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Abscisic acid regulates root growth under osmotic stress conditions via an interacting hormonal network with cytokinin, ethylene and auxin.

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SUMMARY

- Understanding the mechanisms regulating root development under drought conditions is an important question for plant biology and world agriculture.
- We examine the effect of osmotic stress on ABA, cytokinin and ethylene responses and how they mediate auxin transport, distribution and root growth through effects on PIN proteins. We integrate experimental data to construct hormonal crosstalk networks to formulate a systems view of root growth regulation by multiple hormones.
- Experimental analysis shows that (1) ABA-dependent and ABA-independent stress responses increase under osmotic stress, but cytokinin responses are only slightly reduced; (2) inhibition of root growth under osmotic stress does not require ethylene signalling, but auxin can rescue root growth and meristem size; (3) osmotic stress modulates auxin transporter levels and localisation, reducing root auxin levels; (4) PIN1 levels are reduced under stress in an ABA-dependent manner, overriding ethylene effects; and (5) the interplay between ABA, ethylene, cytokinin and auxin is tissue-specific, as evidenced by differential responses of PIN1 and PIN2 to osmotic stress.
- Combining experimental analysis with network construction reveals that ABA regulates root growth under osmotic stress conditions via an interacting hormonal network with cytokinin, ethylene and auxin.

Key words: Arabidopsis thaliana; root development; osmotic stress**;** PIN proteins**;** abscisic acid; cytokinin; ethylene**;** auxin**;** hormonal crosstalk; systems biology.

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INTRODUCTION

Increasing food security for a growing global population is a major challenge facing humanity. Modulation of root system architecture is a key feature of plant responses to drought, potentially leading to yield benefits (Comas *et al.*, 2013; Uga *et al.*, 2013). Understanding the mechanisms regulating root development under drought conditions is therefore an important question for plant biology and world agriculture.

Soils form a complex environment, and roots under drought stress face multiple challenges that can alter their development. As well as osmotic stress, plants may also encounter reduced nutrient uptake and mechanical impedance (Alam, 1999; Whalley *et al.*, 2005). Less clear are the mechanisms by which these stresses mediate developmental changes.

Classic studies have shown that abscisic acid (ABA) biosynthesis and accumulated levels increase under drought stress (Zhang & Davies, 1987), and this response pathway is conserved among vascular and non-vascular land plants, including bryophytes (Takezawa *et al.*, 2015). Low levels of applied ABA or osmotic stress can increase root growth, whilst high levels can inhibit growth. Other hormones also play roles under drought - perturbation of cytokinin, auxin or ethylene pathways can affect survival or development under osmotic stress (Tran *et al.*, 2007; Nishiyama *et al.*, 2011; Cheng *et al.*, 2013; Shi *et al.*, 2014; Cui *et al.*, 2015; Kumar & Verslues, 2015). How ABA and osmotic stress interact with other hormones remains poorly defined (van der Weele *et al.*, 2000; Liu *et al.*, 2014).

Extensive research has been carried out to understand the crosstalk between ethylene and ABA. Ethylene-deficient and -insensitive mutants display increased ABA biosynthesis and responses, but exhibit reduced ABA-mediated inhibition of root growth (Beaudoin *et al.*, 2000; Ghassemian *et al.*, 2000; Cheng *et al.*, 2009). Phenotypic analysis of ethylene and ABA mutants has revealed little crosstalk between the signalling pathways directly (Cheng *et al.*, 2009), but ethylene regulates root growth by altering auxin transport and biosynthesis and several auxin transport mutants show reduced sensitivity to ABA in root length assays (Ruzicka *et al.*, 2007; Swarup *et al.*, 2007; Thole *et al.*, 2014).

Drought and ABA reduce *trans*-zeatin-type cytokinin levels by modulating expression of cytokinin biosynthetic/metabolic enzymes (Dobra *et al.*, 2010; Nishiyama *et al.*, 2011).

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Moreover, it is known that cytokinin can inhibit auxin biosynthesis (Nordstrom *et al.*, 2004) and promote ethylene biosynthesis (Vogel *et al.*, 1998; Stepanova *et al.*, 2007). Furthermore, ethylene promotes auxin biosynthesis (Ruzicka *et al.*, 2007; Stepanova *et al.*, 2007) and auxin can induce a rapid downregulation of cytokinin biosynthesis (Jones & Ljung, 2011; Nordstrom *et al.*, 2004). It is also known that ethylene and cytokinin concentrations, and expression of the associated regulatory and target genes, are interlinked (e.g. To *et al.*, 2004; Shi *et al.*, 2012). Therefore, the effects of osmotic stress on cytokinin biosynthesis will also interplay with auxin and ethylene signalling. In addition, cytokinin-deficient or -insensitive mutants display reduced ABA concentrations but increased ABA sensitivity, and drought induction of ABA biosynthesis has been shown to be similar to wildtype (Nishiyama *et al.*, 2011).

Therefore the metabolic and signalling responses of ABA, auxin, cytokinin and ethylene all play their roles in developmental changes effected by osmotic stress. Previously, we have constructed a network describing the interactions between auxin, ethylene, cytokinin and the POLARIS peptide (PLS) (required for correct auxin, ethylene and cytokinin signalling in Arabidopsis; Casson *et al*., 2002; Chilley *et al*., 2006), revealing a hormonal crosstalk circuit that regulates root growth (Liu *et al.*, 2010). This model has been expanded to include auxin transport via the PIN-FORMED (PIN) efflux transporters (Paponov *et al.*, 2005) and has been implemented into a spatiotemporal model, which can reproduce the patterning of various hormones and response genes (Liu *et al.*, 2013; Moore *et al.*, 2015). In brief, our previous research has shown that Arabidopsis root development and response under standard laboratory growth conditions involves a complex hormonal crosstalk network of overlapping interactions between auxin, ethylene and cytokinin (Liu *et al.*, 2010, 2013, 2014; Moore *et al.*, 2015). One of the important properties of hormonal crosstalk in root development is that a change in one signalling component leads to changes in other signalling components.

Therefore, in order to understand the roles of plant hormones in root development, one of the key questions to address is how hormone concentrations and the expression of associated regulatory and target genes are mutually related. For example, we investigated how the crosstalk between auxin, ethylene and cytokinin is established via the function of the *PLS* gene. We showed that crosstalk between hormones occurs in that, in the *pls* mutant, auxin levels are reduced, cytokinin level increased and ethylene remains

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approximately unchanged. Moreover, increasing the level of either ethylene or cytokinin inhibits *PLS* gene expression while increasing auxin levels promotes *PLS* gene expression (Casson *et al.*, 2002; Chilley *et al.*, 2006). This example clearly demonstrates that auxin, ethylene, cytokinin, and *PLS* gene functions are interrelated. Although we have previously demonstrated how Arabidopsis root development is regulated by hormonal pathways exhibiting crosstalk (Liu *et al.*, 2010; 2013; 2014; Moore *et al.*, 2015), the crosstalk network we previously developed does not include the effects of osmotic stress.

Here, we examine the effect of osmotic stress on ABA, cytokinin and ethylene responses and how they mediate auxin transport, distribution and root growth through effects on PIN proteins. We show that under osmotic stress, Arabidopsis seedlings display increased ABA responses, and demonstrate the effects on auxin transport to the primary root meristem through altered PIN1 levels.

We then use this information to construct a new network to integrate the effects of osmotic stress and ABA with auxin, ethylene and cytokinin. This network develops novel insights into how an integrated system of ABA, auxin, ethylene and cytokinin is formed due to the repression of ethylene effects by ABA to limit auxin accumulation in the meristem. This brings new understanding to the control of root development under stress.

MATERIALS AND METHODS

Plant material

Arabidopsis thaliana wildtype seeds were from lab stocks of the Columbia (Col-0) or C24 ecotypes, originally obtained from Lehle Seeds (Texas, USA). *polaris (pls)* mutant seeds were previously generated by GUS promoter trapping in the C24 background (Topping *et al.*, 1994; Topping & Lindsey, 1997). *proPLS::PLS:GFP* and *35S::PLS* seeds (*PLSox*) in Col-0 background were previously generated by floral dipping (Casson *et al.*, 2002).

pDR5rev::3xVENUS-N7 (Heisler *et al.*, 2005), *35S::DII-VENUS-N7* (Brunoud *et al.*, 2012) and *pTCS::GFP* (Muller & Sheen, 2008), all Col-0 background, were obtained from the Nottingham Arabidopsis Stock Centre (NASC).

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proAUX1::AUX1-YFP(116) was obtained courtesy of Dr. Ranjan Swarup (Nottingham University, UK).

proPIN1::PIN1::GFP (Benkova *et al.*, 2003), *proPIN2::PIN2::GFP* (Xu & Scheres, 2005) and *proPIN4::PIN4::GFP* (Vieten *et al.*, 2005) were obtained courtesy of Prof. Ben Scheres (Wageningen University, Netherlands). *proARR5::GFP* and *proARR5::GUS* (Ws background) were obtained courtesy of Prof. Joseph Kieber (University of North Carolina, USA).

proRGA::RGA::GFP (Silverstone *et al.*, 2001), Col-0 background was obtained courtesy of Dr. Ari Sadanandom (Durham University, UK).

