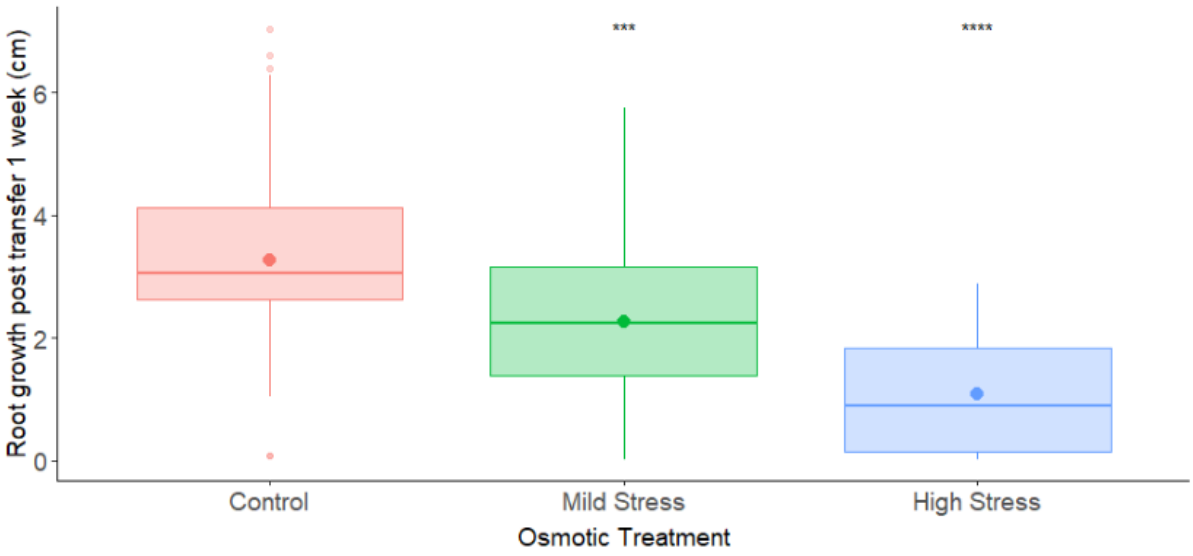

Figure 4.1

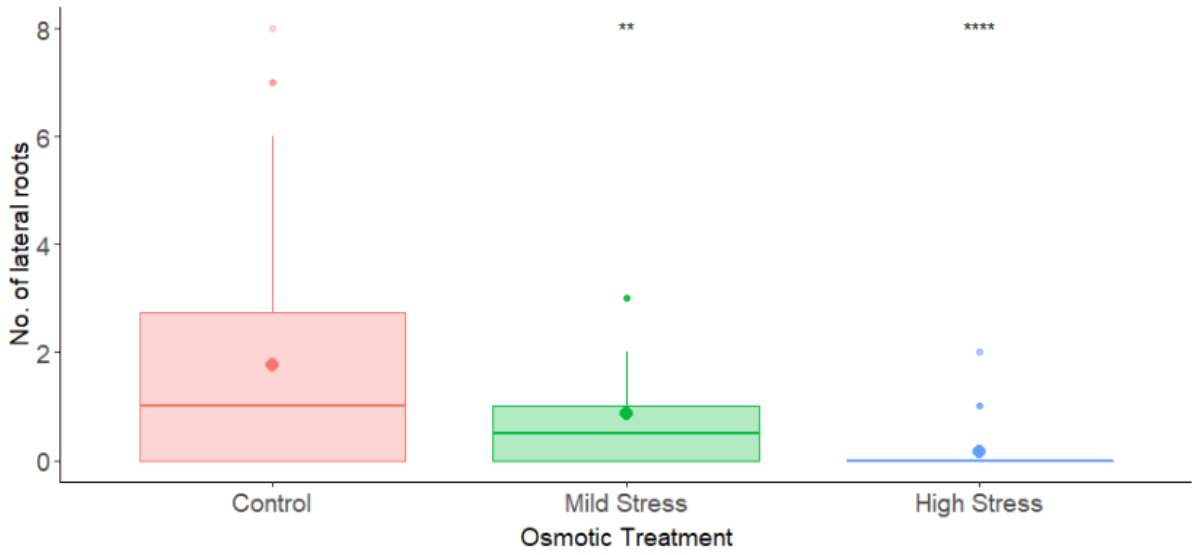

Figure 4.2

Figure 4.1 The primary root length of Col-0 (WT) seedlings following 1 week of osmotic stress treatment.
Figure 4.2 The number of lateral roots on Col-0 (WT) seedlings following 1 week of osmotic stress treatment. For boxplots, upper and lower boundaries of the box indicate the interquartile range (IQE), a line within the box represents the median, and the whiskers represent the min and max excluding outliers. Small circles represent outliers. Stars indicate significance from t-test of treatment compared to Control conditions (NS = not significant, *<0.05, **<0.01, ***<0.001). Anova performed with significance detailed.

Under mild and high osmotic stress, we see a significant reduction in length of primary root growth after 1 week, when compared to control conditions (Figure 4.1) ($p<0.05$). There is also a reduction in the number of lateral roots under both mild and high osmotic stress conditions when compared to control conditions (Figure 4.2) ($p<0.05$). This indicates that osmotic stress has a strong negative impact on root development, especially under high stress conditions.

4.2.2 R2D2 response under osmotic stress

R2D2 (Ratiometric version of 2 D2s) is a useful auxin reporter which contains a combination of RPS5a-driven DII fused to 3xVenus and RSP5-driven mDII fused to ntdTomato on a single transgene (Liao *et al.* 2015). It is possible to visualise auxin accumulation via the reduction of the Venus signal relative to the dTomato signal. Changes in auxin levels are possible to be measured via the change in the ratio of fluorescence between the two channels.

Using the R2D2 reporter it was possible to study the response of auxin within minutes of osmotic stress stimuli, in the form of 10% PEG solution in which the seedlings were placed. Within the 10 - minute osmotic stress treatment, there is a significant increase in the mDII-ndTomato/DII-n3xVenus ratio in R2D2 when compared to control (Figure 4.3) ($p<0.01$). This indicates a significant increase in auxin levels in the short term in response to osmotic stress.

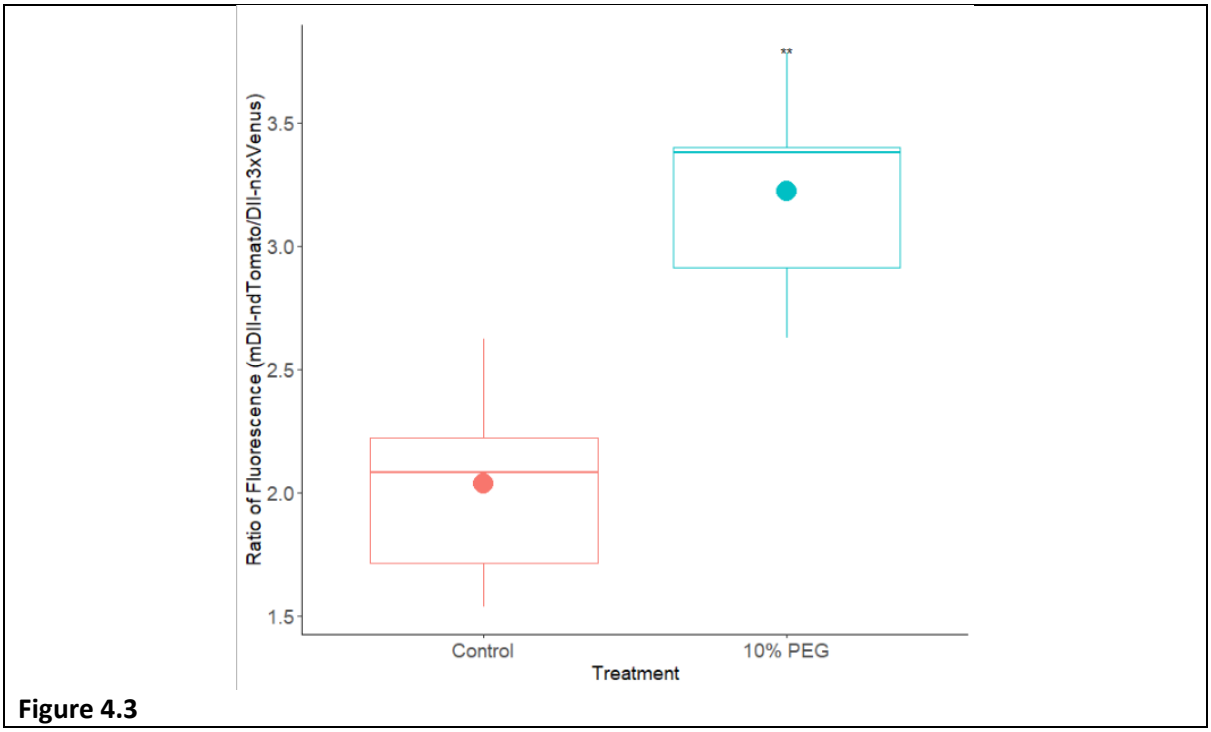

Figure 4.3

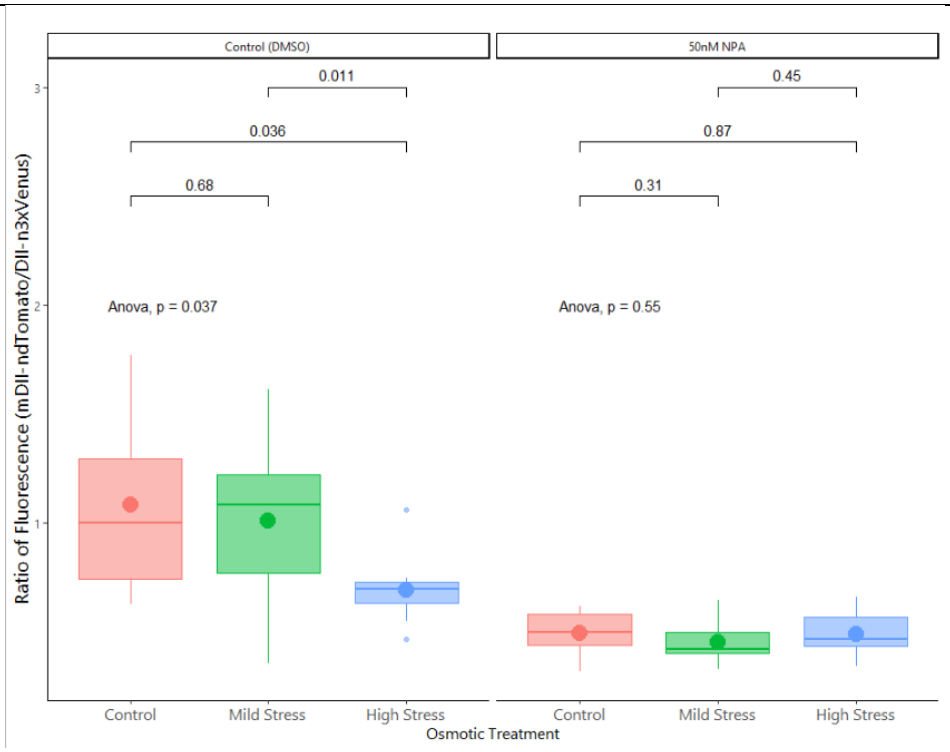

Figure 4.4

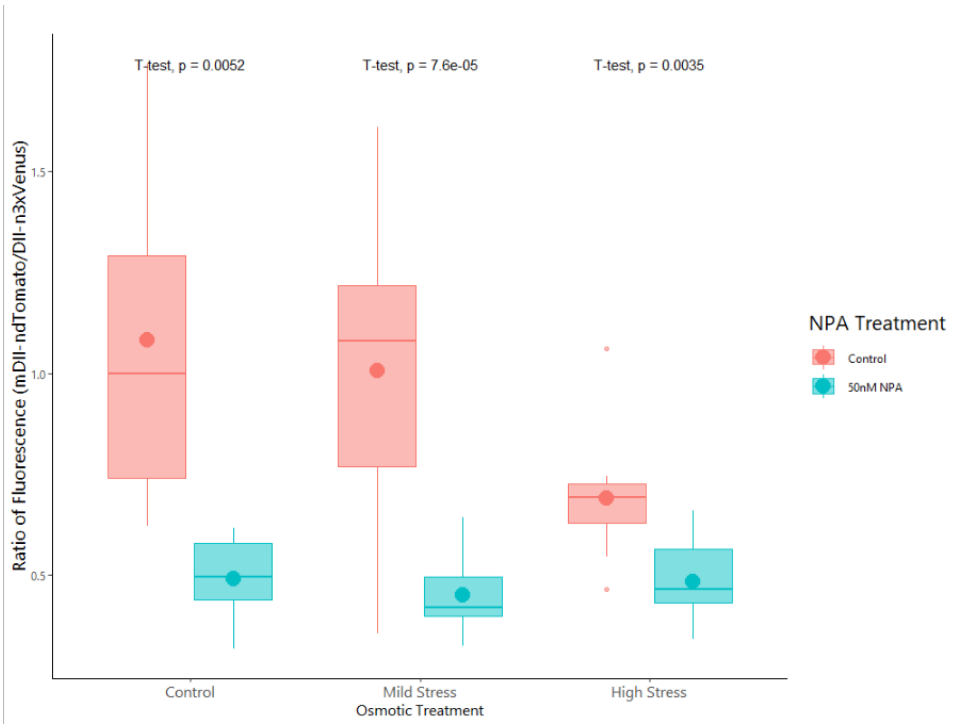

Figure 4.5

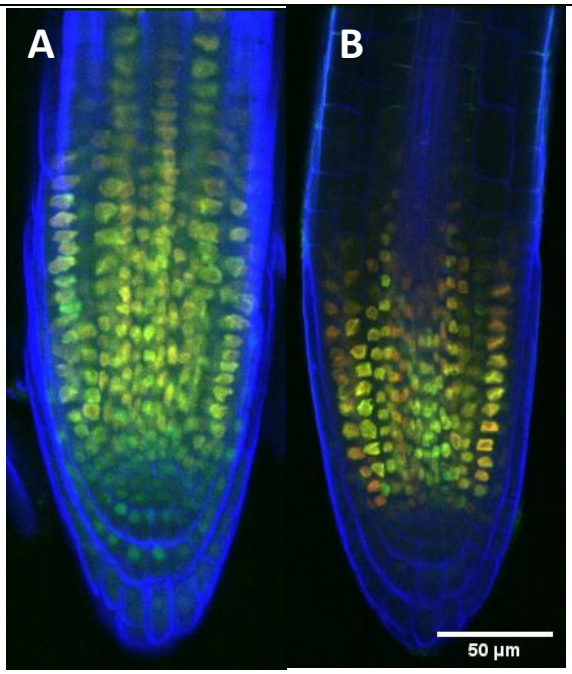

Figure 4.6

Figure 4.3. The change in R2D2 mDII-ndTomato/DII-n3xVenus ratio after 10 minutes of control and 10% PEG treatment. **Figure 4.4 & 4.4**. The levels of R2D2 mDII-ndTomato/DII-n3xVenus ratio after 24hrs or control, mild and high osmotic stress conditions. In combination with control (DMSO) or 50nM NPA treatment. **Figure 4.5**. 20x Confocal images of R2D2 under 24hr **(A)** control and **(B)** high osmotic stress conditions (Red = mDII-ndTomato, Green = DII-n3xVenus, Blue = Calc W staining). For boxplots, upper and lower boundaries of the box indicate the interquartile range (IQE), dark circle within the box represents the mean, a line within the box represents the median, and the whiskers represent the min and max excluding outliers. Small circles represent outliers. Stars and brackets indicate significance from t-test of treatment compared to control (NS = not significant, *<0.05, **<0.01, ***<0.001). Anova performed with significance detailed.

Under 24 hours of high osmotic stress treatment, when compared to the control we see a significant decrease in the mDII-ndTomato/DII-n3xVenus ratio, indicating a decrease in auxin levels under longer term osmotic stress (Figure 4.4) ($p<0.05$). Mild osmotic stress treatment appears to have little impact on auxin levels after 24hrs, represented by little change in R2D2 levels.

Treatment with direct PIN-transport inhibitor N-1-napthylphtalalmic acid NPA (50nM) has a significant reduction on the mDII-ndTomato/DII-n3xVenus ratio in R2D2, in control, mild and high stress osmotic conditions when compared with their respective control (DMSO) treatments (Figure 4.5) ($p<0.01$, $p<0.01$, $p<0.01$). This indicates that treatment with NPA leads to a significant reduction in auxin at the root tip.

In 50 nM NPA-treated plants, the root tip experiences little change in the Tomato/Venus fluorescence ratio after 24 hr mild and high osmotic stress conditions when compared to the control. This indicates that osmotic stress has little impact on auxin levels within NPA treated roots as auxin levels are already heavily reduced.

4.2.3 proPIN3:PIN3:GFP and proPIN7:PIN7:GFP analysis under osmotic stress

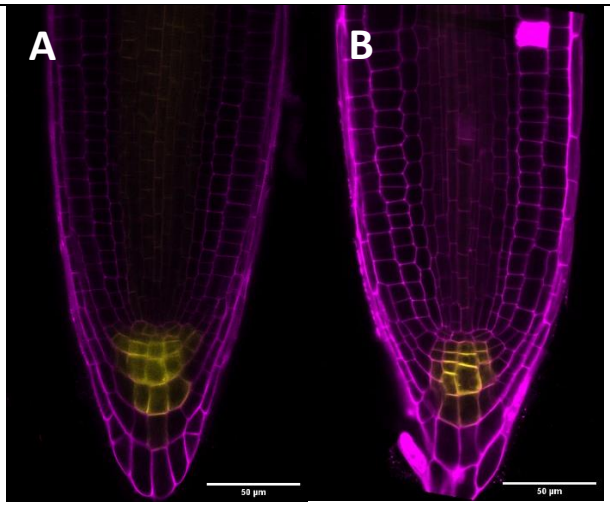

Figure 4.7

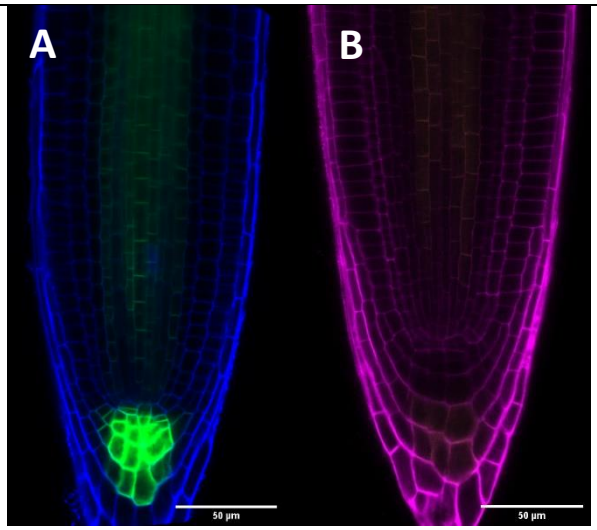

Figure 4.8

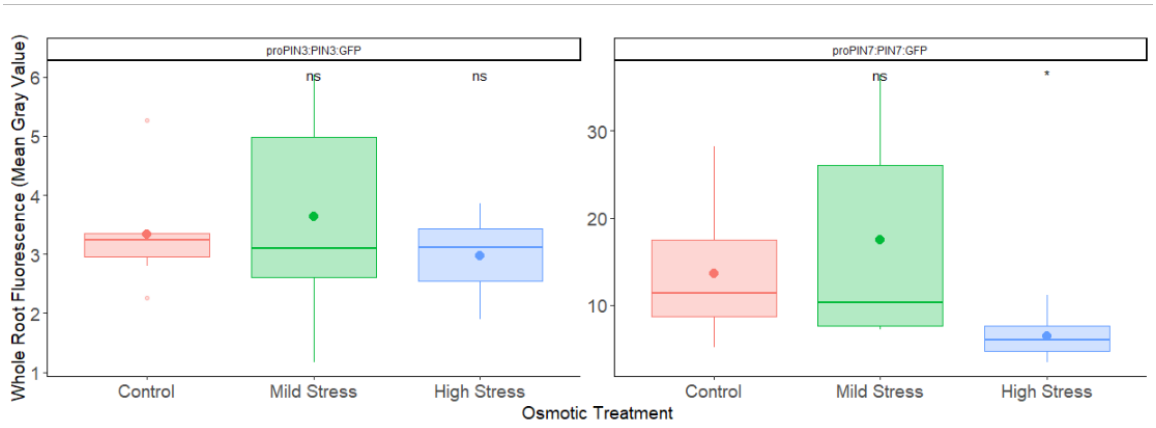

Figure 4.9

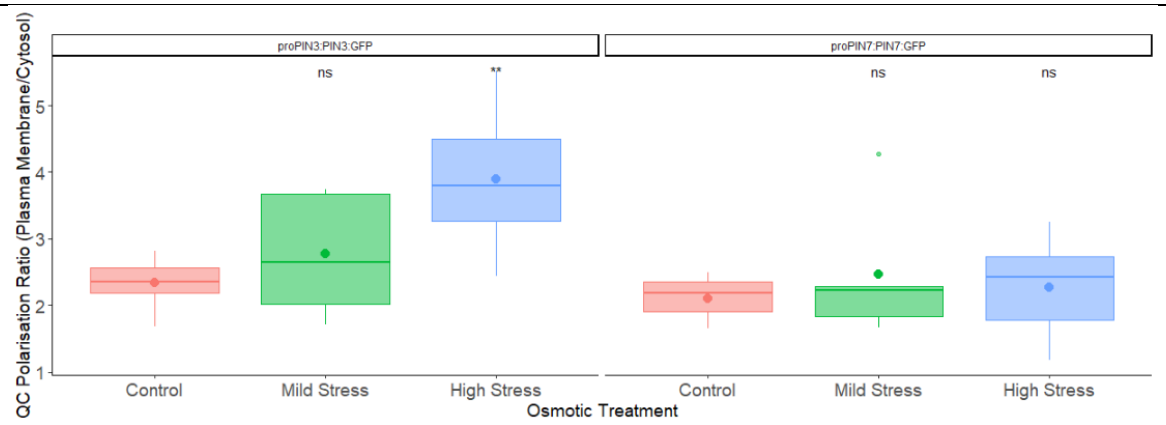

Figure 4.10

Figure 4.7 20x Confocal Images of proPIN3:PIN3:GFP under control **(A)** and high **(B)** osmotic stress conditions, roots were stained with propidium iodide prior to imaging. **Figure 4.8** (1) 20x Confocal Images of proPIN7:PIN7:GFP under control **(A)** and high **(B)** osmotic stress conditions, roots were stained with propidium iodide prior to imaging. **Figure 4.9** PIN3:GFP and PIN7:GFP whole root fluorescence (assessed via mean grey value on ImageJ) under 24hr control, mild and high osmotic stress conditions. **Figure 4.10** Level of polarisation (represented by membrane/cytosol ratio) in PIN3:GFP and PIN7:GFP in the quiescent centre (QC) of the root tip under 24hr control, mild and high osmotic stress conditions. For boxplots, upper and lower boundaries of the box indicate the interquartile range (IQE), dark circle within the box represents the mean, a line within the box represents the median, and the whiskers represent the min and max excluding outliers. Small circles represent outliers. Stars indicate significance from t-test of treatment compared to control (NS = not significant, *<0.05, **<0.01, ***<0.001).

The role of PIN3 and PIN7 in auxin transport under osmotic stress has not previously been studied. Seedlings expressing *proPIN3:PIN3:GFP* and *proPIN7:PIN7:GFP* were analysed under 24 hr osmotic stress conditions using confocal microscopy, to determine whether PIN3 or PIN7 levels or distribution changes to facilitate the reduction in auxin levels. PIN3:GFP showed little change in total fluorescence in the root tip under osmotic stress, indicating PIN3 levels may remain constant under osmotic stress (Figure 4.9). To examine whether PIN3 may be shifted from the cytosol to the plasma membrane, fluorescence was measured in both the plasma membrane and the cytosol in regions of the root tip. The mean level of membrane fluorescence was divided by mean cytosol fluorescence to achieve a membrane/cytosol ratio, which represents the level of PIN polarisation. In the quiescent centre (QC) of the root tip, PIN3:GFP distribution see a significant increase in membrane polarisation under high osmotic stress conditions when compared to the control (Figure 4.10) ($p<0.01$). Under mild stress we see an increase in polarisation, but it is not statistically significant.

