The Mechanisms of Gravity-Dependent Non-Vertical Growth in Higher Plants

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Submitted in accordance with the requirements for the degree of Doctor of Philosophy

The University of Leeds

School of Biology

Centre for Plant Sciences

September 2018

The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others.

Acknowledgements

I would like to thank my supervisor Stefan Kepinski for his support, insight and guidance throughout this project and for both curbing, encouraging and contributing to some of my slightly more unusual methods of growing plants to obtain the data we needed.

I would also like to thank Suruchi, Marta, Rob, Martin, Adam and all the members of the Kepinski lab, past and present, that I've had the pleasure of working with over the last four years for their advice, help and support, especially when things haven't been going so well in the lab.

Thanks must also go to the undergraduate and masters students who've helped me along the way with carrying out the two huge phenotypic screens that this work involved, and to Michael Wilson for his bioinformatics wizardry when it came to identifying our causal mutation. Also to our collaborators at the John Innes Centre, Cristobal Uauy and Olu Shorinola, for providing seeds and for guidance through the minefield of researching Wheat.

To the rest of the Leeds CPS, cheers for making it a pleasant, fun and interesting environment to work in, even if the lunchtime conversations did get a little random and abstract at times.

I would like to thank the BBSRC for funding this work.

And last, but most certainly not least, I would like to thank my Mum and Dad for supporting me not just through my PhD but in everything else I've ever attempted, academic or otherwise, and for putting up with me and my plant obsession for the last 25 years. To the rest of my family for being there when I've needed them and to my friends for sticking with me and helping me blow off some steam when things have gotten a bit stressful.

Science and the obtaining of knowledge about the universe around us is a wondrous thing and I'm grateful I've had the opportunity to make my contribution.

Abstract

Plants are sessile organisms that cannot move their location in response to environmental stimuli and so, instead, react by modifying their physiology and the orientation of their growth. These alterations in growth direction are known as tropisms. Of the environmental stimuli perceived by plants, gravity is the only one that is constant and as such, gravitropism represents a base regulator of plant architecture, ensuring, among other things, that roots grow downwards and shoots typically grow upwards. The regulation of plant architecture is critical for the acquisition of resources such as light, water, and nutrients. In this context, the non-vertical growth of shoot and root branches allows the plant to optimise the orientation of lateral growth for resource capture. Much of the work carried out on gravitropic response has focused on the vertically growing primary organs, however recent work has sought to understand the maintenance of root and shoot branches at specific angles with respect to gravity, known as gravitropic setpoint angles (GSAs). Non-vertical GSAs are particularly interesting because their maintenance requires that the root and shoot branches can bend both with and against gravity. In this work, the existence of GSAs in the cereal crops rice and wheat was demonstrated and forward genetic screens were performed on mutagenised populations of wheat and Arabidopsis to identify genes regulating root GSA. Two wheat mutants were identified that maintained their altered root angles in a field trial. In Arabidopsis, a dominant point mutation was found, in a conserved region of a gene that has been previously demonstrated to regulate GSA in rice, that resulted in more vertical lateral roots. These novel genetic resources could be used to enhance nutrient and water acquisition in food crops resulting in improved yields to meet the needs of the growing world population.

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Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
AGO	Antigravitropic Offset
ATS	*Arabidopsis thaliana* salts
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
EDTA	Ethylene diaminetetraacetic acid
EMS	Ethyl Methanesulphonate
EtOH	Ethanol
GSA	Gravitropic Setpoint Angle
Hr/Hrs	Hour/ Hours
IAA	Indole-3-Acetic Acid
LB	Luria-Bertani
NAA	1-Napthaleneacetic acid
NPA	1-N-Napthylphthalamic acid
PCR	Polymerase Chain Reaction
PEO-IAA	α-(phenylethyl-2-one)-IAA
RH	Relative Humidity
RNA	Ribonucleic acid
RPH	Revolutions per hour
RPM	Revolutions per minute
RSA	Root System Architecture
SCD	Steep, Cheap and Deep
SEM	Standard Error of Mean
WT/Wt	Wild Type

Chapter 1 : General Introduction

1.1 Overview

The many and varied forms of plants have been of fascination to people for centuries. Across the plant kingdom there is wide variation in plant form, from single celled photosynthetic algae and phytoplankton to vast vascular plants such as the giant redwoods and sequoias (*Sequoia sempervirens* and *Sequoiadendron giganteum*) that are the tallest and (arguably) most massive, respectively, living organisms on the planet. To the average person perhaps the most dramatic illustration of the variation in growth habit is that of the branches of trees when they are laid bare in winter, even within a single tree species, for example *Ginkgo biloba,* a myriad of growth forms can be observed from fastigiate (upright and columnar) to pendulous (weeping).

The eventual shape of each individual plant is a consequence of both its genetics and how it has reacted to external stimuli throughout its life. As plants are sessile organisms an adaptation to overcome their lack of motility is plastic development. This means that they can modify the way that they grow in response to a change in their environment or in reaction to a stimulus. Growth towards or away from a stimulus is known as a tropism, a commonly seen example of this is that of a plant placed in a shady room growing towards the light coming from the window, this is known as phototropism. Other examples of tropisms include hydrotropism (in response to water), chemotropism (in response to a chemical signal such as nutrients available in the soil) and thigmotropism (in response to touch or pressure). Plants can respond to a stimulus positively (by growing towards it) or negatively (by growing away from it).

Of all external stimuli gravity is the only one constantly acting upon the plant therefore a response to gravity is central to the form of the whole plant. Plants must be able to react to changes in their orientation with respect to the direction

of gravity, a growth response induced by gravity is known as gravitropism (or geotropism). This is the mechanism by which plants ensure that their roots grow down and the shoots grow up, for example if a field of corn is flattened in a storm it is gravitropism that enables the plant to grow back towards the vertical.

Much of the work done so far has focused on roots that grow vertically downwards and shoots that grow vertically upwards such as the primary root and shoot of *Arabidopsis thaliana* (henceforth Arabidopsis). However, a large part of the overall shape of the plant is made up of roots and shoots that stably grow at non-vertical angles such as the lateral roots and cauline branches of Arabidopsis*,* the mechanisms behind this are less well understood. Non-vertical growth is an important adaptation that allows the plant to spread and gather resources for growth from a greater area. Many non-vertically growing organs are maintained at an angle with respect to the gravity vector, these angles are known gravitropic setpoint angles or GSAs. When these organs are displaced either upward or downward tropic growth is induced to return them to their original GSAs, the mechanisms behind this phenomenon are just beginning to come to light (Roychoudhry et al., 2013).

A number of methods have been employed to study gravitropism, for over a century devices such as a clinostat, a wheel rotating perpendicular to the gravity vector upon which the plant is placed, have been used to disrupt the plants reference to the direction of gravity (Meidner, 1985) and since the beginnings of space flight plants have been sent into the low gravity environment of the earth's orbit (Johnson and Tibbitts, 1968, Lyon, 1968). More recent work has focused on how the genetic makeup and resulting molecular biology of plants affects their growth habit and response to gravity. A number of genes have been found that alter the growth angle, for example overexpression of the gene *DEEPER ROOTING 1 (DRO1)* in a shallow rooting rice cultivar results in a steeper root angle (Uga et al., 2013) and a knockout of the gene *LAZY1* in Arabidopsis results in less vertically growing lateral branches (Yoshihara et al., 2013, Yoshihara and Spalding, 2017, Taniguchi et al., 2017).

It is hoped that with a greater understanding of gravitropism and growth angle control we will be able to produce crops with a greater ability to capture resources to produce higher yields. Already the gene *DRO1* has been used to create rice that produces improved yields under moderate drought conditions (Uga et al., 2013). As more genes are discovered and the pathways become better understood this should pave the way for crops optimised for yield and suboptimal growth conditions hopefully aiding in alleviating the world food crisis.

1.2 Historical work on gravitropism

Much of the initial work on gravitropism was carried out in the 19th and early 20th centuries although some observations were being made as early as the late 17th century. The plant anatomist Nehemiah Grew (1641-1712) observed that shoots uniformly grew upwards and roots uniformly downwards in seeds germinating without soil (Meidner, 1985). This was followed with experiments by Thomas Knight who's letter to Joseph Banks in the Philosophical Transactions of the Royal Society in January 1806 outlines his thoughts and experiments on gravitropism (Knight, 1806). He placed bean seeds around the circumference of a horizontally rotating wheel and noted that at 250 revolutions per minute the root and radicle deviated 80° from the vertical, below 80 revolutions per minute the root and shoot deviated 45° from the vertical, he hypothesised that this was caused by the centrifugal force counteracting the force of gravity (Knight, 1806). He went on to give an early description of asymmetric growth during gravitropic bending of the shoots: "the vessels and fibres on the underside of the germen invariably elongate much more rapidly than those on its upper side; and thence it follows that the point of the germen must always turn upwards" (Knight, 1806).

Julius Von Sachs also used a device based upon a rotating wheel to study gravitropism, this was an early version of what we know today as a clinostat (Meidner, 1985). A clinostat is a rotating wheel upon which the plant is mounted perpendicular to the direction of the gravity vector, this subjects the plant to omnilateral gravitational stimulation and disrupts the plants reference to gravity (Roychoudhry et al., 2013). He also postulated that the streams of "shoot forming substances" are altered in an inverted *Opuntia* (Prickly pear) and that

this results in changes in its growth pattern (Sachs, 1887), this is perhaps an early allusion to the flows of hormones such as auxin through the plant being directors of its growth. Albert Bernhard Frank coined the term “geotropism” in 1868, he also recognised that unequal growth on opposing sides of an organ is the mechanism for curvature (Meidner, 1985).

In their book “The Power of Movement in Plants” Charles and Francis Darwin present their idea that geotropism, apogeotropism (negative gravitropism) and diageotropism (growing at a right angle to the gravity vector) are modified forms of circumnutation (Darwin, 1880). They also noted that in experiments with phototropism the perception of light in one part of a grass coleoptile results in an “influence” that is transported resulting in bending towards the light in another part of the coleoptile, this again could be an early allusion to the flows of hormones throughout the plant (Darwin, 1880). They also expanded on and verified experiments earlier carried out by the Polish scientist Theophil Ciesielski who first noted that when he removed the root cap the root was no longer able to respond to gravity (Darwin, 1880), he also postulated that there was an “influence” transmitted from the root cap that was necessary for gravitropic bending (Tivendale et al., 2014). These experiments showed that the root cap was essential for both graviperception and gravitropic bending of the root (Darwin, 1880).

By the early 20th century the field of plant hormone research was in its infancy and the first auxinic compounds were being discovered, this was facilitated using the *Avena* bioassay developed by Went in the late 1920s (Went, 1928). An agar block containing the compound to be tested was placed on one side of the tip of an excised *Avena sativa* coleoptile and the curvature it induced was measured (Went, 1928). The first compounds identified as auxins were auxenotriolic acid and auxenolonic acid, indeed Indole-3-acetic acid (IAA) (that we now know to be the predominant natural auxin involved in driving tropic growth (Mockaitis and Estelle, 2008)) was not isolated from higher plants until the early 1940s although its auxinic properties had been known for some time (Enders and Strader, 2015). The discovery of auxinic compounds led to the proposal that upon perception of an asymmetric stimulus such as reorienting

the plant with respect to the direction of the gravity vector or a change in the direction of light, an asymmetry in auxin concentration is produced by lateral transport across the organ. This results in asymmetric growth on opposing sides of the organ which leads to bending, this became known as the Cholodny-Went model of tropic bending (Cholodny, 1927, Went, 1928, Lomax, 2007, Firn et al., 2000).

Since the advent of molecular biology in the latter half of the 20th century a large amount of phytohormone research has been carried out that has proven auxin to be central to the regulation of many plant growth processes (Teale et al., 2006) and laid the foundations of our understanding of plant hormones today. Since the advent of the first plant genome sequence, *Arabidopsis thaliana,* in 2000 (Initiative, 2000) our understanding of the genetic control of many plant growth processes has increased rapidly, this has opened the doors to unlock more of the mechanisms behind gravitropism and the control of plant growth angle.

1.3 Gravity sensing and statocytes

Plants sense gravity using specialised cells known as statocytes, these contain starch filled amyloplasts which act as statoliths allowing the plant to perceive the direction of gravity (Baldwin et al., 2013). The starch-statolith hypothesis is currently the best-supported explanation for how plants detect the direction of the gravity vector. Mutants such as *phosphoglucomutase 1* (*pgm1*) that lack a functional starch synthesis enzyme show a reduced gravitropic response in both roots and shoots (Kiss et al., 1989, Weise and Kiss, 1999), due to lack of starch the statoliths do not sediment in the direction of gravity or when subject to centrifugal force (Kiss et al., 1989). However, whilst starch filled amyloplasts are needed for full gravitropic response, in the roots some gravitropic capacity is maintained in the starchless mutants (e.g. *pgm1*) (Kiss et al., 1989) suggesting an additional mechanism for gravity sensing in the root (Morita and Tasaka, 2004).

In the roots of both dicots, such as Arabidopsis and monocots, such as the cereal crops wheat and rice, the statoliths are found in the columella cells of the root cap (Figure 1.1A) (Kaufman et al., 1995, Leitz et al., 2009), this is in agreement with the conclusions that Darwin and Ciesielski drew relating to the root cap being essential for gravity response (Darwin, 1880). Within the columella of the Arabidopsis root cap, there are three horizontal and four vertical files of columella cells, it was found through laser ablation that the cells of the second tier of the columella from the root tip made the greatest contribution to root gravitropic response (Blancaflor et al., 1998, Morita and Tasaka, 2004).

In the shoots of dicots the statocytes are found along the length of the shoot in the innermost layer of the cortex (also known as the starch layer or endodermis) which surrounds the vasculature (Figure 1.1D), this means that they lie on the boundary between the site of transport and the site of growth (the outer layers of the cortex) (Sack, 1991, Fukaki et al., 1998). Characterisation of the mutants *shoot gravitropism 1* and *shoot gravitropism 7* in Arabidopsis has shown that these mutants lack the starch layer, this provides evidence that this layer is needed for shoot gravitropism (Fukaki et al., 1998).

In contrast to dicots, cereal crops such as rice (*Oryza sativa*) and wheat (*Triticum aestivum*) possess specialised gravisensitive organs at points along their stems; these are known as pulvini (Figure 1.1B) (Kaufman et al., 1987). The pulvinus is a swollen disc of tissue that appears as a characteristic “bump” on the stem (Clore, 2013), there are two types of pulvinus found in grass species, the stem/culm pulvinus and the leaf sheath pulvinus. Different families of grasses can possess either one or both of these types of pulvinus, for example the *Festucoideae* have no culm pulvinus but a specialised leaf sheath pulvinus whereas the *Panicoideae* have a well developed culm pulvinus but a poorly developed leaf sheath pulvinus (Brown et al., 1959).

A pulvinus is made up of an internal and external epidermis, parenchyma, collenchymatous tissue and vascular bundles. The parenchyma cells around

the vascular bundles function as the statocytes (although all parenchyma cells contain plastids) (Dayanandan et al., 1976) placing them on the boundary between the sites of transport and growth in a similar manner to the shoot statocytes of dicots (Sack, 1991, Fukaki et al., 1998). Prior to stimulation there is almost no elongation in the cells of the pulvinus (Figure 1.1C) (Arslan and Bennet-Clark, 1960), once stimulated there is an asymmetric growth response similar to that outlined by the Cholodny-Went model. Cells apart from those on the upper part of the pulvinus elongate to varying degrees in a graded fashion with those closest to the upper side elongating less than those closer to the bottom side of the pulvinus (Dayanandan and Kaufman, 1984), this creates the bending of the joint.

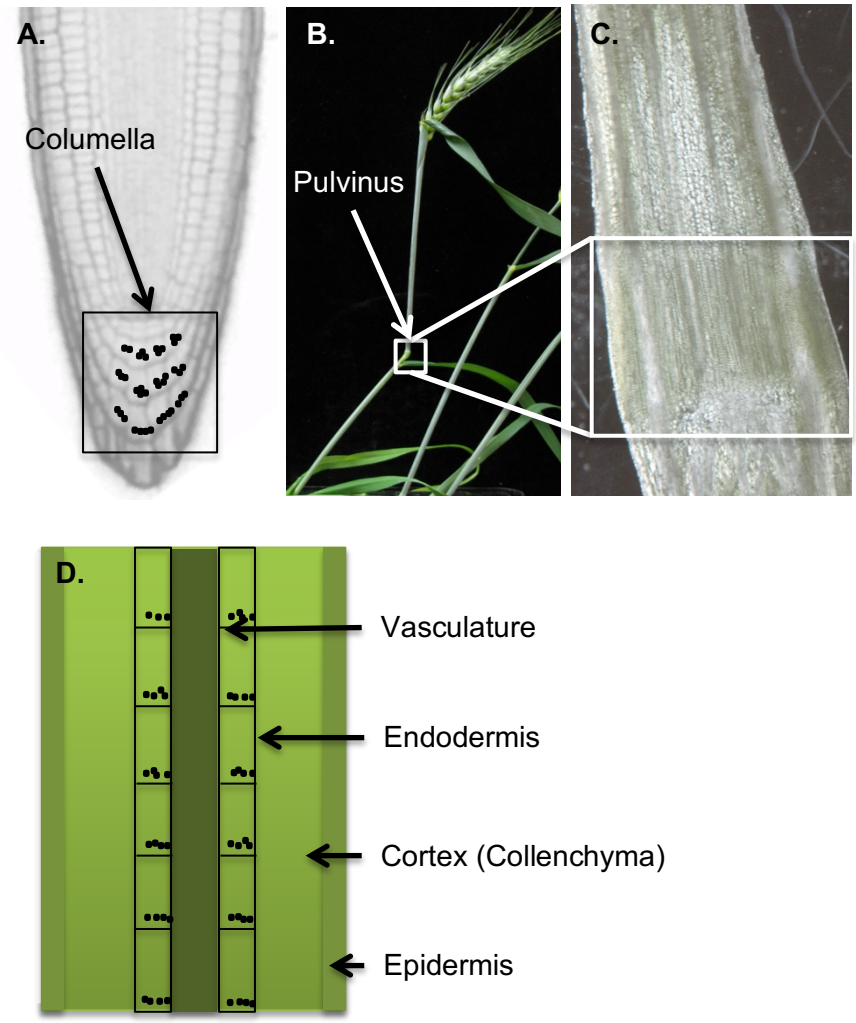


Figure 1.1: Gravity sensing tissues in monocots and dicots
In the roots of both monocots and dicots gravity is sensed in the columella cells of the root tip (**A**, statoliths have been added for illustrative purposes). In the shoots of monocots such as wheat, gravity is sensed in specialized organs known as pulvini (**B**), the response to gravity also occurs in the pulvinus tissue, prior to gravistimulation there is almost no cell elongation within the pulvinus (**C**). In the shoots of dicots such as Arabidopsis gravity is sensed in the endodermis situated between the vasculature and the cortex (**D**).

The ability of a pulvinus to respond to a gravitational stimulus is time dependent, in order to respond, a pulvinus must be relatively free of lignin (which confers structural rigidity and mechanical strength to plant cells (Whetten and Sederoff, 1995)). It is noted that there is a delay in the lignification of the pulvinus when compared with the other tissues of the plant however, once lignified, the pulvinus can no longer respond to a stimulus (Kaufman et al.,

1987, Dayanandan et al., 1977). It also needs to be taken into account that a young pulvinus that does not possess sufficient amyloplasts also cannot respond to a gravitational stimulus, for example, the pulvini of *Avena fatua* (wild oats) do not begin to respond until they have formed 14-16 statoliths per statocyte (Wright, 1986), this means that there is a window of response for each pulvinus. When a plant is lodged (prostrated by wind or rain), it takes the collective response of a number of pulvini in order to bring the plant back upright. In *Zea mays* (maize) for example, it takes 3-4 pulvini bending over the course of 6 days to allow the plant to reach the vertical again, each pulvinus bends no more than 30 degrees and the whole bend is shared between the pulvini (Collings et al., 1998). Interestingly, excised pulvini of barley and oats are capable of making a full 90-degree bend; this perhaps indicates some sort of cross talk between pulvini (Kaufman et al., 1987). An individual pulvinus is capable of responding to a stimulus a number of times, if a gravistimulated pulvinus is allowed to respond and then rotated through 180 degrees it will re-respond and bend in the opposite direction to the first response (Kaufman et al., 1987). It is capable of doing this a number of times (this has been tested up to five reorientations) however the time it takes to achieve similar curvatures increases with each successive reorientation and the pulvinus itself appears to increase in total length (Kaufman et al., 1987).

The columella cells have a highly polarised structure with the nucleus remaining in the “top” (in relation to the direction of the gravity vector) of the cell and a large amount of endoplasmic reticulum (ER) in the “bottom” of the cell, the central region is relatively free of ER to allow the statoliths to sediment with the direction of gravity (Leitz et al., 2009, Sievers, 1991, Kiss et al., 1989). There are a number of models as to how the sedimentation of the statoliths confers gravitational information to the cells; many of these involve interaction of the statoliths with either the cytoskeleton or the ER (Staehelin et al., 2007).

Experiments with microtubule disrupting drugs such as Latrunculin B showed that actin disruption does not inhibit gravitropism in both stems and roots; in hypocotyls the speed of gravity response was increased (Yamamoto and Kiss, 2002). This leaves the ER as the best candidate for transmission of the gravity

stimulus into a growth response; it has been hypothesised that the sedimentation of the amyloplasts onto the ER could mediate gravity sensing (Volkmann, 1979, Hensel and Sievers, 1980) and this triggers a number of downstream signalling responses (Baldwin et al., 2013). The role of the ER in gravity sensing is supported by the recent discovery of a unique kind of ER, Nodal ER, observed exclusively in the columella cells. Nodal ER consists of a central rod-like structure with a diameter of around 100 nM; this is composed of oblong subunits to which several sheets of rough ER are attached along their margins. Patches of nodal ER can be found at the boundaries of the relatively empty central region of the cell and the ER on the periphery of the cell (Zheng and Staehelin, 2001). However as nodal ER is unique to columella cells (Staehelin et al., 2007) it is unlikely to be involved in the core gravitropic response as the mechanism of gravity perception would likely be common to both root and shoot statocytes.

1.4 The gravity signalling pathway

Whilst the evidence described above provides a case for the sedimentation of the statoliths being a key step in gravity sensing, how the directional information of statolith sedimentation is transformed into a growth response is still relatively unknown.

When the statoliths sediment onto the ER it is known that the ER is bent and distorted by their weight, this not only provides evidence of the importance of the ER in gravity sensing but opens a number of possibilities as to signal transduction. The first is that of mechanosensitive or stretch-mediated ion channels that are opened up in the ER when it is distorted by the statoliths or the movement of the statoliths causes actin microfilaments to tug on the ER and open the channels, another is that of ligand-receptor interactions formed between elements on the statolith membrane and the ER membrane (Staehelin et al., 2007, Leitz et al., 2009, Baldwin et al., 2013).

Once open a mechanosensitive ion channel would change the local concentration of an ion within a cell, the lanthanide elements lanthanum and

gadolinium are known to be general inhibitors of cation selective stretch activated ion channels (Caldwell et al., 1998) and they have been found to inhibit gravitropism both strongly in the case of gadolinium and weakly in the case of lanthanum (Ding and Pickard, 1993). However, all stretch mediated ion channels thus far identified in Arabidopsis do not contribute to the gravitropic response though there may be as yet unidentified channels that are involved in the response (Baldwin et al., 2013). The possibility of the actin cytoskeleton being involved in opening mechanosensitive ion channels for the gravitropic response is unlikely as the addition of the microtubule disrupting drug Latrunculin B results in an enhancement of the speed of the gravitropic response in hypocotyls (Yamamoto and Kiss, 2002), also, the roots of germinating flax seeds gain the ability to respond to gravity after the formation of the statoliths but before the formation of the actin cytoskeleton (Ma and Hasenstein, 2006).

A likely candidate for a cation that could be involved in gravitropism would be Ca^{2+}, calcium has long been thought to be involved in the gravitropic response and has the ability to modulate and transduce a wide variety of signals. Calcium signalling is easily modulated by calmodulins and other chelating agents allowing the formation of both transient and prolonged signalling responses. The ER acts as an intracellular store of Ca^{2+} and the concentration within is of 3 to 4 orders of magnitude of that found free in the cytosol (Sinclair and Trewavas, 1997), this again makes calcium an ideal candidate to be involved in the gravitropic response. However, so far no changes in cytoplasmic Ca^{2+} levels have been detected in gravistimulated columella cells (Legué et al., 1997) although the high expression levels of calmodulins in the columella cells (Stinemetz et al., 1987) could suggest that changes in cytoplasmic Ca^{2+} could either be small or highly localised therefore undetectable with the resolution of current detection strategies (Baldwin et al., 2013). Changes in Ca^{2+} concentrations have been detected in other gravistimulated tissues (hypocotyls and petioles but not roots) but the results of Toyota et al and Plieth and Trewavas suggest that these changes are downstream of auxin redistribution as these changes are attenuated by the auxin transport inhibitors napthylphthalamic acid (NPA) and 2,3,5-triiodobenzoic acid (TIBA) (Toyota et

al., 2008, Plieth and Trewavas, 2002), this throws doubt on the idea that Ca^{2+} changes are involved in the early graviresponse.

However there may be more evidence supporting the role of Ca^{2+} in a later role in the graviresponse. Inositol triphosphate ($InsP_3$) is a phosphorylated sugar formed by the cleavage of Phosphatidylinositol 4,5-bisphosphate (a membrane lipid) into two fragments, $InsP_3$ and diacylglycerol (DAG) (Baldwin et al., 2013), there is evidence to suggest that $InsP_3$ acts as a signalling molecule in plants and that it is involved in the control of Ca^{2+} levels. An injection of photoactivatable $InsP_3$ into the guard cells results in an influx of Ca^{2+} into the cytosol causing the stomata to close (Gilroy et al., 1990, Blatt et al., 1990). Fluctuations of $InsP_3$ have been detected in graviresponding pulvini of both oats (*Avena sativa*) and maize (*Zea mays*) and in the inflorescence stems of Arabidopsis (Perera et al., 1999, Perera et al., 2001, Perera et al., 2006). In the pulvini of maize within 10s of gravistimulation a transient 5-fold increase in $InsP_3$ concentration was observed in the lower half of the pulvinus, this then fluctuated between the upper and lower halves for 30 minutes before between 2 and 4 hours settling at higher levels on the lower half. Within 8-10 hours the bending became visible and $InsP_3$ concentrations returned to basal levels across the pulvinus (Perera et al., 1999), this suggests that $InsP_3$ has role in the early stages of gravibending both before and during the initiation of cell elongation. Overexpression of a human derived inositol triphosphatase, which hydrolyses $InsP_3$, causes gravitropic defects in both roots and stems further demonstrating the role of $InsP_3$ in gravitropic response (Perera et al., 2006). Additionally, the knockout mutant of the Arabidopsis Inositol polyphosphate 5-phostphatase *5PTase13*, which thus has higher levels of $InsP_3$ as it cannot be broken down, shows an increased gravitropic response in roots and is less sensitive to treatment with NPA suggesting that 5PTase 13 is involved in gravitropic auxin redistribution (Wang et al., 2009b).

The second possibility for transducing the physical signal of statolith sedimentation into a chemical response is that of a ligand-receptor complex being formed between elements on the statolith membrane and on the ER membrane. The interaction between a ligand and a receptor can trigger a

number of changes in a cell these can include phosphorylation cascades and changes in signalling molecule concentration such as those already discussed (Baldwin et al., 2013). Support for the idea of a ligand-receptor complex being involved in graviperception can be found in the rhizoids of the alga *Chara globularis* (Limbach et al., 2005), when the weight of the sedimented statoliths is increased using lateral centrifugation it does not enhance the gravitropic response whereas intermittently interrupting the interaction between the statoliths and the plasma membrane by quickly inverting the cells reduces the response considerably. This suggests that contact is more important to the response than weight on the membrane (Limbach et al., 2005) suggesting that a ligand-receptor complex is the more likely method for graviperception in *Chara* than that of a mechanosensitive ion channel. However, so far the proteins involved in this have not been identified and the statoliths of *Chara,* filled with $BaSO_4$, do not share a common ancestry with the starch filled statoliths of vascular plants therefore it is possible that two entirely different mechanisms of graviperception evolved in *Chara* and vascular plants (Baldwin et al., 2013).

It has been suggested that the Translocon of the Outer Membrane of Chloroplasts (TOC) complex may have a role in root gravitropism (Stanga et al., 2009, Strohm et al., 2014). The gene *ALTERED RESPONSE TO GRAVITY 1 (ARG1)* encodes a J-domain protein that takes part in early gravity signal transduction in statocytes (Baldwin et al., 2013, Boonsirichai et al., 2003), the knockout mutant *arg1* displays gravitropism defects. Two enhancer mutants to *arg1* were discovered using EMS mutagenesis that react normally to the application of phytohormones such as auxin and are not involved in phototropism, their roots grow in random directions and they do so only when the *arg1* mutation is present (Stanga et al., 2009). These two mutants were named *modifier of arg1 (mar) 1* and *mar2*. The *mar1* mutation maps to the *TOC75-III* gene and is the result of an amino acid change (G658R), knockout mutants of this gene are embryo lethal (Baldwin et al., 2013, Stanga et al., 2009). The *mar2* mutation also maps to a component of the TOC complex, *TOC132* and results in the formation of a premature stop codon preventing the formation of its C-terminal membrane anchor domain, the *mar2-1* single mutant

is gravitropically similar to wild type, its gravitropic defects only arise in combination with the *arg1-2* mutation (Strohm et al., 2014, Stanga et al., 2009). It is likely that *ARG1* and *MAR2* function in the early part of gravity sensing as they respond normally to application of exogenous auxin and the auxin transport inhibitor NPA (Stanga et al., 2009). The TOC complex allows the import of nuclear encoded proteins into plastids, it consists of a pore (Toc75/MAR1), a receptor from the Toc159 family (these include: Toc159, Toc132/MAR2, Toc120 and Toc 90) and a receptor from the Toc34 family (these include Toc33 and Toc34/PPI3) (Strohm et al., 2014). ARG1 is a peripheral membrane protein that localises to some of the same components of the vesicle trafficking pathway as the PIN auxin efflux carriers but not to plastids (Boonsirichai et al., 2003). From their localisation it is not obvious why these mutations would link to additive gravitropism defects, this has led to a number of hypotheses: Direct interaction, Toc132 acts as a ligand on the plastid that interacts with a receptor on the ER, the activity or localisation of the receptor is controlled by ARG1(Strohm et al., 2014). Targeted interaction, Toc132 mediates the import (via Toc75) and localisation of a molecule that acts as a ligand on the plastid that interacts with a receptor on the ER membrane, the activity or localisation of the receptor is controlled by ARG1 (Strohm et al., 2014). Indirect interaction, Toc132 mediates the import of a molecule onto the plastid that does not act as a ligand but is nonetheless required for gravitropism in an ARG1 background (Strohm et al., 2014). The results of Strohm et al indicate that the direct interaction model is incorrect as mutations in other components of the TOC complex than Toc132 also enhance the gravitropism phenotype of *arg1* implying that protein import function into the plastid is necessary for gravitropism, this is supported as when a truncated version of Toc132 lacking its cytoplasmic domain but retaining its import capacity is transformed into the *arg1-2 toc132* mutant its phenotype is rescued (Strohm et al., 2014). They also examined the effect of a *pgm1-1 arg1-2 mar2-1* mutant and found that *arg1-2 mar2-1* enhanced the gravitropic defects of *pgm1-1* suggesting that amyloplast sedimentation is not required for the gravitropism defects of *arg1-2 mar2-1* and that these two mutations function in separate pathways, this makes the indirect interaction model the most likely although the targeted interaction cannot be fully ruled out as the gravitropic defect enhancement was only slight (Strohm et al., 2014).

Although the method of signal transduction is still unclear there are a number of physiological changes known to occur after gravitropic stimulation. Before the relocalisation of the auxin efflux proteins that direct growth there is a rapid alkalinisation of the cytoplasm within the columella cells but not elsewhere in the root, this raises the pH from7.2 to 7.6, this rise in pH is not apparent in the *arg1-2* mutant (Boonsirichai et al., 2003, Fasano et al., 2001, Baldwin et al., 2013). In conjunction with the increase in cytoplasmic pH of the columella cells, the apoplast around the cells acidifies, the pH decreases from 5.4 to 4.7, this suggest that protons are being pumped from the columella cells and into the apoplast (Fasano et al., 2001).

1.5 Auxin response

Once a gravity signal has been detected the gravitational information gained from the statoliths, by whatever mechanism, must be translated into tropic growth to reorient the plant. Plants do not exhibit cell migration and as such must use chemical messages in the form of hormones or other signals (e.g. ions such as Ca^{2+}) passed from cell to cell either by active or passive transport to convey messages from one part of the plant to another (Esmon et al., 2004). There are a number of important plant hormones that control various processes throughout the plant, these include: Cytokinins, Gibberellins, Brassinosteroids and Auxins. Auxins (from the Greek “to increase”) were the first substances to be functionally identified as hormones in plants (Esmon et al., 2004), they, in particular Indole-3-Acetic Acid (IAA, further mention of auxin will refer to IAA unless otherwise specified) modulate many aspects of plant growth and development including many fundamental cellular processes such as division, differentiation and expansion (Mockaitis and Estelle, 2008). It is the ability to control cell expansion that makes auxin the main hormone that drives tropic growth (although fluctuations in other hormones and small molecules are observed and thought to be involved (Baldwin et al., 2013)). Its asymmetric distribution across an organ wanting to carry out a bend from a low concentration on the upper side to a high concentration on the lower side allows the cells to expand in a graded fashion (Chen et al., 1999, Firn et al., 2000). Auxin has opposing effects in roots and shoots; in shoots auxin promotes cell

elongation whereas in roots it inhibits cell elongation. Therefore in shoots the cells with higher concentrations of auxin expand more than those with lower concentrations this means that the organ will bend in the correct direction and vice-versa in roots, this is consistent with the Cholodny-Went model of tropic growth (Chen et al., 1999, Firn et al., 2000).

It is thought that auxin is primarily synthesised from the amino acid tryptophan, mainly via the indole-3-acetamide (IAM) and indole-3-pyruvic acid (IPA) pathways, although other pathways do exist including a tryptophan independent pathway, it is thought that the IAM and IPA pathways are the major biosynthetic routes to IAA (Mano and Nemoto, 2012). Tryptophan is synthesised from chorismate in a six step biosynthetic pathway that is well conserved across all kingdoms (although animals and some eubacteria lack the ability to synthesise tryptophan for themselves and must obtain it from external sources) (Radwanski and Last, 1995). Through the IAM pathway there are a further two steps to convert tryptophan into IAA, tryptophan is first converted into IAM by tryptophan-2-monooxygenase and then this is converted into IAA by indole-3-acetamide hydrolase (Camilleri and Jouanin, 1991, Mano and Nemoto, 2012). Through the IPA pathway tryptophan is converted into IPA by multiple enzymes including *TRYPTOPHAN AMINOTRANSFERASE RELATED 1 (TAR1)* and *TRYPTOPHAN AMINOTRANSFERASE of* ARABIDOPSIS *1/TRANSPORT INHIBITOR RESPONSE 2*(*TAA1/TIR2*), the IPA is then converted into IAA by *YUCCA1 (AtYUC1* in Arabidopsis*)* (Mano and Nemoto, 2012).

Once inside the cell response to auxin is mediated by a number of different proteins. Auxin responsive genes are activated and repressed by a number of proteins that bind to auxin responsive elements in their promoters to allow or prevent transcription. Auxin response elements at their most basic may consist of a TGTCTC motif that can be present multiple times and at different spacings within the promoter allowing different levels of repression and activation of the gene (Guilfoyle et al., 1998). The auxin response elements are bound by a family of proteins known as the AUXIN RESPONSE FACTORs (ARFs) of which there are 23 in Arabidopsis, few of the loss of function mutants display a visible phenotype, this suggests a functional redundancy within the family (Roosjen et

al., 2017, Salehin et al., 2015, Okushima et al., 2005). ARFs can act as both activators and repressors of transcription and contain four domains, domain I is responsible for DNA binding, domain II is responsible for activation/repression of transcription and domains III/IV are responsible for dimerization with both AUX/IAAs and other ARFs. ARFs 5,6,7,8 and 19 have a glutamine rich domain II and function as activators of transcription whereas the rest have a domain II rich in proline, serine or leucine/glycine and are thought to act as repressors (Salehin et al., 2015, Guilfoyle and Hagen, 2012). Mutations in the ARFs can impair many auxin related processes, for example: mutations in ARF7 impairs the expression of many auxin induced genes and negatively impacts differential growth needed for organ bending in response to a stimulus (Harper et al., 2000), mutations in ARF5 give rise to a rootless phenotype known as *monopteros*. It has also been found that ARFs 1 and 2 regulate floral senescence and ARFs 7 and 19 regulate leaf expansion and lateral root development, this demonstrates that the ARFs are involved in many plant growth processes (Teale et al., 2006).

The AUX/IAAs are short-lived proteins that act as transcriptional repressors (or co-repressors when paired with a repressive ARF) (Kepinski and Leyser, 2005). In Arabidopsis there are 29 AUX/IAA genes each consisting of four conserved domains, the role of domain I is unknown, domain II is a highly conserved sequence known as the degron which is recognised by the SCF^{TIR1} ubiquitin ligase complex and domains III/IV act as dimerization domains facilitating the interaction between the AUX/IAAs and the ARFs (Kepinski and Leyser, 2005, Zenser et al., 2001, Tan et al., 2007). IAA17 also possesses the ability to dimerise with itself (Kepinski and Leyser, 2005, Reed, 2001). IAAs 1 and 2 are reported to contain putative nuclear localisation signals (Abel et al., 1994). Different AUX/IAAs are expressed in a tissue specific manner and auxin induces their effects to different degrees, this may give some functional specificity to each AUX/IAA (Reed, 2001). The degron is an important domain in terms of response to auxin; it is the site of recognition between the auxin receptor TIR1 and the AUX/IAA, the recognition causes the AUX/IAA to be targeted for degradation and allows for transcription of the gene under control of an activating ARF (Kepinski and Leyser, 2005). The degron contains a 13

amino acid consensus sequence needed for interaction with TIR1, this consensus sequence is QVVGWPPVRSYRK, and of this sequence the central GWPPV residues are essential for the interaction with TIR1 (Kepinski and Leyser, 2004). Mutations in the degron of a number of the AUX/IAAs has produced a number of dominant or semi-dominant gain of function mutants, these include *shy2-1*(IAA3)*, bodenlos* (IAA12) and the *axr* mutants (IAAs 7 and 17). For example the *axr3-1* mutant contains an amino acid change in the centre of the degron of IAA17, instead of the GWPPV sequence it has a single amino acid change to GWPLV (Rouse et al., 1998), it is thought that mutations such as this stabilise the AUX/IAA and prevent its degradation (Reed, 2001), this strengthens its repression of auxin activated genes.

In order for auxin responsive genes to be transcribed their repressive AUX/IAAs must be removed; this is done via degradation of the AUX/IAA by the 26S proteasome, in order to be degraded the AUX/IAAs must be targeted for degradation. In the case of AUX/IAAs this is via an E3 ubiquitin ligase that is a member of the SCF-type ubiquitin-protein ligase family, SCF type ligases are found in many protein degradation dependent pathways throughout eukaryotes (Kepinski and Leyser, 2005, Deshaies, 1999, Moon et al., 2004). The SCF ligases consist of many subunits and it is from these subunits that they derive their names, they contain a SKP1 protein, a Cullin, an F-Box protein and RBX1. SKP1 links the F-Box protein to the Cullin and RBX1 links the E2 ubiquitin ligase to the Cullin, the RBX-Cullin dimer catalyses the transfer of ubiquitin from the E2 ligase to the substrate bound to the F-box protein, it is the F-Box protein that give the SCF ligase complex its substrate specificity (Deshaies, 1999, Kepinski and Leyser, 2005, Moon et al., 2004). The F-Box protein TIR1 acts as an auxin receptor, the binding of auxin into the receptor pocket stimulates the interaction between the AUX/IAA and TIR1 acting as a kind of “molecular glue”, this allows the AUX/IAA to be ubiquitinated and degraded (Figure 1.2) (Kepinski and Leyser, 2005, Tan et al., 2007).

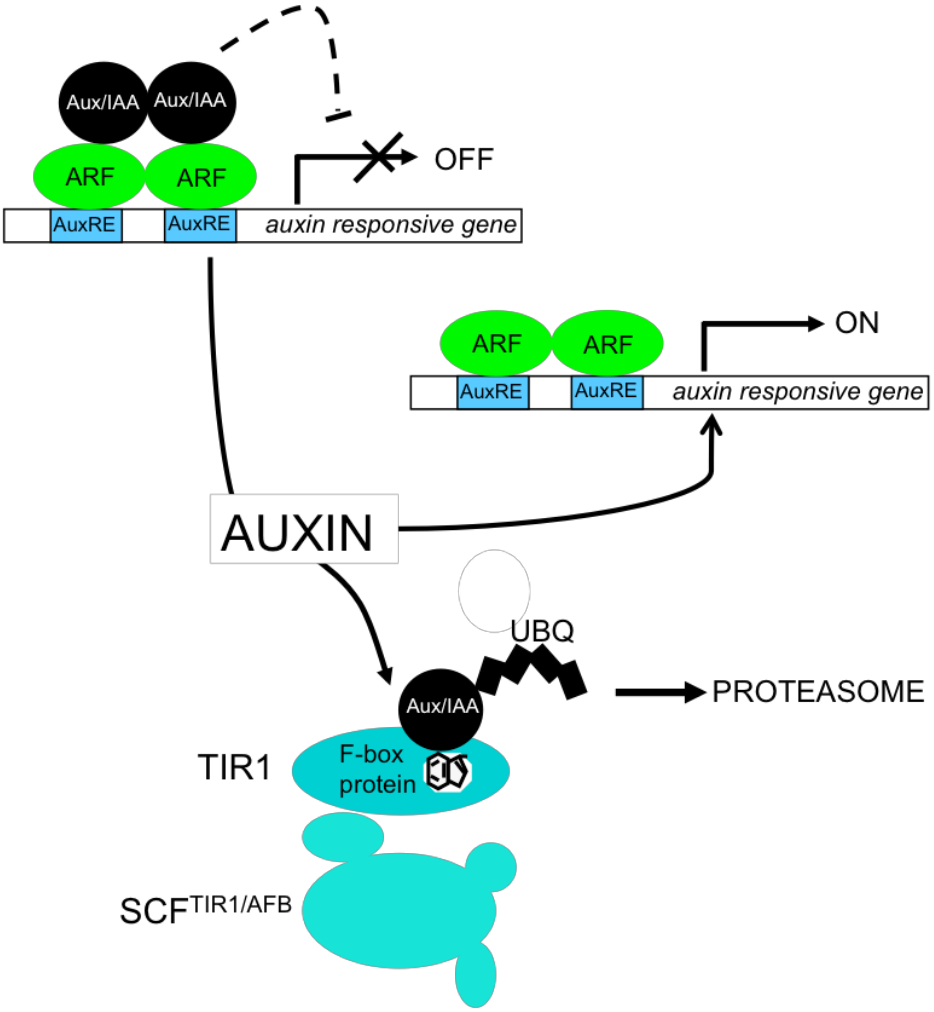


Figure 1.2: The auxin response pathway
An ARF is bound to the promoter of an auxin inducible gene via its DNA binding domain, an Aux/IAA is bound to this ARF preventing it from activating the transcription of the gene. The binding of auxin into the pocket of TIR1 promotes the interaction between the SCF/TIR1 complex and the Aux/IAA by acting as a kind of “molecular glue”, the SCF ligase complex ubiquitinates (UBQ) the Aux/IAA targeting it for degradation by the 26S proteasome. Lower levels of the Aux/IAA result in removal of the repression allowing the gene to be transcribed.

1.6 Auxin transport

Auxin is thought to be primarily synthesised in the shoot tips, young leaves and developing seeds of plants (Ljung et al., 2005, Normanly et al., 1993, Friml and Palme, 2002), some de novo synthesis of auxin can also be observed in other tissues such as at the root tips of both primary and lateral roots (Ljung et al., 2005). Active, free auxin synthesised in the aerial parts of the plant is transported basipetally in a unidirectional manner from the shoot apex in a process known as Polar Auxin Transport (PAT), this flow of auxin from the apex has long been linked to phenomena such as apical dominance and tropisms (Lomax et al., 1995). In roots the auxin stream continues down from the shoots towards the root tip in an acropetal manner where some of it is then redirected back towards the elongation zone, the acropetal movement of auxin from the shoot to the root is thought to be involved in lateral root development (Reed et al., 1998, Rashotte et al., 2000) and the basipetal movement of auxin back from the root tip to the elongation zone is implicated in the gravitropic response (Rashotte et al., 2000, Rashotte et al., 2001).