Plant growth conditions

Seeds were sterilised for 30 s with 70% (v/v) ethanol and 10 min with 20% commercial bleach containing 0.1% Tween-20, then washed five times with sterile distilled water.

Seeds were placed on 10 cm round plates containing half strength Murashige and Skoog medium (Sigma, 2.2 g/l) with agar (Sigma, 5 g/l) and MES (Sigma, 6 mM, 1.2 g/l) and sealed with Micropore tape. To ensure simultaneous germination, seeds were stratified for 4-7 days at 4°C before transfer to a growth room (22°C, 18h photoperiod). Plates were orientated horizontally except for root length assays, when they were orientated vertically.

Five days after germination (DAG), seedlings were transferred to Poly(ethylene glycol) (PEG)-infused ½ MS agar plates with water potentials (ψ_w) of ca. -0.14, -0.37 or -1.2 MPa, adapted from Verslues *et al.*, (2006). The plates were sealed with Micropore tape and placed in a growth room.

Preparation of Polyethylene Glycol-infused plates

The method is adapted from (Verslues *et al.*, 2006). Essentially an overlay solution containing PEG is poured over ½ MS agar plates and PEG is allowed to diffuse into the medium.

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Both the agar medium and overlay solution contained ½ MS salts (Sigma, 2.2 g/l) and MES buffer (Sigma, 6mM, 1.2 g/l) and were adjusted to pH 5.7 by adding 0.1M KOH solution. High gel strength agar (Melford Laboratories, 5 g/l) was added to the base medium before autoclaving. No sucrose was used, as it affects ABA signalling and to minimise the chance of bacterial/fungal contamination. After autoclaving, PEG-8000 (Sigma) was added to the liquid overlay solutions depending on the desired osmotic pressure of the plate (0g/l for -0.14 MPa, 250g/l for -0.37 MPa, 550 g/l for -1.2 MPa). 40 ml of medium was poured onto 10 cm square plates and allowed to set, after which 60 ml of the appropriate overlay solution added. The plates were sealed with Parafilm, allowed to equilibrate for 15-24 h and the overlay solution removed before transferring seedlings and resealing with Micropore tape.

Medium water potentials were verified using a Wescor 5600 osmometer (ELITech, Berkhamsted, Herts., UK); the large sample chamber was used to allow direct measurements of solid medium. Osmolarity data were verified in 10 independent measurements for each treatment.

RNA extraction and cDNA synthesis

100 mg (approximately 30 seedlings at 5 or 6 DAG) were flash-frozen in liquid nitrogen. Seedlings were ground on dry ice whilst still frozen and RNA was extracted using a Sigma Spectrum Total RNA kit (Sigma Aldrich), and DNase digestion was performed with the Sigma On-column DNase kit (Sigma Aldrich). RNA concentration was determined with a Nanodrop ND1000 Spectrophotometer (ThermoFisher Scientific, Hemel Hempstead, UK).

5 ng of RNA in a 20 µl reaction mixture was used for cDNA synthesis, using the Invitrogen Superscript III First Strand Synthesis System (Invitrogen Ltd, Paisley, UK).

cDNA was diluted 1:4 for PCR and qPCR. cDNA was tested for genomic DNA contamination by PCR amplification of *ACT2*, using primers designed over an intron (Table S1). Samples contaminated with genomic DNA were treated with Promega RQ1 DNase, which was then denatured before the cDNA synthesised again.

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Quantitative real-time polymerase chain reaction (qPCR)

SYBR Green Jumpstart Taq Readymix (Sigma Aldrich) was used for with a Corbett Scientific Rotorgene Q (Qiagen, Manchester, UK).

Expression of each gene was calculated using the Rotorgene Q Series software v1.7, using the ΔΔCT method relative to expression of a paired reference gene amplification, according to the manufacturer's instructions. Amplification efficiencies of the genes of interest were checked to ensure they were all within 5% of the reference gene amplification efficiency. Melt curves were used to check for nonspecific/unwanted products and primer dimers. Stabilities of reference genes were verified by ΔΔCT comparison between all samples and the control. All sample amplifications were done in triplicate for technical repetition, with three biological replicates. AT5G15710 was selected as a reference gene, due to its stable expression pattern under osmotic stress, under hormone applications and at various developmental stages (Czechowski *et al.*, 2005). Primer sequences are listed in Table S1.

Compound light microscopy

After 6 days on media containing combinations of PEG and IAA, root tips were mounted in Hoyer's solution (Anderson, 1954) and imaged on a Zeiss Axioskop microscope (Carl Zeiss, Cambridge, UK), fitted with a Retiga 2000R camera (Photometrics, Marlow, UK) and using the 20X Neoflu lens and DIC. At least 3 roots of each treatment were imaged, and the representative images were compiled in GIMP 2.8 (www.gimp.org).

Confocal laser scanning microscopy

Before transferring to osmotic stress plates, plants were selected as being the same developmental stage and screened for fluorescence under a Leica stereo dissecting microscope with fluorescence (www.leica-microsystems.com). After 24 h osmotic treatment, roots were imaged. Whole seedlings were transferred to a propidium iodide solution (0.5 µg/mL) for 1.5 min and washed for the same time in deionised water. Root tips were then removed with a razor blade and transferred to a slide. Roots were imaged

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with a Leica SP5 laser scanning confocal microscope (www.leica-microsystems.com). Gain, line averaging, detection frequencies and other microscope settings were altered between fluorescent marker lines to optimise image quality, but not between roots of the same marker line, to ensure comparability. YFP was excited with the 514 nm band of the argon laser, GFP excited with the 488nm band of the argon laser and propidium iodide was excited at 548nm. Sequential scans were used and detection spectra were optimised to minimise crossover between different fluorophores.

Image Analysis

Meristem size determinations and cell counts were performed using ImageJ (www.imagej.nih.gov/ij/). Meristem size was assayed by measuring the distance along the cell file from the quiescent centre to the first cell that is double the length of the previous cell.

Mean relative fluorescence was calculated with ImageJ for PIN1:GFP, proARR5::GFP, pTCS::GFP and DII:VENUS. CellSet (Pound *et al.*, 2012) was used to measure PIN2:GFP and AUX1::YFP relative fluorescence. In quantifying fluorescence across replicate experiments (n = between 5 and 14, according to the experiment), individual dead cells were excluded to ensure data reflect hormonal outputs.

Statistical analysis

All statistical tests were performed in Microsoft Excel 2010, using the Real Statistics add in (http://www.real-statistics.com/). The 0.05 level of significance was used.

RESULTS

Osmotic stress inhibits primary root growth, modulated by ABA

---Figure 1 ---

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Osmotic stress was induced by growing seedlings on 1/2MS agar containing high molecular weight PEG (van der Weele *et al.*, 2000; Verslues *et al.*, 2006). This allowed us to examine the effects of osmotic stress independently of the ion stresses that mannitol/sorbitol/salt may cause or the mechanical impedance that can result from soil drying. Two stress treatments were chosen - a moderate stress (-0.37 MPa) and a severe stress (-1.2 MPa), both of which were verified using a vapour pressure osmometer (Figure 1a). Control plates lacking PEG were found to have an osmotic pressure of -0.14 MPa. Root cells are able to maintain a more negative water potential and cell turgor at moderate osmotic stress (-0.5 MPa) (Shabala & Lew, 2002) and root length assays demonstrated that plants were able to maintain at least some root growth under all three regimes.

As with previous studies, primary root growth was reduced under osmotic stress (Figure 1d), and lateral root number was also adversely affected (Figure S1, van der Weele *et al.*, 2000; Deak & Malamy, 2005).

The effect of osmotic stress on primary root length is known to be modulated by ABA (Xiong *et al.*, 2006). Low concentrations (0.1 μM) of exogenous ABA have a tendency to increase Arabidopsis primary root growth, whereas higher concentrations (>1 μM) inhibit growth (Figure 1d, Ghassemian *et al.* 2000). Inhibiting ABA biosynthesis with fluridon was found to rescue root elongation under moderate stress, suggesting that ABA is inhibiting root growth under stress.

Under osmotic stress, we observed a reduction in both meristem size and the number of cells in the primary root, which may be the cause of the reduction in growth (Figure 1 b,c). DELLA proteins such as RGA are inhibitors of growth and elongation, and are regulated by gibberellic acid, auxin, ethylene, ABA and stress, to modulate growth (Achard *et al.*, 2003; Fu & Harberd, 2003; Achard *et al.*, 2006). These proteins have been implicated in regulating meristem size and cell expansion in the elongation zone (Ubeda-Tomás *et al.*, 2008; Ubeda-Tomas *et al.*, 2009). To determine whether the effects of osmotic stress are mediated by signalling pathways rather than non-specific cell damage effects, we used DELLA expression as a marker of growth-related signalling changes in the root,by monitoring GFP:RGA expression in roots subjected to osmotic stress. As the root meristem becomes smaller with fewer cells as osmotic stress is increased, we found that GFP:RGA levels increased under stress (Figure 1 e,f). This evidence provides a link

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between osmotic stress and DELLA expression, and suggests that root growth is inhibited at the level of hormone signalling, rather than by a root elongation failure due to a lack of cell turgor or cell death.