Under 24 hour high osmotic stress, we see a significant decrease in total PIN7:GFP fluorescence levels in the root tip when compared to control conditions (Figure 4.9) ($p<0.05$). This indicates that PIN7 expression in the root tip decreases under 24 hrs of high osmotic stress. Under mild osmotic stress conditions we do not see a significant change in PIN7:GFP levels. There is no significant change

in PIN7 polarisation in the QC under osmotic stress conditions, indicating that there is little shift in PIN7 from cytosol to plasma membrane (Figure 4.10).

4.2.4 *PIN3* and *PIN7* expression under osmotic stress

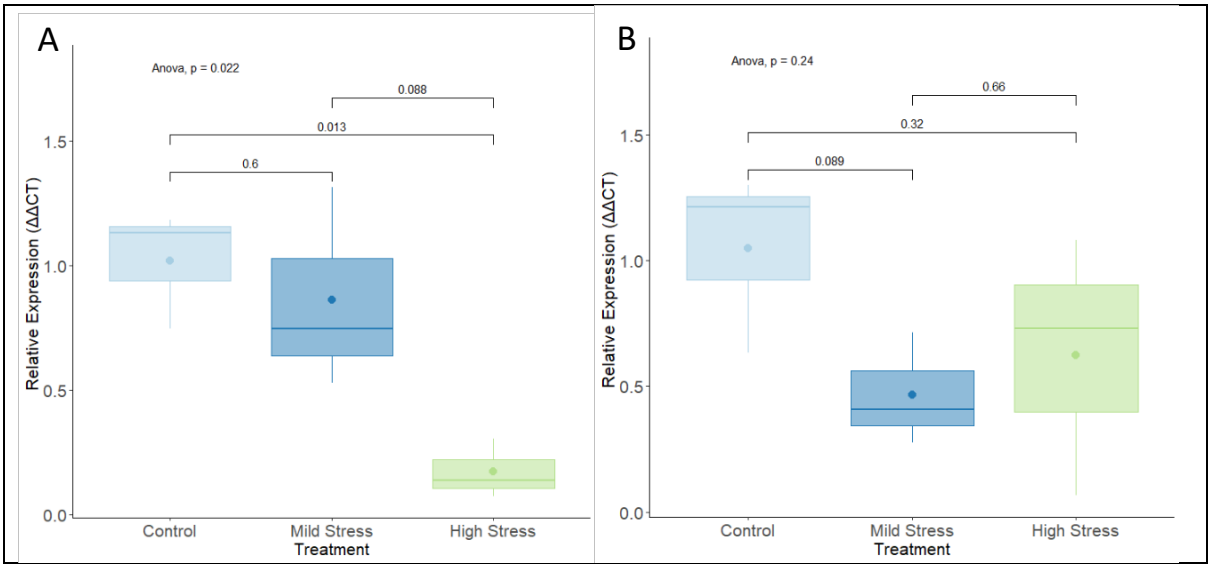

Figure 4.11 Normalized fold expression of *PIN3* **(A)** and *PIN7* **(B)** under control, mild and high osmotic stress conditions. Expression levels were normalized using osmotic stress independent reference gene AT5G15710, relative fold expression was then calculated via ΔΔCT transformation, comparing expression to the relative control sample. For boxplots, upper and lower boundaries of the box indicate the interquartile range (IQE), dark circle within the box represents the geometric mean, a line within the box represents the median, and the whiskers represent the min and max excluding outliers. Small circles represent outliers. Brackets indicate significance from t-test of treatment compared to other treatment (Significant at $p<0.05$).

To further understand the role of PIN3 and PIN7 under osmotic stress conditions, quantitative RT-PCR was conducted studying the transcript levels of both under control, mild and high osmotic stress conditions. We see a significant decrease in *PIN3* expression under high osmotic stress conditions when compared to the control ($p<0.05$), and a non-significant decrease under mild stress. *PIN7* shows a decrease in expression under mild and high osmotic stress although it was not significant when compared to the control (Figure 4.11).

4.2.5 *pin3, pin7, pin3/pin7 and dao1* mutants primary root growth under osmotic stress

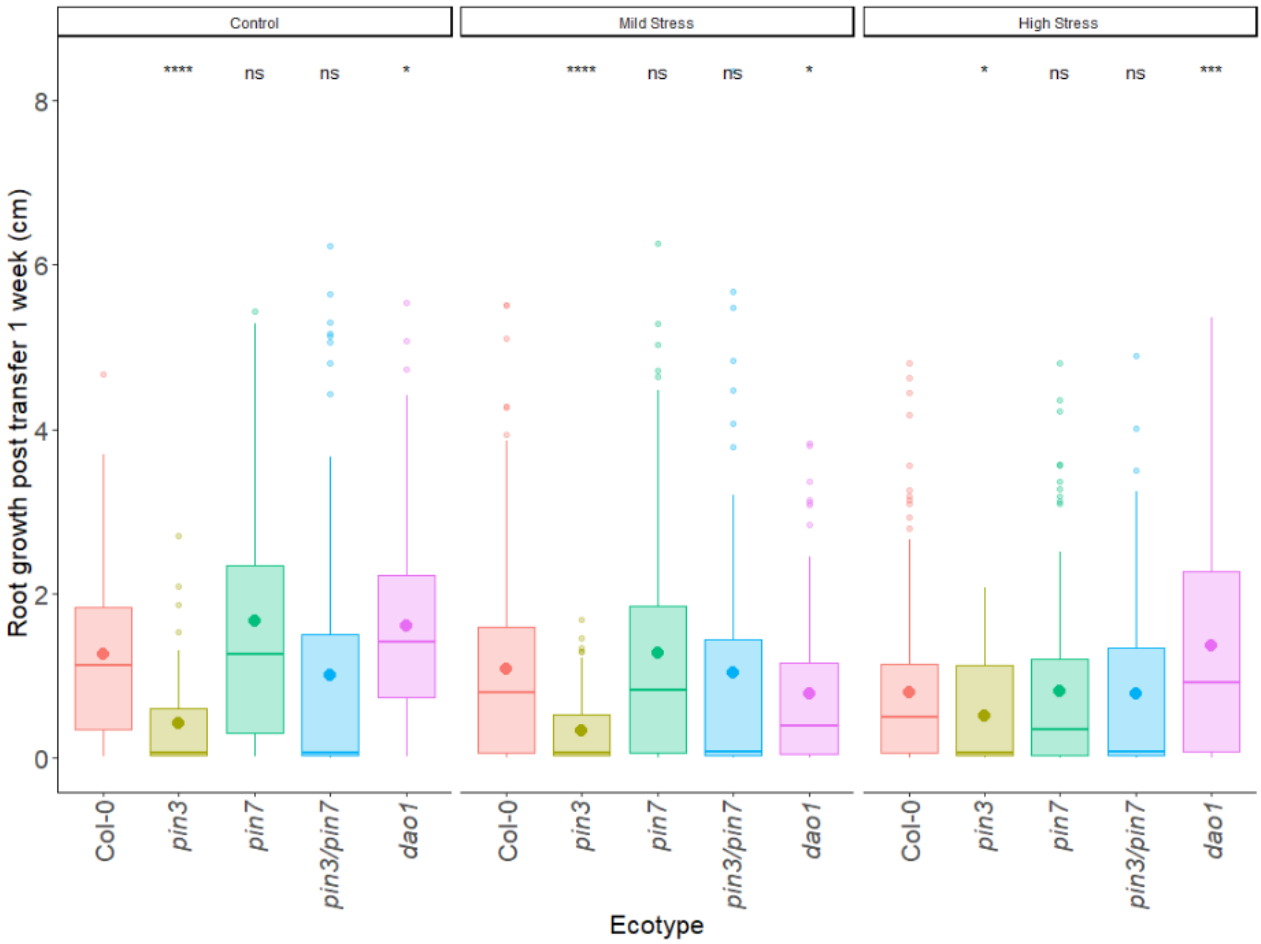

Figure 4.12A

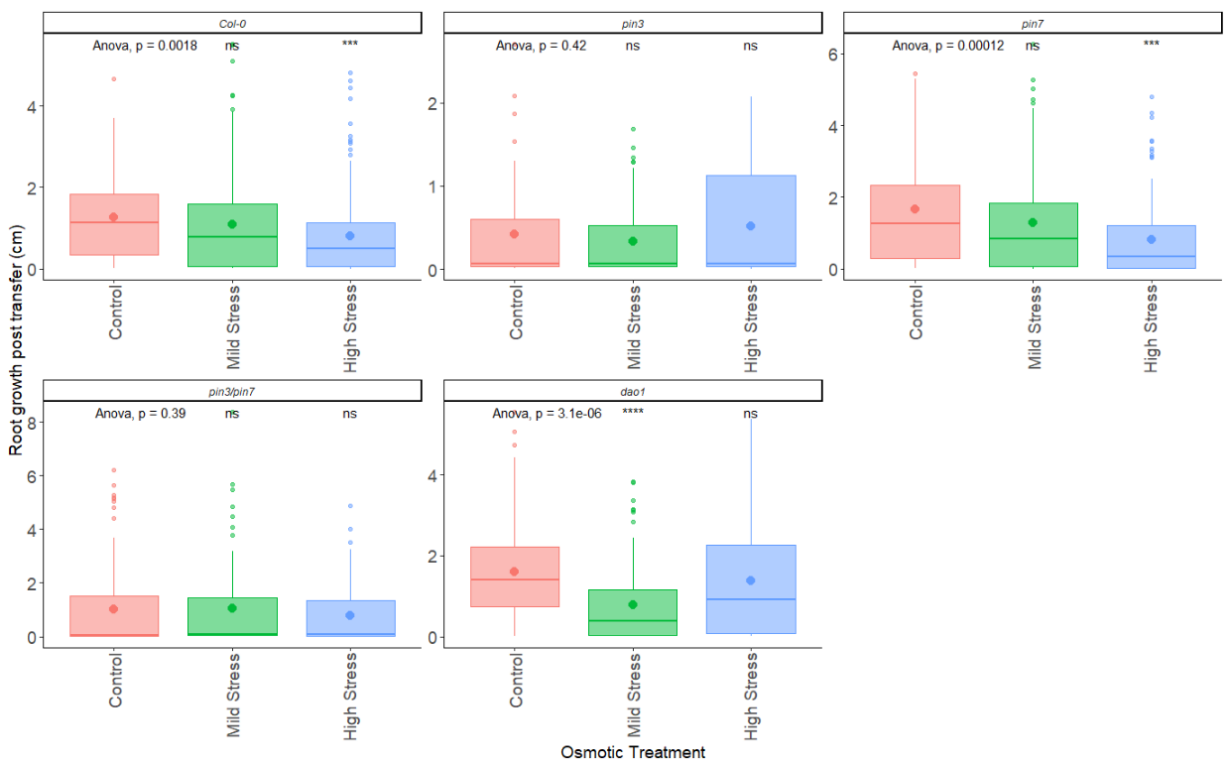

Figure 4.12B

Figure 4.12A. Auxin transport and oxidation mutants primary root length after one week under control, mild and high osmotic stress conditions compared against Col-0 (WT). Col-0, *pin3, pin7, pin3/pin7* and *dao1* were transferred to osmotic treatment plates for 1 week. Stars indicate significance from t-test of treatment compared to Col-0 (WT) **Figure 4.12B** Auxin transport and oxidation mutants (Col-0, *pin3*, *pin7, pin3/pin7* and *dao1)* primary root length after one week under control, mild and high osmotic stress conditions. For boxplots, upper and lower boundaries of the box indicate the interquartile range (IQE), a line within the box represents the median, and the whiskers represent the min and max excluding outliers. Small circles represent outliers. Stars indicate significance from t-test of treatment compared to Col-0 (WT)(NS = not significant, *<0.05, **<0.01, ***<0.001). Anova performed with significance detailed.

The primary root length of auxin transport mutants was analysed under osmotic stress conditions and compared against the performance of Col-0 (WT). Root systems were imaged 1 week after transfer to treatment plates. When compared to Col-0 WT, the *pin3* mutant performs significantly worse in root growth under control, mild and high osmotic stress conditions (Figure 4.12A) ($p<0.05$). Osmotic stress appears to have no impact on the *pin3* mutant, with root growth remaining constant between all three conditions (Figure 4.12B). The *pin7* mutant does not appear to play a strong role in osmotic stress response as there is little difference when compared to the WT in all stress conditions (Figure 4.12A). The *pin7* mutant shares a similar response to osmotic stress as the WT, with high osmotic stress have a significant impact on root growth (Figure 4.12B) ($p<0.001$). The *pin3/pin7* double mutant appears to have little difference in root length after one week under osmotic stress when compared to WT (Figure 4.12A). This is a surprising result as the individual *pin3* mutant performs significantly worse. Interestingly the *pin3/pin7* double mutant has limited response to osmotic stress, with little difference between control, mild and high osmotic stress (Figure 4.12B).

DAO1 plays a key role in the irreversible oxidation of IAA-amino acids conjugates. The auxin oxidation mutant *dao1-1* performs significantly better under control and high osmotic stress conditions when compared to Col-0 WT (Figure 4.12A) ($p<0.05$). Interestingly at mild osmotic stress, the *dao1-1* mutant performs significantly worse than the WT ($p<0.05$). The *dao1-1* mutant is significantly altered by mild osmotic stress ($p<0.05$), but under high osmotic stress shows no difference from the mutant under control conditions (Figure 4.12B).

4.2.6 The role of RBOHC and ROP2 in auxin response to osmotic stress

Comparing the expression of auxin-responsive gene *IAA2* in Col-0, *rbohc* and *rop2* mutants it appears there is little difference between them. This indicates that under control conditions, both RBOHC and ROP2 play little role in maintaining auxin levels (Figure 4.13).

In the WT (Col-0) there is a significant decrease in auxin-responsive gene *IAA2* expression under high osmotic stress when compared to control conditions, indicating that auxin levels are decreasing (Figure 4.14) ($p<0.05$). Mild stress has a near significant negative impact on *IAA2* expression levels.

In the *rbohc* mutant we see a non-significant increase in *IAA2* in response to mild and high osmotic stress. This indicates that RBOHC may play a key role in the reduction we see in *IAA2* in the wildtype under osmotic conditions as this is a significant shift in performance (Figure 4.14).

In the *rop2* mutant, we see a significant increase in *IAA2* expression levels under high osmotic stress when compared to control conditions (Figure 4.14) ($p<0.05$). Under mild stress we also see an increase in expression, although not significant. This is a large change from the reduction of auxin we see in the wildtype (Col-0). ROP2 may play a key role in the reduction of auxin under osmotic stress, without its presence auxin levels appear to increase.

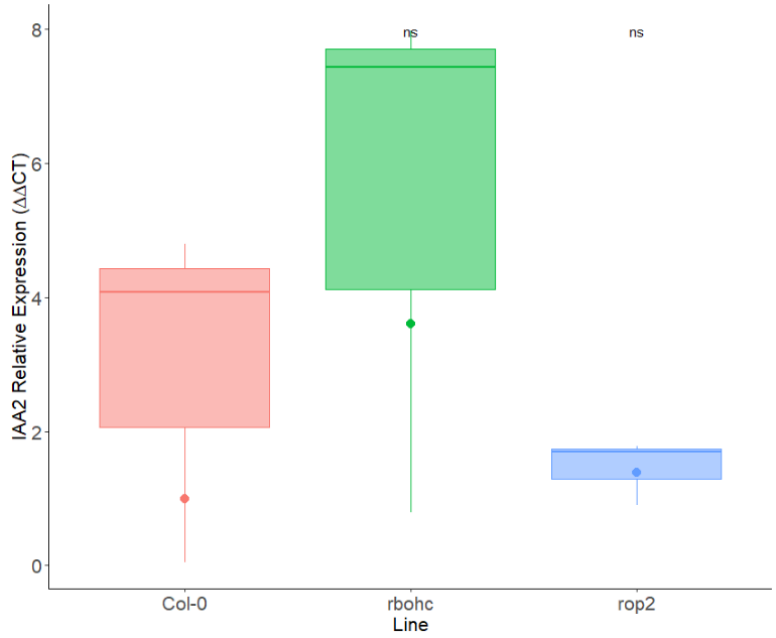

Figure 4.13

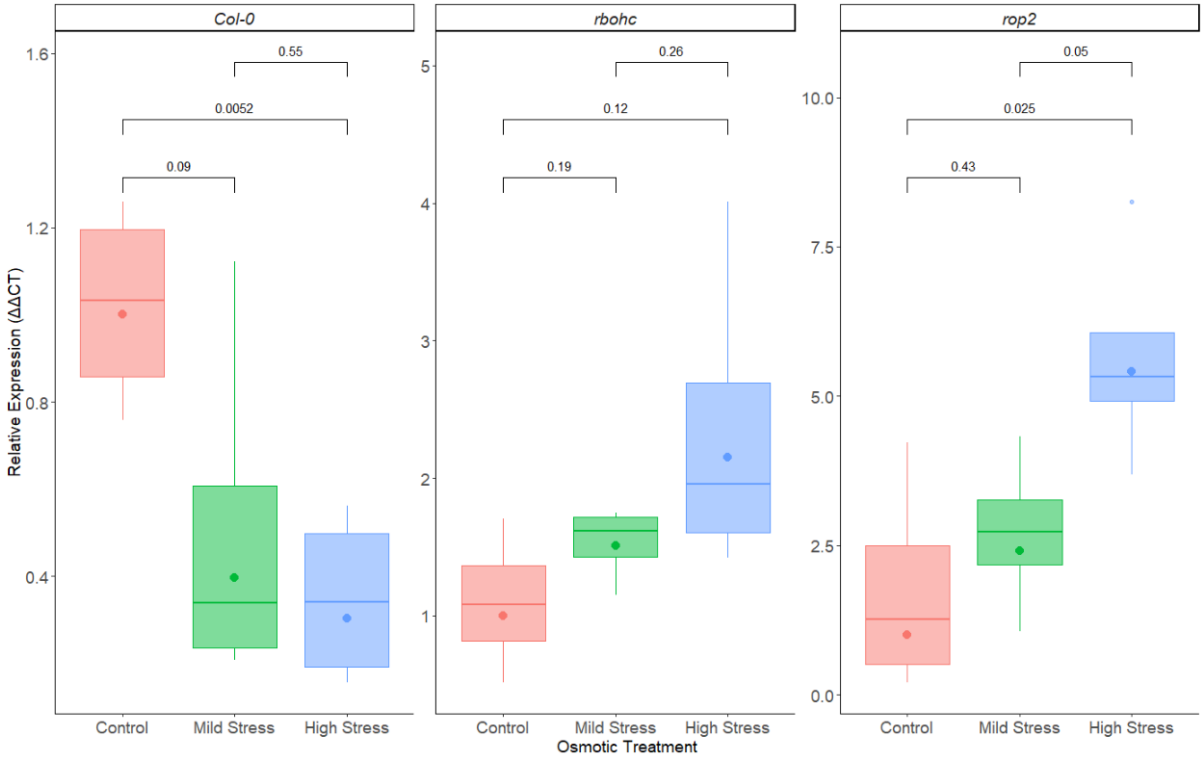

Figure 4.14

Figure (4.13) Normalized fold expression of auxin-responsive gene *IAA2* when compared to Col-0 (WT) in *rbohc* and *rop2* loss-of-function mutants under control conditions. Expression levels were normalized using reference gene AT5G15710, relative fold expression was then calculated via ΔΔCT transformation, comparing expression to the relative control sample. **Figure (4.14)** Normalized fold expression of auxin-responsive gene *IAA2* under 24hr control, mild and high osmotic stress treatment in Col-0 (WT), *rbohc* and *rop2* loss-of-function mutants. Expression levels were normalized using reference gene AT5G15710, relative fold expression was then calculated via ΔΔCT transformation, comparing expression to the relative control sample. For boxplots, upper and lower boundaries of the box indicate the interquartile range (IQE), dark circle within the box represents the geometric mean, a line within the box represents the median, and the whiskers represent the min and max excluding outliers. Small circles represent outliers. Brackets indicate significance from t-test of treatment compared to control (NS = not significant, *<0.05, **<0.01, ***<0.001)

4.2.7 Summary

In both one week mild and high osmotic stress conditions there is a significant reduction in primary root growth and lateral root number (Figure 4.1, Figure 4.2), indicating that both mild and high osmotic stress have a negative impact on root development.

Within 10 mins of high osmotic stress treatment, there is a significant increase in the ndTomato/n3xVenus ratio in R2D2, indicating a significant increase in auxin levels in the short-term response to osmotic stress (Figure 4.3).

Following 24 hours of high osmotic stress there is seen a significant decrease in the ndTomato/n3xVenus ratio in R2D2 indicating a reduction in auxin in the root tip. Mild osmotic stress has a limited impact on R2D2 levels, suggesting auxin levels are not altered by mild osmotic stress (Figure 4.4).

Treatment with PIN-transport inhibitor NPA leads to a significant reduction in ndTomato/n3xVenus ratio in R2D2 in both control, mild and high osmotic stress conditions when compared to their control (DMSO) treatments. In the NPA-treated roots, there is little change in the ndTomato/n3xVenus ratio in 24 hr mild and osmotic stress conditions when compared to the NPA-treated control. These results indicate that the application of NPA leads to a significant reduction in auxin at the root tip in all conditions, furthermore, when roots have been treated with NPA there is little change under osmotic stress conditions as the auxin levels are already heavily reduced (Figure 4.4, Figure 4.5).

proPIN3:PIN3:GFP plants display a significant increase in PIN3 membrane/cytosol fluorescence ratio indicating a significant increase in polarisation under high osmotic stress conditions. Under mild stress there is an increase in polarisation but it is not statistically significant (Figure 4.10). There is a significant decrease in proPIN7:PIN7:GFP expression levels under high osmotic stress, indicating a reduction in PIN7-mediated auxin transport (Figure 4.9).

PIN3 expression is significantly reduced under high osmotic stress conditions when compared to the control. Under mild osmotic stress there is a reduction in *PIN3*, but it is not significant. There is also a non-significant reduction in *PIN7* expression under mild and high osmotic stress (Figure 4.11). Both these results indicate there is a reduction in PIN3 and PIN7 auxin transport under osmotic stress.

Auxin transport protein PIN3 appears to have a significant impact in on root development in normal conditions and under osmotic stress, as the *pin3* mutant performs significantly worse in root growth under one week control, mild and high osmotic stress conditions when compared to Col-0. Osmotic stress appears to have no impact on *pin3* mutants root growth, as under all conditions the root length remained constant (Figure 4.12).

PIN7 does not appear to have a significant role in root development or in the root response to osmotic stress. The *pin7* mutant in all conditions shares similar primary root length to the Col-0 control after one week (Figure 4.12).

The *pin3/pin7* double mutant performance under control, mild and osmotic stress conditions is similar to that of the Col-0 WT. The double mutant does have a more limited response to osmotic stress conditions as mild and high osmotic stress does not have a significant impact when compared to the control (Figure 4.12).

The role of auxin oxidation appears to be critical in root development in control conditions and under osmotic stress conditions. The *dao1-1* mutant has significantly greater primary root growth under control and high stress conditions compared to the Col-0 WT. The *dao1-1* mutant is unaltered in root growth by high osmotic stress but under mild osmotic stress displays significantly reduced root growth compared to the control (Figure 4.12).

In Col-0 WT, the auxin-responsive gene *IAA2* is significantly reduced under high osmotic stress, indicating that auxin levels are decreasing. In *rbohc* and *rop2* mutants we see a non-significant and significant increase respectively in *IAA2* levels under high osmotic stress. This indicates that RBOHC and ROP2 have an important role in the reduction of auxin seen under osmotic stress (Figure 4.14).

