Root responses to mechanical impedance and the role of ethylene signalling.

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Abstract

Plant roots encounter a number of physical stresses in the soil and must be able to respond their growth appropriately. One such stress is mechanical impedance, which becomes an increasing problem in drying soils as soil strength increases with decreasing water content. In addition, the use of larger, heavier farming machinery leads to soil compaction, further increasing soil strength. Mechanical impedance has previously been shown to reduce root elongation and may have a negative impact on crop yields. It is therefore important to understand how root development is affected and growth regulated in response to mechanical impedance.

This thesis investigates the effect of mechanical impedance on root growth of *Arabidopsis thalina* and focuses on the role of the plant hormone ethylene in mediating this response. In particular the role of ethylene signalling in mediating root growth via crosstalk with auxin is examined. In addition the involvement of other plant hormones such as ABA, cytokinin and gibberellin is also briefly investigated. Experiments were carried out using a previously developed method whereby seedlings grown on horizontally orientated, dialysis membrane covered agar experience sufficient mechanical impedance to induce a response.

Mechanically impeded roots exhibited a characteristic ethylene response, with decreased primary root growth, increased diameter and root hair growth occurring closer to the tip. Analysis of mutants with altered responses to ethylene and auxin, and the effect of inhibitors of ethylene signalling and auxin demonstrated that both correct ethylene signalling and auxin transport are required for a mechanical impedance response. Confocal microscopy demonstrated that under mechanical impedance, auxin is redistributed at the root tip with increases in the expression of the transporters PIN1 and PIN2. ABA signalling is not required for a response to mechanical impedance and cytokinin responses appear to be reduced.

Contents

Declaration and statement of copyright

I declare that the work presented in this thesis is the result of my own work. No part of this thesis has previously been submitted for a higher degree.

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1. Introduction

Plant roots are able to respond to a range of environmental cues and rely on flexible growth to adapt to any stressful conditions they encounter. Root growth is therefore important for maintaining crop yields and root growth traits are of great interest to plant breeders (Gewin, 2010). Physical stresses in the soil limit root elongation. These include insufficient water or oxygen and mechanical impedance (Bengough et al., 2006). As plant roots are required to navigate through barriers in the soil, their roots must be able to respond to mechanical impedance. In addition there is a strong interaction between soil strength and water content, meaning mechanical impedance becomes an increasing problem in drying soils (Whalley et al., 2005; Jin et al., 2013). Soils dry as a result of the evapo-transpirational demand from the crop canopy, causing soil water content to decrease and strength to increase. This effect is exacerbated by increased soil compaction through the use of larger and heavier farming machinery (Jin et al., 2013).

Soil strength is typically measured using penetrometer resistance. This is a measure equal to the force needed to push a metal cone through the soil divided by the cross sectional area (Bengough et al., 2011). Penetrometer resistance has been shown to correlate with root elongation with a penetrometer resistance of 2MPa used as an indicator for the soil strength at which mechanical impedance limits root growth (Whitmore and Whalley, 2009; Bengough et al., 2011). Such levels of soil strength can occur even in relatively moist soils. Mechanical impedance has been shown to limit root growth in soils as wet as -100kPa (Whalley et al., 2005).

Early studies have also shown that mechanical impedance in the soil environment affects leaf growth due to signalling between the root and shoot. In hard soils, leaf expansion decreases (Masle and Passioura, 1987; Young et al., 1997). There is

therefore an agronomic relevance to understanding the response of roots to mechanical impedance, as the effects of soil drying and compaction can result in decreased crop yields (Whalley et al., 2008).

Due to its importance in agriculture, most studies investigating root elongation in relation to mechanical stress have focused predominantly on crop species. Early studies in particular focused on maize as it is experimentally convenient and an important worldwide crop (Bengough et al., 2011). There have been comparatively fewer studies that have investigated the response of *Arabidopsis thaliana* roots to mechanical impedance. By studying the response of *Arabidopsis* roots, more details about the molecular basis of root responses to mechanical impedance can be determined.

1.1 The structure of the *Arabidopsis* root and its use as a model system

Arabidopsis thaliana is widely used in plant research as the model organism and standard reference for plant biology and was originally adopted due to its usefulness for genetic experiments. Important features that make *Arabidopsis* a useful experimental system include its small size and short generation time, making it easy to grow, as well as its ability to self-pollinate and produce a lot of seed (Koornneef and Meinke, 2010). In addition *Arabidopsis* has a small genome and was the first plant genome to be sequenced (Arabidopsis Genome Initiative, 2000). The root of *Arabidopsis* has a largely fixed cellular organisation and is amenable to experimental manipulation, making it a useful model for studying developmental processes such as patterning and hormone responses (Scheres and Wolkenfelt, 1998).

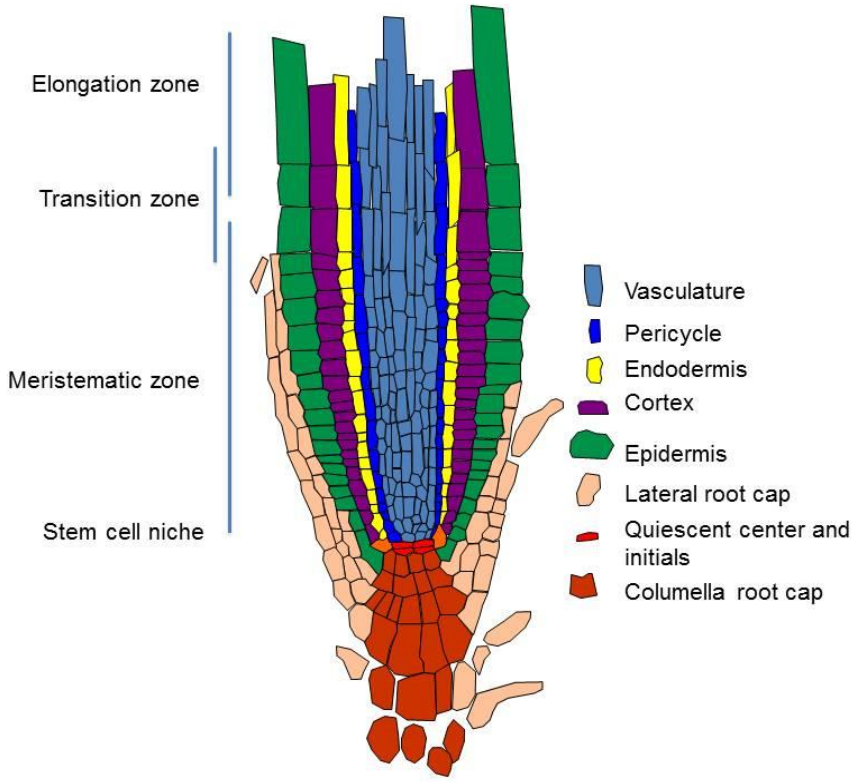

Figure 1. **Structure of the *Arabidopsis* primary root tip, taken from (Jaillais and Chory, 2010).**
The *Arabidopsis* root has a highly ordered structure made up of concentric rings of cell files (epidermis, cortex, endodermis, pericycle and vasculature). Root tissue is derived from stem cells in the apical meristem. Cells divide first in the meristematic zone before passing through the transition zone into the elongation zone.

The *Arabidopsis* root displays a radial organisation, with concentric rings of cell files that are easily recognizable by their morphology (Figure 1). From the outside to the inside, these are made up of the epidermis, cortex, endodermis, pericycle and finally central vascular tissue (phloem and xylem). At the root tip an additional cell layer above the epidermis forms the lateral root cap (Dolan et al., 1993). The root tip can be divided into distinct developmental zones displaying different cellular behaviours. The root apical meristem (RAM) generates the primary root and is the zone of cell division. The RAM is surrounded by a protective cell layer made up of the columella and lateral root cap. In the elongation zone the rate of division decreases and cells undergo rapid elongation. Finally in the differentiation zone elongated cells mature and begin to form root hairs (Dolan et al., 1993).

Root tissue is derived from stem cells in the RAM, with specific initials giving rise to the different cell files. These stem cell initials are arranged adjacently to the quiescent centre (QC), a region of four cells with a low frequency of division that is essential for the maintenance of undifferentiated initials (Dolan et al., 1993; van den Berg et al., 1997). Together they form the stem cell niche. The epidermis, cortex, endodermis and stele are derived from initials on the shootward and lateral sides of the QC. They undergo a process of division and elongation at the root tip before reaching maturation. Repetition of this process forms the basis of primary root growth (Petricka et al., 2012).

1.2 Mechanisms of root elongation and the effect of mechanical impedance

Roots elongate through a process of cell division and expansion. Active cell division occurs in the meristem before passing through the transition zone into the elongation zone, where they undergo rapid elongation (Ubeda-Tomas et al., 2012). Here the cells expand until they reach a mature cell length. It is generally believed that cell expansion is driven by water influx into the cell generating a turgor pressure. Cell turgor pressure therefore generates growth pressure, which is equal to the soil pressure that opposes root elongation (Jin et al., 2013).

Root elongation has been shown to decrease in strong soils in a number of crop species. For example increasing soil strength (penetrometer resistance) resulted in a decrease in root elongation in peanuts and cotton (Taylor and Ratliff, 1968), maize (Bengough and Mullins, 1991), pea (Croser et al., 1999; Iijima and Kato, 2007) and tobacco (Alameda et al. 2012). In a study by Croser et al. (1999 and 2000) roots were subjected to mechanical stress by being grown in compressed sand. Under mechanical impedance cell length was reduced and the length of the elongation zone shortened.

The slower rate of elongation in mechanically impeded roots is therefore likely due to a reduced rate of axial cell elongation and production (Croser et al., 2000; Jin et al., 2013). Cell wall tension in the axial direction opposes root elongation. In response to mechanical impedance tension is increased by the stiffening of cell walls in the elongation zone and with a corresponding shortening of the elongation zone (Bengough et al., 2006).

1.3 Effects of mechanical impedance on root morphology and physiology

1.3.1 Root thickening

As well as reducing root elongation, mechanical impedance has other impacts on root morphology and physiology. It has been widely reported that strong soils induce thickening in the root (Clark et al., 2002; Hanbury and Atwell, 2005; Jin et al., 2013). Comparisons suggest that root thickening facilitates root penetration in hard soils and better maintains root elongation (Bengough et al., 2011). It is thought root thickening enables the reduction of axial stress at the tips of roots (Hettiaratchi, 1990; Jin et al., 2013). This is consistent with the observation that root elongation is insensitive to radial pressure (Kolb et al., 2012) but can be very sensitive to axial pressure (Bengough et al., 2012).

1.3.2 Root hair growth

Changes in root hair growth also appear to play a role in the root's response to mechanical impedance. Root hairs are likely to be involved in anchorage of the root tip to allow expanding tissue to advance into the new soil (Bengough et al., 2011). It has been shown that root hairs proliferate closer to the root tip of mechanically impeded barley roots (Goss and Russel, 1980). In *Arabidopsis* it appears that root hairs elongate only when cell elongation has ceased (Bengough et al., 2010).

Although root hairs do not contribute to pull-out resistance of the root, it was hypothesised early that they may contribute to anchorage (Stolzy and Barley, 1968; Bailey et al., 2002). Root hair growth in *Arabidopsis* has been shown to be affected by mechanical impedance, with root hair growth occurring closer to the root tip (Okamoto et al., 2008).

1.3.3 The root cap

Peak stress occurs at the point adjacent to the apex of the root cap and the root cap plays an important role in determining mechanical action between the root and soil (Kirby and Bengough, 2002; Bengough et al., 2006) In maize roots, removing the root cap halves elongation rate in compacted soils due to an increase in root penetration resistance (Iijima et al., 2003). The intact root cap and associated border cells facilitate root elongation by decreasing friction between the root tip and the soil (Bengough et al., 2006). The rates of border cell and mucilage production are found to increase with increasing mechanical impedance (Iijima et al., 2000). Analysis of particle movement along maize roots has shown that lubrication by the root cap allows sand particles to move more easily along the epidermis of the elongation zone than for mutants with the root cap removed (Vollsnes et al, 2010). As the root tip is the area at which peak stress occurs and axial pressure has been observed to affect cell elongation more strongly than radial pressure, it is possible that the root tip plays a role in sensing mechanical impedance. Soil strength may be sensed by the effect of axial pressure on the root tip causing to root to adjust the rate of growth accordingly (Jin et al., 2013). Control of growth is then likely to be mediated through the control of cell flux and elongation.

1.4 Hormonal control of root growth and development

There are a number of plant hormones involved in the regulation of root growth and development including auxin, ethylene, cytokinin, abscisic acid, gibberellin and brassinosteroids. Plant hormones and their signalling systems interact both antagonistically and synergistically to control cell division, growth and differentiation (Takatsuka and Umeda, 2014). Hormones form a network with their associated target genes, with genes regulating hormone activities and hormones regulating gene expression (Moore et al., 2015). The concentration of hormones in a cell is the result of a number of factors, including changes in biosynthesis and short- or long-range transport and by activation, inactivation and degradation (Del Bianco et al., 2013; Moore et al., 2015). For roots to develop correctly there must be a specific patterning of responses to hormone signalling and gene expression. For example, cellular patterning in the *Arabidopsis* root requires the establishment of an auxin concentration maximum close to the QC (Sabatini et al., 1999; Moore et al., 2015). Hormonal crosstalk is also vital for plants to able to respond to stress. Under stress, levels of plant hormones are altered in order to coordinate a change in development and response to stress (Liu et al., 2014). For example in response to osmotic stress abscisic acid (ABA) levels increase in order to maintain normal growth. It is therefore important to understand how the different plant hormones interact to control root growth and what changes may occur in response to stress.

1.4.1 The ethylene signalling pathway

Ethylene is a gaseous hormone that regulates a number developmental processes including fruit ripening, organ senescence and root growth (Ju and Chang, 2015). It also functions as a stress hormone and is induced in response to wounding,

flooding, cold and nutrient stress as well as mechanical stimulation (Buer at al., 2003; Yamamoto et al., 2008; Lin et al., 2009).

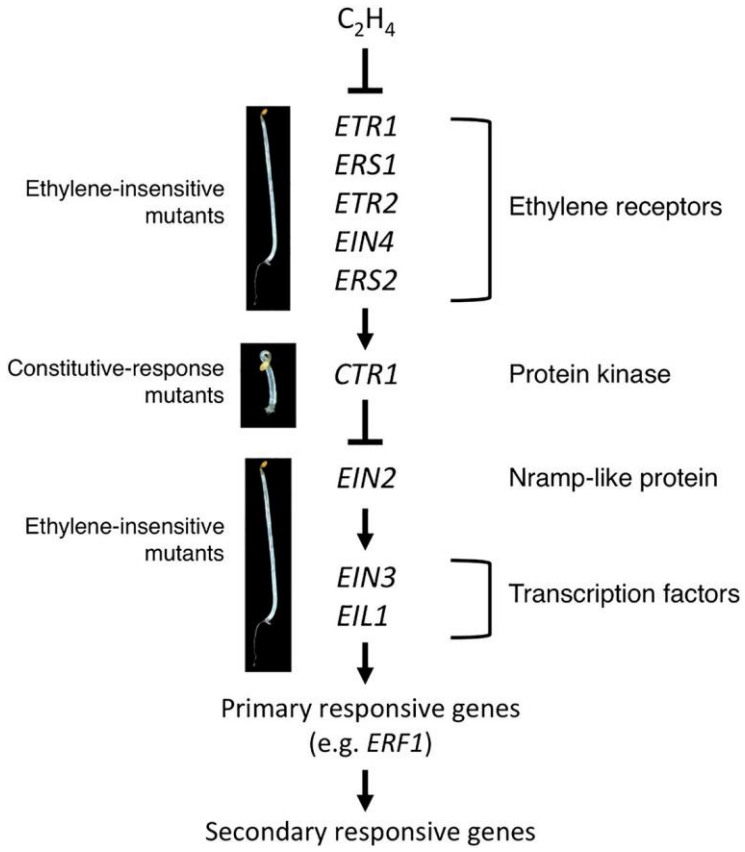

Figure 2. **The core ethylene signalling pathway, taken from (Ju and Chang, 2015).** Ethylene represses activity of the ethylene receptors (ETR1, ERS1, ETR2, EIN4, and ERS2) which would otherwise prevent an ethylene response through the negative regulator CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1). EIN2 and EIN3 are positive regulators of the ethylene response. Mutant seedling phenotypes in the triple response assay are shown. Arrows indicate activation and T-bars repression of the pathway.

The core ethylene signalling pathway is shown in Figure 2. Perception of ethylene occurs at the endoplasmic reticulum (ER) membrane, with signal transduction leading to a transcription cascade that results in changes in cellular, physiological and metabolic responses (Ju and Chang, 2015). *Arabidopsis* has five ethylene receptors (ETR1, ERS1, ETR2, EIN4, and ERS2) that act as negative regulators of the ethylene response (Chang et al., 1993; Hua et al., 1995; Hua et al., 1998; Sakai et al., 1998; Hua and Meyerowitz, 1998). In the absence of ethylene, the receptor

interacts with and activates CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1), a Raf-like kinase (Kieber et al., 1993). CTR1 is a negative regulator of the ethylene response and its activation results in repression of the downstream pathway. When ethylene binds to the receptor it represses it's signalling, preventing the activation of CTR1 (Clark et al., 1998; Hua and Meyerowitz, 1998). ETHYLENE INSENSITIVE TWO (EIN2), a positive regulator of ethylene signalling, is then free to signal to the nucleus (Alonso et al., 1999). Here the transcription factor EIN3 is stabilised (An et al., 2010) and initiates a transcription cascade involving ETHYLENE RESPONSE FACTOR 1 (ERF1) and other genes to promote the ethylene response (Chao et al., 1997; Solano et al., 1998).

1.4.2 Ethylene and hormonal crosstalk during root development

One important aspect of root development is the establishment of an auxin gradient. Auxin is transported towards the root tip, where is it required for meristem maintenance and pattern formation (Van de Poel et al., 2015). High concentrations of auxin in the root inhibit growth and the directional transport of auxin relies on the distribution of PIN efflux proteins (Grieneisen et. al., 2007; Mironova et al., 2010). Ethylene has been shown to play an important role in the maintenance of this auxin gradient and has been shown to mediate root growth through both auxin biosynthesis and transport (Ruzicka et al., 2007; Swarup et al., 2007).