ABA-dependent and ABA-independent stress responses increase under osmotic stress, but cytokinin signalling responses have limited change

To verify that ABA-dependent and ABA-independent drought stress responses were active under our experimental osmotic stress regime, qPCR was carried out to monitor the expression of the genes *RD29B* and *DREB2B*. *RD29B* expression is highly ABA-responsive but not responsive to ABA-independent signalling, whereas *DREB2B* is inducible as an early response to dehydration but not to ABA treatment (Nakashima *et al.*, 2000; Jia *et al.*, 2012).

RD29B expression shows a very large (ca. 100-fold) increase under moderate and severe osmotic stress at 6 and 24 h (Figure 2b). *DREB2B* expression increases significantly under severe stress at 6 h, but not under moderate stress, returning to near unstressed levels by 24 h (Figure 2c). These results show that both osmotic treatments elicited expression changes in stress response genes.

The cytokinin receptor mutant *ahk3* maintains root growth under drought stress and altering cytokinin signalling/levels has been shown to alter survival of plants under drought (Tran *et al.*, 2007; Werner *et al.*, 2010; Kumar & Verslues, 2015). Therefore we also examined cytokinin responses under our specific osmotic stress treatments.

ARABIDOPSIS RESPONSE REGULATOR 5 (ARR5) is a type-A negative regulator of cytokinin responses that displays increased expression under cytokinin treatment (Brandstatter & Kieber, 1998). Under osmotic stress treatment, we found that there was a small but statistically non-significant decrease in *ARR5* transcript abundance, although proARR5::GFP fluorescence decreased significantly (Figure 2 a,d,e). As *ARR5* expression can also be negatively regulated by ethylene, we also examined the responses of pTCS::GFP, a fluorescent protein under the control of a synthetic cytokinin responsive promoter (Muller & Sheen, 2008; Shi *et al.*, 2012). *pTCS::GFP* expression showed a downward trend, but no statistically significant change, in fluorescence under stress (Figure 2 a,f). Therefore under osmotic stress, Arabidopsis seedlings exhibit an increased

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ABA-dependent and -independent stress responses, and possibly a small reduction in cytokinin responses.

---Figure 2 ---

Inhibition of root growth under osmotic stress does not require ethylene signalling, but auxin can rescue root growth and meristem size

---Figure 3 ---

As both ethylene and auxin have been implicated in affecting survival and development under stress, we examined the growth responses of different ethylene and auxin mutants under osmotic stress. Auxin can either promote root growth by increasing meristem size, or reduce root growth by inhibiting expansion in the elongation zone (Dello Ioio *et al.*, 2008). Ethylene inhibits root growth by increasing auxin biosynthesis and basipetal auxin transport to the elongation zone via the efflux carrier PIN2 and influx carrier AUX1 (Ruzicka *et al.*, 2007; Swarup *et al.*, 2007).

EIN2 is required for ethylene responses (Guzman & Ecker, 1990) and AUX1 is required for auxin influx into cells (Swarup *et al.*, 2001; Yang *et al.*, 2006). *ein2*, *aux1-7* and *eir1-1/pin2* mutants display a similar reduction in primary root growth to wildtype under osmotic stress (Figure 3a, d). Supplementing growth medium with the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) was found to inhibit further wildtype root growth, regardless of stress (Figure 3b). This indicates that ethylene growth inhibition by basipetal auxin transport acts in a separate pathway to osmotic stress growth inhibition.

The reduction in root length however is not completely auxin-independent. Supplementing growth medium with a low concentration of auxin (1 nM indole-3-acetic acid, IAA) is mildly inhibitory to root growth (Evans *et al.* 1994). However, under moderate osmotic stress this concentration of auxin was found to rescue root growth, and can partially rescue root growth under severe stress, suggesting that root length may be modulated through auxin responses under stress (Figure 3c). This is supported by the observation that 1 nM IAA leads to a larger root meristem in roots subject to moderate and severe osmotic stress (Figure 3e). These observations are also consistent with the growth responses of other auxin and ethylene mutants examined (Figure S2). The *axr3-1*

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line has reduced sensitivity to auxin (Leyser *et al.*, 1996) and displays an exaggerated reduction in root growth and meristem size under osmotic stress (Figure 3d, S3). Other ethylene mutants such as *pls* and the *PLS* overexpressor line (PLSox) also display near wildtype responses to osmotic stress (Figure S2; Casson *et al.*, 2002; Chilley *et al.*, 2006).

---Figure 4 ---

Abiotic stresses, including osmotic stress and drought, can increase ethylene biosynthesis (Ichimura *et al.*, 2000; Spollen *et al.*, 2000; Joo *et al.*, 2008), and various stress responses such as compatible solute accumulation and regulation of leaf growth are dependent on ethylene signalling (Skirycz *et al.*, 2011; Cheng *et al.*, 2013; Cui *et al.*, 2015). To determine whether ethylene responses were altered under the osmotic stress conditions applied, we monitored expression of two genes associated with ethylene signalling, *ERF1* and *PLS*. *ERF1* expression is activated directly by ethylene signalling (Solano *et al.*, 1998). Its expression increases under many abiotic stresses, and it can bind to GCC and DRE promoter elements to activate stress responsive gene expression (Cheng *et al.*, 2013). Our results show a trend of increased level of *ERF1* expression under moderate stress (ANOVA P = 0.09; Figure 4a). *PLS* transcription has previously been shown to increase under auxin treatment and decrease in response to ACC treatment (Casson *et al.*, 2002; Chilley *et al.*, 2006). Under increasing osmotic stress, our results show a trend of a reduction in PLS:GFP fluorescence levels (Figure 4b,c), indicating lower auxin or higher ethylene signalling or both in stressed root tips.

---Figure 5---

Several papers have recently implicated a role for auxin in drought resistance and growth responses (Xu *et al.*, 2013; Shi *et al.*, 2014), but the precise role of auxin transport and distribution in these responses is unclear. Auxin increases meristem size, promoting growth, whilst cytokinin antagonises auxin signalling, reducing meristem size and increasing cell differentiation (Dello Ioio *et al.*, 2007; Dello Ioio *et al.*, 2008; Moubayidin *et al.*, 2010). ABA recently has been shown to act in coordination with ethylene and auxin to affect root growth, requiring basipetal auxin transporters PIN2 and AUX1 to inhibit root growth (Thole *et al.*, 2014). ABA decreases levels of *PLETHORA* (*PLT*) gene expression and levels of PIN1, PIN2 and AUX1 in a ROS dependent manner, and the

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ABA responsive transcription factor ABI4 has been shown to down-regulate PIN1 expression (Shkolnik-Inbar and Bar-Zvi, 2010; Yang *et al.*, 2014). Meristem size is reduced under osmotic stress, due to premature differentiation in an ABA-dependent manner (Ji & Li, 2014; Ji *et al.*, 2014). As our results showed no detectable increase in cytokinin signalling in response to osmotic stress (Figure 2), we hypothesised the reduction in meristem size may be due to altered auxin levels.

To examine the effect of osmotic stress on auxin distribution in the root, transgenic auxin biosensors and reporters were used. Under severe osmotic stress the fluorescence of the *DR5::YFP* auxin sensor, which is activated in the presence of auxin (Sabatini *et al.*, 1999; Heisler *et al.*, 2005), decreased under osmotic stress (Figure 5a), suggesting a reduced auxin response in the root tip. In agreement with this, *35S::DII:VENUS:N7*, which is rapidly degraded in the presence of auxin (Brunoud *et al.*, 2012), was found to increase significantly in root tips under osmotic stress, indicating a decrease in root tip auxin signalling (Figure 5a,b).

The auxin transporters PIN1 and PIN4 are localised to the membrane of the vascular tissues and root meristem respectively in *Arabidopsis* and funnel auxin from the stele into its concentration maxim around the quiescent centre and columella initials (Galweiler *et al.*, 1998; Friml *et al.*, 2002). We found that, following qPCR analysis, both *PIN1* and *PIN4* gene transcript abundances decreased under osmotic stress, with associated reductions in PIN1:GFP and PIN4:GFP fluorescence (Figure 5a,c). PIN1::GFP also showed reduced polarity under osmotic stress and accumulated in bodies similar to BFA bodies (Figure 5a, S4 ; Geldner *et al.*, 2001). This is consistent with work at the root apical meristem, where PIN1 internalisation has been reported under a mannitol induced loss of turgor (Nakayama et al., 2012)

Under moderate osmotic stress, an increase in *PIN2* transcript and fluorescent protein levels was observed, as found in previous studies (Figure 5a,c,d; Xu *et al.*, 2013). The auxin influx carrier AUX1, which is expressed in many of the same tissues as PIN2, showed decreased expression and fluorescence under osmotic stress (Figure 5a,c).

These results suggest that a reduced auxin response in the root tip under osmotic stress, seen as reduced DR5::YFP and increased DII:VENUS expression, may be the consequence of altered PIN protein expression to limit auxin supply and remove auxin

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from the root meristem. Given that exogenous auxin application can rescue root growth under stress (Figure 3), we investigated further the regulation of auxin accumulation and response in the root under osmotic stress.