Chapter 5. The response of ABA under osmotic stress

5.1 Introduction

ABA has shown to play a crucial role in abiotic stress response, in particular in root development under osmotic stress. ABA levels were found to increase under osmotic stress, leading to a reduction in PIN1 levels in the root tip, overriding the effects of ethylene (Rowe *et al.* 2016). Despite this work, there remain a number of areas that require more study if we are to have a better understanding of osmotic stress response. It is important to study how quickly ABA response develops in the roots; to confirm whether the increase that we see within 24 hrs is a process that take place rapidly or a slow increase. It is important to assess how other signals such as ROS or the absence of ROPs alters ABA response to osmotic stress.

In order to understand how quickly ABA response develops in *Arabidopsis* roots, ABAleonSD1-3L21 was studied at 10 minutes under osmotic stress conditions, and after the application of H_2O_2. To further elucidate ABA response to osmotic stress and ROS, ABAleonSD1 was also studied after 24hrs of osmotic stress, 10mM H2O2 application, and after treatment with RBOH-inhibiting diphenylene iodonium (DPI).

To determine the system-wide ABA response to osmotic stress, and the influence of ROS production and ROP function in this response, ABA-responsive gene *RD29B* was studied in Col-0 (WT) and in *rbohc, rbohi* and *rop2* mutants after 24 hrs of mild and high osmotic stress treatment.

5.2 Results

5.2.1 ABAleonSD1-3L21 response to osmotic stress

Without ABA present, ABAleon is capable of FRET occurring between mTurquoise to cpVenus. ABA triggers an increase in the distance between probes, resulting reduced FRET efficiency (Waadt *et al.* 2014). An increase in ABA leads to an increase in mTurquoise and a decrease in cpVenus173 emission, resulting in a decrease in emission ratio (Waadt *et al.* 2014). Low ratios indicate high ABA concentrations and high ratios indicate low ABA concentrations (Waadt *et al.* 2014).

There is a small non-significant decrease in ABAleonSD1-3L21 levels, representing a small increase in ABA levels, within 10 minutes under osmotic stress (10% PEG solution), indicating that ABA levels are increasing but not by a large margin. Exogenous H_2O_2 treatment (10mM) leads to a significant decrease in ABAleonSD1 therefore a clear increase in ABA within 10 minutes (Figure 5.1 A) ($p<0.05$)

Under 24hrs of osmotic stress and 10mM H_2O_2 treatment, there is a significant decrease in emission ratio of ABAleonSD1-3L21 when compared to the control (Figure 5.1 B) ($p<0.05$). This indicates that there is a significant increase in ABA levels under 24hr high osmotic stress and 10mM H_2O_2 treatment in the root tip.

The decrease in ABAleonSD1-3L21 ratio (representing ABA increase) that is seen under osmotic stress in 24hrs is not seen under RBOH inhibitor Diphenylene Iodonium (DPI) treatment (10µM DPI). In fact, under 10 µM DPI we see a much lower level of ABAleonSD1-3L21 ratio in the non-osmotic treatment when compared to the control (DMSO) ($p<0.05$) and a slight increase the ratio following osmotic stress treatment (Figure 5.1 C). This suggests that DPI treatment causes an increase in ABA levels. It appears that osmotic stress is unable to alter ABA levels in DPI treated roots, in fact causes a small non-significant decrease in ABA.

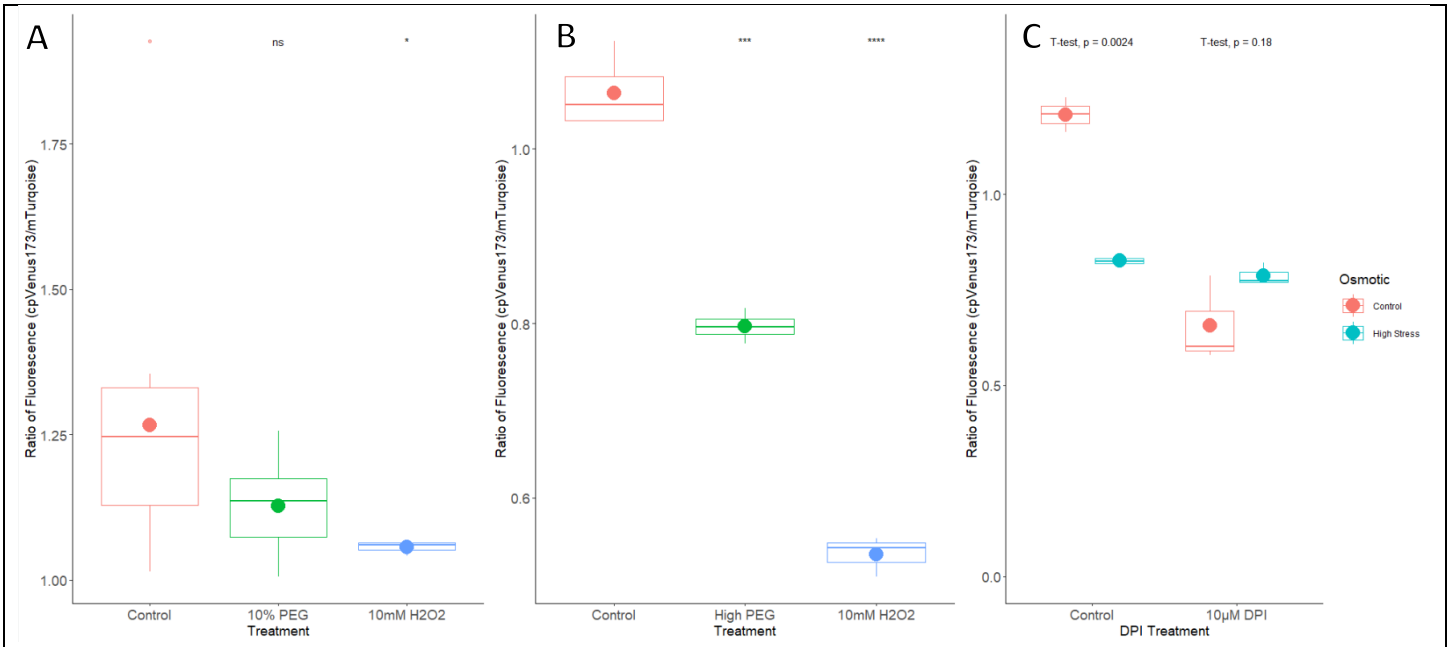

Figure 5.1

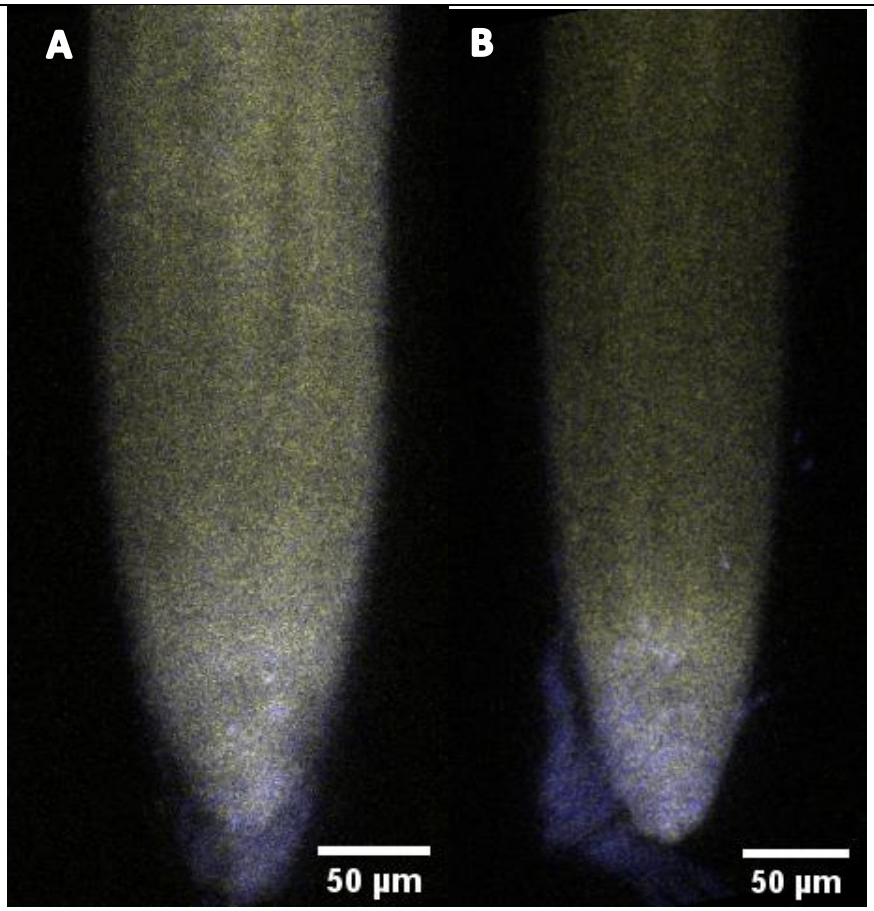

Figure 5.2

Figure 5.1 Analysis of ABAleonSD1-3L21 in the root tip under osmotic stress conditions. The level of ABA is calculated by ratio mTurquoise/cpVenus173 emission ratio. Low ratios indicate high ABA concentrations and high ratios indicate low ABA concentrations. **(A)** ABAleonSD1-3L21 levels under short term response under 10 minutes of 10% PEG and 10mM H_2O_2 treatment. **(B)** ABAleonSD1-3L21 response under 24hr High PEG and 10mM H_2O_2 treatment. **(C)** ABAleonSD1-3L21 levels following 24 hours Control, High PEG, 10µM DPI and High PEG + 10µM DPI treatment). **Figure 5.2** ABAleonSD1-3L21 Confocal Images at 20x. 24hr Control conditions **(A)** and High PEG **(B)**. Excited at 458 nm, ratio was calculated between cpVenus173 (Yellow, 520-550nm) divided by mTurquoise (Blue, 465-505nm). Decrease in cpVenus (Yellow)/mTurquoise (Blue) ratio indicates increase in ABA.

For boxplots, upper and lower boundaries of the box indicate the interquartile range (IQE), a line within the box represents the median, and the whiskers represent the min and max excluding outliers. Small circles represent outliers. Stars indicate significance of t-test of treatment compared to Control (NS = not significant, *<0.05, **<0.01, ***<0.001)

5.2.2 ABA-Responsive gene *RD29B* under osmotic stress

Comparing the expression of ABA-responsive gene *RD29B* in Col-0 (WT), *rbohc, rbohi* and *rop2* mutants under control conditions, it appears there is little difference between the Col-0 and the loss-of-function mutants. This suggests that RBOHC, RBOHI and ROP2 do not have a significant impact on ABA signalling under control conditions (Figure 5.3).

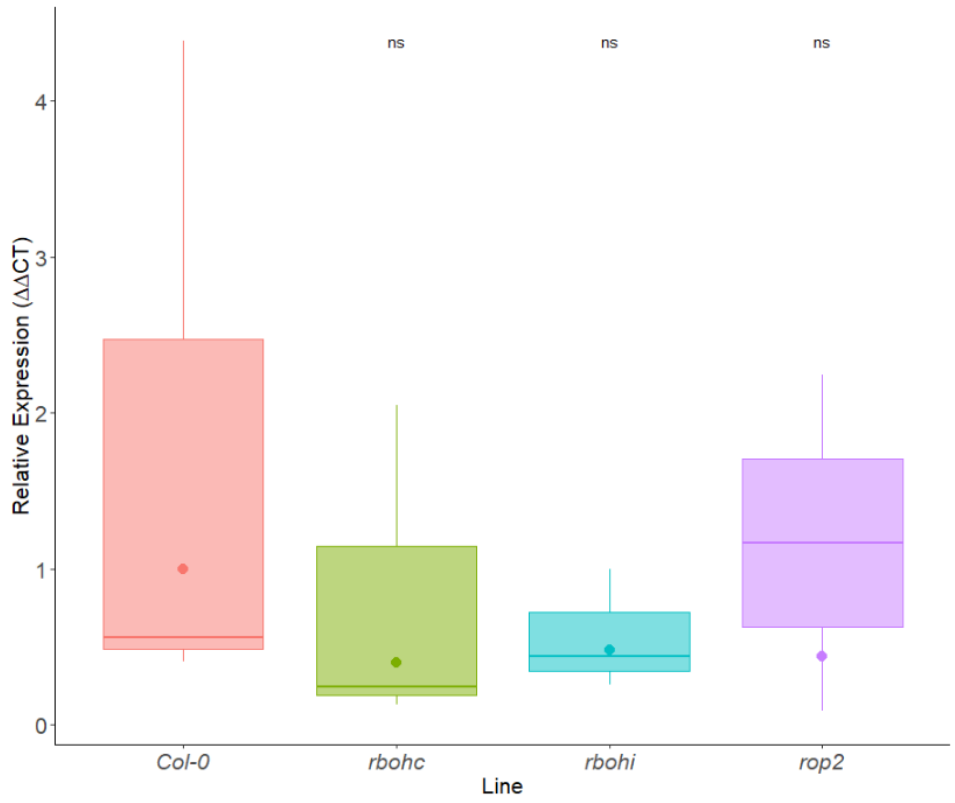

Figure 5.3

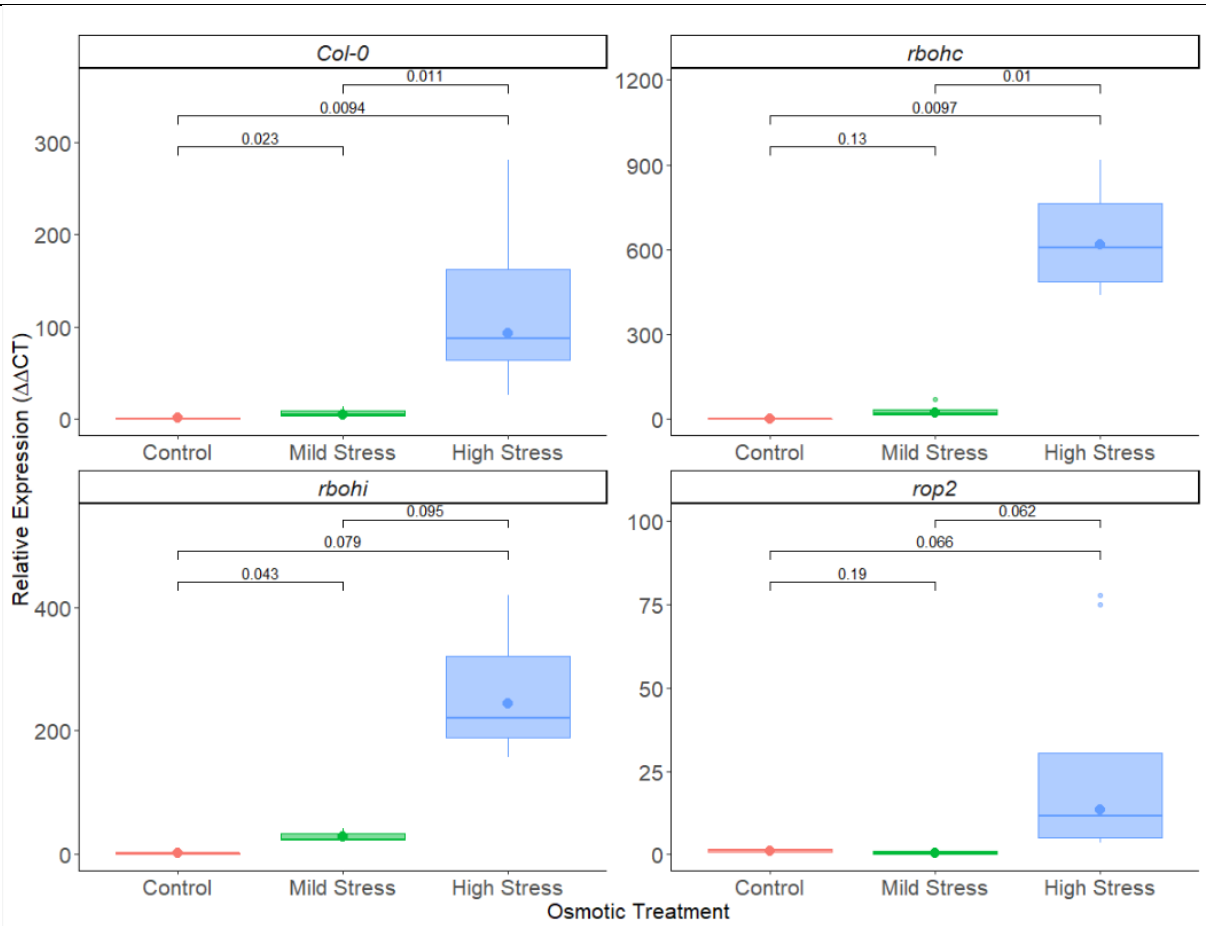

Figure 5.4

Figure 5.3 Normalized fold expression of ABA-responsive gene *RD29B* when compared to Col-0 in *rbohc*, *rbohi* and *rop2* loss-of-function mutants under control conditions. Expression levels were normalized using reference gene AT5G15710, relative fold expression was then calculated via ΔΔCT transformation, comparing expression to the relative control sample. **Figure (5.4)** Normalized fold expression of ABA-responsive gene *RD29B* under 24hr control, mild and high osmotic stress treatment in Col-0 (WT), *rbohc, rbohi* loss-of-function mutants. Expression levels were normalized using reference gene AT5G15710, relative fold expression was then calculated via ΔΔCT transformation, comparing expression to the relative control sample. For boxplots, upper and lower boundaries of the box indicate the interquartile range (IQE), dark circle within the box represents the geometric mean, a line within the box represents the median, and the whiskers represent the min and max excluding outliers. Small circles represent outliers. Brackets indicate significance from t-test of treatment compared to control (Significant = $p<0.05$)

In Col-0 WT, there is a significant upregulation of ABA-responsive gene *RD29B* under 24hr mild (near 10-fold) and high (near 100-fold) osmotic stress compared to the control (Figure 5.4) ($p<0.05$). There is also a significant upregulation in *RD29B* when comparing between mild and high osmotic stress ($p<0.05$). This indicates that ABA responses are increased under mild and high osmotic stress, and that there is an even greater response under high stress.

In the *rbohc* mutant, there is a highly significant increase in *RD29B* expression under high osmotic stress (over 600-fold) increase. Although we see an increase in *RD29B* under mild osmotic stress (20-fold), it is not significant (Figure 5.4). There is a significant difference in *RD29B* between mild and

high osmotic stress in the *rbohc* mutant, indicating a large relative increase in ABA after 24 hrs of high osmotic stress.

In *rbohi* loss-of-function mutant there is a significant increase in *RD29B* under mild osmotic stress ($p<0.05$) and a large near-significant increase under high osmotic stress (over 200-fold) when compared to control conditions (Figure 5.4). Both *rbohI* and *rbohc* mutants appear to have increased ABA response to 24hr osmotic stress, suggesting that ABA response may be enhanced by reduced ROS production.

The *rop2* mutant appears to have a non-significant increase in *RD29B* expression under mild and high osmotic stress conditions when compared to control conditions (Figure 5.4). In the *rop2* mutant, *RD29B* expression increases 20-fold in response to high osmotic stress when compared to the control. This indicates a reduced ABA response to osmotic stress, as a 20-fold increase is low when compared to the near 100-fold increase in the WT. This indicates the ABA pathway may not be fully functioning without ROP2's presence.

5.3 Summary

After 10 mins of osmotic stress treatment, there is a small non-significant decrease in ABAleonSD1 ratio, indicating a potentially rapid small increase in ABA. Following 10 minutes of exogenous H_2O_2 treatment there is a significant decrease in ABAleon ratio, indicating exogenous ROS application leads to a rapid ABA increase (Figure 5.1A).

Following 24 hrs of both high osmotic stress treatment and 10mM H2O2, there is a significant decrease in the emission ratio of ABAleonSD1 when compared to control conditions (Figure 5.1B). This indicates that there is a significant increase in ABA levels after osmotic stress treatment and following the application of exogenous ROS.

Under control conditions, 24hr treatment with 10µM RBOH-inhibitor DPI led to a decrease in ABAleonSD1 ratio, indicating that DPI treatment results in an increase in ABA levels (Figure 5.1C). The decrease in ABAleonSD1 ratio seen under osmotic stress in control seedlings does not take place when 10µM DPI is applied, indicating that ABA levels do no longer increase under osmotic stress in DPI-treated seedlings (Figure 5.1C). These results suggest that DPI treatment leads to an increase in ABA levels, and that osmotic stress is unable to alter ABA levels in DPI treated roots.

The ABA-responsive gene *RD29B* expression does not significantly change in *rbohc*, *rbohi* and *rop2* mutants when compared to the expression in Col-0 (WT) (Figure 5.3). This indicates that RBOHC, RBOHI and ROP2 do not have a significant impact on ABA levels in control conditions.

In Col-0 (WT) seedlings, there is a significant upregulation in the ABA-responsive gene *RD29B* after 24hr mild and high osmotic stress conditions (Figure 5.4). In the *rbohc* mutant, there is non-significant increase in *RD29B* under mild osmotic stress, and a significant increase in *RD29B* expression under high osmotic stress (Figure 5.4). The *rbohi* loss-of-function mutant displays a significant increase in *RD29B* under mild osmotic stress conditions, and a large non-significant increase under high osmotic stress conditions (Figure 5.4). Both the *rbohc* and *rbohi* mutant have larger fold increases in *RD29B* expression under high osmotic stress, indicating that ABA response could be enhanced under reduced ROS production.

Expression of *RD29B* in the *rop2* mutant displays a non-significant increase under mild and high osmotic stress when compared to control conditions (Figure 5.4). When compared to the large 100-fold increase in *RD29B* seen under high osmotic stress in Col-0 (WT), it appears the ABA pathway may not be functioning properly without the presence of ROP2.

Chapter 6. The role of ROS in osmotic stress response in the root system

6.1 Introduction

ROS play a critical role in rapid abiotic stress response, where they are rapidly upregulated in a form of ROS wave, acting as a signal that spreads throughout the plant system (Huang *et al.* 2016, 2019). ROS also play a key role in development, controlling processes such as cell polarity and expansion. The temporal-spatial distribution of ROS has significant impact on key development areas such as the QC and RAM (Tsukagoshi *et al*. 2016).

The role of ROS in the root response to osmotic stress has received little attention, both at the short time frame of stress signalling and how ROS alters root development over a longer period. A variety of techniques including microscopy, qRT-PCR and loss-of-function mutant analysis were used to study ROS under osmotic stress.

In order to understand how rapidly ROS signals can develop under osmotic stress, the ROS sensor roGFP2-Orp1 was studied under 10 minutes of osmotic stress (PEG solution and sorbitol treatment). The application of RBOH-inhibitor DPI and ABA-biosynthesis inhibitor fluridone along with the osmotic stress treatment was used to investigate the role RBOH proteins and the ABA pathway might have in the rapid ROS response.

roGFP2-Orp1 was also studied under 24 hr osmotic stress treatment in combination with DPI and fluridone treatment. This can help determine how ROS patterning can develop in the root system over a longer period of osmotic stress, and the role of RBOH proteins and the ABA pathway in this response.

The expression levels of key proteins in the ROS production pathway, namely *PERK4, RBOHC* and *RBOHI* were assessed under osmotic stress conditions via qRT-PCR. This aimed to determine what regions of ROS production may be upregulated under osmotic stress.

The primary root growth of loss-of-function mutants *rbohd, rbohf, rbohd/rbohf, rbohc, perk4-1, upb1.1* and *upb1.2* was assessed under control, mild osmotic stress, and high osmotic stress conditions. These proteins play key roles in ROS production, as a result this loss-of-function study can help determine the role of ROS production in root development and during osmotic stress response.

Col-0 (WT) seedlings primary root length was analysed under osmotic stress in combination with the exogenous application of H_2O_2 and different concentrations of DPI. Using these two treatments we can determine how either enhancing the level of ROS (via H_2O_2 application) or restricting ROS production (via DPI application) can lead to alterations in root development under osmotic stress. The application of both H_2O_2 and DPI together under control and osmotic stress conditions can help

reveal whether exogenous ROS application can rescue the root system phenotype displayed under RBOH-inhibition via DPI application.