Other hormones are involved in the control of root growth that also interact with ethylene. These include giberellins (GAs), cytokinins (CKs) and abscisic acid (ABA). GAs are responsible for degrading DELLA proteins, which inhibit root growth in the elongation zone (Daviere and Achard, 2013). The transport of GAs is likely to be mediated by ethylene (Shani et al., 2013). CKs have both been shown to regulate

ethylene biosynthesis and have inhibitory effects on root growth (Zďárská et al., 2013). CKs are also known to interact antagonistically with auxin (Dello Ioio et al., 2008).

1.4.3 POLARIS (PLS)

POLARIS (PLS) is a 36 amino acid peptide that interacts with both auxin and ethylene responses (Casson et al., 2002; Chilley et al., 2006; Liu et al., 2013). Its expression is strongest at the root tip where it acts as a negative regulator of the ethylene response. The *pls* mutant shows developmental defects including a short root with reduced cell elongation (Casson et al., 2002). PLS expression in *Arabidopsis* is induced by auxin but repressed by ethylene (Casson et al., 2002; Chilley et al., 2006). Expression of PLS influences the abundance of the auxin transport proteins PIN1 and PIN2, with the *pls* mutant showing reduced levels of both (Liu et al., 2013). In combination with other experimental data it has been shown that interactions between PLS and PIN proteins are important for hormonal crosstalk. The PLS peptide and PIN1/PIN2 form an interacting network with auxin, ethylene and cytokinin (Liu et al., 2013). It is important to understand the function of PLS when investigating the role ethylene signalling and hormonal crosstalk in response to abiotic stress.

1.5 The role of ethylene signalling in the response to mechanical impedance

Previous studies have demonstrated the role of plant hormones in response to mechanical stimulation, in particular the role of ethylene and auxin (Masle, 2002; Braam, 2005; Okamoto et al., 2008; Yamamoto et al., 2008). However, the exact nature of the signalling mechanism involved remains unknown. Studies have

shown that changes in root morphology due to mechanical impedance resemble changes in morphology when exposed to ethylene (Masle 2002, Buer et al. 2003).

Root thigmotropic responses have previously been shown to be regulated by ethylene. Hard (1.5% as opposed to 1%) agar plates inclined at an angle provide a touch stimulus for *Arabidopsis* roots. The response seen is a wavy growth pattern in roots due to the mechanical stimulus avoidance response (Okada and Shimura, 1990). Using this method, Buer et al. (2003) demonstrated that ethylene modulates this response. Furthermore ethylene suppressed gravity-dependent responses such as root-looping. When *Arabidopsis* is grown in medium consisting of a normal layer and a harder layer, the root can show a bending response at the lower, harder layer. The bending or non-bending response of roots has been shown to depend on ethylene (Yamamoto et al., 2008).

It has been demonstrated also that ethylene plays a role root growth responses to mechanical impedance. In maize seedlings grown in a dense medium, decreased root elongation and increased diameter increased was accompanied by increasing levels of ethylene production (Sarquis et al. 1991). In tomato seedlings, inhibition of ethylene limits root penetration. In seeds germinated in the presence of an ethylene inhibitor, roots maintained positive gravitropism, making contact with the growth medium, but were unable to penetrate it (Santisree et al., 2011). A reduced ethylene perception mutant also showed an inability to penetrate soil. The role of ethylene signalling has also been demonstrated in mechanically impeded *Arabidopsis* roots (Okamoto et al., 2008). Roots undergoing mechanical impedance showed a phenotype characteristic of an ethylene response similar to what has been previously observed in roots growing in hard soils. Roots were shorter, increased in diameter, had decreased cell elongation and formed root hairs closer

to the root tip. Ethylene insensitive mutants and seedlings grown in the presence of inhibitors of ethylene signalling showed a reduced response (Okamoto et al., 2008).

It is possible that ethylene signalling mediates the response of roots to mechanical stress through coaction with auxin. Auxin has been shown to be involved in the response of roots to mechanical impedance in tomato seedlings (Santisree et al., 2011) and in *Arabidopsis* (Okamoto et al., 2008). It has previously been demonstrated that the effect of ethylene on root growth is mediated through regulation auxin biosynthesis and localisation of auxin transporters. It is likely that through this mechanism ethylene inhibits cell elongation and promotes cell expansion (Ruzicka et al., 2007; Strader et al., 2010). So far the evidence suggests that root responses to mechanical impedance are the result of an ethylene response mediated through auxin signalling.

1.6 Project aims

This project aims to investigate how mechanical stress on roots causes a change in root growth and the mechanism by which this occurs. We hypothesise that changes in root growth under mechanical stress occurs due to an activation of ethylene responses which in turn regulates the effect of auxin.

In order to investigate the signalling mechanism involved in changes in primary root growth, *Arabidopsis* roots need to undergo constant mechanical impedance. I am using a previously developed method that ensures roots undergo constant mechanical stimulation (Okamoto et al., 2008). *Arabidopsis* seedlings are grown on dialysis membrane covered plates orientated either vertically or horizontally.

When grown horizontally they are mechanically impeded as the roots are unable to penetrate the dialysis membrane as they attempt to grow downwards.

Using this method the response of wild-type *Arabidopsis* roots to mechanical impedance will be characterised (Chapter 3.1.1). In particular, whether they respond to mechanical impedance by exhibiting a characteristic ethylene response (shorter roots, longer root hairs and root growth closer to the tip). As well as investigating the response of the primary root to mechanical impedance, I will also aim to look at the effect on the more overall root architecture, in particular lateral root growth (Chapter 3.1.3). It has been previously reported that mechanical impedance affects lateral root growth (Goss, 1977; Bingham and Bengough, 2003).

Secondly the role of plant hormones in the response of roots to mechanical impedance will be investigated. The main focus will be on the role of ethylene and its effect on auxin; however the role of other plant hormones such as ABA, cytokinins and giberellins will also be briefly looked at (Chapters 3.3.3, 3.5.1 and 3.5.2). Chemical inhibitors and analysis of mutant responses will be used to investigate the role of ethylene and auxin in regulating the mechanical impedance response (Chapters 3.2 and 3.3). Hormonal responses will also be investigated by looking for changes in the expression and localisation of fluorescently labelled reporter proteins (Chapter 3.4 and 3.6). Finally gene expression analysis using quantitative real time (qRT)-PCR will also be used to investigate the role of plant hormones in regulating root growth under mechanical impedance (Chapter 3.6)

2. Materials and Methods

2.1 Plant Materials

Wildtype *Arabidopsis thaliana* seeds were obtained from laboratory stocks of Columbia (Col-0) or C24 ecotypes originally from the Lehle seeds (Texas, US). The auxin resistant mutant *aux1* (Pickett et al. 1990), auxin transport mutant *eir1* (Roman et al. 1995), ethylene insensitive *etr1* and *ein2,* and ethylene overproduction mutant *eto1* (Guzman and Ecker, 1990) were obtained from laboratory stocks. The *polaris* (*pls)* mutant was previously generated by promoter trapping in a C24 background (Topping et al. 1994; Topping and Lindsey 1997).

DR5::VENUS (Heisler et al. 2005) lines used for fluorescent imaging of auxin distribution were obtained from the Nottingham Arabidopsis Stock Centre (NASC) as was TCS::GFP (Müller and Sheen, 2008). ProPIN1::PIN1::GFP (Benkova et al. 2003) and proPIN2::PIN2::GFP (Xu and Scheres, 2005) were obtained courtesy of Ben Scheres (Wageningen University). RGA::GFP (Silverstone et al. 2001) was obtained courtesy of Ari Sadanandom (Durham University).

2.2 Chemical Suppliers

All materials and reagents were obtained from Sigma Aldrich unless otherwise stated.

2.3 Seed Sterilisation

Seed sterilisation was carried out under sterile conditions within a laminar flow hood. Seeds were sterilised in Eppendorf tubes first with 70% (v/v) ethanol for 30s then for 12 minutes in commercial bleach diluted to a 20% solution with water. After removal of the bleach solution seeds were washed with sterile

deionised water at least five times. Seeds were stored in deionised water at 4°C for at least five days before being germinated and used for experiments.

2.4 Growth Conditions

Agar media was made up to contain 2.2 g/l of Murashige Skoog basal salt mixture, 10 g/l sucrose and 5g/l agar. The pH of the media solution was adjusted to pH 5.7 using a 0.1M KOH solution before the addition of agar. Media was autoclaved for 20 min at 120°C. Seeds were first placed on round Petri plates of half strength Murashige Skoog (MS) nutrient agar media, sealed with Micropore tape and maintained in a growth room (22°C, 18 hour photoperiod) as described previously (Casson et al. 2002).

The growing system used to provide continuous mechanical stimulation to roots has been adapted from a previously described method (Okamoto et al. 2008). Three days after germination, seedlings were transferred onto square plates (100x100x20mm, STARSTEDT) containing ½ MS nutrient agar media covered with a dialysis membrane (Dialysis tubing cellulose membrane; molecular weight cut off 14,000; flat width 76mm; Sigma-Aldrich) and the plates were sealed with Micropore tape. Seedlings were grown on dialysis membrane plates orientated either vertically (control) or horizontally (mechanically impeded) for 4 days (22°C, 18 hour photoperiod).

Chemicals and hormones such as ABA, Fluridone, AgS_2O_3 and NPA were filter sterilised and added to agar media as it cooled and before placement of the sterilised dialysis membrane. Stock solutions of 10mM were prepared according to Table 1 and stored for up to one month.

Chemical/Hormone	Stock solution preparation
ABA (Abscisic acid)	22.6 mg of ABA was dissolved in 10ml of Methanol and filter sterilised. Stored at -20°C
Fluridone ($C_{19}H_{14}F_3NO$)	32.9 mg of Fluridone was dissolved in 10ml of Methanol and filter sterilised. Stored at -20°C
NPA (N-1-Naphthylphthalamic acid; Greyhound Chromatography and Allied Chemicals)	29.1 g of NPA was dissolved in 10ml of DMSO and stored at -20°C.
Silver Thiosulphate (AgS_2O_3)	Silver Nitrate solution (5ml at 3.4 mg/ml) was added dropwise to a Sodium Thiosulphate solution (5ml at 12.7 mg/ml) to give 10mM silver ion solution and stored at 4°C.

Table 1. Chemical and hormone stock solution preparation

2.5 Preparation of dialysis membranes and plates

Dialysis tubing (Dialysis tubing cellulose membrane; molecular weight cut off 14,000; flat width 76mm; Sigma-Aldrich) was cut into flat square (10cmx10cm) sections and rinsed with deionised water. Membranes were then treated in a solution of 1mM EDTA at 60°C for 20 min to remove trace metals. Membranes were rinsed for a second time in deionised water and stored in deionised water within glass bottles. Before use membranes were autoclaved for 20 min at 120°C.

Plates were prepared by first pouring liquid agar media into square plates (100x100x20mm, STARSTEDT) and allowing to set. Autoclaved dialysis

membranes were then placed flat on top of the set agar within the plate. Dialysis membranes were handled using forceps sterilised with 95% (v/v) ethanol to avoid contamination. Once dialysis membranes were in place seedlings were transferred on to the plates and placed directly onto the membrane surface.

2.6 Analysis of primary root growth

After 4 days of growth on the dialysis membrane, all plates were photographed alongside a ruler for scale (model; Epson 1680 pro flathead scanner) and primary root length was measured. Root tips were photographed using a Leica stereomicroscope and distance from the root tip to the start of root hair growth measured. Each growth assay was repeated at least three times with at least 15 individuals per treatment. All image analysis was carried out using ImageJ.

2.7 Analysis of lateral root growth

Seedlings were photographed at 9 days old alongside a ruler for scale. Images were analysed using the image analysis programme Smart Root (Lobet et al., 2011; available at http://www.uclouvain.be/en-smartroot). Primary and lateral root lengths and number of lateral roots were recorded for each seedling.

2.8 Root imaging using laser scanning confocal microscopy

Distribution and expression of Auxin and PIN proteins was investigated using the DR5::VENUS, proPIN1::PIN1::GFP and proPIN2::PIN2::GFP reporter lines. In addition the reporter line RGA::GFP and synthetic reporter TCS::GFP were used to investigate the possible role of cytokinin and gibberellin.

Individual 7 day old seedlings were stained with propidium iodide by transferring the seedling to a solution of propidium iodide (0.5 μg/ml) and treating for 1 min

30s. Seedlings were then washed in sterile deionised water for the same time and the root tip removed and mounted in deionised water on the slide. Root tips were imaged using a Leica SP5 laser scanning confocal microscope (www.leica-microsystems.com). For different reporter lines, microscope settings were set to optimise image quality but were consistent between individuals of the same reporter line. Sequential scans were used and detection spectra were chosen to minimise crossover between fluorophores. YFP and propidium iodide were excited using the 514 nm band of the argon laser and GFP with the 488 band of the argon laser.

2.8.1 Analysis of confocal images

Images obtained were processed first using LAS AF LITE software (v2.63 build 8173http://www.leica-microsystems.com/products/microscope-software/life-sciences/las-af-advanced-fluorescence/). ImageJ was then used to analyse images and fluorescence was measured using the "colour histogram" tool. For each treatment images from at least six different individuals were analysed.

2.9 RNA extraction/Dnase/ cDNA synthesis

Whole roots of 7 day old seedlings were removed using a razor blade and frozen in liquid nitrogen. At least 30mg of tissue was then ground whilst frozen and used for RNA extraction. RNA was extracted using a Sigma Spectrum Plant Total RNA kit (Sigma Aldrich). An on-column DNA digest was performed using an On-Column DNase I Digest Set (Sigma Aldrich). Total RNA concentration was then measured using a Nanodrop ND1000 Spectrophotometer (ThermoFisher Scientific, Hemel Hempstead, UK).

For cDNA synthesis, RNA solutions were concentrated to above 300 ng/µl. 3 µg of RNA in a 20 µl solution was used for cDNA synthesis. Reaction mixes were made up and incubated according to Table 2 and used the Superscript III First-Strand Synthesis System (Invitrogen Ltd, Paisley, UK).

RNA mix	**x1 reaction**	**Incubation**
RNA	3 µg in 10µl sdH_20	
OligodT20 (50µm)	1 µl	
dNTPs (10mM)	1 µl	
Total	12 µl	65°C for 5 minutes
cDNA synthesis mix	**x1 reaction**	**Incubation**
10x RT BUFFER	2 µl	
50 mM MgCL2	2 µl	
0.1 DTT	2 µl	
RNase OUT	1 µl	
Superscript III	1 µl	
Incubated RNA mix	12 µl	
Total	20 µl	50°C for 50 mins 85°C for 5 mins

Table 2. Reaction mixes and incubation times used by the Superscript III first strand synthesis system for cDNA synthesis

After cDNA synthesis 1 µl of RNase H was added and the sample incubated for 20 mins at 37°C to remove the RNA template. cDNA solutions were then diluted 1 in 4 and stored at -20°C until required for PCR.

2.10 PCR

The products of cDNA synthesis were tested using a standard PCR amplification of *ACT2* (primer sets Table 6). A standard PCR was also used to test primer sets to be used in qRT-PCR. Each PCR reaction was set up with the following mixes (Table 3) and PCR cycling conditions (Table 4) according to the standard MyTAQ DNA polymerase protocol (Bioline).

PCR mix	X1 reaction
5x MyTAQ Reaction Buffer	10 µl
cDNA template	1 µl
Forward Primer (20µM)	1 µl
Forward Primer (20µM)	1 µl
MyTaq DNA Polymerase	1 µl
Water (sterile ddH_20)	36 µl

Table 3. PCR reaction set-up

Step	Temperature	Time	Cycles
Initial Denaturation	95°C	1min	1
Denaturation	95°C	15s	35
Annealing	55°C	15s	
Extension	72°C	10s	

Table 4. PCR cycling conditions. PCR performed using an Applied G-Storm GS1 PCR machine

PCR products were tested using gel electrophoresis. Gels were made by dissolving agarose in TAE buffer and ethidium bromide added before pouring. A 2.5 µl sample was loaded with 2.5 µl of 5x loading buffer. Each gel also had a separate well

containing 5 µl Hyperladder for fragment size determination. Gels were imaged using a BioRad Gel-Doc 1000.

2.11 qRT-PCR

Quantitative real-time polymerase chain reactions (qRT-PCR) were carried out using a SYBR Green JumpStart Taq Readymix (Sigma-Aldrich) on a Rotorgene Q (Qiagen). The reaction mix for each run was made up according to Table 5 and using the primer sets in Table 6.

Samples from three biological replicates were amplified in each case and each reaction was set up in triplicate for technical repetition. Gene expression was calculated from the average of the three technical repeats relative to the amplification of a reference gene (AT5G15710) using the Rotorgene Q series software v1.7 to perform a comparative quantitation. The relative concentration of each sample is calculated as Amplification^(Calibrator takeoff – Sample takeoff). The reference gene AT5G15710 was chosen due to its relative stability under physical stress conditions (Czechowski et al., 2005). Reference gene stability was tested by comparing expression levels between all sampled of treatment and control. Amplification specificity was checked using melt curve analysis.

qPCR mix	**x1 reaction**
Forward Primer (20 µM)	0.25 µl
Reverse Primer (20 µM)	0.25 µl
cDNA	0.5 µl
2X SYBR JumpStart Readymix	10 µl
sterile dH_20	9 µl
Total	**20 µl**

Table 5. qPCR reaction mix set up

Target gene	Primer Sequences 5'-3'
ACT2	GGATCGGTGGTTCCATTCTTGC AGAGTTTGTCACACACAAGTGCA
AT5G15710	CTCTTTCGCCTCTTGGTTTG TCCTTCCCACGAGAAACAAT
ERF1	GGTATTAGGGTTTGGCTCGG CCGAAAGCGACTCTTGAACT
PIN1	TCGTTGCTTCTTATGCCGTT AGAAGAGTTATGGGCAACGC
PIN2	AATGCTGGTTGCTTTGCCTG CCTTTGGGTCGTATCGCCTT
ARR5	TGTCCTGATTCTTTCGGCTT ACCCATCTTTGTCACTCTTGA
DREB2B	CCCATCAGAGCCAAGACCAA GGACCATTGCCTCAGAACTC
RD29B	GGGGAAAGGACATGGTGAGG GGTTTACCACCGAGCCAAGA

Table 6. Primer sets used for qPCR

2.1 Statistical Analysis

All statistical analysis was carried out using either IBM SPSS Statistics 22.0 or on Microsoft Excel 2010.