PIN1 levels are reduced under stress in an ABA-dependent manner, overriding ethylene effects

Ethylene has been shown to increase, and ABA to repress, *PIN1* expression (Ruzicka *et al.*, 2007; Shkolnik-Inbar & Bar-Zvi, 2010; Liu *et al.*, 2013; Yang *et al.*, 2014). As both ethylene and ABA biosynthesis increase under stress, we therefore decided to examine *PIN1* expression in the context of osmotic stress and these two hormones.

We found that pharmacological treatment of seedlings with the ethylene precursor ACC shows a trend of increasing PIN1:GFP levels, and application of the ethylene perception inhibitor silver thiosulphate (STS) leads to a trend of decreasing PIN1:GFP levels ($P=0.037$), consistent with previous observations. However, as neither ACC nor STS treatment can rescue PIN1:GFP fluorescence under stress (Figure 6a,d), the changes in PIN1:GFP levels under stress appear to be regulated independently of ethylene signalling in these conditions.

---Figure 6---

In proPIN1::PIN1:GFP transgenic seedlings, treatment with ABA led to decreased fluorescence, and treatment with the ABA biosynthesis inhibitor fluridon led to an increased PIN1 fluorescence ($P<0.001$; Figure 6b,e), showing a downregulation of PIN1 fusion protein levels by ABA. proPIN1::PIN1:GFP fluorescence was also affected by osmotic stress ($P<0.0001$), with increasing stress reducing PIN1 levels (Figure 6b,e). Under moderate osmotic stress, fluridon treatment rescues PIN1 levels to untreated levels (Figure 6b,e), indicating a possible interaction between ABA signalling and osmotic stress to regulate PIN1 levels.

To determine whether ABA may therefore be overriding ethylene effects on PIN1 accumulation, the effects of combined hormone applications on PIN1::GFP fluorescence

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were determined. It was found that low concentrations (0.1-1 μM) of exogenous ABA had no significant effect on PIN1 levels, but higher concentrations (10 μM) reduced PIN1 levels significantly. Interestingly, it was also found that low exogenous concentrations of ABA were sufficient to suppress the high levels of PIN1:GFP fluorescence following ACC treatment to untreated levels, indicating ABA can override the effect of ACC on PIN1 levels (Figure 6c,f).

In summary, this experimental analysis shows that (1) ABA-dependent and ABA-independent stress responses increase under osmotic stress, but cytokinin responses are only slightly reduced; (2) inhibition of root growth under stress does not require ethylene signalling, but auxin can rescue root growth and meristem size; (3) osmotic stress modulates auxin transporter levels and localisation, reducing root auxin levels; (4) PIN1 levels are reduced under stress in an ABA-dependent manner, overriding ethylene effects; and (5) the interplay of the four hormones (ABA, auxin, cytokinin and ethylene) is tissue-specific. In particular, PIN1, which is expressed in the stele cells, and PIN2, which is expressed in the epidermis/cortex cells of the root, differentially respond to osmotic stress. Therefore, our experimental data indicate that an analysis of the regulation of root growth under osmotic stress requires a study of the interplay between ABA, auxin, ethylene and cytokinin as an integrative system.

Constructing hormonal crosstalk networks to formulate a systems view of the regulation of root growth by multiple hormones under osmotic stress conditions

To understand better the relationships between the signalling pathways studied under osmotic stress, we developed a network approach, based on our experimental data and evidence in the literature (Figure 7). The rationale for the network construction is described in Notes S1. Integration of available data reveals that ABA regulates root growth under osmotic stress conditions via an interacting hormonal network with cytokinin, ethylene and auxin. Although each hormone has its own signalling module to regulate its downstream gene expression, the signalling of four hormones (ABA, cytokinin, ethylene and auxin) exhibit interplay under osmotic stress conditions. The PIN auxin efflux carriers and influx carrier AUX1 also respond to osmotic stress, and therefore they play important roles in the interaction network. In addition, the interplay of

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the four hormones is tissue-specific. In particular, PIN1, which is localised in the stele cells, and PIN2, which is localised in the epidermis/cortex cells, respond differentially to osmotic stress (Figure 5). Therefore regulation of root growth under osmotic stress conditions must be elucidated as an integrative hormonal crosstalk system in a tissue-specific context.

---Figure 7 here--

We previously developed a hormonal interaction network for a single Arabidopsis cell by iteratively combining modelling with experimental analysis (Liu *et al.*, 2010). We described how such a network regulates auxin concentration in the Arabidopsis root, by controlling the relative contribution of auxin influx, biosynthesis and efflux; and by integrating auxin, ethylene and cytokinin signalling. Recently, we have developed this hormonal interaction network to include PIN1 or PIN2 activities in a single Arabidopsis cell (Liu *et al.*, 2013; Liu *et al.*, 2014), and subsequently moved on to study the spatiotemporal dynamics of hormonal crosstalk in a multi-cellular root structure (Moore *et al.*, 2015). Here we show that, after now incorporating ABA into the existing hormonal crosstalk network, a novel network for osmotic stress conditions can be constructed. Figure 7 describes how ABA, cytokinin, ethylene, auxin, PIN1 and AUX1 interplay in a single stele cell under osmotic stress conditions. Similarly, Figure S5 describes how ABA, cytokinin, ethylene, auxin, PIN2 and AUX1 interplay in a single epidermis/cortex cell under osmotic stress conditions.

The network reveals that under osmotic stress, due to the promotion of biosynthesis or signalling of both ABA and ethylene, expression of *RD29B* and *ERF1* increases since *RD29B* and *ERF1* expression is activated directly by ABA and ethylene signalling respectively (Solano *et al.*, 1998; Jia *et al.*, 2012). Increasing ethylene biosynthesis promotes auxin biosynthesis (Swarup *et al.*, 2001., 2007) that inhibits cytokinin biosynthesis (Nordstrom *et al.*, 2004). However, due to the overriding role of ABA over the regulation of PIN1 by ethylene (Figure 5), the regulation of PIN1 by auxin, ethylene and cytokinin is overridden by ABA under osmotic stress. Consequently, expression of PIN1 is lower under osmotic stress (Figure 5).

However, this overriding ABA effect is tissue-specific. In the epidermis/cortex, PIN2 expression increases under osmotic stress (Figure 5; Figure S5). The decreased PIN1 and

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increased PIN2 expression reduce auxin levels in the root tip under osmotic stress conditions and thus, DII:VENUS levels increase despite the potential for ethylene to increase auxin accumulation. Since auxin promotes and ethylene inhibits the expression of *PLS*, expression of *PLS* is lower under osmotic stress. Since PLS in turn promotes auxin accumulation in the root tip (Liu *et al.*, 2010; Liu *et al.*, 2013), a decreased *PLS* expression correspondingly reduces auxin levels, and this effect is in addition to the effects of a decreased PIN1 expression and an increased PIN2 expression under osmotic stress. A decreased *PLS* expression also enhances the ethylene pathway (Casson *et al.*, 2002; Chilley *et al.*, 2006), promoting *ERF1* expression.

In addition, osmotic stress may inhibit cytokinin biosynthesis (Dobra *et al.*, 2010; Nishiyama *et al.*, 2011). It is known that cytokinin can inhibit auxin biosynthesis (Nordstrom *et al.*, 2004) and promote ethylene biosynthesis (Vogel *et al.*, 1998; Stepanova *et al.*, 2007). Therefore, the effects of osmotic stress on cytokinin biosynthesis also interplay with auxin and ethylene signalling. Although exogenous application of ACC increases *AUX1* expression (Ruzicka *et al.*, 2007), *AUX1* showed decreased expression and protein fusion fluorescence under osmotic stress. This implies that the increased ABA biosynthesis under osmotic stress plays an important role in *AUX1* expression and fluorescence. This reduced *AUX1* expression and fluorescence also interplay with all components in the network (Figure 7) due to the effects on the auxin levels in the root tip.

This work shows that combining experimental analysis with network construction reveals that ABA regulates root growth under osmotic stress conditions via an interacting hormonal network with cytokinin, ethylene and auxin. One of the important properties of this hormonal crosstalk network under osmotic stress conditions is that a change in one signalling component leads to changes in other signalling components. Therefore, elucidating the regulation of root growth under osmotic stress conditions requires the study of multiple hormones as an integrated system.

DISCUSSION

The hormonal crosstalk networks that we have developed (Figures 7, 8, S5, S6) describe the actions of multiple hormones and the associated regulatory and target genes under

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osmotic stress conditions. They provide a means to integrate our experimental analysis with a variety of experimental data in the literature. Such networks formulate a systems view on the regulation of root growth by multiple hormones under osmotic stress conditions. Specifically, the causal regulatory relationships of auxin efflux and influx transporters, concentrations of four hormones (ABA, auxin, ethylene and cytokinin), signalling components we have experimentally measured, and osmotic stress can be understood as an integrative system, as summarised in Figure 8 and Figure S6. Figures 8 and S6 are the simplified descriptions of Figure 7 and S5, respectively. All these figures reveal the nonlinear and complex responses of auxin transporters, hormones and signalling components to osmotic stress.

---Figure 8---

Although ethylene-induced basipetal auxin transport in the root is required for ABA to limit root growth under unstressed conditions (Beaudoin *et al.*, 2000; Ghassemian *et al.*, 2000; Thole *et al.*, 2014), we have shown that osmotic stress limits root growth independently of this mechanism. We present the hypothesis that auxin transport to the root via PIN1 is limited under osmotic stress in an ABA-regulated manner, and together with enhanced PIN2 levels, leads to reduced auxin concentrations in the root meristem. Lower auxin levels lead to a reduction in meristem size and reduced root growth.