6.2 Results

6.2.1 roGFP2-Orp1 response to osmotic stress

After 10 mins of osmotic stress treatments (10% PEG and 300 mM sorbitol) there is a significant increase in oxidation levels in roGFP2-Orp1 when compared to the control (Figure 6.1) ($p<0.05$). The application of fluridone appears to have an impact on the oxidation increase we see under 10% PEG treatment, as the combination of both leads to a similar value to that of the control. The 10-min independent application of fluridone does not lead to any changes in the level of oxidation under osmotic stress in roGFP2Orp1. The application of DPI at both 1 µM and 20 µM concentrations leads to a significant increase in oxidation under 10 mins, when compared to the control (Figure 6.1) ($p<0.05$). The application of DPI (1 uM and 20 uM) does not reduce the increased level of oxidation under osmotic stress. In fact, treatment with DPI leads to a non-significant increase in osmotic stress induced oxidation.

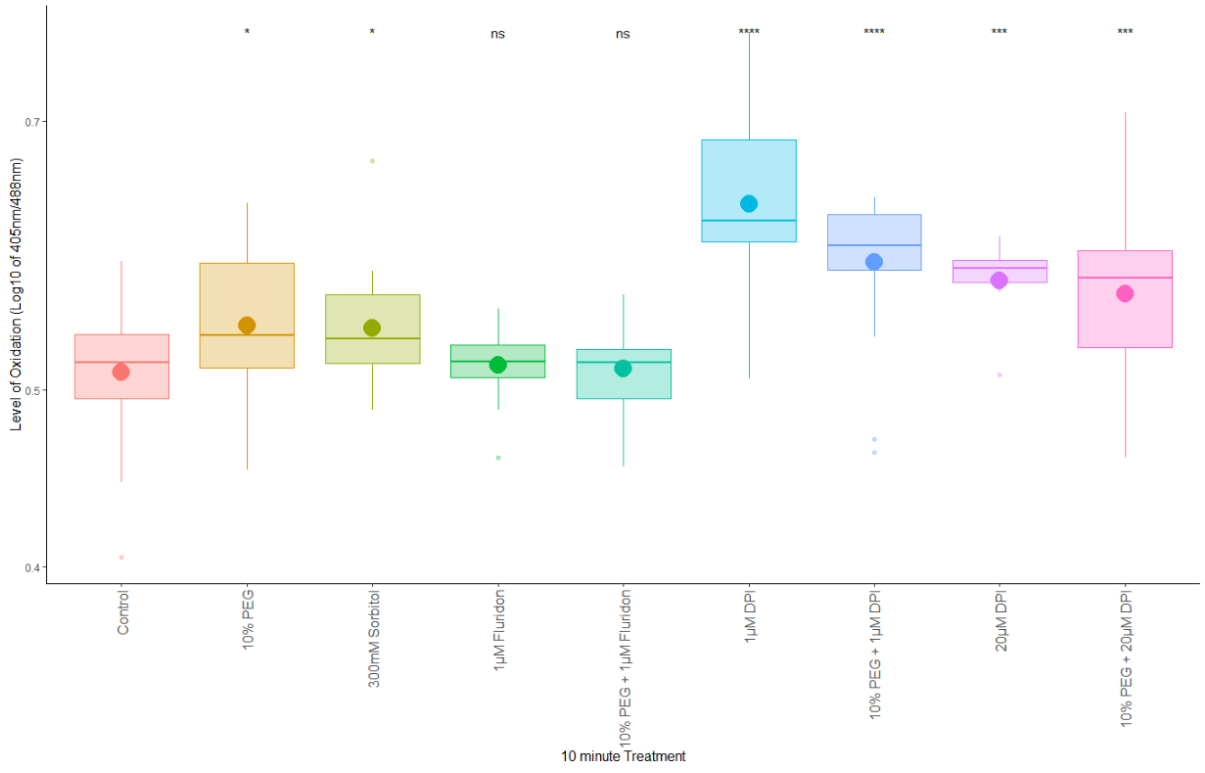

Figure 6.1

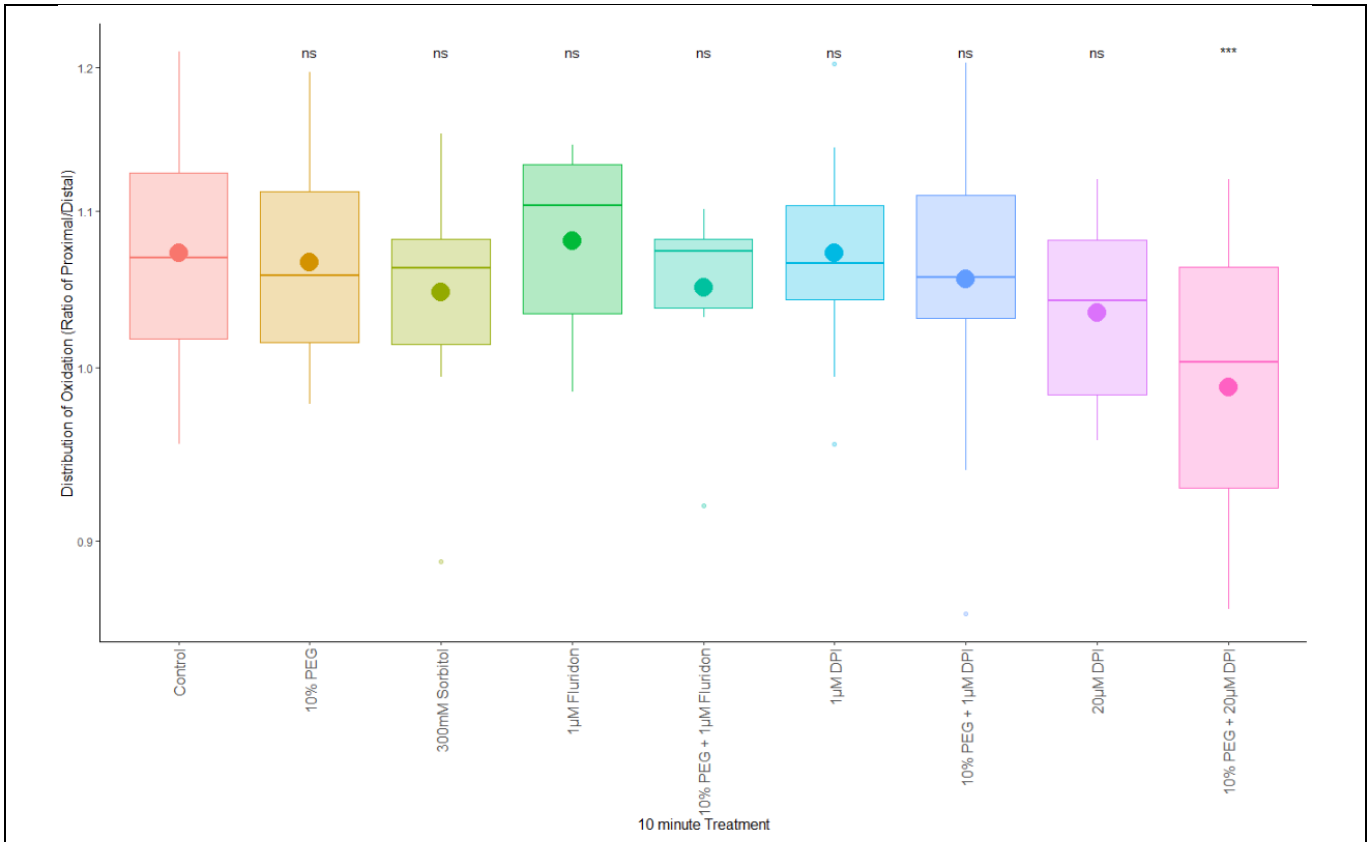

Figure 6.2

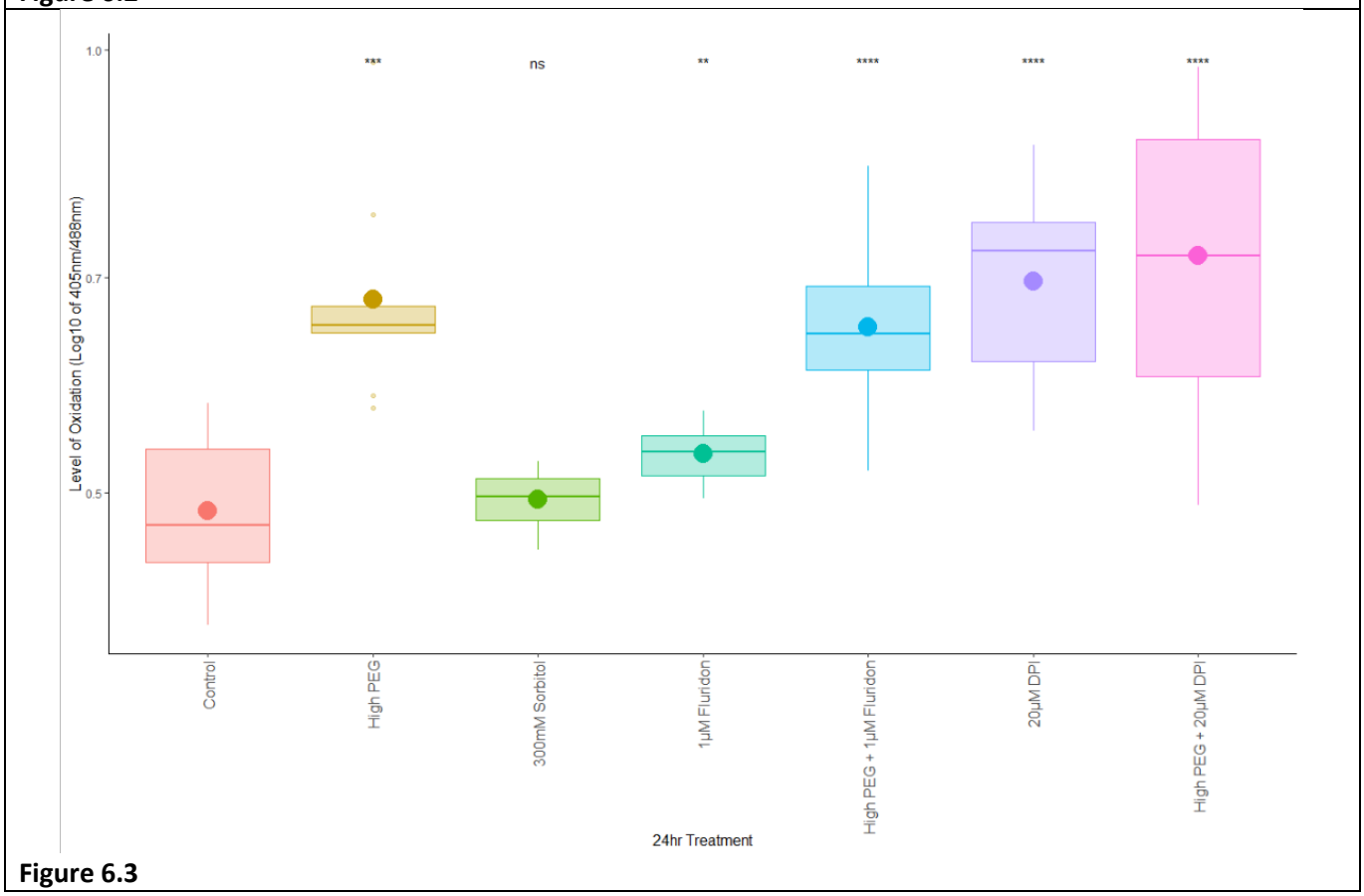

Figure 6.3

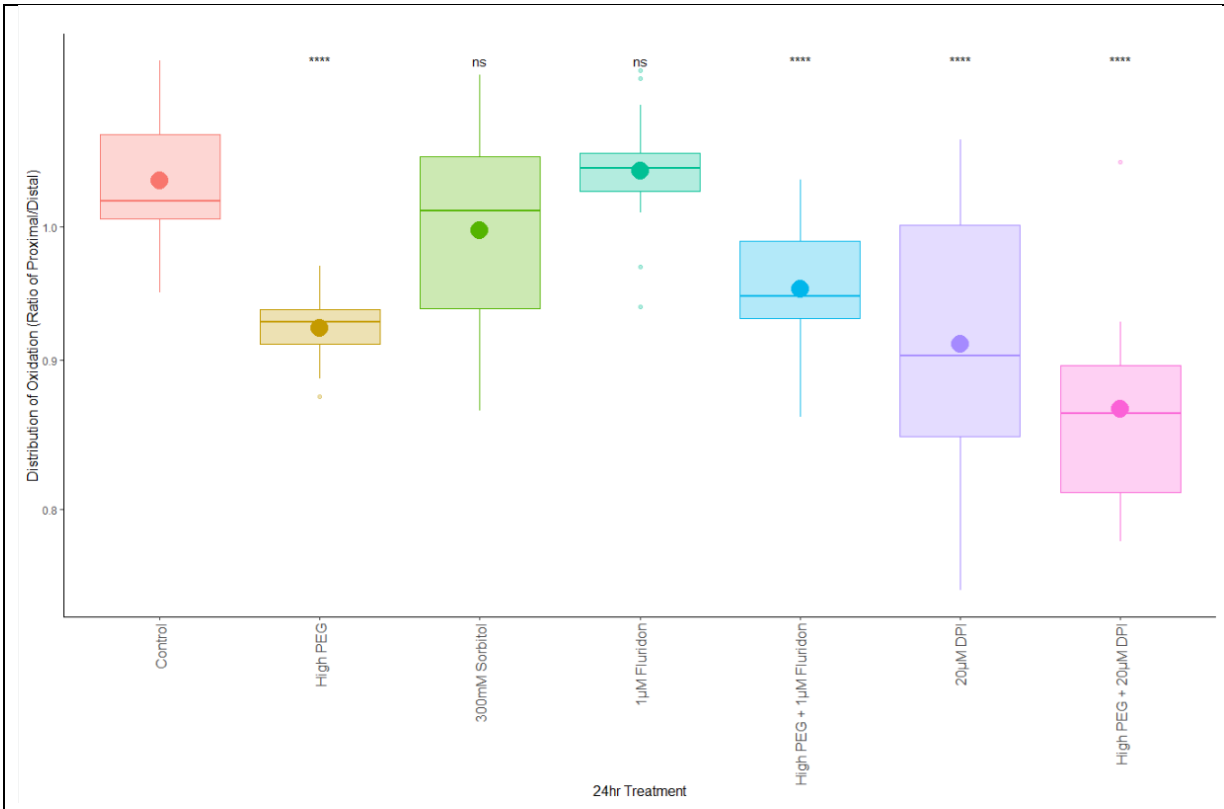

Figure 6.4

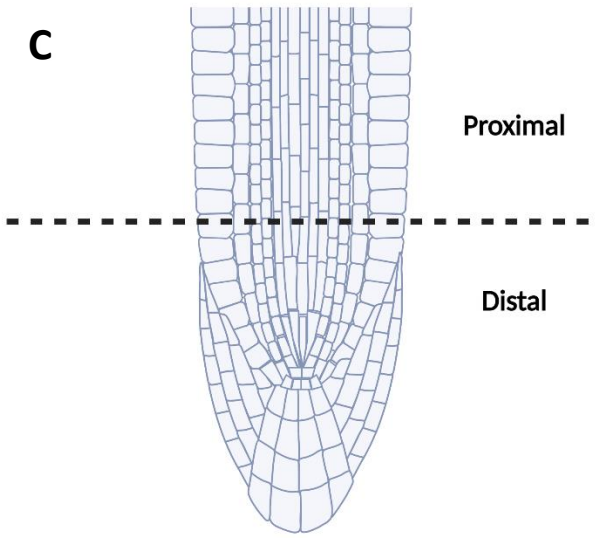

Figure 6.5

Figure 6.1 Levels of oxidation in the root tip via Log10 of the 405nm/488nm ratio in roGFP2-Orp1 upon a variety of 10-minute treatments. An increase in ratio represents an increase in oxidation levels. The 10-minute treatments that took place ranging from Control, 10% PEG, 300 mM Sorbitol, 1 µM Fluridone, 10% PEG + 1 µM Fluridone, 1uM DPI, 10% PEG + 1 µM DPI, 20 µM DPI and 10% PEG + 20 µM DPI. **Figure 6.2** The distribution of oxidation (via a ratio between proximal and

distal root tip oxidation values) in the root tip via reporter roGFP2-Orp1 under 10-minute treatments. The 10-minute treatments that took place ranging from Control, 10% PEG, 300mM Sorbitol, 1µM Fluridone, 10% PEG + 1µM Fluridone, 1 µM DPI, 10% PEG + 1 µM DPI, 20 µM DPI and 10% PEG + 20 µM DPI. Increase in ratio represents the distribution of oxidation shifting down to the root tip. A decrease represents a shift in distribution away from the root tip towards the proximal area of the root. **Figure 6.3** Levels of oxidation in the root tip via Log10 of the 405nm/488nm ratio reporter roGFP2-Orp1 upon a variety of 24-hour treatments. The increase in ratio represents an increase in oxidation levels. A variety of 24hr treatments took place ranging from Control, High PEG, 300mM Sorbitol, 1 µM Fluridone, High PEG + 1 µM Fluridone, 20µM DPI and High PEG + 20µM DPI. **Figure 6.4** The distribution of oxidation (via a ratio between proximal and distal root tip oxidation values) in the root tip via reporter roGFP2-Orp1 under 24-hour treatments. Treatments include Control, High PEG, 300mM Sorbitol, 1 µM Fluridone, High PEG + 1 µM Fluridone, 20µM DPI and High PEG + 20µM DPI. **Figure 6.5** 20x Confocal images of roGFP2-Orp1 24hr Control **(A)** and High PEG **(B)**. Level of oxidation calculated via ratio between 405nm (Blue) Excitation / 488nm (Green) Excitation. Increase in Blue/Green ratio represents increase in oxidation levels in the root tip. **(C)** Diagram depicting how roGFP2-Orp1 distribution was analysed as a ratio between Proximal and Distal levels of fluorescence.

For boxplots, upper and lower boundaries of the box indicate the interquartile range (IQE), a line within the box represents the median, and the whiskers represent the min and max excluding outliers. Small circles represent outliers. Stars indicate significance difference via t-test of treatment compared to Control (WT)(NS = not significant, *<0.05, **<0.01, ***<0.001)

10 minute treatment with 10% PEG, 300 mM Sorbitol, 1 µM Fluridone, 1 µM DPI, 10% PEG + 1 µM DPI and 20 µM DPI did not lead to a shift in proximal/distal distribution of the level of oxidation in roGFP2-Orp1. Only the combined treatment of 10% PEG + 20 µM DPI led to a significant shift in distribution towards the distal region of the root when compared to the control (Figure 6.2) ($p<0.05$).

The 24 hr High PEG treatment led to a significant increase in oxidation when compared to the control (Figure 6.3) ($p<0.05$). Interestingly the treatment of 300 mM Sorbitol did not lead to a similar increase. 24 hr 1 µM Fluridone treatment led to a significant increase in the level of oxidation of the root tip when compared to the control conditions ($p<0.05$). The 1 µM Fluridone treatment did not alter the significant increase in the level of oxidation generated under 24 hr High PEG osmotic treatment. 20 µM DPI treatment independently and in combination with High PEG led to significant increases in oxidation when compared to the control ($p<0.05$). 20 µM DPI treatment did not have an impact on oxidation levels under High PEG treatment.

24 hr high PEG treatment led to a significant shift in the distribution of oxidation towards the distal region of the root tip when compared to the control (Figure 6.4) ($p<0.05$). Osmotic treatment with 300 mM Sorbitol did not lead to a shift in distribution. The application of 1 µM Fluridone also did not lead to a shift in distribution when compared to the control, nor did it lead to a change in oxidation levels under High PEG treatment. 24 hr 20 µM DPI treatment led to a significant shift in the distribution of oxidation down to the root tip ($p<0.05$). The application of 20 µM DPI in combination

with High PEG treatment led to an even greater shift in oxidation distribution towards the root tip when compared to High PEG treatment ($p<0.05$).

6.2.2 Expression of *PERK4*, *RBOHC*, *RBOHI* under osmotic stress

In Col-0 (WT) there is a significant increase in *PERK4* expression under mild osmotic stress conditions and a near-significant increase under high osmotic stress conditions (Figure 6.6). It appears PERK4 expression is both affect by mild and high osmotic stress. *RBOHC* expression levels do not appear to increase under mild or high osmotic stress conditions. We see a significant increase in *RBOHI* expression (over 2-fold) under high osmotic stress compared to control. Mild osmotic stress treatment appears to have no impact on *RBOHI* levels.

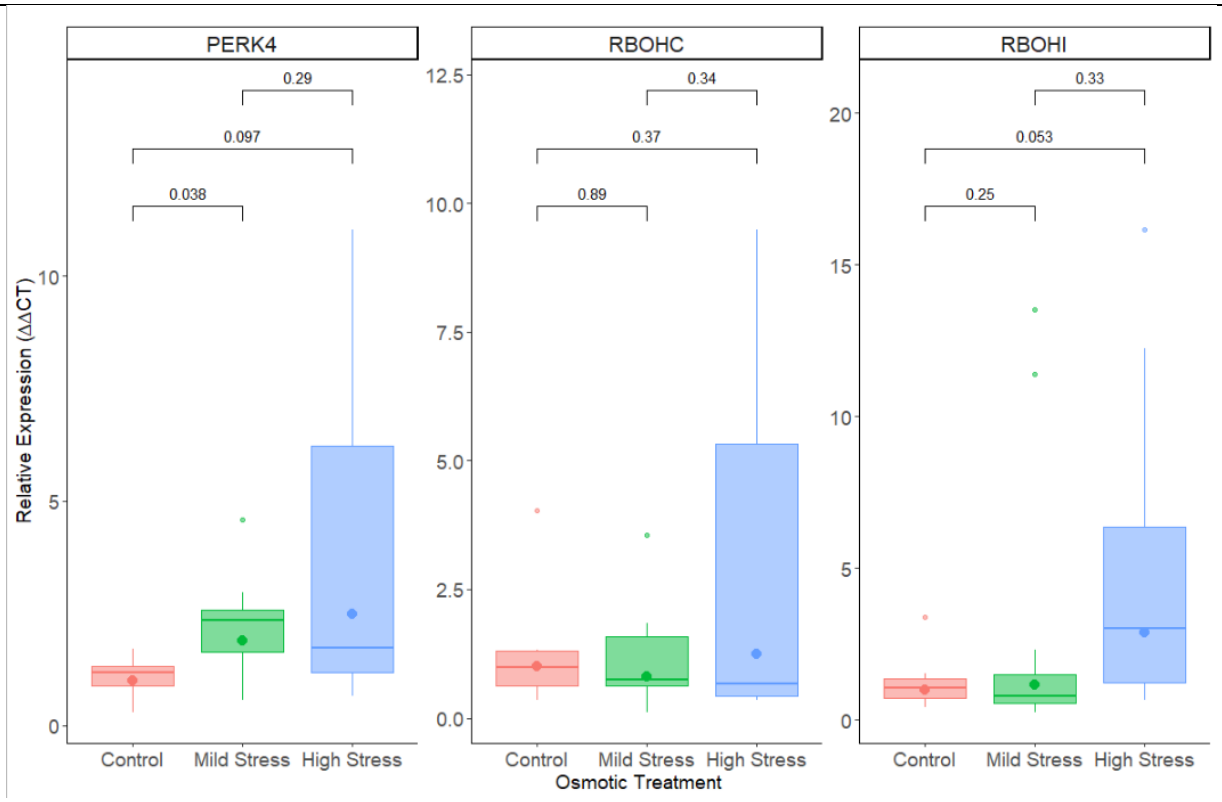

Figure 6.6

Figure 6.6. Normalized fold expression of *PERK4, RBOHC* and *RBOHI* under 24hr control, mild and high osmotic stress treatment in Col-0 (WT). Expression levels were normalized using reference gene AT5G15710, relative fold expression was then calculated via ΔΔCT transformation, comparing expression to the relative control sample. For boxplots, upper and lower boundaries of the box indicate the interquartile range (IQE), dark circle within the box represents the geometric mean, a line within the box represents the median, and the whiskers represent the min and max excluding outliers. Small circles represent outliers. Brackets indicate significance from t-test of treatment compared to control (NS = not significant, *<0.05, **<0.01, ***<0.001)

6.2.3 The role of ROS in primary root length under osmotic stress

The *rbohd* mutant primary root length is significantly smaller than Col-0 under control, mild and high osmotic stress conditions (Figure 6.7) ($p<0.05$). Osmotic stress does appear to have a significant impact on the *rbohd* mutant, with high osmotic stress having a significant impact on primary root growth when compared to the control (Figure 6.7). *rbohf* root growth is also significantly worse than Col-0 under control, mild and high osmotic stress conditions ($p<0.05$). The *rbohf* mutant performs significantly better under mild osmotic stress compared to control ($p<0.05$). High osmotic stress does not have a significant impact on root growth (Figure 6.7).