3. Results

3.1 The effect of mechanical impedance on root growth

3.1.1 Mechanical stress treatment

To examine the effects of mechanical impedance on root growth, seedlings were grown on agar plates covered with a dialysis membrane placed either vertically or horizontally (Okamoto et al. 2008). Growing seedlings in the horizontal plane provides continuous mechanical stimulation as root tips touch the dialysis membrane while attempting to bend downwards. The presence of the dialysis membrane also prevents roots from growing into the agar media and forming coil structures. When seedlings are grown in the vertical plane, they grow down the surface of the membrane without contacting any physical barrier, and they do not experience mechanical impedance.

Root growth of seedlings grown vertically in the presence and absence of a dialysis membrane was compared. This was to ensure that the presence of a dialysis membrane did not have a detrimental effect on root growth under normal conditions. Seedlings were germinated and transferred to the membrane system after 3 days. They were then grown for 4 days in the presence or absence of a dialysis membrane and root growth and morphology was determined. It was found that growing seedlings in the presence of a dialysis membrane does not appear to have a detrimental effect on root growth compared with growing seedlings on just agar media (Figure 3). This suggests that sufficient nutrients and water are available to seedlings even in the presence of the dialysis membrane.

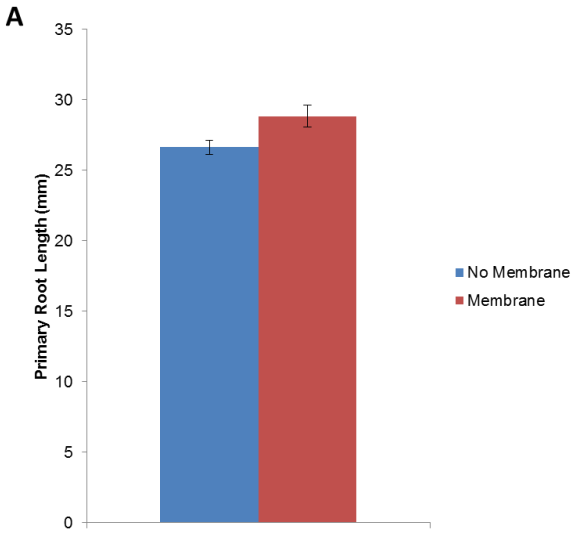

Figure 3. **The effect of the presence of a dialysis membrane on wildtype (Col0) *Arabidopsis* root growth**. (A) The effect of a dialysis membrane on primary root length of vertically grown 7d old seedlings. Error bars show mean +/- SE (n = 32)

3.1.2 The effect of mechanical impedance on primary root growth and morphology

The root growth of vertically orientated and horizontally orientated (mechanically impeded) wildtype (Col0) *Arabidopsis* seedlings was compared. Wildtype seedlings grown horizontally in the presence of a dialysis membrane showed altered root growth and morphology compared to vertically grown seedlings. Root length was significantly reduced in horizontally grown seedlings (t-test, $p < 0.001$; Figure 4A) compared to vertically grown seedlings. In addition initiation of root hair growth was closer to the root tip (t-test, $P<0.001$; Figure 4B), an indicator of meristem degradation with differentiation occurring closer to the root tip (Rost and Baum, 1988; Sanchez-Calderon et al., 2005; Shishkova et al., 2008). Mechanically impeded roots showed an increase in diameter of 10% from a mean thickness of 138 ± 2.23 (SE) µm in vertically grown seedlings to 154 ± 3.23 (SE) µm in horizontally grown seedlings (t-test, $p < 0.001$). The growth of these mechanically impeded roots was characteristic of an ethylene response (Le et al.,

2001; Okamoto et al., 2008), with shorter roots, longer root hairs and root hair growth closer to the tip (Figure 4).

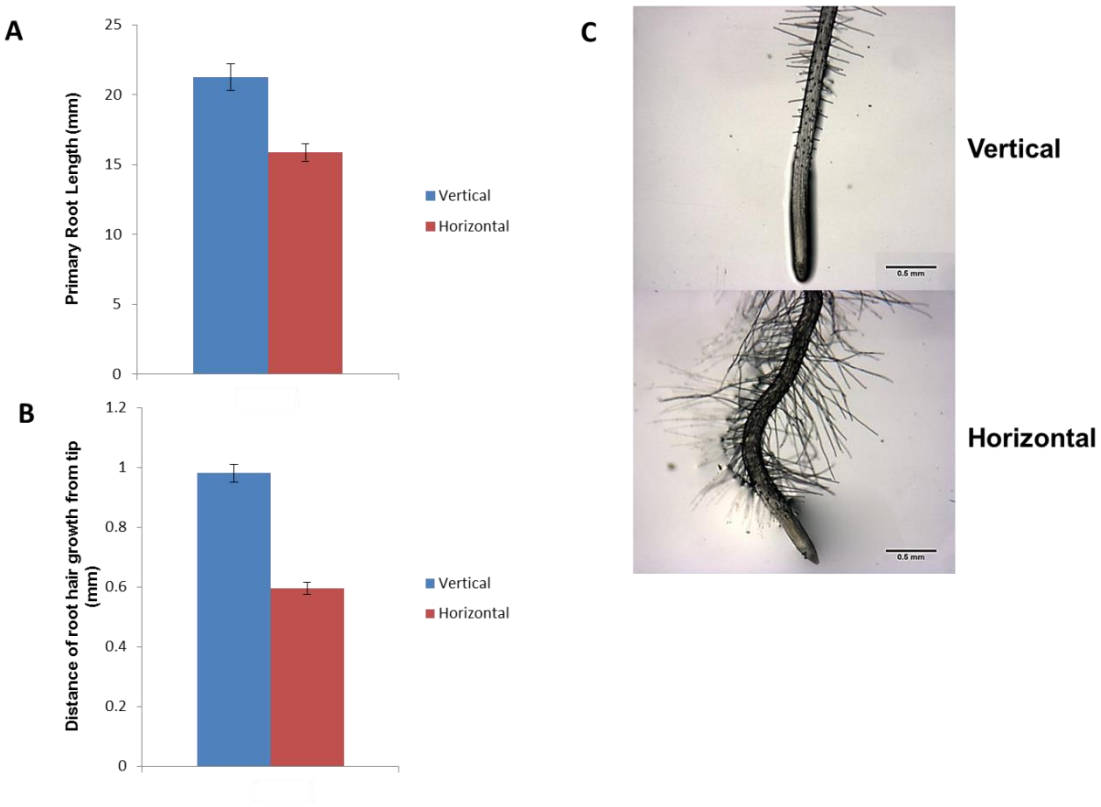

Figure 4. **The effect of mechanical impedance on wildtype (Col0) *Arabidopsis* root growth.** A,B Effect of mechanical impedance on (A) primary root length (t-test, $p < 0.001$) and (B) root hair growth (t-test, $p < 0.001$)of 7d old seedlings. Error bars show mean +/- SE (n = 60). C, Roots of 7d old seedlings grown on dialysis membrane. Scale bar indicates 0.5 mm.

3.1.3 The effect of mechanical impedance on lateral root growth

Previous studies have shown that mechanical impedance may also have an effect on root architecture. Studies on wheat and barley have shown that lateral root growth is affected when roots are mechanically impeded (Goss, 1977; Bingham and Bengough, 2003). In mechanically impeded barley, lateral root length has been shown to increase and the overall density of lateral roots is greater (Goss,

1977). However, lateral root growth has also been reported to decrease in strong soils, although to a lesser extent than the primary root (Bingham and Bengough, 2003). Therefore we examined whether mechanical impedance has an effect on root architecture of *Arabidopsis*.

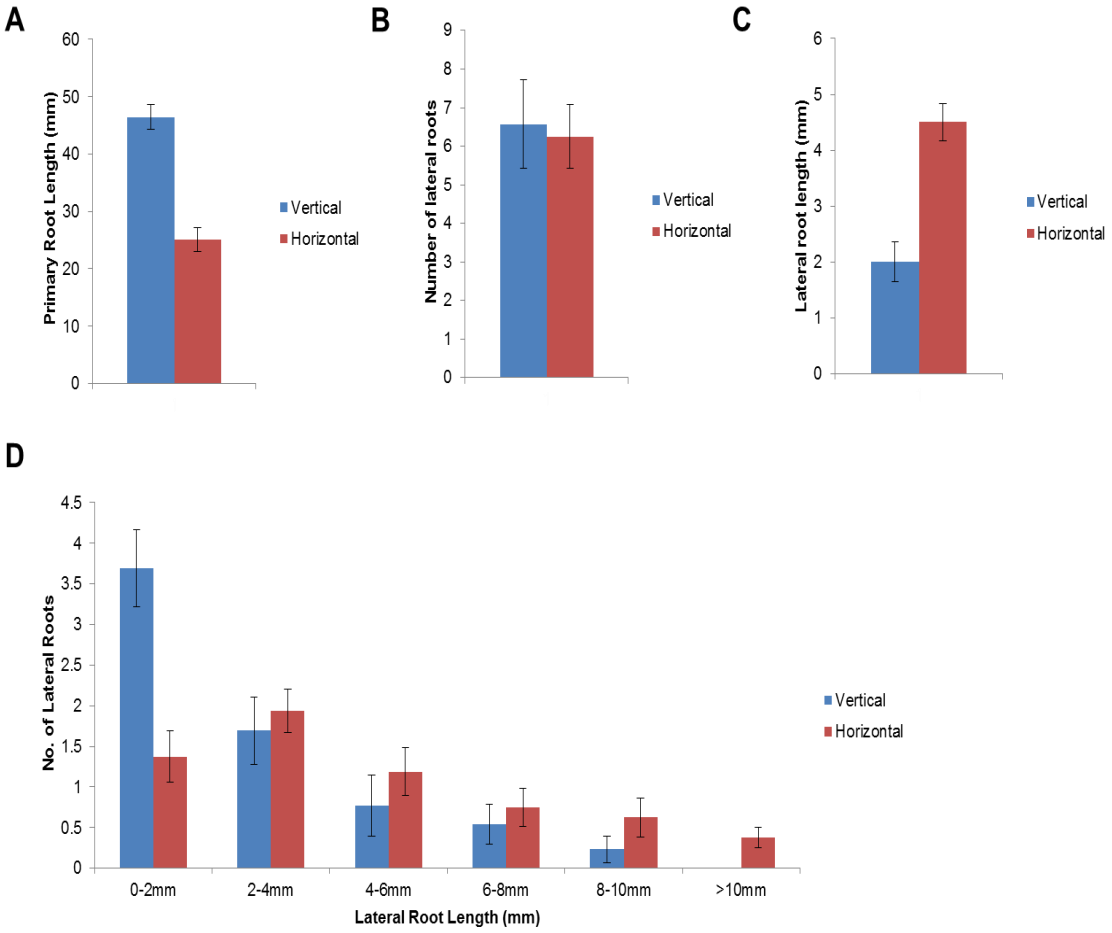

Figure 5. **The effect of mechanical impedance on lateral root growth of wildtype (Col0) *Arabidopsis* seedlings. A-C**, The effect of mechanical impedance on (A) primary root length (t-test, $p < 0.001$) , (B) number of lateral roots (t-test, $p = 2.06$) and (C) lateral root length (t-test, $p < 0.001$) of 9 day old seedlings. **D**, The average number of lateral roots of different lengths of 9 day old seedlings grown vertically or horizontally in the presence of a dialysis membrane. All error bars show mean +/- SE (n = 18)

To investigate whether mechanical impedance may affect lateral root growth, 9 day old seedlings were imaged and the number and length of lateral roots

recorded (Figure 5). Again primary root length was significantly shorter in horizontally grown seedlings compared to vertically grown ones (Student's t-test, $P<0.001$). Although the number of lateral roots did not differ between treatments (Figure 5B), horizontally grown seedlings had significantly longer lateral roots (t-test, $P<0.001$; Figure 5C). On average, vertically grown seedlings have a higher number of shorter (between 0-2mm) lateral roots while horizontally grown seedlings appear to have a higher number of longer roots (Figure 5D).

3.2 How does mechanical impedance affect mutants with altered responses to ethylene and auxin?

3.2.1 The effect of mechanical impedance on ethylene insensitive mutants

Previous studies have shown that ethylene signalling plays a role in the response of roots to mechanical stimulation (Sarquis et al., 1991; Yamamoto et al., 2008; Okamoto et al., 2008). Thigmotropic responses such as root bending and the wavy growth pattern have been shown to be ethylene dependent (Buer et al., 2003; Yamamoto et al., 2008). Studies focusing on the response of roots to mechanical impedance have also demonstrated the possible role of ethylene signalling in both maize (Sarquis et al., 1991) and *Arabidopsis* (Okamoto et al., 2008). Furthermore, the response of mechanically impeded roots resembles the response to ethylene treatment (Le et al., 2001; Okamoto et al., 2008). Based on these findings it is likely that ethylene signalling is involved in the root's response to mechanical impedance.

In order to investigate the role of ethylene signalling in the response of roots to mechanical impedance, the response of mutants insensitive to ethylene was analysed. Two different mutants were used, *etr1* (*ethylene resistant 1,* a gain of

function ethylene receptor mutant) and *ein2* (*ethylene insensitive 2,* an ethylene signalling mutant).

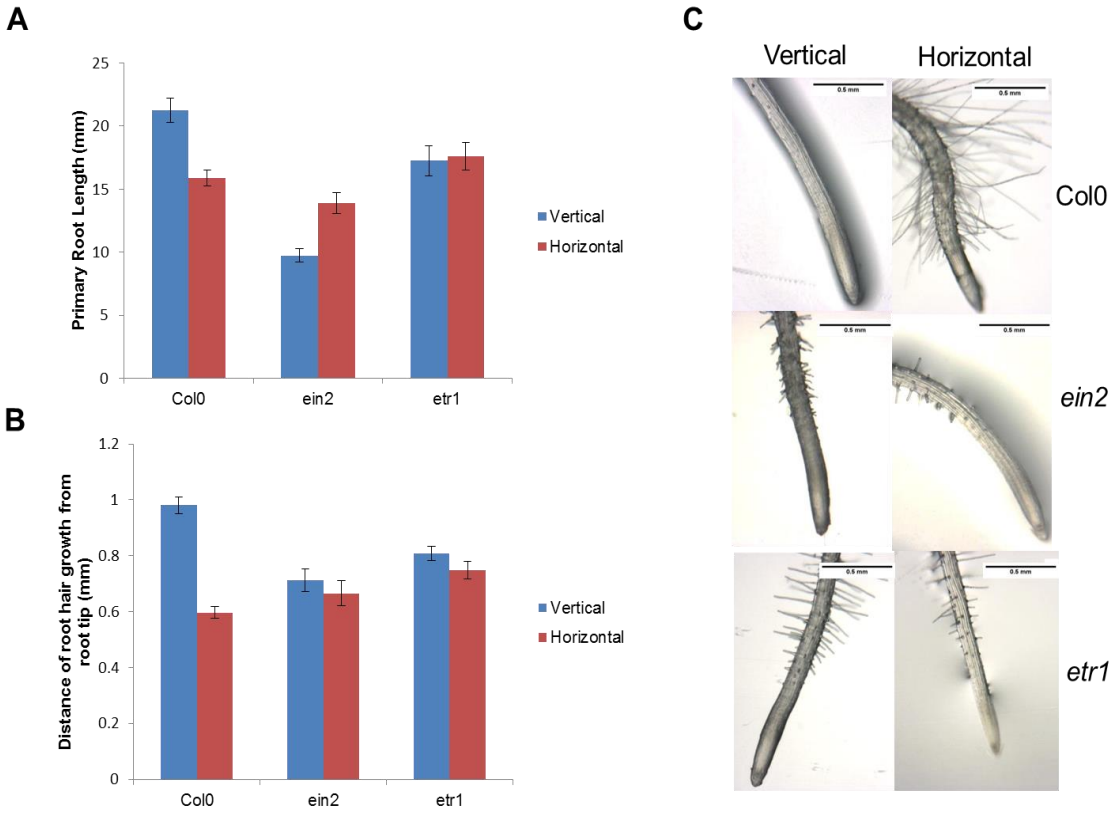

***Figure 6*. The effect of mechanical impedance on the root growth of ethylene resistant *Arabidopsis* mutants.** A,B Effect of mechanical impedance on (A) primary root length (ANOVA, $p < 0.001$) and (B) root hair growth (ANOVA, $p < 0.001$) of 7d old seedlings. Error bars show mean +/- SE (Col0 n = 60, *ein2* n = 52, *etr1* n = 36). Data was collected from a minimum of three replicates with repeats occurring on separate days. Data includes wildtype trend for comparison with mutant response. C, Roots of 7d old seedlings grown on dialysis membrane. Scale bar indicates 0.5mm.

The same experimental procedure used to examine the response of wildtype seedlings to mechanical impedance was used to investigate the mutant response. Seedlings were germinated and transferred to the membrane system after 3 days and grown for 4 days in the presence or absence of a dialysis membrane. At 7 days root growth and morphology was determined. Both *etr1* and *ein2* showed an altered response under mechanical impedance compared to the wildtype (Figure 6). Primary root length was not reduced when the mutant seedlings were

mechanically impeded and in the case of *ein2* increased (Figure 6A). Seedlings genotype was found to have a significant effect on root length (ANOVA, $P<0.001$) with a strong interaction between plate orientation and genotype (ANOVA, $P<0.001$).

In addition, *ein2* and *etr1* did not show any of the other characteristic ethylene response traits observed in the wildtype under mechanical impedance, such as longer root hairs and root hair growth closer to the tip (Le et al., 2001; Okamoto et al., 2008; Figure 6 B,C). As with root length, plate orientation and genotype were found to have an interaction effect on the distance of root hair growth from the root tip (ANOVA, $P<0.001$). These results suggest that ethylene signalling is required for the response of roots to mechanical impedance.