Cytokinin-deficient plants display increased ABA sensitivity, but cytokinin receptor mutants show increased root growth under stress (Nishiyama *et al.*, 2011; Kumar & Verslues, 2015). This would place cytokinin signalling downstream of ABA in regulating root growth under stress.

In Arabidopsis, the auxin:cytokinin ratio is critical in determining the rate of root growth. Cytokinin inhibits root growth by antagonising auxin, to modulate the rate of cell division and differentiation in the root apical meristem (Dello Ioio *et al.*, 2007,, 2008; Moubayidin *et al.*, 2010). As active cytokinin levels and cytokinin signalling are reduced under drought and osmotic stress (as indicated by the reduced expression of the cytokinin-sensitive proARR5::GFP reporter, Figure 2c-e; Dobra *et al.*, 2010; Nishiyama *et al.*, 2011), but the meristem is smaller, it seems likely that meristem size is primarily regulated by altered auxin responses in these conditions. In cytokinin receptor mutants, auxin sensitivity would be predicted to increase, making plants more resistant to root

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553 growth inhibition due to reduced auxin levels. The combination of increased ABA
554 sensitivity and enhanced root growth may account for the increase in drought stress
555 tolerance of cytokinin-deficient plants (Tran *et al.*, 2007; Werner *et al.*, 2010; Nishiyama
556 *et al.*, 2011).

557 Auxin application cannot completely rescue root growth under severe stress so factors
558 other than auxin-mediated regulation of meristem size may also be limiting growth. It is
559 possible that at higher stress levels, cells exhibit reduced expansion due to reduced water
560 availability, or that the high rate of programmed cell death is limiting growth (Duan *et*
561 *al.*, 2010). Under stress, plants must also divert significant resources to protective
562 measures such as compatible solute accumulation, *LATE-EMBRYOGENESIS-*
563 *ABUNDANT* (*LEA*) gene transcription and chaperone transcription so constitutively
564 drought tolerant plants often display dwarf phenotypes (Bray, 1997; Kasuga *et al.*, 1999).
565 It is possible that the balance of growth against protection may be playing a role here,
566 limiting root growth indirectly.

567 Construction of hormonal crosstalk networks (Figures 7, 8 ,S5 and S6) reveals multiple
568 layers of complexity in the regulation of root development by osmotic stress. One layer
569 of complexity is how hormone concentrations and the expression of their associated
570 regulatory and target genes are mutually related. Another layer of complexity is how the
571 interrelated hormones and gene expression quantitatively control root growth. Figures 7,
572 8 , S5 and S6 show that the responses of auxin transporters, hormones and signalling
573 components are linked via hormonal crosstalk networks. Therefore, a change in one
574 response may lead to changes in other responses, and understanding the effects of one
575 component (in Figures 7 , 8 , S5 and S6) requires consideration of how this component
576 affects all other components.

577 Experimentally, it has been shown that mutants in one PIN protein family member
578 change the level or localisation of other remaining PIN proteins under non-stressed
579 growth conditions (Blilou *et al*., 2005). It has also been shown that *pin1* and *pin2* single
580 mutants only display a moderate reduction of root length and root meristem size (Blilou
581 *et al.*, 2005). Our data here show that, under osmotic stress, PIN1 expression decreases
582 and PIN2 expression increases. The decreased PIN1 and increased PIN2 expression work
583 together to reduce auxin levels in the root tip. This example shows that change in auxin
584 level cannot be attributed the function of an individual PIN protein under osmotic stress.

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Although data in Figures 7 , 8 , S5 and S6 show how PIN1 and PIN2 link with ABA, auxin, ethylene and cytokinin under osmotic stress, the hormonal crosstalk for other PIN proteins currently cannot be established. This is because there is insufficient biological knowledge to establish the hormonal crosstalk for other PINs even if no osmotic stress exists.

Furthermore, in order to quantitatively link a mutant gene with root length or root meristem size control, any changes in the level of all relevant hormones must be quantitatively analysed. This is because ABA, auxin, ethylene and cytokinin are involved in root development and a mutant may change all or some of the four hormones to some extent. For example, establishment of a quantitative relationship between the *pin2* mutant and root length needs not only to establish a mathematical model for studying how the *pin2* mutant quantitatively affects other transporters and all four hormones via hormonal crosstalk networks (Figures 7, 8, S5 and S6), but also to establish the quantitative relationship between all hormones and root length by combining both experimental and modelling analysis. However, this is beyond the context of the current work.

The network we have constructed provides new insight into the interactions of phytohormones and how they regulate growth under stress. Based on experimental results (Nordstrom *et al*., 2004), our hormonal crosstalk networks (Figures 7, 8, S5, and S6; Liu *et al*., 2013) describe a negative regulation of auxin biosynthesis by cytokinin. However, Jones *et al*. (2010) have shown that cytokinin positively regulates auxin biosynthesis in young developing tissues (10 DAG). In previous work, our hormonal crosstalk network analysis revealed that both sets of experimental results (Nordstrom *et al.*, 2004; Jones *et al.*, 2010) can be incorporated into the hormonal crosstalk network, leading to the same conclusions about other regulatory relationships of hormonal crosstalk (Liu *et al.*, 2013). Hormonal crosstalk networks can also be constructed for the case where a positive regulation of auxin biosynthesis by cytokinin is described with all other regulatory relationships remaining unchanged.

As we have demonstrated, the network can be used to investigate how an integrated system of ABA, auxin, ethylene and cytokinin is formed under osmotic stress, due to the repression of ethylene effects by ABA via the enhanced transport of auxin away from the meristem and to the elongation zone. Recently, we have shown that spatiotemporal modelling of hormonal crosstalk can simulate and explain the level and patterning of

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hormones and gene expression in Arabidopsis wildtype and mutant roots (Moore *et al.*, 2015). However, that hormonal crosstalk does not include the effects of osmotic stress. Therefore, the novel hormonal crosstalk network developed in the current work provides a framework for spatiotemporal modelling of hormonal crosstalk under osmotic stress conditions, and will allow us to analyse how the patterning of multiple hormones regulate root development under osmotic stress. In particular, this will allow us to examine the mechanisms by which ABA could override ethylene induction of *PIN1* gene expression, whilst still allowing *PIN2* expression to increase.

The hormonal crosstalk network developed in this work will also allow us to further interrogate interactions with other growth-regulating hormones such as the GA/DELLA system. DELLA proteins are degraded as part of the GA signalling pathway and are viewed as master regulators of plant growth (Dill *et al.*, 2001). Levels of the DELLA protein RGA increase under osmotic stress (Figure 1) and ABA has previously been shown to increase RGA stability (Achard *et al.*, 2006). High DELLA levels can reduce cell proliferation and the rate of differentiation to regulate meristem size (Ubeda-Tomás *et al.*, 2008, 2009; Achard *et al.*, 2009). Several models already exist detailing how the GA signalling cascade is regulated by negative feedback loops and how hormone dilution can explain the cessation of cell expansion in the elongation zone (Band *et al.*, 2012; Middleton *et al.*, 2012). By further integrating other hormones into the network, we should in future be able to elucidate how ABA, cytokinin, ethylene, auxin and other hormones such as GA regulate root growth under osmotic stress.

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Figure legends

Figure 1. Experimental setup shows that osmotic stress (OS) leads to reduced root growth, smaller meristem with fewer cells, and that ABA modulates root growth under stress, via increased DELLA. a) Medium osmolarity of PEG-infused agar, measured with a vapour pressure osmometer 24 h after overlay solution is removed. n=10. b) Primary root meristems stained with propidium iodide after 24 h osmotic stress treatment. Arrowheads indicate quiescent centre and approximate end of the meristematic zone. c) Meristematic cell count (ANOVA P=0.002) and meristem size (ANOVA P=0.04) after 24 h osmotic stress treatment. d) The effect of ABA and the ABA biosynthesis inhibitor fluoridon on root growth under osmotic stress (treatment period: 5-7 DAG). Ln transformed 2 factor ANOVA P (stress) < 0.0001, P (hormone) < 0.0001, P (interaction) =0.0049. Blue diamonds: no hormone; black triangles: 0.1 µM ABA, yellow circles: 1 µM ABA; red squares: 0.1 µM Fluoridon. e) proRGA::GFP:RGA under osmotic stress. f) GFP:RGA fluorescence under osmotic stress. Measured in ImageJ, ANOVA P=0.015. U: Unstressed (-0.14 MPa), M: Moderate stress (-0.37 MPa), S: Severe stress (-1.2 MPa). For confocal images, scale bars indicate 50 µm. Error bars indicate S.E.M. Letters indicate significance with a Tukey Pairwise comparison.