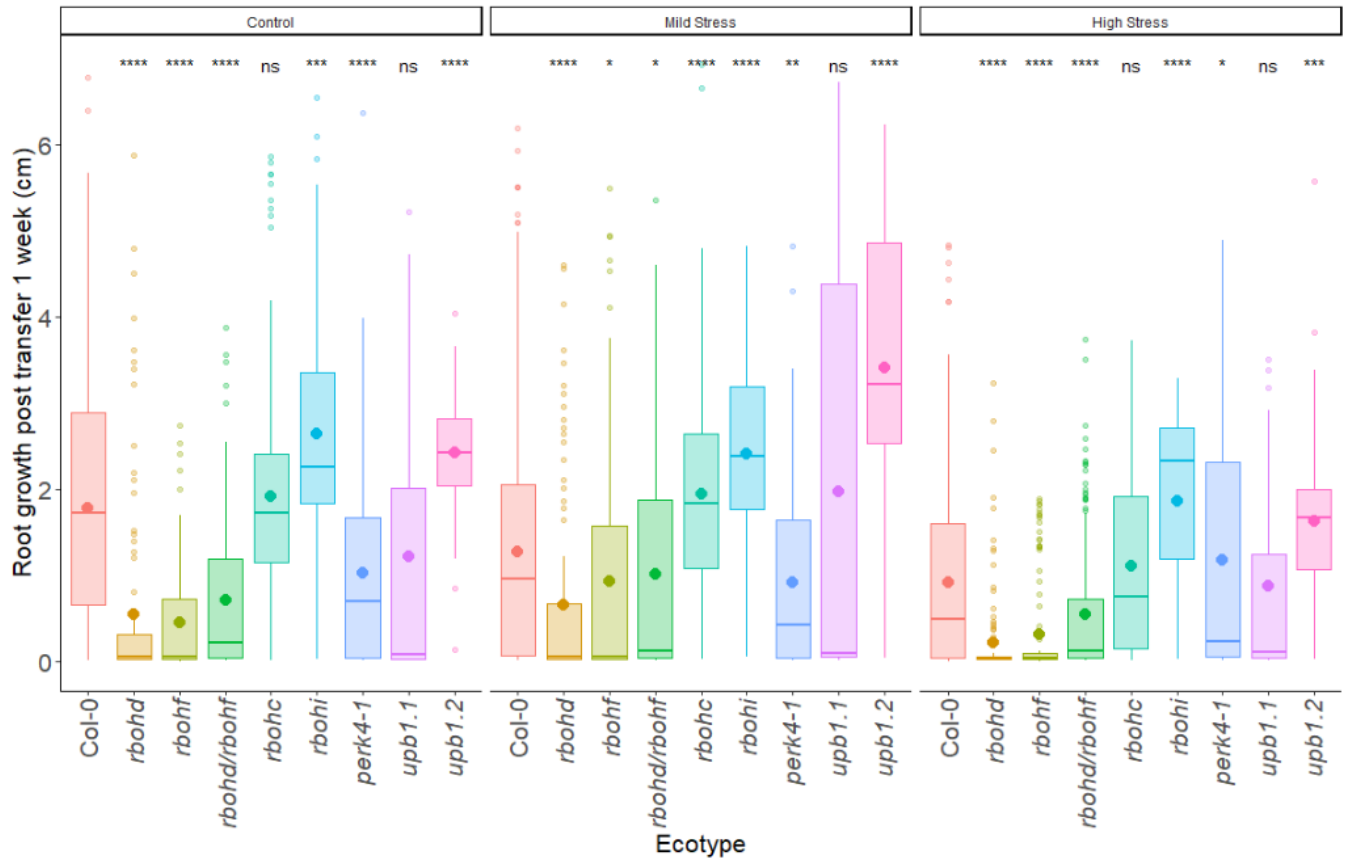

Figure 6.7

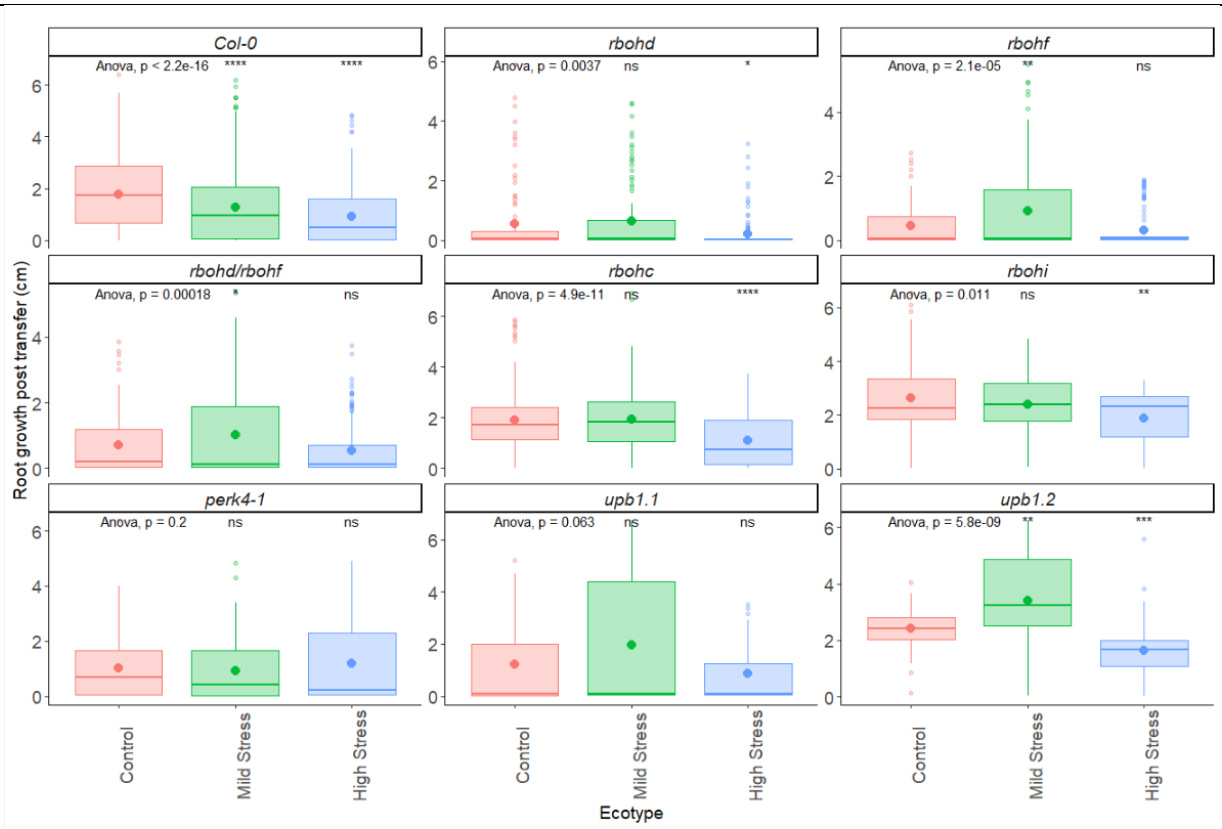

Figure 6.8

Figure 6.7 ROS mutants primary root length after one week's growth under control, mild and high osmotic stress conditions compared against Col-0 (WT). Col-0, rbohd, *rbohf, rbohd/rbohf, rbohc, rbohi, perk4-2, upb1.1 and upb1.2* seedlings were transferred to osmotic treatment plates and measured after1 week. **Figure 6.8** ROS mutants primary root length after one week's growth under control, mild and high osmotic stress conditions compared against control conditions. For boxplots, upper and lower boundaries of the box indicate the interquartile range (IQE), a line within the box represents the median, and the whiskers represent the min and max excluding outliers. Small circles represent outliers. (NS = not significant, *<0.05, **<0.01, ***<0.001). Anova performed with significance detailed.

The *rbohd/rbohf* double mutant has significantly shorter primary roots than Col-0 under control, mild and high osmotic stress (Figure 6.7) ($p<0.05$). The double mutant performs significantly better under mild stress conditions when compared to the performance under control conditions ($p<0.05$). Under high osmotic stress there is little difference between control condition performance.

The *rbohc* mutant has significantly longer primary roots than Col-0 under mild stress (Figure 6.7) ($p<0.05$). Under control and high stress conditions, the *rbohc* mutant has a similar performance to that of Col-0. The *rbohc* mutant has significantly reduced root growth performance under high stress conditions when compared to the control (Figure 6.8).

The *rbohi* mutant has significantly longer primary roots under control, mild and high stress conditions when compared to Col-0 (Figure 6.7) ($p<0.05$). High osmotic stress has a significant impact on root growth in the *rbohi* mutant when compared to control conditions (Figure 6.8) ($p<0.05$).

The *perk4-1* has significantly lower primary root length than Col-0 in both control and mild stress conditions. Interestingly *perk4-1* also has significantly longer roots under high osmotic stress (Figure 6.7) ($p<0.05$). *perk4-1* shows no response in root growth to mild or high osmotic stress when compared to control conditions (Figure 6.8).

Under control, mild stress and high osmotic stress conditions, there is little difference in root growth between the *upb1.1* mutant and Col-0 (Figure 6.7). The *upb1.1* mutant does see an increase in root growth under mild stress, but it is not significant when compared to control conditions.

The *upb1.2* mutant has a significantly longer root system than Col-0 under control, mild and high osmotic stress conditions (Figure 6.7) ($p<0.05$). Under mild stress, the *upb1.2* mutant has significantly longer roots compared to control ($p<0.05$). Whereas under high osmotic stress, the mutant's roots are significantly shorter compared to the control (Figure 6.7) ($p<0.05$)

6.2.4 The impact of H_2O_2 and DPI treatment on primary root length under osmotic stress

Exogenous application of 1 mM H_2O_2 had a significantly negative impact on primary root length in Col-0 in all conditions when compared the control (Figure 6.9) ($p<-0.05$). All forms of exogenous DPI treatment (5 µM DPI, 10 µM DPI, 20 µM DPI) also had a significantly negative impact on root growth when compared to Col-0 under all conditions ($p<0.05$). The combination of 1 mM H_2O_2 treatment combined with 20 µM DPI treatment also led to significantly reduced root growth in all conditions when compared to Col-0 ($p<0.05$) (Figure 6.9).

Seedlings treated with 1 mM H_2O_2 were still responsive to osmotic stress, with mild and high osmotic stress significantly reducing root growth when compared to control conditions (Figure 6.10) ($p<0.05$). Treatment with 5 µM DPI, 10 µM DPI, 20 µM DPI and 1 mM H_2O_2 + 20 µM DPI all led to a similar response in which root growth saw a small but significant improvement under mild osmotic stress conditions when compared to control ($p<0.05$). High osmotic stress in these treatments saw no impact on root growth when compared to the control (Figure 6.10).

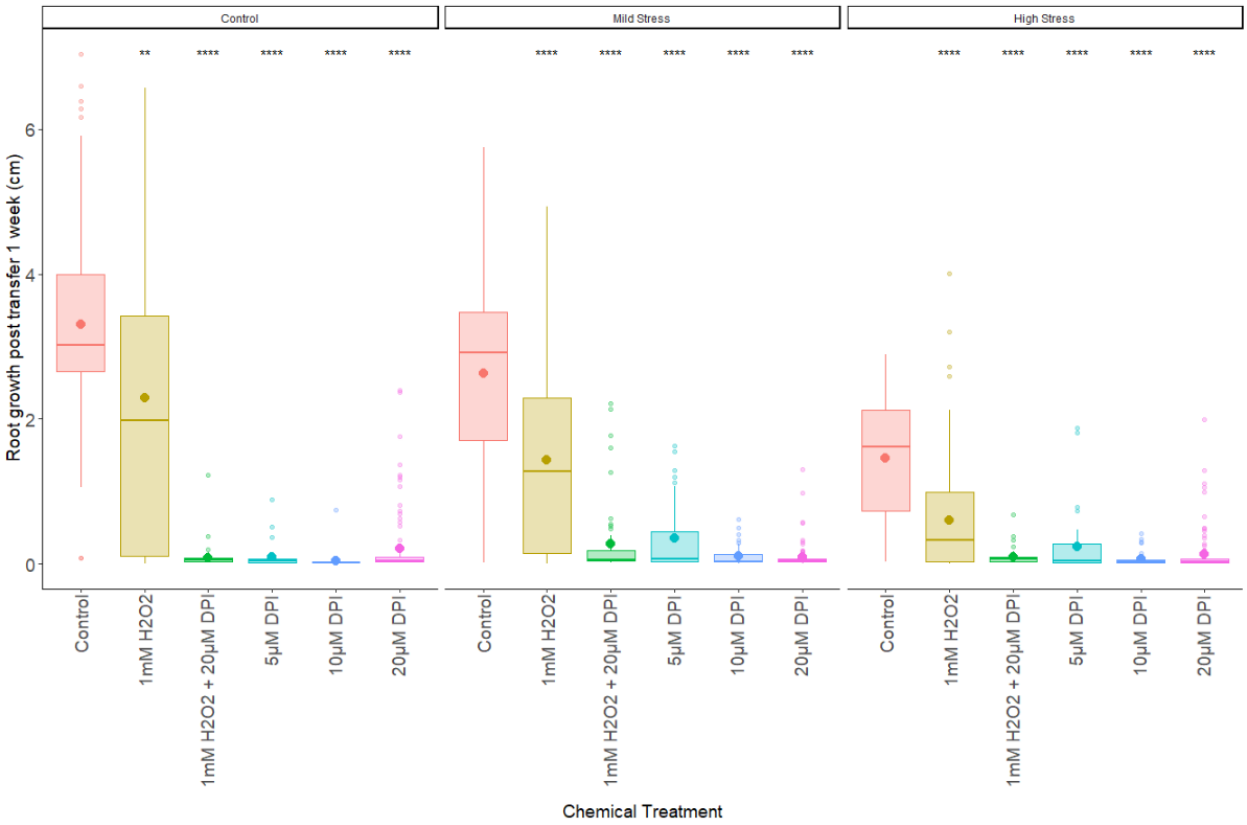

Figure 6.9

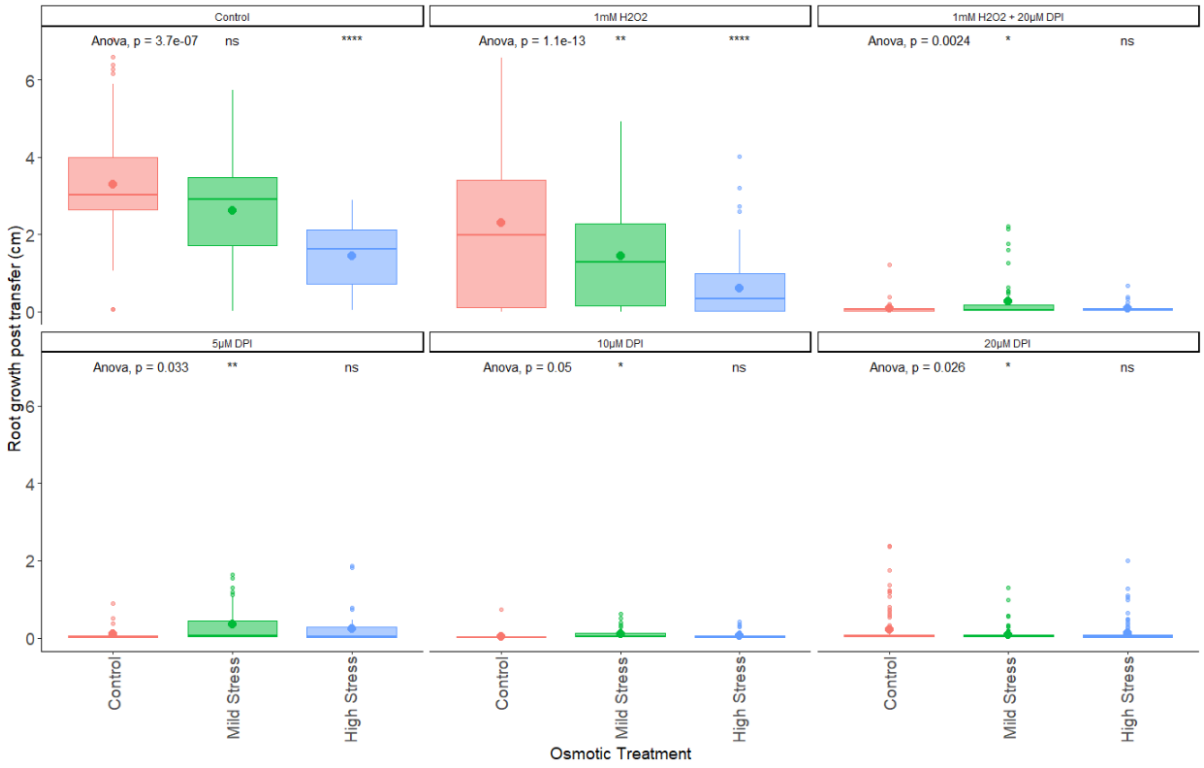

Figure 6.10

Figure 6.9 Col-0 (WT) root growth for one week under control, mild and high osmotic stress conditions with treatments of 1mM H_2O_2, 1mM H_2O_2 + 20µM DPI, 5µM DPI, 10µM DPI and 20µM DPI compared to control conditions. **Figure 6.10** Col-0 (WT) primary root growth after 1 weeks growth under control, mild and high osmotic stress conditions, separated by chemical treatments of 1mM H_2O_2, 1mM H_2O_2 + 20µM DPI, 5µM DPI, 10µM DPI and 20µM DPI. For boxplots, upper and lower boundaries of the box indicate the interquartile range (IQE), a line within the box represents the median, and the whiskers represent the min and max excluding outliers. Small circles represent outliers. Stars indicate significance from t-test of treatment compared to Col-0 (WT)(NS = not significant, *<0.05, **<0.01, ***<0.001).

6.3 Summary

ROS patterns can develop quickly under osmotic stress as following 10 minutes of osmotic stress (10% PEG and 300 mM sorbitol) there is a significant increase in oxidation levels in roGFP2-Orp1 when compared to control conditions (Figure 6.1). The combination of ABA-biosynthesis inhibitor fluridone application with osmotic stress leads to a similar level of oxidation as the control, indicating that the oxidation spike seen under 10% PEG is limited by fluridone. The sole application of fluridone for 10 mins leads to no change in oxidation level in roGFP2-Orp1. The application of RBOH-inhibitor DPI at both 1 µM and 20 µM concentrations leads to a significant increase in oxidation compared to the control. The application of DPI (1 µM and 20 µM) leads to a non-significant increase in the level of oxidation seen under osmotic stress.

It appears that spatial patterns of oxidation are not formed quickly under osmotic stress or exogenous chemical treatment, as the 10 min treatment of 10% PEG, 300 mM sorbitol, 1 µM Fluridone, 1 µM DPI, 20 µM DPI and the combination of 10% PEG with 1 µM DPI do not lead to a shift in the distribution of oxidation. Only the combined treatment of 10% PEG and 1 µM DPI led to a significant shift in the proximal/distal distribution ratio when compared to the control (Figure 6.2).

The longer 24 hr treatment of osmotic stress (High PEG) led to a significant increase in the level of oxidation in roGFP2-Orp1, whereas the 24 hr treatment of 300 mM Sorbitol did not. The application of 1 µM fluridone in combination with High PEG treatment resulted in an increase level of oxidation compared to the control, indicating fluridone has little impact on the effects of High PEG. Interestingly, the sole application of 24 hr 1 µM fluridone treatment led to a significant increase in oxidation. 24 hr 20 µM DPI application led to a significant increase in oxidation. The combination of 24 hr 20 µM DPI application and High PEG leads to a significant increase in oxidation when compared to control conditions (Figure 6.3)

Following 24 hr High PEG treatment there is a significant shift in the spatial distribution of oxidation levels towards the distal region of the root tip. Application of 1 µM Fluridone treatment in combination with High PEG treatment also led to a significant shift to the distal region when compared to control conditions. 24 hr 300 mM Sorbitol treatment did not lead to this shift in

oxidation distribution or did the sole application of 24hr 1uM Fluridone. 24 hr 20 µM DPI application led to a significant shift in distribution of oxidation down the root tip. Combining 24 hr 20 µM DPI treatment with High PEG treatment led to an even greater shift in oxidation to the root tip (Figure 6.4).

It an attempt to determine where this increase in ROS production could originate, it was revealed that in Col-0 (WT) the expression of ROS-production linked *PERK4* increases significantly under mild osmotic stress and a near-significant increase under high osmotic stress. ROS-producing *RBOHI* expression significantly increases under high osmotic stress but sees no change under mild osmotic stress. *RBOHC* expression do not change under either mild or high osmotic stress conditions (Figure 6.6).

Via a loss-of-function mutant study it was possible to determine that ROS-production enzymes *rbohd, rbohf* and the *rbohd/rbohf* double mutant have significantly smaller primary root growth under control, mild and high osmotic stress conditions. The *rbohc* mutant has a similar performance to that of Col-0 (WT) under control and high osmotic stress conditions, whereas under mild conditions the primary root growth is significantly greater than Col-0. Interestingly, the *rbohi* mutant has significantly longer primary roots under control, mild and high osmotic stress conditions than Col-0 (WT) (Figure 6.7).

The *perk4-1* mutants primary root length is significantly smaller that Col-0 under both control and mild stress conditions. Under high osmotic stress, *perk4-1* has significantly longer roots compared the control. The *perk4-1* mutant is somewhat unresponsive to osmotic stress, displaying similar root lengths under all three control, mild stress, and high stress conditions. The *upb1.2* has significantly longer roots compared to Col-0 (WT) under control, mild and high osmotic stress conditions. *upb1.2* is also responsive to both mild and high osmotic stress, with the former resulting in significantly longer roots that control conditions, and the latter leading to significantly shorter roots (Figure 6.7).

To understand the role of ROS in root development, the exogenous application of key ROS signal H_2O_2 and RBOH-inhibitor DPI was studied under osmotic stress conditions. 1 mM H_2O_2 application had a significantly negative impact on primary root length in Col-0 under all conditions. The application of DPI (5 µM, 10 µM and 20 µM) under all conditions also had a significant negative impact on primary root growth. The combination of 1 mM H_2O_2 and 20 µM DPI also led to significantly reduced root growth under all conditions compared to Col-0 (WT) (Figure 6.9).

Roots treated with 1 mM H_2O_2 remained responsive to osmotic stress, under both mild and high osmotic stress seedlings were significantly smaller compared to control conditions. Treatment with

DPI (5 µMI, 10 µM and 20 µM) independently and in combination (1 mM H_2O_2 + 20 µM DPI) all led to similar unresponsive phenotype under osmotic stress. Mild stress leads to small but significant improvement in root length, and high osmotic stress has no impact on root growth compared to control conditions (Figure 6.10).

Chapter 7. The role of ROPs in the osmotic stress response

7.1 Introduction

Rho of plant (ROP) proteins perform a vast range of functions, including acting as switches to convert intracellular and extracellular stimuli leading to localized regulation of intracellular responses (Feiguelman *et al.* 2018). ROPs have been shown to regulate the activation of RBOHs and the signalling of ROS, ABA and auxin (Wu *et al.* 2011, Liao *et al.* 2017).