Families of influx and efflux carrier proteins mediate the generation of the gradients of auxin that drive tropic growth and other responses; it is an energy dependent process (Friml and Palme, 2002). The amino acid permease like transporter AUX1 and the AUX1/AUX1-LIKE (LAX) family of transporters are involved in auxin influx into the cell (Kramer, 2004). A p-glycoprotein-like ABC transporter sequence, AtPGP4 has also been reported to have auxin influx activity (Santelia et al., 2005, Terasaka et al., 2005) however its role in auxin influx is controversial as it has also shown activity against a number of different structurally unrelated substrates (Terasaka et al., 2005).

Auxin efflux is controlled by the PIN-FORMED (PIN) family of proteins, these are a set of transporters whose asymmetric distribution around the cell membrane directs the flow of auxin inside plants (Kramer, 2004, Friml, 2003, Benková et al., 2003). In Arabidopsis there are genes encoding 8 PIN proteins each with overlapping, tissue specific expression patterns (Ganguly et al.,

2012). It is thought that rice (*Oryza sativa*) contains at least 12 PINs that are evolutionarily related to the Arabidopsis PINs, these include four PIN1, one PIN2, three PIN5, one PIN8 and three monocot specific PIN proteins, PIN9, PIN10a and PIN10b (Wang et al., 2009a), in maize (*Zea mays*) there are 14 PIN or PIN-like genes (Villiers and Kwak, 2012). A truncation in the rice PIN2 (*OsPIN2*) results in a reduced gravitropic response and larger root angle, the Arabidopsis *pin2* mutant can be rescued by transformation using the full-length coding sequence of *OsPIN2* under the control of the *AtPIN2* promoter, this highlights the similarities and conservation between the PIN proteins of different species (Wang et al., 2017).

The PINs are membrane proteins consisting of ten hydrophobic transmembrane regions (5 N-terminal and 5 C-terminal) and a hydrophilic loop of varying length, the Arabidopsis PINs can be separated into two groups according to loop length. The long loop PINs (1,2,3,4,6 and 7) have a hydrophilic loop of between 298 and 377 residues whereas the short loop PINs (5 and 8) have a loop of between 27 and 46 residues, the long loop PINs are found on the plasma membrane whereas the short loop PINs are found in the ER though their auxin transport capacity and function in the ER have yet to be determined (Ganguly et al., 2012, Ganguly et al., 2010).

In the roots of Arabidopsis, PIN1 localises to the basal end of the vascular cells (though weak signals can be detected in the epidermis and cortex), PIN2 localises apically in the epidermal and lateral root cap cells but basally in the cortical cells, PIN3 is expressed in the cells of tiers two and three of the columella without notable polarity, it is also expressed at the basal side of vascular cells and on the lateral side of the pericycle cells in the elongation zone. PIN4 is found in the quiescent centre and to the basal side of the provascular cells, PIN7 also localises to the basal side of the provascular cells in the meristem and elongation zone but shares its distribution with PIN3 in the columella cells (Ganguly et al., 2010, Blilou et al., 2005, Müller et al., 1998). It is this tissue and membrane specific expression and localisation of the different PIN proteins that creates the “fountain” of auxin transport in the roots of plants that is crucial to their development and ability to carry out tropisms (Petrášek

and Friml, 2009). In shoots, PIN1 is important in the regulation of phyllotaxis (the pattern with which organs emerge from the SAM), it is expressed in the vasculature of leaf primordia and in flower primordia and its expression and localisation changes throughout organ formation and outgrowth. The *pin1* mutant is still capable of forming leaves but their size, shape and positioning around the stem are abnormal (Reinhardt et al., 2003).

PINs 3 and 7 are the PINs primarily involved in gravitropic response in Arabidopsis roots (Friml et al., 2002, Guyomarc'h et al., 2012, Palme et al., 2006). The *pin3* single mutant shows a reduced gravitropic response but differential growth is not completely abolished (Friml et al., 2002), the *pin3pin7* double mutant shows a greater reduction in gravitropic response than was observed for the single mutant (Kleine-Vehn et al., 2010). There is evidence of functional redundancy between similar PINs (Vieten et al., 2005), due to their similar expression patterns and the additive effects of the double mutant it is thought that there is redundancy between PIN3 and PIN7 in the gravitropic response, indeed an asymmetry can be seen in the distribution of both PINs in the columella cells upon gravistimulation (Kleine-Vehn et al., 2010). It has also been reported that in *pin* mutants the expression patterns of the remaining PINs can be altered to compensate for the lost PINs, for example in the *pin3pin7* double mutant the expression of PIN4 expands into the lateral root cap and in the *pin3pin4pin7* triple mutant PIN2 expression has been detected at the basal end of provascular cells where PIN3 and PIN7 are normally found (Blilou et al., 2005).

PIN proteins are constitutively cycled between the plasma membrane and endosomal compartments (Kleine-Vehn et al., 2010). GNOM is the endosomal regulator of vesicle budding, it encodes a guanine nucleotide exchange factor for adenosyl ribosylation factors (ARF-GEF) (Kleine-Vehn et al., 2008). It is thought to be important in PIN targeting to a basal localisation but not to an apical localisation, ARF-GEF GNOM is sensitive to the fungal toxin brefeldin A (BFA) and when treated with BFA there is an accumulation of the PIN proteins in BFA compartments due to inhibition of ARF-GEF PIN cycling indicating the need for functional ARF-GEF GNOM for PIN recycling from plasma membrane

to endosomes and vice-versa (Kleine-Vehn et al., 2008). Current findings suggest that upon gravity stimulation it is internalisation of the PIN proteins that plays a key role in establishing PIN polarity in the columella cells as there is no evidence for increased degradation of both PIN3 and PIN7 (Kleine-Vehn et al., 2010). When treated with BFA, accumulation of PIN3-GFP is observed in BFA compartments in gravity stimulated roots whereas in unstimulated roots this is only observed occasionally and with a weaker fluorescence intensity than in stimulated roots, this along with the use of mutants with a weak allele of GNOM (GNOM knockouts are seedling lethal) suggests that gravity stimulation recruits PIN3 into an ARF-GEF GNOM dependent polar recycling pathway to generate an asymmetric plasma membrane localisation (Kleine-Vehn et al., 2010). Experiments using FRAP (Fluorescence Recovery After Photo bleaching) and the protein synthesis inhibitor CHX have been used to confirm that it is the movement of PIN3 from one side of the cell to another (this can be termed Transcytosis) as opposed to de novo synthesis and targeting of PIN3 to the new “bottom” of the cell upon gravistimulation that produces the asymmetry in PIN3 needed to produce the auxin asymmetry needed to mount a gravitropic response. Similar asymmetries are observed in PIN7 indicating a functional redundancy between PIN3 and PIN7 during gravity response (Kleine-Vehn et al., 2010).

The reversible phosphorylation of PIN proteins plays an important role in modulating auxin transport and its dependent processes (Sukumar et al., 2009, Zhang et al., 2010, Benjamins et al., 2001). Phosphorylation of PINs has been shown to target them for cycling into a trafficking pathway that leads to an apical/shootward polarity (Dhonukshe et al., 2010), the PIN phosphorylation sites are located upon the hydrophilic loop and are highly conserved across the long-loop PINs (Zhang et al., 2010, Barbosa et al., 2014). Phosphorylation and dephosphorylation are mediated by a number of Kinases and Phosphatases. The kinases known to phosphorylate PINs are PINOID, WAG1 and WAG2 (PID/WAGs) and the D6 PROTEIN KINASEs (D6PK and D6PK-LIKE), these are members of an evolutionarily conserved family of serine/threonine protein kinases that are related to the mammalian cAMP-dependent, cGMP-dependent and Ca^{2+} dependent protein kinases but sit within their own structurally distinct

family found in Arabidopsis known as the AGCVIII family (Barbosa and Schwechheimer, 2014, Santner and Watson, 2006, Benjamins et al., 2001, Christensen et al., 2000). All of these kinases are associated with the plasma membrane and knockout mutants share similar phenotypes to those of *pin* knockouts. The *pinoid* mutant shares a phenotype with the *pin1/pin-formed* mutant which exhibits a “pin-like” inflorescence stem with either no or heavily deformed flowers (Okada et al., 1991), the *d6pk* mutants share a phenotype with *pin2* of slight agravitropism and the *wag/pid* mutants share a phenotype with the *pin3, pin4* and *pin7* mutants (Barbosa and Schwechheimer, 2014), the *wag* mutants also display enhanced root waving on inclined plates (Santner and Watson, 2006). The phosphatase complex PP2A is thought to act antagonistically to PINOID as loss of function mutants display gravitropism defects and a basal to apical shift in PIN polarity in the roots (Michniewicz et al., 2007).

Huang et al (2010) have shown that PINOID (and possibly the other AGCVIII kinases) phosphorylate PIN1 in three evolutionarily conserved TPRXS(N/S) (where X is any amino acid) motifs, the loss of ability to phosphorylate these sites (as shown through using site directed mutagenesis to change the phosphorylatable serines into unphosphorylatable alanines) results in a number of dominant defects, especially that of three cotyledon leaves, this phenotype is also characteristic of the *pid* mutant. Phosphomimic lines were also created by changing the serines into glutamic acid, these phosphomimic lines were unable to rescue the phenotype of the *pin1* inflorescence defects indicating that reversible phosphorylation of these sites is required for normal *pin1* function (Huang et al., 2010). Ganguly et al show that the two phosphorylation sites on PIN3 for PINOID are RKSNASRRSF(/L) and TPRPSNL (the latter of which is similar to that found on PIN1 by Huang et al (Huang et al., 2010)), it was found that mutations in the serines of the first site had the most marked effect when introduced into the *pin3* mutant line as mutations in the first site failed to rescue the defective gravitropism phenotype of *pin3* (Ganguly et al., 2012). Single or double mutations of the five S/T residues had little effect on the membrane targeting of PIN3 but an increase in phenotypic effects is seen with triple, quadruple and quintuple mutants suggesting a redundancy in their roles in PIN3

trafficking (Ganguly et al., 2012). Interestingly these mutations did not impede WAG1 phosphorylation as WAG1 was able to phosphorylate both the wild type and phosphosite mutant lines to a similar level suggesting that WAG1 has a different phosphosite on PIN3 (Ganguly et al., 2012). Through observing PIN3 trafficking in the phosphomutants in different cell types, the authors pose that the phosphorylation sites are interpreted differently in different cells types and that cell type specific factors in combination with PIN species may be key to the control of PIN polarity in different cells of the plant (Ganguly et al., 2012). We have yet to fully understand the extent to, and the mechanisms by which PIN phosphorylation contributes to the gravity response.

1.7 The gravitropic setpoint angle

Much of the work thus far carried out on gravitropic response in plants has been carried out on primary roots and shoots. However, if we are to truly understand the mechanisms that govern plant architecture we must also understand the growth angle control of the lateral organs.

In a recent review Roychoudhry and Kepinski separate growth angle into two broad classes; those that are maintained with respect to gravity independently of other parts of the plant and those that are not (Roychoudhry and Kepinski, 2015). The latter is a broad group and could include many different angles found within plants such as the angle of the leaves in the leaf rosette of Arabidopsis or the angles of the leaf veins in the leaves of many species. A third grouping could be added to this to include angles that are set with respect to gravity but are not maintained. For the first group, those whos angles are set and maintained with respect to gravity, the angle that they form with direction of the gravity vector (0° is vertically downwards) is known as the Gravitropic Setpoint Angle (GSA) (Roychoudhry and Kepinski, 2015, Digby and Firn, 1995). The GSA concept was originally proposed by Digby and Firn as “the angle with respect to gravity at which an organ shows no gravity induced differential growth in order to correct its orientation” (Digby and Firn, 1995). This is a good way to define GSA as it separates GSA, which is a growth angle, from gravitropism, which is a growth response. When a branch is reoriented above

its GSA it induces differential growth to bend downwards to return the branch to its GSA, similarly, if a branch is reoriented to below its GSA differential growth will be induced to cause the branch to bend upwards to return to its GSA (Figure 1.3). Vertical growth upwards (for example the primary shoot) can be defined as having a GSA of 180° and vertical growth downwards (for example the primary root) would have a GSA of 0° (Roychoudhry and Kepinski, 2015). Recent work has shown that when decapitated (the primary shoot apical meristem/SAM is removed) many plants respond by allowing the branch of the subapical node to become the new primary (Cline, 1996). This results in a shift in the GSA of that branch from its initial non-vertical GSA to a near vertical GSA of around 180°. This change is suppressed by application of exogenous auxin to the stump of the severed leader, simulating the synthesis in, and flow of from, auxin from the now severed SAM, which confirms the involvement of auxin in the maintenance of GSA and indicates the presence of a switch with features similar to that of the mechanism that controls bud outgrowth (Cline, 1996, Roychoudhry et al., 2013).

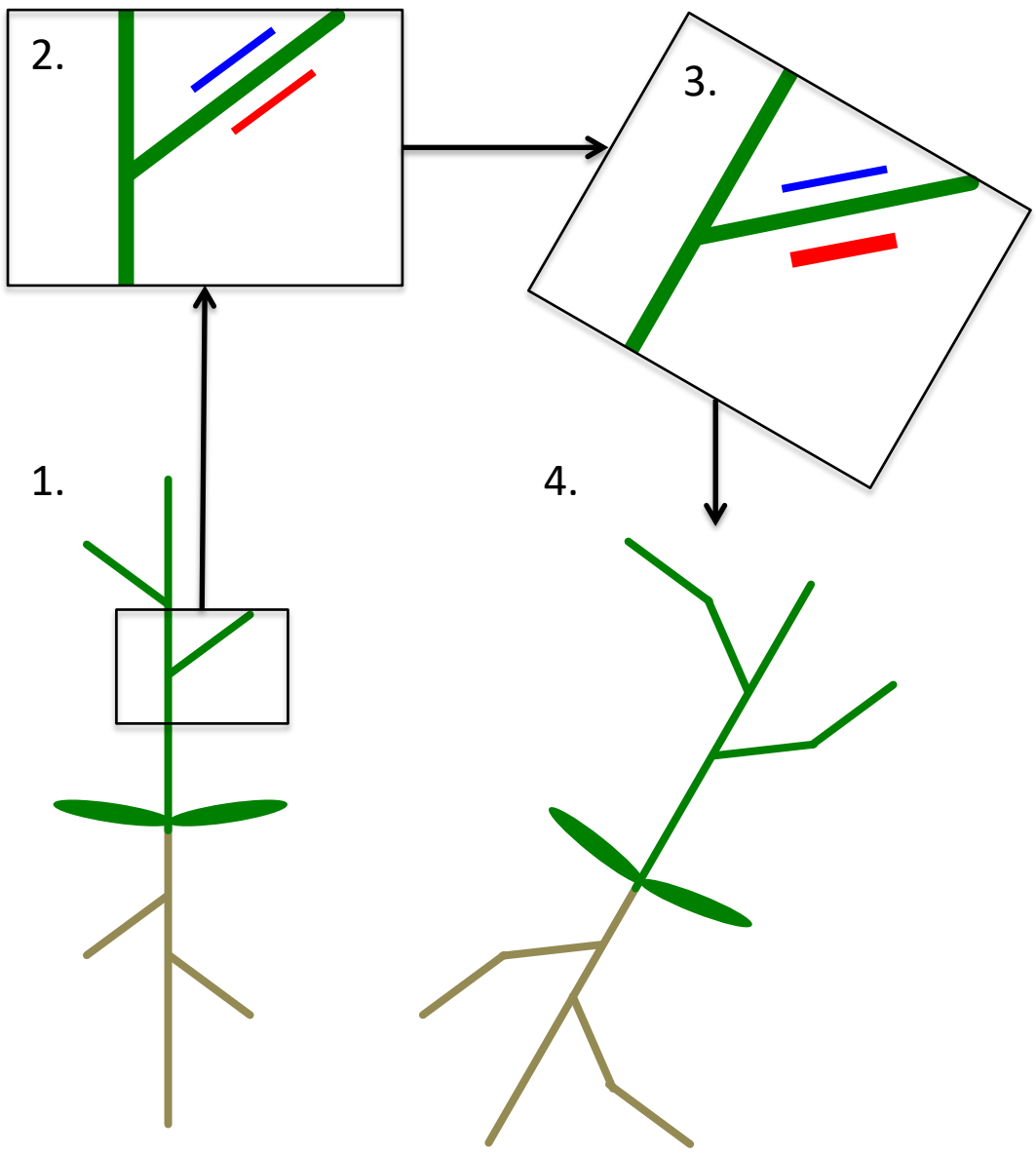


Figure 1.3: GSA maintenance in a lateral shoot
1. The lateral shoot is growing at its GSA, 2. the gravitropic and antigravitropic growth components are balanced and the shoot is growing stably at a non-vertical angle. 3. The plant is reoriented and senses this, more auxin is redistributed to the bottom side of the shoot causing the cells on this side of the shoot to elongate more. 4. Differential growth is induced to return the shoot to its original angle with respect to the gravity vector.

Clinorotation of Arabidopsis produces a rapid outward bending response in the lateral organs but not in the primary root and shoot. As clinorotation subjects the plant to omnilateral gravitational stimulation and disrupts the plants reference to the gravity vector, this suggests that the maintenance of a non-vertical GSA is dependent on an angle offset mechanism that is still active when a “gravitropic growth component” is removed, this mechanism can be described as the

“Antigravitropic Offset” (AGO) component that acts in tandem with the gravitropic component to produce balanced non-vertical growth. Importantly this offset mechanism is not active in the primary axis, this results in its near vertical growth (Roychoudhry et al., 2013). The idea of an antigravitropic growth component is not a new one; it was posed by De Vries in 1872 following some of the earliest clinorotation experiments where an outward bending of lateral organs was observed (Vries, 1872) and again following experiments in space in the late 1960s (Johnson and Tibbitts, 1968, Lyon, 1968). De Vries termed this growth epinasty and its cause has long been a subject of debate as it was thought to be an ethylene induced stress response due to clinorotation (Salisbury and Wheeler, 1981, Lyon, 1972). Through clinorotation of an ethylene insensitive mutant, *ein2-1*, Roychoudhry et al demonstrated that the outward bending of the lateral organs was not due to ethylene induced stress but the plant reacting to the withdrawal of normal gravity stimulation. The authors went on to demonstrate that the AGO is auxin dependent by carrying out clinorotation on both the roots and shoots of plants treated with the auxin transport inhibitor 1-N-Naphthylphthalamic acid (NPA). Upon these treatments plants showed significantly reduced outward bending upon clinorotation (Roychoudhry et al., 2013).

As a result of their findings Roychoudhry et al pose a model of lateral growth in which the angle at which a lateral organ grows is the product of opposing gravitropic and antigravitropic growth components. These components act in tandem to produce a net balance in auxin flux across the organ resulting in maintained lateral growth (Roychoudhry et al., 2013). When the organ is at its GSA the magnitude of both of these components is equal but there is a difference in the regulation of the magnitude of the components. The magnitude of the gravitropic component is constantly changing dependent on where the branch is in relation to the gravity vector; in contrast, the magnitude of the antigravitropic component is constant (Roychoudhry et al., 2013). For example, if the organ is placed above its GSA the magnitude of the gravitropic component will increase to bring it back down to its GSA. If the branch is placed below its GSA the magnitude of the antigravitropic component does not increase, instead the magnitude of the gravitropic component decreases allowing the antigravitropic component to bring the branch back up to its GSA. This explains

why outwards bending of the lateral organs is observed when the plants reference to gravity is removed (Roychoudhry et al., 2013).

Through the study of both loss and gain of function mutants, a number of genes are known to modulate growth angle and the magnitude of the GSA. It has been shown that the loss of function *tir1-1* mutant displays less vertical growth angles and the gain of function *axr3-10* mutant displays more vertical growth angles in both roots and shoots, by reorienting the altered growth angles of both mutants these have been proven as GSAs (Roychoudhry et al., 2013). In addition to these, mutations in many of the other genes in the auxin response pathway also result in changes to the GSA of the roots and shoots, these include mutants with lower levels of auxin or auxin response such as *wei8 tar2* and mutants with higher levels of auxin or auxin response such as *yucca1-1D* (Roychoudhry et al., 2013). Other genes have been found that are known to be involved in growth angle control, these include *TAC1* (the knockout mutant of which results in more vertical lateral shoots in Arabidopsis) (Dardick et al., 2013) and the *LAZY* group of genes of which there are six in Arabidopsis (Yoshihara and Spalding, 2017). Both the *LAZYs* and *TAC1* are in the IGT gene family as they contain a conserved IGT (GφL(A/T)GT) motif in their second region of conserved sequence, however, where the *LAZYs* typically contain 5 regions of conserved sequence, *TAC1* lacks region V meaning it is not a part of the *LAZY* family of genes (Yoshihara and Spalding, 2017).

“lazy” mutants were first described in the early 20th century in rice and maize, the mutant plants display a prostrate growth habit when mature though, in both cases in the early stages of growth their habit is normal (Jones and Adair, 1938, Jenkins and Gerhardt, 1931). It was initially thought that the prostrate growth was a result of a lack of strength of the culm due to differences in cell wall composition (Jenkins and Gerhardt, 1931), however, it was later found that the phenotype was due to a “gravitational indifference” (Overbeek, 1936). Further research found that the “lazy’” phenotype in rice could be attributed to a recessive mutation in a single gene (Abe et al., 1996), this was later cloned and named *LAZY1* (Li et al., 2007). *lazy1* loss of function mutants have altered polar auxin transport and the resulting change in the distribution of IAA in the tillers (Yoshihara and Iino report no auxin asymmetry in gravistimulated *lazy1* tillers

(Yoshihara and Iino, 2007)) leads to the reduced gravitropism and spreading phenotype typical of the mutants described in earlier studies (Li et al., 2007, Jones and Adair, 1938, Abe et al., 1996). It was originally thought that *LAZY1* was a grass specific gene (Li et al., 2007) however a LAZY1-like protein was found in Arabidopsis (Yoshihara and Iino, 2007), despite a low sequence similarity to the rice LAZY1 the *atlazy1* knockout mutant displays a similar lax shoot habit and impaired gravitropic response to that seen in the rice *lazy1* mutant (Yoshihara and Iino, 2007, Yoshihara et al., 2013). Further sequence analysis found that in Arabidopsis there are 6 genes in the *LAZY* family, these are *AtLAZY1* (At5g14090), *AtLAZY2* (At1g17400), *AtLAZY3* (At1g19115), *AtLAZY4* (At1g72490), *AtLAZY5* (At3g24750), *AtLAZY6* (At3g27025) (Yoshihara and Spalding, 2017, Yoshihara et al., 2013). There are a number of papers that report these same genes under different names, these names are *AtNGR1* and *AtDRO3* for *AtLAZY2*, *AtNGR2* and *AtDRO1* for *AtLAZY4* and *AtNGR3* and *AtDRO2* for *AtLAZY3* (Guseman et al., 2017, Ge and Chen, 2016) (see Table 1.1), for the remainder of this work I will refer to them by their corresponding “LAZY” name.

Table 1.1: Naming of the Arabidopsis *LAZY* family of genes

AGI Number	***LAZY* Name**	**Other Names**
AT5G14090	*LAZY1*	*ATLAZY1*
AT1G17400	*LAZY2*	*ATLAZY2, ATNGR1, DRO3*
AT1G19115	*LAZY3*	*ATNGR3, DRO2*
AT1G72490	*LAZY4*	*ATNGR2, DRO1*
AT3G24750	*LAZY5*	
AT3G27025	*LAZY6*	

The Arabidopsis LAZYs are characterized by 5 regions of conservation that were used to define the family, the only predicted domain structures that could be inferred from the sequence of *LAZY1* were nuclear localization sequences and an EAR motif. EAR motifs are usually found in proteins that act as transcriptional regulators and along with the nuclear localization sequences could suggest involvement in the control of gene expression. This is further supported by subcellular localization studies using *Nicotiana benthamiana*, when transiently expressed the p35S:AtLAZY1-eGFP construct fluorescence localizes to the plasma membrane and the nucleus (Yoshihara et al., 2013). Signals for pLAZY4:LAZY4-mCherry co-localise with PIN1-GFP signals when co-expressed in Arabidopsis protoplasts indicating that LAZY4 is plasma membrane localized but not nuclear localized (Taniguchi et al., 2017). A sequence of 14 amino acids at the C-terminus is relatively well conserved across the family, this sequence has been designated the Conserved C-Terminus in LAZY family proteins (CCL). It is thought that this region is important for LAZY function as when genomic fragments of *LAZY2* and *LAZY4* lacking the CCL are expressed in the at*lazy1 atlazy2 atlazy4* triple mutant (henceforth referred to as *atlazy124*) they do not rescue the gravitropism phenotype. When fluorescent protein constructs of LAZY4 and LAZY2 lacking the CCL domain are transiently expressed in Arabidopsis protoplasts fluorescence is still localized to the plasma membrane, this suggests that whilst the CCL is important for LAZY function it is not important for localization (Taniguchi et al., 2017).

Through a series of promoter:GUS fusions Yoshihara and Spalding (2017) report the expression of the 6 lazy genes in light grown seedlings to be the following: *AtLAZY1* is expressed throughout the shoot including the vasculature, there is some expression in the root. *AtLAZY2* is highly expressed in the hypocotyl, and the root tip, it is also expressed in the cotyledons in dark grown seedlings. *AtLAZY3* is expressed in the root tip. Highest expression of *AtLAZY4* was seen in the root tip but in dark grown seedlings expression was also noted in the cotyledons, hypocotyl stele and root stele. *AtLAZY5* is expressed throughout the root except the root apex and also in the cotyledons of dark grown seedlings. *AtLAZY6* expression was seen in the petioles and in dark

grown seedlings also around the shoot apical meristem (Yoshihara and Spalding, 2017). The expression of the rice ortholog of *AtLAZY4* known as *OsDRO1* is negatively regulated by auxin (Uga et al., 2013), it is currently unknown whether the same is true in Arabidopsis.

The *LAZY* genes may have some involvement in PIN localization, in the lateral roots of wild type plants PIN3:GFP signal intensity is much higher in the lower columella cells than in the higher columella cells whereas in the lateral roots of *atlazy124* the signal intensity is much higher in the columella cells on the upper side than those on the lower side (Taniguchi et al., 2017), the PIN proteins are known to have a role in growth angle control (Rosquete et al., 2013).

atlazy mutants exhibit gravitropism defects the severity of which increases with mutating more genes, roots of the *atlazy2 atlazy3 atlazy4* (henceforth *atlazy234*) triple mutant often grow horizontally or completely upside-down (Yoshihara and Spalding, 2017). Etiolated hypocotyls usually respond rapidly to a gravitational stimulus, the at*lazy1 atlazy2 atlazy3 atlazy4* quadruple mutant hypocotyls only respond weakly to the gravistimulus however they respond rapidly to a light stimulus indicating that the *LAZY* genes are not involved in the phototropic response and suggesting that they act upstream of where phototropism has influence on the bending of organs (Yoshihara and Spalding, 2017). It has been observed that in the inflorescence stems of at*lazy124* the accumulation of starch in the endodermis is normal and when compared with wild type the sedimentation of the amyloplasts after 5 minutes is normal, this suggests that the *AtLAZY* genes are involved in a process downstream of gravity perception by amyloplast sedimentation (Taniguchi et al., 2017). It is thought that AtLAZY1, AtLAZY2 and AtLAZY4 play redundant roles in root gravitropism, *atlazy1, atlazy2* and *atlazy4* single mutants and the *atlazy1 atlazy2* double mutant display root phenotypes similar to that of wild type whereas the *atlazy124* triple mutant has roots that grow upwards (Ge and Chen, 2016).

It is also thought that the LAZYs involvement in gravitropism is upstream of auxin redistribution. Using the DR5rev:GFP auxin reporter it was found that after enlargement of the central S2 columella cells in roots of wild type plants there is a greater GFP signal on the lower side of the columella cells than on the upper side indicating a greater amount of auxin on the lower side of the root. In roots of the same developmental stage in the at*lazy124* mutant greater GFP signal was observed on the upper side of the columella cells (Taniguchi et al., 2017). Similar results were also found using the DII-VENUS reporter which emits a fluorescent signal inversely proportional to the auxin signalling activity, upon reorientation in wild type roots, a greater signal is seen on the upper flank of the root than the lower flank, this auxin gradient drives gravitropic bending. In reoriented *atlazy2 atlazy3 atlazy4* (*atlazy234*) plants DII-VENUS also shows the formation of a small auxin gradient but in the opposite direction, consistent with this the roots of the *atlazy234* mutant also displays reverse gravitropic bending upon reorientation (Yoshihara and Spalding, 2017).

It is not clear as to whether the altered growth angles displayed by the *lazy* mutants represent a change in the GSA of the lateral organs or are simply as a result of an altered gravitropic capacity. Taniguchi et al demonstrated that when four-day-old seedlings of *atlazy124* are rotated by 180°, the lateral roots generated grow against the direction of gravity mirroring the phenotype of an *atlazy124* plant that had not been reoriented (Taniguchi et al., 2017) suggesting that the *LAZYs* at least have a role in the setting of the lateral root growth angle though the angle maintenance needed to confirm these as GSAs (i.e. bending both towards and against gravity to return to an angle) as defined by Digby and Firn (Digby and Firn, 1995) and Roychoudhry et al (Roychoudhry et al., 2013) has yet to be demonstrated (Taniguchi et al., 2017, Yoshihara and Spalding, 2017, Ge and Chen, 2016).

1.8 Summary and project aims

Our picture of gravitropic response in plants is as yet incomplete, whilst the sensing of gravity and the mounting of a growth response is reasonably well understood; there are still many gaps in our knowledge, especially surrounding

gravitropic response in the lateral roots. Much of the work done thus far, especially that carried out on the molecular mechanisms governing gravitropism, has been carried out in the model species *Arabidopsis thaliana* and due to the wide array of plant growth forms and architectures the knowledge gained cannot be assumed to be true for all plants. Gravitropic setpoint angles form a major component of the architecture of Arabidopsis and using knowledge of their maintenance mutations can be introduced that alter those angles, it is currently unknown if GSAs exist in other species, especially crop species where the modification of the GSAs could be advantageous to yield through improvement of resource capture. Recently ideotype crop architectures have been proposed that emphasize a change in root angle as a key trait to improve yields and tolerance to suboptimal conditions (Lynch, 2013).

It has been found that within lateral roots an antigravitropic offset mechanism results in the maintenance of stable, non-vertical growth and that it is likely this offset is not active in the primary root (Roychoudhry et al., 2013). However, little is known about how the angle of non-vertical growth is set, the offset is initiated and importantly what defines a non-vertically growing lateral root from a vertically growing primary root, in essence what makes a lateral, lateral. Furthermore, the extent to which growth angles in other species, such as the cereal crops rice and wheat, are maintained as GSAs is unknown. To address these questions a number of approaches have been taken including both physiological approaches, such as reorientations and clinorotation, and genetic approaches, such as forward genetic screens and site directed mutagenesis.

Specifically the aims of this project were:

- To confirm the existence of GSAs in species beyond Arabidopsis, specifically crop species.
- To investigate further the mechanisms behind the setting of GSAs in both Arabidopsis and other species.
- To investigate what, in a gravitropism sense, defines a lateral root. In essence what makes a lateral, lateral.

Chapter 2 : General methods and recipes

2.1 Sterilisation of seeds using chlorine gas

All steps were carried out in a fume hood. Seeds were placed in a 1.5 ml microcentrifuge tube and placed inside a desiccator jar with the lid of the tube open. 3 ml of 37% Hydrochloric acid was added to a beaker containing 100 ml thick household bleach (Hospec), this was placed inside the desiccator jar and the jar was sealed using Parafilm (Bemis). After 3 hours seeds were removed from the desiccator jar and left to air in sterile conditions under a laminar flow hood.

2.2 Preparation of Hoagland's No. 2 plant growth media

Hoagland's No. 2 plant growth medium was prepared according to the following recipe; ingredients were dissolved in dH_2O. Media for rice growth was autoclaved before use.

Table 2.2.1: Composition of Hoagland's No. 2 plant growth media

Ingredient	**mg/Litre**
Potassium Nitrate	606.6
Calcium Nitrate	656.4
Magnesium Sulphate	240.76
Ammonium Phosphate Monobasic (Ammonium Dihydrogen Phosphate)	115.03
Manganese Chloride Tetrahydrate	1.81
Boric Acid	2.86
Molybdenum Trioxide	0.016
Zinc Sulphate (Heptahydrate)	0.22
Copper Sulphate (Pentahydrate)	0.08
Ferric Tartrate	5

2.3 Preparation of *Arabidopsis thaliana* Salts (ATS) plant growth media

ATS solid plant growth media was made up to the following specifications:

Table 2.3.1: Composition of ATS plant growth media

Ingredient	Concentration
KNO_3	5 mM
KH_2PO_4	2.5 mM
$MgSO_4$	2 mM
$Ca(NO_3)_2$	2 mM
Fe-EDTA	50 mM
Micronutrients Solution (See below)	1 ml/L
Sucrose	1%
Plant Agar (Duchefa)	0.80%

It was made up from the following six stock solutions:

Table 2.3.2: Amounts of ATS stock solutions

Solution	ml/L
1 M KNO_3	5
1 M KH_2PO_4 buffer (Adjusted to pH5.5 using K_2HPO_4)	2.5
1 M $MgSO_4$	2
1 M $Ca(NO_3)_2$	2
20 mM Fe-EDTA	2.5
Micronutrients	1

The composition of the micronutrients solution is as follows:

Table 2.3.3: Composition of ATS micronutrients solution

Ingredient	Concentration
H_2BO_3	70 mM
$McCl_2$	14 mM
$CuSO_4$	0.5 mM
$ZnSO_4$	1 mM
$NaMoO_4$	0.02 mM
NaCl	10 mM
$CoCl_2$	0.01 mM

After the addition of the sucrose and agar dH_2O was added to volume, the solution was autoclaved before use.

2.4 Preparation of Luria-Bertani (LB) bacterial growth media

LB bacterial growth medium was prepared as below, all media was autoclaved before use.

Table 2.4.1: Composition of LB bacterial growth media

Ingredient	Amount (g/L)
Tryptone	10
Yeast Extract	5
Sodium Chloride	10
Agar (For solid medium)	15

Solid medium was used for plates, liquid medium for bacterial cell cultures.

2.5 Preparation of 10 mM dNTPs for PCR

10 mM dNTPs for use in PCR were prepared as below:

Table 2.5.1: Preparation of 10 mM dNTPs for PCR

Stock	Amount (µl)
100 mM dATP	10
100 mM dTTP	10
100 mM dCTP	10
100 mM dGTP	10
dH_2O	60

2.6 Preparation of TAE buffer for agarose gel electrophoresis

50 X TAE buffer for agarose gel electrophoresis was prepared according to the table below, for use at 1 X dH_2O was used to dilute the 50 X stock.

Table 2.6.1: Composition of 50 X TAE buffer for agarose gel electrophoresis

Ingredient	Amount
Tris Base	242 g
Glacial Acetic Acid	57.1 ml
0.5 M EDTA pH 8.0	100 ml
dH_2O	to 1 L

2.7 Antibiotic stock solutions

The following antibiotic stock solutions were used to add antibiotics to LB bacterial growth media:

Table 2.7.1: Preparation of antibiotic stock solutions

Antibiotic	Stock Concentration (mg/ml)	Solvent	Working Concentration (µg/ml)
Kanamycin	100	dH_2O	40
Gentamycin	100	dH_2O	25
Rifampicin	50	DMSO	100
Spectinomycin	70	70% EtOH	70

2.8 Hormone stock solutions

The following stocks of plant hormones were used for the addition of hormones to plant growth media:

Table 2.8.1: Preparation of plant hormone stock solutions

Hormone	Solvent	Stock Concentration (mM)
IAA	70% EtOH	1,10 and 100
PEO-IAA	DMSO	100
NPA	DMSO	25
2,4-D	70% EtOH	100

2.9 List of plant materials used

Table 2.9.1 List of plant materials used, their background and sources

Species	Plant Line	Background	Source
Triticum aestivum	Bobwhite	N/A	Suruchi Roychoudhry, Kepinski Lab, Leeds
Triticum aestivum	Cadenza	N/A	Cristobal Uauy, John Innes Centre
Triticum aestivum	TILLING lines x 397	Cadenza	Cristobal Uauy, John Innes Centre
Oryza sativa ssp. japonica	Nipponbarre	N/A	Suruchi Roychoudhry, Kepinski Lab, Leeds
Oryza sativa ssp. indica	IR64	N/A	International Rice Research Institute, Philippines
Solanum pennellii	N/A	N/A	Tomato Genetics Resource Centre, UC Davis, California
Solanum lycopersicum	Alicante	N/A	Wilko Ltd., UK
Capsicum annuum	Jalapeno	N/A	Thompson and Morgan, UK
Solanum melongena	Czech Early	N/A	Thompson and Morgan, UK
Nicotiana benthamiana	N/A	N/A	Suruchi Roychoudhry, Kepinski Lab, Leeds
Arabidopsis thaliana	Col-0 (Columbia)	Col-0	Suruchi Roychoudhry, Kepinski Lab, Leeds
Arabidopsis thaliana	Cvi-0 (Cape Verde Islands)	Cvi-0	Suruchi Roychoudhry, Kepinski Lab, Leeds
Arabidopsis thaliana	*lazy4*	Col-0	Suruchi Roychoudhry, Kepinski Lab, Leeds
Arabidopsis thaliana	80.2.1 MV	Col-0	This project
Arabidopsis thaliana	71.14.4 MV	Col-0	This project
Arabidopsis thaliana	68.8.5 Wavy	Col-0	This project
Arabidopsis thaliana	27.3.2 Short/Jagged Leaves	Col-0	This project
Arabidopsis thaliana	56.23.5 Wavy	Col-0	This project
Arabidopsis thaliana	63.23.5 Wavy	Col-0	This project
Arabidopsis thaliana	*pLAZY4:LAZY4*	Col-0	This project
Arabidopsis thaliana	*pLAZY4:LAZY4* 80.2.1 MV	Col-0	This project
Arabidopsis thaliana	*pLAZY4:LAZY4* R145A	Col-0	This project

Arabidopsis thaliana	*pLAZY4:LAZY4* R145K	Col-0	This project
Arabidopsis thaliana	*pLAZY4:LAZY4* R145E	Col-0	This project
Arabidopsis thaliana	*tac1*	Col-0	Suruchi Roychoudhry, Kepinski Lab, Leeds
Arabidopsis thaliana	UKID96	UKID96	NASC
Arabidopsis thaliana	St-0	St-0	NASC
Arabidopsis thaliana	Kyoto	Kyoto	NASC
Arabidopsis thaliana	Ei-2	Ei-2	NASC

Chapter specific methods can be found in individual sections at the end of each chapter.

Chapter 3 :The maintenance of gravitropic setpoint angles in the cereal crops rice (*Oryza sativa*) and wheat (*Triticum aestivum*)

3.1 Introduction

Gravity is the only force that is constantly acting upon the plant and therefore response to gravity is central to plant form, the shape of a plant is key to its ability to gather the resources it needs to grow and, in the case of crops, produce the desired product. Non-vertical growth such as that seen in the lateral roots and shoots of Arabidopsis is a key adaptation that allows plants to exploit the largest area possible for resource gathering. The cereal crops rice (*Oryza sativa*) and wheat (*Triticum aestivum*) each contribute around 20% of the worlds calorie consumption (Dubcovsky and Dvorak, 2007, Abdullah et al., 2006) and therefore maintaining and improving yields is critical to providing food for the growing global population. The green revolution of the mid 20th century showed that there is a direct link between modifying plant architecture and improving crop yields (Lynch, 2007), a greater knowledge of how gravity governs the architecture of rice and wheat could lead to the development of cultivars with a greater yields as a result of more efficient resource capture.

Gravity is sensed in specialized cells known as statocytes, these contain starch filled amyloplasts which, by sedimenting, act as statoliths allowing the plant to perceive the direction of gravity (Baldwin et al., 2013). In the roots of both the model dicot Arabidopsis and monocots such as the cereal crops rice and wheat, the statocytes are found within the columella cells of the root cap (Leitz et al., 2009, Kaufman et al., 1995). In the shoots of dicots, the statocytes are found along the length of the shoot in the innermost layer of the cortex (also known as the starch layer or endodermis). This surrounds the vascular tissue meaning the statocytes lie on the boundary between the sites of transport and growth (Sack, 1991, Fukaki et al., 1998). In contrast, in the shoots of cereal crops the statocytes are found in specialized gravisensitive organs at points along their stems, these are known as pulvini and appear as a characteristic “bump” on the stem at the base of each node (Kaufman et al., 1987, Clore, 2013). Within the

pulvinus it is the parenchyma cells surrounding the vascular bundles that function as the statocytes (Dayanandan et al., 1976).

Many of the non-vertical growth angles found in the lateral organs of the plant are maintained with respect to the direction of the gravity vector (0º is vertically downwards and 180º is vertically upwards). These angles are known as Gravitropic Setpoint Angles (GSAs). A GSA can be defined as “the angle with respect to gravity at which an organ shows no gravity induced differential growth in order to correct its orientation” (Digby and Firn, 1995). If a lateral organ that is being maintained at a GSA is displaced from its GSA, differential growth will be induced in order to correct its displacement and bring the organ back to its original growth angle.

A model of GSA maintenance in dicots involving opposing gravitropic and anti-gravitropic auxin fluxes has been proposed (Roychoudhry et al., 2013). This study provides evidence of an antigravitropic growth offset (AGO) component that is acting in the absence of the gravitropic growth component in the lateral organs growing at non-vertical angles. This antigravitropic component acts in tandem with the gravitropic growth component to produce balanced non-vertical growth. Importantly, this AGO is not active in the primary axis resulting in its near vertical growth (Roychoudhry et al., 2013).

The root system of Arabidopsis consists of a primary root that maintains a near vertical GSA of around 0º. From this primary root, lateral roots arise that maintain non-vertical GSAs. In contrast, wheat produces a number of seminal roots directly from the seed which can vary in number between 3 and 5 depending on the cultivar. In wheat, the primary seminal root (or radicle) grows vertically in a 2D system, the other seminal roots grow at non-vertical angles that can also vary between cultivars. Adventitious/nodal roots can also be produced from the nodes at the base of the primary shoot and the tillers. Additionally, lateral branches can arise from all of these roots (Manschadi et al., 2008). In contrast to wheat, rice produces only one seminal root from which lateral roots can later arise. Thus, in rice seedlings, the majority of the root

system consists of nodal and crown roots both of which develop from nodes. However, crown roots develop from nodes below the soil surface, whereas nodal roots develop from nodes above the soil surface. All of these roots are capable of forming first and second order lateral roots (Inukai et al., 2005, Morita and Nemoto, 1995).

A number of root and shoot angle modifying quantitative trait loci (QTLs) and mutants have been documented in cereal crops. In rice, mutations in the gene LAZY1 result in a prostrate growth habit with a much wider tiller angle than that of the background lines that the mutant plants were derived from, "*lazy*" mutants have been found in both in *japonica* and *indica* cultivars of rice. The tillers of the rice *lazy1* mutant lines also have an impaired gravitropic response (Yoshihara and Iino, 2007). TILLER ANGLE CONTROL1 (TAC1) is another gene that has been identified to modify tiller angle in rice. A previous study showed that high expression levels of TAC1 result in a wider tiller angle than the wild type in the *japonica* cultivar "Nipponbarre", while lower expression levels or mutations in TAC1 result in a narrower tiller angle. Using the OsTAC1 cDNA sequence a BLAST search has revealed short expressed sequence tags that could make up the full-length coding and 3'UTR sequence of TAC1 in Wheat, maize (*Zea* mays) and sorghum (*Sorghum bicolour*) suggesting that TAC1 could have a role in regulating the tiller angle of many other plants in the family Graminae (Yu et al., 2007). In roots, a QTL has been found that has some control of root system architecture (RSA). High expression levels of DEEPER ROOTING1 (DRO1) were found to induce more vertical root growth in rice (Uga et al., 2013). A number of QTLs have been identified in wheat that are involved in control of seminal root angle however it was found that individually these QTLs have little effect (Christopher et al., 2013), this may be due to the complexities of wheat genetics and issues surrounding ploidy and redundancy.

GSAs are already documented in the model dicot Arabidopsis (Roychoudhry et al., 2013, Mullen and Hangarter, 2003, Rosquete et al., 2013). However, it is currently unknown if the non-vertical growth habits of monocot organs are actively maintained as GSAs, moreover, the mechanisms that regulate non-vertical growth in monocots remain unidentified. In this work the existence of

GSAs in wheat and rice was investigated. Using clinorotation it was shown that there is an AGO present in the seminal roots of wheat and crown roots of rice, when reoriented, growth is induced towards the roots original angle. The addition of exogenous auxin makes these roots more vertical showing the involvement of auxin in the control of root angle in rice and wheat. Clinorotation of the tillers of both rice and wheat suggests the presence of an AGO in the tillers, when reorienting the tillers of rice and wheat to the vertical it was also shown that growth was induced to move the tiller back towards its original angle.