Figure 2. ABA-responsive genes (e.g. *RD29B*) and ABA-independent stress genes (e.g. *DREB2B*) are up-regulated by OS; cytokinin response genes (*ARR5*, *TCS*) may go down slightly. a) proARR5::GFP (top panels) and pTCS::GFP (bottom panels) after 24 h osmotic stress treatment. b) *RD29B* expression under osmotic stress. Ln transformed 2 factor ANOVA: P (stress) < 0.0001, P (time) = 0.42, P (interaction) = 0.15 Red: six hour treatment. Blue: 24 h treatment. c) *DREB2B* expression under osmotic stress. 2 factor ANOVA P (stress) = 0.0014, P (time) = 0.0014, P (interaction) = 0.3, Red: six hour treatment. Blue: 24 h treatment. d) *pARR5::GFP* fluorescence after 24h osmotic stress treatment. Measured in ImageJ, ANOVA P=0.0015. e) *ARR5* transcript abundance. f) pTCS::GFP fluorescence after 24 h osmotic stress treatment, measured in imageJ. ANOVA P=0.44. U: Unstressed (-0.14 MPa), M: Moderate stress (-0.37 MPa), S: Severe stress (-1.2 MPa). For confocal images, scale bars indicate 50 µm. Error bars indicate S.E.M. Letters indicate significance with a Tukey Pairwise comparison,

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Figure 3. Auxin and ethylene regulation of root length under osmotic stress. a) Root growth of Col-0 (blue diamonds) and the ethylene insensitive mutant *ein2* (red squares) under osmotic stress (treatment: 5-8 DAG) Ln transformed 2 factor ANOVA P (stress) < 0.0001, P (mutant) = 0.71, P (interaction) = 0.063 b) The effect of 1-aminocyclopropane-1-carboxylic acid (ACC, black circles) and silver thiosulphate (STS, red squares) on root growth under osmotic stress, in Col-0 (treatment: 5-11 DAG). Blue diamonds: no hormone treatment. 2 factor ANOVA P (stress) < 0.0001, P (hormone) < 0.0001, P (interaction) = 0.12 c) The effect of indole-3-acetic acid (IAA) on root growth under osmotic stress. Blue diamonds: no hormone treatment, black circles: 0.1nM IAA, red squares 1nM IAA. Treatment: 5-11 DAG. Ln transformed 2 factor ANOVA, P (hormone) =0.43, P (stress) <0.0001, P (interaction) = 0.036 d) The effect of osmotic stress on root growth on wildtype (Col-0: blue triangles), auxin transport mutants (*eir1-1/pin2*: red squares and *aux1-7*: black circles) and an auxin resistant mutant (*axr3-1*: yellow triangles). Ln transformed 2-factor ANOVA P (stress) <0.0001, P (mutant) <0.0001, P (interaction) <0.0001. Treatment: 5-8 DAG. Error bars indicate S.E.M. e) Root meristems under combined IAA and osmotic stress treatments (5-11 DAG). Arrowheads indicate the position of the quiescent centre and the end of the meristematic zone. U: Unstressed (-0.14 MPa), M: Moderate stress (-0.37 MPa), S: Severe stress (-1.2 MPa).

Figure 4. Ethylene response to osmotic stress. Osmotic stress causes an increased ethylene response, seen as increased expression of ethylene responsive genes (eg *ERF1*) and suppression of genes down-regulated by ethylene such as *PLS*. a) Relative transcript abundance of *ERF1* after 24 h osmotic stress treatment. ANOVA P=0.09. b) Relative fluorescence of proPLS::PLS:GFP after 24 h osmotic stress treatment. ANOVA P=0.23 c) proPLS::PLS:GFP after 24 h osmotic stress treatment Green: GFP, Magenta: propidium iodide. Error bars indicate S.E.M. Scale bars indicate 50µm. U: Unstressed (-0.14 MPa), M: Moderate stress (-0.37 MPa), S: Severe stress (-1.2 MPa).

Figure 5. Response of auxin transport and responses to osmotic stress. Osmotic stress modulates auxin transporter levels, reducing root auxin levels. a) pDR5rev::3xVENUS-N7, 35S::DII:VENUS-N7, proPIN4::PIN4:GFP, proPIN1::PIN1:GFP, proPIN2::PIN2:GFP and proAUX1::AUX1:YFP after 24 h osmotic stress treatment. b) DII:VENUS fluorescence under osmotic stress. ANOVA P= 0.003. c) Auxin transporter relative expression under osmotic stress. ANOVA PIN1 P=0.05, PIN4 P=0.05 PIN2

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P=0.33, AUX1 P= 0.05. d) proPIN2::PIN2:GFP fluorescence under osmotic stress ANOVA P=0.003. Letters indicate significance with a Tukey's pairwise comparison. Green:GFP/YFP, Magenta: propidium iodide. Scale bars indicate 50 µm. U: Unstressed (-0.14 MPa), M: Moderate stress (-0.37 MPa), S: Severe stress (-1.2 MPa).

Figure 6. Relationship between osmotic stress, ABA and auxin/ethylene. ABA application reduces PIN1 expression further to osmotic stress, and overrides the effect of ethylene in increasing PIN1 levels, indicating that ABA suppresses the ethylene response in the root. a) proPIN1::PIN1:GFP under osmotic stress with either the ethylene precursor ACC or the perception inhibitor STS. Green: GFP. b) proPIN1::PIN1:GFP under osmotic stress with either ABA or the biosynthesis inhibitor fluoridon. Green: GFP. c) proPIN1::PIN1:GFP under combined ACC and ABA treatment. d) proPIN1::PIN1:GFP fluorescence under osmotic stress treatment with either: no hormone (blue bars), 1 µM ACC (red bars) or 10 µM STS (green bars). e) proPIN1::PIN1:GFP fluorescence under osmotic stress treatment with either: no hormone (blue bars), 1 µM ABA (red bars) or 1 µM fluridon (green bars). f) proPIN1::PIN1:GFP fluorescence under combined ABA and ACC treatment. Error bars indicate S.E.M. U: Unstressed (-0.14 MPa), M: Moderate stress (-0.37 MPa), S: Severe stress (-1.2 MPa).

Figure 7. A hormonal crosstalk network for the regulation of root growth under osmotic stress conditions, in a vascular cell expressing PIN1, revealing that ABA regulates root growth under osmotic stress conditions via an interacting hormonal network with cytokinin, ethylene and auxin. **Symbols: Auxin:** auxin, **Ra**: inactive auxin receptor, **Ra*** active auxin receptor, **DR5m**: *DR5* regulated *YFP* mRNA transcript, **DR5p**: *DR5* regulated YFP protein, **DIIp**: DII-VENUS protein, **PIN1m**: *PIN1* mRNA transcript, **PIN1p**: PIN1 transporter protein, **AUX1m**: AUX1 mRNA transcript, **AUX1p**: AUX1 transporter, **PLSm**: *POLARIS* mRNA transcript, **PLSp**: POLARIS peptide, **ET:** ethylene, **Re**: inactive ethylene receptor, **Re***: active ethylene receptor, **CTR1**: inactive CTR1 kinase, **CTR1*** active CTR1 kinase, **X** : the unknown factor that regulates auxin transport from the aerial tissues, **ERF1m**: *ERF1* mRNA transcript, **ABA**: abscisic acid, **Raba**: inactive abscisic acid receptor, **Raba***: active abscisic acid receptor, **RD29Bm**: *RD29B* mRNA transcript **CK**: Active cytokinin, **Rck**: inactive cytokinin receptor, **Rck*:** active cytokinin receptor **ARR5m**: *ARR5* mRNA transcript, **ARR5p**: ARR5 protein, **Osmotic stress**: The osmotic stress imposed by the growth medium.

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Figure 8. A simplified representation of the hormonal crosstalk network for the regulation of root growth under osmotic stress conditions, in a vascular cell expressing PIN1, demonstrating that the responses of auxin transporters, hormones and signalling components to osmotic stress are nonlinear and complex. **Symbols: Auxin:** auxin, **Ra**: inactive auxin receptor, **Ra*** active auxin receptor, **DR5m**: *DR5* regulated *YFP* mRNA transcript, **DR5p**: *DR5* regulated YFP protein, **DIIp**: DII-VENUS protein, **PIN2m**: *PIN2* mRNA transcript, **PIN2p**: PIN2 transporter protein, **AUX1m**: AUX1 mRNA transcript, **AUX1p**: AUX1 transporter, **PLSm**: *POLARIS* mRNA transcript, **PLSp**: POLARIS peptide, **ET:** ethylene, **Re**: inactive ethylene receptor, **Re***: active ethylene receptor, **CTR1**: inactive CTR1 kinase, **CTR1*** active CTR1 kinase, **X** : the unknown factor that regulates auxin transport from the aerial tissues, **ERF1m**: *ERF1* mRNA transcript, **ABA**: abscisic acid, **Raba**: inactive abscisic acid receptor, **Raba***: active abscisic acid receptor, **RD29Bm** : *RD29B* mRNA transcript, **CK**: Active cytokinin, **Rck**: inactive cytokinin receptor, **Rck*:** active cytokinin receptor **ARR5m**: *ARR5* mRNA transcript, **ARR5p**: ARR5 protein, **Osmotic stress**: The osmotic stress imposed by the growth medium