The role of ROPs in osmotic stress response in the root has not received much attention despite their key role in regulating stress signalling. As a result, both ROP2 and ROP6's role in osmotic stress response in the root was investigated. To determine their role in root development under osmotic stress, the primary root length of both *rop2* and *rop6* loss-of-function mutants were studied under osmotic stress. The expression of *ROP2* was assessed via qRT-PCR to determine whether it is upregulated in osmotic stress response.

7.2 Results

7.2.1 ROP2 and ROP6 role in root development under osmotic stress

The *rop2* loss-of-function mutant has significantly longer primary roots under control, mild stress and high stress conditions when compared Col-0 (Figure 7.1) (t-tests, $p<0.05$). Mild and high osmotic stress has little impact on *rop2* root growth when compared to control conditions (Figure 7.2).

The *rop6* mutant has significantly longer roots than Col-0 under high osmotic stress conditions (Figure 7.1) (t-test, $p<0.05$). Under control and mild stress conditions, *rop6* has a similar performance to that of Col-0. *rop6* has little change in root growth under mild and high osmotic stress conditions when compared to control conditions (Figure 7.2).

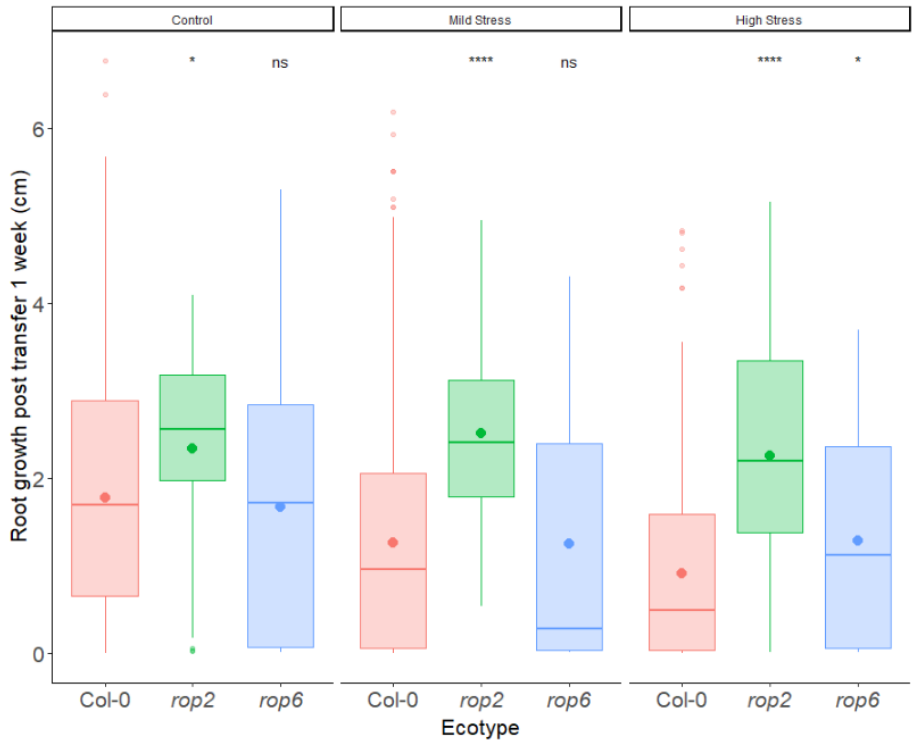

Figure 7.1

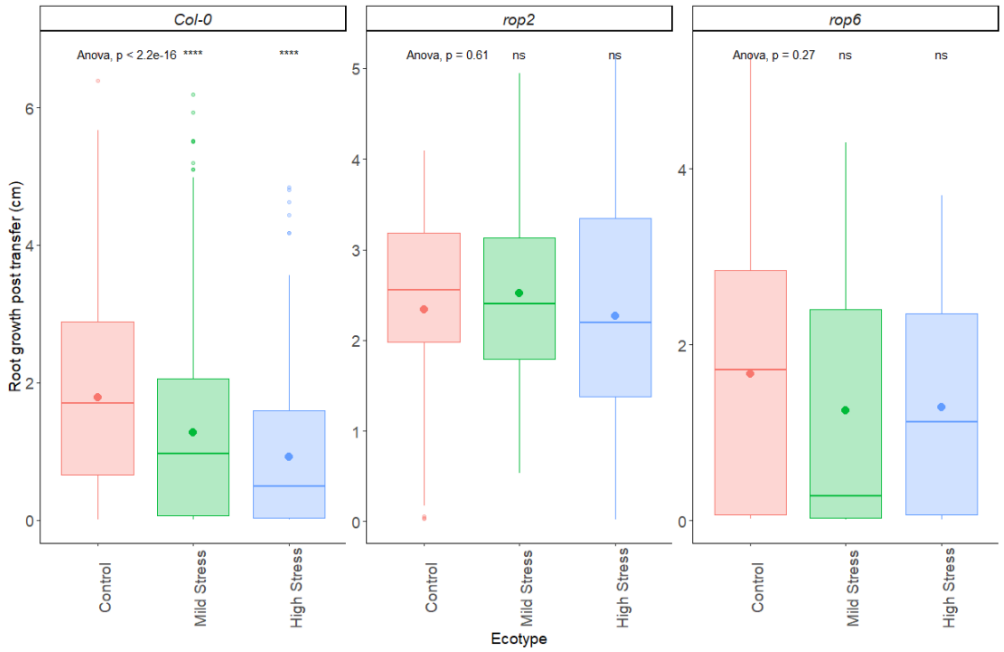

Figure 7.2

Figure 7.1 *rop2 and rop6* loss-of-function mutants primary root length after one week's growth under control, mild and high osmotic stress conditions compared against Col-0 (WT). Seedlings were transferred to osmotic treatment plates and measured after1 week. **Figure 7.2** *rop2* and *rop6* mutants primary root length after one week's growth under control, mild and high osmotic stress conditions compared against control conditions. For boxplots, upper and lower boundaries of the box indicate the interquartile range (IQE), a line within the box represents the median, and the whiskers represent the min and max excluding outliers. Small circles represent outliers. Stars indicate significance from t-test of treatment compared to Col-0 (WT)(NS = not significant, *<0.05, **<0.01, ***<0.001). Anova performed with significance detailed.

7.2.2 *ROP2* expression under osmotic stress

In Col-0 (WT) we see a non-significant increase in *ROP2* expression under osmotic stress (Figure 7.3). Although on average, it was an over 8-fold increase in *ROP2* expression, the data was highly variable. This indicates that *ROP2* expression may not change under osmotic stress.

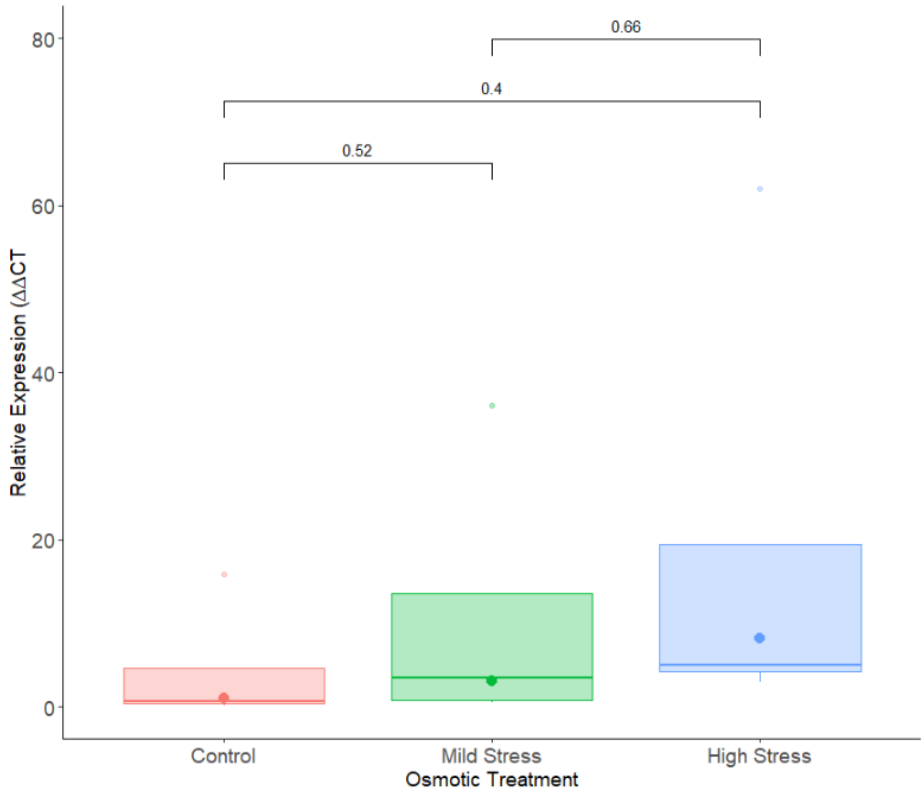

Figure 7.3 Normalized fold expression of *ROP2* under 24hr control, mild and high osmotic stress treatment in Col-0 (WT). Expression levels were normalized using reference gene AT5G15710, relative fold expression was then calculated via ΔΔCT transformation, comparing expression to the relative control sample. For boxplots, upper and lower boundaries of the box indicate the interquartile range (IQE), dark circle within the box represents the geometric mean, a line within the box represents the median, and the whiskers represent the min and max excluding outliers. Small circles represent outliers. Brackets indicate significance from t-test of treatment compared to control (NS = not significant, *<0.05, **<0.01, ***<0.001)

7.3 Summary

In an attempt to determine the role of ROP2 and ROP6 in root development under osmotic stress. It appears the *rop2* mutant has significantly longer roots under all conditions compared to Col-0. The

loss-of-function mutant is unresponsive to osmotic stress, with mild and high stress having little impact on *rop2* root growth. The *rop6* mutant's primary root length is significantly greater under only high osmotic stress conditions and is similar to *rop2* in appearing unresponsive to osmotic stress.

There is no significant increase in *ROP2* expression under osmotic stress, although there is a small increase, indicating it does not change in expression.

Chapter 8. Discussion

8.1 The role of POLARIS in plant development

POLARIS (PLS) acts an inhibitor of ethylene response, where it colocalises to the same location as ETR1 in the endoplasmic reticulum, where there is evidence of direct interaction (Mudge 2016). PLS is able to bind to copper (Cu) *in vitro*. Copper is required for ETR1 as a co-factor so that it is able to bind to ethylene (Mudge 2016, Rodriguez *et al.* 1999). Flooding *pls* mutants plants with copper can rescue short root phenotype indicating its inhibition of ETR1 involves the limiting of the copper co-factor (Mudge 2016).

Previously, the study of PLS under osmotic stress has focused on a mutant in the background of C24. The C24 mutant has been shown to be more tolerant to abiotic stress conditions (Bechtold *et al.* 2018). In order to remove this factor from the study of PLS, a mutant was developed in the Col-0 background via CRISPR-Cas9 and named *pls-2*.

The *pls* CRISPR mutant (*pls-2)* exhibits an enhanced ethylene response via the reduction of root and shoot growth during growth in the dark (Figure 3.1). This has been previously shown in Chilley *et al.* (2006) which revealed the role of PLS in ethylene signalling and auxin transport. This work is important to confirm previous research into the role PLS plays in plant development.

Unfortunately, due to time-constraints it was not possible to study the *pls-2* mutant response to osmotic stress. However, this *pls-2* mutant will be a key tool in the future when developing an understanding of how PLS influences plant development. Using *pls-2* we could determine the role of PLS in root development under osmotic stress via studying the phenotypic response and performing qRT-PCR of critical responsive genes in the ABA, ROS and Auxin pathways,

Recently work has shown that PLS regulates the expression levels of various ethylene-responsive genes and plays an important role in ethylene signalling (Figure 8.1). The PLS peptide has also been shown to be essential for ethylene-induced auxin biosynthesis in the *Arabidopsis* root tip (Shen 2019). Since ethylene interplays with other hormones such as auxin and cytokinin, PLS is also important for hormonal crosstalk to regulate root development.

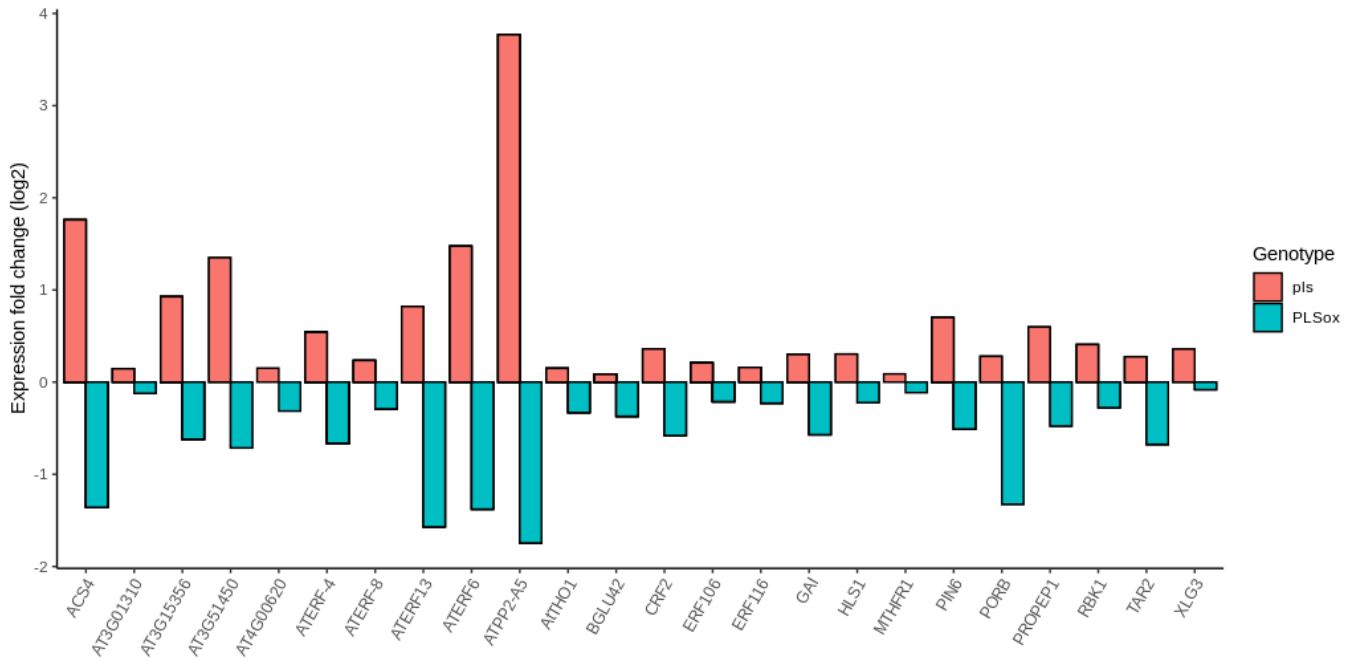

Figure 8.1. Expression levels of 24 ethylene-responsive genes in the *pls-1* mutant and in *PLS* overexpressing seedlings (PLSox) both from the C24 *Arabidopsis* background compared to wildtype (Col-0) levels. Taken from Shen (2019).

Studying both TCSn:GFP and *pls-3*/TCSn:GFP, we can see that there is a significant increase in levels of TCSn:GFP fluorescence in the QC of the *pls-3/*TCSn:GFP mutant (Figure 3.2). This indicates that if PLS is no longer functioning, there is a significant increase in cytokinin levels in the QC. This data correlates with previous research that indicates that in the *pls* mutant we see an increase in expression of cytokinin-inducible gene ARR5 (Chilley *et al.* 2006).

From these data, we can suggest that PLS plays a key role in inhibiting cytokinin levels in auxin-cytokinin homeostasis, through ethylene signalling crosstalk. This reduction of cytokinin when in PLS present is likely due to the inhibitory impact of PLS on ethylene response, which has been shown to promote cytokinin levels (Chilley *et al.* 2006).

8.2 The phenotypic response to osmotic stress

Under mild and high osmotic stress, we see a reduction in primary root growth and a reduction in the number of lateral roots (Figure 4.1). This correlates with previous research that under osmotic stress there is a decline in RAM size, due to a reduction in cell division and cell size, leading to a reduction in root growth (Rowe *et al.* 2016, Cajero Sanchez *et al.* 2019, Yuan *et al*. 2021). Under osmotic stress, the anatomy of the SCN is unaltered, enabling plants to restore once osmotic stress is removed (Cajero Sanchez *et al.* 2019).

8.3 The role of auxin and its transporters under osmotic stress

8.3.1 Rapid spike in auxin levels within minutes of osmotic stress

Despite its key role in plant development, there has been little study of the auxin response within minutes of abiotic stress application. Given that auxin has been shown to act as a rapid non-transcriptional signal, causing changes in plasma membrane potential and triggering cytosolic Ca^{2+} spikes and a rise of apoplastic pH (Dubey *et al.* 2021), auxin could potentially act as a rapid systemic signal triggering a stress response similar to that of a ROS wave.

Interestingly, a significant increase in mDII-ndTomato/DII-n3xVenus ratio in R2D2 is seen after 10 mins of osmotic stress treatment (Figure 4.3). This suggests that within the first few minutes of osmotic stress there is a rapid spike in auxin levels. The increase in auxin could be acting as a rapid osmotic stress signal as this significant increase in a short period of time could trigger a variety of responses. For example, an increase in auxin levels could lead to a spike in ROS production, triggering further stress responses as exogenous IAA has been found to increase ROS levels in root tissues (Zwiekwa *et al.* 2019). Previously ROS has been found to function downstream of auxin mediated signal transduction in gravitropism (Hee Joo *et al.* 2001). Auxin can promote PINOID kinase levels, leading to increased RBOH activity and ROS production (Saini *et al.* 2017). An increase in PINOID levels could also lead to enhanced ABA production. An increase in auxin could also promote flavonoid accumulation, which play a variety of roles in osmotic stress (Peer and Murphy 2007, Saini *et al.* 2007).

An increase in auxin can cause very rapid changes in membrane potential, for example IAA has been shown to rapidly induce plasma membrane depolarization (Dindas *et al.* 2018, Serre *et al.* 2021). This is likely through auxin transport processes, active signalling responses and a higher concentration of weak acids (IAA). Membrane depolarization has been shown to be tightly correlated with rapid root

growth inhibition and AFB1 auxin receptor function (Serre *et al.* 2021). It is possible that membrane depolarization could act as an immediate signal to inhibit root growth in response to osmotic stress.

Root apoplastic pH could be a key factor in this early signalling phase. Auxin regulates the phosphoactivation of AHA2, a plasma membrane H^+-ATPase which controls apoplastic pH (Takahashi *et al.* 2012). Potentially changes in pH can lead to the promotion of RBOH production which leads to ROS wave. Apoplastic alkalization can have an important impact on ROS signalling specificity as cysteine residues are readily oxidized at higher pH levels. An increase in pH leads to increased reactivity of cysteines with ROS (Geilfus *et al.* 2017).

The mechanism leading to the rapid spike of auxin is not clear. Potentially the spike is promoted by the rapid ROS wave or other signals such as Ca^{2+} or ABA. In certain cases, ROS such as H_2O_2 have been found to promote auxin accumulation, in particular in the short term in areas such as the root meristem (Zwiewka *et al.* 2019). Further research is required to elucidate what occurs downstream of this spike in auxin and what could be causing it. Potentially there is a direct link between osmosensors and this rapid auxin increase.

8.3.2 PIN proteins plays a critical role in auxin transport under osmotic stress

In the longer-term study (24 hrs), there was a reduction in R2D2 ratio levels under high osmotic stress conditions compared to both control and mild stress conditions (Figure 4.4). Under mild stress we did not see a significant reduction in R2D2 levels compared to control. Similar to previous research (Rowe *et al.* 2016) this indicates that under high osmotic stress conditions there is a significant reduction in the auxin maximum in the root tip. This reduction of auxin at the root tip is likely contributing to the reduction in root growth we see under osmotic stress (Rowe *et al.* 2016). The reduction in auxin is further confirmed by the decrease in expression levels of *IAA2* (an auxin responsive gene) in both mild and high osmotic stress (Figure 4.13).

N-1-napthylpthalamic acid (NPA) has recently been shown to be a direct PIN transport inhibitor, independent of other auxin transport proteins (Abas *et al.* 2021). The application of NPA results in a significant reduction in R2D2 levels in the root tip indicating a reduction of auxin content due to reduced PIN transport (Figure 4.4). In all three treatments (control, mild and high osmotic stress) NPA has a significant impact on R2D2 levels when compared to the control (DMSO) treatment. This indicates that a critical level of auxin transport via PINs to maintain an auxin maximum is still taking

place even at high osmotic stress. Under NPA treatment, mild and high osmotic stress has no impact on R2D2 levels compared to the control NPA treatment, indicating that once PIN-mediated auxin transport is inhibited, we see no further reduction in auxin levels under osmotic stress (Figure 4.4). This suggests that PIN-mediated auxin transport is critical for auxin reduction we see in osmotic stress response. Furthermore, these results suggest that local auxin biosynthesis has little impact on levels of auxin under osmotic stress as once PIN-mediated auxin transport is removed there is little change in auxin levels. Auxin biosynthesis in the root tip may still have some effect but not to the level that is detectable via the R2D2 auxin reporter.

8.3.3 PIN3 and PIN7 under osmotic stress

Studying proPIN3:PIN3:GFP expression under osmotic stress, there was seen little overall change in total fluorescence but there was an increase in PIN3:GFP localization to the plasma membrane (Figure 4.9, Figure 4.10). PIN polarization has been shown to direct auxin flux and plays a key role in plant phototropism and gravitropism (Wisniewska *et al.* 2006, Rakusova *et al.* 2015, Ding *et al.* 2011). An increase in plasma membrane localization suggests that auxin transport out of the root tip is potentially increasing under osmotic stress, leading to the reduction in auxin maximum (Rowe *et al.* 2016).

PIN3 expression was significantly reduced under osmotic stress conditions in *Arabidopsis* (Figure 4.11). A reduction in *PIN3* expression, in particular in areas such as the stele, may lead to a reduction of auxin transport acropetally to the root tip and a reduction of the auxin maximum. The contrasting results between PIN3:GFP and *PIN3* expression might be due to a whole plant system reduction of *PIN3* expression and no decrease in the root tip. Recently Yuan *et al.* (2021) has shown that *PIN3* expression is reduced under osmotic stress due to repression by increased levels of HAT2 transcription factor. This research also found that PIN3:GFP expression was reduced under mannitol treatment. Interestingly HAT2 expression is induced by auxin but is not altered by ABA, ethylene or BR treatment. More research is required investigating how ROS could cause HAT2 and PIN3 expression changes.

Under control, mild and high osmotic stress conditions, the *pin3* mutant appears to perform worse than WT, with shorter roots (Figure 4.12A). This indicates that PIN3 plays an important role in root growth. With PIN3 no longer functioning it is likely that auxin transport down in the root tip is reduced, leading to a reduction in auxin at the root tip therefore a reduction in root growth.

Although performing worse than compared to Col-0 under all conditions, the *pin3* mutant appears to be insensitive to PEG treatment (4.12B). For example, there is little difference in the mutant's performance under control or under high stress conditions. This phenotype is similar to that of Yuan *et al.* (2021) which found that *pin3* mutant roots were less sensitive to mannitol treatment. This indicates that the inhibition of root development via osmotic stress requires the normal operation of PIN3. When normally functioning, PIN3 likely performs a critical in auxin transport to reduce auxin in the root tip leading to the osmotic stress phenotype previous described.

proPIN7:PIN7:GFP transgenics show a decrease in fluorescence in the root under osmotic stress, indicating that PIN7 levels reduce under high osmotic stress response in the root (Figure 4.9). This reduction likely leads to a slowing of auxin transport down into the root tip leading to the reduction of the auxin maximum. Using qRT-PCR, *PIN7* expression saw a non-significant reduction in both mild and high osmotic stress (Figure 4.11). Potentially *PIN7* expression is only reduced in the root system, as indicated by PIN7:GFP. As a result, the expression in the whole seedling is not significantly altered.

The *pin7* mutant did not have a significantly different root growth under control or osmotic stress conditions when compared to the WT (Figure 4.12A). Furthermore, the *pin7* mutant was responsive to osmotic stress and was significantly reduced in root growth under mild and high stress conditions (Figure 14.2B). This indicates that PIN7 may not have a significant role in root growth, or in osmotic stress response. Potentially the absence of PIN7 is rescued by redundancy from other PIN proteins.