3.2.2 The effect of mechanical impedance on ethylene sensitive mutants

The response to mechanical impedance of mutants with enhanced ethylene biosynthesis or signalling responses was also investigated. Two ethylene mutants were used, *eto1*, an ethylene overproduction mutant (Guzman and Ecker, 1990), and *polaris (pls),* an ethylene signalling mutant (Chilley et al., 2006). The response of the wildtype ecotype C24 was also investigated as it is the wildtype background for the *pls* mutant

Both *eto1* and *pls* seedlings showed a similar response to the wildtype ecotypes under mechanical impedance, with reduced root length and increased root hair growth closer to the tip (Figure 7). In the case of *eto1*, horizontally grown seedlings exhibited a more pronounced response than the wildtype. For example the difference in root length between vertically and horizontally grown seedlings was greater for the *eto1* mutant than for the wildtype (Figure 7A).

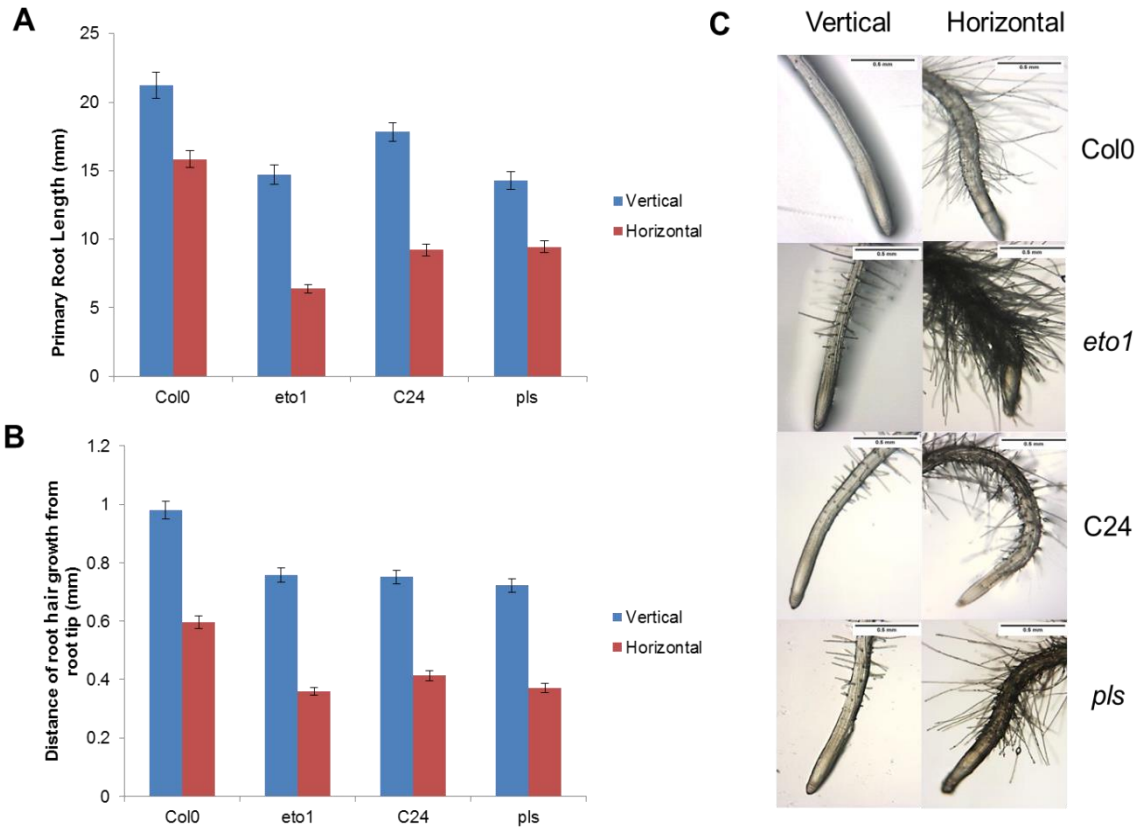

***Figure 7*. The effect of mechanical impedance on the root growth of ethylene sensitive *Arabidopsis* mutants.** A,B Effect of mechanical impedance on (A) primary root length and (B) root hair growth of 7d old seedlings. Error bars show mean +/- SE (Col0 n = 60, *eto1* n = 43, C24 n = 44, *pls* n = 43). Data was collected from a minimum of three replicates with repeats occurring on separate days. Data includes wildtype trend for comparison with mutant response. C, Roots of 7d old seedlings grown on dialysis membrane. Scale bar indicates 0.5mm.

The reduced response of the ethylene insensitive mutants under mechanical impedance, together with the enhanced response of the *eto1* mutant compared with the wildtype, suggest that ethylene signalling plays an essential role in regulating root development in response to mechanical impedance.

3.2.3 The effect of mechanical impedance on auxin transport mutants

The reduced response of ethylene insensitive mutants to mechanical impedance supports the role of ethylene signalling in the response of roots to mechanical impedance. Ethylene has been shown to control root growth through the regulation of auxin biosynthesis and transport (Ruzicka et al. 2007, Strader et al. 2010). To investigate whether this is true for the response to mechanical

impedance, two auxin transport mutants were investigated. The response of *aux1,* an auxin influx mutant, and *eir1* (*pin2*), an auxin efflux mutant, under mechanical impedance were investigated.

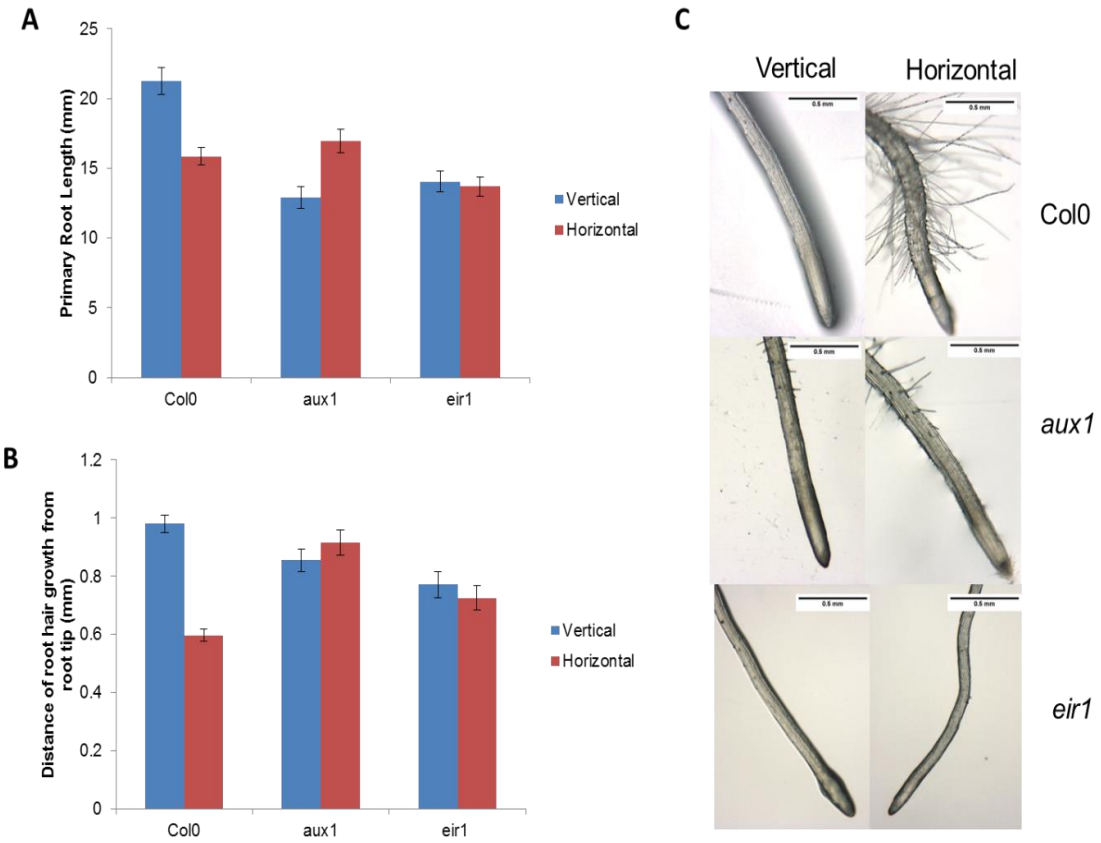

Figure 8. **the effect of mechanical impedance on the root growth of auxin transport *Arabidopsis* mutants.** A,B Effect of mechanical impedance on (A) primary root length (ANOVA, $p < 0.001$) and (B) root hair growth (ANOVA, $p < 0.001$) of 7d old seedlings. Error bars show mean +/- SE (Col0 n = 60, *aux1* n = 59, *eir1* n = 43). Data was collected from a minimum of three replicates with repeats occurring on separate days. Data includes wildtype trend for comparison with mutant response. C, Roots of 7d old seedlings grown on dialysis membrane. Scale bar indicates 0.5mm

Horizontally grown *aux1* and *eir1* did not exhibit the characteristic mechanical response phenotype observed in the roots of wildtype seedlings. Primary root length of *aux1* and *eir1* was not reduced in response to mechanical impedance, with *aux1* showing an increase in length and *eir1* showing no difference in length when compared with the vertical control (Figure 8A). Root hair growth in mechanically impeded *aux1* and *eir1* also appeared to be unaffected by mechanical impedance. When mechanically impeded, neither of the mutants showed the

enhanced growth of root hairs seen in the response of the wildtype (Figure 8 B,C) Genotype was found to have a strong effect on root length (ANOVA, $P<0.001$) with a strong interaction between genotype and plate orientation (ANOVA, $P<0.001$), indicating an altered response of the auxin mutants to mechanical impedance. In addition, the distance of root hair growth from the root tip was also significantly affected by genotype (ANOVA, $P=0.001$) with an interaction between genotype and plate orientation (ANOVA, $P<0.001$). Neither *aux1* nor *eir1* showed a decrease in the distance between the root tip and root hair growth (Figure 8B).

The reduced response of *aux1* and *eir1* to mechanical impedance does indicate that auxin signalling and transport are involved in the root response to mechanical impedance. It is possible therefore that ethylene mediates the response of roots to mechanical impedance through auxin signalling.

3.3 The effect of chemical and hormone treatments on the response of *Arabidopsis* roots to mechanical impedance.

Analysis of the root growth response of various ethylene and auxin insensitive mutants suggests that ethylene signalling coupled with auxin transport is involved in the root response to mechanical impedance. To further investigate this, the effects of chemical inhibitors of ethylene signalling and auxin transport on root growth in response to mechanical impedance were studied.

3.3.1 The effect of Ag^+ treatment on the root response to mechanical impedance

To confirm the role of ethylene signalling, a Silver Thiosulphate solution (AgS_2O_3) was added to the agar media before seedlings were transferred onto the dialysis membrane plates. Silver ions inhibit ethylene responses in plants by occupying the

copper binding site of the ethylene receptor complex (Beyer, 1976; Binder et al., 2007).

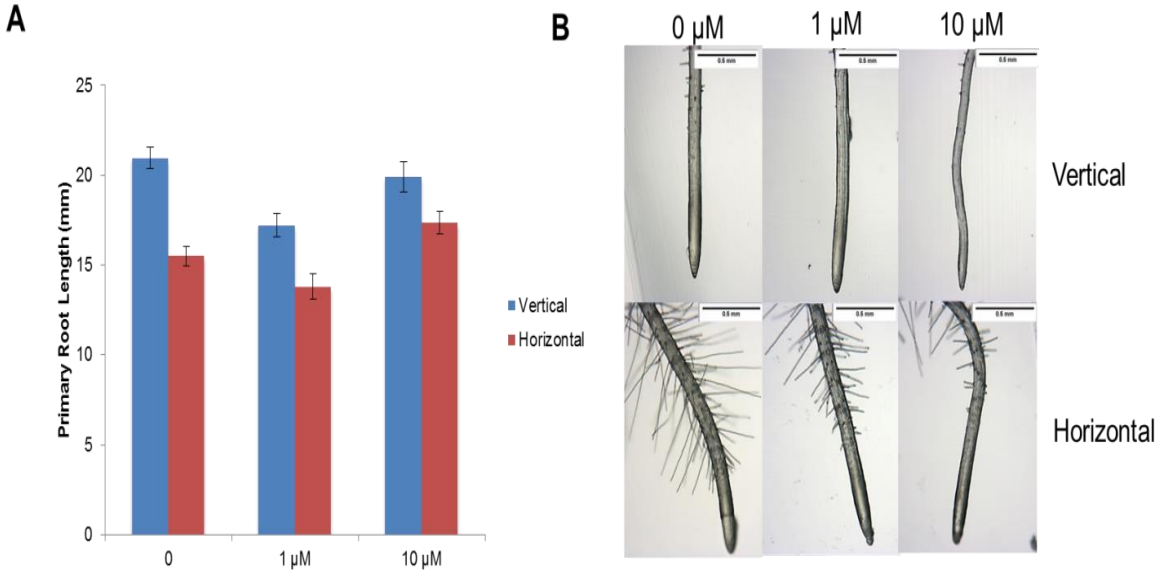

***Figure 9*. The effect of Ag^+ on the response of wildtype *Arabidopsis* to mechanical impedance. A,** Primary root length of 7 day old seedlings grown vertically or horizontally in the presence of a dialysis membrane (ANOVA, $p < 0.001$). Error bars show mean +/- SE ($n = 75$). Data was collected from three replicates with repeats occurring on separate days.. **B,** Roots of *Arabidopsis* grown in the presence of a dialysis membrane. Scale bar indicates 0.5mm.

Seedlings grown in the presence of 1 µM and 10 µM Ag^+ showed reduced root growth compared to the control with ethylene treatment having a significant effect on root length (ANOVA, $P<0.001$) The difference in root length between horizontally and vertically grown seedlings was reduced in the presence of Ag^+, particularly in the presence of 10 µM Ag^+ (Figure 9A). Overall horizontally grown seedlings exposed to Ag^+ showed a less pronounced mechanical impedance phenotype response compared to the control. When mechanically impeded, root hairs of Ag^+ treated seedlings were shorter and growth was initiated further from the root tip than untreated seedlings (Figure 9B).

3.3.2 The effect of NPA on the root response to mechanical impedance

Ethylene is thought to mediate the response of roots in part at least through changes in auxin transport (Ruzicka et al., 2007; Swarup et al., 2007). Investigating the response of auxin transport mutants demonstrated the role of auxin transport in the root response to ethylene signalling, with mutants showing a reduced response under mechanical impedance (Figure 8). To further confirm this, the effect of a chemical inhibitor of auxin transport on the response of roots to mechanical impedance was also investigated. *Arabidopsis* seedlings were exposed to NPA (1-N-Naphthylphthalamic acid), a chemical inhibitor of auxin efflux (Fujitu and Syono, 1996).

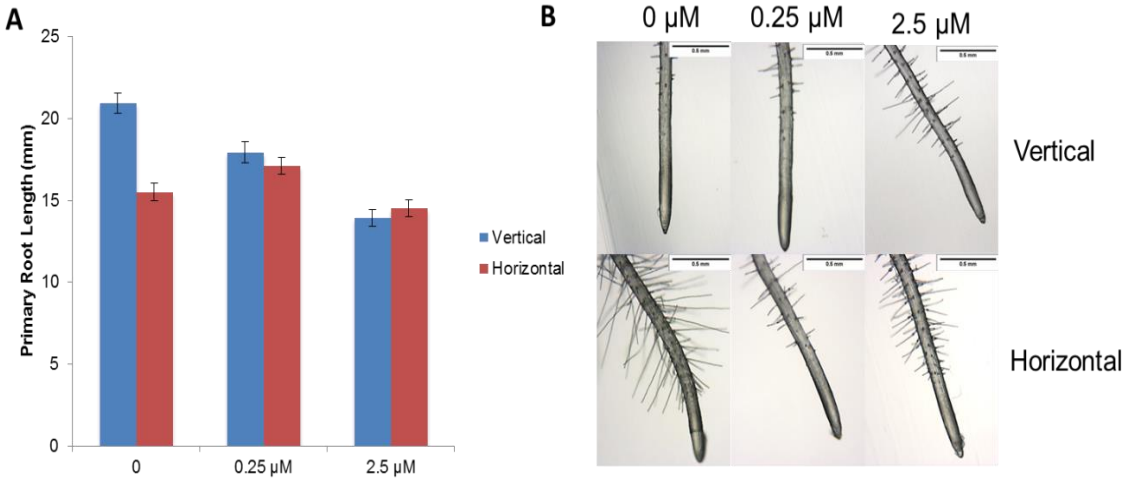

***Figure 10*. The effect of NPA on the response of wildtype *Arabidopsis* to mechanical impedance. A,** Primary root length of 7 day old seedlings grown vertically or horizontally in the presence of a dialysis membrane (ANOVA, $p < 0.001$). Error bars show mean +/- SE ($n = 64$). Data was collected from three replicates with repeats occurring on separate days. **B,** Roots of *Arabidopsis* grown in the presence of a dialysis membrane. Scale bar indicates 0.5mm

Seedlings exposed to 0.25 µM and 2.5 µM NPA had shorter roots than the control and showed a reduced response to mechanical impedance (Figure 10). The effect of NPA treatment on root growth is significant (ANOVA, $P<0.001$) with a strong interaction between NPA treatment and plate orientation (ANOVA, $P<0.001$).

For example, when exposed to even small amounts of NPA (0.25 μM), horizontally grown seedlings exhibited no change in root length compared to vertically grown seedlings (Figure 10A).

Taken together, the reduced response of *Arabidopsis* roots to mechanical impedance when exposed to Ag+ and NPA further confirms the role of ethylene signalling and auxin transport.

3.3.3 The role of ABA in the root response to mechanical impedance

Previous studies have detected a potential role for ABA in the response of roots to mechanical impedance in soils. ABA concentration in xylem sap has been shown to increase in plants whose roots are subject to mechanical impedance (Hartung et al. 1994, Hurley and Rowarth. 1999). However it is unclear whether this is due to mechanical stress or a lower water potential. For example root xylem ABA concentration has been shown to correlate better with root water potential than with soil strength (Dodd et al. 2010). The dialysis membrane system used in the current work separates the effects of mechanical impedance and osmotic stress, by only inducing mechanical impedance without changing the osmotic potential. Therefore we examined the effect of both inhibiting ABA and adding ABA on the root response to mechanical impedance.