3.2 Results

3.2.1 Wheat seminal roots and rice crown roots bend outwards upon clinorotation.

In Arabidopsis the maintenance of GSAs is thought to be controlled by a balance of a gravitropic growth component and an antigravitropic offset component (AGO). It has been shown that clinorotation, which removes the plants reference to the gravity vector, results in a rapid outward bending of the lateral roots and shoots. It was decided to clinorotate the roots of wheat and rice to confirm the presence of this offset in cereal crops.

Upon clinorotation both wheat seminal roots (Figure 9.1A and B) and rice crown roots (Figure 9.1 C and D) exhibited an outward bending, this confirms the presence of an AGO in the non-vertically growing seminal and crown roots of these monocots. This outward bending is also seen in the primary seminal root of wheat and the primary root of rice both of which appear to grow vertically in the 2D pouch system.

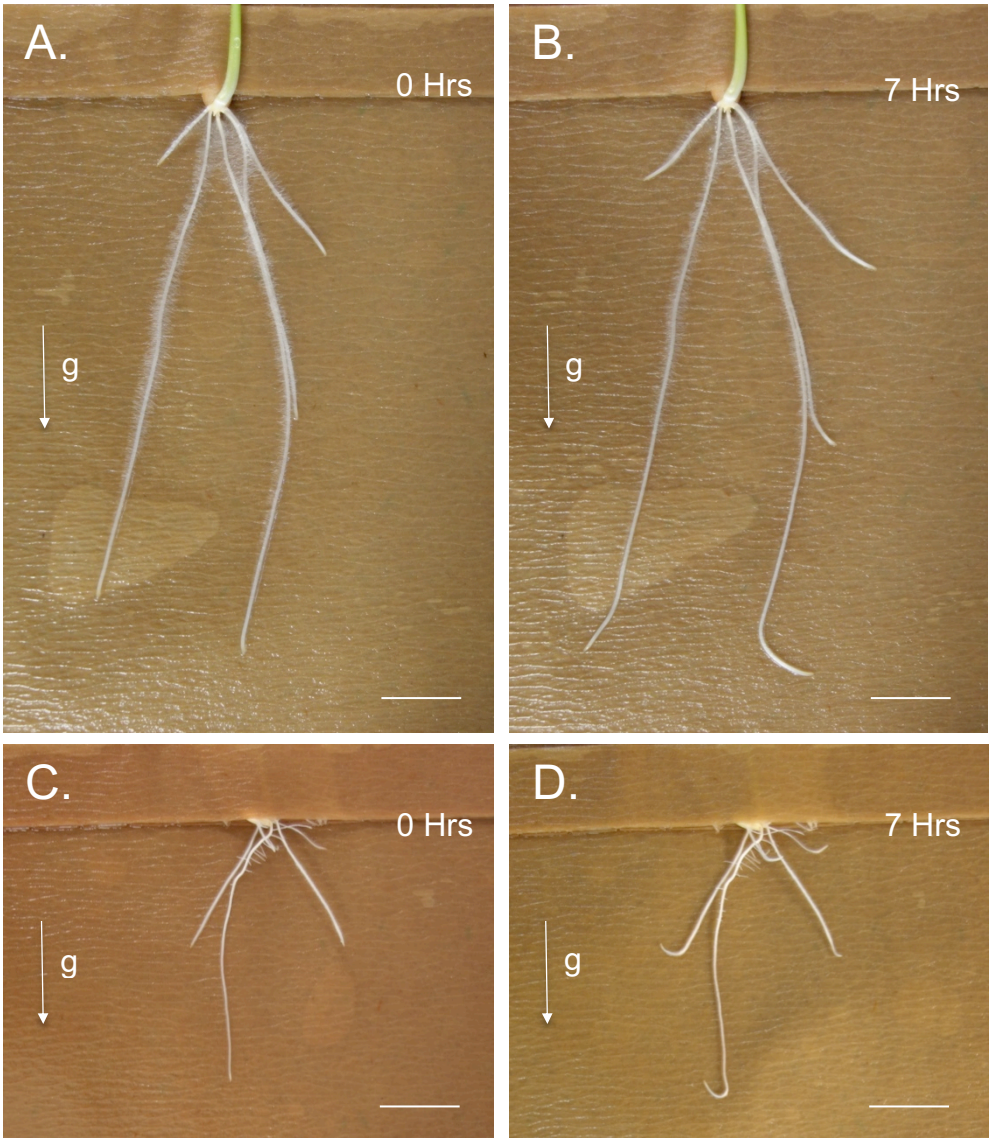


Figure 3.1: Wheat seminal roots and rice crown roots bend outwards upon clinorotation.

A and **B**. Wheat seminal roots bend outwards upon clinorotation for 7 hours. **C** and **D**. Rice crown roots bend outwards upon clinorotation for 7 hours. Scale Bars represent 10 mm, ”g” represents the direction of gravity as it was before clinorotation.

3.2.2 Wheat seminal roots and rice crown roots return to close to their original angles after reorientation

In the model dicot Arabidopsis, if it is reoriented, gravity induced differential growth is initiated in the non-vertically growing lateral roots to return them to close to their original angle, these angles are therefore maintained with respect to gravity making them GSAs. To investigate the non-vertically growing roots of the cereal crops wheat and rice for GSA maintenance, wheat and rice plants

were first reoriented to determine if any of the non-vertical root angles are maintained with respect to gravity.

Vertically growing wheat seedlings in a 2D germination pouch based system were reoriented by 30° for a 24-hr period and measured the angles of seminal root tips before and after reorientation. In these assays, wheat seminal roots reoriented themselves in both upwards and downwards directions. It was found that roots that were placed below their original angle on average return to within 15º of their original angle by bending upwards. Roots that were placed above their original angle on average return to within 4° of their original angle by bending downwards (Figure 3.2 A and Figure 3.3). In both cases the average tip angle 24 hours after reorientation was more vertical than the angle before reorientation. A control experiment was carried out on non-reoriented seminal roots of wheat, it was found that the non-reoriented roots do become more vertical over time. The average tip angle decreases by 9.2° within the first 24 hours and by a further 5.9° between 24 and 48 hours on wheat plants of the same age as those used for the reorientation assay (Figure 3.5B).

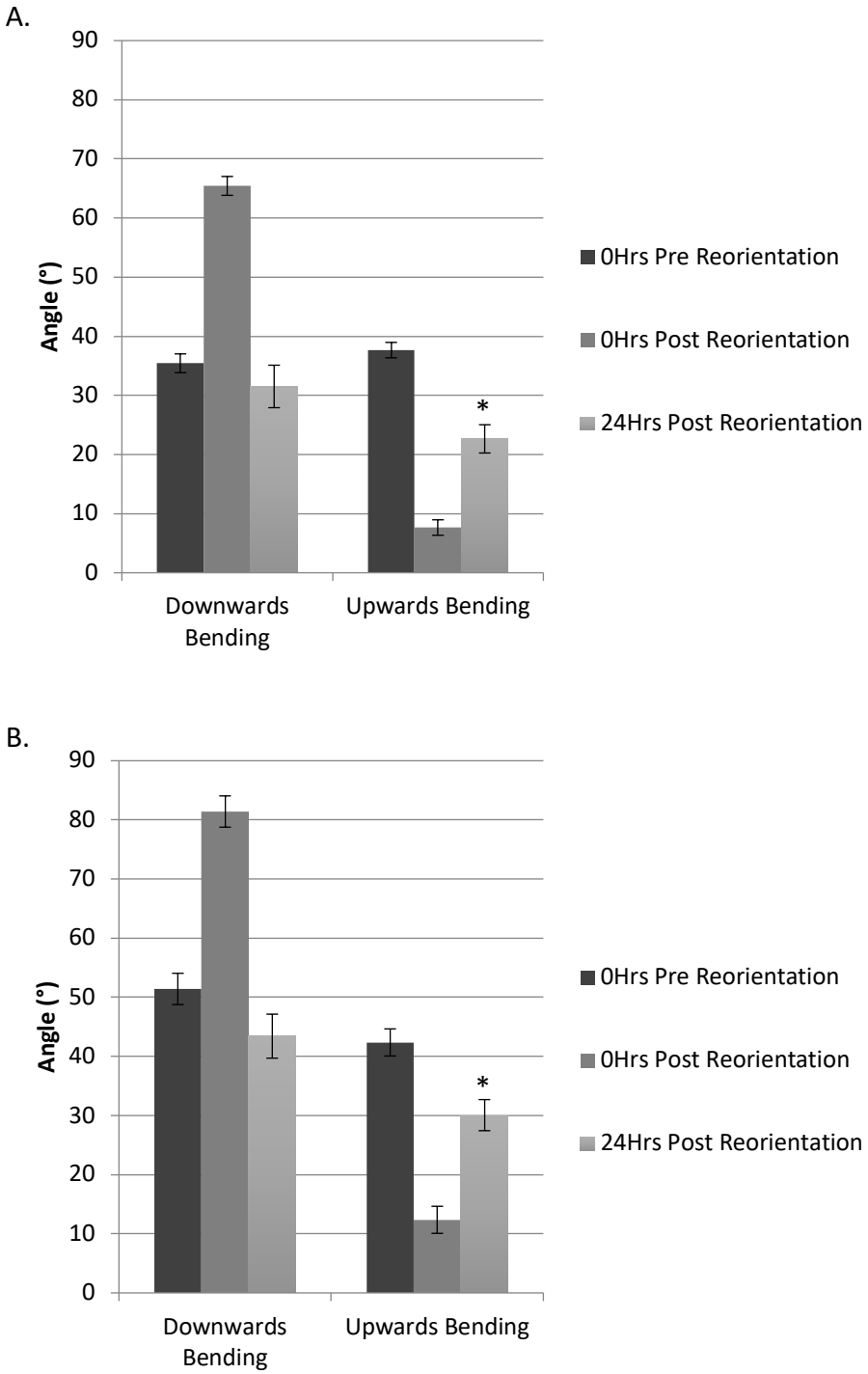


Figure 3.2: Wheat seminal roots and rice crown roots return to close to their original angles after reorientation

A. Wheat Seminal Roots return to close to their original angles after reorientation by 30° by both upwards and downwards bending, downwards bending n=37, upwards bending n=45. **B**. Rice crown roots return to close to their original angles after reorientation by 30° by both upwards and downwards bending, downwards bending n=43, upwards bending n=45. Error bars represent SEM. *=$p<0.05$ (Students T-test between 0Hrs pre-reorientation and 24Hrs post reorientation).

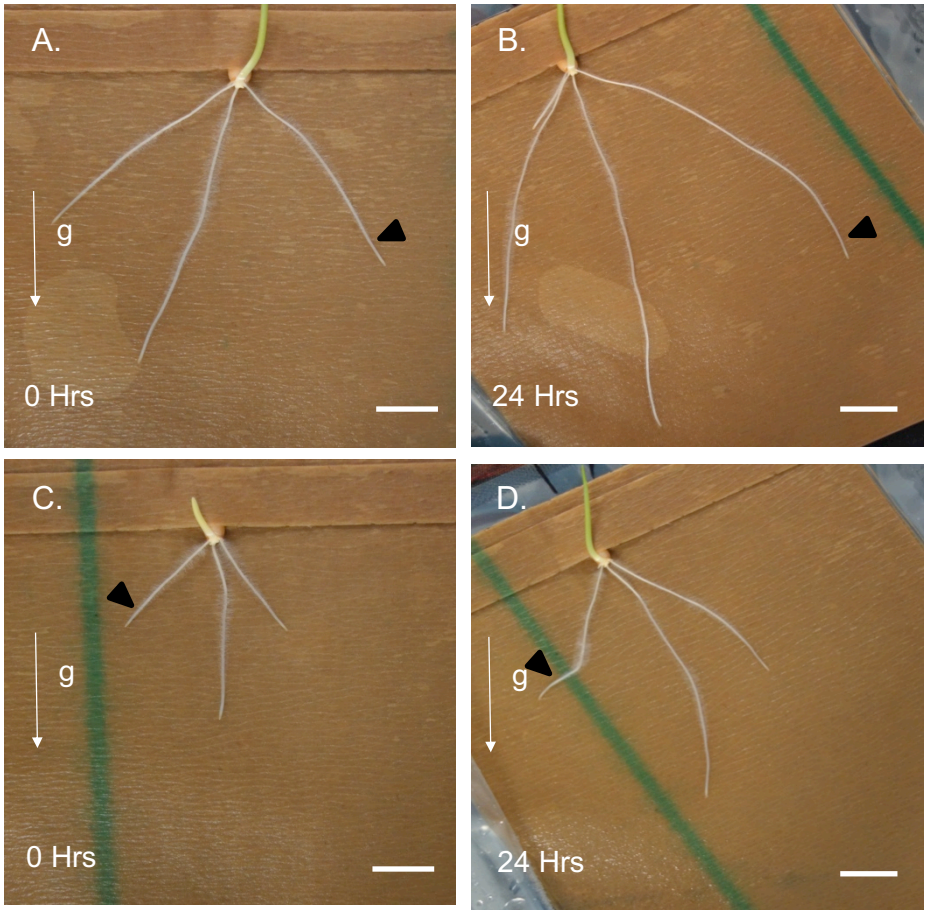


Figure 3.3: Wheat seminal roots return to close to their original angles after reorientation

A and **B**. Wheat seminal root returns to a GSA of around 27° by bending downwards. **C** and **D**. Wheat seminal root returns to a GSA of around 33° by bending upwards. Scale bars represent 10 mm. ”g” represents the direction of gravity.

Similar experiments were carried but with rice crown roots. Similarly to wheat, roots placed below their original angle on average returned to within 8° of their original angle by bending upwards and roots placed above their original angle returned on average to within 12.3° of their original angle by bending downwards (Figure 3.2 B and Figure 3.4). Again, in both cases the average tip angle 24 hours post reorientation was more vertical than that prior to reorientation. As a control an additional experiment was also carried out on rice, non-reoriented crown roots of rice also become more vertical over time. The average tip angle decreases by 11.7° in the first 24 hours and by a further 2.3° between 24 and 48 hours on rice plants the same age as those used for the reorientation assay (Figure 3.5 A).

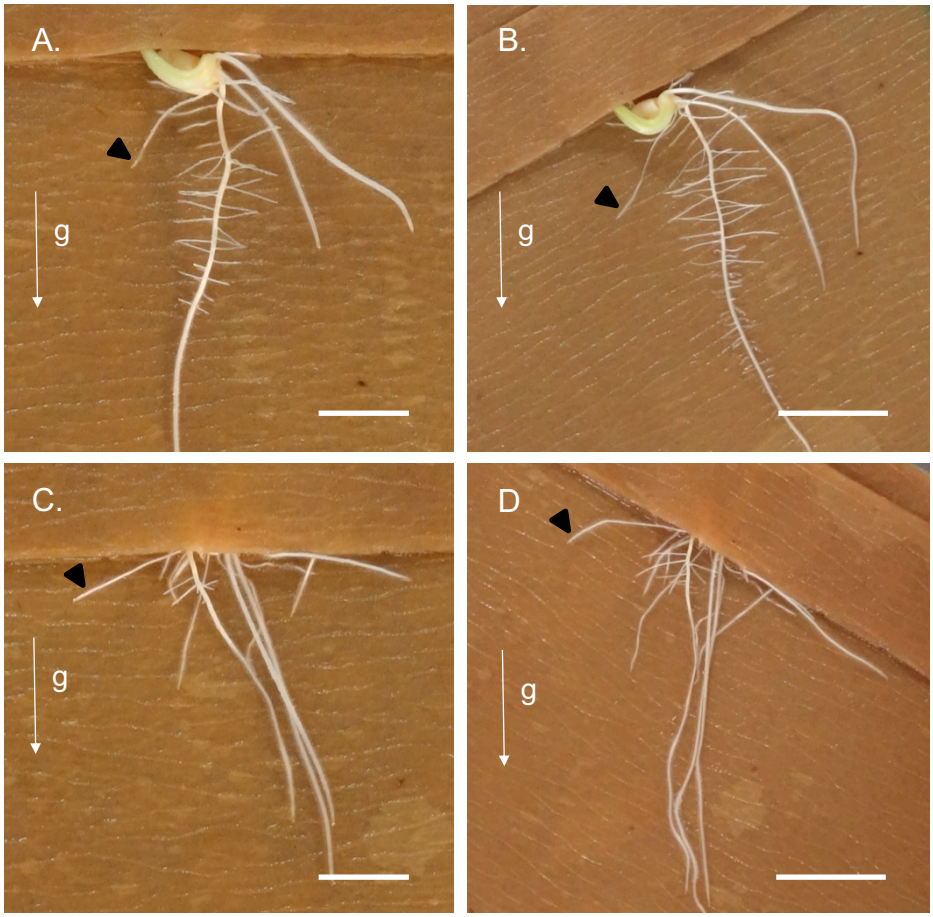


Figure 3.4: Rice crown roots return to close to their original angles after reorientation

A and **B**. Rice crown root returns to a GSA of around 41° by bending upwards. **C** and **D**. Rice crown root returns to a GSA of around 56° by bending downwards. Scale bars represent 10 mm. ”g” represents the direction of gravity.

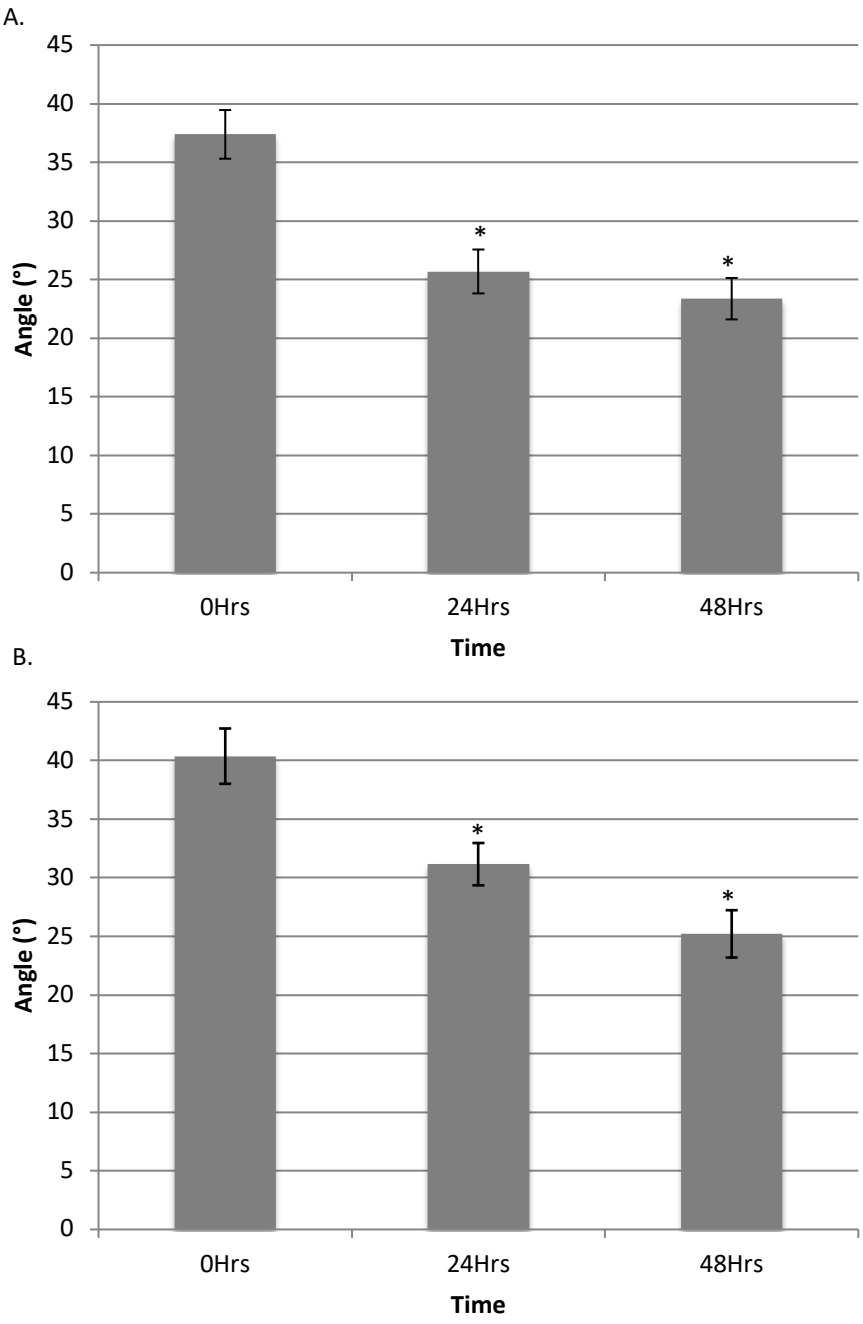


Figure 3.5: Non-reoriented wheat seminal roots and rice crown roots become more vertical over time

A. Non- reoriented rice crown roots become more vertical over time, the tip angle decreases by 11.7° in the first 24 hours and by 2.3° between 24 and 48 hours, n=147. **B**. Non-reoriented wheat seminal roots become more vertical over time, the tip angle decreases by 9.2° in the first 24 hours and by 5.9° between 24 and 48 hours, n=73. Error bars represent SEM, * indicates p=<0.05 (Students T-test).

3.2.3 Wheat seminal roots and rice crown roots become more vertical when treated with exogenous IAA

In Arabidopsis the GSA of the lateral roots becomes more vertical when grown on plates containing the natural auxin indole-3-acetic acid (IAA). This demonstrates a role for auxin in the maintenance of GSAs. It was decided to test whether auxin has the same effect on the non-vertically growing roots of wheat and rice.

Wheat and rice seedlings were treated with increasing concentrations of IAA. In wheat, the average tip angle of the 1st pair of seminal roots became more vertical. Similarly, rice crown roots also adopted a more vertical growth orientation upon auxin treatment. This demonstrates that auxin also has an effect on GSA maintenance in monocots (Figure 3.6), this could either be via a direct mechanism or through other pathways linked with auxin such as the ethylene response pathway. Clinorotation of the ethylene insensitive mutant *ein2-1* in Arabidopsis has shown that ethylene does not have a role in the control of the antigravitropic offset and is unlikely to be involved in GSA maintenance in Arabidopsis (Roychoudhry et al 2013), experiments with ethylene insensitive mutants in rice and wheat would need to be carried out to confirm this in monocots.

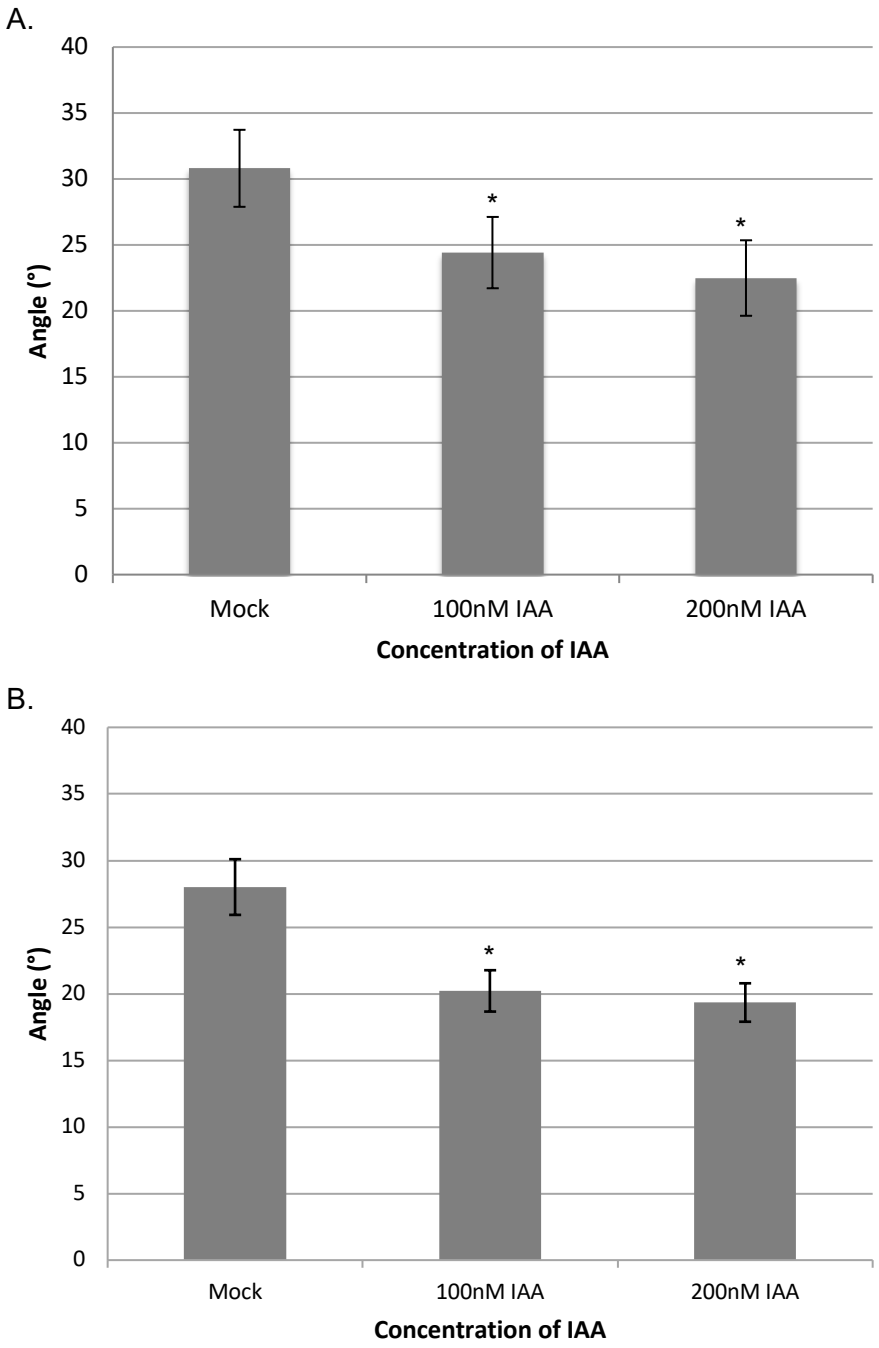


Figure 3.6: Wheat seminal roots and rice crown roots become more vertical when treated with IAA

A. Wheat seminal roots become more vertical when treated with IAA, n=60 for mock treatment, n=60 for 100 nM IAA treatment, n=57 for 200 nM treatment. **B.** Rice crown roots become more vertical when treated with IAA, n=104 for mock treatment, n=80 for 100 nM IAA treatment, n=78 for 200 nM IAA treatment. *=$p<0.05$ (Students T-test). Error bars represent SEM.

3.2.4 Wheat and rice tillers bend outwards upon clinorotation

The gravitropism biology of the shoots of cereal crops is different from that of dicots such as Arabidopsis. In cereal crops the gravity sensing cells are found at discrete points along the stem known as pulvini.

A number of mutations affecting tiller angle in rice have been described, these include *lazy1*, *tac1, prog1* and *loose plant architecture 1* (Yoshihara and Iino, 2007, Yu et al., 2007, Tan et al., 2008, Wu et al., 2013) . We decided to investigate the maintenance of GSAs in rice and wheat tillers as this may give some insight into the mechanisms behind these mutants and how they fit in with gravitropism in cereal crops.

In Arabidopsis the non-vertically growing lateral shoots also exhibit an outward bending upon clinorotation. The shoot systems of both wheat and rice were clinorotated to determine whether an AGO was present in the tillers of rice and wheat.

When clinorotated there is a widening of the tiller angle in both rice and wheat. This is most dramatic in wheat with all tillers exhibiting a pronounced outward bending facilitated initially by the tissue at the base of the stem and then by the pulvini once gravitropically competent pulvini had developed (Figure 3.7A and B). In rice the primary culm does not show an outward bending suggesting that unlike in wheat the AGO is only present in the non-vertically growing tillers (Figure 3.7C and D).

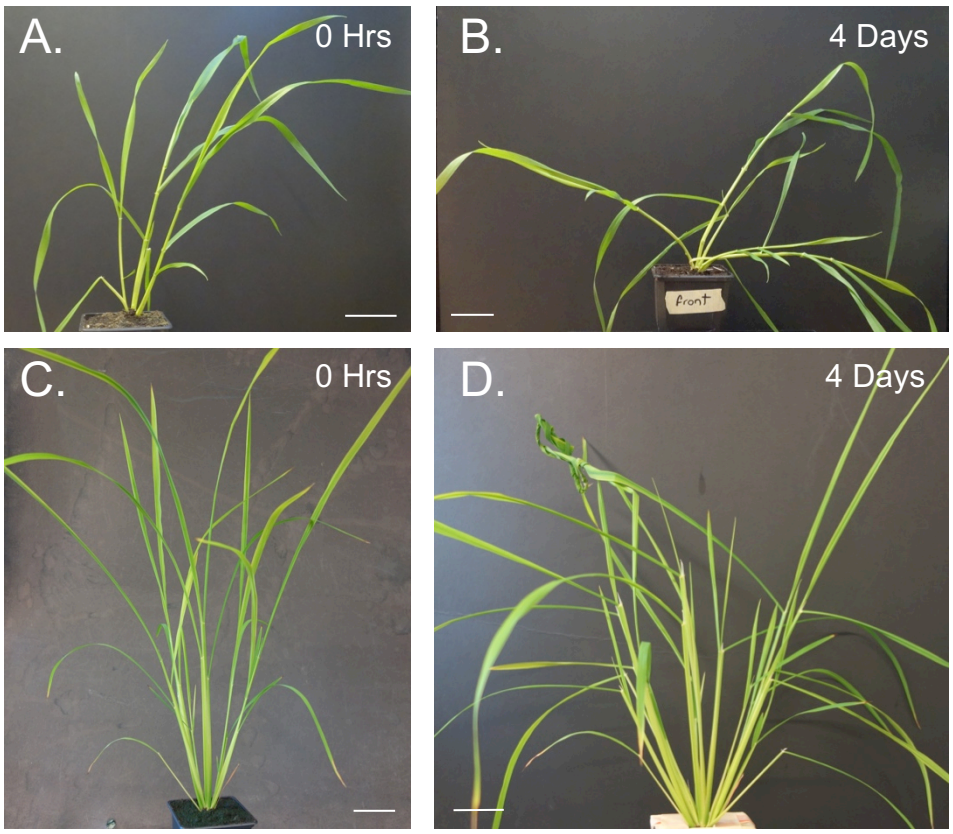


Figure 3.7: Wheat and rice tillers bend outwards upon clinorotation. **A** and **B**. The tillers of a 5 week-old wheat plant bend outwards upon clinorotation for 4 days at 4RPH, scale bars represent 5 cm. **C** and **D**. The tillers of a 6 week-old rice plant bend outwards upon clinorotation for 4 days at 4 RPH, scale bars represent 5 cm.

3.2.5 Wheat and Rice tillers return to close to their original angles after reorientation to the vertical

Target tillers growing at non-vertical angles were chosen on 6-week old plants of the indica rice cultivar “IR64” and on 5-week old plants of the wheat cultivar “Bobwhite”. The plants were reoriented to place this tiller at 180º. After 4 days it was found that the tillers had moved to place themselves closer to their original angle. On average rice tillers returned to within approximately 8º from their original angle (Figure 3.8), wheat tillers returned to within approximately 2º of their original angle (Figure 3.9). It is likely that this bending has occurred in the tiller base, as there is no visible pulvinus facilitated bending upon the rice and wheat stems at this age. This demonstrates that the tillers of rice and wheat are maintained at GSAs.

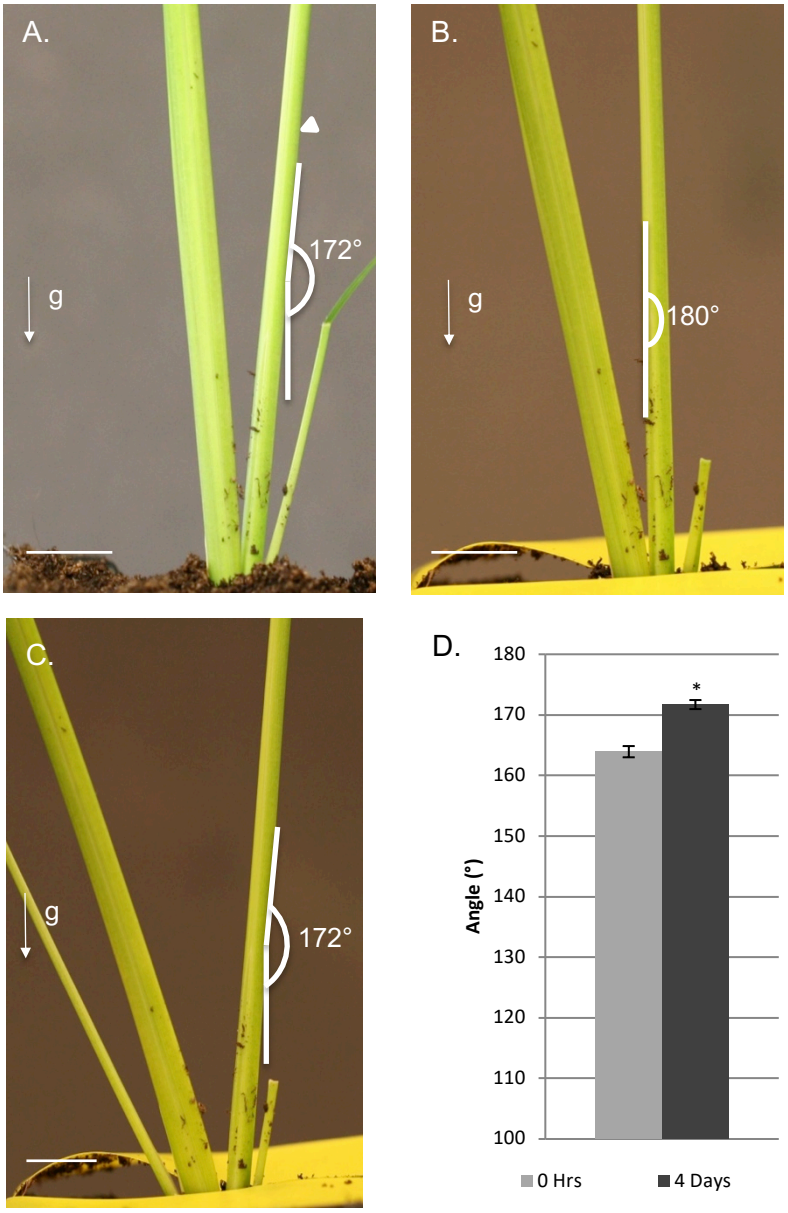


Figure 3.8: Rice tillers return to close to their original angles after reorientation to the vertical

A. 6 week old representative rice plant before reorientation, angle of the target tiller relative to gravity is 172°. **B.** Rice plant is reoriented so that the target tiller is at 180° with respect to gravity. **C.** After 4 days the target tiller has returned to 172° relative to gravity therefore it has returned to its GSA. **D.** Average tiller angle of rice before (0 Hrs) and after (4 Days) reorientation (error bars represent SEM)), n=50 , *=P<0.05 (Students T-test), Scale bars represent 10mm .

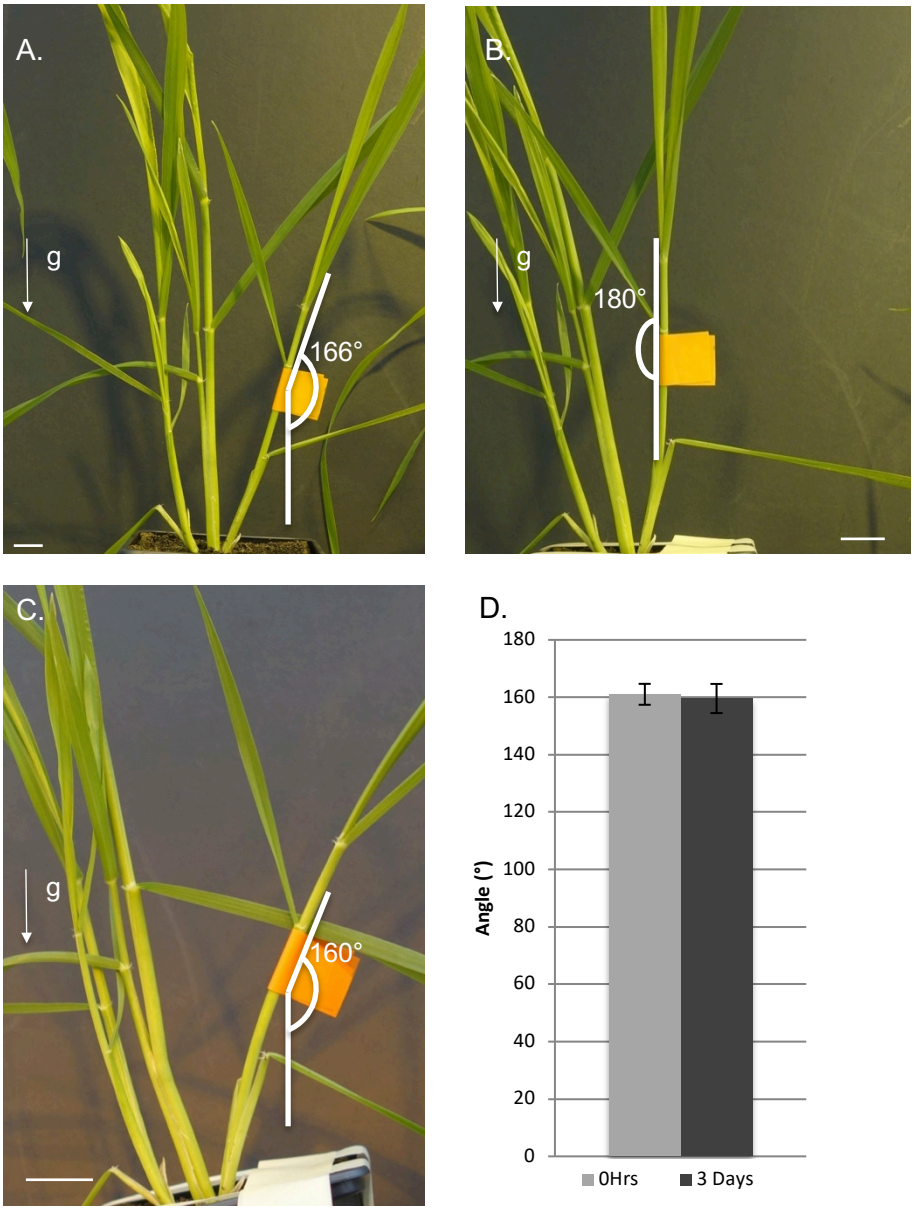


Figure 3.9: Wheat tillers return to close to their original angles after reorientation to the vertical

A. A representative 5 week old wheat plant before reorientation, angle of the target tiller (marked with tape) relative to gravity is 166°. **B**. The plant is reoriented so that the target tiller is at 180° with respect to gravity. **C**. After 3 days the target tiller has returned to an angle of 160° therefore it has returned to its GSA. **D**. Average tiller angle of wheat before (0 Hrs) and after (3 Days) reorientation (error bars represent SEM)) n=14, P>0.05 (Students T-test) Scale bars represent 10 mm.

3.3 Discussion

Since the “Green Revolution” of the mid 20th century introduced dwarf cultivars of rice and wheat, it has been known that plant architecture is of major agronomic importance. As the world population increases, this only increases the pressure upon the land to produce high yielding crops to satisfy its food requirements (Lynch, 2007).

The existence of Gravitropic Setpoint Angles (GSAs), a major factor in overall plant architecture, has already been demonstrated in the model dicot *Arabidopsis thaliana* (Roychoudhry et al., 2013, Mullen and Hangarter, 2003). This work set out to investigate the existence of GSAs outside of Arabidopsis, specifically in the cereal crop species wheat (*Triticum aestivum*) and rice (*Oryza sativa*), and whether these GSAs are maintained in a similar way.

In Arabidopsis*,* GSAs are maintained in the non-vertically growing lateral roots and shoots (Roychoudhry et al., 2013, Mullen and Hangarter, 2003, Rosquete et al., 2013). Due to the difference in plant architecture and gravitropism biology between Arabidopsis and cereals we had to decide which angles to investigate as potential GSAs. It was decided that those roots that were growing non-vertically in a 2D system would be investigated - the 1st and 2nd pairs of seminal roots in wheat and the crown roots in rice. In the shoots it was decided to investigate the tiller angles of both rice and wheat.

Roychoudhry et al proposed a model of GSA maintenance that is a balance of two opposing growth components: a gravitropic growth component and an antigravitropic offset (AGO) component acting in opposition to result in balanced non-vertical growth. They demonstrated the existence of this AGO using clinorotation, this exposes the plant to omnilateral gravitational stimulation and disrupts the plants reference to gravity, in the absence of the gravitropic component the AGO continues to act upon the plant resulting in outward bending of the non-vertically growing organs (Roychoudhry et al., 2013). It was found that when the seminal roots of wheat and the crown roots of rice were clinorotated they also displayed this outwards bending, however in both wheat

and rice the primary seminal root also exhibits some outwards bending. Although in wheat this root grows vertically downwards in the 2D pouch system, when grown in a clear gel medium in 3D the primary root is slightly non-vertical, the primary root and 1st pair of seminal roots forming a “tripod” like structure. Uga et al also showed that the tip of the rice primary seminal root does not always demonstrate a vertical growth angle (Uga et al., 2013). This together with the outward bending upon clinorotation suggests that there is an AGO present in the primary seminal root of wheat and primary root of rice (Figure 3.1).

It was found that when the plants were reoriented, both the seminal roots of wheat and the crown roots of rice undergo a large amount of bending in order to return the angle of the tip to close to the angle it was growing at before reorientation (Figure 3.2, 3.3 and 3.4) this demonstrates active maintenance of the tip angle with respect to the gravity vector. This implies that before reorientation the root was undergoing no tropic growth in order to correct its orientation with respect to gravity and fulfils Digby and Firn’s original definition of a GSA (Digby and Firn, 1995). The average tip angle of both wheat seminal roots and rice crown roots is more vertical after reorientation than before for both upwards and downwards bending roots, this could be due to weakening of the AGO as both the seminal roots of wheat and crown roots of rice become more vertical over time (Figure 3.5), this is also seen in Arabidopsis where lateral roots and cauline branches emerge at very shallow, non-vertical GSAs that become increasingly more vertical as they grow and lengthen (Roychoudhry et al., 2013).

Roychoudhry et al also showed that when the auxin transport inhibitor NPA is added to the growth medium this prevents the outward bending of the lateral roots of Arabidopsis upon clinorotation demonstrating that the gravitropic growth component and the AGO are both auxin fluxes (Roychoudhry et al., 2013).

The application of exogenous auxin has been shown to make the GSAs of the lateral roots of Arabidopsis more vertical (Roychoudhry et al., 2013). In this

work it has been shown that treatment with 100 nM IAA (a natural auxin (Mockaitis and Estelle, 2008)) results in a decrease in average tip angle when compared with mock treated roots, of 6.39º and 7.80º in wheat and rice respectively (Figure 3.6). In wheat, treatment with a greater concentration of IAA results in further decreases to the root GSA, treatment with 200 nM IAA results in a decrease in average tip angle of 8.32º compared with mock treated roots. In rice increasing the concentration of IAA to 200 nM also results in a further decrease in tip angle, a reduction of 8.67° when compared with mock treated roots. There is however little difference between the reduction in angle caused by 100 nM and 200 nM IAA, this could suggest both a maximum reduction in GSA by the addition of exogenous IAA and a difference in IAA sensitivity between rice and wheat.

In Arabidopsis a number of mutants are known that modulate the GSA of the lateral roots (Roychoudhry et al., 2013, Mullen and Hangarter, 2003), for example the mutant *axr3-10* which is predicted to have a higher level of auxin response than wild type also has more vertical lateral root GSAs, the mutant *tir1-1* (an auxin receptor (Kepinski and Leyser, 2005)) which is predicted to have a lower level of auxin response that wild-type also has less vertical lateral root GSAs (Roychoudhry et al., 2013). The ability for certain mutations to modulate root GSA has potentially very exciting implications for cereal crops, if mutants can be found in cereals that also modulate root GSA this opens up the possibility to improve root architectures to improve crop yields, potentially even to tailor the root architecture for specific soil types e.g. more vertical resulting in deeper roots to increase drought tolerance in dry soils or areas with low rainfall or an increase in shallow, less vertical roots for thin soils or soils with higher nutrient content in the upper layers.

Recently a major QTL controlling root angle was discovered in rice, this was named *DEEPER ROOTING 1 (DRO1) (Uga et al., 2013).* A study was carried out on near isogenic lines derived from a cross between the deeper rooting rice cultivar Kinandang Patong (KP) and the shallow rooting rice cultivar IR64. Those lines homozygous for the KP allele of *DRO1* (*DRO1-kp*) in the IR64 genetic background (*DRO1-NIL*) display deeper rooting. The shallow rooting

IR64 cultivar has a truncated version of *DRO1*, higher expression levels of full length *DRO1* result in deeper rooting and faster gravitropism kinetics. *DRO1* expression is repressed by auxin. From this study it is unclear how *DRO1* is related to the maintenance of GSAs in rice roots, the main expression of DRO1 is in the distal elongation zone so it is possible that as a positive regulator of gravitropic response it acts to increase the asymmetric elongation of the cells on the upper and lower sides of the roots during gravitropic bending (Roychoudhry and Kepinski, 2015). Another possibility is that whilst DRO1 may be involved in the setting of the angle it is not involved directly in its maintenance beyond aiding in the expansion of cells required for tropic growth. This would raise the point that there should be a clear separation between a root growth angle and a GSA, a root growth angle being set in relation to gravity but not maintained, perhaps belonging to a sub-group of the second class of angles defined by Roychoudhry and Kepinski (Roychoudhry and Kepinski, 2015), whereas a GSA is set and maintained in relation to gravity. DRO1 could therefore be considered to be involved in the setting of the root growth angle but not in the maintenance of the GSA.