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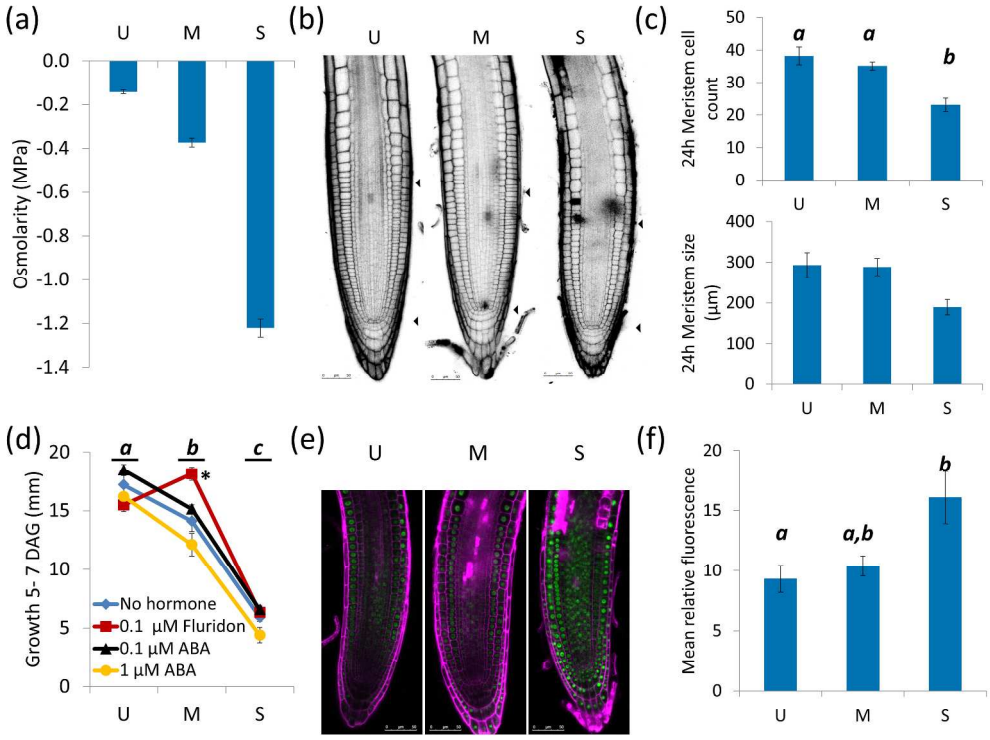

Revised Fig. 1
300x229mm (260 x 260 DPI)

Review

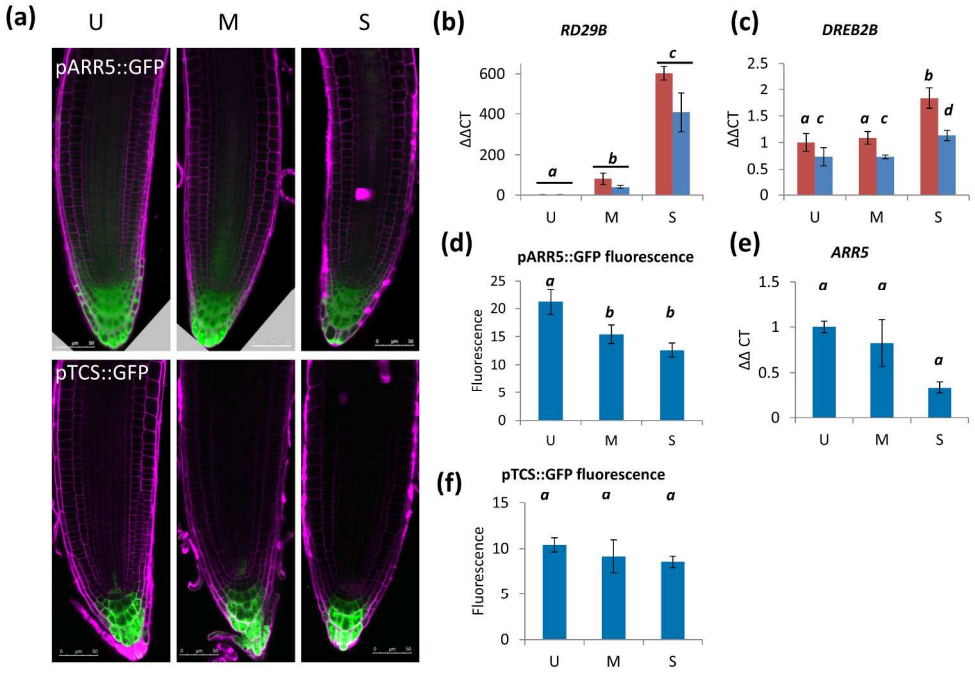

Fig. 2
249x176mm (300 x 300 DPI)

Review

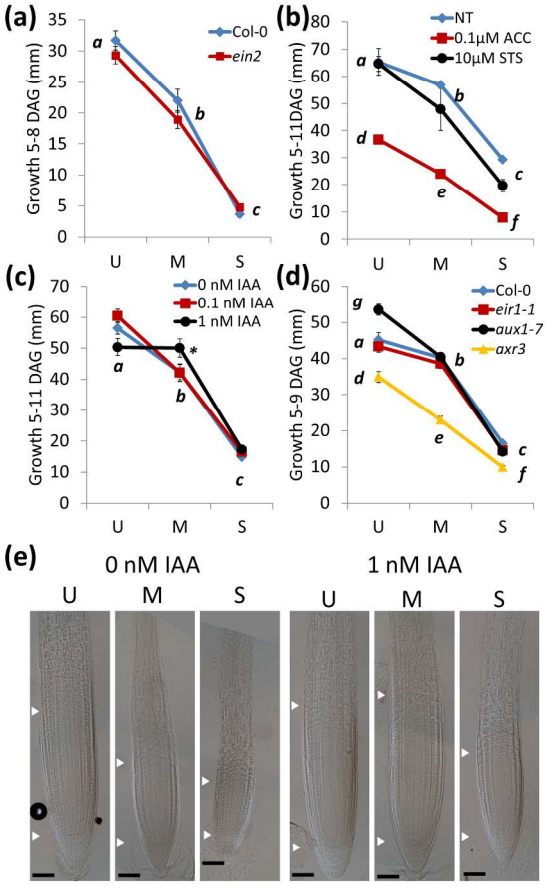

Fig. 3
299x400mm (195 x 195 DPI)

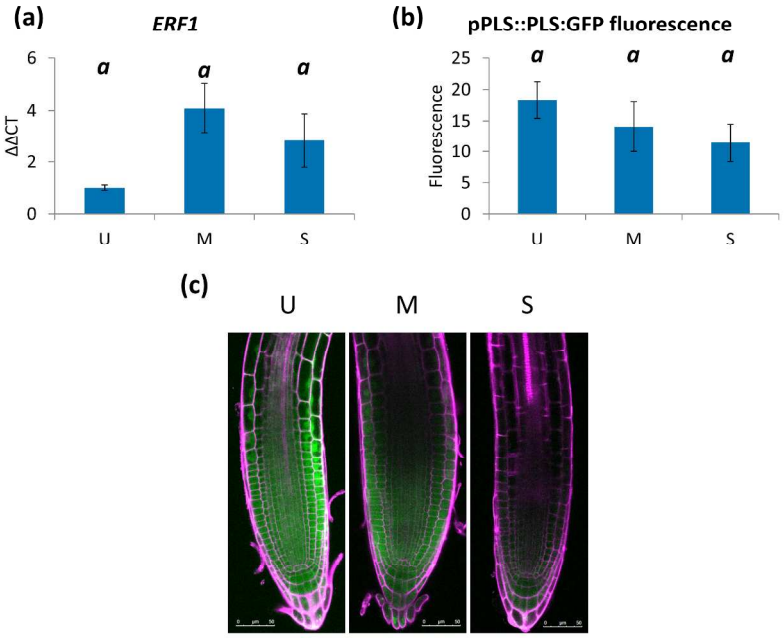

Fig. 4
254x190mm (300 x 300 DPI)