To investigate whether ABA signalling is involved in the root response to mechanical impedance, seedlings were treated with Fluridone, an inhibitor of abscisic acid biosynthesis (Moore and Smith, 1984). Fluridone was added to the agar media before seedlings were transferred onto the dialysis membrane plates to determine the effect on the roots of vertically and horizontally grown seedlings.

Seedlings treated with 100nM fluridone had shorter roots but showed the same response under mechanical impedance as seedlings as untreated seedlings. While both ABA treatment (ANOVA, P<0.001) and plate orientation (ANOVA, P<0.001) had a significant effect on root length there was no interaction between the two (ANOVA, P = 0.171). Horizontally grown seedlings had shorter roots than vertically grown seedlings, even under fluridone treatment and still exhibited characteristics of the ethylene response (Figure 11 A,B). This suggests that ABA is not required for the root response to mechanical impedance.

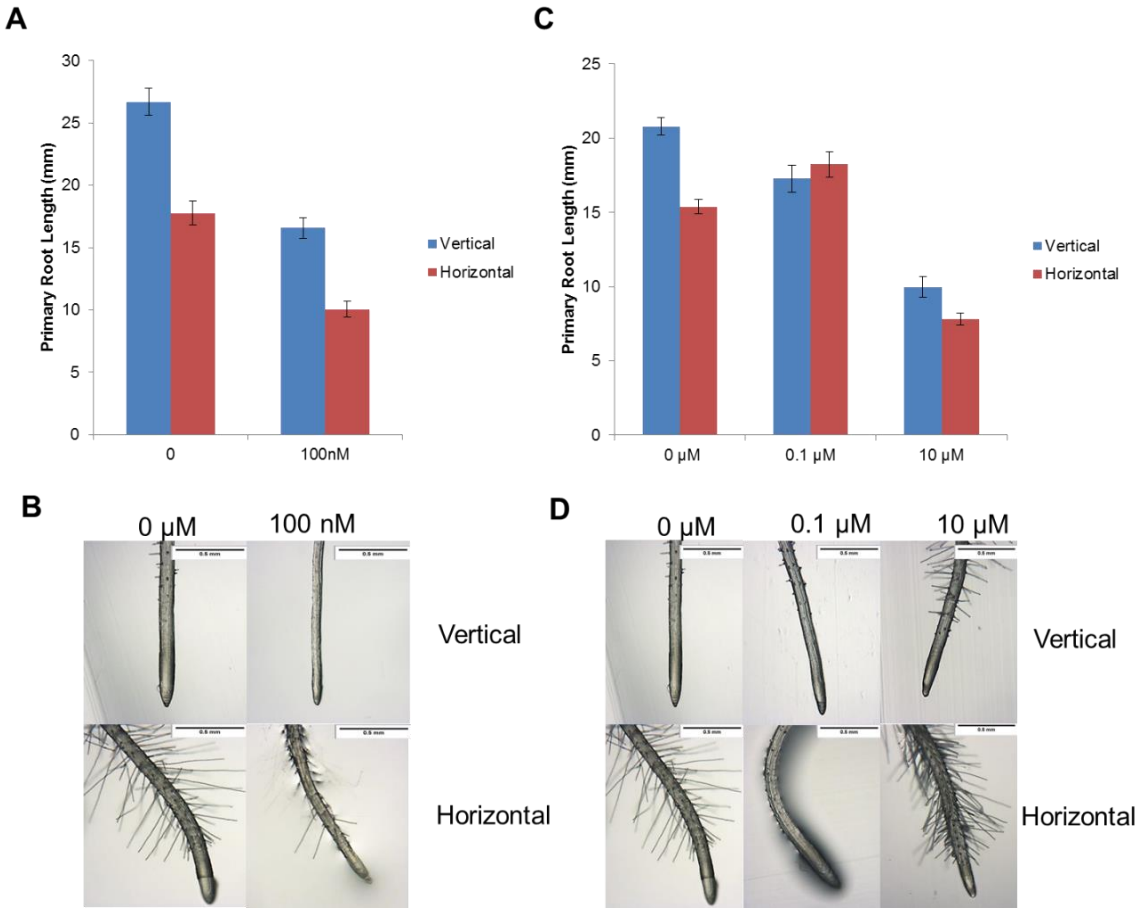

***Figure 11*. The effect of Fluridone and ABA treatment on the response of wildtype *Arabidopsis* to mechanical impedance. A,C** Primary root length of 7 day old seedlings treated with (A) Fluridone or (C) ABA (ANOVA, $p < 0.001$) grown vertically or horizontally in the presence of a dialysis membrane. Error bars show mean +/- SE (Fluridone n = 40, ABA n = 41). Data was collected from three replicates with repeats occurring on separate days. **B,D** Roots of Fluridone (B) or ABA (D) treated *Arabidopsis* grown in the presence of a dialysis membrane. Scale bar indicates 0.5mm

To further examine the effects of ABA on the root response to mechanical impedance, seedlings were exposed to ABA added to the agar media. Seedlings were grown on either a relatively low concentration (0.1 μM) or larger concentration (10 μM) of ABA. Small concentrations (0.1 μM) stimulate root growth, whereas larger concentrations inhibit it (Ghassemian et al., 2000).

Seedlings treated with ABA showed a different response to mechanical impedance depending on the concentration (Figure 11 C,D). ABA treatment significantly affected root length (ANOVA, $P<0.001$) with a strong interaction between ABA treatment and plate orientation (ANOVA, $P<0.001$). Seedlings treated with 0.1 μM ABA exhibited a reduced response to mechanical impedance. When mechanically impeded, root length did not differ between mechanically impeded and vertically grown seedlings treated with 0.1 μM of ABA. The root length of mechanically impeded seedlings treated with 0.1 μM ABA was greater than mechanically impeded, untreated seedlings (Figure 11C). In addition, root hair growth of seedlings treated with 0.1 μM ABA did not differ between the vertically and horizontally grown seedlings, with mechanically impeded roots showing no increase in root hair growth (Figure 11D). In contrast seedlings treated with 10 μM ABA exhibited a similar response to untreated seedlings, where root length decreased in horizontally grown seedlings (Figure 11C). Overall root length in both vertically and horizontally orientated seedlings was shorter when treated with 10 μM ABA than when untreated. Seedlings treated with 10 μM exhibited the same increase in root hair growth when mechanically impeded as the untreated control (Figure 11D). This suggests that roots still responded to mechanical impedance when treated with 10 μM of ABA. Taken together these results suggest that ABA signalling is not essential in the root response to mechanical impedance. However very small amounts of ABA can nullify the response.

3.4 The effect of mechanical impedance on the localisation and expression of auxin responsive genes.

The reduced response of auxin transport mutants *aux1* and *eir1* to mechanical impedance demonstrates the role of auxin signalling in the root's response to mechanical impedance (Figure 8). This is further supported by evidence that NPA, an inhibitor of auxin transport, can also prevent a root response to mechanical impedance (Figure 10). Auxin inhibits root growth and its directional movement is determined by the distribution of PIN proteins (Wiśniewska et al. 2006). Therefore it would be expected that the expression and localisation of auxin at the root tip may change in roots that are mechanically impeded. In addition, the expression of PIN proteins involved in the transport of auxin might also be expected to change. Confocal fluorescence microscopy was used to reporter lines containing fluorescent auxin reporters and PIN:GFP fusion proteins to examine both hormone distribution patterns and hormone regulated gene expression.

3.4.1 The effect of mechanical impedance on the localisation of the auxin reporter DR5:Venus

To analyse the hormone distribution pattern of auxin at the root tip under mechanical impedance, seedlings expressing the auxin reporter DR5:Venus (Heisler et al. 2005) were imaged using confocal laser scanning microscopy.

Horizontally grown roots showed a change in the localisation and relative fluorescence of the DR5:Venus reporter (Figure 12). Vertically grown seedlings exhibited DR5:Venus expression in quiescent centre, columella and stele whereas horizontally grown seedlings showed DR5:Venus located in the lateral root cap and epidermis (Figure 12A). In horizontally grown seedlings, relative fluorescence

decreased in the columella cells (t-test, P=0.05) and increased in the lateral root cap (t-test, P=0.001). This suggests a redistribution of auxin to the lateral root cap under mechanical impedance associated with the reduced root length observed in horizontally grown seedlings.

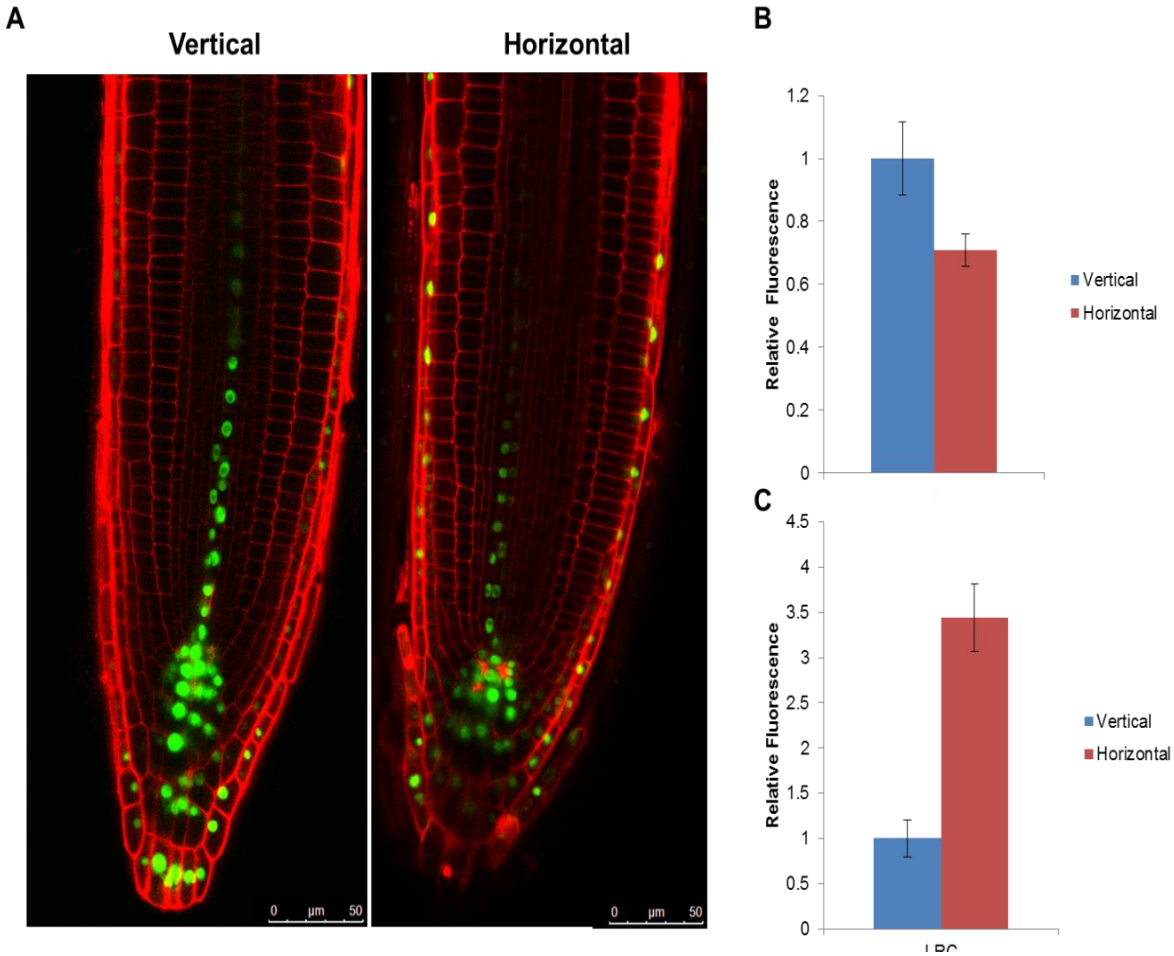

Figure 12. **The effect of mechanical impedance on DR5:Venus distribution. A,** Laser scanning confocal image of *Arabidopsis* root expressing DR5:Venus. Green: DR5:Venus, Red: propidium iodide stain. Scale bar indicates 50 µm. **B-C,** Relative expression of DR5:Venus in the (B) collumella (t test, p = 0.05) and (C) lateral root cap (t-test, p = 0.001) of *Arabidopsis* roots. Error bars show the mean +/- SE. Seedlings were grown in the presence of a dialysis membrane and imaged at 7 days old. Representative of 6 images per treatment.

3.4.2 The effect of mechanical impedance on PIN:GFP and PIN2:GFP expression

Under mechanical impedance, DR5:Venus is redistributed, with an increased signal in the lateral root cap. As PIN proteins are responsible for the directional movement of auxin (Wis´niewska et al., 2006), the expression of fluorescently

tagged PIN proteins was also analysed. Changes in PIN protein levels have been linked to changes in PIN gene expression (Casson et al., 2009). The expression of two PIN proteins were investigated, PIN1 which is located in vascular tissue and PIN2, which is located in cortical, epidermal and lateral root cap cells (Figure 13 and 14).

PIN1:GFP was observed at the root tip in the vascular tissue of both vertically and horizontally grown seedlings (Figure 13A). PIN2:GFP was observed mainly in the epidermis and lateral root cap (Figure 14A). Although statistically insignificant, a small trend increase in the relative fluorescence of PIN1:GFP (Figure 13, t-test, P=0.286) and PIN2:GFP (Figure 14, t-test, P=0.085) was consistently observed in horizontally grown roots.

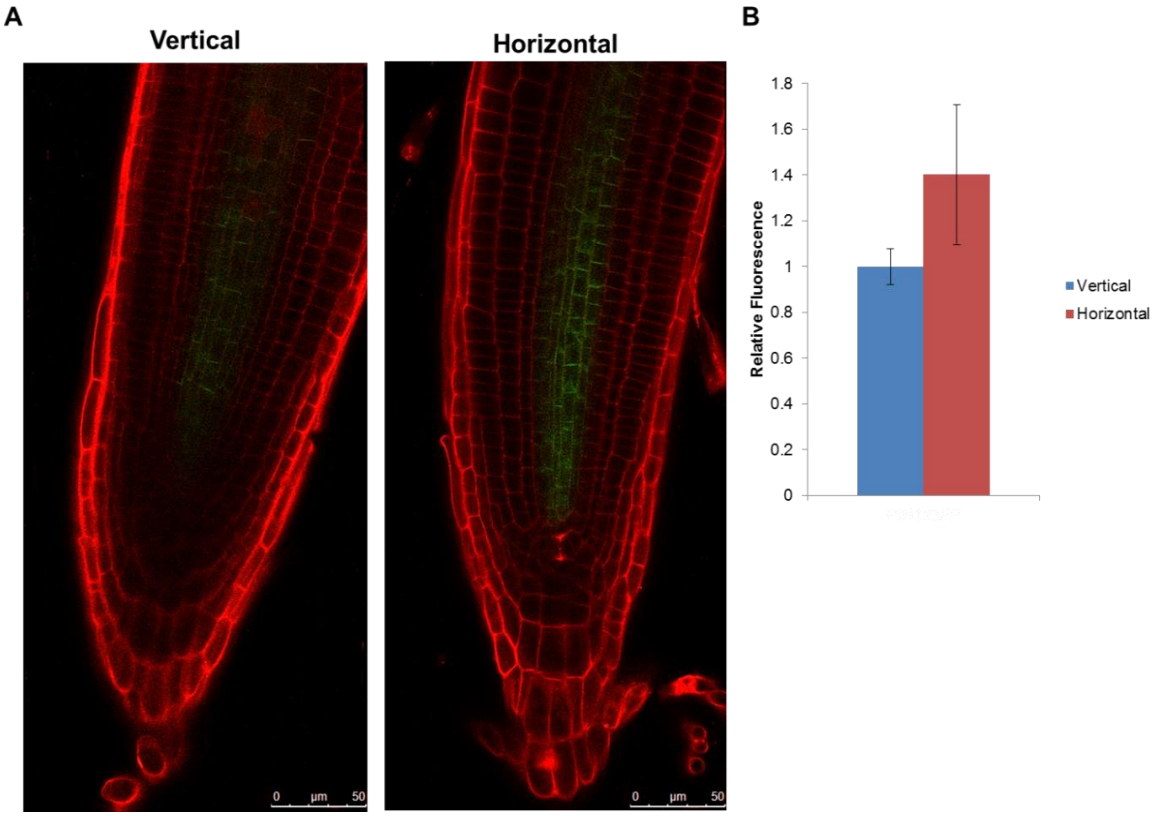

***Figure 13*. The effect of mechanical impedance on PIN1:GFP expression. A,** Laser scanning confocal image of *Arabidopsis* root expressing PIN1:GFP. Green: PIN1:GFP Red: propidium iodide stain. Scale bar indicates 50 µm. **B** Relative expression of PIN1:GFP in the root tip of 7d old *Arabidopsis* seedlings (t- test, p = 0.286). Error bars show the mean +/- SE. Seedlings were grown in the presence of a dialysis membrane and imaged at 7 days old. Representative of 6 images per treatment.