The tillers of rice and wheat were investigated for GSA maintenance. The rice cultivar “IR64” (as opposed to “Nipponbarre” that was used for the root experiments) was specifically used for the shoot experiments due to its wider tiller angles and hence less vertical tillers, the wheat cultivar “Bobwhite” was used for the wheat tiller reorientations. Rice generally have very vertical tillers, indeed the lowest starting tiller angle of those tillers used for reorientation was 151.56º, this means that by the nature of the tillers the growth response is small when they are reoriented to 180º. It was observed that for those individual tillers that were less vertical the difference between the angles before and after reorientation was greater, perhaps it would require longer than the 4 days the experiment was carried out over for those less vertical tillers to bend the amount necessary to return fully to their original angles due to the speed of the growth response. Another possibility is that the developing pulvini further up the stem begin to share the bending with those in the tiller as although excised pulvini are capable of executing full 90º bends in whole cereal grass shoots this is usually shared over a number of pulvini, a maximum of 4 in wheat and rice and 6 to 8 in

larger grasses such as sugarcane and bamboo (Kaufman et al., 1987). The tillers of wheat are generally less vertical than rice and are organised in a "tripod" like fashion, the least vertical tiller was recorded at an angle of 146.31º however the angles of most tillers lie between 160º and 170º. Wheat seems to return to closer to its original angle than rice (within 2º), this could be due to a difference in bending kinetics between rice and wheat or a difference in the speed or capacity of gravisensing between the two species.

A number of shoot growth angle mutants have been identified in rice, these include TAC1 (Yu et al., 2007), LAZY1 (Yoshihara and Iino, 2007) and PROG1 (Tan et al., 2008). *lazy1* plants display a wider tiller angle and impaired gravitropic response, *lazy1* coleoptiles exhibit no circumnutation and the changes in directional auxin flow found in wild type coleoptiles are absent in *lazy1*. *LAZY1* encodes a novel protein LAZY1 that is expressed in gravity sensitive shoot tissues such as pulvini, little expression is detected in roots, the results of Yoshihara and Iino also show that auxin redistribution and asymmetry must occur downstream of LAZY1. Loss of function mutants of *PROG1* also display wider tiller angles, the *PROG1* gene is thought to encode a putative transcription factor with high expression levels in the leaf sheaf pulvinus, the tiller base and the lamina joint (Tan et al., 2008). In contrast to LAZY1 and PROG1, TAC1 knockout mutants (*tac1*) display narrower tiller angles. TAC1 is highly expressed in the tiller base and is thought to be a member of a novel gene family involved in tiller angle control in a number of grass species (Yu et al., 2007). Phylogenetic analysis of TAC1 orthologues from a number of species show another TAC1-like clade of genes which includes LAZY1, as the two genes have opposing effects it would be tempting to assume that they act as antagonists to each other, indeed their expression patterns indicate that their expression is co-ordinately regulated however the complexities of shoot branching in dicots such as trees cannot be fully explained by this model. The function of these genes in relation to GSA maintenance in the tillers of rice is still unclear, however it seems unlikely that LAZY1 is involved in the active maintenance of the GSA as it is thought to act downstream of the auxin redistribution that would be required for GSA maintenance (Yoshihara and Iino, 2007). As both TAC1 and LAZY1 are expressed in higher order shoots of trees

such as peach (*Prunus persica*) whose angles appear to be set with no reference to gravity (it is thought that the angles of these branches are set in relation to the other branches) it again makes it unlikely for these two genes to be involved in the active maintenance of GSAs (Dardick et al., 2013).

To conclude, response to gravity is central to plant form. Many non-vertical angles are maintained with respect to the direction of the gravity vector, these are known as GSAs. It has been found that these GSAs are maintained in the non-vertically growing roots of both wheat and rice and the non-vertically growing tillers of wheat and rice. It is known that mutations in a number of genes can modulate these GSAs to be more or less vertical (Roychoudhry et al., 2013), the potential to modulate the non-vertical growth angles of crop species opens up a large number of possibilities to optimize the plant architecture resulting in increased yields or increased tolerance to sub-optimal conditions. This could help to alleviate the growing world food crisis.

3.4 Methods

3.4.1 Wheat seminal root reorientations

Seeds of *Triticum aestivum* “Bobwhite” were placed on moist filter paper and cold treated at 4ºC for two days. Seeds were placed into cyg seed germination pouches (Mega-International, Minneapolis, US) with the embryo oriented so that the germ was facing outwards and downwards. Pouches were placed upright in a reservoir of Hoagland’s No. 2 basal salt solution (Hoagland and Arnon, 1950) and plants were allowed to grow for 5 days. Growth conditions were as follows: 22ºC Day, 15ºC Night, 16 Hour photoperiod. Pouches were wrapped in aluminium foil to exclude light from the roots. Plants were photographed and reoriented by 30º, plants were allowed to grow for 24 hours and then a second photograph was taken. Angles before and after reorientation were measured using RootNav (Pound et al., 2013), measurements were then processed using Microsoft Excel, all roots that showed no growth or the starting angle was below 25º were excluded from the analysis as reorientation would have placed the roots beyond the vertical (0°) and transposed them to the other side. All images were captured using a Sony Cyber-Shot DSC RX100.

3.4.2 Rice crown root reorientations

Seeds of *Oryza sativa japonica* “Nipponbarre” were de-husked, placed onto moistened filter paper and cold treated for 2 days at 4ºC. Seeds were then placed at 27ºC 12-hour photoperiod for 3 days to allow germination before transferring to cyg seed-germination pouches. Pouches were wrapped in aluminium foil to exclude light. Plants were allowed to grow for 48 hours before being photographed and reoriented by 30º. After 24 hours, a second photograph was taken. The angles were measured and the data processed as for the wheat seminal root reorientations.

3.4.3 Wheat and rice angle change assays

Both wheat and rice were grown as for the reorientation experiments however after photographing at 0 hours the pouches were re-wrapped in foil and placed back into their respective growth conditions for 24 hours before being

photographed again, this was repeated to give a total growth time of 48 hours after the initial photo was taken. Plants were not reoriented. Roots were measured as before using RootNav.

3.4.4 Wheat and rice root clinorotation

Plants were grown as above but instead of reorientation plants were placed upon a 1 RPM clinostat in their respective growth conditions. Plants were photographed after clinorotation. Both wheat and rice were clinorotated for 7 hours, wheat was clinorotated at room temperature and rice was clinorotated at 27°C, the shoots of both wheat and rice were in the light whereas light was excluded from the roots.

3.4.5 Wheat and rice auxin treatments

Plants were grown as for reorientations but were placed in a reservoir of Hoagland's No 2 containing IAA to a given concentration, plants were not reoriented. Wheat plants were photographed after 6 days of growth in the pouch, rice plants were photographed after 4 days of growth in the pouch. Angles of both wheat and rice were measured using RootNav.

3.4.6 Wheat and rice shoot clinorotation

Wheat and rice were grown at 20ºC 16Hr photoperiod and 28ºC 12 Hr Photoperiod respectively, all experiments were carried out under these growth conditions. 4 and 5-week-old wheat plants and 4-week-old rice plants were placed upon a 4rph clinostat (Phillip Harris Education, England), for 4 Days, photographs of the plants were taken before and after clinorotation.

3.4.7 Rice tiller vertical placement

Seeds of *Oryza sativa indica* "IR64" were de-husked and placed into moist compost in cell trays, seeds were cold treated for 2 days at 4ºC before being placed at 27ºC, 12 hour photoperiod to grow. At 6-weeks-old a target tiller was chosen, a photograph was taken and the plant was reoriented to place this

target tiller at 180º. After 4 days a second photograph was taken. The tiller angles before and after reorientation were measured using imageJ.

3.4.8 Wheat tiller vertical placement

Seed of the wheat cultivar “bobwhite” was placed into moist compost, seeds were cold treated at 4ºC for 24 hours before being placed at 20ºC constant, 16 Hr photoperiod for growth. At 5-weeks-old a target tiller was chosen, a photograph was taken and the plant was reoriented to place this target tiller at 180º. After 4 days a second photograph was taken. The tiller angles before and after reorientation were measured using imageJ.

Chapter 4 : Mutations affecting the angle of seminal root growth in wheat

4.1 Introduction

In the previous chapter, it was demonstrated that GSAs are maintained in the cereal crops rice and wheat and that in the roots the mechanisms of their maintenance are similar to that of Arabidopsis i.e. the presence of an antigravitropic offset component to produce stable non-vertical growth and the ability of auxin to control the GSA. The tillers were also shown to maintain GSAs and this shares some commonalities with shoot GSA maintenance in Arabidopsis such as outwards bending of the non-vertical tillers upon clinorotation.

Previous work has shown that introducing mutations into a number of genes has the ability to modulate the GSA in the model plant *Arabidopsis thaliana*; these include mutations in many genes in the auxin response pathway such as the Aux/IAAs and TIR1 (Roychoudhry et al., 2013). Additionally, genes modulating root and shoot growth angle have also been found in cereal crops, these include LAZY1 (Yoshihara and Iino, 2007), TAC1 (Yu et al., 2007), PROG1 (Tan et al., 2008) and DRO1 (Uga et al., 2013). The above referenced studies focused on rice but these genes have also been documented in other cereal crops such as Maize (*Zea mays*) (TAC1 (Ku et al., 2011) and LAZY1 (Dong et al., 2013)).

Forward and reverse genetic screens have long been used in the discovery of novel genes and in assigning function to known genes. In the model plant Arabidopsis both approaches have been widely used to great success in many different areas to unpick the molecular and genetic workings of plant biology. Arabidopsis is diploid and this is to the advantage of its use in both approaches as it means knockouts are easily generated (e.g. through T-DNA insertion mutagenesis) and it is easy to see mutant phenotypes caused by recessive

mutations (e.g. those that may be caused by chemical mutagenesis such as those that are due to EMS (Kim et al., 2006, Lawrence and Pikaard, 2003)). However, a number of important crop species are polyploid these include: bread wheat, pasta wheat, coffee, potato, sugarcane, canola and cotton (Lawrence and Pikaard, 2003). Polyploids can arise either by the duplication of a single genome (autopolyploidy) or combining the genomes of two progenitors (allopolyploidy). It is thought that ecological or population fitness advantages may be the reason for the success of natural polyploids (Lawrence and Pikaard, 2003). This could be due to the pairing of homologous chromosomes in allopolyploids ensuring the transmission of each set of chromosomes to the next generation, this gives a kind of “forced heterozygosity” that could result in permanent hybrid vigour (Lawrence and Pikaard, 2003). It also could be that the presence of multiple copies of the same gene acts as a kind of insurance policy in case of deleterious mutations in one of the copies or that different copies of the same gene from different progenitors allows polyploids to exploit the differing activities of the proteins they encode allowing the polyploid to thrive in a greater range of habitats (Lawrence and Pikaard, 2003).

Wheat is one of the world’s most important food crops, worldwide there are around 650 million tonnes produced annually and this accounts for around 20% of the calories consumed by humans. 95% of wheat produced is common wheat (*Triticum aestivum)* with the remaining 5% being durum wheat (*T. turgidum ssp. durum)* (Dubcovsky and Dvorak, 2007). Both common and durum wheat are polyploids with durum wheat being tetraploid and common wheat being hexaploid, they are allopolyploids formed by the coming together of two and three progenitor genomes respectively. It is thought that their polyploidy arose as a result of domestication (Dubcovsky and Dvorak, 2007). Around 10,000 years ago the transition from hunter gathering to agriculture in western Asia (around modern day Turkey) led to the domestication of three cereals: einkorn wheat (*Triticum monococcum*), emmer wheat (*Triticum turgidum ssp. dicoccon*) and barley (*Hordeum vulgare*) (Dubcovsky and Dvorak, 2007). Einkorn wheat is diploid (containing genomes A^mA^m) and emmer wheat is tetraploid (genomes BBAA), it is thought that the initial domestication of emmer wheat led to the evolution of the modern durum wheat (also a tetraploid containing genomes

BBAA) in the eastern Mediterranean as durum wheat is closely related to domesticated emmer also found in that region (Luo et al., 2007). As the cultivation of domesticated emmer spread northeast into Europe it came into contact with *Aegilops tauschii* (genomes DD), this close contact resulted in sympatry and the production of the hexaploid common wheat (*Triticum aestivum)* that we know today, common wheat contains genomes BBAADD (Dubcovsky and Dvorak, 2007). There is a positive correlation between polyploidy in wheat and its success as a crop; in areas where einkorn and domesticated emmer were cultivated together the emmer eventually superseded the einkorn. In terms of grain size and yield potential, under optimum conditions, there is little to differentiate tetraploid durum wheat and hexaploid common wheat to explain common wheat's dominance over durum in the amount produced annually (Dubcovsky and Dvorak, 2007). However the additional D genome taken from *Ae.tauschii* has given hexaploid common wheat a greater adaptability in terms of photoperiod, vernalization requirements, increased tolerance to suboptimal temperatures and soil conditions and greater resistance to a number of pests and diseases thus allowing it to be grown successfully in a greater area than durum wheat (Dubcovsky and Dvorak, 2007).

Whilst this polyploidy has led to agricultural advantages for a number of crops, including (as evidenced above) wheat, it has thus far made them poor choices for genetic analyses and forward genetic screens as the phenotypic effects of single gene knockouts are easily masked by homeologous genes present on the other genomes (Uauy et al., 2009, Lawrence and Pikaard, 2003). Reverse genetics (using mutations in a gene to link a gene to a phenotype in order to determine the genes function) is an important way of determining which genes functions give certain traits such as those that may be important to agriculture. In diploid Arabidopsis and rice T-DNA insertion mutagenesis has been used to produce large collections of gene knockouts that can be used to probe gene function, however, this cannot be used successfully in wheat due to its polyploidy. RNA interference has been used successfully in the polyploid *Arabidopsis suecica* to knock down a gene found on both its progenitor genomes (Lawrence and Pikaard, 2003). RNA interference (RNAi) involves the

formation of a dsRNA that leads to the degradation of homologous mRNAs through the RNA induced silencing complex (RISC), this results in post-transcriptional gene silencing. RNAi has already successfully been used in wheat, early uses include using particle bombardment leading to transient expression in leaf cells to co-transform an in-vitro synthesized dsRNA with the *gusA* reporter gene, this reduced the expression of GUS in the co-transformed cells (Schweizer et al., 2000, Fu et al., 2007). RNAi was later used to show that reduced expression of the wheat germin-like protein TaGLP4 results in decreased resistance to the cereal powdery mildew *Blumeria graminis* demonstrating that this gene is involved in the resistance response (Fu et al., 2007, Christensen et al., 2004). It has been suggested that it would be possible to use RNAi to knockdown all three homeologous genes in wheat as they share at least 95% sequence similarity making it possible to design an RNAi trigger sequence (a sequence homologous to the sequence of the target gene cloned in both sense and antisense orientations separated by a linker sequence to allow the formation of the dsRNA needed for RNAi) that can be used to sufficiently silence all three genes (Fu et al., 2007).

However, whilst both T-DNA insertion and RNAi, when used in the correct context, can be incredibly useful in elucidating gene function, they are both techniques based on transgenic transformation which limits their use in crop improvement due to strict regulatory controls. Another reverse genetic approach well suited to use on polyploids is that of combining Ethyl Methanesulphonate (EMS) mutagenesis with Targeted Induced Local Lesions in Genomes (TILLING), as the mutations are introduced using a chemical mutagen this means that the mutations are stably inherited and allows the alleles that are generated to be used in traditional crop breeding programmes (Uauy et al., 2009).

Once a mutagenized population has been produced using EMS, DNA can be extracted from multiple mutant individuals and pooled, this allows for a high throughput. A PCR is then carried out on the pools of DNA using primers to amplify a target region of the genome, the PCR product is then heated, this allows heteroduplexes to form between wild-type and mutant DNA. The

mismatches between mutant and wild-type base pairs form a single stranded bulge that is recognized by a nuclease originally extracted from celery called CEL1 (indeed crude celery juice extract can be used instead of the expensive purified enzyme (Wang et al., 2012)), the PCR products are then cleaved at these mismatched sites and the products visualized using size separation on a polyacrylamide gel (Slade et al., 2005, Uauy et al., 2009). After the pools containing the mutant individuals are identified a second screen can be carried out to identify the individual plants that contain putative mutations, after these individuals have been identified the PCR products can be sequenced to determine the location and nature of the mutation. Once the mutations have been found gene function can be assigned using the phenotype of the mutant line (Uauy et al., 2009).

The root architecture of wheat is known to have a great influence on its yield in water limited environments, root traits thought to be important in drought tolerance in wheat include: the depth of rooting (Hurd, 1975), root distribution at depth (Hurd, 1968, O'Brien, 1979, Manske and Vlek, 2002), root elongation rate (O'Brien, 1979), xylem vessel diameter (Richards and Passioura, 1989) and the angle of the seminal roots (Nakamoto and Oyanagi, 1994, Manschadi et al., 2006). The contribution of each of these effects alone is highly dependent on soil type and the pattern of drought stress. For example, in situations where the crop is dependent on high levels of stored soil moisture such as where there is high winter rainfall but low summer rainfall, a major risk to the yield is down to running out of water before grain filling is completed. Richards and Passioura found that decreasing the diameter of the xylem vessels, therefore increasing the hydraulic resistance, resulted in improved post-anthesis water availability and a better grain yield, they used selection for this trait in a breeding program to develop wheat with an improved yield in drier environments (Richards and Passioura, 1989, Passioura, 1972). Using a known drought tolerant cultivar of wheat, SeriM82, and a standard wheat cultivar, Hartog, Manschadi et al showed that the drought tolerant cultivar of wheat had a much more compact root system than the standard wheat cultivar whose distance spread from the stem base was 28% more than that of the drought resistant cultivar. They also found that when compared with Hartog, SeriM82 allocated less root growth laterally

and produced a greater root length at depth (Manschadi et al., 2006). A larger root architecture screen was also carried out on 26 wheat cultivars chosen for their use in different water-limited environments in Australia, it was found that the average angle of the primary seminal root and of the second pair of seminal roots was roughly constant between the cultivars (Manschadi et al., 2008). However, there was a large variation in the average angle of the first pair of seminal roots with the largest angle being 56.3° in the cultivar Diamondbird and the smallest angle being 36.2° in the cultivar SeriM82 (already known for its drought tolerance), a subset of these cultivars were then taken forward to be grown in soil filled chambers and their whole root systems analysed 40 days after planting using fractal analysis (Manschadi et al., 2008). Fractal analysis superimposes a grid on the root system and takes into account the number of boxes that are intercepted by roots, it was found that those cultivars that displayed more vertical seminal roots also had a greater concentration of roots directly below the plant whereas those with less vertical seminal roots spread more laterally (Manschadi et al., 2008). These results and the results of Oyanagi (Oyanagi, 1994) and Nakamoto and Oyanagi, who found that the growth angle of the nodal roots was related to the growth angle of the seminal roots (Nakamoto and Oyanagi, 1994), emphasize the link between the growth angle of the root axes and the spatial distribution of the roots in the soil and, whilst little is known about the architecture of the mature wheat root system, these results suggest that the seminal root angle and early rooting characteristics are major determinants of the architecture and functioning of the mature root system (Manschadi et al., 2008).

In rice it has already been found that modifications to the RSA can have an effect on yield. A quantitative trait locus (QTL) was found that has a role in controlling the root growth angle of rice, DEEPER ROOTING 1 (DRO1) was found using recombinant inbred lines derived from a cross between the shallow rooting rice cultivar IR64 and the deeper rooting cultivar Kinandang Patong (KP) (Uga et al., 2011). It was found that lines homozygous for the KP allele of DRO1 had the same root dry weight in the upper layers of the soil as IR64 but a significantly greater root dry weight in the deeper layers (Uga et al., 2011). A near isogenic line (NIL) of the DRO1 allele from KP was developed in the IR64

background (DRO1-NIL), the maximum root depth of this NIL was more than twice that of wild type IR64. It was later found when the sequences were analysed from the DRO1 alleles of KP and IR64 there was a single base pair deletion in exon 4 of the IR64 allele that resulted in a premature stop codon, transforming a genomic fragment from KP containing DRO1 into IR64 resulted in increased deep rooting (Uga et al., 2013). When subjected to three levels of drought stress; no drought, moderate drought and severe drought, the DRO1-NIL line and IR64 produce similar yields per plant under no drought conditions. Under moderate drought conditions the DRO1-NIL line produces almost the same yield per plant as under no drought conditions whereas IR64 produces 43% of the yield it produces under no drought conditions, under severe drought conditions IR64 produces almost no yield per plant whereas DRO1-NIL produces around 30% of its no drought yield. Further experiments were carried out in which the flow of water from the lower soil layers to the upper soil layers was blocked using a layer of gravel, IR64 roots did not penetrate the gravel to access the water from the lower soil layer whereas DRO1-NIL plants did, the DRO1-NIL plants had a higher yield than the IR64 plants (Uga et al., 2013). It was also found that deeper rooting due to DRO-1 also increased yield (when comparing the DRO1-NIL line to IR64) in paddy field conditions; this was due to an increased thousand-grain weight and percentage of ripened grains due to better grain filling (Arai-Sanoh et al., 2014). Plant carbohydrates are produced through photosynthesis; it is thought that high nitrogen is important to maintaining a high photosynthetic rate. It is thought that DRO1-NIL may be able to better supply the grain with carbohydrates after heading due its increased nitrogen uptake from the lower soil layers post heading (Arai-Sanoh et al., 2014). Nitrogen in the upper soil layers is often insufficient at later growth stages, it is thought that the ability of DRO1-NIL to access nitrogen in the lower soil layers is advantageous to its growth at these later stages, particularly during the grain filling period where it would be able to maintain a higher photosynthetic efficiency. It was found that twenty days after heading, the flag leaves of DRO1-NIL contained more nitrogen than those of IR64 and at maturity the total nitrogen content of the plant was higher in DRO1-NIL (Arai-Sanoh et al., 2014).

Thus far QTLs have been identified in wheat that are thought to be involved in seminal root number, deep root ratio, elongation rate and hydrotropism but a QTL involved specifically in seminal root growth angle has yet to be found (Hamada et al., 2012). In this work we aimed to screen through an EMS mutagenized TILLING population of wheat to find lines with altered seminal root growth angles, determine if there are relationships between the different root architecture traits (e.g. does having long roots give a greater hull area or is having a wider growth angle a greater contributor) and to potentially identify some genes that could be involved in the control of seminal root growth angle in Wheat. We then aimed to determine the heritability of these traits and if the phenotypes seen in the 2D system used for the screen translated into soil based field trials. The potential to alter the seminal root growth angle of wheat could have a large impact upon its yield as it could improve its ability to gather resources from specific soil types or its resistance to drought.

4.2 Results

4.2.1 Deciding upon a screening system

Twenty seeds from a single spike of a total of 397 individual TILLING lines, each carrying a number of mutations, were obtained from Dr Cristobal Uauy (John Innes Centre, Norwich, UK). Lines were screened in the M_5 generation post EMS mutagenesis. Before beginning screening of the lines for altered root architecture traits it was decided to establish a method of screening to minimize variability among plants and with a high enough throughput to allow the lines to be easily and effectively screened. Through growth in low percentage gelling agent media inside a 400 ml Media bottle it was discovered that the wild type wheat root system is a 3-dimensional structure with a “tripod” like shape formed between the first pair of seminal roots and the primary root (Figure 4.1 A, B and C). In contrast, when grown in a 2-dimensional system such as a cyg-seed germination pouch (Mega-International, Minnesota, USA) the tripod like structure is replaced by a vertically (occasionally skewed) growing primary root followed by one or two successively less vertical pairs of seminal roots (Figure 4.1 D and E). The growth in the gel filled Schott bottle system was notably slower (3 Weeks) than that in the seed germination pouches (6 Days) to obtain plants from which root traits could be measured. It was decided that for ease of measurement of the root traits, and to maintain a high throughput, that growth in seed germination pouches was better for the purposes of screening.

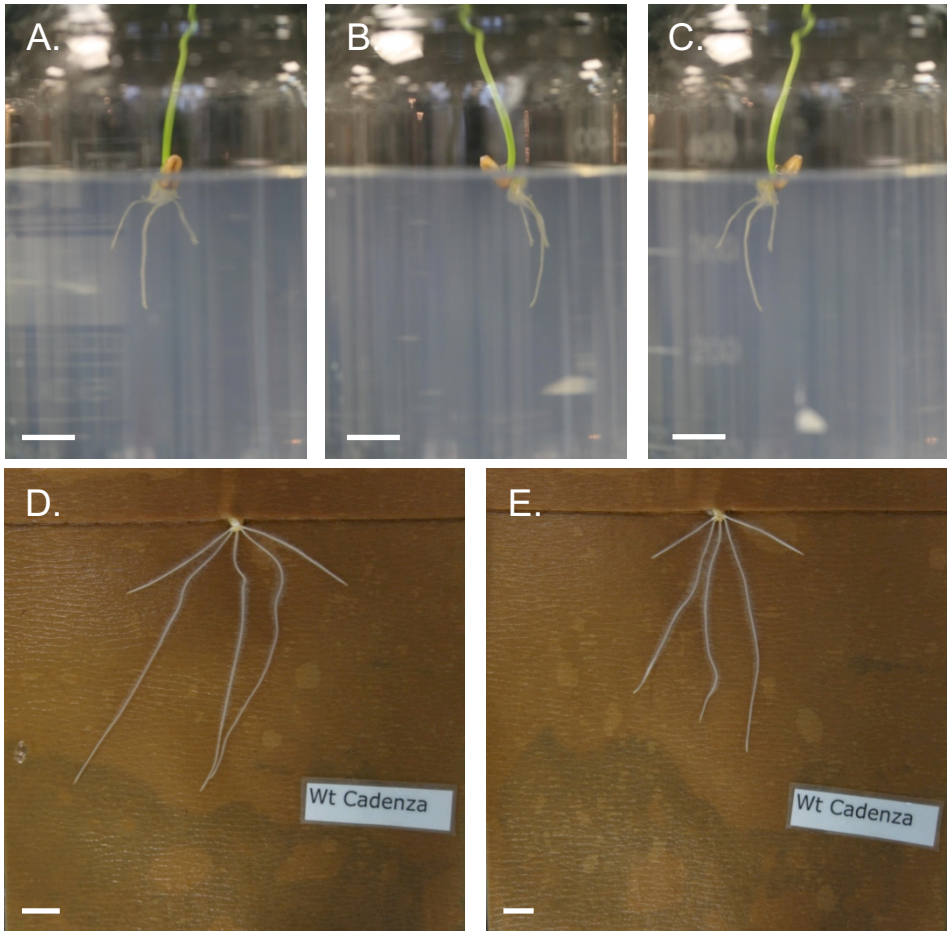


Figure 4.1: A comparison of wheat root systems grown in two and three dimensions

When grown in a 3D system the wheat root system forms a tripod like structure with both the primary root and the first pair of seminal roots growing at non vertical angles (**A**,**B** and **C**), when grown in a 2D system this tripod like structure is replaced by a vertically growing (occasionally skewed) primary root and successively less vertical pairs of seminal roots (**D** and **E**). Scale bars represent 1 cm.

4.2.2 Seed weight has no significant effect upon the tip angle of the seminal roots

To minimize variability amongst plants of individual lines and to better aid comparison of lines with differing average seed weights, it was decided to determine if the weight of the seeds had an effect upon the seminal root tip angle of wheat. Wt Cadenza seeds were separated into three weight classes: <30 mg, 30-40 mg and > 40 mg, these classes were decided based upon the median weight of a group of 50 seeds of Wt Cadenza. Fourteen seeds in each weight class were grown for 6 days in seed germination pouches before measurement of the tip angles of the seminal roots using RootNav. There was no significant difference in the seminal root tip angle between the different seed weight classes of both the first and second pairs of seminal roots although the tip angles of the second pair of seminal roots are slightly less vertical than those of the first pair (Figure 4.2). It was therefore decided that seed representative of each line could be selected visually (i.e. not abnormally small or large when compared with other seed of that line) for screening and that individual lines of differing average seed weights would be comparable for seminal root tip angle.

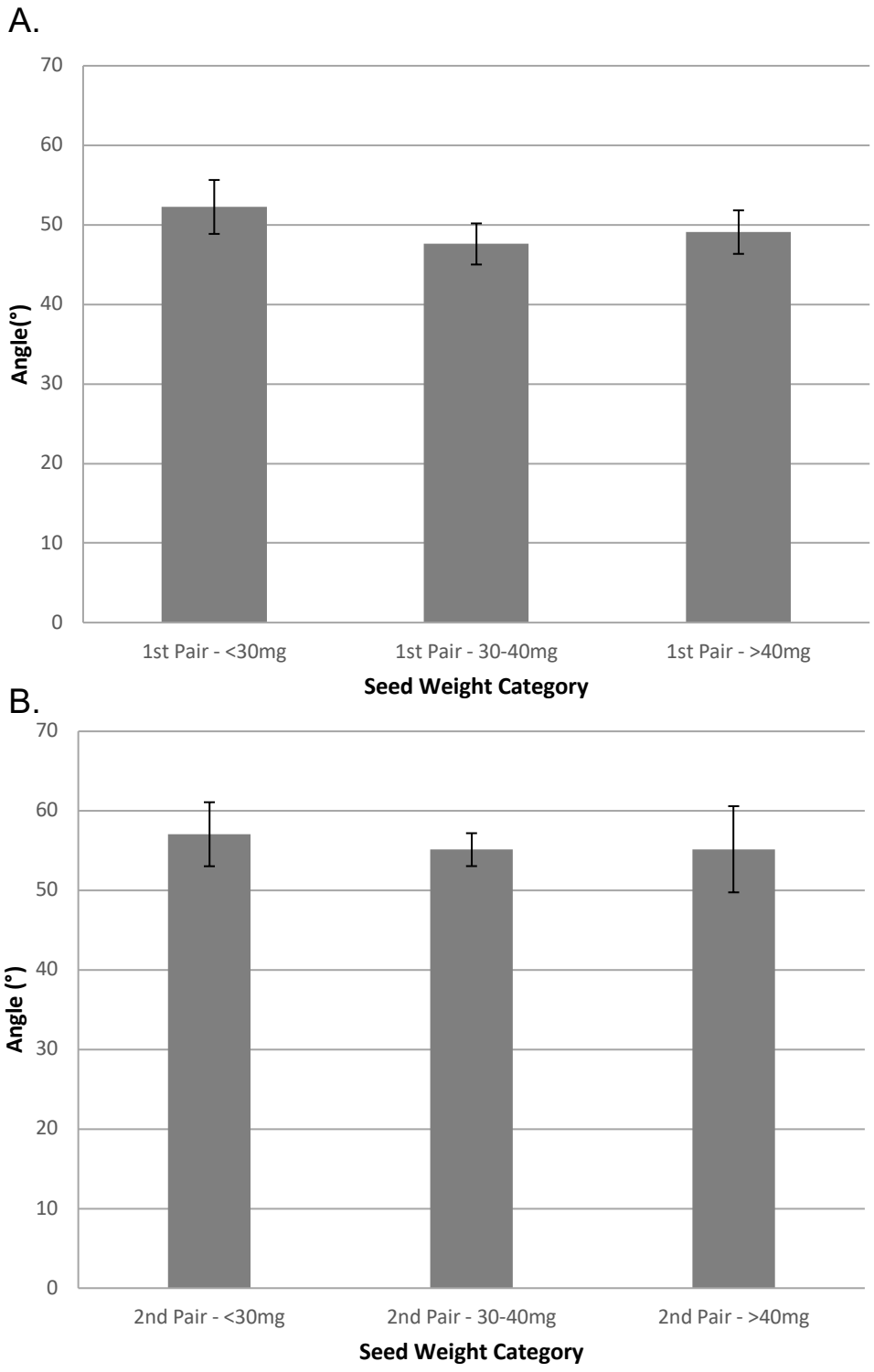


Figure 4.2:There is no difference in the tip angle of the seminal roots between seeds of different weight categories

The weight of the seed makes no significant difference to the tip angle of both the 1st (a) and 2nd (b) of seminal roots in wheat. 1st pair: <30 mg n=28, 30-40 mg n=28, >40 mg n=32. 2nd pair: <30 mg n=11, 30-40 mg n=4, >40 mg n=11. P>0.05 for all comparisons.

4.2.3 Comparison of root architecture traits across all the screened TILLING lines

Once all 397 TILLING lines had been screened and measured, the relationships between the different root architecture traits were explored to determine how each contributed to the overall root architecture, and how the individual traits were linked to give a better idea of the role of each trait in generating the root architecture of an average wheat plant.

4.2.4 There is a positive correlation between the length of the primary seminal root and 1st pair seminal roots

Across all lines there is a positive correlation between the length of the primary seminal root and the first pair of seminal roots (Figure 4.3) (The second pair of seminal roots were omitted from comparison as not all lines consistently produced two pairs of seminal roots), the equation of the trend line has a gradient of 0.3917 and a y-intercept of 55.557. This indicates there is little to no discrepancy between the growth rates of the primary and 1st pair of seminal roots and that root growth rate is uniform between the different root types.

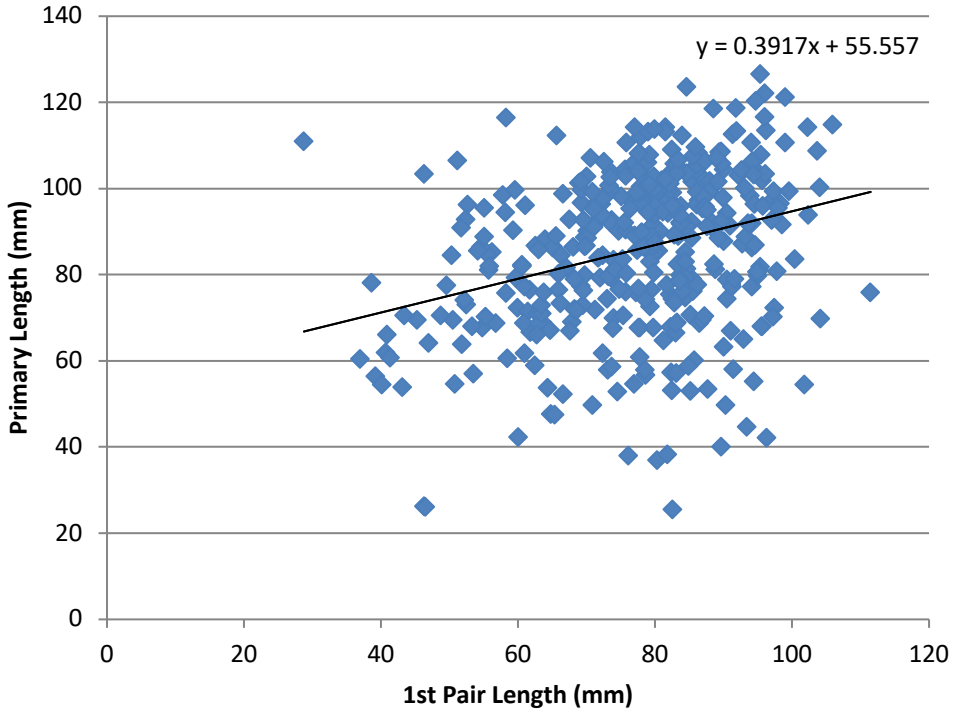


Figure 4.3: There is a positive correlation between primary seminal root length and 1st pair length

There is a positive correlation between primary length and 1st pair length, this indicates that there is little to no discrepancy in growth rate between the primary and the 1st pair of seminal roots. For each point n=10 for primary roots and n=20 for 1st pair of seminal roots. R^2= 0.092.

4.2.5 There is weak to no correlation between first pair tip angle and either first pair length or first pair emergence angle

There is weak to no correlation between the tip angle of the first pair of seminal roots and both the length and emergence angle of those roots (Figure 4.4). The equations of the trend lines (0.0458x+33.233 for 1st pair length against tip angle and 0.0887x+32.191 for 1st pair emergence angle against tip angle) suggest a weakly positive correlation in both cases however the large spread of the data and weakness of the correlation would indicate that these root architecture traits are not linked and can therefore be treated as independent of each other.

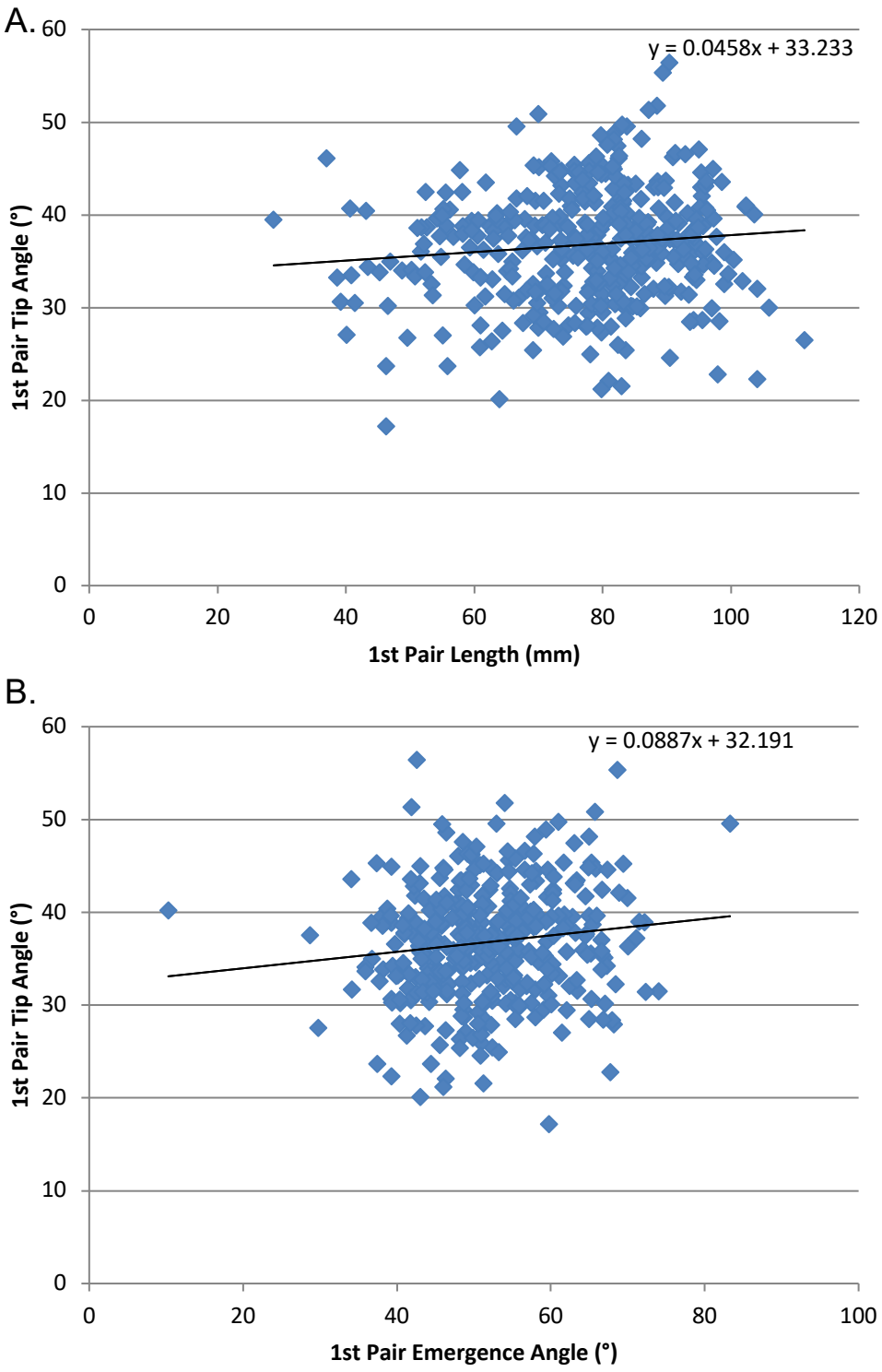


Figure 4.4:There is weak to no correlation between first pair tip angle and either first pair length or first pair emergence angle

There is little to no correlation between both the (A) first pair tip angle and first pair length and (B) the first pair tip angle and first pair emergence angle. This indicates that these traits are not linked. For each point n=20 for 1st pair of seminal roots. R^2 = 0.012 for A and 0.017 for B.

4.2.6 There is a positive correlation between first pair tip angle, first pair length and primary length and the hull area.

The hull area is a measurement of the area encompassed by the root system (indicated by the red dotted line on Figure 4.5) and therefore the area of soil from which the root system can gather resources. A positive correlation was found between hull area and a number of other traits. The strongest correlation was shown between the hull area and the length of the first pair of seminal roots (Figure 4.6 B), the second strongest correlation was between the hull area and the tip angle of the first pair of seminal roots (Figure 4.6 A), the weakest correlation was between hull area and the length of the primary root (Figure 4.6 C). This suggests that all three traits play a role in determining the hull area of the wheat root system however the extent to which each has an effect is variable, for example the length and angle of the first pair of seminal roots play a greater role in determining the hull area than the length of the primary root, this highlights the importance of the non-vertically growing seminal roots in plant resource gathering.

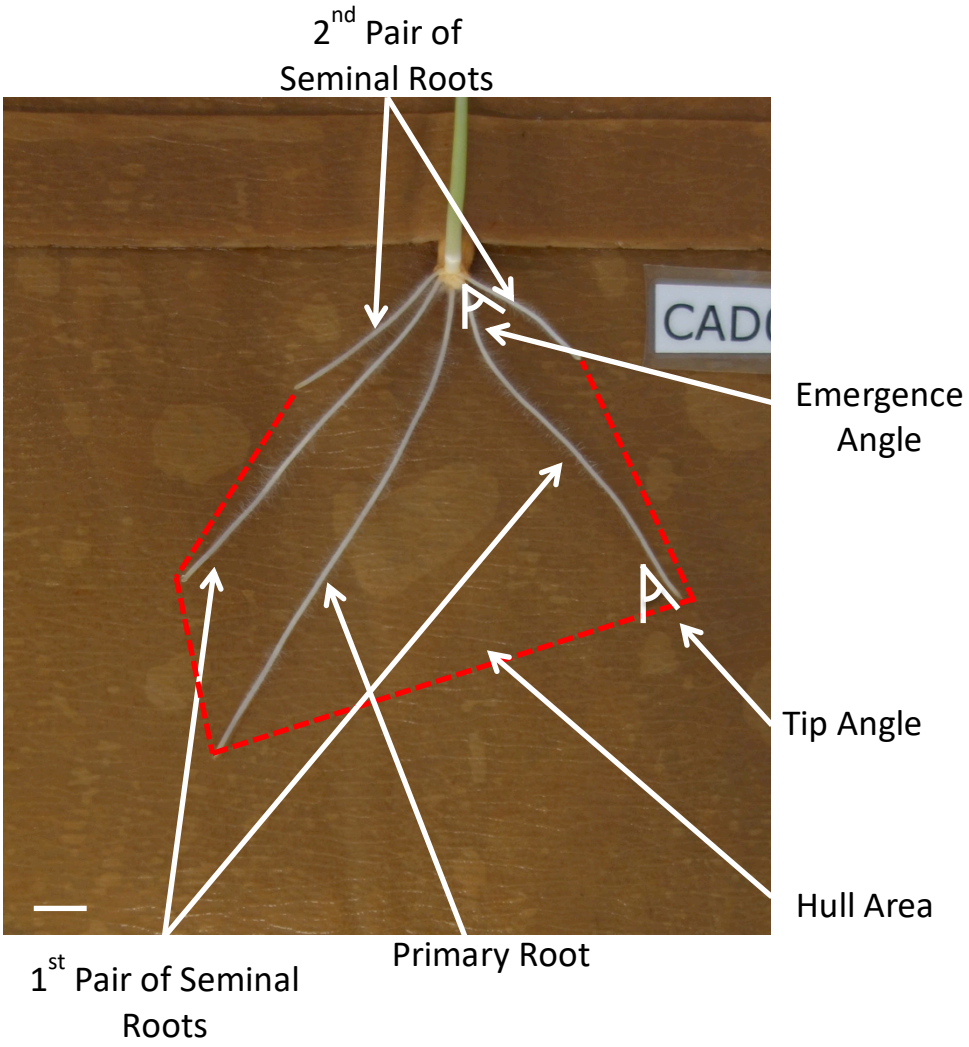


Figure 4.5: The anatomy of the juvenile wheat root system grown in a 2D system

The anatomy of the juvenile root system (around 5 days old) showing: (a) the first pair of seminal roots, (b) primary root, (c) the hull area – the area encompassed by the root system, (d) the tip angle, (e) the emergence angle and (f) the second pair of seminal roots. Scale bar represents 1 cm.