Review

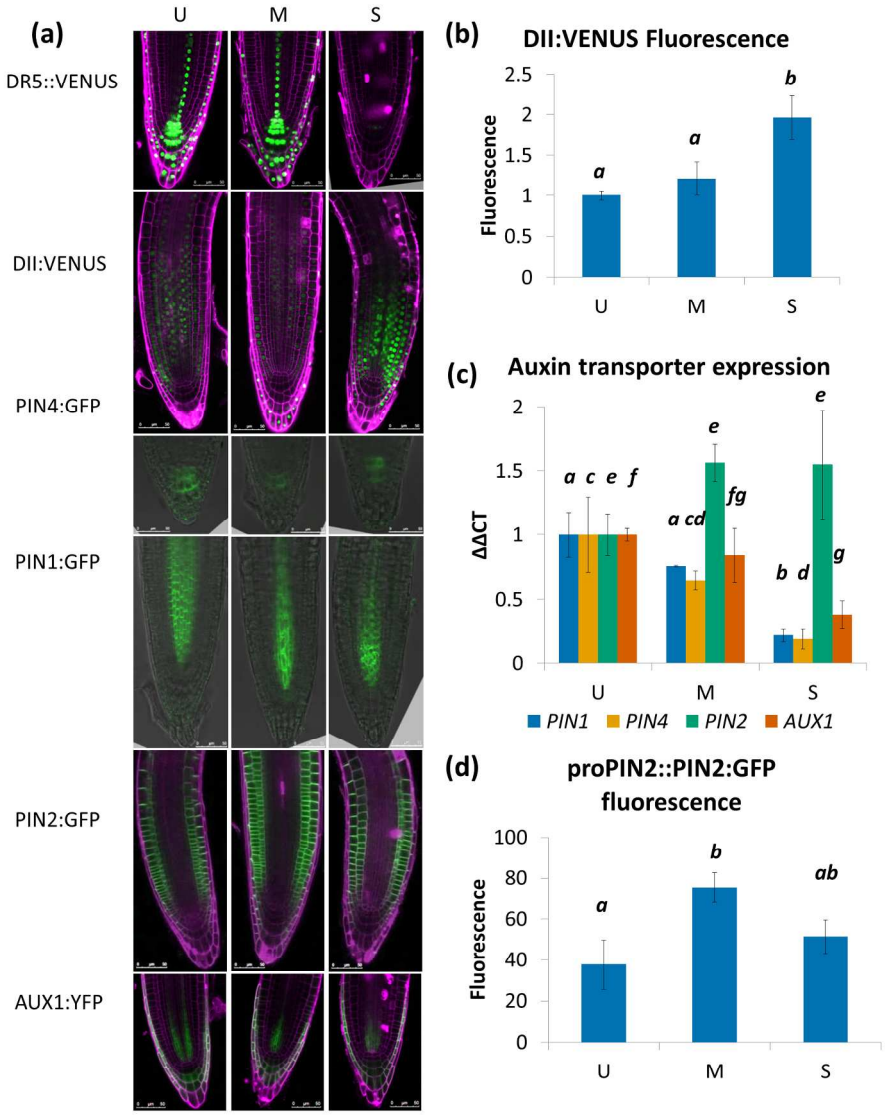

Fig. 5
355x451mm (156 x 156 DPI)

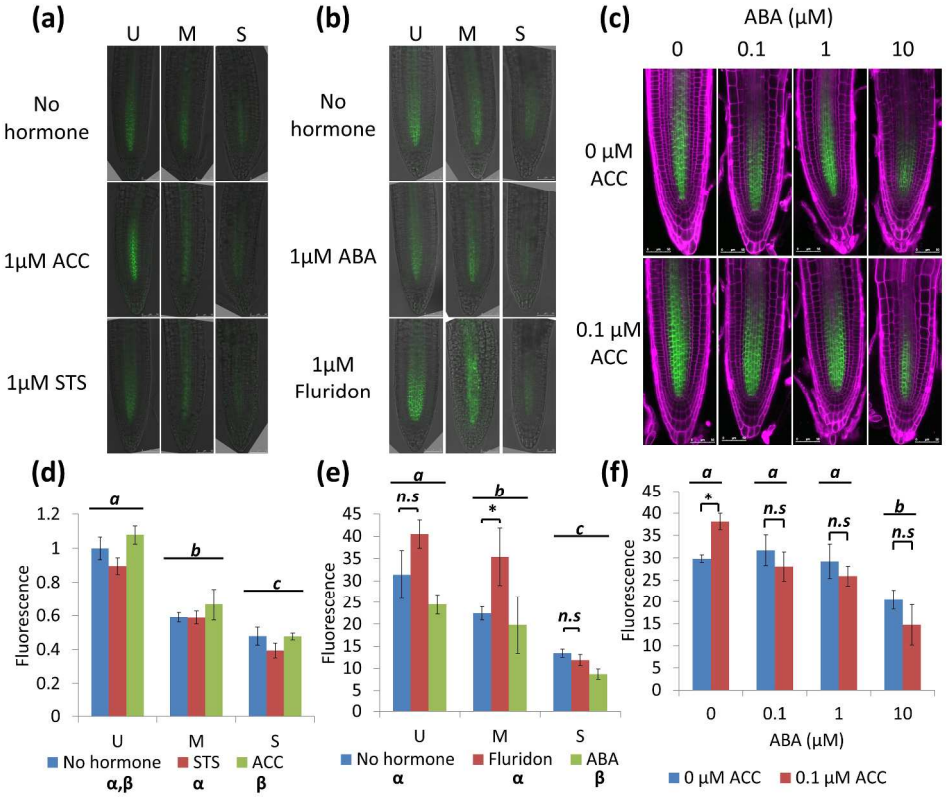

Fig. 6
369x299mm (211 x 211 DPI)

For Review

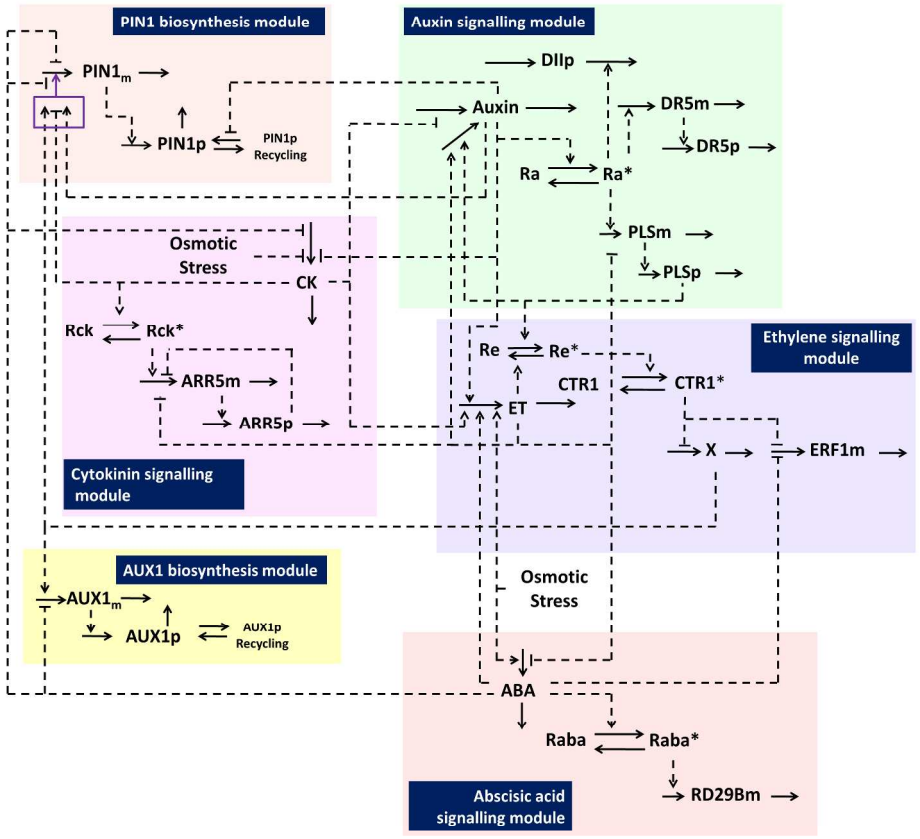

Fig. 7 revised
359x359mm (217 x 217 DPI)

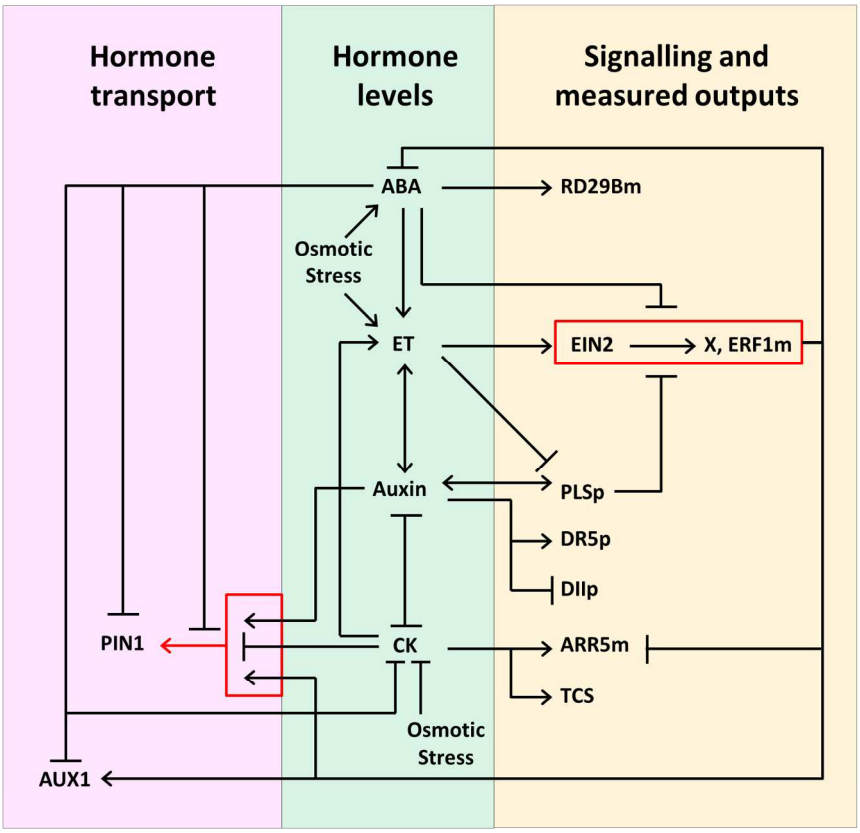

Fig. 8
308x294mm (156 x 156 DPI)