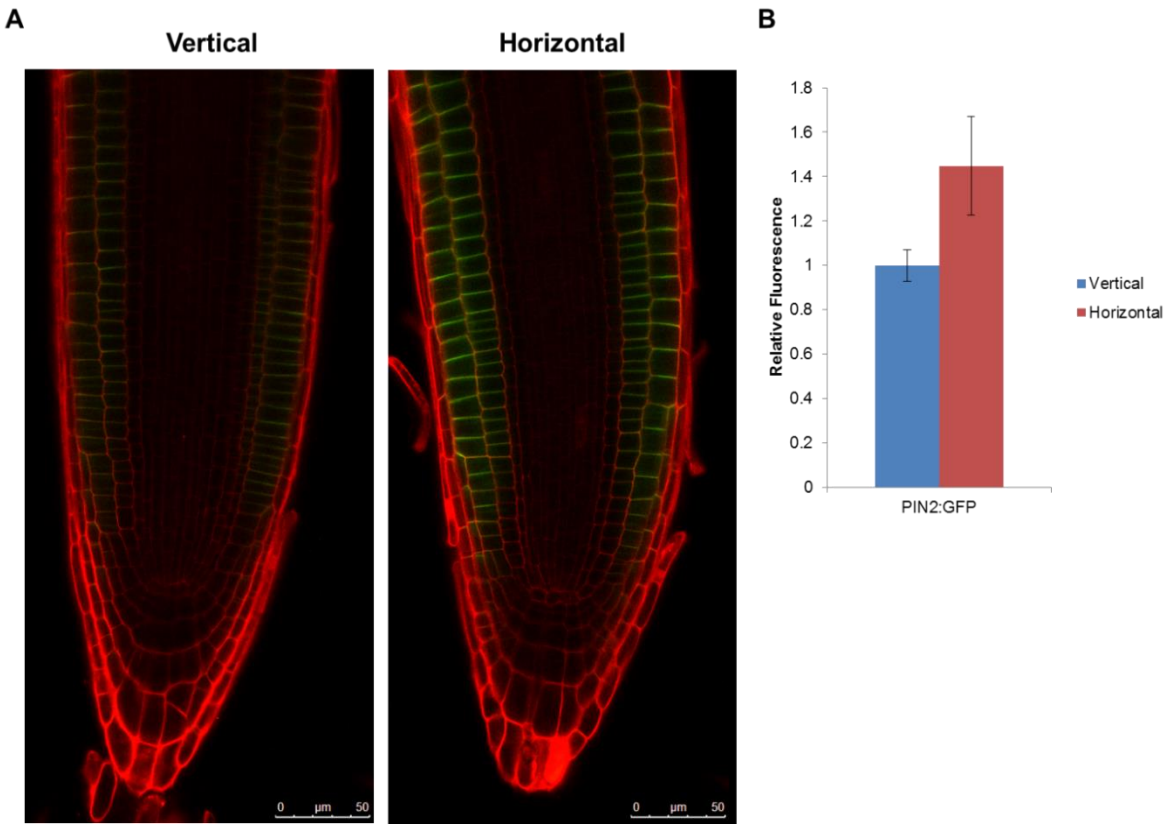

Figure 14. **The effect of mechanical impedance on PIN2:GFP expression. A,** Laser scanning confocal image of *Arabidopsis* root expressing PIN1:GFP. Green: PIN1:GFP Red: propidium iodide stain. Scale bar indicates 50 µm. **B** Relative expression of PIN1:GFP in the root tip of 7d old *Arabidopsis* seedlings (t-test, p = 0.085). Error bars show the mean +/- SE Seedlings were grown in the presence of a dialysis membrane and imaged at 7 days old. Representative of 6 images per treatment.

Overall, confocal imaging has shown that auxin distribution changes in roots responding to mechanical impedance. DR5:Venus expression indicated that auxin increases in the lateral root cap (Figure 12). This is consistent with the observation that mechanically impeded roots show reduced growth, as auxin inhibits growth in roots. Analysis of fluorescently tagged PIN protein expression revealed that both PIN1 and PIN2 show a subtle increase (Figure 13 and 14.) It is likely that this increase in expression leads to the redistribution of auxin. In conclusion, confocal analysis of auxin responsive genes further demonstrated the role of auxin signalling in facilitating the root response to mechanical impedance.

3.5 The effect of mechanical impedance on the expression of cytokinin and gibberellin responsive genes.

As well as ethylene and auxin, other plant hormones are involved in the regulation and development of roots, including cytokinins and giberellins. Cytokinins interact antagonistically with auxin, which is important for maintaining root meristem size and the position of the transition zone (Dello Ioio et al., 2008). Auxin has also been shown to downregulate cytokinin biosynthesis (Nordström et al., 2004). Gibberellins are also involved in many aspects of plant development including root growth. For example, gibberellins have been shown to increase the stability of PIN1, PIN2 and PIN3 (Willige et al., 2011) and gibberellin biosynthesis is linked to PIN1 mediated transport of auxin (Saini et al. 2013). As both cytokinins and giberellins are involved in regulation of root development, they may be involved in the root's response to mechanical impedance. Therefore, expression of cytokinin and gibberellin responsive genes under mechanical impedance was investigated using confocal microscopy.

3.5.1 The effect of mechanical impedance on expression of TCS:GFP

In order to examine the possible effect of mechanical impedance on cytokinin signalling, seedlings expressing the synthetic reporter TCS:GFP were imaged using confocal microscopy. TCS:GFP is a fluorescently tagged synthetic reporter of the cytokinin response (Müller and Sheen, 2008).

TCS:GFP expression was primarily localised in the columella of the root tip in both vertical and horizontally grown seedling (Figure 15A). Horizontally grown seedlings showed a significant decrease the relative fluorescence of TCS:GFP

compared to vertical seedlings (Figure 15B; t-test, P=0.003). This indicated that cytokinin signalling was reduced in roots responding to mechanical impedance.

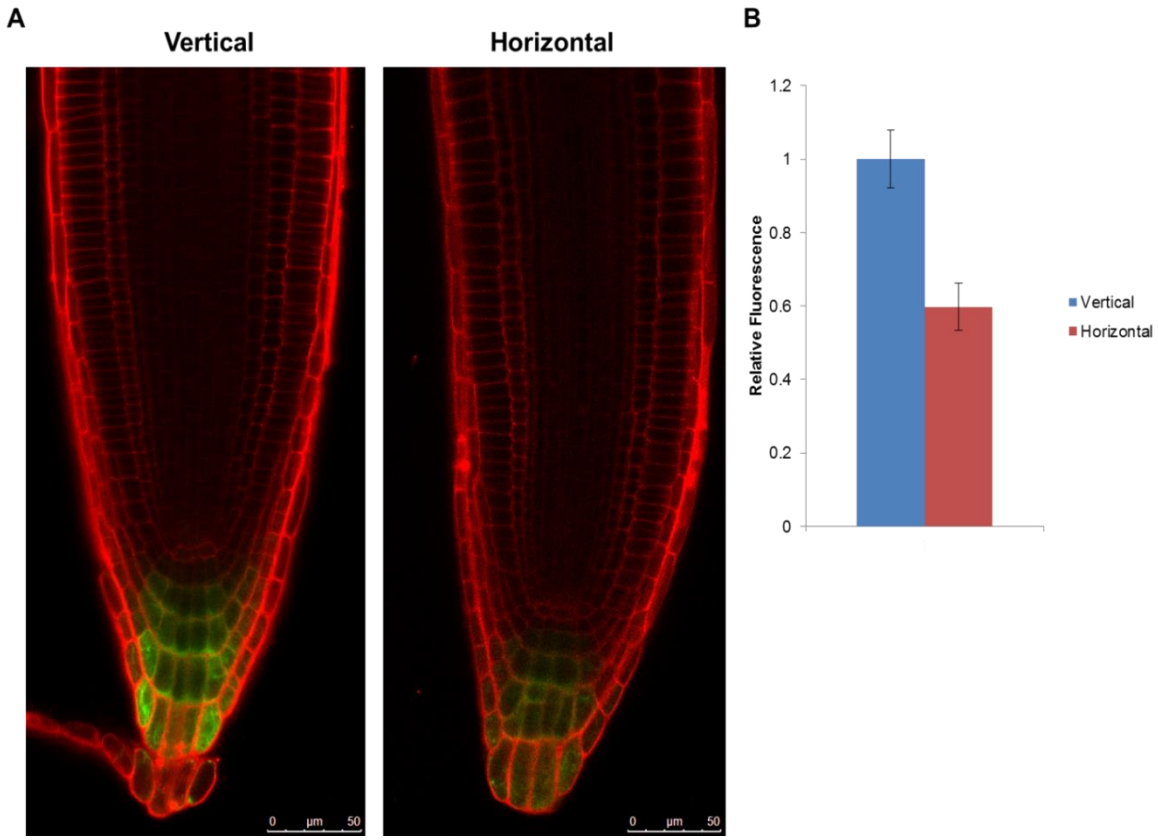

***Figure 15*. The effect of mechanical impedance on TCS:GFP expression. A,** Laser scanning confocal image of *Arabidopsis* root expressing TCS:GFP. Green: TCS:GFP Red: propidium iodide stain. Scale bar indicates 50 µm. **B** Relative expression of TCS:GFP in collumella cells of the root tip of 7d old *Arabidopsis* seedlings (t-test, p = 0.003). Error bars show the mean +/- SE Seedlings were grown in the presence of a dialysis membrane and imaged at 7 days old. Representative of 8 vertical treatment and 6 horizontal treatment images.

3.5.2 The effect of mechanical impedance on expression of RGA:GFP

In order to examine the effect of mechanical impedance on gibberellin signalling, seedlings expressing RGA:GFP (Silverstone et al. 2001) were imaged using a confocal microscope. RGA is a DELLA protein involved in inhibition of growth, such as inhibiting growth in the root elongation zone (Daviere and Achard, 2013). RGA is degraded in response to gibberellic acid (GA), therefore a decrease in RGA:GFP florescence would indicate an increased response to GA (Silverstone et al., 2001).

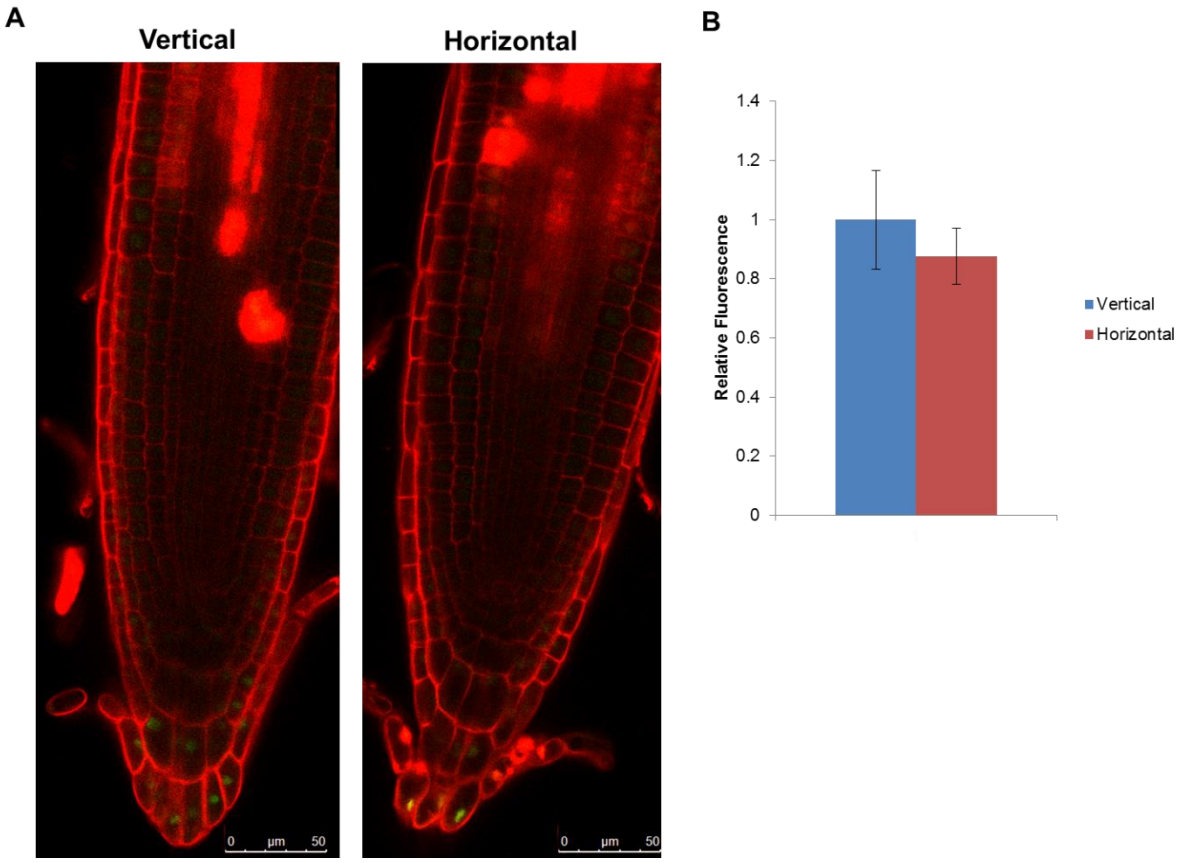

***Figure 16*. The effect of mechanical impedance on RGA:GFP expression. A,** Laser scanning confocal image of *Arabidopsis* root expressing RGA:GFP. Green: TCS:GFP Red: propidium iodide stain. Scale bar indicates 50 µm. **B,** Relative expression of TCS:GFP in collumella cells of the root tip of 7d old *Arabidopsis* seedlings . Error bars show the mean +/- SE Seedlings were grown in the presence of a dialysis membrane and imaged at 7 days old. Representative of 6 vertical treatment and-8 horizontal treatment images.

RGA:GFP was weakly expressed in the root tip, predominantly in the root cap and epidermis (Figure 16A). There was no observable difference in the relative florescence of RGA:GFP in horizontally grown seedlings compared to vertically grown ones (Figure 16B) This suggests that GA signalling is not involved in the root's response to mechanical impedance.

Analysis of the expression of cytokinin- and gibberellin-responsive genes through confocal microscopy has shown whether these hormones are involved in the root's response to mechanical impedance. Horizontally grown roots showed a decrease in fluorescence of the synthetic reporter TSC:GFP. This suggests cytokinin

signalling may be repressed during the mechanical impedance response. Gibberellin signalling however, does not appear to be affected, as the relative fluorescence of RGA:GFP did not significantly change. It appears that the root response to mechanical impedance may alter, or may be mediated by, effects of plant hormones in addition to auxin and ethylene.

3.6 The effect of mechanical impedance on the expression of genes responsive to ethylene, auxin and physical stress.

In order to further understand the how mechanical impedance affects gene expression in root tissue, qRT-PCR was used to quantify changes in gene expression. A variety of genes were investigated including those responsive to ethylene and auxin, as well as those that are known to change expression under physical stress. Firstly, to further investigate the role of ethylene signalling, the expression of *ETHYLENE RESPONSE FACTOR 1 (ERF1)*, a transcription factor involved in the ethylene signalling cascade (Solano et al., 1998), was quantified. To investigate auxin signalling, the expression of genes encoding the auxin efflux carriers PIN1 and PIN2 was quantified. The expression of *ARR5*, a cytokinin responsive gene, was also investigated to further determine the effect of mechanical impedance on cytokinin signalling. In addition *DREB2B* and *RD29B* expression was also investigated, as these genes have previously been shown to be responsive to other abiotic stresses, in particular osmotic stress. Expression of the target genes was calculated relative to the amplification of a reference gene (AT5G15710).

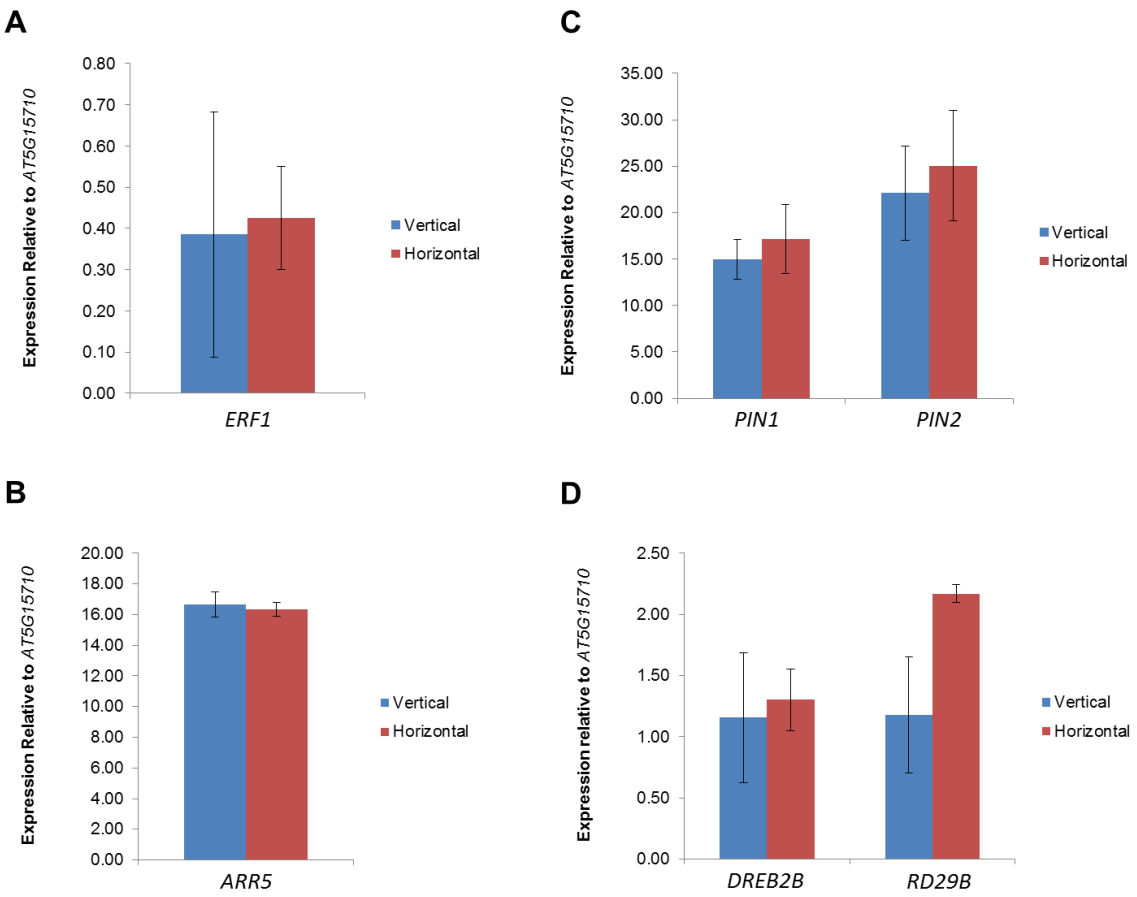

Figure 17. **The effect of mechanical impedance on the expression of genes responsive to auxin, ethylene and abiotic stress.** Quantitative real-time PCR analysis of the expression of ERF1 (A), ARR5 (B), PIN1 and PIN2, (C) DREB2B and RD29B (D). Relative expression of the genes of interest were calculated relative to the expression of a reference gene (AT5G15710). Error bars show the mean +/- SE for the relative expression of three separate experiments.