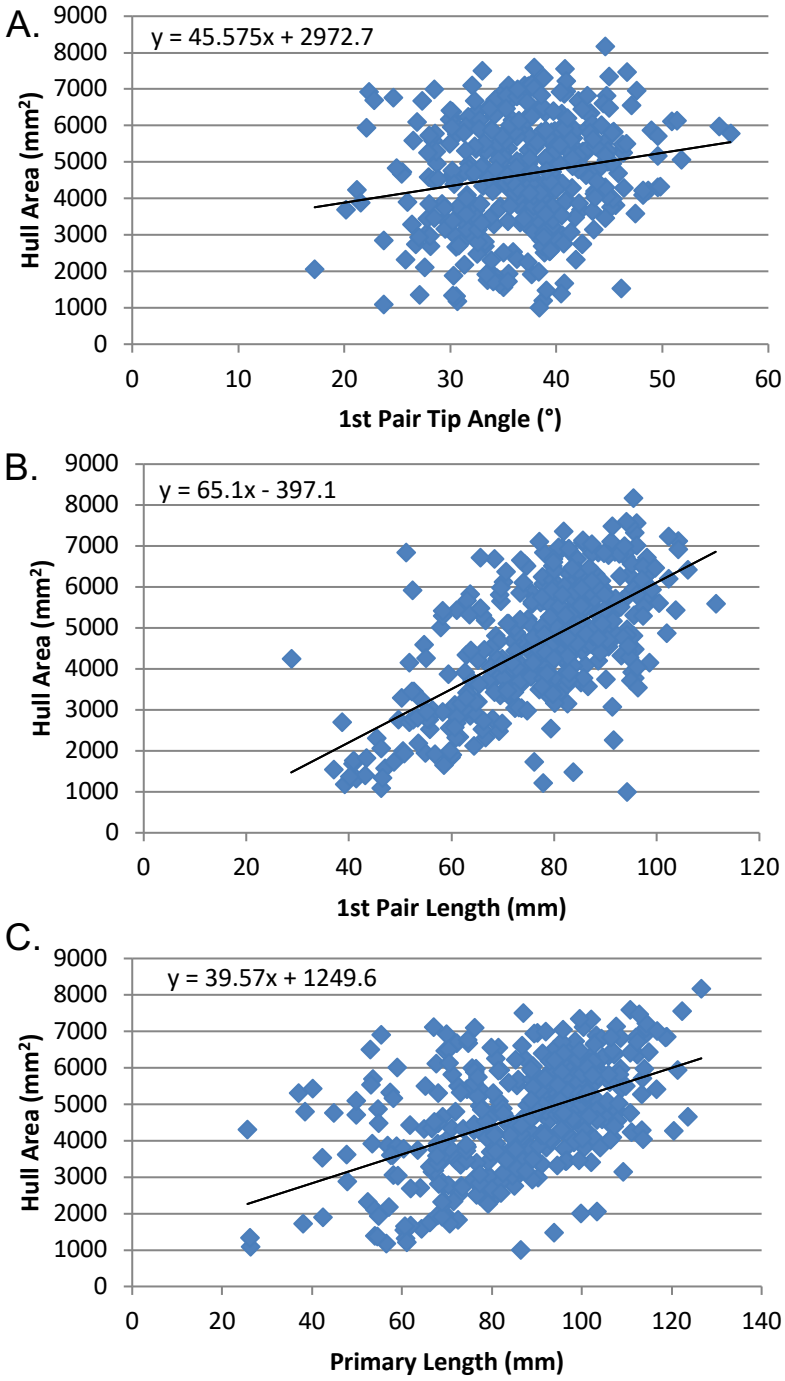


Figure 4.6: There is a positive correlation between first pair tip angle, first pair length and primary length with hull area.

There is a positive correlation between first pair tip angle (a), first pair length (b) and primary length (c) and hull area, this correlation is strongest for first pair length followed by primary length. This indicates that the length of the seminal roots plays a bigger role in resource gathering for the plant at this stage of its development that root angle but that all three traits contribute to resource capture. n=10 for hull area and primary roots, n=20 for 1st pair of seminal roots. R^2 = 0.03 for A, 0.39 for B and 0.24 for C.

4.2.7 Length is a greater contributor to the size of the hull area than root angle

To determine which root traits made the biggest overall contribution to hull area a number of lines were selected that belonged to three different hull area categories: the lowest hull areas (CAD0209, 0001, 0015, 0017, 0214), mid-range hull areas (CAD0073, 0181, 0236, 0117, 0174) and the highest hull areas (CAD0179, 0184, 0118, 0203, 0110, 0200, 0148, 0201, 0198) (Figure 4.7 A). Comparison of a number of traits showed that those lines with the highest hull areas also displayed long roots (both primary and 1st pair seminal) and those with the lowest hull areas displayed short roots whereas the tip angles (primary and 1st pair seminal) were variable across a range of hull areas (Figure 4.7 B) suggesting that root length is a greater contributing factor to the size of the hull area than root angle.

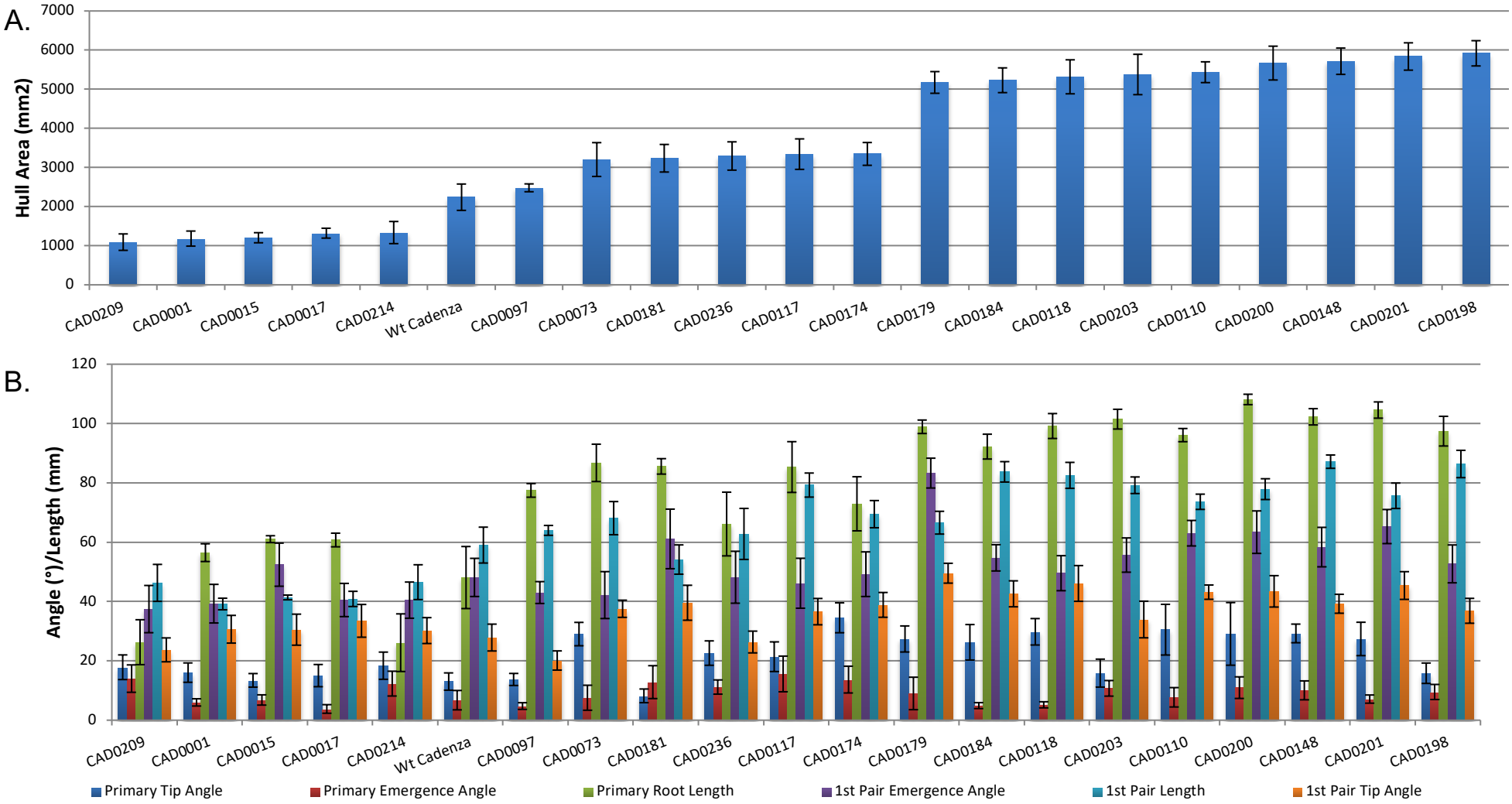

A.
Hull Area (mm2)
7000
6000
5000
4000
3000
2000
1000
0
CAD0209
CAD0001
CAD0015
CAD0017
CAD0214
Wt Cadenza
CAD0097
CAD0073
CAD0181
CAD0236
CAD0117
CAD0174
CAD0179
CAD0184
CAD0118
CAD0203
CAD0110
CAD0200
CAD0148
CAD0201
CAD0198
B.
Angle (°)/Length (mm)
120
100
80
60
40
20
0
Primary Tip Angle
Primary Emergence Angle
Primary Root Length
1st Pair Emergence Angle
1st Pair Length
1st Pair Tip Angle

Figure 4.7: Length is a greater contributor to the size of the hull area than root angle

A. A number of lines were selected displaying a range of hull areas from amongst the smallest (CAD0209) to the largest (CAD0198) of all the lines screened. **B.** A number of their root architecture traits were compared to determine which had the greatest impact on the overall hull area, the graph shows that those lines with the largest hull areas had the longest roots whereas lines with the largest hull areas did not always have the least vertical roots indicating that root length is a greater contributor to hull area than root angle. Error bars represent SEM.

4.2.8 The root system architectures can be split into four different archetypes

As it was found that both the length and angle of the first pair of seminal roots contribute to the size of the hull area it was decided that the RSAs could be divided into four different archetypes based upon these traits (Figure 4.8): Long and more vertical, long and less vertical, short and more vertical and short and less vertical. These archetypes could then be used to select lines to take forwards for further testing along with those that displayed the extremes of root angle (most and least vertical), these lines are listed in Table 4.1. Four lines that were among the most vertical and four lines that were among the least vertical were selected along with four with long and less vertical roots, four with long and more vertical roots, one with short and less vertical roots and one with short and more vertical roots. These lines were then taken forwards for further testing.

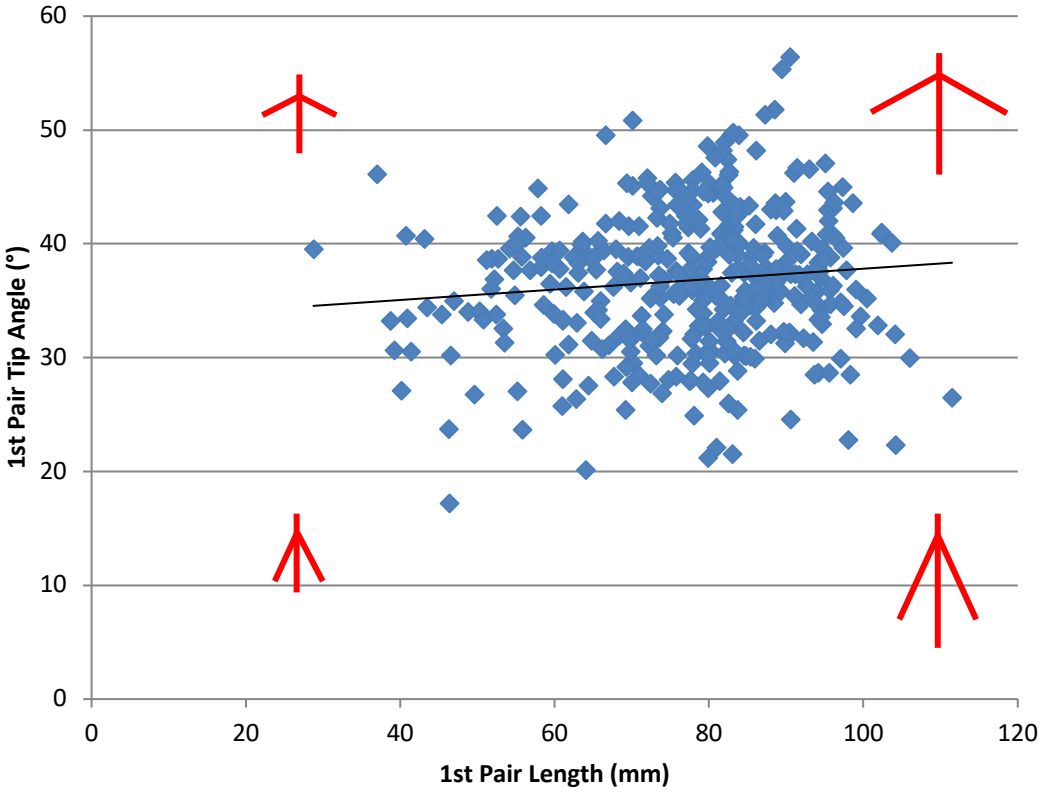


Figure 4.8: The root system architectures can be split into four different archetypes

Based on the traits of seminal root length and seminal root angle (both important contributors to the overall hull area) the root systems of the individual lines can be split into four archetypes (clockwise from top left): Short and less vertical, long and less vertical, long and more vertical and short and more vertical. A total of 397 lines were screened. For each measurement n=20.

Line	Selected on Basis of	Phenotype from Screen
CAD0111	Angle	Less Vertical
CAD0923	Angle	Less Vertical
CAD0359	Angle	Less Vertical
CAD0727	Angle	Less Vertical
CAD0224	Angle	More Vertical
CAD0157	Angle	More Vertical
CAD0190	Angle	More Vertical
CAD0092	Angle	More Vertical
CAD0052	Architecture	Long Roots, Less Vertical
CAD0647	Architecture	Long Roots, Less Vertical
CAD0674	Architecture	Long Roots, Less Vertical
CAD0097	Architecture	Long Roots, More Vertical
CAD0548	Architecture	Long Roots, More Vertical
CAD0251	Architecture	Long Roots, More Vertical
CAD0334	Architecture	Short Roots, Less Vertical
CAD0757	Architecture	Short Roots, More Vertical

Table 4.1: The Wheat TILLING lines selected to be taken forwards for further work

The wheat TILLING lines that were selected to be taken forwards for further work, lines were selected on the basis of their phenotype from the original screen based on either first pair tip angle alone or their root archetype based on the categories described in Figure 4.8.

4.2.9 The seminal root traits are not consistent on all spikes

For each of the lines chosen three individual spikes were selected and seed extracted from each one, ten plants were grown from each spike and their root architecture traits measured to see if each plant matched the phenotype found for that line from the original screen. For lines selected on the basis of architecture individual plants did not generally display both the phenotypic characteristics that defined the archetype they were selected for. Two lines (CAD0334 and CAD0757) displayed a “half phenotype” in which the angle phenotype was correct on some plants but no plants displayed the root length phenotype, this suggests that in these lines that the phenotypes of the two different traits are affected by mutations in different genes. For those lines selected on the basis of angle, individual spikes were found to be phenotypically consistent or nearly so for their selected trait (Figure 4.9). It was decided that for a field trial it would be best to use lines selected on the basis of angle alone and from these lines two more vertical lines and two less vertical lines were selected and the remaining seed from those spikes that were found to be phenotypically consistent (or nearly so) was taken forwards to be used in the field trial.

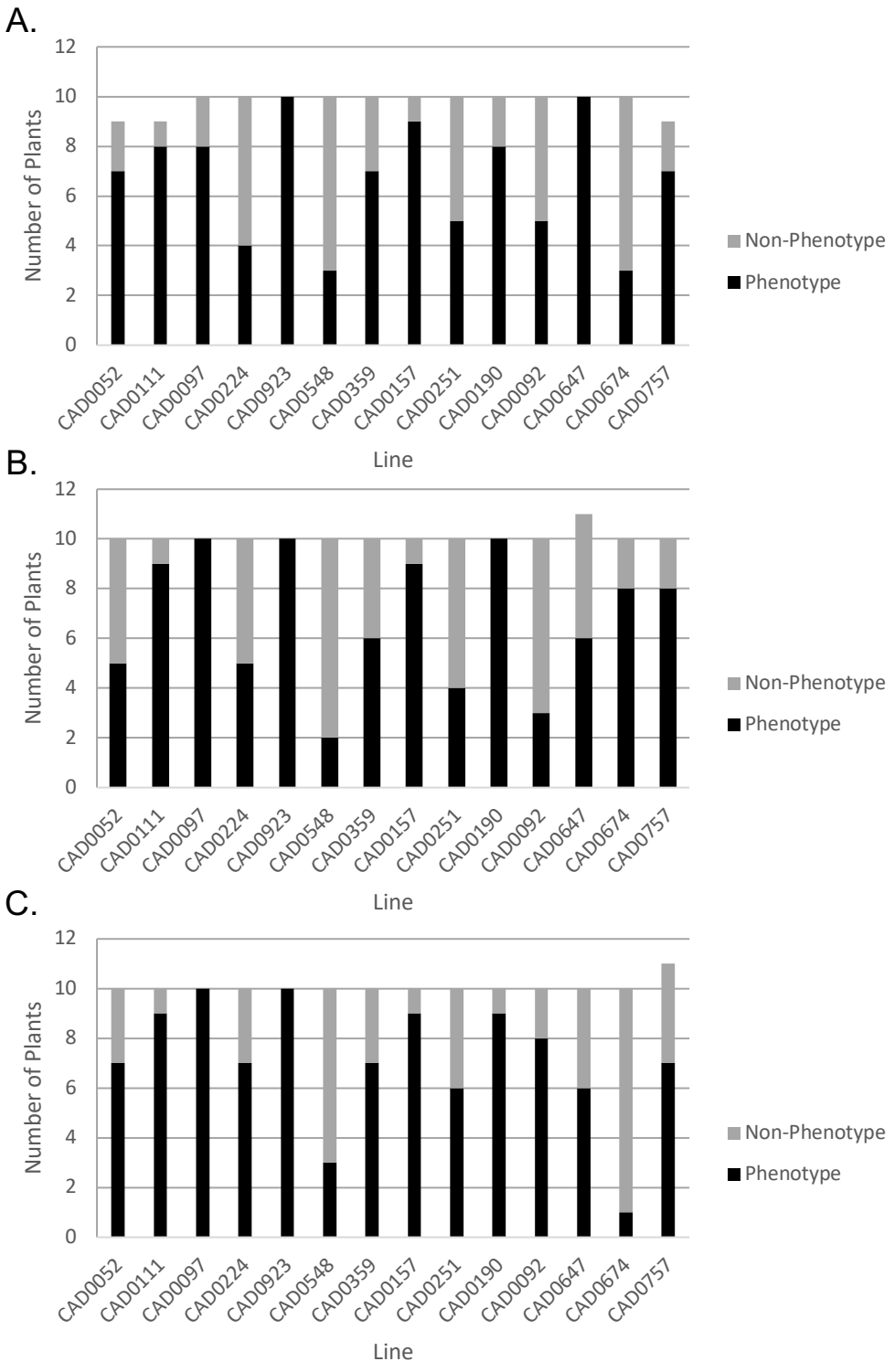


Figure 4.9: The seminal root traits are not consistent on all spikes
The root characteristics for which the lines were selected were not consistent for all lines and not on all spikes within a line, three spikes were tested for each line, spike 1 (a), spike 2 (b) and spike 3 (c), seed from phenotypically consistent (or nearly so) spikes was taken forwards for the field trial.

4.2.10 Some phenotypes from the initial screen carry through into the soil

To determine if the phenotypes found in the original screen carried through into a field environment, for the selected angle phenotype lines a seed from a phenotypically consistent (or nearly so) spike was placed into the centre of a kitchen colander that had been sunk into the ground up to its rim and backfilled with soil. The colanders had 16 tiers of holes in the sides and 8 concentric rings of holes in the base; a 3 mm border of plastic separated the holes. The plant was then allowed to grow until maturity before the colander was lifted from the field and the holes that roots emerged from were marked, the number of roots emerging from each tier was counted and the root emergence angle from each tier was calculated as shown in Figure 4.10. The average number of roots per line was variable, ranging from an average of 116.14 roots for CAD0111 to 48 roots for CAD0190, the results suggest that the number of roots is not linked to the angle phenotype for which the lines were selected (Figure 4.11). To normalize for the different numbers of roots produced by each line, the percentage of the plants total number of roots emerging at each angle was calculated. CAD0190 had a greater percentage of its root system emerging at more vertical angles and CAD0923 had a greater percentage of its root system emerging at less vertical angles when compared with Wt Cadenza (Figure 4.12), this matches the phenotypes that each of these lines demonstrated in the original 2D screen (Table 4.2), phenotypes were based upon average 1st pair tip angle of ten 6-day old plants from each line, when compared with Wt Cadenza.

Table 4.2: The phenotypes in the original screen on which the field trial lines were chosen

Line	Phenotype in Original Screen
CAD0111	Less Vertical 1st Pair Seminal Roots
CAD0923	Less Vertical 1st Pair Seminal Roots
CAD0157	More Vertical 1st Pair Seminal Roots
CAD0190	More Vertical 1st Pair Seminal Roots

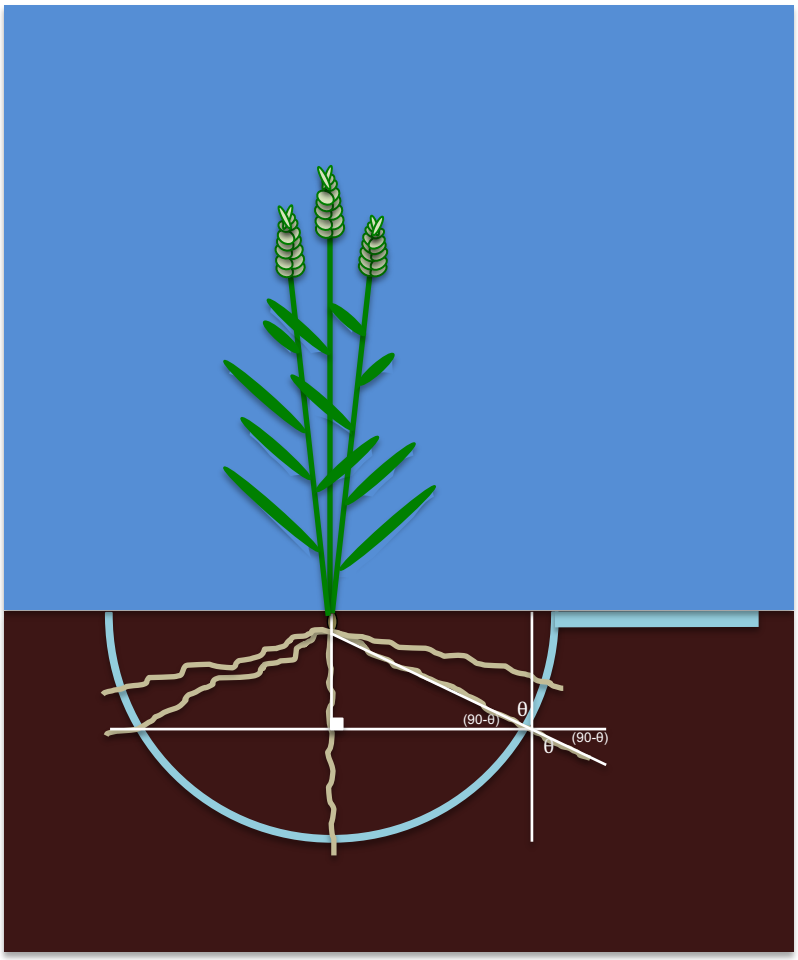


Figure 4.10: Diagram showing how the angle of the root emerging from the colander was calculated.

The diameter across the colander and the depth from the rim of the colander were measured for each row of holes. From this the radius of the colander could be calculated, this would act as the adjacent, the depth would act as the opposite. The length of the hypotenuse was calculated and used to work out (90-θ) in the equation “Adjacent/Hypotenuse= Cosine (90-θ)”. From this the value of θ was calculated, this is the angle at which the roots emerged from the colander at the row of holes.

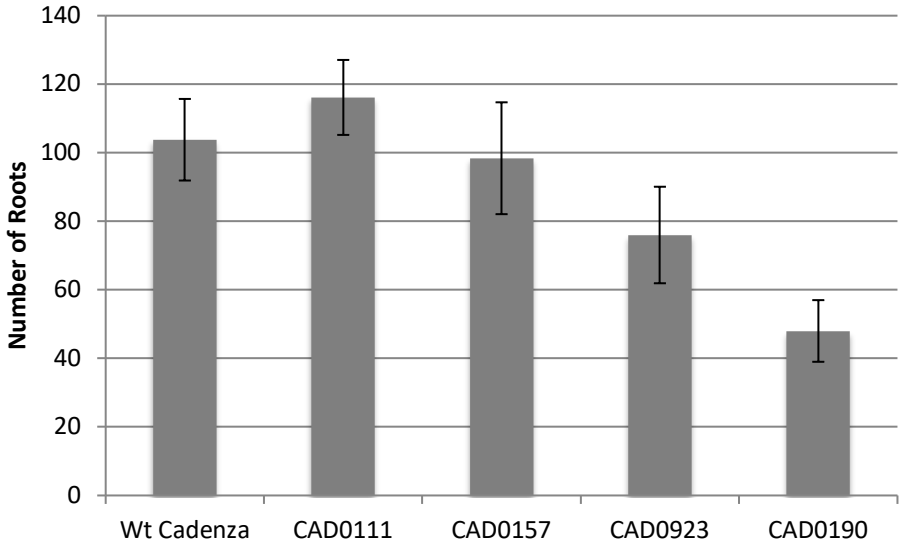


Figure 4.11: Variation in the total number of roots produced by each line in the field trial

There was a wide variation in the average total number of roots produced by each line in the field trial ranging from 116.14 roots produced by CAD0111 to 48 roots produced by CAD0190. (CAD0111 n=7, CAD0157 n=5, CAD0923 n=6, CAD0190 n=5), there does not appear to be a link between the root number phenotype and the angle phenotype they were selected for, error bars represent SEM.

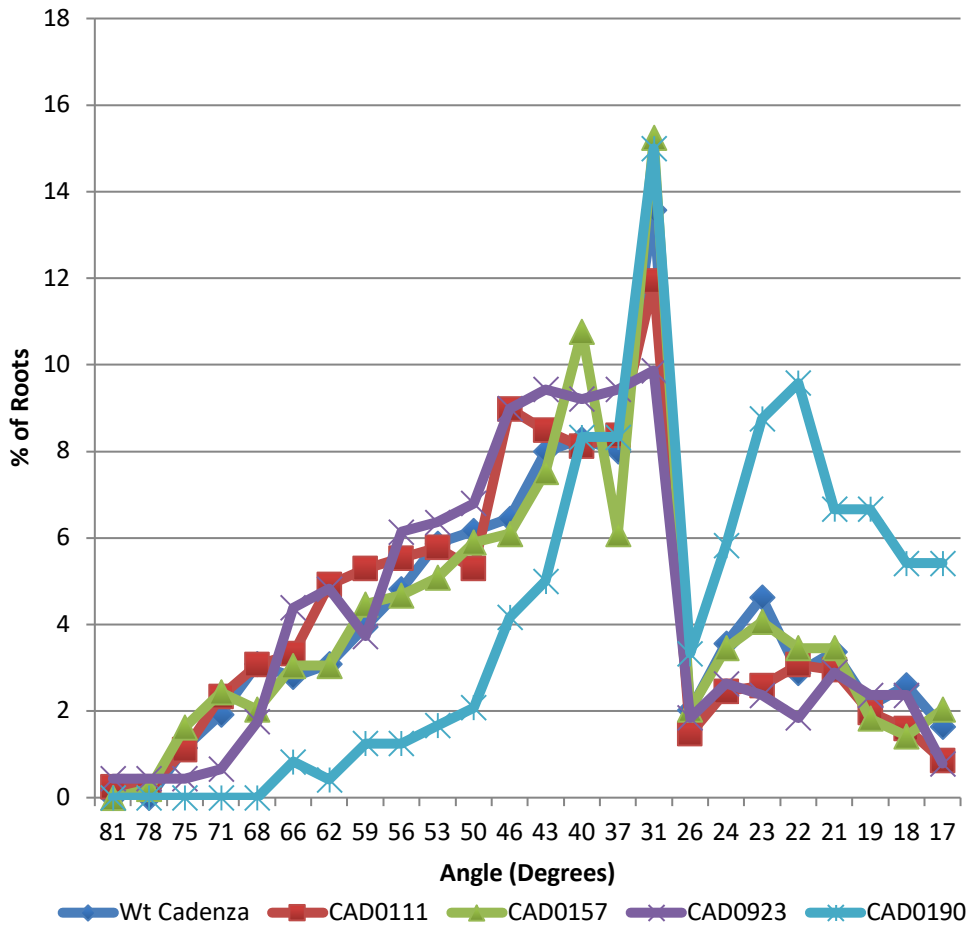


Figure 4.12 :In the field trial CAD0190 had a greater percentage of roots more vertical and CAD0923 had a greater percentage of roots less vertical than wild type cadenza

In the field trial CAD0190 had a greater percentage of roots more vertical than wild type, this line was chosen on the basis of its more vertical angle in the original screen and this shows that this phenotype carries through from the 2D pouch system to soil. CAD0923 shows a greater percentage of roots less vertical than wild type, this line was chosen as in the original screen it showed a less vertical angle phenotype, this demonstrates that this phenotype also carries through from the 2D pouch system to soil. Wt Cadenza n=10, CAD0111 n=7, CAD0157 n=5, CAD0923 n=6, CAD0190 n=5

4.2.11 There is no significant difference in angle between Wt Cadenza and the CAD0190 back-cross F_1

As CAD0190 showed the strongest phenotype in the field trial it was decided to back-cross the line with Wt Cadenza in order to work towards mapping the causal mutation for its more vertical root system. It was found that there was no significant difference in 1st pair tip angle between the CAD0190 back-cross F_1

and Wt Cadenza, however the difference in 1st pair root length between the two was significant (Figure 4.13). This is in agreement with the length phenotypes shown during the original screen where the average 1st pair length of Wt Cadenza was 59 mm and the average 1st pair length of CAD0190 was 74.7 mm. This suggests that whilst the angle phenotype of CAD0190 may be recessive or a result of a combination of a number of mutations, the length phenotype may be dominant.

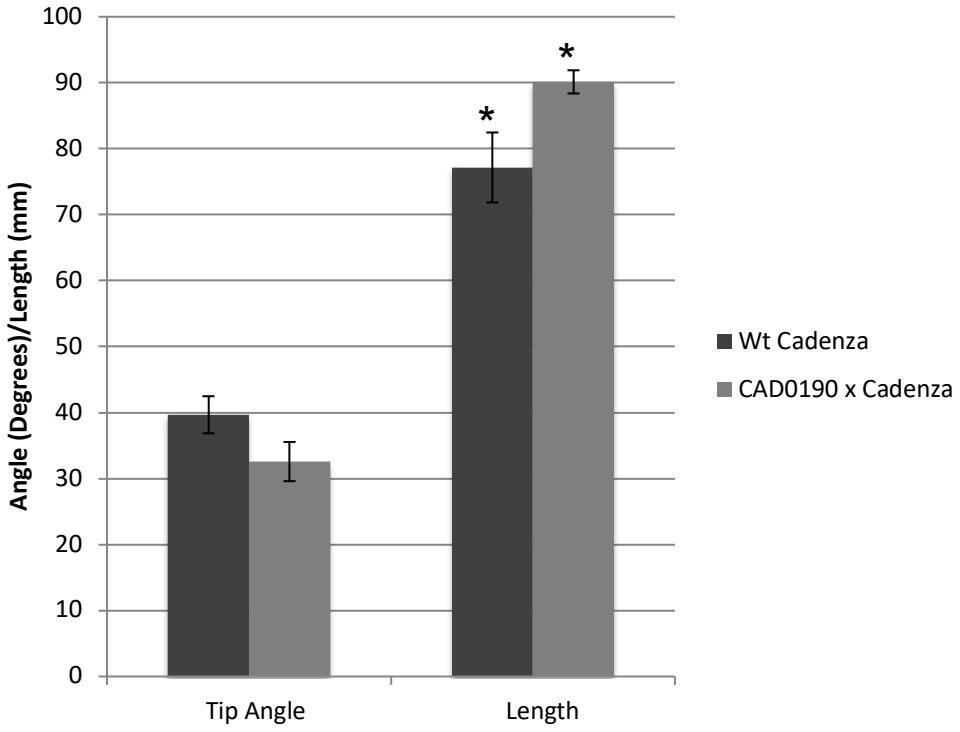


Figure 4.13: There is no significant difference in angle between Wt Cadenza and the CAD0190 back-cross F_1 but there is a significant difference in length.

There is no significant difference in angle between Wt Cadenza and the CAD0190 back-cross F_1 ($P>0.05$, Students T-test) but there is a significant difference in length ($P<0.05$, Students T-Test, indicated with a *) n=20 for Wt Cadenza, n=18 for CAD0190 back-cross F_1, error bars represent SEM.

4.3 Discussion

Due to its high importance as a food crop worldwide there is constant demand for improved wheat cultivars. Be it in an improvement in yield or greater tolerance to suboptimal conditions, the rapidly growing world population is fuelling the need to produce more food with only limited scope for increasing agricultural land. Another area of concern is the environmental impact of high intensity, high input agriculture on the land and the planet as a whole. As soils become depleted in nutrients the demand for chemical fertilisers such as nitrates, produced by the Haber process, a high-energy demand method of fixing nitrogen (Ahlgren et al., 2008, Dawson and Hilton, 2011), and phosphates, mined from a rapidly dwindling number of mineral sources (Dawson and Hilton, 2011), increases, the production of these fertilisers is not sustainable at current levels and to meet the growing demand for more food. The ability to produce crop cultivars that are capable of producing high yields in lower input systems and suboptimal soil types will become a key focus of plant science in the coming years. Root architecture has been found to be of great importance in soil exploration and therefore nutrient and water acquisition of crop plants, it is hoped that a greater understanding of the genetic control of root architecture traits such as root growth angle and length will lead to an ability to modify these traits to produce improved crop cultivars and alleviate both the humanitarian and environmental pressures of the growing food crisis (Lynch, 2007).

TILLING is a powerful genetic resource for the determination of gene function and the introduction of new phenotypes into traditional breeding programs. Mutations caused by chemical mutagenesis such as using EMS are stable, heritable and non-transgenic meaning that advantageous mutant phenotypes can be easily and rapidly introduced into breeding lines (Uauy et al., 2009) allowing research to directly feed into food production and circumventing many of the issues surrounding the use of transgenic crop plants. In this work, a TILLING population of the common wheat cultivar “Cadenza” was screened for root architecture phenotypes with the aim to study the links between different root architecture traits, find mutant lines with phenotypes that could be

advantageous in crop breeding programs and explore the underlying genetics behind these phenotypes.

Prior to screening the TILLING lines a system had to be established that would provide a rapid and efficient means of looking at the root architecture traits between different lines and give uniformity between plants of the same line. Growth in a three-dimensional system based upon a media bottle filled with a low gelling agent percentage media (Figure 4.1) reveals the wheat seedling root system to be a highly three dimensional, tripod-like structure. However, plant growth in this system proved to be slow, 3 weeks to produce a plant of a size analysis could be carried out upon, the space and resources required to screen through many plants high (limiting throughput), and the ability to extract quantitative data from plants grown in this manner difficult. In a number of other studies a two-dimensional screening system has been used to great effect for high throughput screening of wheat (Manschadi et al., 2008, Manschadi et al., 2010, Atkinson et al., 2015, Bai et al., 2013), barley (Manschadi et al., 2008, Bengough et al., 2004) and maize (Zhu et al., 2005, Hund et al., 2009). A range of systems have been used from those based on thin, soil filled rhizoboxes (Bengough et al., 2004, Manschadi et al., 2008), to those based on a thin gel between two pieces of Perspex (Bengough et al., 2004, Manschadi et al., 2008, Manschadi et al., 2010) and a number based upon germination paper (Atkinson et al., 2015, Hund et al., 2009, Zhu et al., 2005, Bai et al., 2013). These 2D systems all have their own advantages and disadvantages, for example, in a rhizobox style system only the roots that are against the Perspex sides can be visualized and the full architecture cannot be seen unless a pin board is inserted and the soil is washed away (Bengough et al., 2004). However, inevitably some of the RSA is disturbed by this and the accuracy of some of the measurements is lost, so whilst soil is perhaps the most natural or “field like” way of growing the plants, for screening, the difficulty of visualizing the roots to obtain accurate data make it a less than ideal method to use unless mature plants are required to be screened. It has been found that there is little difference between the growth and architecture of young root systems of barley grown in a soil filled rhizobox style system and those grown in an air gap between gel and a piece of Perspex (Bengough et al., 2004), this gel and

Perspex system has a number of advantages over the rhizobox system. Firstly, it is much easier to visualize the entire root system as the plants grow as all roots can be seen. Secondly, it is easier to image the roots and gather accurate data from the root architecture as the plates can be scanned without disturbing the plants. Finally, this non-destructive method of imaging means that it is possible to image the root system at multiple stages in its development (Bengough et al., 2004). However, within many gel based culture systems such as that commonly used for Arabidopsis there is a need to maintain sterile growth conditions around the plant in order to prevent contamination, this is dependent on the contents of the growth media and can be difficult to achieve for larger plants such as wheat. The gelling agent percentage of the media can also have effects on the growth of the roots; for example, the gravitropic response of wheat is enhanced on media containing 1.6% agar when compared to wheat grown on 0.8% agar (Personal communication – Dr Marta Del Bianco). Germination paper based systems have been used successfully in a number of different studies from those based on root hairs in maize (Zhu et al., 2005), to those based on other seedling root characteristics in maize (Hund et al., 2009) and in wheat (Atkinson et al., 2015, Bai et al., 2013). A number of these studies use a “cigar roll” type system whereupon seeds or seedlings of wheat are placed upon a moistened sheet of germination paper that is then rolled up and sat in a reservoir of liquid growth media (Zhu et al., 2005), this system could however be problematic for the determination of architecture traits such as angle as the rolling of the paper could constrain the roots meaning thigmotropism could possibly override gravitropism thus not allowing the roots to assume their natural GSA. For screening the TILLING lines, it was decided to use a pouch based system similar to that used by Hund et al (Hund et al., 2009) and Atkinson et al (Atkinson et al., 2015) as the flat pouches make it easy to achieve a high throughput in limited space without constraining the roots to the same degree as the “cigar roll” method. It was also decided to photograph the pouches, as opposed to scanning as Hund et al had (Hund et al., 2009), to increase speed of imaging whilst keeping image quality high enough for analysis using RootNav (Atkinson et al., 2015, Pound et al., 2013). It was decided not to pre-germinate seeds before placing into pouches so as to avoid root disturbance and maintain root emergence and growth angles as set by the plant. In order to achieve a more soil-like environment in future experiments, a

thin layer of soil of about the thickness of a wheat root could be placed between two sheets of Perspex, this should ensure that the majority of the roots could be seen without having to disturb them by washing as in Bengough et al (Bengough et al., 2004).

It was important to ensure that within individual lines there was uniformity between plants in order to get an accurate root phenotype for the line. A variable to be taken into consideration was that of seed weight, it has already been shown in both rice and wheat that seed size is important in determining seedling vigour and that there is a positive correlation between seed weight and the total dry mass of the young plants produced (Roy et al., 1996, Evans and Bhatt, 1977). In their trial of Barley in a gel and Perspex system Bengough et al found that in all of the three varieties they tested, a higher grain weight correlated with a greater seminal root number, in two of the varieties, angular spread of the roots was correlated with grain weight and in one of the varieties primary seminal root length also correlated with grain weight (Bengough et al., 2004). It was decided to determine if the grain weight had an effect on seminal root angle in the wheat cultivar “Cadenza” from which the TILLING lines were produced, it was found that there was no significant difference in the tip angle of both the first and second pairs of seminal roots of plants between different seed weight classes (Figure 4.2) although the tip angles of the second pair of seminal roots were on average slightly less vertical than those of the first pair of seminal roots. This was also found by Manschadi et al in their screen of Australian wheat cultivar root architectural traits, on average the root angles of the second pairs of seminal roots did not differ significantly between the different cultivars and all had an average growth angle of around 78°. However, there was a wide variation in the growth angles of the first pair of seminal roots between the different cultivars with the largest angled cultivar displaying seminal root angles of around 56° and the smallest angled cultivar displaying seminal root angles of around 36° (Manschadi et al., 2008). In light of this and in addition to the fact that not all lines consistently produced a second pair of seminal roots it was decided to concentrate on the first pair of seminal roots for comparison between the different TILLING lines, it was also decided that as the average seed weight varied between lines and that it had no bearing on root tip angle that seeds

could be selected visually (i.e. not abnormally small or large) that were representative of that line. Further analysis of the subset of lines that consistently produced a second pair of seminal roots could be used to determine if the trends and links with other traits seen in the first pair of seminal roots also apply to the second pair despite their different growth angles.

The hull area (also known as convex hull area) is the smallest area that encloses the whole root system (Atkinson et al., 2015) and as such is a good indicator of the area of soil from which a plant can gather resources. In theory a plant with a larger hull area should be better able to gather what it needs from its soil environment. The overall hull area of a root system is determined by contributions from a number of root architectural traits some of which are linked to each other and some of which are independent. It was found that across all 397 TILLING lines there was no correlation between the tip angle of the first pair of seminal roots and both the length and emergence angle of those roots, this suggests that tip angle is not linked to either of these traits and that these traits could be controlled by different mechanisms and different sets of genes (Figure 4.4). It was found that there is a positive correlation between the length of the primary root and the first pair of seminal roots (Figure 4.3), this could indicate that the control mechanisms for root length in both the primary and first pair seminal root axes are the same. A number of traits contribute to the overall hull area, it was found that first pair length, tip angle and primary length all correlate positively with hull area, of these the strongest correlation was between hull area and first pair length, this was followed by first pair tip angle and finally primary length with the weakest correlation of the three (Figure 4.6). Whilst all three traits contribute positively to the hull area the stronger correlation of the traits from the first pair of seminal roots highlights the importance of the non-vertically growing roots in determining the area from which the root system can gather resources. Through comparison of individual lines across the range of hull areas displayed in the screen it was found that traits related to length had the greatest effect overall on the hull area as those lines with a high hull area had consistently long roots and those with a low hull area had shorter roots whereas first pair tip angle varied across the different lines (Figure 4.7). However, as all plants were measured once, and at the same age, those with a

greater root length could just display greater seedling vigour and therefore a faster growth rate. A number of factors are known to affect seedling vigour including seed weight (Roy et al., 1996, Evans and Bhatt, 1977), seed protein content (Evans and Bhatt, 1977) and osmotic stress (Landjeva et al., 2008), although osmotic stress should not have been a limiting factor due to the pouches being stood in a reservoir of liquid growth media, the size of the seeds, although kept visually consistent within the same line, varied between lines, whilst this may have no effect on the root angle (Figure 4.2) it could result in variation in seedling vigour between the different lines. Although within the screen length related traits were found to have the largest effect on hull area, the work of both Manschadi and Oyanagi suggests that root angle at the seedling stage (such as that measured in the screen) is an important determining factor in the shape of the mature root system (Manschadi et al., 2008, Oyanagi, 1994) as both found that wheat cultivars with steeper seminal root angles at the seedling stage displayed a higher number of roots at greater soil depths (Manschadi et al., 2008, Manschadi et al., 2006, Oyanagi, 1994).

Whilst hull area is a measure of the total area under the root system which the plant can gather resources from it may not be the best trait for determining which lines would perform best in a field environment. The soil is a heterogeneous environment with the different resources that the plant requires for growth found at their greatest concentrations within different soil layers and each resource has a different mobility within the soil. For example, nitrogen (in the form of nitrate) and water are highly mobile within the soil and are generally found at greatest concentrations within the deeper layers of the soil (Lynch, 2013), in contrast, phosphorus is relatively immobile and can be found at greatest concentrations in the upper layers of the soil either from deposition from rotting plant matter and other organic material or from application of artificial fertilisers (Lynch, 2013, Lynch and Brown, 2001). Modification of plant root architecture to increase “topsoil foraging” of phosphate in low phosphate soils has already been explored in soy bean (*Glycine max*) and common bean (*Phaseolus vulgaris*) (Lynch, 2013, Lynch and Brown, 2001, Wang et al., 2010), indeed plants are known to modify their own root architecture in response to deficiencies in both nitrates and phosphates, with nitrate deficiency in

Arabidopsis resulting in the plant adopting a less vertical lateral root GSA and phosphate deprived plants adopting a more vertical one (Roychoudhry et al., 2017). Additional experiments could be carried out to determine how nutrient deficiency affects the root GSA of wheat; further screens could then be carried out on the TILLING population to find mutants with aberrant responses to this. These mutants could then be used to search for QTLs and genes that control root GSA response to nutrient deficiency in wheat.