The *pin3/pin7* double mutant did not have significantly different root growth when compared to WT in all conditions indicating that their combined deletion does not result in a significant change to osmotic stress response (Figure 14.2A). This is particularly interesting as *pin3* mutant shows significant changes to root growth. Again, perhaps there may be redundancy between PIN proteins, leading to the unaltered phenotype.

Considering PIN3 and PIN7 roles in the acropetal transport of auxin to form a 'reverse fountain' pattern of auxin distribution (Friml *et al.* 2003), it can be suggested that these changes in patterning, expression and polarization likely contribute to the reduction in auxin levels seen in the root tip under osmotic stress. It appears that PIN3 plays a more critical role in root development than PIN7 under these conditions. More work is required to analyse their precise role in osmotic stress response and what pathways could be causing these changes in expression and patterning.

8.3.4 The role of DAO1 under osmotic stress

DOA1 is a key enzyme in auxin metabolism. If DAO1 is no longer functioning, oxIAA-Asp and oxIAA-Glu are no longer being formed, a key step in the GH3-IRL1-DAO pathway (Hayashi *et al.* 2021). As these oxidised forms can no longer be formed there is a large amount of IAA-Asp and IAA-Glu leading to IAA release and increased free IAA in the plant system (Hayashi *et al.* 2021).

The *dao1* mutant has significantly more root growth when compared to the WT under control conditions (Figure 4.12A). Under high stress conditions the *dao1* mutant also has significantly improved root growth. Interestingly, under mild stress conditions the mutant performs worse than the WT. The *dao1* mutant is sensitive to mild osmotic stress, with root growth significantly reduced (Figure 4.12B). High osmotic stress appears to have no impact on the *dao1* mutant, with no difference between *dao1* root growth in control and high osmotic stress conditions.

It is likely that increased free IAA in the *dao1* mutant is improving plant performance under high osmotic stress as exogenous IAA has been found to improve root performance in previous studies (Rowe *et al.* 2016, Yuan *et al.* 2021). It is unclear why *dao1* mutant would perform much worse under mild osmotic stress whilst not responding to high osmotic stress. Perhaps the accumulation of free IAA under mild stress has negative consequences on root development. More work is required to study the expression of *DAO1* under osmotic stress and look at how the *dao1* mutant alters other hormonal pathways.

8.4 The dynamics of ABA under osmotic stress

8.4.1 ABAleonSD1-3L21 analysis of osmotic stress response

Within 10 mins of osmotic stress treatment, there is an increase in ABA levels (represented by a decrease in ratio between cpVenus173/mTurquoise ratio in ABAleonSD1-3L21), though this is not statistically significant (Figure 5.1A). This correlates with previous work indicating ABA levels rise under osmotic stress conditions (Rowe *et al.* 2016). Previously ABA has been shown to induce ROS accumulation within minutes (Postiglione and Muday 2020). A rapid increase in ABA could lead to an enhanced ROS wave through the plant system in response to osmotic stress along with altering other stress signals and hormones such as auxin and ethylene.

Application of exogenous H_2O_2 (10 mM H_2O_2) for 10 mins leads to a significant decrease in cpVenus173/mTurquoise ratio, signifying an increase in ABA levels (Figure 5.1A). This indicates that ABA levels rapidly increase in the root tip under elevated ROS levels. Previously a strong positive feedback loop between ABA and ROS has been proposed (Song *et al.* 2014, Mittler & Blumwald 2015). These results reinforce this idea and indicate that this process could occur within minutes.

Under 24 hrs of osmotic stress, there is a significant increase in ABA levels (represented by a decrease in cpVenus173/mTurquoise ratio in ABAleonSD1) (Figure 5.1B). This indicates there is a long-term increase in ABA in response to osmotic stress, correlating with previous research (Rowe *et al.* 2016). Treatment with exogenous H_2O_2 (10 mM) led to a significant increase in ABA levels, higher than that following the osmotic stress treatment. This indicates along with the previous 10-min treatment, that there is a strong relationship positive relationship between ABA and elevated ROS.

Upon treatment with RBOH inhibitor diphenyleneiodonium (DPI, 10 µM) for 24 hrs, there is seen a significant increase in ABA (represented by decrease in ABAleonSD1 ratio) when compared to control conditions (Figure 5.1C). This indicates that ABA levels are increased when RBOH ROS production is limited by DPI. It is possible that this increase in ABA is due to a stress response from the DPI treatment, whereby DPI may have a toxic impact on the plant system at a 10 µM concentration or ROS levels may regulate ABA concentrations. There are other pathways where ROS levels could be increasing including the Fe/Asc pathway (Martiniere *et al.* 2019), leading to an ABA increase.

Under DPI treatment, osmotic stress appears to reduce the level of ABA compared to the control DPI treatment (Figure 5.1C). This indicates that the osmotic stress induced increase in ABA is no longer possible when RBOH ROS production is limited. Potentially this might be due to the levels of ABA

already being high under the 10 µM DPI treatment. Comparing the cpVenus173/mTurquoise ratio at high osmotic stress between control (DMSO) and 10 µM DPI treatment it appears little has changed in ABA levels. The lack of difference between ABA levels in control and DPI treatment is likely due to other pathways producing high ABA levels under osmotic stress other than RBOH ROS production.

8.4.2 ABA-Responsive *RD29B* under osmotic stress

From analysing the expression of *RD29B*, an ABA-responsive gene, it is possible to estimate the levels of ABA response within the plant. In WT (Col-0) there was a significant increase in *RD29B* expression under both mild and high osmotic stress (Figure 5.4). Under high osmotic stress there is a near 200-fold upregulation in expression. This indicates that under high osmotic stress we see a significant increase in ABA responses, likely indicative of high ABA concentrations. This correlates with previous research indicating that *RD29B* expression along with ABA levels increase under osmotic stress (Rowe *et al.* 2016).

8.5 The dynamics and role of ROS in osmotic stress response

8.5.1 The level of oxidation in the root tip increases under osmotic stress

Developed by Nietzel *et al.* (2019), the fluorescent protein sensor cytosolic roGFP2-Orp1 is capable of monitoring oxidation levels in the cytosol. Using this reporter, it is possible to study the levels of oxidation within the plant root and help determine ROS dynamics under osmotic stress.

From studying roGFP2-Orp1, there is a significant increase in oxidation levels within 10 minutes of osmotic stress treatment following both 10% PEG and 300 mM sorbitol treatments (Figure 6.1). This rapid increase in oxidation is likely an increase in ROS acting as a signal, triggering responses within the root system, potentially travelling up the root in a shootward direction as a signal to the rest of the plant. Previous research has shown that ROS is rapidly upregulated as a stress signal under abiotic stress in a form of a ROS wave leading to an increase in oxidation (Fichman and Mittler 2020).

The exogenous application of the ABA biosynthesis inhibitor fluridone for 10 minutes under control conditions did not lead to a change in oxidation levels. However, the application of fluridone appears to have a significant impact on the level of oxidation under 10% PEG treatment, indicating that local carotenoid biosynthesis (which leads to ABA) might play a role in rapid osmotic stress signalling and the resulting oxidation increase we see under osmotic stress (Figure 6.1).

Both 10-min treatments with RBOH inhibitor DPI (1 µM & 20 µM) surprisingly lead to an increase in oxidation levels (Figure 6.1). Perhaps the inhibition of RBOH leads to ROS production elsewhere in the plant system in a form of homeostasis or there is a reduction in antioxidant capacity such as lowered glutathione pool. Inhibiting other ROS pathways such as the Fe/Asc pathway in combination with DPI might prove useful in elucidating why we observe a rise in oxidation. Inhibiting the Fe/Asc pathway is possible with treatment with bathophenanthrolinedisulfonic acid (BPDS), a membrane-impermeant Fe^{2+} chelator that depletes free Fe^{2+} in the cell apoplastic space. The inhibition or a deliberate increase of antioxidant production could help determine what is causing the rise of oxidation in roGFP2-Orp1.

20 µM DPI treatment leads to less oxidation than that of the 1 µM DPI treatment under 10 mins (Figure 6.1). Considering the increase in oxidation we see under both 1 µM and 20 µM treatments, perhaps both of these treatments are not at high enough concentrations for full RBOH inhibition. Further work with difference concentrations of DPI is needed in order to fully understand how the inhibition RBOH production alters oxidation in osmotic stress response.

1 µM and 20 µM DPI treatments led to a small increase in the level of oxidation under 10% PEG conditions which are already increased compared to the control (Figure 6.1). This suggests that DPI may cause even greater levels of oxidation, perhaps in a stress response. Interestingly this combination of 10% PEG treatment coupled with DPI treatment (both 1µM and 20µM) led to a small decrease below just the level of oxidation at relative DPI treatment. This is challenging to explain and requires more research.

We see an even greater increase in roGFP2-Orp1 ratio under the 24-hr osmotic stress treatment, indicating that root tip oxidation has risen over the longer period of osmotic treatment (Figure 6.3). Interestingly sorbitol treatment did not lead to an increase in ratio. This might be explained by different effects that PEG and sorbitol have upon plant cells (plasmolysis versus cytorrhysis) (Verslues *et al.* 2006).

24-hr treatment with 1 µM fluridone led to a significant increase in roGFP2-Orp1 levels (Figure 6.3). As there is a large amount of evidence indicating ABA leads to ROS increase (e.g. Watkins *et al*. 2017, Postiglione and Muday 2020), it is unlikely that ABA is inhibiting ROS levels. Fluridone inhibits carotenoid biosynthesis which indirectly lowers ABA content. Lowered carotenoids can lead to reduction in protection from photobleaching (Gamble and Mullet 1986). Fluridone treatment also is not sufficient to reduce ABA content completely as there is a large pool of epoxy carotenoids (Srivastava *et al.* 2002). As a result, increased oxidation in response to 24 hr fluridone treatment

could be caused by increased light-stress response or due to other sources of ABA still increasing under osmotic stress.

When compared to the control, 24-hr High PEG treatment combined with 1 µM fluridone led to an increase in roGFP2-Orp1 oxidation levels (Figure 6.3). It is clear that fluridone application has little impact on oxidation levels under 24 hr High PEG. This indicates that inhibition of carotenoid biosynthesis and part of the ABA pathway does not have an impact on the increase in oxidation under 24 hr osmotic stress. As previously mentioned, fluridone can lead to enhance light-stress along with other ABA pathways are still functioning. Interestingly, fluridone treatment does appear to hinder the increase in oxidation under osmotic stress in the 10-min treatment but not under the 24-hour treatment. Perhaps after 24 hrs, fluridone-mediated stress has had an impact, leading to an increase in stress signalling and ROS-mediated oxidation. As a result, the impact of reduced carotenoid biosynthesis on ROS levels/oxidation is negated.

24-hr treatment with DPI also led to an increase in oxidation, indicating that the increase in oxidation under 10-minute DPI treatment was not only a short-term response (Figure 6.3). The increase indicates that DPI might cause stress to the plant leading to an overproduction in ROS via other forms of production such as the Fe/Asc pathway or a reduction in antioxidant capacity. The presence of osmotic stress treatment did not lead to a change in oxidation under 24 hr DPI treatment. This could be explained by DPI treatment causing an increase in oxidation that osmotic stress may not be able to exceed.

The rise in oxidation seen in both 10 min and 24 hr osmotic treatment of roGFP2Orp1 is likely due to the increased production of ROS; as previous work of ROS in abiotic stress response has indicated that this could be the case ((Mittler *et al.* 2004, Shabala *et al.* 2015, Baxter *et al.* 2014, Martiniere *et al.* 2019). However, it is important to consider this may also be due to a reduction in antioxidant capacity such as glutathione or catalase (Dumanovic *et al.* 2021). As a result, more work is required to confirm this increase in oxidation is due to ROS production. Using other forms of ROS analysis such as CellRox DeepRed or DAB (3,3'-Diaminobenzidine) staining may help.

8.5.2 Change in the pattern of oxidation in the root tip under osmotic stress

In the 10-min short term treatments there is little change in the proximal/distal distribution of oxidation in the root tip, as shown by 10% PEG, 300 mM sorbitol, 1 µM fluridone, 1 µM DPI, 10% PEG + 1 µM DPI and 20 µM DPI treatments compared to the control (Figure 6.2). Only under the 10% PEG + 20 µM DPI treatment there is a significant difference in distribution; oxidation is greater in the higher regions of the root system (proximal region). The shift in oxidation is potentially due to the

reduction of RBOH production in the root tip whilst ROS production is increased further up the root system. Changes in oxidation can also be caused by changes in reduction rate due to lowering of antioxidant production

Over the longer term 24-hr treatments there are distinct changes in the proximal/distal distribution of roGFP2-Orp1 (Figure 6.4). Under High PEG treatment we see a significant increase in oxidation higher up the root in the EZ area when compared to the meristematic zone. Along with the broad increase in oxidation in the root tip, this shift in patterning could have key ramifications for plant root development. A more oxidized elongation zone, and a more reduced environment in the apical meristem leads to an inhibition in root growth (Tsukagoshi *et al.* 2010, Tsukagoshi *et al.* 2016, Mabuchi *et al.* 2018). This shift of distribution to an increasingly oxidized EZ under osmotic stress could account for the observed reduction in cell elongation, leading to reduced root growth. Under sorbitol treatment there is a similar shift in distribution although not significant.

The application of fluridone does not appear to have affect the pattern of roGFP2-Orp1 expression in control conditions or under osmotic stress (Figure 6.4). Despite this, it is possible that ABA may have an impact on the distribution of oxidation under osmotic stress. As previously described, there are other active ABA pathways together with the potential for fluridone application to cause stress to the plant.

There are large shifts in roGFP2-Orp1 proximal/distal distribution with increased oxidation higher up the root system under 24-hour DPI treatment (Figure 6.4). This shift in distribution could be caused by the lack of apoplastic ROS production via RBOH inhibition, leading to increased ROS in regions away from the root tip. DPI application during high stress treatment results in an even greater shift in distribution. More research is required into the location of RBOH ROS production in the root tip, and the effects of DPI upon ROS distribution.

8.5.3 ROS and ROS inhibitor treatment alters root growth under osmotic stress

In the wildtype (Col-0), exogenous treatment with H_2O_2 (1 mM) leads to a reduction in root growth under control, mild and high osmotic stress conditions (Figure 6.9). This indicates that elevated ROS levels in seedlings can lead to decreased root growth under osmotic stress conditions. The exogenous application of H_2O_2 likely leads to increased ABA among other responses that likely contribute to the reduced root growth. Increasing the level of ROS past a certain threshold concentration can also lead to cell death (Duan *et al.* 2010, Sofo *et al.* 2015).

The application of different concentrations of exogenous DPI (5 µM, 10 µM and 20 µM DPI) led to almost a complete reduction in root growth in all treatments (Figure 6.9). This suggests that apoplastic ROS production via RBOH enzymes is critical for root growth in all conditions.

Combining these results with the increase in oxidation and ABA levels that we see under DPI treatment it is highly possible that these concentrations of DPI treatment are damaging to the plant leading to a stress response and cell death. As a result, these results along with the DPI treatment data in ABAleonSD1 and roGFP2Orp1 should be treated with caution.

The combination treatment of H_2O_2 with DPI had little impact on the high negative effect of DPI on root growth. This indicates that the harmful impact on DPI on root growth may not be due to the reduction of ROS in the root tip as its replacement with exogenous H_2O_2 treatment has little impact.

8.5.4 RBOHD and RBOHF play a key role in root growth and osmotic stress response

The *rbohd* and *rbohf* single mutants and *rbohd/rbohf* double mutant appear to have significantly reduced root growth in all conditions (Control, Mild and High stress) (Figure 6.7). This indicates that both RBOHD and RBOHF are critical for root growth under control conditions and under osmotic stress. Interestingly, these three mutant ecotypes performed better under mild osmotic stress conditions, when compared to control and high stress (Figure 6.8). Furthermore, they are tolerant to high osmotic stress conditions when compared with the stunted control response. Previously RBOHD and RBOHF have been found to play a key role in ABA-signalling, ABA-mediated growth inhibition and in stress-induced ROS accumulation (Kwak *et al.* 2003, Jiao *et al.* 2013, Martiniere *et al.*2019). It is clear that tight regulation of these pathways is critical for growth under normal conditions, but also under osmotic stress. It can be concluded that RBOHD and RBOHF act as negative regulator of growth inhibition under osmotic stress.

8.5.5 RBOHC has a complex role in root osmotic stress response

The *rbohc* mutant has a similar growth response to Col-0 (WT) under control and high osmotic stress conditions but grows significantly better under mild stress (Figure 6.7). The *rbohc* mutant remains sensitive to strong osmotic stress, with high stress having a significant impact on its root growth (Figure 6.8). Mild stress does not have a significant impact when compared to control conditions. This indicates that RBOHC may not be critical for root growth under standard conditions or response to high osmotic stress. Under mild stress, ABA-stimulated ROS production may be limited as RBOHC

is no longer functional (Ma *et al.* 2019). This reduced ROS production may not have the same level inhibitory impact and could lead to improved root performance and tolerance to mild osmotic stress. Potentially as ABA-induced ROS production is limited, other pathways are less inhibited, improving root growth.

RBOHC expression does not appear to increase under osmotic stress (Figure 6.6). This may indicate that ROS production in RBOHC is not responsive to osmotic stress. Potentially RBOHC activity could be altered via its localisation or by post-translational modification in response to osmotic stress. Previous studies have identified that RBOHD and RBOHF spatio-temporal expression is finely tuned in response to biotic stress (Morales *et al.* 2016). Other members of the RBOH family such as RBOHC could have a similar level of intricate patterning that cannot be detected by the whole-seedling qRT-PCR that was performed.

The *rbohc* mutant displays a significant increase in *RD29B* expression under osmotic stress (near to 600-fold) suggesting a large increase in ABA levels under osmotic stress, much larger than that seen in the WT (Figure 5.4). Loss of RBOHC function appears to limit ABA-mediated inhibition of root growth (Ma *et al.* 2019). This may lead to ABA levels further increasing, in a form of homeostasis, reflected in the observed high expression of *RD29B*. Interestingly, the loss-of-function of RBOHC does not appear to alter ABA homeostasis under control conditions as *rbohc* has similar levels of *RD29B* to Col-0 under control conditions (Figure 5.3).

Auxin response gene *IAA2* expression does not show significant changes under osmotic stress conditions in the *rbohc* mutant, while WT seedlings show a significant decrease in *IAA2* levels under osmotic stress (Figure 4.14). This indicates that RBOHC is critical and therefore RBOHC-mediated ROS production is required for the auxin response under osmotic stress. Perhaps as RBOHC-mediated ROS production is no longer possible, auxin levels are no longer able to be adjusted under osmotic stress. The impact of RBOHC on *IAA2* levels appears only osmotic stress related as the *rbohc* mutant has a similar level of expression to that of Col-0 (Figure 4.13).

As ABA levels are still increasing but IAA levels are no longer altered by osmotic stress in the *rbohc* mutant, it is concluded that RBOHC may act as a link between ABA and auxin signalling under osmotic stress.

8.5.6 *PERK4* expression increases under osmotic stress where it plays critical role

PERK4 has been suggested to be a link between ABA and ROS production in limiting root growth (Ma *et al.* 2019). ABA accumulation is thought to drive PERK4 to increase the expression of NADPH

oxidases such as RBOHC, leading to ROS production that inhibits root growth. RBOHC is thought to be a vital enzyme for ROS formation in the primary root in response to ABA (Ma *et al.* 2019). *perk4* mutants were found to display attenuated sensitivity to ABA inhibition of root growth, as they were incapable of generating ROS in response to ABA (Ma *et al.* 2019)

PERK4 expression significantly increases under mild osmotic stress and has a very near to significant two-fold increase under high stress (Figure 6.6). This indicates that PERK4 may be involved in osmotic stress response. Increased ABA levels under osmotic stress likely increase *PERK4* expression, leading to increased expression of RBOHs and increased ROS production, which leads to reduced root growth. Interestingly, there was not the same increase in expression of *RBOHC* under osmotic stress that was seen for *PERK4*. RBOHC is suggested as the primary mechanism behind PERK4-mediated ABA inhibition of root growth (Ma *et al.* 2019). Although *RBOHC* expression may not increase, PERK4 might alter RBOHC performance in some other form or regulate (i.e increase) the expression of other RBOH genes under osmotic stress.

The *perk4-1* mutant root grows significantly less than wildtype under control and mild osmotic stress conditions, whilst growing longer under high osmotic stress conditions (Figure 6.7). The results also show that the *perk4* mutant is less sensitive than the wild type to osmotic stress, similar to results present by Ma *et al.* (2019) (Figure 6.8). This indicates that PERK4 is critical for root growth under standard conditions and also plays a role in the osmotic stress response. When PERK4 function is lost, ABA-induced expression of RBOH enzymes, leading to ROS production is reduced, resulting in altered root growth under control conditions and improved root growth under high osmotic stress. PERK4 is likely an important link between ABA and ROS under osmotic stress.

8.5.7 RBOHI plays critical role in root growth inhibition and sensitivity to osmotic stress

RBOHI has been identified to play a key role in under osmotic stress conditions (Huan *et al.* 2017). Mannitol stress was found to significantly increase *RBOHI* expression at the transcript level, whereas ABA application was found to decrease RBOHI expression. Loss of function mutant *rbohi* displayed enhanced sensitivity to mannitol stress. Overexpression of RBOHI lead to drought tolerance in *Arabidopsis*, along with an increase in H_2O_2 compared to wildtype (Huan *et al.* 2017).

In the current study, *RBOHI* expression levels has very near significant two-fold increase under 24-hours of high osmotic stress treatment (Figure 6.6). Under mild osmotic stress, this upregulation is not seen. This indicates that *RBOHI* expression is likely upregulated leading to an increase ROS production under intense osmotic stress conditions. In the *rbohi* mutant under osmotic stress, there

is a near significant increase in the ABA-responsive gene *RD29B* (about 300-fold) (Figure 16.2). This indicates that ABA response under osmotic stress is still possible with *RBOHI* no longer functioning. In fact, ABA responses may be enhanced as this increase in *RD29B* is greater than we see in wildtype, indicating that RBOHI acts as repressor of ABA responses under osmotic stress. Similar to the response we see in *rbohc*, ABA levels may increase in *rbohi* to accommodate for restricted ROS production in the osmotic stress response. Along with the *rbohc* mutant, the loss-of-function *rbohi* mutant has similar levels of *RD29B* expression to that of Col-0, indicating RBOHI may not have a function in ABA-homeostasis.

Interestingly *rbohi* exhibited significantly better root growth that wildtype under all conditions (Control, mild stress, and high stress), indicating that RBOHI acts to inhibit root growth and particularly under osmotic stress (Figure 19.1). The potential reduction in ROS production from the loss of RBOHI may improve root length. The *rbohi* mutant is tolerant to osmotic stress treatment seeing little change between control and stress treatments. This is in contrast to what Haun *et al.* (2017) described in their research, with *rbohi* mutant displaying enhanced sensitivity to mannitol. The difference in effects of mannitol and PEG could explain the large discrepancy between these results. Mannitol is toxic and can cause plasmolysis, while PEG does not. More work is required to analyse the role of RBOHI in osmotic stress to explain the difference between studies.

8.5.8 The role of UPB1 under osmotic stress

UBP1, a bHLH transcription factor, plays a key role in maintaining ROS balance between meristematic and elongation zones (Tsukagoshi *et al*. 2010). UPB1 upregulates ROS homeostasis by repressing the expression of class III peroxidases in the elongation zone. The *upb1-1* mutant has a larger meristematic zone, owing to an increase in the number of cells, which accumulates O_2^-as UPB1-targeted peroxidase expression is not suppressed. In the current study *upb1-1* showed little difference in root length when compared to the WT, however *upb1-2* displayed a significant improvement in root length in all conditions (Figure 6.7). These data are similar to Tsukagoshi *et al.* (2010) who showed that UPB1 is a negative regulator of root growth; although my results indicate this is the case even under osmotic stress conditions. Loss of UPB1 function, leading to altered ROS balance and an increased O_2^- distribution in the root tip improves root growth under control and osmotic stress conditions. ROS mutants such as *rbohd, rbohf, rbohi* and *perk4-1* displaying decreased/improved root growth likely have some form of shift in ROS balance between meristematic and elongation leading to their change in growth. Both *upb1-1* and *upb1-2* appear to be near-significantly and significantly sensitive to osmotic stress respectively. Both mutants show increases in root length under mild osmotic stress and reduced root length under high osmotic stress

(Figure 6.8). This indicates that under mild stress, UPB1 may negatively regulate root growth as its loss of function leads to improved root growth.