The results from qRT-PCR analysis of the expression of the genes of interest are shown in Figure 17. *ERF1* did not appear to show any significant change in relative expression between vertically and horizontally grown seedlings (Figure 17A), as did *ARR5* (Figure 17C). Although not statistically significant (Figure 17B; t-test, p = 0.64 and p = 0.73 *PIN1* and *PIN2* respectively) *PIN1* and *PIN2* both showed a small increase in relative expression under mechanical impedance. *DREB2B* showed no change in relative expression in horizontally grown seeds whereas *RD29B* showed an increase, however this was statistically insignificant (Figure 17D; t-test, p =0.17). These results suggest that the genes tested did not display any change in mRNA levels under mechanical impedance.

4. Discussion

4.1 The response of primary root growth to mechanical impedance resembles the response of roots to ethylene

When grown horizontally in the presence of a dialysis membrane, *Arabidopsis* roots experience mechanical impedance to their growth. When mechanically impeded, *Arabidopsis* roots show a change in root growth and morphology that resembles an ethylene response. Mechanically impeded roots are shorter, exhibit a small increase in diameter and root hair growth is closer to the tip (Figure 4). This result corresponds to previous reports of the nature of the root's response to mechanical impedance in a number of species including maize (Sarquis et al., 1991) and *Arabidopsis* (Okamoto et al., 2008).

A decrease in root elongation is a well-documented, characteristic response to mechanical impedance (Okamoto et al., 2008; Jin et al., 2013). This is likely due to a decrease in cell elongation, with cell length reported to be shorter in mechanically impeded roots (Croser et al., 2000; Okamoto et al., 2008). A decrease in root elongation is observed in roots treated with exogenous ethylene (Kays et al., 1974; Sarquis at al., 1991) and ethylene is known to have an inhibitory effect on cell elongation in the root elongation zone (Le et al., 2001).

Root thickening and radial swelling of cells is another characteristic widely reported in mechanically impeded roots (Clark et al. 2002; Jin et al., 2013). In the results described in this thesis only a small increase in root thickening of about 10% was observed, although previous studies have reported a greater increase in radial expansion of roots. Okamoto et al. (2008) observed an increase of around 30% in mechanically impeded *Arabidopsis*, however root diameter was measured

when seedlings were four days old as opposed to seven. This could indicate that change in root diameter is more pronounced at earlier stages of root development. Root thickening has also been reported in a number of crop species including maize, rice and barley (Sarquis et al., 1991; Clark et al., 2002; Haling et al., 2013). This response is thought to facilitate root elongation through strong soils by reducing axial stress at the root tip (Bengough et al.; 2011). It is likely that species differences can account for differences in the amount of thickening. It may also be likely that greater levels of mechanical resistance are needed to induce radial expansion than were present in the current experimental system.

Radial swelling of cortical cells has been shown to accompany reduced cell elongation in mechanically impeded roots (Croser et al., 2000). Ethylene has previously been shown to facilitate radial swelling of cells through affecting microtubule orientation (Le et al., 2004). It is likely therefore that re-orientation of microtubules is required for root thickening in mechanically impeded roots. As well as in crease in cell diameter, an increase in the number of cortical cell layers has been observed in impeded barley (Wilson et al., 1977) and pea (Croser et al., 1999) roots. Further investigation is needed into how mechanical impedance and ethylene signalling result in the radial swelling of roots. It needs to be determined whether an increased number of cortical cell layers also contributes to root thickening in response to mechanical impedance.

Root hair growth is altered in mechanically impeded roots. It was found in this thesis that mechanically impeded roots show elongated root hairs and root hair growth closer to the tip (Figure 4). A change in root hair growth is another previously reported characteristic of roots responding to mechanical impedance (Goss and Russel, 1980; Okamoto et al., 2008). Application of exogenous ethylene

has a similar effect of root hair growth, with the induction of ectopic root hairs (Le et al., 2001). Ethylene is known to stimulate root hair development (Tanimoto et al., 1995) and facilitate cell elongation in root hairs (Pitts et al., 1998). An increase in root hair growth has been hypothesised to contribute to anchorage of the primary root (Bengough et al., 2011). Evidence exists that root hairs offer an advantage for root penetration into high strength soil layers. For barley, it has been shown that genotypes that possess root hairs are more successful at growing through high strength soil than genotypes with no root hair growth (Haling et al., 2013). The increased growth of root hairs further indicates the strong role of ethylene in the root response to mechanical impedance. It is also likely that increased root hair growth offers an advantage to roots growing in strong soils (Bengough et al., 2011; Lynch and Wojciechowski, 2015).

Root hair growth relatively close to the root tip is an indication that differentiation is occurring closer to the root tip either as a result of decreased meristem activity or the shortening of the elongation zone (Rost and Baum, 1988; Sanchez-Calderon et al., 2005; Shishkova et al., 2008). It is unclear however whether mechanical impedance affects both cell elongation (shorter elongation zone) and cell division (shorter root apical meristem). Okamoto et al. (2008) reported a shortening of the elongation zone in *Arabidopsis* roots impeded by a membrane. However meristem length and number of cells was unchanged in impeded roots. This suggests that cell division in unaffected by mechanical impedance. In contrast, Croser et al. (1999) reported that both cell elongation and cell division was affected in impeded pea roots. As well as a shorter elongation zone, mechanically impeded roots had a longer cell doubling time and fewer cells undergoing mitosis. It has also been shown that mechanically impeded maize roots show a disturbed cell pattern in the root apical meristem (Potocka et al. 2011).

Ethylene has been shown to control cell division in the quiescent centre (QC) and is involved in the control of root apical meristem size and cell number (Van de Poel., 2015). Increased ethylene levels result in increased numbers of QC cells, resulting in extra columellar layers (Ortega-Martninez et al., 2007). Further study with *Arabidopsis* is needed to determine whether a similar effect is observed in mechanically impeded roots. Ethylene has also recently been shown to inhibit cell proliferation in the root apical meristem, causing a reduction in meristem size (Street et al., 2015). It is clear that mechanical impedance results in the reduction in the size of the root elongation zone (Croser et al., 1999; Okamoto et al., 2008), resulting in cell differentiation, and therefore root hair growth, occurring closer to the root meristem. However, it is unclear what the effect of mechanical impedance is on cell division and the root apical meristem. Meristem activity in impeded roots should be investigated in future studies, for example through examining the activity of CYCB1;2:GUS, a marker for cell division (Bulankova et al., 2013).

4.2 Mechanical impedance affects lateral root growth

Mechanical impedance affects not just primary root growth, but overall root architecture. Although primary root growth was reduced in mechanically impeded roots, the number of lateral roots did not change (Figure 5 A,B). Therefore the density of lateral roots is greater in mechanically impeded roots. In addition, the laterals of mechanically impeded roots were on average longer than the laterals of unimpeded roots (Figure 5 C,D). Previous studies have observed compensatory growth of lateral roots in response to mechanical impedance of the primary root axis. In barley, lateral roots grew longer when primary root growth was mechanically impeded (Goss, 1977). Although the number of laterals decreased in mechanically impeded roots, their density was reported to double. This resulted in

an overall reduction in the distance between laterals and an increase in length. Goss (1977) also reported that changes in lateral root growth could result in the total dry weight of the root system being unaffected by mechanical impedance. However increased lateral root length was only observed when pore size of the growth medium was large enough to allow lateral root growth. At smaller pore sizes growth of lateral roots was also impeded. Bingham and Bengough (2003) observed such a decrease in lateral root length in impeded roots. However lateral root growth was shown to be inhibited to a lesser extent than primary roots and the overall ratio of lateral root length: primary root length increased.

The increase in lateral root growth and density of lateral roots could be due to an increase in curvature of the primary root in response to a barrier. An increase in lateral root emergence occurs on the convex side on curving roots (Fortin et al., 1989; Smet et al., 2007). Mechanically inducing roots to bend results in lateral root formation and this can be independent of a gravitropic response (Richter et al., 2009). It is possible therefore that curvature in root growth caused by the presence of the dialysis membrane in the horizontal treatment may contribute to the observed changes in lateral root growth.

Overall it appears that lateral root growth is likely to increase in mechanically impeded roots, if their growth is not itself severely impeded, perhaps to compensate for the stunted growth of the primary root (Goss, 1977). Such an increase in lateral root growth was observed in mechanically impeded roots (Figure 5C). Lateral root growth is initially agravitropic, with gravitropism being acquired slowly after emergence (Guyomarch et al., 2012). It is possible that in the horizontally grown seedlings, the initially agravitropic lateral roots did not attempt to grow down into the membrane and therefore did not perceive a barrier.

Therefore their growth was initially unimpeded, allowing for the compensatory growth of lateral roots in response to mechanical impedance of the primary root. It is possible that lateral roots may enhance anchorage and soil exploration, providing an advantage in strong soils (Goss, 1977; Richter et al., 2009).

Mechanical impedance has a number of effects on root growth using the membrane system described in this thesis. Primary roots are shorter and exhibit characteristics of an ethylene response (Figure 4). However, more work is needed to determine how mechanical impedance affects cell elongation and division in the root tip. Ethylene is known to have an inhibitory effect on cell elongation (Le et a., 2001), at least in part through effects on auxin distribution and accumulation to growth-inhibitory levels in the elongation zone (Swarup et al., 2007; Ruzicka et al., 2007). In addition, previous studies have shown that cell elongation and length of the elongation zone is inhibited under mechanical impedance (Croser et al., 2000; Okamoto et al., 2008). However the effect on cell division and the apical meristem is still unclear. Lateral root growth is also affected but appears to be less sensitive than primary root growth to mechanical impedance. Lateral roots of mechanically impeded seedlings were longer than the vertical control (Figure 5C) However, changes in the root architecture of *Arabidopsis* needs to be examined over a longer period of time to see whether the enhanced growth of lateral roots is sustained in older seedlings.

4.3 Ethylene signalling is required for the response to mechanical impedance.

We examined the physiological response of *Arabidopsis* mutants that are insensitive to ethylene. When mechanically impeded, the roots of *etr1* and *ein2* did not exhibit the characteristic response seen in the wildtype (Figure 6). Most

notably there was no reduction in root length between vertically and horizontally grown mutant seedlings that characterises the wildtype response, with *ein2* roots in fact increasing in length under mechanical impedance. In addition, wildtype *Arabidopsis* grown in the presence of silver ions also exhibited a reduced response to mechanical impedance. Silver ions occupy the copper binding sites of the ethylene receptor ETR1, inhibiting ethylene signalling (Beyer, 1976; Binder et al., 2007). When treated with silver thiosulphate, there was a smaller difference in root length between the vertical and horizontal treatments (Figure 9A). The observed physiological response to mechanical impedance of *etr1, ein2* and silver-treated wildtype seedlings demonstrates the requirement of an intact ethylene signalling system and further demonstrates the role of ethylene in the root's response to mechanical impedance. These results confirm previous reports that ethylene signalling mutants and Ag+ treatment of wildtype seedlings results in inhibition of the response to mechanical impedance (Okamoto et al., 2008).

Further evidence for the role of ethylene comes from the response of the ethylene overproduction mutant *eto1* to mechanical impedance. Mechanically impeded *eto1* mutants showed an exaggerated response compared to the wildtype, with an even greater reduction in root length and increase in root hair growth (Figure 7). In addition the response of the *pls (polaris)* mutant was investigated. The POLARIS (PLS) peptide acts as a negative regulator of ethylene signalling (Chilley et al., 2006). Therefore under mechanical impedance it would be expected that the *pls* mutant should show a response similar to or greater than the wildtype, as the negative regulation of ethylene signalling is inhibited. Mechanically impeded *pls* mutant seedlings responded similarly to the wildtype, exhibiting an ethylene response (Figure 7). Additional experiments could be performed to examine

whether *PLS* expression is altered under mechanical impedance, as ethylene downregulates *PLS* expression (Chilley et al., 2006).

The evidence presented in this thesis and elsewhere suggests that ethylene signalling is involved in the root's response to mechanical impedance. The decrease in root growth is likely due to the inhibitory effect of ethylene on cell elongation (Okamoto et al., 2008; Strader et al., 2010). Although the role of ethylene signalling has been confirmed, the role ethylene biosynthesis remains unclear. Sarquis et al. (1991) reported that a strong correlation between mechanical impedance and ethylene production, with an increase in ethylene production preceding the observed morphological changes of the root. In addition mechanically impeded roots accumulated 1-aminocyclopropane-1-carboxylic acid (ACC), a precursor to ethylene, and ACC synthase activity increased (Sarquis et al., 1992). However in mechanically impeded *Arabidopsis* no increase in ethylene accumulation was observed (Okamoto et al., 2008). Okamoto et al. (2008) also argue that if changes in ethylene biosynthesis are required, the *eto1* mutant could be expected not to respond to mechanical impedance, as it already produces high levels of ethylene. That the *eto1* mutant does exhibit a response to mechanical impedance could be evidence that ethylene biosynthesis plays a minor role. Further investigation is needed to determine the role of ethylene biosynthesis in the response to mechanical impedance and whether it plays a more minor role than ethylene signalling. It should be noted that the time course over which ethylene production was measured differs greatly between studies. While Sarquis et al. (1991) investigated ethylene production over a period of eight hours, Okamoto et al. (2008) measured total ethylene accumulated over three days. It could be possible that ethylene biosynthesis plays a greater role in the early

response of roots to mechanical impedance. A time course of ethylene production in mechanically impeded *Arabidopsis* could be used to determine whether ethylene production increases after initial stimulation by mechanical impedance.

4.4 Correct auxin transport is required for the response to mechanical impedance.

Ethylene signalling plays an important role in the response of roots to mechanical impedance. This shown by the physiological response of mechanically impeded roots resembling an ethylene response (Figure 4) and the reduced response observed in ethylene signalling mutants (Figure 6). Ethylene is thought to regulate root growth through cross talk with auxin (Stepanova et al., 2007; Swarup et al., 2007; Ruzicka et al., 2007). We therefore also investigated the role of auxin in the response to mechanical impedance.

The auxin transport mutants *aux1* and *eir1* did show reduced root growth when mechanically impeded with *aux1* mutants actually showing enhanced root growth (Figure 8A). In addition they did not exhibit any of the characteristics of an ethylene response seen in the wildtype, such as root hair growth closer to the tip (Figure 8 B,C). When wildtype seedlings were grow in the presence of 1-N-naphthylphthalamic acid (NPA), an inhibitor of auxin efflux, they also exhibited a reduced response to mechanical impedance (Figure 10). These results strongly suggest that auxin transport is required for the response of roots to mechanical impedance. This corresponds with the findings of Okamoto et al. (2008) who reported that the *aux1* mutant was insensitive to mechanical impedance.

As auxin transport is required for the response to mechanical impedance it would be expected that auxin synthesis and distribution should alter in roots responding

to mechanical impedance. In mechanically impeded roots, DR5:Venus accumulates in the lateral root cap (Figure 12). Previous studies using GUS expression analysis have shown auxin distribution to be altered, with a spreading of DR5:GUS expression from the root tip towards the outer cells of the meristem (Okamoto et al. 2008). In addition, both PIN1:GFP and PIN2:GFP showed increased an increase in fluorescence (Figures 13 and 14). As changes in PIN protein levels have been show to correlate with changes in gene expression (Casson et al., 2009), it can be assumed this is due to increases in the level of *PIN1* and *PIN2* gene expression. These results suggest that that the inhibition of root growth in response to mechanical impedance by ethylene requires changes in auxin transport and signalling.

These results fit with previous observations that ethylene controls root growth through interaction with auxin (Stepanova et al., 2007; Swarup et al., 2007; Ruzicka et al., 2007). The reduction of root growth by ethylene requires correct functioning of auxin biosynthesis, transport and signalling. It has also been shown that under exogenous application of ethylene or treatment with ACC, auxin is redistributed towards the root meristem and elongation zone (Swarup et al., 2007; Ruzicka et al., 2007). The ethylene-induced response of auxin therefore occurs in specific regions of the root tip and this requires specific delivery of auxin to these regions (Ruzicka et al., 2007). The transport of auxin from the root tip to the meristem and elongation zone requires basipetal transport of auxin via AUX1 and PIN2. Auxin transport mutants *aux1* and *eir1* show a reduced response to treatment by exogenous ethylene or ACC (Stepanova et al. 2007). In addition the ectopic expression of DR5 under ACC treatment in not observed in these mutants (Ruzicka et al., 2007). This corresponds with our observation that *aux1* and *eir1* show a reduced response to mechanical impedance. Although ethylene signalling is

unimpaired in these mutants, their growth remains unaltered under mechanical impedance as auxin transport is impaired. Therefore the basipetal transport of auxin is required for the root's response to mechanical impedance.

Ethylene has also been shown to modulate auxin responses through expression of transport proteins. The expression of *PIN1* and *PIN2* are both upregulated by ethylene (Ruzicka et al. 2007). It is likely that ethylene has this stimulatory effect on the expression of auxin transporters when roots respond to mechanical impedance. The expression of both *PIN1* and *PIN2* exhibited a small increase in roots responding to mechanical impedance (Figures 11 and 12). However more work is needed to determine to what extent the capacity of auxin transport is regulated by ethylene under mechanical impedance. For example the expression of *AUX1* has been shown to increase under ethylene treatment (Ruzicka et al., 2007). The directional movement of auxin depends also on the polar localisation of PIN proteins (Wis´niewska et al., 2006). It should be investigated whether mechanical impedance affects the polar localisation of PIN proteins. It is possible that PIN protein localisation is altered at the subcellular level to allow for the altered directional transport of auxin under mechanical impedance.

As well as controlling root growth through auxin transport, ethylene has been shown to stimulate local auxin biosynthesis at the root apex (Swarup et al., 2007; Ruzicka et al., 2007). Okamoto et al. (2008) reported increased expression of *ANTHRANILATE SYNTHASE*(AS)-α and AS-β, along with increased levels of IAA in mechanically impeded roots. The AS enzyme catalyses the rate limiting step of Trp biosynthesis, a precursor of IAA (indole-3-acetic acid), a common active form of auxin. The transcription of both subunits has been shown to be regulated by ethylene (Stepanova et al., 2005).