An ideotype for RSA in maize has been proposed (although elements of this have been shown to still be applicable in other cereal crop species such as wheat) to enhance water and nitrate acquisition, the “Steep, Cheap and Deep (SCD)” ideotype proposes a root system comprising of a deep growing primary root with few laterals, steep angled seminal roots with few but long laterals and crown roots with many laterals (Lynch, 2013)). Along the lines of the SCD ideotype described by Lynch (Lynch, 2013) it was decided to divide the lines from the TILLING screen into a number of different archetypes based upon the length and tip angle of the first pair of seminal roots, these archetypes included long and more vertical, long and less vertical, short and more vertical and short and less vertical (Figure 4.8). Each archetype would present its own advantages and disadvantages for the wheat plant in different soil types with the idea that cultivars could be bred from each archetype to be used in soils where their phenotype would be most advantageous to yield, for example in phosphate rich but nitrate poor soils a long and more vertical root system would be best suited to produce the highest yield. It was decided to select a few lines at the extremes of each archetype along with lines that sat at either end of the range of first pair tip angles in preparation for a field trial. Before the final selection of lines for the field trial could be carried out around ten individual plants from three separate spikes from each line were phenotyped to determine if the traits segregated and were uniformly carried on each plant of the spike (Figure 4.9). It was found that for the lines selected on the basis of architecture, often, individual plants would not carry the phenotype for which they were selected; two lines displayed a “half phenotype” with the correct angle phenotype but the not correct length phenotype. This could be as a result of wheat’s polyploidy, potentially a phenotype altering mutation in a gene on one

of the genomes could be masked by non-mutant copies on the other genomes being expressed (Uauy et al., 2009, Lawrence and Pikaard, 2003), EMS creates random mutations throughout the genome (Kim et al., 2006) and although each TILLING line carries a large number of mutations the probability of the same mutation occurring in all six copies of a gene in the same line is extremely low. Differing expression levels of the other non-mutant copies could explain the different degrees of phenotypes displayed, also the lack of correlation between first pair length and tip angle (Figure 4.4A) suggests that each trait is controlled by a different set of genes and are not linked, therefore could segregate producing plants that display an angle phenotype but not a length phenotype and vice versa. As a result of this it was decided to take forward only lines selected on the basis of an angle phenotype for the field trial, two more vertical and two less vertical lines that had spikes where all or nearly all plants displayed the desired phenotype were chosen.

For the field trial a method similar to that used by Oyanagi (1994) was chosen, a plastic mesh dish (in this case a kitchen colander) was sunk into the ground to its rim and backfilled with soil, a single seed from a homozygous spike was inserted into the middle of the colander and the plant allowed to grow to maturity. Plants were arranged in 12 blocks of three surrounded by wild type plants, the lines were arranged to minimize positional effects on the averaged results. It was found that CAD0190 (more vertical in the original screen) had a greater percentage of its root system emerging from the lower holes of the colander than wild type and CAD0923 (less vertical in the original screen) had a greater percentage of its root system emerging from higher holes in the colander (Figure 4.12). This, along with the results of Manschadi and Oyanagi confirms that seedling root architectures have a bearing on the architecture of the mature root system and can be used in screens for selection of more favourable root architectures for use in the field (Oyanagi, 1994, Manschadi et al., 2008). However it also highlights the importance of using a 3D soil based system to validate the phenotypes found in the 2D system as only two of the four lines tested in the field trial maintained their phenotype from the original 2D screen.

As CAD0190 displayed the strongest phenotype in the field trial it was decided to backcross this line with wild type Cadenza to work towards determining the causal mutation for its root angle phenotype. It was found that when the F_1 generation of plants were phenotyped they did not show any significant difference in first pair tip angle from wild type cadenza, however, the roots were significantly longer than those of wild type cadenza (a phenotype also seen in the original screen) (Figure 4.13), this could again be a result of the polyploid nature of wheat coupled with the dominance of the mutations. For example, the root angle phenotype could be recessive and therefore easily masked by the non-mutant copies on the other genomes (Uauy et al., 2009, Lawrence and Pikaard, 2003) whereas the root length phenotype could be dominant and therefore unaffected by the presence of non-mutant copies. In order to map the causal mutation of CAD0190 a large number of F_2 individuals could be planted in a much larger field trial, genomic DNA samples collected from each individual and then each individual plant phenotyped as before using the colanders. Using the DNA from plants carrying the more vertical root phenotype and the existing map of mutations for each line the causal mutation could then be found.

Beyond the scope of this work, additional field trials could be carried out in a similar manner to the one carried out here but in different locations and on different soil types to determine if the phenotypes seen here translate onto other soils. Further TILLING lines could be tested to build up a suite of altered root angle mutants whose phenotypes can be seen both in the pouches and in soil. By mapping the causal mutations of these lines an understanding of the genes and mechanisms involved in root growth angle control in wheat could be gained that would allow the production of ideotype varieties in a similar manner to that of the steep, cheap and deep ideotype described for maize (Lynch, 2013) and varieties able to better gather water and nutrients in different soil types such as has been achieved in beans whose roots have been altered to increase “topsoil foraging” (Lynch, 2013, Lynch and Brown, 2001, Wang et al., 2010). Additional screens across the TILLING population could be carried out using soil filled cores and CT imaging methods (Perret et al., 2007) to monitor and quantify the change in the root architecture of the different lines through their life cycle, this could perhaps be used to find QTLs and genes involved in the control of RSA at

specific points in the life cycle e.g. grain filling. Knowledge of the genetic control of the chronological succession of root types and architectures could be used to induce architectures advantageous for each specific stage of the life cycle.

4.4 Materials and Methods

4.4.1 Wheat growth in seed germination pouches for the TILLING lines screen

Wheat seeds were placed on moist filter paper and cold treated at 4˚C for two days. Seeds were placed into cyg seed germination pouches (Mega-International, Minneapolis, US) with the bottom slit open, the embryo was oriented so that the germ was facing outwards and downwards. Pouches were placed upright in a reservoir of Hoagland's No. 2 basal salt solution and plants were allowed to grow for 6 days. Growth conditions were as follows: 22˚C Day, 15˚C Night, 16 Hour photoperiod. Pouches were wrapped in aluminium foil to exclude light from the roots in batches of five. Plants were photographed using a Sony Cyber-Shot DSC RX100. Root architecture parameters such as hull area, tip angle, length and emergence angle were measured using RootNav (Pound et al., 2013) as described below and comparative analysis was carried out in Microsoft Excel. For screening of the TILLING lines ten pouches were grown and measured for each line.

4.4.2 Wheat seed weight effects on root growth angle test

Seeds of Wt Cadenza were separated into three weight categories before being placed onto moist filter paper, the categories were as follows: <30 mg, 30-40 mg and >40 mg. Plants were grown as for the TILLING line screen. Plants were photographed as above. Tip angles were measured using ImageJ (NIH), comparative analysis was carried out using Microsoft Excel.

4.4.3 Wheat growth in 3D

Wheat seeds were sterilized using chlorine gas before being placed onto sterile filter paper soaked in sterile water inside a Petri dish and sealed using Parafilm, all these steps were carried out under sterile conditions. Seeds were cold treated for two days at 4˚C. Using sterile forceps the seeds were placed into a 400 ml media bottle containing either ATS or Hoagland's No. 2 basal salt

solution with 0.25% phytagel, seeds were placed on end with the embryo facing downwards into the media. Sterile sand was used to cover the surface of the gel and the bottle was wrapped in aluminium foil below the gel level and placed at 20˚C constant, 16 Hour photoperiod for three weeks. Bottles were then photographed.

4.4.4 Measurement of root system architecture traits using RootNav

The file size of the images was reduced using ImageJ (NIH) before images were loaded into RootNav. The point where the roots emerged from the seed was designated the source, the primary seminal root (radicle) was designated the primary and the non-vertically growing seminal roots were designated laterals. Laterals were selected so as to make it easier to separate the 1st and 2nd pairs of seminal roots. The RootNav data output tables were copied into Microsoft Excel for further analysis. Roots were then separated into primary, 1st pair of seminal roots and 2nd pair of seminal roots before averages were calculated for each trait for each TILLING line.

4.4.5 Selection of lines for crossing

Lines were selected on the basis of either being consistently at the extremes of 1st pair seminal root angle or adhering to a particular root archetype in both the Leeds TILLING line screen and the parallel screen being carried out at the John Innes Centre (JIC, Norwich, UK). Four archetypes were selected for: long roots with a low 1st pair seminal root angle, long roots with a high 1st pair seminal root angle, short roots with a low 1st pair seminal root angle and short roots with a high 1st pair seminal root angle. 16 lines were chosen to take forwards (see Table 4.1).

For each line chosen to take forwards for crossing, seed was taken from three separate spikes, 10 to 12 seeds from each spike were placed into cyg seed germination pouches as detailed above and grown and analysed as for the screening of the TILLING lines. It was then decided how many of the plants from each spike carried the desired phenotype to determine the zygosity of the

spikes. Plants carrying the desired phenotype for each line were taken to JIC for backcrossing with Wt cadenza.

4.4.6 Investigation of root growth in soil

20 cm diameter kitchen colanders were buried in the trials field (Norwich, UK) to the rim and backfilled with the soil. Seed from 5 lines was placed into the centre of the colanders, one seed per colander. There were ten wild type containing colanders and between 5 and 7 colanders for each line. The colanders were positioned in 12 blocks of three surrounded by wild type plants to protect from wind damage. The lines were arranged so that each line wouldn't sit in the same position within a block more than twice to try to negate any effects of position upon the growth of the plant. A net was placed over the plants to prevent predation from pests. Seeds were planted in March 2017. Colanders were lifted in August 2017 when the plants had produced seed, the colanders were lifted from the soil and a mark was made next to each hole from which a root was emerging, using trigonometry the angle at which the root emerged from the colander was calculated (See Figure 4.10).

Chapter 5 : An EMS Mutagenesis based screen for Gravitropic Setpoint Angle mutants

5.1 Introduction

It is known that mutations in a number of genes can modulate the gravitropic setpoint angle (GSA) of the lateral roots and shoots of Arabidopsis. For example in a number of the genes involved in the auxin response pathway such as the *tir1-1* mutant and the *axr3-10* (a stabilized version of IAA17) mutant show a more vertical lateral root GSA (Roychoudhry et al., 2013). Also, mutations in some genes in the *LAZY* gene family affect the growth angle of the lateral organs, for example at*lazy1*, *lazy1 atlazy2* and *atlazy1 atlazy3* display less vertical shoot branch GSAs and the knockout mutant at*lazy4* displays a less vertical lateral root angle (Yoshihara and Spalding, 2017); the closely related *attac1* mutant displays a more vertical shoot branch GSA (Dardick et al., 2013, Yoshihara and Spalding, 2017).

Ethyl Methanesulphonate (EMS) is a chemical mutagen that randomly modifies nucleotides across the genome that upon DNA replication results in mispairing and base changes (Kim et al., 2006). In the case of EMS, the majority of the time the EMS alkylates guanine residues resulting in the formation of O^6-ethylguanine that can base pair with Thymine but not with the usual Cytosine, upon replication this results in a base pair change from G/C to A/T(Kim et al., 2006, Greene et al., 2003). EMS mutagenesis can be used to search for loss-of or gain-of function mutations, to understand the role of specific amino acid residues in proteins and to undertake suppressor screens to find interacting partners or other genes involved in a pathway (Kim et al., 2006).

EMS mutagenesis can be used for both forward and reverse genetic approaches to finding new mutants. In the case of forward genetics, the

mutagenized population is screened for novel phenotypes and a number of methods are employed to determine the causal mutation, by the use of targeting induced local lesions in genomes (TILLING) it can also be used for a reverse genetic screening approach (Kim et al., 2006).

Forward genetic screens such as those that be carried out on EMS mutagenized populations are a popular method for identifying genes involved in a developmental or physiological process. However, mapping the mutation by traditional methods has always been a labour intensive and time-consuming process. Map based cloning has long been the method of choice for the mapping of mutations, this involves the use of restriction fragment length polymorphisms (RFLPs) coupled with either a hybridisation or PCR based approach to mapping the gene (Chang et al., 1988, Konieczny and Ausubel, 1993, Jander et al., 2002), all of these require an outcross between the mutant carrying the gene to be determined and another ecotype (Jander et al., 2002, Konieczny and Ausubel, 1993, Chang et al., 1988). Using either pre-determined RFLP markers (Chang et al., 1988) or, since the sequencing of the Arabidopsis genome, mapping onto the Col-0 reference sequence, the single nucleotide polymorphisms (SNPs) between the ecotypes used in the outcross (Jander et al., 2002) allow the position of the mutation to be found using linkage to the SNPs or RFLPs (Jander et al., 2002). However to do this requires large numbers of plants to be grown (up to 4000 plants to fine map a mutation (Jander et al., 2002)) and requires multiple generations of plants post-outcross meaning it can take over a year to map a mutation (Jander et al., 2002, Konieczny and Ausubel, 1993).

Recent advances in deep DNA sequencing has allowed the process of causal mutation identification to be sped up and simplified (Allen et al., 2013). The use of whole genome sequencing to identify causal mutations in forward genetic screens has become a much more attractive prospect as technological advancement has pushed the cost of sequencing down and increased the speed with which it can be done. Also, recent studies have explored using a back-cross to the mutant progenitor line instead of an outcross to another ecotype (Allen et al., 2013), this has a number of advantages. It eliminates

phenotypic variation that can be introduced by crossing to a divergent genome; this prevents loss of subtle phenotypes and simplifies the process of identifying how the mutation segregates (Allen et al., 2013). It also simplifies identification of the mutation if the mutagenesis was carried out on a complex background such as that of a transgenic or previously mutagenized line, this would have needed to be introgressed into the divergent genome in the case of an outcross (Allen et al., 2013, James et al., 2013). Mutation identification relies on the principle of bulk segregant analysis; the genomes from many individuals (up to thousands) can be sequenced simultaneously. In a back-crossed population all the mutagen induced mutations except the causal mutation and those closest to it will segregate as these will be fixed in a pool that is selected for the mutant phenotype (James et al., 2013). Using the frequency and position of mutagen-induced SNPs in a pool of mutant F_2 plants the causal mutation can be identified (Austin et al., 2011). In theory, a recessive causal mutation will always be homozygous in plants carrying the phenotype whereas the homozygosity of other SNPs will decrease with increasing distance away from the causal mutation due to recombination events, the zygosity and position of the linked SNPs can then be used to find the causal mutation (Allen et al., 2013). In order to map a dominant mutation, to ensure that the causal mutation will always be homozygous in the pool of plants with the phenotype, tissue would need to be collected individually from a large number of F_2 plants, these plants would then need to be taken through to F_3 to determine which of them was homozygous for the causal mutation and which were heterozygous and only F_2 tissue from homozygous plants used for bulk segregant analysis.

The *LAZY* genes are known to be involved in the control of lateral root and shoot growth angle (Yoshihara and Spalding, 2017, Yoshihara et al., 2013, Taniguchi et al., 2017, Guseman et al., 2017, Ge and Chen, 2016), they typically contain 5 regions of conserved sequence, are only found in land plants and are members of the IGT gene family (Yoshihara and Spalding, 2017). In Arabidopsis there are 6 genes in the *LAZY* family, these are *AtLAZY1* (At5g14090), *AtLAZY2* (At1g17400), *AtLAZY3* (At1g19115), *AtLAZY4* (At1g72490), *AtLAZY5* (At3g24750), *AtLAZY6* (At3g27025) (Yoshihara and Spalding, 2017) (see Table 1.1).

The expression patterns of the different *LAZYs* are consistent with their knockout mutant phenotypes. *AtLAZY1* is expressed throughout the shoot with some expression also in the root (Yoshihara and Spalding, 2017), the *atlazy1* mutant displays a less vertical shoot phenotype (Yoshihara and Spalding, 2017, Yoshihara et al., 2013, Taniguchi et al., 2017). *atlazy2* has no shoot phenotype and slightly less vertical lateral roots (Taniguchi et al., 2017, Yoshihara and Spalding, 2017), *AtLAZY2* is expressed mainly in the root tip and hypocotyl (Yoshihara and Spalding, 2017). *AtLAZY3* is expressed in the root tip (Yoshihara and Spalding, 2017) and as expected *atlazy3* shows no shoot phenotype however it shows no root phenotype (Yoshihara and Spalding, 2017, Taniguchi et al., 2017), *AtLAZY4* is also expressed in the root tip (Yoshihara and Spalding, 2017)and shows no shoot phenotype but it displays a less vertical lateral root phenotype (Yoshihara and Spalding, 2017, Taniguchi et al., 2017). *AtLAZY5* is expressed throughout the root apart from the apex and *AtLAZY6* is expressed in the petioles of light grown seedlings, it has been suggested that due to its expression pattern *AtLAZY6* may have a role in petiole angle control (Yoshihara and Spalding, 2017).

Knockouts of multiple *LAZYs* have additive effects on the phenotype of both the roots and the shoots; the *atlazy2 atlazy4* (*atlazy24*) double mutant has no shoot phenotype but a more extreme root phenotype than either of the single mutants (Yoshihara and Spalding, 2017, Taniguchi et al., 2017). Additional knockouts of *atlazy2, atlazy3* and *atlazy4* to the *atlazy1* mutant increase the severity of its shoot phenotype (Taniguchi et al., 2017, Yoshihara and Spalding, 2017) and modulate the root phenotype in accordance with their own root phenotype i.e. the addition of *atlazy4* results in less vertical lateral roots (Taniguchi et al., 2017). The *atlazy1 atlazy2 atlazy4* (*atlazy124*) triple mutant has upwards growing lateral roots and very lax growth in the shoots, both the laterals and the primary shoot tips point downwards (Yoshihara and Spalding, 2017, Taniguchi et al., 2017).

The *atlazy* knockout mutants also exhibit gravitropism defects, the severity of which increases when more genes are mutated (Yoshihara and Spalding, 2017,

Taniguchi et al., 2017) . Of the single mutants only *atlazy2* and *atlazy4* show a slight impairment in primary root gravitropic response when compared to wild type, the gravitropism impairment increases in higher order mutants with the *atlazy24* double mutant and *atlazy 1 atlazy2 atlazy3 atlazy4* (*atlazy1234*) quadruple mutants showing negligible gravitropic response to a 90° reorientation (Yoshihara and Spalding, 2017).

In the gravity signalling cascade it is thought that the *LAZYs* act upstream of auxin redistribution (Taniguchi et al., 2017, Yoshihara and Spalding, 2017) , it has been reported that they may be involved in the localisation of the PIN proteins (Taniguchi et al., 2017) which are known to be involved in the mounting of a graviresponse (Friml et al., 2002, Guyomarc'h et al., 2012, Palme et al., 2006) and have a role in growth angle control (Rosquete et al., 2013). In their model of GSA maintenance Roychoudhry et al pose that it is a balance of two opposing fluxes of auxin that allows lateral organs to be maintained at non-vertical angles (Roychoudhry et al., 2013), the role of the *LAZYs* in the distribution of auxin may explain the altered growth angles that are seen in their knockout mutants (Taniguchi et al., 2017, Yoshihara and Spalding, 2017, Yoshihara et al., 2013). Both the at*lazy24* and at*lazy2 atlazy3 atlazy4* mutants display altered lateral root growth angles with respect to gravity (Yoshihara and Spalding, 2017, Taniguchi et al., 2017) however neither respond to reorientation ((Yoshihara and Spalding, 2017) Personal communication – Adam Binns (Unpublished)) suggesting that gravitropism is impaired to a point which negates their ability to maintain angles. Interestingly the single mutant, *atlazy4*, which also displays an altered lateral root growth angle, is able to respond to gravity and maintain GSAs as when reoriented the lateral roots return to their original angles in the same way as wild type (Personal communication- Suruchi Roychoudhry (Unpublished)). This suggests a potential dual role for the *LAZYs* in both the gravitropic response and the setting of the root growth angle with respect to gravity, further work is needed to clarify the role of the *LAZYs* in the setting and maintenance of GSAs.

In this work an EMS mutagenized population of Arabidopsis was screened for abnormal lateral root growth angle phenotypes, several mutants were selected

and carried forwards to the next generation. In order to determine how their phenotype related to the maintenance of GSAs, tests such as reorientation assays, auxin treatments and clinorotation were carried out, these would inform the selection of a candidate mutant for genotyping. The ideal mutant would display an altered root growth angle but have no gravitropic impairment or alteration in its response to auxin in order to give greater insight into the mechanisms governing the setting of a GSA. Mutants with gravitropic or auxin response defects and those with the mildest phenotypes were excluded from further examination. Two mutants were selected to take forwards, of these the mutant with the strongest and most interesting phenotype was then genotyped using whole genome sequencing; this gave several candidate genes of which the most likely to cause the phenotype (LAZY4/DRO1) was selected. This gene was then cloned from wild-type plants and mutagenized to replicate the mutant, it was then transformed into a knockout of the candidate gene to prove that this would reproduce the phenotype caused by the EMS induced mutation.

5.2 Results

5.2.1 EMS mutagenesis of Arabidopsis “Col-0” Seeds

Around 20,000 seeds of wild type Arabidopsis “Columbia” were exposed to 25 mM EMS for 16 Hrs. The resulting M_1 seed had a germination frequency of 82.5%, this is higher than the desired 75% germination frequency of a successful mutagenesis using EMS as detailed by Leyser (Leyser, 2000). However, the presence of albino sectors on the M_1 plants (also a sign of a successful mutagenesis (Leyser, 2000)) was sufficient evidence of a positive outcome to begin screening . The M_1 plants were split into 80 pools for seed collection and the resultant M_2 plants were screened for abnormal root phenotypes with a focus on the growth angles of the lateral roots, one plate was screened per pool of M_2 seed per round of the screen. Plants showing abnormal root phenotypes were planted and assigned a name based upon the M_2 pool, the round of the screen, the plant number on that specific plate and a brief descriptor of their phenotype e.g. 80 (pool).2 (round of screen).1 (plant on plate) MV (More Vertical). The M_3 plants were subjected to a number of tests to determine the heritability of the trait seen in the M_2 and the nature of the trait with regards to gravitropism and auxin response. The screening process is detailed by the flowchart in Figure 5.1. The screen was carried out over 46 rounds and approximately 18400 M_2 individuals were inspected for abnormal root phenotypes.

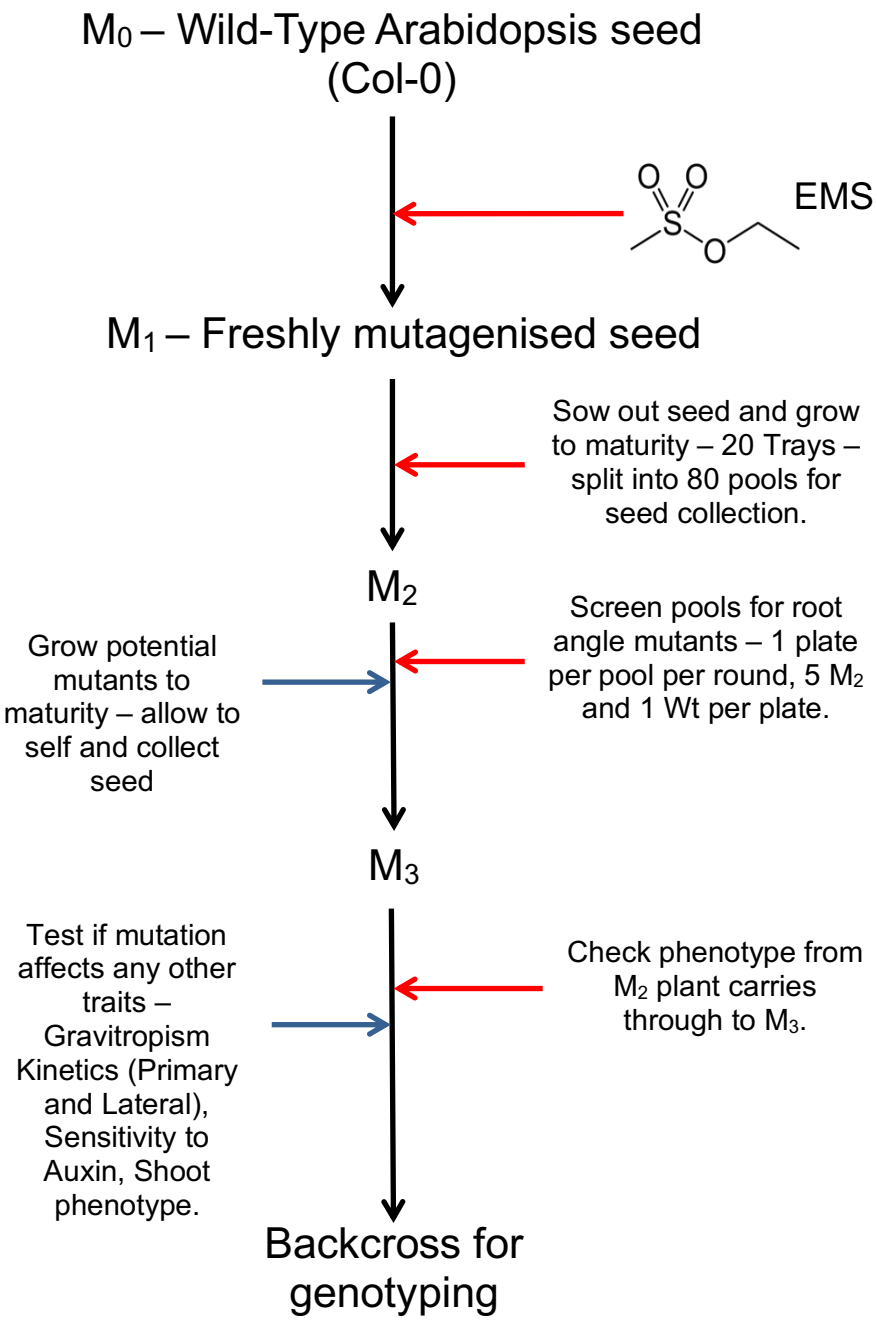


Figure 5.1: A flowchart of the screening process from Wt Col-0 seed to backcrossing for genotyping

A flowchart detailing the screening process from applying the EMS to the unmutagenised M_0 Wt Col-0 seed, screening in the M_2 generation, further tests in the M_3 generation to backcrossing to allow genotyping.

5.2.2 Screening the M2 population

As a result of the M_2 screening twelve mutants with phenotypes heritable into the M_3 were found, these mutants and their phenotypes are listed in Table 5.1. A number of different phenotypes were observed including short, hairy and wavy roots and both more and less vertical lateral roots. Two mutants were selected that had short, more vertical and hairy roots (58.4.4 and 56.4.4) suggesting that they either have a greater amount of auxin or an enhanced auxin response, it could also suggest an increase in ethylene production or response as increased ethylene levels also result in short and hairy roots (Růžička et al 2007), these mutants were therefore discounted from further analysis. A third mutant with short roots (27.3.2) was also found however it was not hairier than wild type and the angle of the roots were normal suggesting that the phenotype was not as a result of a change in the amount of or response to auxin, however as the angles of the roots were normal this mutant was excluded from further analysis. Three mutants were found with wavy roots (68.8.5, 56.23.5 and 63.23.5), they showed a strong and consistent phenotype but as were unrelated to angle were not given further analysis. Of the mutants selected on the basis of lateral root angle alone three were less vertical and three more vertical than wild type. The less vertical lines had mild (29.23.2 LV) or inconsistent (11.2.4 LV and 11.12.4 LV) phenotypes that did not present in every plant or in every set of plants grown and were therefore not taken forwards for further analysis. Of the three more vertical lines one had a consistent but mild phenotype (27.1.1 MV) and was not subject to further analysis, two (80.2.1 MV and 71.14.4 MV) had stronger phenotypes and were taken forwards for further phenotypic tests.

Table 5.1: Phenotypes of mutants selected from the M_2 screen

Mutant	Phenotype Selected for	Secondary Phenotypes	Notes
56.4.4	Hairy and short roots	More vertical	Potential auxin mutant
58.4.4	Hairy roots	Short, more vertical roots	Potential auxin mutant
11.2.4	Less vertical	None	Inconsistent phenotype
11.12.4	Less vertical	None	Inconsistent phenotype
29.23.2	Less vertical	None	Mild phenotype
80.2.1	More vertical	None	Strong, consistent phenotype
71.14.4	More vertical	None	Consistent phenotype
27.1.1	More vertical	None	Consistent but mild phenotype
27.3.2	Short roots	Jagged leaf edges	No angle phenotype, not auxin?
68.8.5	Wavy roots	Wavy shoots	Consistent phenotype
56.23.5	Wavy roots	Wavy shoots	Consistent phenotype
63.23.5	Wavy roots	Wavy shoots	Consistent phenotype

5.2.3 80.2.1 MV has more vertical lateral roots than Wt Col-0

Early in the M_2 screening process a mutant was found that had dramatically more vertical lateral roots, this mutant was found in pool 80 in the second round of the screen and was subsequently named 80.2.1 MV (More Vertical). The lateral roots are consistently more vertical (Figure 5.2 B) than those of Wt Col-0 (Figure 5.2 A). When the angles with respect to gravity of 0.5 mm sections of the first 3 mm of root were measured there is a significant difference between Wt Col-0 and 80.2.1 MV in the angle of each root section (Figure 5.2 C), on average the roots of 80.2.1 MV are 23.4° more vertical than those of Wt Col-0.

In contrast to the dramatic root phenotype, there is no visible phenotypic difference between the shoots of 80.2.1 MV (Figure 5.3 B) and those of Wt Col-0 (Figure 5.3 A). When the angle with respect to gravity of 5 mm sections of the first 3 cm of the lateral shoots are measured there is no significant difference between the angles of 80.2.1 MV and Wt Col-0 at each section (Figure 5.3 C). This suggests that the causal mutation for 80.2.1 MV's phenotype affects a gene that is either not expressed or not active in the angle control machinery of the shoots.

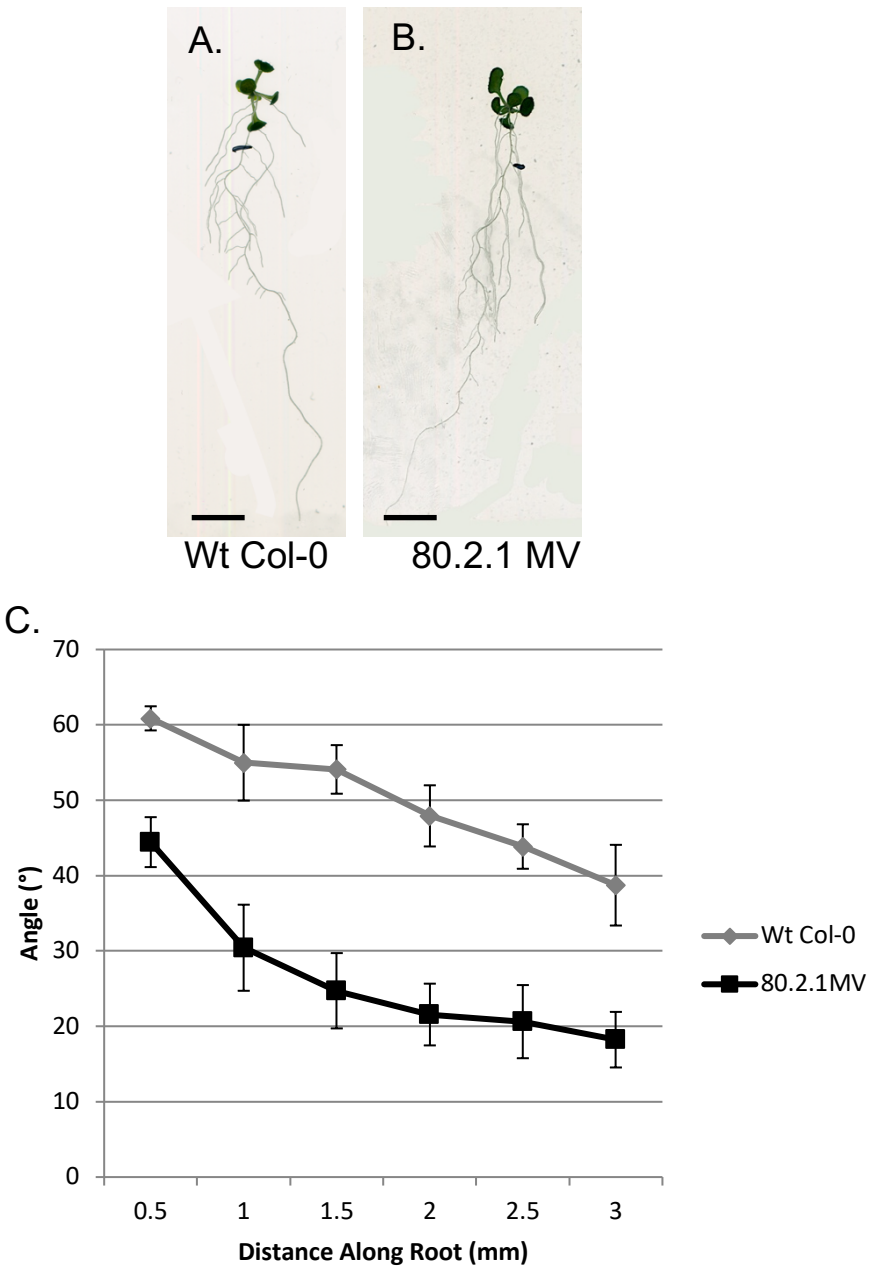
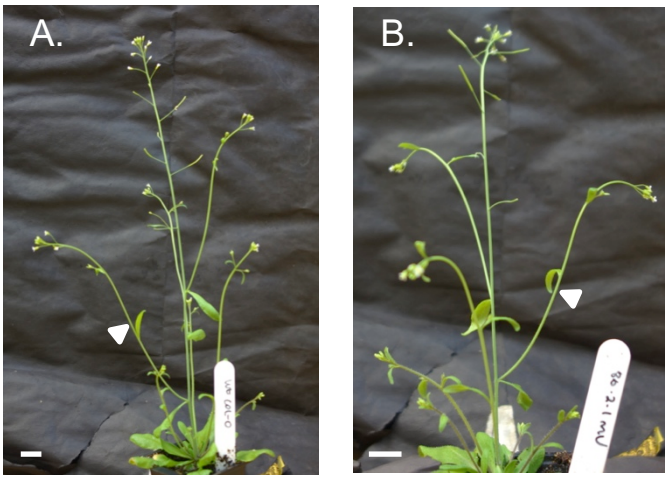


Figure 5.2: 80.2.1 MV has more vertical lateral roots than Wt Col-0
A. The root system of a 12 day old Wt Col-0 plant. **B.** The root system of a 12 day old plant of 80.2.1 MV, the lateral roots are visibly more vertical than Wt Col-0. **C.** The root angle of the first 3 mm of root of both Wt Col-0 and 80.2.1 MV, on average the angles of Wt Col-0 range from 60.8° at 0-0.5 mm to 38.7° at 2.5-3 mm from the origin, the angles of 80.2.1 MV range from 44.4° at 0-0.5 mm to 18.2° at 2.5-3 mm from the origin. $P<0.05$ for all points (Students T-test) n=10 for each point. Scale bars represent 1.5 cm, error bars represent SEM.



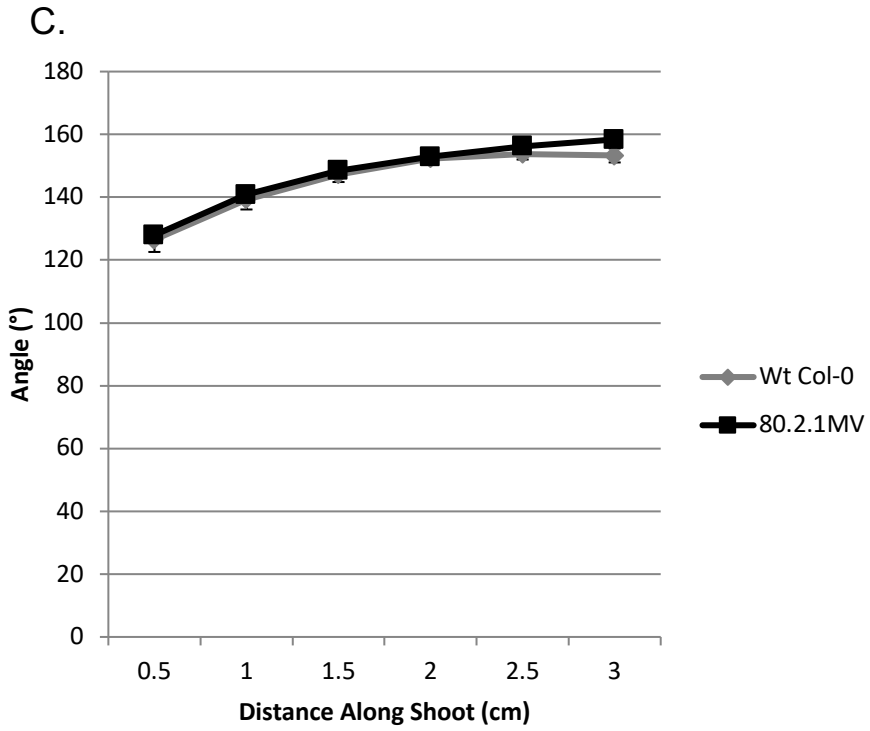


Figure 5.3: The shoots of 80.2.1 MV have no angle phenotype when compared with Wt Col-0

A. The shoots of a 4-week old Wt-Col-0 plant **B.** The shoots of a 4-week old plant of 80.2.1 MV, there is no visible phenotypic difference in the shoots between Wt Col-0 and 80.2.1 MV, examples of the branches measured are indicated with a white arrow **C.** The shoot angles of the first six 0.5 cm sections of shoot of both Wt Col-0 and 80.2.1 MV were measured with respect to gravity, on average the angles of Wt Col-0 range from 126.5° at 0-0.5 cm to 153.3° at 2.5-3 cm from their origin, the angles of 80.2.1 MV range from 127.9° at 0-0.5 cm to 158.4° at 2.5-3 cm from their origin. n=10 branches per line. P>0.05 for all points (Students T-test). Error bars represent SEM.

5.2.4 The kinetics of gravitropic bending of Wt Col-0 and 80.2.1 MV are similar in both primary and lateral roots

To test for gravitropism defects in 80.2.1 MV and to determine whether the more vertical lateral root phenotype was a result of increased gravitropic capacity or lack of an AGO, the kinetics of gravitropic bending were measured in both the primary and lateral roots. No significant difference in the speed of gravitropic bending was found between Wt Col-0 and 80.2.1 MV in primary roots displaced 90° from the vertical (Figure 5.4 A). Similarly, in lateral roots there was no significant difference between Wt Col-0 and 80.2.1 MV in the speed of gravitropic bending of both upwards bending (Figure 5.4 B) and downwards bending (Figure 5.4 C) roots when reoriented 45° from their original angles. This suggests that the lateral root phenotype is not due to an increase in gravitropic capacity or lack of an AGO. The presence of a normal AGO in 80.2.1 MV is further supported as similar levels of outwards bending are observed between Wt Col-0 (Figure 5.5 A and B) and 80.2.1 MV (Figure 5.5 E and F) when grown for 6 hours on a clinostat rotating at 1 revolution per minute (RPM).

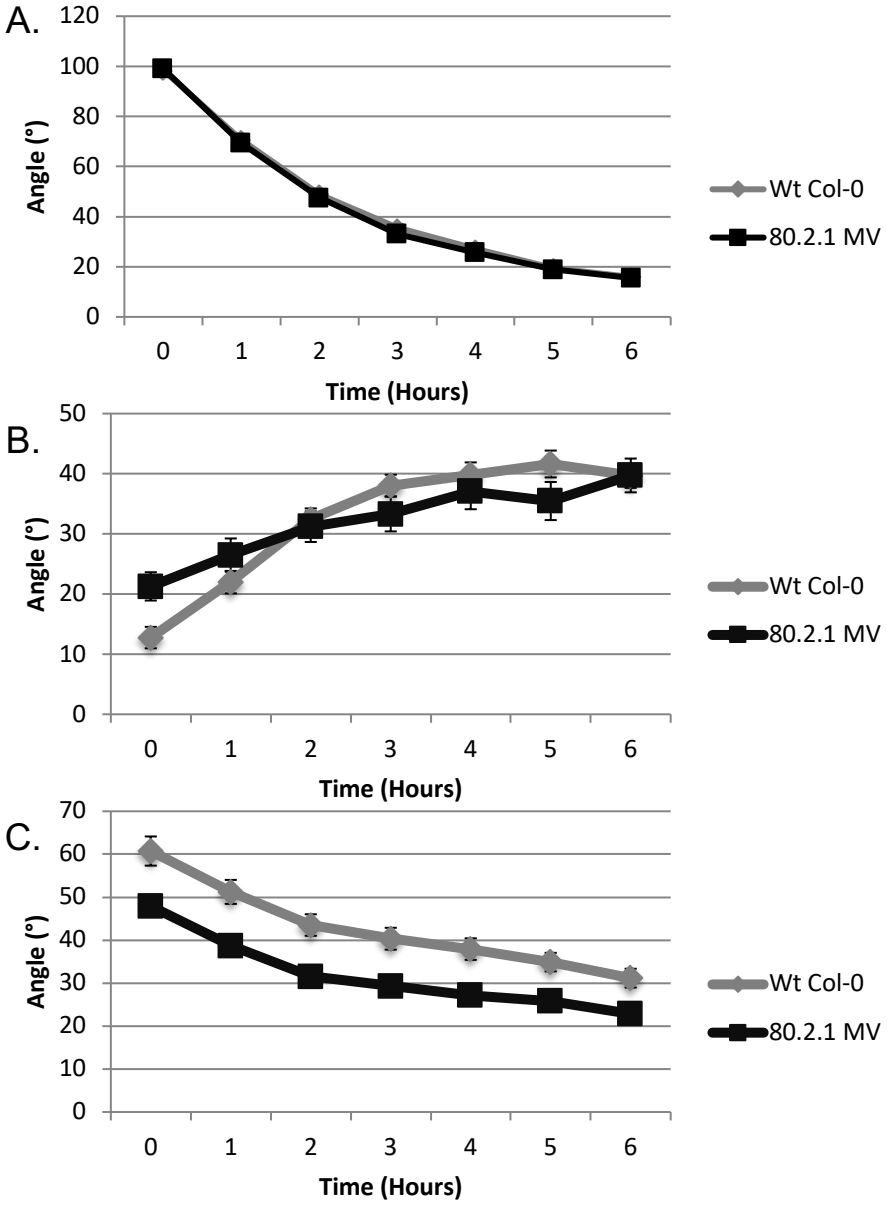


Figure 5.4: The kinetics of gravitropic bending of Wt Col-0 and 80.2.1 MV are similar in both primary and lateral roots

A. The primary roots of 80.2.1 MV bend at a similar rate when reoriented by 90° from the vertical, the average bend rate of Wt Col-0 is 13.8° per hour and the average bend rate of 80.2.1 MV is 13.9° per hour. (Wt n=142, 80.2.1 MV n=142) **B.** The upwards bending lateral roots of 80.2.1 MV bend at a similar rate to those of Wt Col-0 when reoriented by 45° from the vertical, the average bend rate of Wt Col-0 roots is 4.5°per hour and for 80.2.1 MV is 3.1° per hour. (Wt n=42, 80.2.1 MV n=32) **C.** The downwards bending lateral roots of 80.2.1 MV bend at a similar rate to those of Wt Col-0 when reoriented by 45° from the vertical, the average bend rate of Wt Col-0 roots is 4.9°per hour and for 80.2.1 MV is 4.2° per hour (Wt n=93, 80.2.1 MV n=102). The difference in bend rate between Wt Col-0 and 80.2.1 MV was not statistically significant for both upwards and downwards bending lateral roots and downwards bending primary roots ($P>0.05$ Students T-Test). Error bars represent SEM.