8.5.9 The role of ROPs in osmotic stress response

ROP proteins play a key role in development and stress responses, acting as molecular switches triggering signalling cascades (Feiguelman *et al.* 2018) Previously ROP6 activation under osmotic stress has been shown to trigger the nanoclustering of RBOHD and RBOHF leading to ROS production (Smokvarska *et al.* 2020) along with playing a role in auxin signalling (Wu *et al.* 2011).

The *rop2* mutant displays root growth similar to WT under control conditions but performs significantly better under osmotic stress when compared with WT (Figure 7.1). Furthermore, the *rop2* mutant is also insensitive to osmotic stress, with seedling length in both mild and high osmotic stress conditions similar to that under control conditions (Figure 7.2). These results indicate that ROP2 plays an essential role in promoting root growth inhibition under osmotic stress. Loss of ROP2 function leads to root systems being longer and more tolerant to osmotic stress.

Although *RD29B* expression increases under osmotic stress conditions in the *rop2* mutant, it is not a significant increase, and when compared to the WT it is comparatively much lower (20-fold compared to 200-fold) (Figure 5.3). This suggests that ROP2 is critical for the osmotic stress-induced ABA response. Interestingly there is evidence that other ROPs (ROP 10 & ROP11) suppress ABA responses (Yu *et al.* 2012). In the *rop2* mutant we see an increase in *IAA2* levels in mild and high osmotic stress indicating an increase in auxin under osmotic stress (Figure 4.14). This is directly opposite to the decrease in auxin that has been previously characterised in Col-0 under osmotic stress (Rowe *et al.* 2016) and seen in our own *IAA2* WT results; and indicates that ROP2 is required for repression of auxin responses during osmotic stress. The role of ROP2 in both *RD29B* and *IAA2* expression may be strictly osmotic stress related, as the *rop2* mutant does not have significantly different levels of either gene when compared to the Col-0 mutant under control conditions.

It is therefore possible to suggest that in the WT, ROP2 activation via osmotic stress leads to increased ABA levels which in turn leads to a decrease in auxin accumulation in the root tip. Given the role for ROP6 in inducing RBOHD and RBOHF ROS production under osmotic stress (Smokvarska *et al.* 2020); increased ROS production via ROP2 activation may lead to an increase in ABA levels. In the *rop2* mutant, ABA levels do not increase as greatly therefore auxin levels are able to increase.

Previously studies have shown that the perturbation of ROP activity leads to auxin-related phenotypes. This is likely due to ROPs effects on PM localization and the trafficking of PINs which, in turn, regulates auxin fluxes and concentrations (Dubey *et al.* 2021). For example, PIN2 recycling in

roots is dependent on ROP6 activation by its GEF protein SPIKE1 (Lin *et al.* 2012). Plants showing ROP6 overexpression or the expression of the constitutively active of form ROP6 bend their roots faster and to a greater degree than WT plants (Platre *et al.* 2019). It is also possible that the impact *rop2* mutant has on *IAA2* levels is independent of ROS or ABA pathways. ROP2 mutation was found to have little impact on ROS accumulation under high sorbitol concentration (Smokvarska *et al.* 2020). This suggests that ROP2 has may have direct control of auxin levels under osmotic stress.

Interestingly *ROP2* expression does not increase significantly under osmotic stress (Figure 7.3). This may not be critical as found with ROP6, and the activation state may be more important than the level of expression under osmotic stress (Smokvarsak *et al.* 2020). It is likely that its activations state may be altered under osmotic stress conditions. More research is required to analyse ROP2's role in osmotic stress and its impact on ABA and auxin levels under osmotic stress conditions.

ROP6 activation under osmotic stress has been shown to trigger the nanoclustering of RBOHD and RBOHF leading to ROS production (Smokvarska 2020). The *rop6* mutant performs significantly better that WT under high osmotic stress (Figure 7.1). This correlates with previous research that *rop6.2* mutants grew faster under high sorbitol conditions (Smokvaska *et al.* 2020). The rop6 mutant appears to be insensitive to osmotic stress, having a similar level performance at control compared to high osmotic stress (Figure 7.2). As osmotically stress ROS production is reduced due the absence of ROP6 (Smokvarska *et al.* 2020). This reduction in ROS production under osmotic stress leads to a better performance of root growth and tolerance to osmotic stress. Further research is required to analyse the role of ROP6 osmotic stress-induced ROS production on auxin and ABA levels.

8.6 Hormonal crosstalk under osmotic stress

Previous work has shown that ABA plays a critical role in regulating root growth under osmotic stress via interacting with auxin, cytokinin and ethylene (Rowe *et al.* 2016). Specifically, osmotic stress modulates auxin transporter levels and localisation leading to reduced root auxin concentrations, and PIN1 levels are reduced in an ABA-dependent manner (Rowe *et al.* 2016). However there remains large gaps in our understanding of root growth regulation under osmotic stress.

8.6.1 Short Term Signalling

The majority of research into abiotic stress signalling has focused on plant systems above the soil such as stomata, and there has been little work on visualising the short-term signalling that takes place in the root under osmotic stress.

By studying the ratiometric auxin reporter R2D2, results in this thesis show that within minutes of stress there is a rapid spike in auxin levels in the root tip (Figure 4.3). In this short time frame, auxin may be acting initially as a stress signal, altering plasma membrane polarization and apoplastic pH, leading to downstream osmotic stress responses such as immediate root growth inhibition (Serre *et al*. 2021). Increased auxin could increase ROS levels, causing ROS wave and increase the level of stress signalling in the root system (Peer *et al.* 2013, Zwiewka *et al.* 2019).

ABA levels, represented via ABAleonSD1-3L21, also appear to increase (non-significantly) within minutes of osmotic stress treatment (Figure 15.1 A). A rapid change in ABA could act as stress signals throughout the plant system, and immediately begin to alter hormonal pathways such as auxin, cytokinin and ethylene. ABA levels rapidly increase within minutes under exogenous treatment of H_2O_2, combining this with previous evidence, it is apparent there is a strong positive relationship between ABA and ROS that can operate within minutes (Figure 15.1 A).

Studying fluorescent reporter roGFP2Orp1, we see a rapid rise in oxidation in the root tip within 10 minutes of osmotic stress (Figure 17.1). When considering previous analysis, it is likely that this is a spike in ROS causing the increase in oxidation, rather than a reduction in antioxidant capacity. A spike in ROS could act as a stress signal in the form of ROS wave (Fichman and Mittler 2020). An increase in ROS will have numerous downstream consequences including increased ABA levels, which lead to osmotic stress response. The application of ABA biosynthesis inhibitor fluridone led to a reduction in the spike of oxidation we see under 10 mins of osmotic stress treatment, indicating that increasing ABA production may have a role in the increase in ROS under osmotic stress (Figure 17.1).

From these data based on the analysis of fluorescent reporters, we can propose that auxin, ROS and ABA all increase within 10 minutes of osmotic stress treatment, leading to downstream signalling (Figure 24). Both ABA and ROS share a positive feedback relationship whereby increases in one will increase the levels of the other. Auxin and ROS have also been shown to promote each other; increased ROS leads to increased auxin concentrations and auxin application can lead to increase ROS levels (Liao *et al.* 2015, Zwiekwa *et al.* 2019). ABA has been found to negatively influence auxin levels under osmotic stress (Rowe *et al.* 2016), so it is unlikely it is behind the rapid increase in auxin levels.

With all three hormones increasing rapidly in a short window of osmotic stress, it is difficult to determine what comes first, potentially generating the other signals. Potentially all three stress signals are triggered synchronously via an osmo-sensing system, although the nature of the osmo-

sensing system is unclear. Potentially a homolog of the calcium-gated channel OSCA1 could be behind this rapid cascade (Liu *et al.* 2018).

How ABA, ROS and auxin all interact in this short time frame needs further research. ROP2 and RBOHC should be studied to determine the role they have in short-term signalling. There remain further questions on what is occurring downstream of these increases in ABA, ROS and auxin. More research is required in this area if we are to reach a better understanding of short-term osmotic stress signalling.

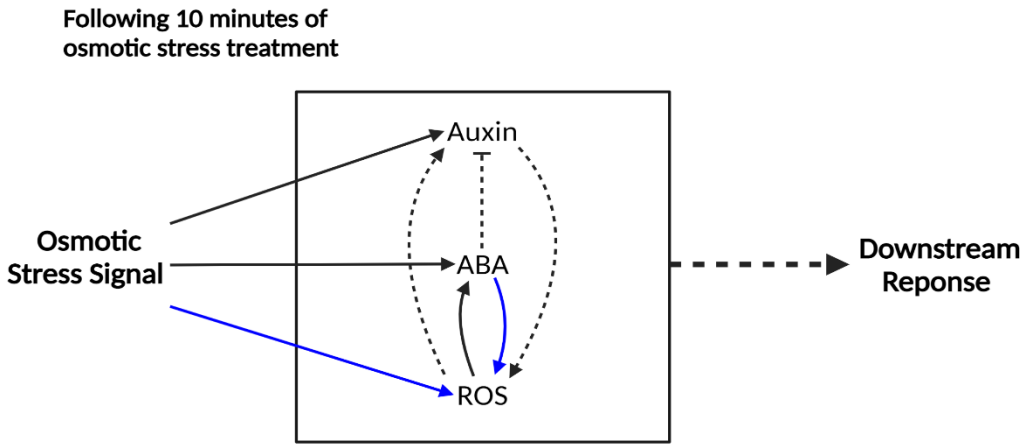

Figure 8.2 A network depicting rapid auxin, ABA and ROS signalling interaction within 10 minutes of osmotic stress in the root tip. Solid black arrows represent direct evidence presented in this thesis of promotion of accumulation. Solid blue arrows represent the hypothesised promotion of accumulation of ROS represented by an increase in oxidation in roGFP2Orp1. Dotted arrows represent indirect evidence from literature that promotion of accumulation could occur. Dotted flat-headed arrows represent indirect evidence from literature that inhibition of accumulation that could occur. Direct evidence is supported by fluorescent reporter results (R2D2, ABAleonSD1-3L21 and roGFP2Orp1). Promotion of ROS accumulation by osmotic stress is supported by Martinere *et al.* (2019). ABA promotion of ROS accumulation is supported by Yang *et al.* (2014) Potential positive feedback cycle of ROS and auxin is supported by Liao *et al.* (2015), Zwiekwa *et al.* (2019). Potential short-term ABA-mediated inhibition of auxin is supported by Rowe *et al.* (2016). Downstream response of auxin, ABA and ROS supported by Serre *et al.* (2021), Fichman and Mittler (2020), Rowe *et al.* (2016).

8.6.2 Longer Term Signalling (24 hr)

Studying signalling over on a longer time frame of osmotic stress treatment (24hrs) was also important as there remains large gaps of knowledge that require more work. Previously Rowe *et al.* (2016) presented evidence that auxin transport is altered under osmotic stress response by ABA and ABA-independent means.

A reduction in auxin response under osmotic stress was found that is consistent with previous research (Rowe *et al.* 2016). It is clear that polar auxin transport is critical for auxin maximum

maintenance under osmotic stress. When PIN transport is inhibited by the application of NPA, auxin levels are reduced in the meristem under control and osmotic stress conditions (Figure 10.3). This indicates that polar auxin transport is still taking place even under high osmotic stress conditions. Under NPA treatment, auxin responses are no longer sensitive to osmotic stress, indicating that auxin biosynthesis or conjugation has little contribution to auxin levels in the root tip in these conditions (Figure 10.2). PIN3 and PIN7 levels, localization and polarization are altered under osmotic stress, likely enhancing the reduction of auxin we see under osmotic stress (Figure 11.3, Figure 11.4, Figure 12). PIN3 plays a critical role in root development under control conditions and osmotic stress, whereas for PIN7 this is less clear. How ABA and ROS alter PIN3 and PIN7 levels to control root development under osmotic stress requires study.

As indicated in other research (Rowe *et al.* 2016), ABA responses were found to increase under 24hr osmotic stress (Figure 15.1 B, Figure 16.2). ABA responses were also shown in this thesis to increase under exogenous H_2O_2 treatment, supporting the view that ABA and ROS have a strong positive relationship (Figure 15.1 B).

From the data collected, it is clear that ROS have a critical role in the osmotic stress response. Analysing roGFP2Orp1, there is a rise in the levels of oxidation in the root tip after 24hr osmotic stress, either through an increase in the level of ROS in the root tip or a reduction in the level of antioxidation (Figure 17.3). We also see a shift in the distribution of oxidation under osmotic stress; to a more oxidised elongation zone and reduction in the apical meristem (Figure 17.4). This shift likely contributes to the inhibition of root growth we see under osmotic stress (Mabuchi *et al.* 2018).

DPI treatment led to an increase in both ABA and in oxidation in (ABAleon and roGFP2Orp1) (Figure 15.B, Figure 17.3). Considering the significant inhibitory impact that DPI treatment had on root growth, it is likely that this form of DPI treatment is causing significant stress to the plant leading to a rise in both stress hormones. As a result, it is difficult to interpret these DPI results in the context of signalling under osmotic stress. More research is required to fully analyse how ROS production via RBOH enzymes can alter ABA and ROS levels under osmotic stress.

Auxin, ABA, and ROS all play critical roles in plant development. Auxin decreasing likely caused by the increase in ROS and ABA under osmotic stress. ROS and ABA likely have a strong positive relationship, increasing each other to reduce the level of auxin in the root tip. Before we can develop a network including ROS into the osmotic stress signalling network, more work is required to understand how ROS interacts with auxin and ABA. For example, how ROS alters the impact of ABA on PIN transport shown in Rowe *et al.* (2016). Also determining how inhibiting the ROS pathway via some other form of treatment than DPI alters osmotic stress response.

8.7 The role of ROS in root development under osmotic stress

The role of ROS in root development has previously been studied, including controlling cell polarity and expansion, and the development of root hairs and Casparian strip (Huang *et al.* 2016). However, the control ROS exerts on root development under osmotic stress has not received a critical analysis.

ROS producing enzymes RBOHD and RBOHF play a critical role in plant development and osmotic stress response. Both have enzymes have previously been found to play key roles in abiotic stress response (Smokvaska *et al.* 2020). This study reveals that when either or both proteins are no longer functioning, it results in reduced root growth, indicating their role in positively regulating root development (Figure 19.1). The mutants with reduced root growth appear to be insensitive to osmotic stress, indicating that RBOHD and RBOHF may play some role in osmotic stress root development (Figure 19.2).

RBOHI appears to have a significant role in inhibiting root development, both in control conditions and under osmotic stress (Figure 19.1). Loss of function of RBOHI led to improved primary root length under all conditions. With RBOHI absent, ABA levels still increase, indicating it may have no role in the ABA pathway under osmotic stress (Figure 16.2). Potentially RBOHI is involved in ABA-independent osmotic stress response.

Although the RBOHC mutant appears to have little role in root growth under control and high stress conditions, its absence does appear to improve root growth under mild osmotic stress (Figure 19.1). In the RBOHC mutant we see a significant change in auxin signalling under osmotic stress. There is no longer a reduction in auxin responsive *IAA2* which is seen in the control under osmotic stress (Figure 14.2). ABA-responsive *RD29B* sees a large increase under osmotic stress similar to that of the WT, suggesting RBOHC does not alter ABA response too greatly (Figure 16.2). Despite the fact that RBOHC expression does not see a change under osmotic stress (Figure 18), RBOHC localisation and production may change, leading to changes in ROS production and altering auxin levels in the root tip. The lack of a reduction in auxin may explain why the *rbohc* mutant performs better under mild stress conditions.

ABA-regulated inhibition of root growth under control conditions has been shown to be partially mediated by PERK4-stimulated ROS accumulation, likely through RBOHC (Ma *et al.* 2019). PERK4 expression significantly increases under osmotic stress (Figure 18). Loss of function of PERK4 results in significant changes to root development under osmotic stress, with longer primary roots under high stress whilst displaying reduced root growth in control and mild conditions (Figure 19.1). The loss of PERK4 also results in tolerance to osmotic stress as determined by root growth (Figure 19.2).

PERK4 may be critical for root development for its role in mediating ABA signal to ROS production. A possible pathway under osmotic stress is increasing PERK4 expression under increased ABA levels results in ROS production from RBOH's. This increased level of ROS results in reduced root growth. RBOHC may not be the only RBOH enzyme that is altered by increased PERK4 expression. More work is required to analyse auxin, ABA and ROS response in the *perk4* mutant under control and osmotic stress conditions.

The balance of ROS in the root tip is critical for plant development. Mutation in UPB1 leads to improved root growth in all conditions including osmotic stress, likely due to altered ROS distribution in the root tip (Figure 19.2).

8.8 A key role for ROP2 in osmotic stress signalling

ROP2 and ROP6 clearly have a key role in root development under osmotic stress conditions. Both *rop2* and *rop6* appear insensitive to osmotic stress, performing better under high stress conditions when compared to the WT (Figure 21.1).

ROP2 also has a clear position in auxin and ABA signalling under osmotic stress. Auxin responses appear to increase under osmotic stress in the *rop2* mutant but decrease in the WT (Figure 14.2). The increase in ABA responses appears to be reduced in comparison to the WT (Figure 16.2). From these data it is proposed that under osmotic stress, ROP2 activates, leading to an increase in ABA response which in turn reduces auxin accumulation in the root tip, leading to a reduction in root growth.

ROP2 may regulate ABA and auxin through the production of ROS, as ROP6 has previously been shown to be responsible for ROS production via RBOHD and RBOHF under osmotic stress. Further work is required to understand how ROP2 is controls root development under osmotic stress. More work is also required to analyse auxin and ABA signalling in the *rop6* mutant.

8.9 Perspectives

With rapidly changing environments, plants must be able to respond immediately to changes in conditions. Plants have developed a variety of responses to the damaging consequences of osmotic stress (Bhargava and Sarwant 2013, Nezhadahmadi *et al.* 2015). In root systems, promoting or inhibiting root growth is a critical developmental choice that a plant must make, grow deeper for more moisture or inhibition of growth to prevent exposing the root further to the loss of water (Cajero-Sanchez *et al.* 2019). With increasing global temperatures and greater frequencies of drought, understanding how plants make these decisions and respond to osmotic stress is critical.

Previously ABA and auxin roles in osmotic stress have been partly characterised. It is clear that ROS also play a role in the early phase of osmotic signalling along with in the development of root growth under water-deficit. More research must focus on how ROS interacts with ABA and auxin leading to osmotic stress phenotypes. Potentially plant breeders can consider how ROS pathways including RBOH-mediated ROS production could be harnessed to improve plant tolerance to drought stress; as well as exploiting early osmotic stress signalling.

8.10 Future Work

Root response to osmotic stress is a critical topic that the scientific community needs to understand if we are to resolve food security issues in the face of climate change. There are various areas that require more research if we are to understand how plants control the roots development under osmotic stress and improve drought performance.

More effort is required to analyse the short-term response of auxin to osmotic stress. For example, how does inhibiting PIN transport with NPA alter this rapid auxin spike? Potentially this auxin signal generates a number of downstream responses that could be analysed. Further study could look at how PIN expression and distribution changes in the short-term osmotic stress response. This could be achieved via LSCM and qRT-PCR. Further study could look at the immediate response of ROS and ABA to exogenous auxin treatment to simulate this rapid auxin spike.

The control of PIN3 and PIN7 under osmotic stress needs to be further analysed. For example, it is unclear whether it is an increase ABA and/or ROS levels that is responsible for the changes in expression and distribution that we see. This can be achieved by studying PIN3 and PIN7 under the exogenous application of ABA, fluridone and other treatments. More research could reveal how ROS influences other PINs that have been found to be critical in osmotic stress response.

As previously mentioned, it is important to clarify the increases in oxidation we see under osmotic stress in roGFP2Orp1 is a result of increase ROS production or a reduction in antioxidant capacity; or a combination of both. More evidence can be achieved via the use of CellRox and DAB staining as well as the inhibition of ROS production via other means than DPI treatment. The crossing of loss-of-function RBOH mutants with roGFP2-Orp1 could create an effective line to study if ROS production via RBOH is responsible for the spike in oxidation.

Further work is required to analyse the role of ROS production by RBOHs under osmotic stress, and how this leads to changes in ABA, ROS and auxin levels. More work is needed to study how RBOH expression and distribution is altered under osmotic stress. Determining how these RBOH proteins are regulated, for example through ubiquitination could reveal critical answers. It is important to analyse how PERK4 may alter auxin, ABA and ROS under osmotic stress; potentially through increasing RBOH apoplastic ROS production.

The role of *rbohi* in root development under osmotic stress requires further analysis considering the difference between this study's results and Huan *et al.* (2017). More research is required into how RBOHI alters ABA, auxin and ROS response to osmotic stress. Potentially this could involve the use of mannitol and PEG treatment in comparison with each other, this may reveal why we see such contrasting results between studies.

Work is required to analyse whether ROP2's impact on root development under osmotic stress is through the ROS pathway, or whether the impacts to auxin and ABA independently. It is important to analyse whether ROP2 alters RBOH ROS production. This could be achieved via qRT-PCR of RBOH enzymes in the loss-of-function *rop2* mutant under osmotic stress conditions. How ROP6-mediated osmotic stress production (Smokvaska *et al.* 2020) alters ABA and auxin is also an area that requires more work. The role of RHOGDI's in regulating ROP response to osmotic stress response is also interesting (Garcia-Mata *et al.* 2011) and could be studied further.

More research is required to fully understand what is causing the application of diphenyleneiodonium chloride (DPI) to have a negative and potentially toxic impact on root development. Using a smaller range of DPI treatments may answer as well as treating with a shorter time period. Potentially there are other chemical sources of inhibiting RBOH production that could be identified and used.

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Appendix

Primers for qRT-PCR

Gene	Primer sequences	Tm (C°)	Notes
AT5G15710	Fw – CTCTTTCGCCTCTTGGTTTG Rv - TCCTTCCCACGAGAAACAAT	53.9 53.9	Housekeeping Gene for osmotic stress (Czechowski *et al.* 2005)
RD29B	Fw - GGG GAA AGG ACA TGG TGA GG Rv - GGT TTA CCA CCG AGC CAA GA	60.03 59.96	ABA-responsive gene
IAA2	Fw – GAAGAATCTACACCTCCTACCAAAA Rv - CACGTAGCTCACACTGTTGTTG	54.3 56.2	Auxin-responsive gene
ROP2	Fw – GGTGTTCCCATTATCCTTGTTG Rv - TCACAAGAACGCGCAACGGTTC	53.9 60.7	ROP2 expression
RBOHI	Fw – GGTACCGCAAAACGGTATGG Rv - ACATGCGTAGCACAACAACG	56.3 56.3	RBOHI expression
RBOHC	Fw – TCACCAGAGACTGGCACAATAAA Rv - GATGCTCGACCTGAATGCTC	56.3 55.8	RBOHC expression
PERK4	Fw – ACGTTTACGCCCCGAATGAC Rv - CACCTTAGTAGATTGGGCTCGG	58.0 57.0	PERK4 expression
PIN3	Fw – GAGGGAGAAGGAAGAAAGGGAAAC Rv - CTTGGCTTGTAA TGTTGGCATCAG	60.8 60.8	PIN3 expression (Wang *et al.* 2015)
PIN7	Fw – GTCCGTTAGGCACTTCCTTTACCC Rv - TCAAGGCGGTGCAAAAGAGATTCG	64.5 64.5	PIN7 expression (Wang *et al.* 2015)

Table 3. Primers used for qRT-PCR analysis of gene expression