Further investigation is needed to determine the extent to which auxin biosynthesis is induced, and the role it plays, in mechanically impeded roots.

Overall the evidence suggests that both ethylene and auxin have key roles in the root's response to mechanical impedance. Both ethylene signalling and auxin transport are required for roots to respond to mechanical impedance, as shown by the altered response of ethylene signalling (*etr1* and *ein2*) and auxin transport (*aux1* and *eir1*) mutants. The response of roots to mechanical impedance results in the basipetal transport of auxin from the root tip towards the outer cells of the meristem and elongation zone. It is likely that the reduction in root growth is a result of ethylene controlling root growth through auxin transport. Our results fit with previous models of ethylene action on root growth through coaction with auxin (Ruzicka et al., 2007).

4.5 The role of other plant hormones

Our results show that the plant hormone ethylene signalling through coaction with auxin is required for a response to mechanical impedance in roots. However, it is likely that other plant hormones may be involved in the response to mechanical impedance. Previous literature has focused predominantly on the role of ethylene in the response to mechanical impedance, and more work is needed to determine how other hormones are involved. For example abscisic acid (ABA), cytokinins (CKs) and gibberellins (GAs) all interact with the ethylene signalling pathway, mostly acting upstream of ethylene, to control root growth (Van de Poel et al., 2015). The role of these hormones in the response to mechanical impedance was therefore also investigated.

4.5.1 ABA signalling is not required for a mechanical impedance response

ABA interacts with ethylene to control root development and is involved in the response of roots to abiotic stress. ABA acts upstream of ethylene having a dual role on ethylene biosynthesis (Van de Poel et al., 2015). ABA can have an inhibitory effect on root growth mediated through ethylene signalling (Ghassemian et al., 2000; Thole et al., 2014) and increasing biosynthesis (Luo et al., 2014). However ethylene and ABA also show an antagonistic action. ABA can have an inhibitory effect on ethylene production (Li et al., 2011; Ludwikow et al., 2014) and ethylene in turn can limit ABA synthesis (Cheng et al., 2009). ABA is involved in the response of roots to osmotic stress, a feature that accompanies mechanical impedance in drying soils. Under moderate osmotic stress ABA maintains root growth and limits ethylene production (Xu et al., 2013). There are few studies examining the role of ABA to the response of mechanical impedance. Increased levels of ABA have been reported in roots responding to mechanical impedance (Hartung et al., 1994; Hurley and Rowarth, 1999). However it has been suggested that changes in ABA concentration are better correlated with water potential than soil strength (Dodd et al., 2010).

We examined how ABA was involved in the response to mechanical impedance through chemical inhibition of ABA synthesis by fluridone and application of exogenous ABA. Our results show that ABA signalling is not required for a response to mechanical impedance. Disrupting ABA with fluridone did not affect the response (Figure 11 A,B). Although roots were shorter in both the control and treatment, the overall response to mechanical impedance remained. In addition, high exogenous concentrations of ABA, although reducing overall root length, did not result in preventing a response to mechanical impedance. The observed

increase in ABA observed in Hartung et al. (1994) and Hurley and Rowarth (1999) could perhaps be linked to reduced water content in drying soils. It is also possible that although ABA concentrations increase in xylem tissue, this does not affect growth at the root tip. ABA might perhaps act instead as a root to shoot signal, as plant hormones can also act as a signal of an altered soil environment to the shoot (Jin et al., 2013).

In contrast to high exogenous ABA, low levels of exogenous ABA can nullify the response, maintaining root growth under mechanical impedance (Figure 11 C,D). It has previously been shown that low levels of ABA enhance root elongation, however this was shown to be independent of ethylene signalling (Ghassemian et al., 2000). ABA is also known to enhance root elongation under moderate osmotic stress (Xu et al., 2013). This result demonstrates that ABA can prevent ethylene from reducing root growth and nullify the ethylene response. More work is needed to further demonstrate whether ABA is involved in the root response to mechanical impedance, such as expression analysis of ABA responsive genes. It should also be investigated whether ABA might still play a role in the response of other parts of the plant, such as shoot growth, to mechanical impedance.

4.5.2 Mechanical impedance appears to alter cytokinin signalling but not affect giberellin signalling.

Cytokinins (CKs) and gibberellins (GAs) both interact with ethylene to control root growth and, like ABA, this is often upstream of ethylene (Van de Poel., 2015). For example, CKs can induce ethylene biosynthesis to inhibit root growth (Zd'árská et al., 2013). CKs are also known to interact with antagonistically with auxin, an interaction that has strong implications for root development, such as meristem size (Dello Ioio et al., 2008). GAs too have been shown control aspects of auxin

signalling, for example increasing the stability of PIN1, PIN2 and PIN3 (Willige et al. 2011). Both CK and GA signalling is influenced in plants responding to abiotic stress. CKs are known to have both positive and negative effects on stress tolerance. CK levels tend to decrease overall under prolonged periods of moderate stress although high levels may be maintained under severe stress (Zwack and Rashotte, 2015). In general, it also appears that GA signalling is suppressed under abiotic stress (Colebrook et al., 2014). As both ethylene and auxin interact with GA and CK to control root growth it is likely that CK and GA signalling is affected in roots responding to mechanical impedance. However their role in the mechanical impedance response is unclear.

Firstly our results suggest that CK is negatively regulated under mechanical impedance. Expression of TSC:GFP, a synthetic reporter of CK, was significantly decreased in mechanically impeded roots (Figure 15). This could be due to the increased levels of auxin, as auxin acts antagonistically with CK, downregulating CK biosynthesis (Nordström et al., 2004). This also corresponds with the observed trend that CK levels tend to decrease under prolonged moderate stress. However a decrease in CK under moderate stress is thought to be necessary to maintain root elongation (Zwack and Rashotte, 2015).

The expression of RGA:GFP did not differ between control and mechanically impeded seedlings (Figure 16). GA induces the degradation of DELLA proteins, preventing their inhibition of root growth (Daviere and Achard, 2013). That there is no change in RGA:GFP expression suggests that GA degradation of DELLA proteins does occur at the root tip under mechanical impedance. Ethylene is known to inhibit root growth via DELLA proteins and delays the GA-mediated degradation of DELLAS (Achard et al., 2003). The maintenance of RGA:GFP

expression could indicate that ethylene is preventing GA-mediated degradation. However, the overall fluorescence of RGA:GFP was low meaning that changes in DELLA protein levels may not be detected. Expression of RGA:GFP was only examined at the root tip and will need to be examined in other parts of the root, particularly the elongation zone, in order to determine their role in the mechanical impedance response.

Further study is needed to determine how both CKs and GAs interact with auxin and ethylene and how they are involved in the mechanical impedance response.

4.6 Gene expression analysis using qRT-PCR showed no significant change in the expression of target genes under mechanical impedance.

As well as the analysis of fluorescently tagged reporter lines, gene expression under mechanical impedance was examined using quantitative reverse transcription (qRT)-PCR. We investigated the effect of mechanical impedance on the expression of a of genes involved in the response to ethylene and auxin as well as other those responsive to other hormones such as cytokinin (CK) and ABA.

As ethylene and auxin appear to be involved in the mechanical impedance response, the expression genes involved in ethylene signalling and auxin transport were investigated. To further investigate the role of ethylene signalling expression of *ETHYLENE RESPONSE FACTOR 1 (EFR1)* was investigated. ERF1 is a transcription factor involved in the ethylene signalling cascade and acting downstream of EIN2. Considering the apparent role of ethylene signalling in the mechanical impedance response it would be expected that expression of *ERF1* might increase in mechanically impeded roots. However our results showed no change in the expression of ERF1 between horizontally grown mechanically

impeded roots and the vertical control (Figure 17A). This is inconsistent with previous reports that the expression of ERF1 was over five times greater in mechanically impeded roots (Okamoto et al., 2008). It also does not fit with our observations that ethylene signalling is required for the response to mechanical impedance.

The expression of the auxin efflux transporters *PIN1* and *PIN2* was also investigated and in both cases showed a small but statistically insignificant increase (Figure 17C). This is consistent with the observed increase in expression of PIN1:GFP and PIN2:GFP (Figures 11 and 12). Ethylene has previously been shown to upregulate expression of PIN proteins. Seedlings treated with ACC show enhanced expression of *PIN1* and *PIN2* (Ruzicka et al., 2007).

Other genes investigated included the CK-responsive *ARR5* and drought responsive *DREB2B* and *RD29B*. ARR5 is a transcription repressor that is induced in response to CK (Brandstatter and Kieber, 1998). Our results suggest that *ARR5* expression is unchanged in response to mechanical impedance (Figure 17B). However, as we observed a decrease in expression of the CK reporter TCS:GFP (Figure 15) it might be expected that the expression of *ARR5* should decrease also. *ARR5* has been shown to be negatively regulated by ethylene signalling in response to cold stress (Shi et al., 2012). Therefore an increase in ethylene signalling in response to mechanical impedance might be expected to induce a decrease in *ARR5* expression. Similarly *RD29B* and *DREB2B* are both induced in response to abiotic stress, notably water deprivation. *RD29B* expression is regulated by ABA whereas *DREB2B* is involved in ABA-independent signalling in response to stress. Chemical inhibition of ABA biosynthesis demonstrated that ABA is not required for a response to mechanical impedance (Figure 11 A,B). However expression of *RD29B*

appeared to increase in mechanically impeded roots, although the increase was not significantly different. In addition there was no significant change in expression of *DREB2B* in mechanically impeded roots (Figure 17D).

It is possible that qRT-PCR is not sensitive enough to detect small changes in gene expression that occur only at the tip. As whole root tissue was used for RNA extraction, it may be that specific and localised changes are not detected. Ruzicka et al. (2007) found observed increases in *PIN2* and *AUX1* expression under ethylene treatment observed using imaging techniques could not be confirmed using qRT-PCR, even though only root tissue from the last 2 mm of the root tip was used. They suggest that the effect of ethylene on transcript levels of auxin transporters may be weak and may not be detected by qRT-PCR. Therefore, in the case of *PIN1* and *PIN2*, changes in expression might not be detected at statistically significant levels. Future work should continue to examine changes in gene expression under mechanical impedance, particularly in relation to hormone signalling and abiotic stress. Expression should be examined in seedlings of different ages and exposed to mechanical impedance for different lengths of time to understand how these factors may affect changes in gene expression in response to mechanical impedance.

4.7 Further questions and future work

4.7.1 Is ethylene biosynthesis involved in the mechanical impedance response?

Our research has confirmed that ethylene signalling is involved in the response of roots to mechanical impedance, however the role of ethylene biosynthesis has not been confirmed. So far the evidence for whether ethylene biosynthesis increases in mechanically impeded roots is conflicting. Early studies on maize report increases

in ethylene production and ACC synthase activity in mechanically impeded roots (Sarquis et al., 1991, 1992). However in *Arabidopsis* no change in ethylene production was found between mechanically impeded and control roots (Okamoto et al., 2008). It is possible that the response to mechanical impedance is due primarily to changes in ethylene signalling components rather than biosynthetic components. Further investigation is needed to determine whether ethylene biosynthesis alters in mechanically impeded roots. This can be achieved through measuring the ethylene production of mechanically impeded seedlings using methods such as gas chromatography. Ethylene production should be measured across a series of time points to determine whether it plays a role in primarily in the early response to mechanical impedance. It could also be investigated whether 1-aminocyclopropane-1-carboxylic acid (ACC), a precursor of ethylene, is accumulated under mechanical impedance. In addition it should be examined whether changes in expression of genes involved in ethylene biosynthesis, such as ACC synthase and ACC oxidase, occurs in response the mechanical impedance.

4.7.2 Does ethylene and auxin signalling induce changes in cytoskeletal organisation in mechanically impeded roots?

Cytoskeletal organisation is an important factor in determining plant growth. Microtubule orientation has been linked to cellulose microfibril orientation, which in turn affects the orientation of cell growth (Baskin et al, 2001). It has previously been shown that ethylene promotes radial expansion of cells through the reorientation of microtubules (Roberts et al., 1985; Le et al., 2004). In mechanically impeded maize roots, microfibril orientation of cortical cells has been shown to change (Veen, 1982). It is therefore likely that microtubule organisation could differ in the cells of mechanically impeded roots. Future work should examine to

what extent microtubule organisation is disrupted and whether this is localised to specific tissues. It should also be investigated whether changes in the cytoskeleton of mechanically impeded roots relies on ethylene and auxin signalling. Microtubule organisation can be visualised using fluorescence microscopy in plant expressing MICROTUBULE ASSOCIATED PROTEIN 4 (MAP4):GFP. By treating seedlings with inhibitors of ethylene and auxin signalling, it can be determined whether these hormones are involved in changes to microtubule organisation in mechanically impeded roots.

4.7.3 How are other plant hormones involved in the mechanical impedance response?

It is clear from these results and previous reports that ethylene and auxin crosstalk is a key component in regulating root growth in response to mechanical impedance. The roles of other plant hormones however are less well known. We have made initial investigations into the role of other hormones, for example demonstrating that ABA signalling is not required for a response to mechanical impedance (Figure 11 A,B) and that cytokinin may be downregulated (Figure 15). Further investigation is needed to confirm whether ABA is required and to understand how crosstalk with other plant hormones mediates the response to mechanical impedance. This can be achieved through examining the response of ABA insensitive mutants, such as *abi1* and *ani2*. to mechanical impedance. In addition, the expression of ABA responsive genes under mechanical impedance should be examined. It could also be determined whether changes in auxin distribution occur in mechanically impeded roots when ABA signalling is inhibited.

4.7.4 How do roots respond when mechanical impedance and osmotic stress is combined?

As mechanical impedance is often a feature of drying soils, plant roots are likely to experience and respond to both mechanical impedance and osmotic stress (Whalley et al., 2005). Studies have now been conducted that examine the response of roots and the role of plant hormones to osmotic stress and mechanical impedance separately. Future work should now be conducted to look at how plants respond to the combination of mechanical impedance and osmotic stress. Agar plates infused with polyethylene glycol (PEG) to lower the water potential can be used to investigate the response of *Arabidopsis* to osmotic stress (Verslues et al., 2006). The use of PEG infused plates could be combined with dialysis membranes to produce a response to both osmotic stress and mechanical impedance. In this way the response of *Arabidopsis* to both stresses could be investigated.

4.8 Conclusions

Using a method previously described in Okamoto et al. (2008), we have been able to subject *Arabidopsis* roots to mechanical impedance and investigate the root response and the role of ethylene signalling. In response to mechanical impedance *Arabidopsis* roots exhibit reduced growth along with other characteristics of an ethylene response. These include thicker roots, longer root hairs and root hair growth closer to the tip. Lateral root growth may also be affected, with lateral roots growing longer if not severely impeded themselves.

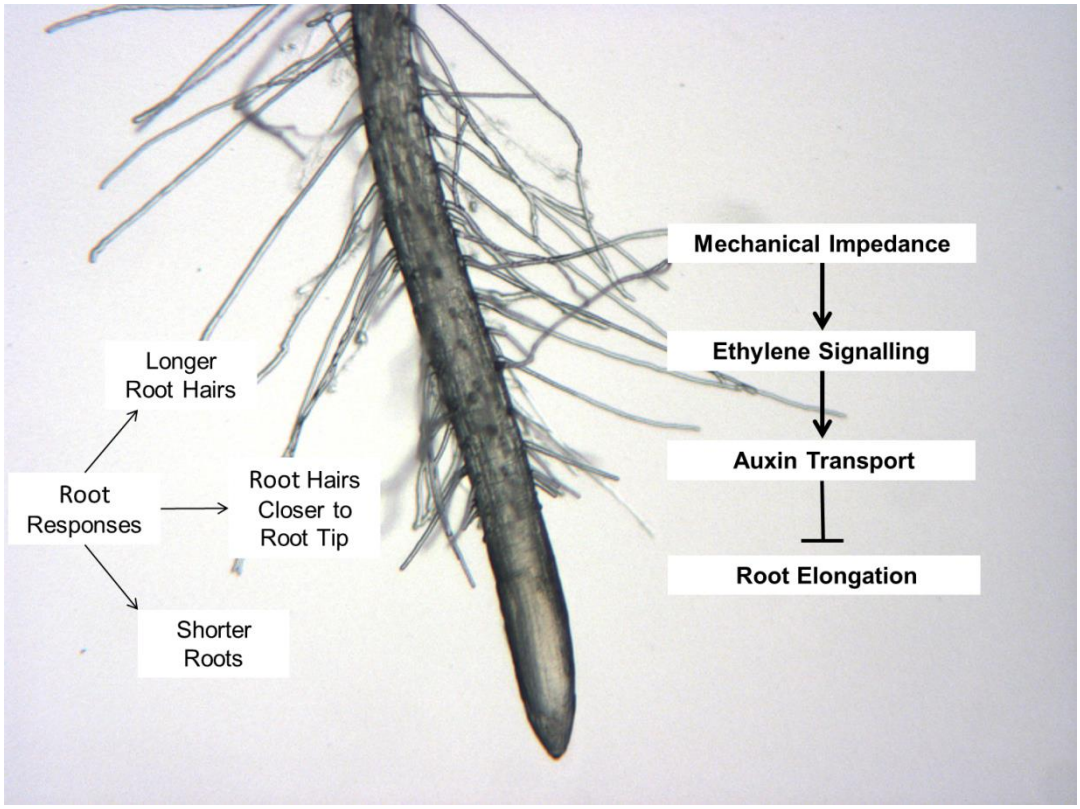

Figure 18. **The effect of mechanical impedance on root growth and the role of ethylene signalling.**

Ethylene signalling plays a major role in the root response to mechanical impedance, as shown by analysis of mutant responses and chemical inhibitors of ethylene signalling. Auxin transport is also required for a response and the expression and distribution of auxin responsive genes is altered in mechanically impeded roots. Our results suggest that ethylene signalling controls root growth in mechanically impeded roots through altering the transport of auxin (Figure 19). This fits with previous studies on how ethylene controls root growth, which demonstrated that ethylene controls root growth through crosstalk with auxin (Ruzicka et al., 2007).

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