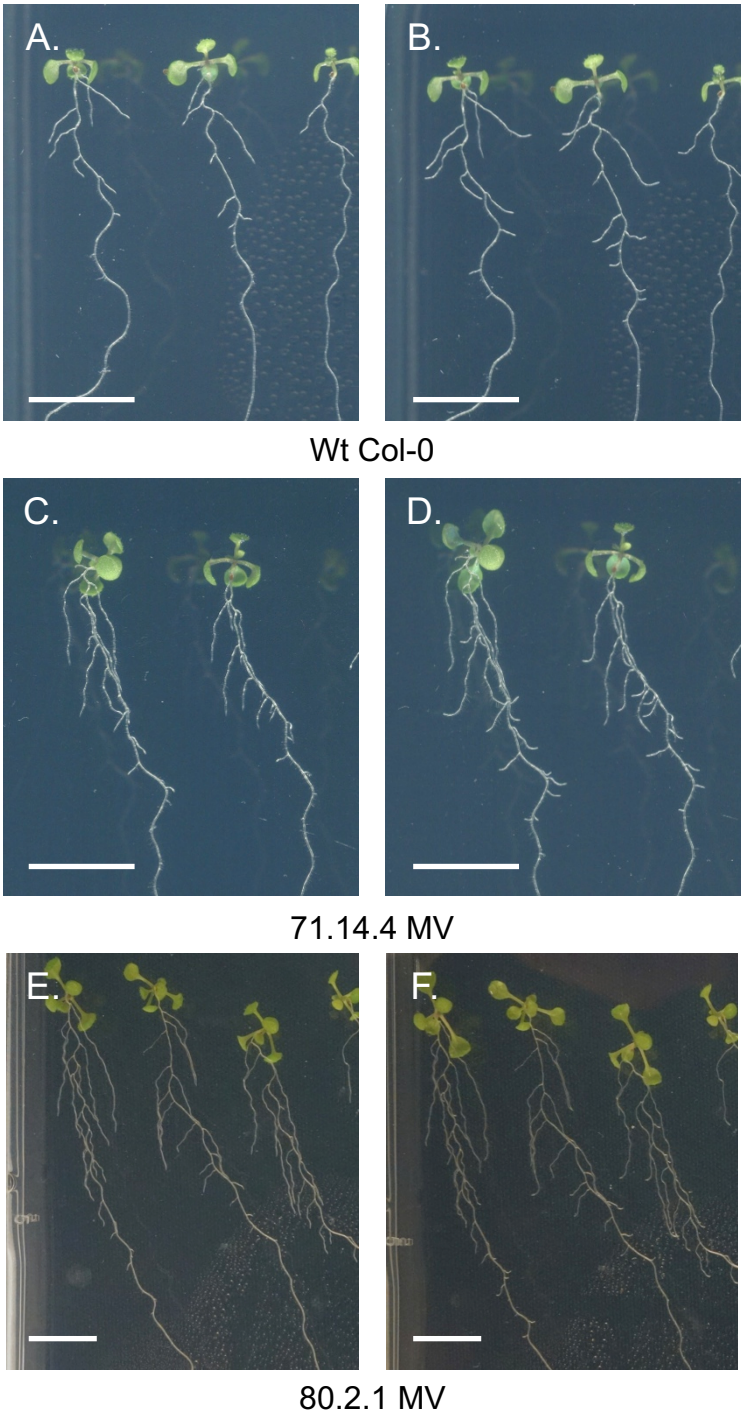


Figure 5.5: Both 80.2.1 MV and 71.14.4 MV display outwards bending of the roots upon clinorotation

The roots of Wt Col-0 bend outwards upon clinorotation, **A** shows the roots before clinorotation, **B** shows the same plants after 6 hours of clinorotation. The roots of 71.1.4 MV also display a similar outwards bending, **C** shows before clinorotation **D** shows the roots after 6 hours of clinorotation. The lateral roots of 80.2.1 MV also exhibit similar bending, **E** shows the roots before and **F** shows the roots after 6 hours of clinorotation. This suggests that the more vertical lateral root phenotype is not due to a lack of or weakening of the AGO. Scale bars represent 1 cm.

5.2.5 Auxin has different effects on the root growth angle but similar effects on elongation rate between 80.2.1 MV and Wt Col-0

When treated with exogenous auxin the lateral roots of Wt Col-0 become more vertical (Figure 5.6 A, B and E) and the roots elongate less than those left untreated (Figure 5.6 F). To determine if 80.2.1 MV's lateral root angle response to auxin was similar, 5 day-old plants of both Wt Col-0 and 80.2.1 MV were transferred onto plates containing 50 nM IAA and allowed to grow for a further 6 days before the lateral root angles were measured. It was found that the lateral roots of 80.2.1 MV do become slightly more vertical when treated with IAA (Figure 5.6 C, D and E), this was not shown to be statistically significant, however it is worth noting that mock treated roots of 80.2.1 MV are already slightly more vertical than Wt Col-0 roots treated with 50 nM IAA (Figure 5.6 E). When placed upon 50 nM and 100 nM IAA it was found that primary root elongation of both Wt Col-0 and 80.2.1 MV was similarly inhibited at both concentrations (Figure 5.6 F), this together with the lateral root phenotype suggests that 80.2.1 MV is able to respond to auxin in a similar way to Wt Col-0 and that the causal mutation is not within the auxin response machinery e.g. Aux/IAAs, ARFs etc.

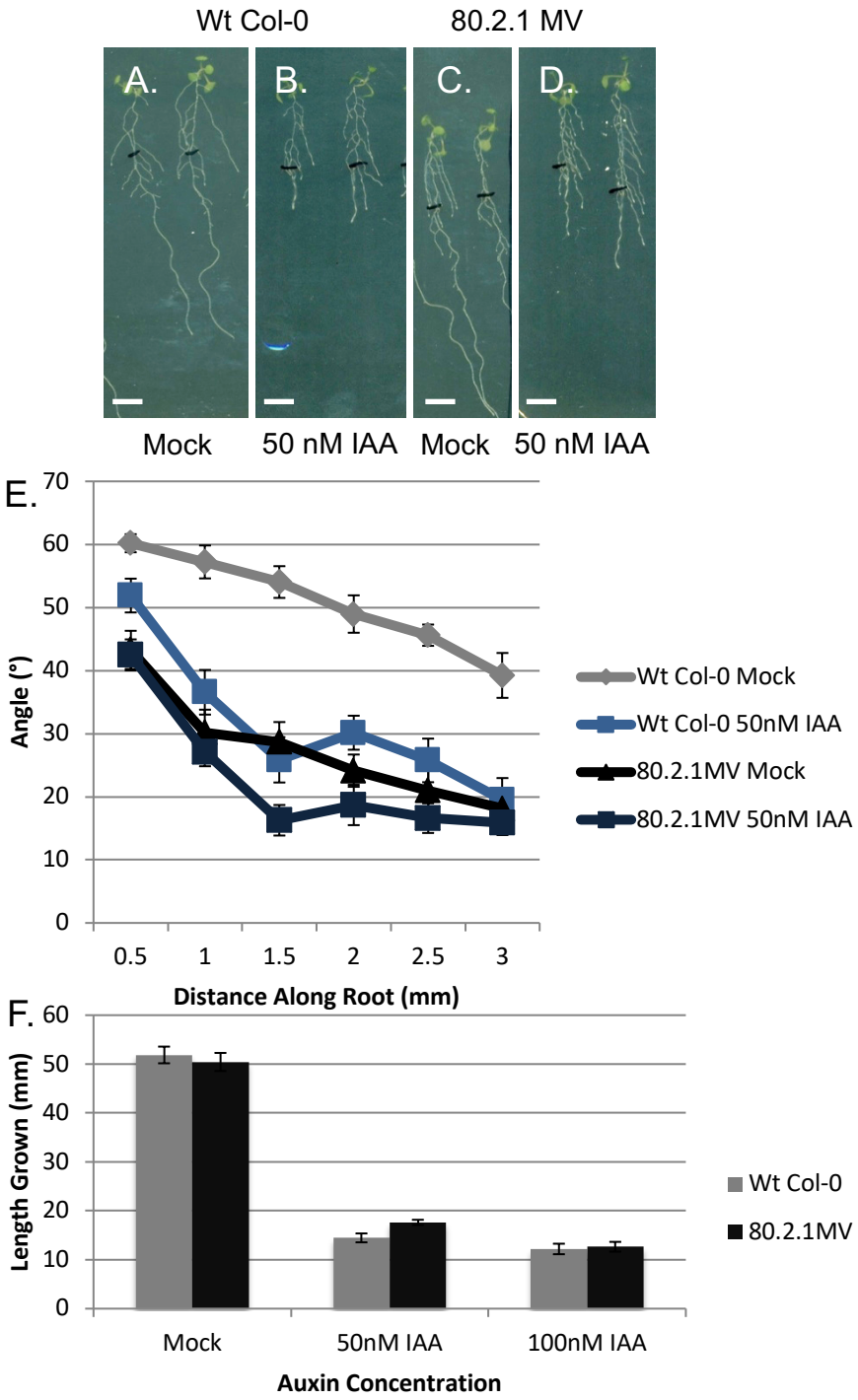


Figure 5.6: Auxin has different effects on the root growth angle but similar effects on elongation rate between 80.2.1 MV and Wt Col-0

A. 11day old mock treated plant of Wt Col-0. **B.** 11 day old Wt Col-0 plant treated with 50 nM IAA. **C.** 11 day old mock treated plant of 80.2.1 MV. **D.** 11 day old 80.2.1 MV plant treated with 50 nM IAA. **E.** The lateral roots of both Wt Col-0 and 80.2.1 MV become more vertical when treated with 50 nM IAA. The difference is significant ($P<0.05$ Students T-test) between mock and treated roots of Wt Col-0 but not between mock and treated roots of 80.2.1 MV, n = 20 roots per treatment. **F.** The primary root elongation of both Wt Col-0 and 80.2.1 MV is equally inhibited upon treatment with 50 nM and 100 nM IAA, n=16 roots per treatment. Scale bars represent 1 cm, error bars represent SEM.

5.2.6 80.2.1 MV is a dominant mutation caused by a single gene

In preparation for genotyping 80.2.1 MV was back-crossed to Wt Col-0, the phenotype of the resulting F_1 progeny would determine if the mutation was dominant or recessive. All the F1 plants grown have the more vertical lateral root phenotype of 80.2.1 MV (Figure 5.7 B and C). There is no significant difference between the lateral root angle of 80.2.1 MV and the 80.2.1 MV x Wt Col-0 F_1 (Figure 5.7 D). In the F_2 generation, out of a total of 216 plants 164 displayed the phenotype and 54 did not display the phenotype, this is a segregation of 3:1 phenotype to no phenotype (Figure 5.8 A), of those plants displaying the phenotype in F_2, in the F_3 generation out of 164 lines, 54 lines were homozygous for the phenotype and 110 lines were heterozygous for the phenotype (Figure 5.8 B). This means that of all the plants grown in the F_2 this gives a segregation of 25%:24%:51% Homozygous no phenotype: Heterozygous phenotype: Homozygous phenotype, roughly 1:1:3 (Figure 5.8 C). The phenotype of the F_1 generation and the segregation of the F_2 and F_3 generations suggests that the causal mutation in 80.2.1 MV is dominant and is a mutation in a single gene.

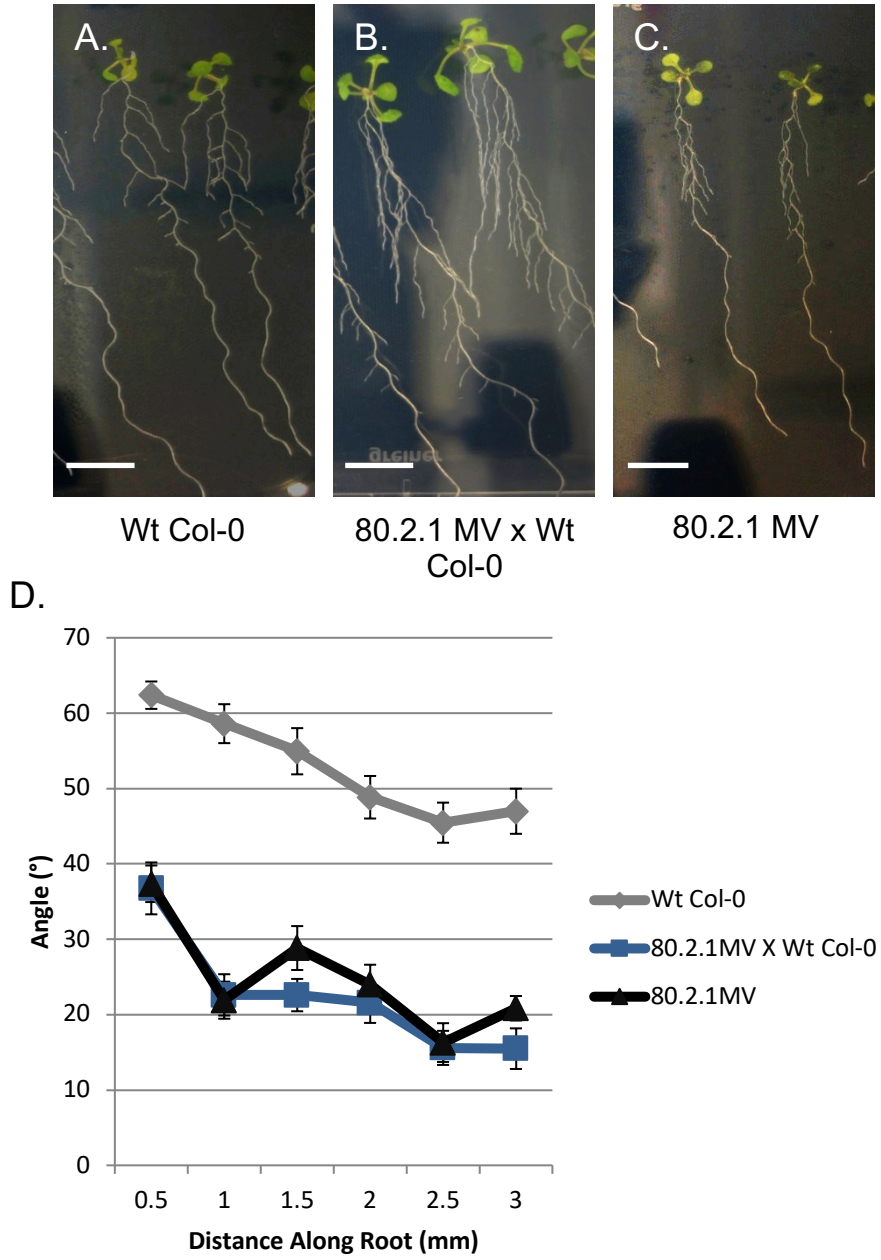


Figure 5.7: 80.2.1 MV is a dominant mutation

A. Root system of 10 day old plants of Wt Col-0. **B.** Root system of 11 day old plants of 80.2.1 MV x Wt Col-0 F_1 plants, the appear more vertical than roots of wild type plants and closely resemble the roots of 80.2.1 MV (**C.**). **D.** The angle of the first 3 mm of root of the 80.2.1 MV x Wt Col-0 is more vertical than Wt Col-0 and is similar to that of 80.2.1 MV. There is no significant difference between 80.2.1 MV and 80.2.1 MV X Wt Col-0 at any point ($P>0.05$ Students T-test, n=20 roots for each line). Scale bars represent 1 cm, error bars represent SEM.

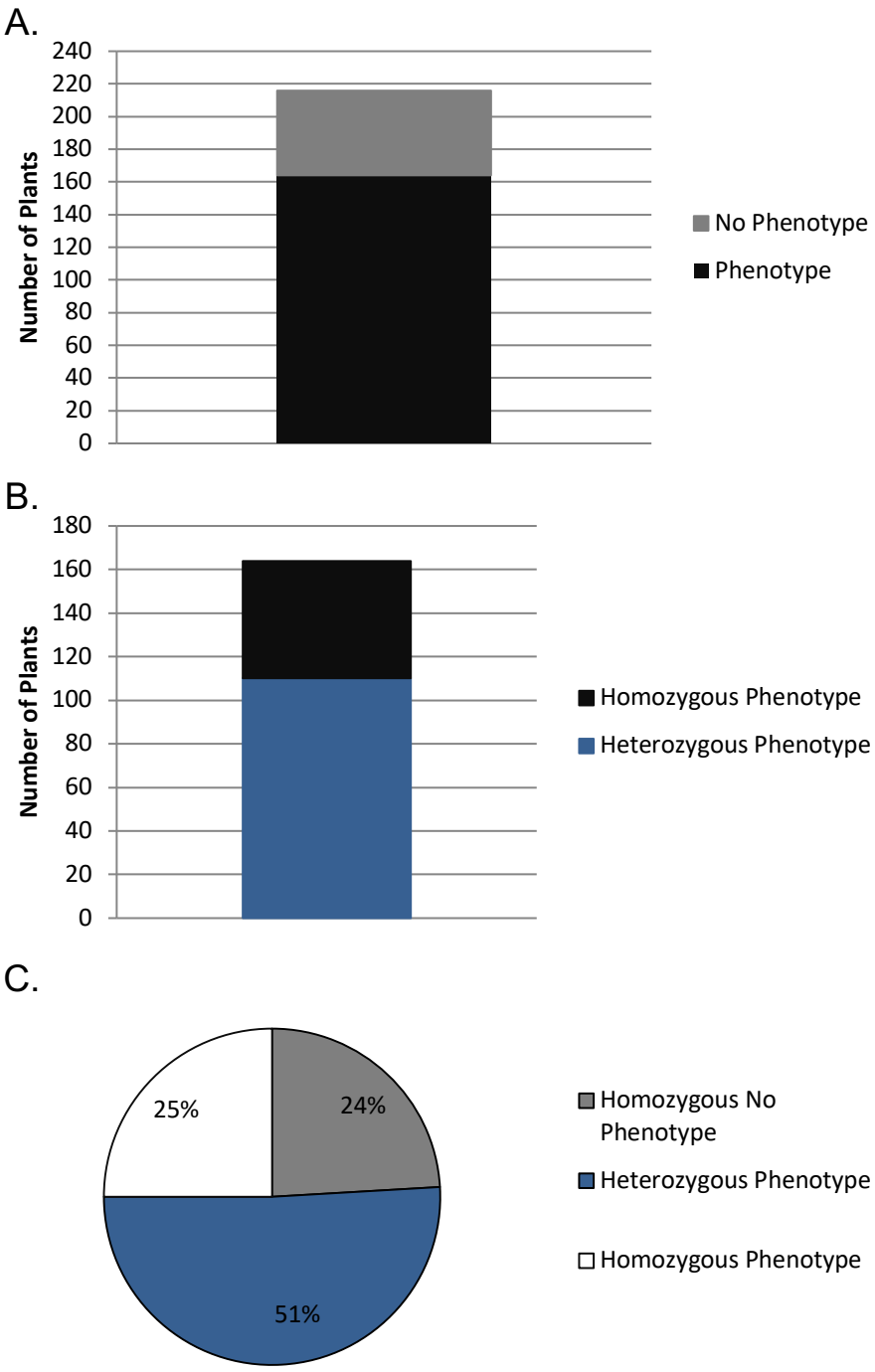


Figure 5.8: Segregation indicates that the phenotype of 80.2.1 MV is caused by a single gene

A. In the F_2 generation out of a total of 216 plants 164 displayed the phenotype and 54 did not display the phenotype, this is a segregation of 3:1 phenotype to no phenotype. **B.** Of those plants that displayed the phenotype in the F_2, in the F_3, 54 lines were homozygous for the phenotype whereas 110 lines were heterozygous for the phenotype. **C.** Of all the plants grown in the F_2 this gives a segregation of 25%:24%:51% Homozygous no phenotype: Heterozygous phenotype: Homozygous phenotype, roughly 1:1:3.

5.2.7 71.14.4 MV has more vertical lateral roots than Wt Col-0

In later stages of the M_2 screen a second mutant with more vertical lateral roots was found in pool 71 in the 14th round of the screen and was named 71.14.4 MV. The lateral roots are also consistently more vertical (Figure 5.9 B) than those of Wt Col-0 (Figure 5.9 A). When the angles with respect to gravity of 0.5 mm sections of the first 3 mm of root were measured, there is a significant difference between Wt Col-0 and 71.14.4 MV in the angle of root sections at 0-0.5, 0.5-1, 1.5-2 and 2.5-3 mm from the primary root (Figure 5.9 C), on average the roots of 71.14.4 MV are 14.2° more vertical than those of Wt Col-0.

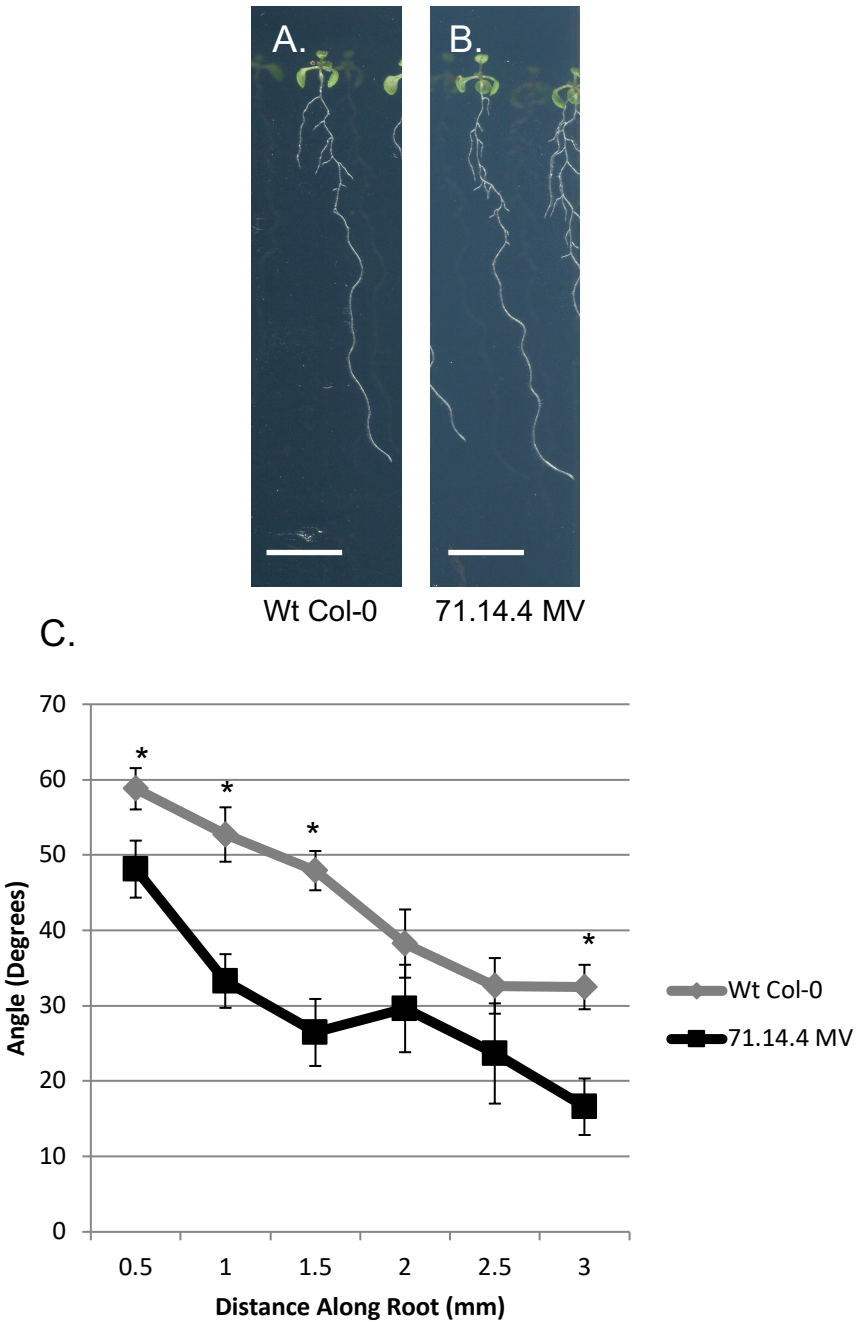


Figure 5.9: 71.14.4 MV has more vertical lateral roots than Wt Col-0
A. The root system of a 10 day old Wt Col-0 plant. **B.** The root system of a 10 day old plant of 71.14.4 MV, the lateral roots are visibly more vertical than Wt Col-0. **C.** The root angle of the first 3 mm of root of both Wt Col-0 and 71.14.4MV, on average the angles of Wt Col-0 range from 58.8° at 0-0.5 mm to 32.5° at 2.5-3 mm from the origin, the angles of 71.14.4 MV range from 48.1° at 0-0.5 mm to 16.6° at 2.5-3 mm from the origin. 10 roots were measured from each line, error bars represent SEM, $P<0.05$ at all points marked with * (Students T-test). Scale bars represent 1 cm.

5.2.8 The shoots of 71.14.4 MV have no angle phenotype when compared with Wt Col-0

Similarly to 80.2.1 MV, in contrast to the root phenotype, 71.14.4 MV has no angle related shoot phenotype when compared with Wt Col-0 (Figure 5.10 C). When the first 3 cm of shoot were measured there was no significant difference ($P<0.05$) in the angle between 71.14.4 MV and Wt Col-0 at all points except those at 0-0.5 cm and 2.5-3 cm. This suggests that either the causal mutation for the angle phenotype is in a gene that has different roles and is involved in different pathways in roots and shoots or that the causal mutation is in a gene that is not expressed in the shoots.

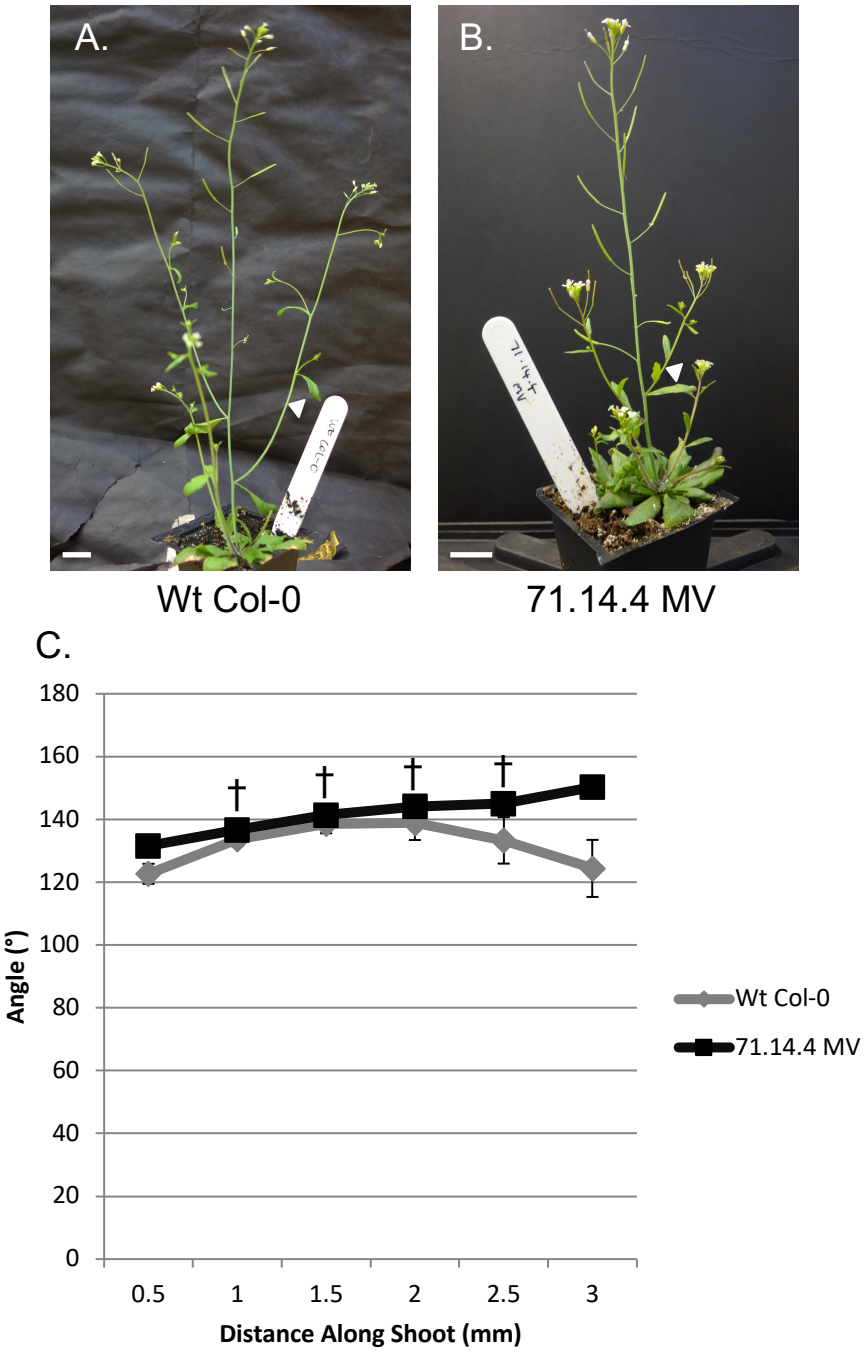


Figure 5.10: The shoots of 71.14.4 MV have no angle phenotype when compared with Wt Col-0

A. The shoots of a 4-week old Wt-Col-0 plant **B.** The shoots of a 4-week old plant of 71.14.4 MV, there is no difference in angle phenotype between Wt Col-0 and 71.14.4 MV, a representative branch is indicated with a white arrow. **C.** The shoot angle of the first 3 cm of shoot of both Wt Col-0 and 71.14.4 MV, on average the angles of Wt Col-0 range from 122.7° at 0-0.5cm to 124.4° at 2.5-3 cm from their origin, the angles of 71.14.4 MV range from 131.6° at 0-0.5 cm to 150.3° at 2.5-3 cm from their origin. P>0.05 for all points marked with † (Students T-test). Scale bars represent 1 cm. Error bars represent SEM.

5.2.9 The Kinetics of gravitropic bending of Wt Col-0 and 71.14.4 MV are similar in both primary and lateral roots

As with 80.2.1 MV, the gravitropism kinetics of the primary and lateral roots of 71.14.4 MV were tested to determine if there were differences in gravitropic capacity and AGO when compared with Wt Col-0. No significant difference in the speed of gravitropic bending between Wt Col-0 and 71.14.4 MV in primary roots displaced 90° from the vertical was detected (Figure 5.11 A). Similarly, in lateral roots there was no significant difference between Wt Col-0 and 71.14.4 MV in the speed of gravitropic bending of both upwards bending (Figure 5.11 B) and downwards bending (Figure 5.11 C) roots when reoriented 45° from their original angles. This suggests that the more vertical lateral root phenotype in 71.14.4 MV is also not due to an increase in gravitropic capacity or lack of an AGO. The presence of a normal AGO in 71.14.4 MV is further supported as similar levels of outwards bending are also observed between Wt Col-0 (Figure 5.5 A and B) and 71.14.4 MV (Figure 5.5 C and D) when grown for 6 hours on a clinostat rotating at 1 RPM.

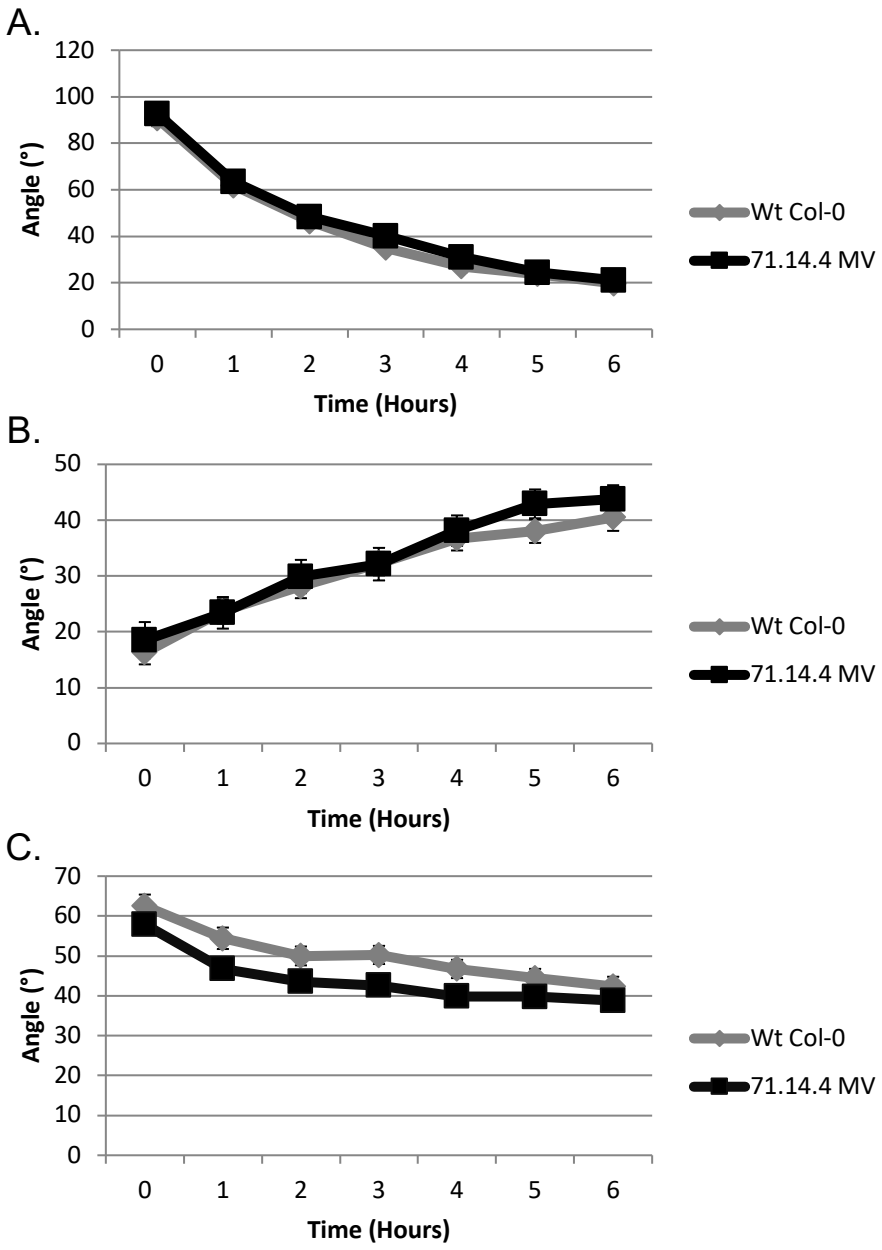


Figure 5.11: The Kinetics of gravitropic bending of Wt Col-0 and 71.14.4 MV are similar in both primary and lateral roots

A. The primary roots of 71.14.4 MV bend at a similar rate when reoriented by 90° from the vertical, the average bend rate of Wt Col-0 is 11.8° per hour and the average bend rate of 71.14.4 MV is 11.9° per hour. (Wt n=31, 71.14.4 MV n=28) **B.** The upwards bending lateral roots of 71.14.4 MV bend at a similar rate to those of Wt Col-0 when reoriented by 45° from the vertical, the average bend rate of Wt Col-0 roots is 4°per hour and for 71.14.4 MV is 4.2° per hour. (Wt n=50, 71.14.4 MV n=47) **C.** The downwards bending lateral roots of 71.14.4 MV bend at a similar rate to those of Wt Col-0 when reoriented by 45° from the vertical, the average bend rate of Wt Col-0 roots is 3.4°per hour and for 71.14.4 MV is 3.2° per hour. (Wt n=71, 71.14.4 MV n=99). There is no significant difference in bend rate between Wt Col-0 and 71.14.4 MV for all kinetics tests carried out (P>0.05 Students T-test). Error bars represent SEM.

5.2.10 Auxin has similar effects on the root growth angle and elongation rate of 71.14.4 MV as on Wt Col-0

To test for defects in auxin response, 71.14.4 MV was also treated with exogenous auxin. The lateral roots of both Wt Col-0 and 71.14.4 MV both become significantly more vertical (Figure 5.12 E) when treated with 50 nM IAA. When transferred to 50 nM and 100 nM IAA it was found that there was no significant difference in primary root elongation between 71.14. MV and Wt Col-0 in each treatment (Figure 5.12 F), this together with the lateral root phenotype suggests that 71.14.4 MV is also able to respond to auxin in a similar way to Wt Col-0 and that the causal mutation is not within the auxin response machinery.

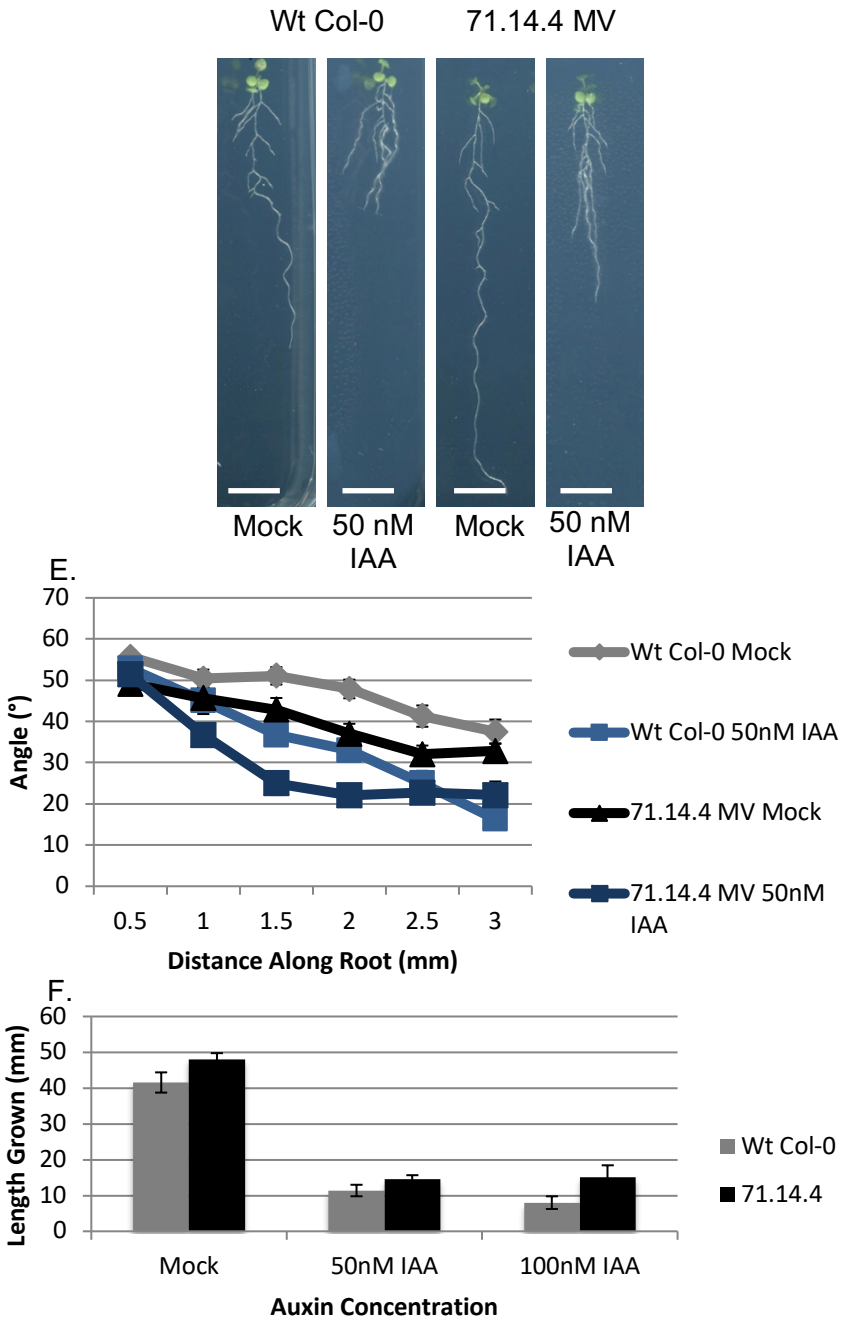


Figure 5.12: Auxin has similar effects on the root growth angle and elongation rate of 71.14.4 MV as on Wt Col-0

A. 11day old mock treated plant of Wt Col-0. **B.** 11 day old Wt Col-0 plant treated with 50 nM IAA. **C.** 11 day old mock treated plant of 71.14.4 MV. **D.** 11 day old 71.14.4 MV plant treated with 50 nM IAA. **E.** The lateral roots of both Wt Col-0 and 71.14.4 MV become more vertical when treated with 50 nM IAA. There is a significant difference between mock and treated for all points except 0.5 mm ($P<0.05$ Students T-test) **F.** The primary root elongation of both Wt Col-0 and 71.14.4 MV is equally inhibited upon treatment with 50 nM and 100 nM IAA. Error bars represent standard error, n= 20 roots per treatment for lateral root angle, n = 8 roots per treatment for primary elongation, there is no significant difference between 71.14.4 MV and Wt Col-0 in all treatments. Scale bars represent 1 cm, error bars represent SEM.

5.2.11 71.14.4 MV is a recessive mutation

71.14.4 MV was also back-crossed to determine the dominance of the mutation. The lateral roots of all F_1 plants have the lateral root phenotype of Wt Col-0 (Figure 5.13 B) as opposed to 71.14.4 MV. There is a significant difference between Wt Col-0 and 71.14.4 MV at all points (except 0-0.5 mm), the roots of 71.14.4 MV X Wt Col-0 F_1 are less vertical than those of 71.14.4 MV and there is no significant difference between Wt Col-0 and 71.14.4 X Wt Col-0 F_1 (Figure 5.13 D), this suggests that 71.14.4 MV is a recessive mutation.

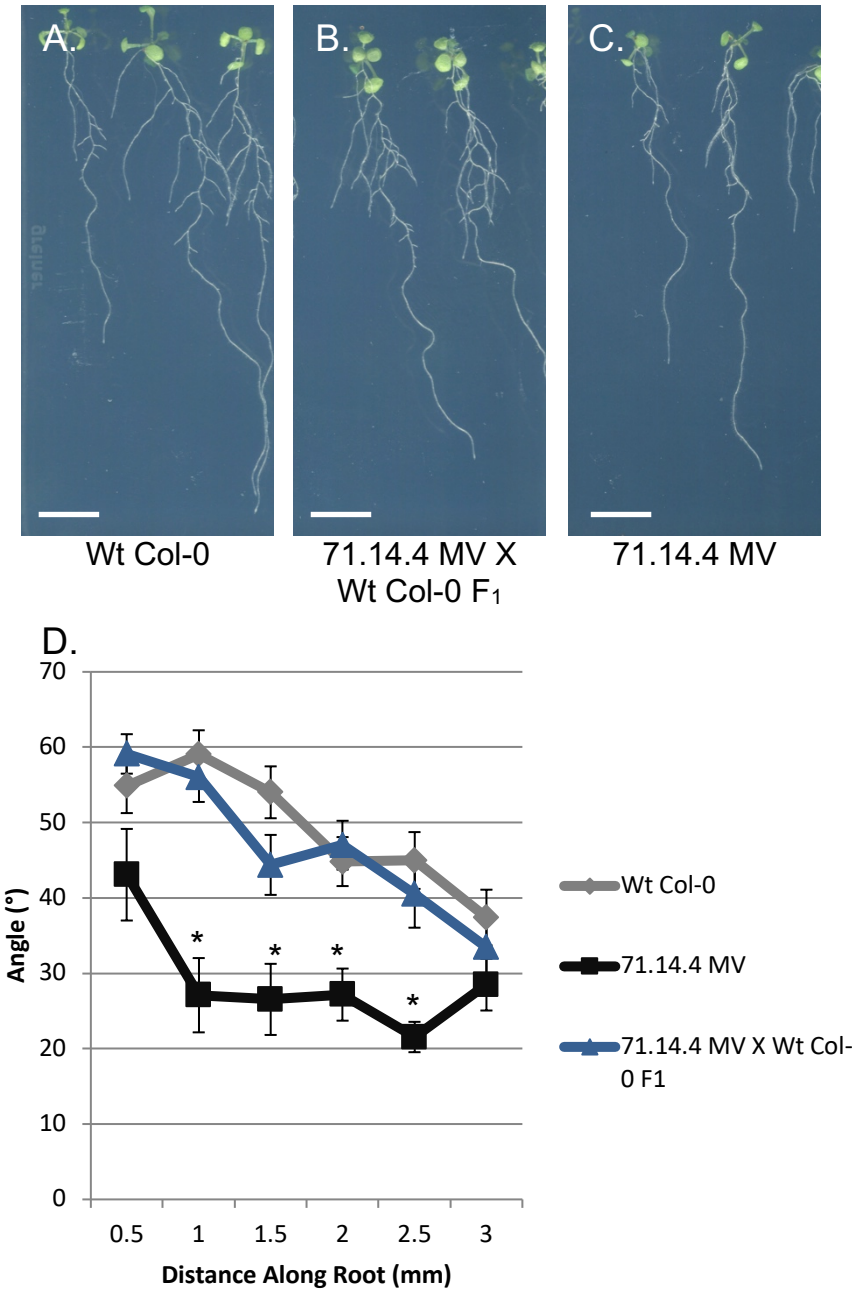


Figure 5.13: 71.14.4 MV is a recessive mutation

A. Root system of 10 day old plants of Wt Col-0. **B.** Root system of 10 day old plants of 71.14.14 MV x Wt Col-0 F_1 plants, they appear more vertical than roots of 71.14.4 MV plants and closely resemble the roots of Wt Col-0 (**C.**). **D.** The angle of the first 3 mm of root of the 71.14.4 MV x Wt Col-0 F_1 is less vertical than 71.14.4 MV and is similar to that of Wt Col-0. There is a significant difference ($P<0.05$ Students T-test) from wild type Col-0 at all points marked with *, n=10 roots for each line. Error bars represent SEM.

5.2.12 Genotyping 80.2.1 MV by whole genome sequencing

As 80.2.1 MV had the strongest phenotype of the two it was decided to genotype it, this was to be done by whole genome sequencing. First it was back-crossed to Wt Col-0, the resulting F_1 were allowed to self and produce seed. The F_2 generation were grown and separated into two pools: Phenotype and No Phenotype. A piece of leaf tissue was collected from each plant, the plants that were in the Phenotype pool were allowed to self and the F_3 was grown for each plant to determine which F_2 individuals were homozygous for the mutation. Tissue from plants that displayed no phenotype was pooled and tissue from the plants that were homozygous for the phenotype was pooled. DNA was extracted from the two pools and sent for whole genome sequencing (Figure 5.14) using Illumina NextSeq sequencing at the Next Generation Sequencing Facility at St James Hospital, Leeds. Assembly against TAIR10 and subsequent bioinformatics analysis using SNP calling and variant effect prediction produced a list of 25 candidate genes (Table 5.2). Of these candidate genes, based upon the phenotype of the mutant and information available on expression patterns and effects of mutations in these genes, AT1G72490 (*AtLAZY4/DEEPER ROOTING1*) was decided upon as the most likely candidate.

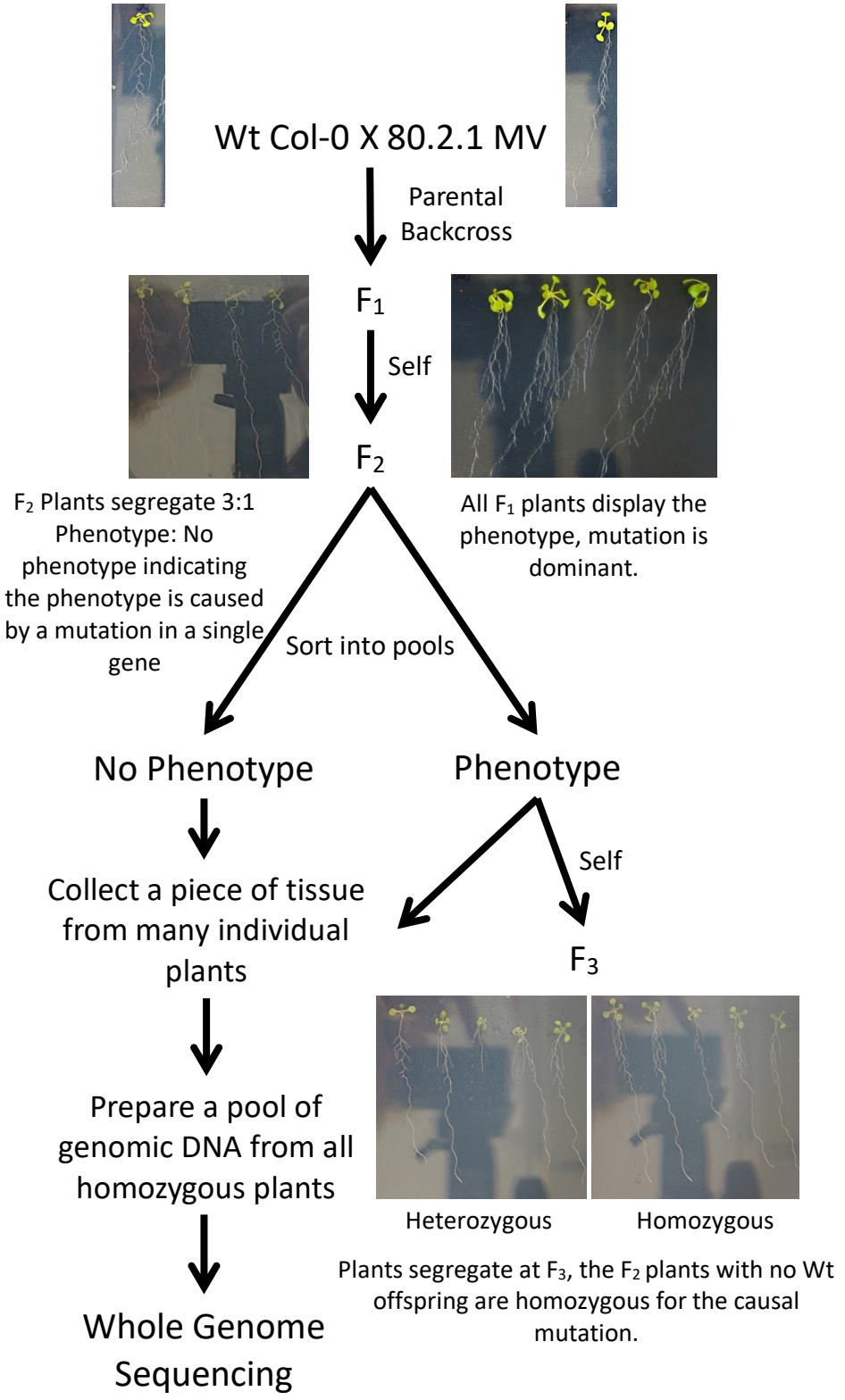


Figure 5.14: Flowchart of progress from parental backcross to whole genome sequencing

A flowchart detailing the stages from the parental backcross of 80.2.1 MV through to the whole genome sequencing used to determine the causal mutation.

Gene	Variant Effect Type	Reference Base	Alternate Base	Gene Encodes	
AT1G05740	Splice donor variant	GTA	G	Eukaryotic protein of unknown function (DUF842)	
AT1G18500	missense variant	A	T	methylthioalkylmalate synthase-like 4	
AT1G33070	missense variant	C	A	MADS-box family protein	
AT1G59510	missense variant	G	A	Carbohydrate-binding protein	
AT1G66480	missense variant	C	T	plastid movement impaired 2	
AT1G71200	Splice region variant	CT	C	basic helix-loop-helix (bHLH) DNA-binding superfamily protein	
AT1G72490	**missense variant**	**C**	**T**	**AtLAZY4/DEEPER ROOTING 1**	
AT1G73290	3’ UTR variant	CAT	C	serine carboxypeptidase-like 5	
AT1G75110	Stop gained	C	A	Nucleotide-diphospho-sugar transferase family protein (RRA2)	
AT1G75420	3’ UTR variant	G	A	UDP-Glycosyltransferase superfamily protein	
AT1G76620	Missense variant	G	C	Protein of unknown function, DUF547	
AT1G78630	Missense variant	G	A	Ribosomal protein L13 family protein	
AT2G25900	Splice region variant	G	T	Zinc finger C-x8-C-x5-C-x3-H type family protein	
AT2G27260	Missense variant	C	A	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family	
AT3G42995	Frameshift variant	GT	G	Transmembrane protein	
AT3G61700	5’ UTR variant	GTA	G	Plant protein 1589 of unknown function	
AT4G19580	Missense variant	C	A	DNAJ heat shock N-terminal domain-containing protein	

AT4G19580	missense variant	T	C	DNAJ heat shock N-terminal domain-containing protein	
AT5G12390	5‘ UTR variant	T	C	Tetratricopeptide repeat (TPR)-like superfamily protein	
AT5G42560	missense variant	G	T	Abscisic acid-responsive (TB2/DP1, HVA22) family protein	
AT5G51270	Splice donor variant	A	T	U-box domain-containing protein kinase family protein	
AT5G52530	3’ UTR variant	A	T	dentin sialophosphoprotein-related	
AT5G52530	3’ UTR variant	C	T	dentin sialophosphoprotein-related	
AT5G65780	missense variant	T	C	branched-chain amino acid aminotransferase 5	
AT5G66620	3’ UTR variant	CAT	C	DA1-related protein 6	

Table 5.2: Possible candidates for the causal mutation in 80.2.1 MV

A list of possible candidates for the causal mutation in 80.2.1 MV, based upon the phenotype of the mutant AT1G72490 (*AtLAZY4/DEEPER ROOTING 1*) is the most likely candidate.

5.2.13 Complementation of the root phenotype shows that an amino acid change in LAZY4 is responsible for the more vertical lateral roots.

As *LAZY4* proved to be the most likely candidate for the gene carrying the causal mutation in 80.2.1 MV it was decided to clone the *LAZY4* coding sequence from both 80.2.1 MV cDNA and from Wt Col-0 cDNA, carrying out site directed mutagenesis on the Wt version to replicate the potential causal mutation in 80.2.1 MV. The mutation in LAZY4 thought to be the cause of the 80.2.1 MV root phenotype was a change at position 145 from an arginine to a lysine (Figure 5.15). A construct was produced in the pALLIGATOR V plasmid containing the native *LAZY4* promoter and the correct version of *LAZY4*, site directed mutagenesis of the Wt version was carried out in the complete *pLZY4::LZY4* construct in pALLIGATOR V. Four constructs were produced: *pLZY4::LZY4* 80.2.1, *pLZY4::LZY4* R145K, *pLZY4::LZY4* R145A, *pLZY4::LZY4* R145E. The constructs were transformed into the *lazy4* knockout line via *Agrobacterium* floral dip and the T_1 seeds selected using seed coat fluorescence from the pALLIGATOR V plasmid backbone under a GFP microscope. The T_1 generation of all four constructs displayed the more vertical lateral root phenotype seen in 80.2.1 MV (Figure 5.16 A) and all lines containing a mutated LAZY4 were significantly more vertical that Wt Col-0 at all points measured (Figure 5.16 B), this confirms that a change in the amino acid residue at position 145 in LAZY4 is the cause of the more vertical lateral root phenotype of 80.2.1 MV. As all mutant constructs produced the same more vertical lateral root phenotype this suggests that it is the loss of the arginine at position 145 that is the cause of the phenotype as opposed to a gain in a different residue. The lateral root phenotype of the *lazy4* mutant transformed with the Wt *pLZY4::LZY4* construct displayed no significant difference from Wt Col-0 in the angles of the lateral roots at any point measured (Figure 5.16 B), this confirms that the construct was functional.

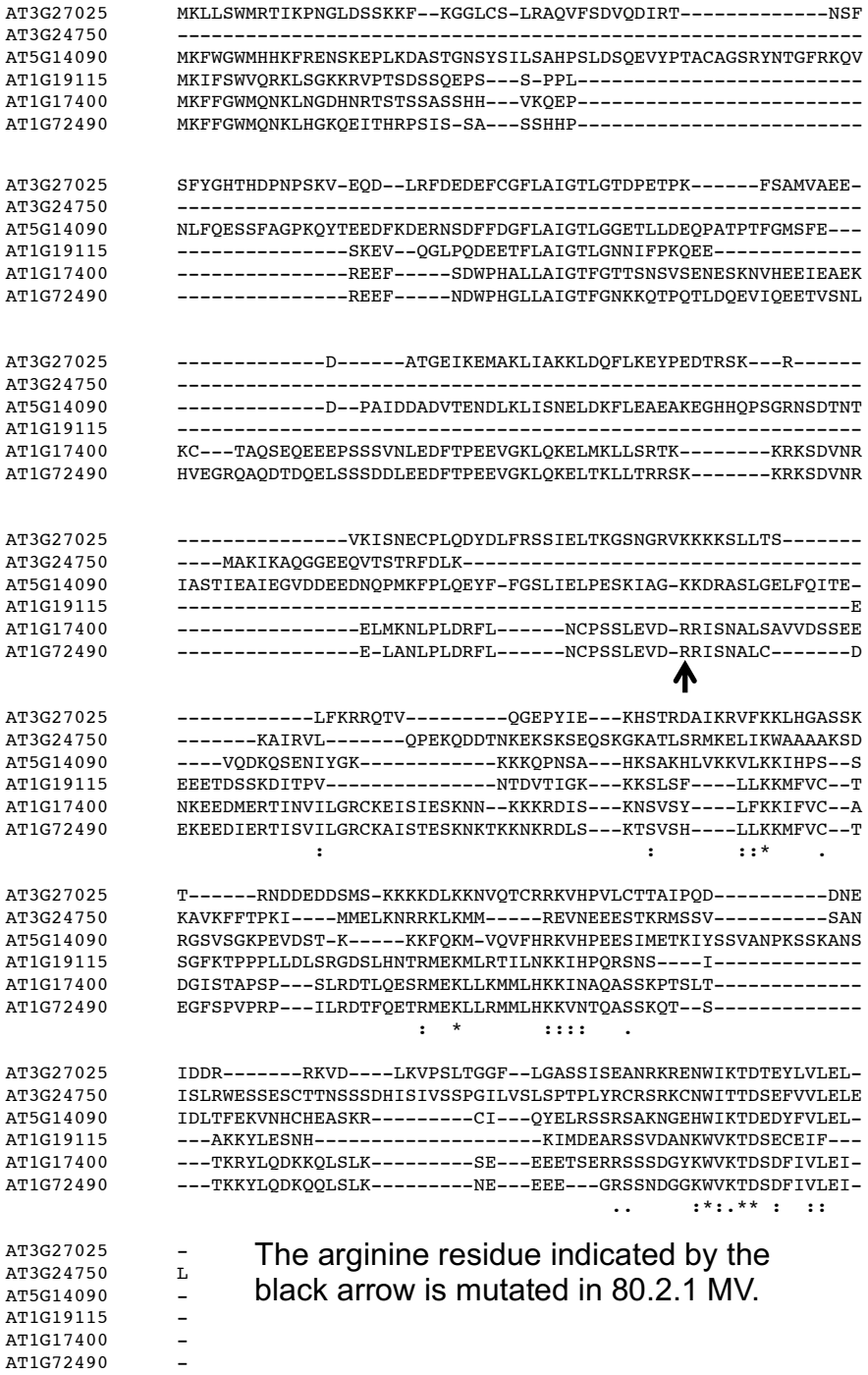


Figure 5.15: An alignment of the Arabidopsis LAZY family protein sequences showing the position of the causal mutation in 80.2.1 MV

An alignment of the Arabidopsis LAZY family showing the position of the causal mutation in 80.2.1 MV, note that this is within a motif conserved in both LAZY4 (At1g72490) and LAZY2 (At1g17400).

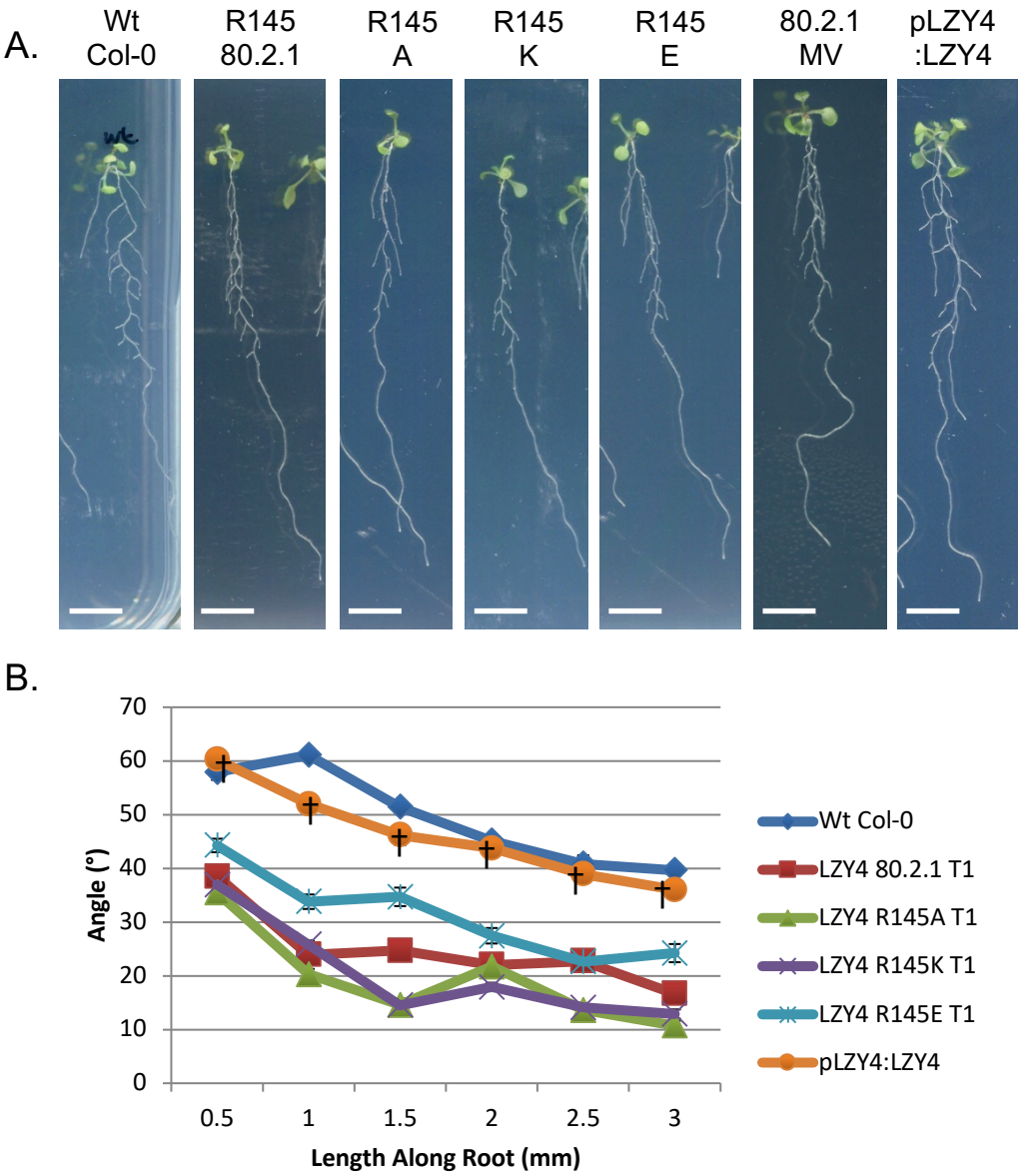


Figure 5.16: The T_1 lateral root phenotype of mutagenised LAZY4 complements that of 80.2.1 MV

In order to determine if the change in R145 of LAZY4 was the causal mutation for the more vertical lateral site directed mutagenesis was carried out on LAZY4 and under the control of the native promoter was transformed into the *lazy4* knockout line. **A**. All of the lines produced carrying a mutation at R145 in LAZY4 displayed a more vertical lateral root phenotype in the T_1 generation similar to that of 80.2.1 MV. **B**. The lateral roots of all transformants carrying the mutated forms of LAZY4 were significantly more vertical than wild type col-0. Error bars represent SEM, scale bars represent 1 cm, † indicates P>0.05. n = 10 roots per line.

5.2.14 The domain containing the causal mutation of 80.2.1 MV is conserved across multiple species

The domain carrying the causal mutation of 80.2.1 MV is found in both LAZY2 and LAZY4 of Arabidopsis. Bioinformatic analysis of LAZY4 and LAZY2 homologs in a number of other species revealed a conserved region of roughly 18 amino acids (LPLDRFLNCPSSLEVDRR) across a wide range of species, both monocots and dicots (Figure 5.17). This suggests that the amino acids in this region are important for the function of LAZY2 and LAZY 4 and that this motif appeared early on in plant evolution.

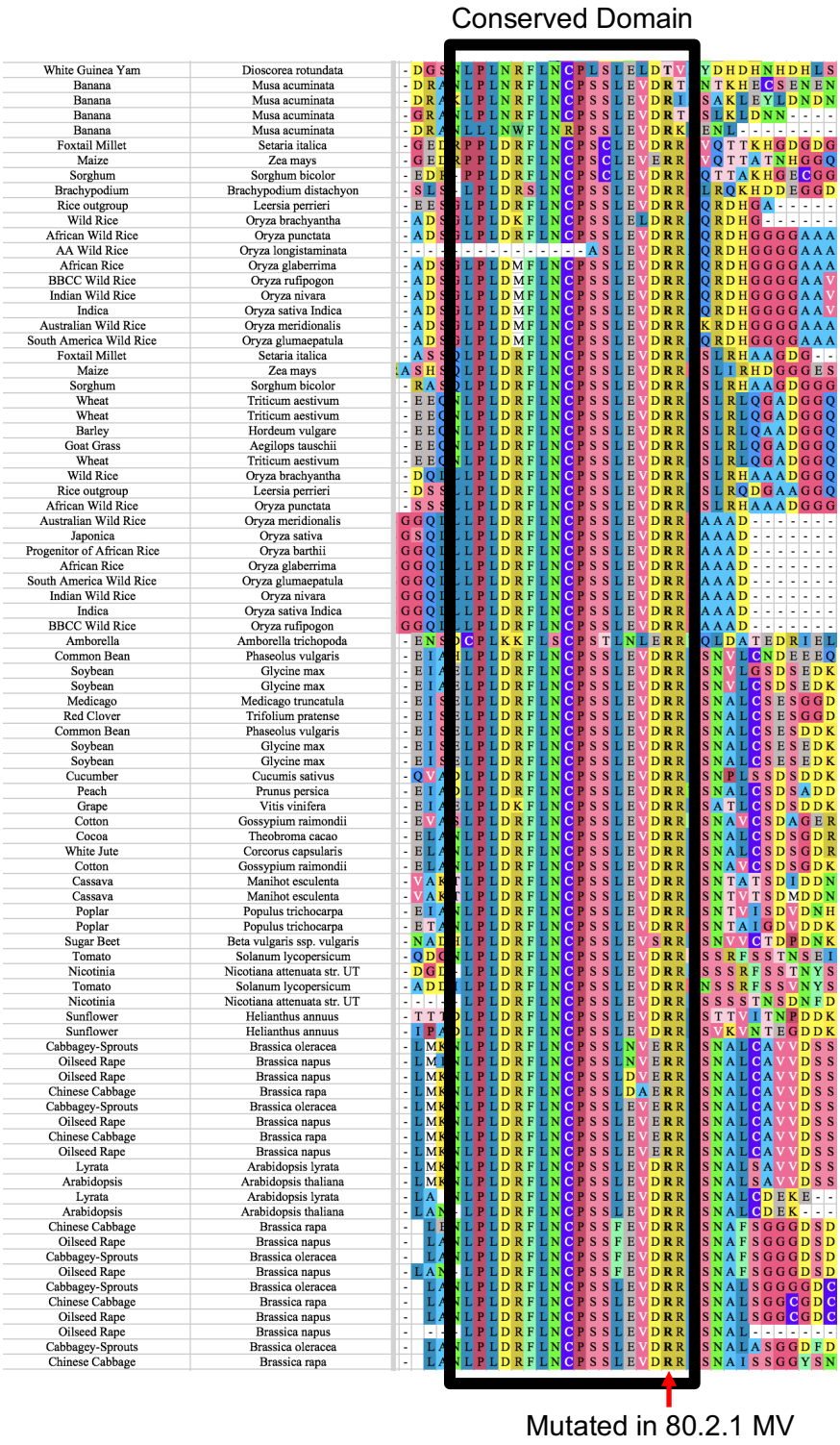


Figure 5.17: The mutation containing domain is conserved across multiple species

The domain containing the causal mutation in 80.2.1 MV is conserved across multiple species, there are 18 amino acids that are highly conserved in LAZY4 across both dicot and monocot species. Alignment courtesy of Dr Michael Wilson.

5.2.15 Ecotypes with an altered amino acid in the conserved domain also have more vertical lateral roots.

A number of ecotypes were found with a natural polymorphism in the conserved domain of LAZY4 that contains the mutation found in 80.2.1 MV. In all ecotypes tested the polymorphism resulted in a V143A change in the LAZY4 protein. The lateral roots of all these ecotypes are more vertical than those of Wt Col-0 (Figure 5.18); this suggests that the whole conserved domain is important for the setting of lateral root angle and that mutations in a number of the residues may have the potential to modulate lateral root angle.

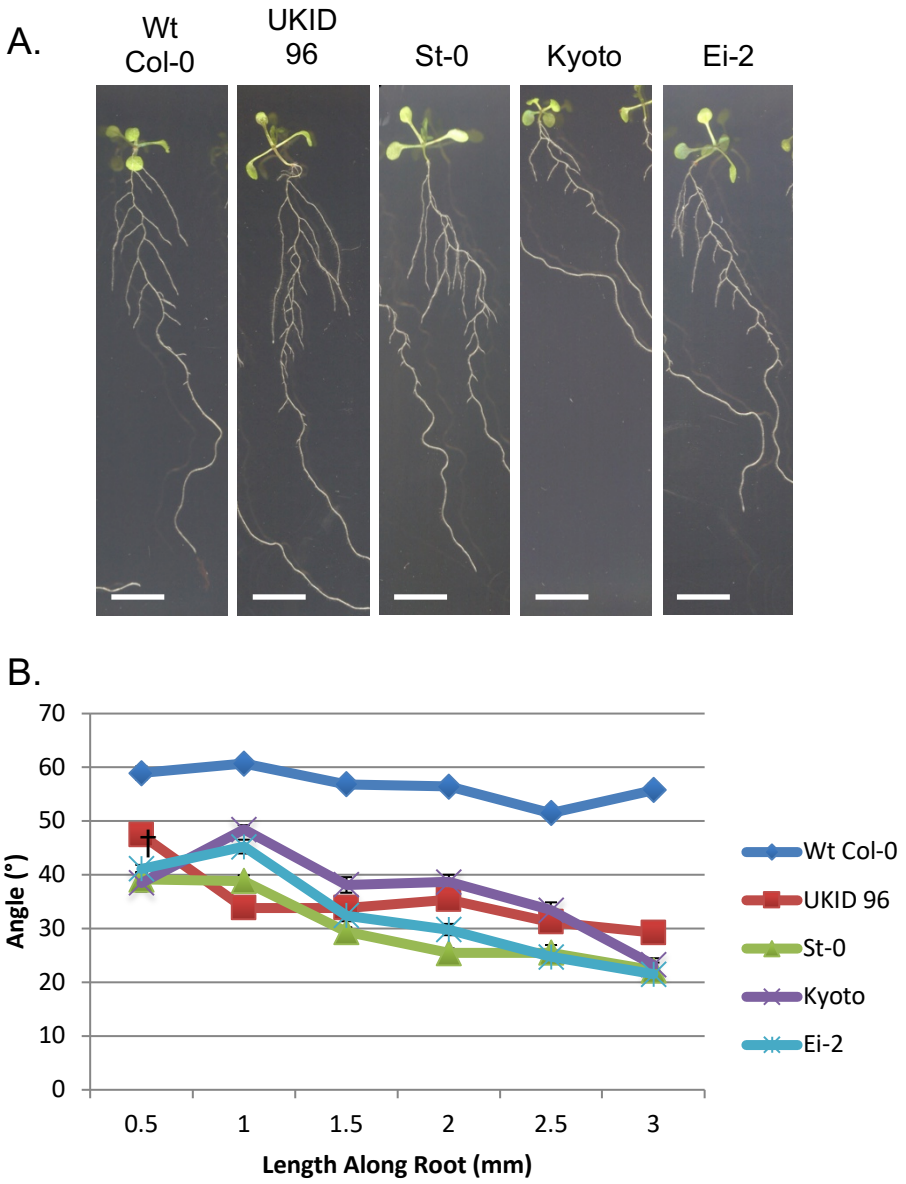


Figure 5.18: Ecotypes with an alternative amino acid in the conserved domain also have more vertical lateral roots.

A. A number of ecotypes were found with polymorphisms in the conserved region surrounding the mutation contained within 80.2.1 MV. All of the ecotypes contain a V143A change in LZY4 when compared with Col-0 **B**. The lateral roots of all ecotypes were significantly more vertical than Col-0. Error bars represent SEM, scale bars represent 1cm, † indicates P>0.05. n=10 roots per ecotype.

5.2.16 Phenotypes of a number of other mutants that were not selected on the basis of angle

In addition to mutants selected upon the basis of lateral root angle, a number of other mutants with potentially interesting root phenotypes were also found, these include a short rooted phenotype (27.3.2 Short) when compared with wild type and a number of mutants with unusually wavy roots (68.8.5 Wavy, 56.23.5 Wavy and 63.23.5 Wavy) (Figure 5.19). Interestingly these mutants also displayed abnormal shoot phenotypes, 27.3.2 Short shows a jagged edged leaf phenotype but otherwise normal cauline branches. The wavy mutants in addition to sharing a root phenotype also have a similar shoot phenotype that comprises of contorted siliques and leaves and increasingly wavy shoots (Figure 5.20), occasionally the lateral shoots form coils.

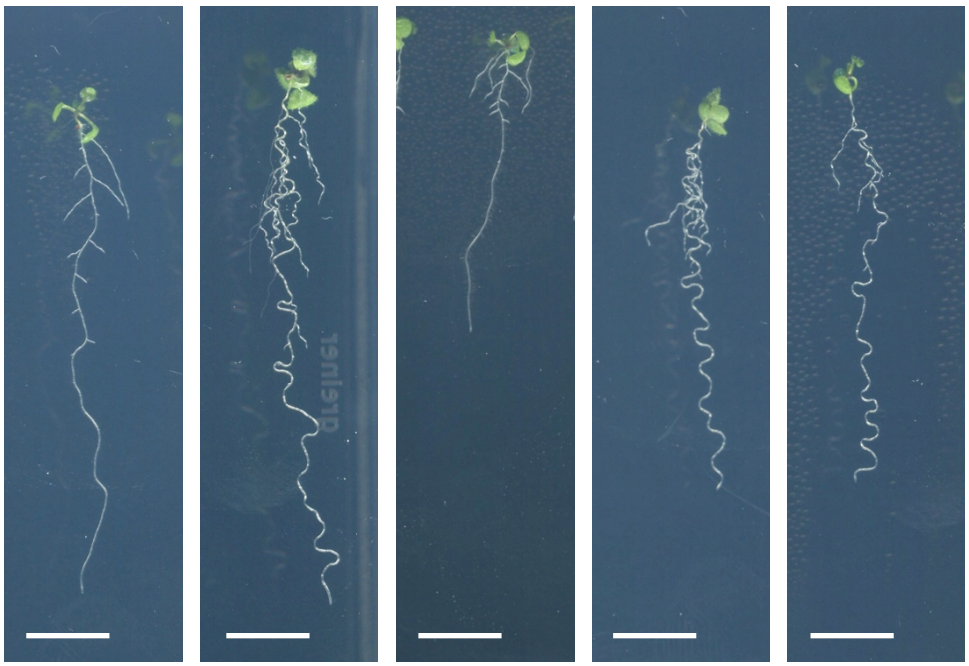


Figure 5.19: Root phenotypes of a number of other mutants that were not selected on the basis of angle

A number of other mutants with root phenotypes not relating to angle were also found during the screen, these included a number of mutants with a root waving phenotype such as 68.8.5, 56.23.5 and 63.23.5 and mutants with shorter roots such as 27.3.2. Scale bars represent 1 cm.

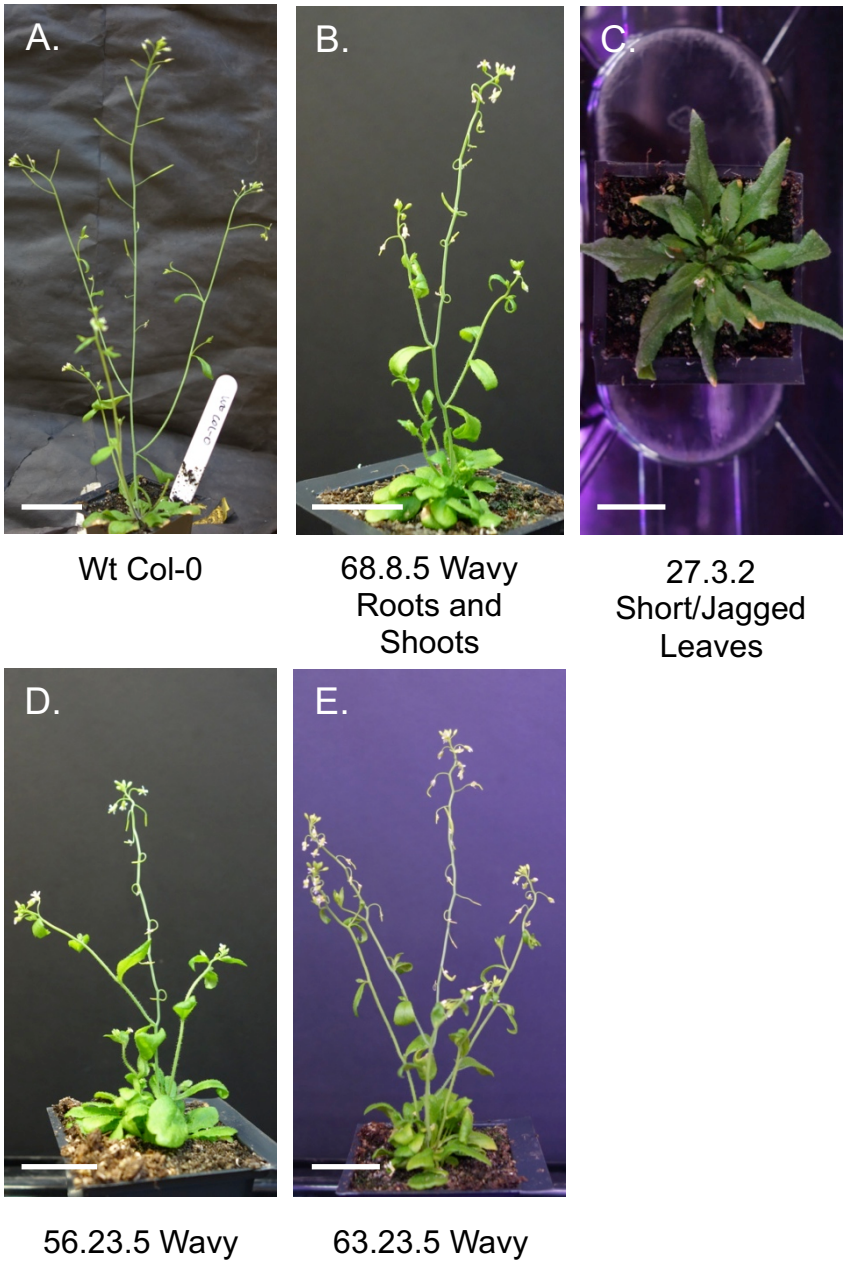


Figure 5.20: Shoot phenotypes of a number of other mutants that were not selected on the basis of angle

The mutants that were selected not on the basis of root angle that can be found in Figure 5.19 also displayed shoot phenotypes, those with a wavy root phenotype also share a similar "wavy" shoot phenotype (**A,B,D** and **E**) in which the siliques and occasionally the cauline branches and leaves curl inwards. 27.3.2 also displayed a jagged leaf phenotype in addition to its shorter roots (**C**). Scale bars represent 2 cm.

5.3 Discussion

The ability of mutations in a number of different genes to modulate the GSA of the lateral roots of Arabidopsis (Roychoudhry et al., 2013) gives exciting prospects for the modification of plant architectures for future research and crop development. An EMS mutagenesis based screen for lateral root angle mutants was carried out to find mutants with altered lateral growth angles that had the potential to further understanding of lateral root angle setting and maintenance, and, with long-term potential for the mutation to be used in the production of crop plants with altered lateral root GSAs.

Over the course of the screen 18400 individual M_2 plants were screened for abnormal root phenotypes. Twelve M_2 lines were found whose phenotypes carried through to the M_3 generation, of those, two lines were selected for further analysis that had consistent, strong, more vertical lateral root angle phenotypes that did not result in severe growth defects, they were named 80.2.1 MV (Figure 5.2) and 71.14.4 MV (Figure 5.9). In contrast to their root phenotypes neither 80.2.1 MV nor 71.14.4 MV display a lateral shoot angle phenotype (Figure 5.3 and 5.10 respectively). These observations indicate that the causal mutations are in genes that are only active in the root and are either not expressed in, or play no role in gravitropic response in, the shoot. A number of genes are known that only affect the angle of the lateral organs in either the root or the shoot. Examples of genes known to only affect lateral shoot angle but not root angle include *LAZY1* and *TAC1* both of which have dramatic shoot phenotypes (Dardick et al., 2013, Yoshihara et al., 2013, Yoshihara and Spalding, 2017, Taniguchi et al., 2017), the knockout mutant of *TAC1* has no lateral root angle phenotype (Supplementary Figure 5.1) despite its more vertical shoot phenotype (Dardick et al., 2013) and AtLAZY1, which has a less vertical shoot angle phenotype (Yoshihara et al., 2013, Yoshihara and Spalding, 2017), has little to no expression in the roots and shows no root gravitropism defects (Yoshihara and Spalding, 2017). A single knockout mutant of *atlazy4* has no shoot angle phenotype and no expression in the shoots of light grown plants (Taniguchi et al., 2017, Yoshihara and Spalding, 2017) but displays a less vertical lateral root phenotype (Supplementary Figure 5.2).

GSA maintenance is dependent on a balance of opposing gravitropic and antigravitropic growth components (also known as the AGO), when reoriented the lateral roots must reorient in both upwards and downwards directions in order to return to their original angles (Roychoudhry et al., 2013). Neither 80.2.1 MV or 71.14.4 MV display defects in the gravitropic bending of their primary roots and both the upwards and downwards bending of their lateral roots (Figure 5.4 and Figure 5.11), in addition to this, both display similar degrees of outwards bending of the lateral roots to wild type Col-0 when subjected to rotation on a 1 RPM clinostat for 6 hours (Figure 5.5). This suggests that the more vertical lateral root phenotypes of both mutants are not due to either an increase in gravitropic capability or the loss of/reduction in magnitude of the AGO. Although in their model of GSA maintenance, Roychoudhry et al (2013) propose that the GSA of an organ is determined by the magnitude of its AGO (Roychoudhry et al., 2013), these results would suggest that there is perhaps another level of control above that of the magnitude of the AGO to determining the growth angle of a lateral root and that maybe there are two different but linked mechanisms that dictate the setting of the growth angle and its maintenance. As they get older and longer lateral roots adopt a more vertical GSA, Roychoudhry et al (2013) showed that upon clinorotation roots and shoots of increasing length displayed less outwards curvature than shorter roots and shoots, they reasoned that this was as a result of a diminishing of the AGO (Roychoudhry et al., 2013). However, this was not observed in these experiments, roots of all ages and lengths displayed a similar capacity for outwards bending upon clinorotation for both mutants and Wt Col-0 (Figure 5.5) again suggesting that there is another level of control above that of the magnitude of the AGO to determining the growth angle of the lateral roots.

An alternative model could include the addition of an upstream element that has the ability to modulate the AGO. In a non-reoriented lateral root, change in the expression or activity of the upstream element could result in the dampening down or suppression of the AGO as the root ages. However, upon gravistimulation (or loss of a gravitropic stimulus), the graviresponse machinery could effect a change in this upstream element that results in the removal of this suppression and allows the AGO to act to return the lateral roots to their original

angle. It is possible that the genes containing the causal mutations in both 80.2.1 MV (*AtLAZY4*) and 71.14.4 MV could be involved in this upstream element as older roots of similar size on both mutants and Wt Col-0 show similar levels of outwards bending upon clinorotation (Figure 5.5) despite their different lateral root GSAs. This is supported by the similarity between the lines and wild type in their ability to bend upwards upon reorientation (Figure 5.4B and 5.11B).

Addition of exogenous auxin causes the lateral roots of wild type plants to assume a more vertical GSA and mutants in the auxin biosynthesis or response pathways also display altered lateral root GSAs (Roychoudhry et al., 2013). In reference to the model outlined above it could be possible that the additional element that controls the magnitude of the AGO could itself be regulated by auxin in terms of either its expression or activity, this could explain the changes in the angle assumed by the lateral roots upon addition of greater levels of auxin or changes in the plants ability to respond to auxin. When grown on plates containing exogenous auxin similar effects are seen in both 80.2.1 MV and 71.14.4 MV as are seen in wild type, the lateral roots of both mutants become more vertical than mock treated roots, not significantly so for 80.2.1 MV but this could be due to how vertical it is to begin with (already more vertical than wild type treated roots) and the difficulty in measuring the lateral roots when they are so tightly packed around the primary (Figure 5.6 and 5.12). The ability of auxin to control cell expansion is key to its role in the gravitropic response, in roots auxin inhibits cell elongation and therefore root elongation (Baldwin et al., 2013, Chen et al., 1999, Firn et al., 2000). When grown on increasing concentrations of auxin root elongation of Wt Col-0 is inhibited (Figures 5.6 and 5.12), it was found that for both 80.2.1 MV (Figure 5.6) and 71.14.4 MV (Figure 5.12) root elongation was inhibited to a similar degree as that of Wt, this indicates that neither mutant has a greater or lesser response to auxin. This, along with the change in the lateral root angles, suggests that the mutation found within 71.14.4 MV is not within the auxin biosynthesis or response pathways and that *LAZY4* does not have a role in auxin response.

In addition to the synthesis of, and response to auxin, its movement around the plant is also crucial to its role in the gravitropic response (Rashotte et al., 2000, Rashotte et al., 2001). Families of influx and efflux transporters such as the AUX1/LAX influx transporters and the PIN efflux proteins mediate the production of the auxin fluxes and gradients that drive tropic growth (Kramer, 2004, Friml and Palme, 2002, Friml, 2003, Benková et al., 2003) and it is the PINs whose asymmetric distribution around the cell directs the flow of auxin around the plant (Kramer, 2004, Friml, 2003, Benková et al., 2003). It is thought that auxin transport is important to GSA control as changes to auxin signalling in the gravity sensing cells alone is able to change growth in cells and tissues that are non-adjacent to the gravity sensing cells (Roychoudhry et al., 2013). The causal mutation of 80.2.1 MV has been confirmed as a single amino acid change in the gene *LAZY4* (Figure 5.15 and 5.16). It is thought that the LAZY genes may be involved in the control of PIN localization as there is a difference in PIN3: GFP signal intensity between higher and lower columella cells in the lateral roots between wild type plants and the triple mutant atlazy124 (Taniguchi et al., 2017). It is possible that PIN localisation is altered in 80.2.1 MV and is a contributing factor towards its more vertical lateral root phenotype. However, this is unlikely to be as a result of interference in PIN cycling and relocalisation, for example through the ARF-GEF GNOM pathway (Kleine-Vehn et al., 2008, Kleine-Vehn et al., 2010), as the gravitropism kinetics are not significantly different to wild-type (Figure 5.4). The gravitropism kinetics of 71.14.4 MV are also not significantly different to wild type (Figure 5.11) it is similarly unlikely that the causal mutation interferes with PIN relocalisation.

When back-crossed with Wt Col-0 80.2.1 MV is a dominant mutation (Figure 5.7) whereas 71.14.4 MV is a recessive mutation (Figure 5.13). Despite a recessive mutation being easier to map it was decided to map the mutation of 80.2.1 MV as opposed to 71.14.4 MV as it has a stronger phenotype, the segregation in the F_2 and F_3 generations suggests it is caused by a single gene (Figure 5.8) and its dominance could make it useful for future crop breeding programs. A number of important food crops are polyploid (Lawrence and Pikaard, 2003) as detailed in the previous chapter, this means that the effects of single gene knockouts are easily masked by the presence homeologous genes

on the other genomes (Lawrence and Pikaard, 2003, Uauy et al., 2009) this could also be true of the effects of recessive mutations meaning that a dominant mutation is of advantage for introduction into these crops to produce a desired phenotype. The phenotype of 80.2.1 MV makes it an ideal candidate mutation to attempt to produce lines of crops that follow the “Steep, Cheap and Deep” ideotype proposed by Lynch (Lynch, 2013) and detailed in the previous chapter.

Whole genome sequencing did not produce a clear-cut answer for the causal mutation in 80.2.1 MV as a number of mutations were identified that were present in the phenotype carrying pool but not the no phenotype pool. A variant effect predictor was used to pick out only those base changes that would cause missense mutations, 3’UTR variants, promoter variants, 5’UTR variants and gains of a stop codon, a list of 25 candidates was identified (Table 5.2). Using a genome browser to visually choose those with obvious clear changes across multiple reads and phenotypic and expression information about the potential candidate genes allowed a conclusion could be reached. It was postulated that the mutation was a single amino acid change in the gene *LAZY4* (also known as *AtDEEPER ROOTING 1*) at position 145, which in the wild type is an arginine (Figure 5.15). The LAZY genes are known to be involved in the control of lateral root and shoot growth angle (Yoshihara et al., 2013, Yoshihara and Spalding, 2017, Ge and Chen, 2016, Taniguchi et al., 2017, Guseman et al., 2017) as *atlazy* mutants exhibit gravitropism defects the severity of which increases with mutating more genes and the roots of *atlazy124* often grow horizontally or completely upside-down (Yoshihara and Spalding, 2017, Taniguchi et al., 2017). It is known that *AtLAZY4* is expressed in the root tip but not expressed in the shoots (although there is some expression in the cotyledons of dark grown seedlings) (Yoshihara and Spalding, 2017) and that changes made in the gravity sensing cells found in the root tip have an effect on the GSA of lateral roots (Roychoudhry et al., 2013). Single knockout mutations of *lazy* genes have little effect upon the phenotype of plants (although the *lazy4* single knockout mutant does display less vertical lateral roots (Supplementary Figure 5.2)) and other than the gravitropism defects and altered growth angles seen in higher order mutants the plants are morphologically similar to wild type (Yoshihara and Spalding, 2017). The combination of this phenotypic and

expression information make *AtLAZY4* an ideal candidate to carry the causal mutation in 80.2.1 MV. Auxin is known to negatively regulate the expression of the rice ortholog of *AtLAZY4* known as *OsDRO1* (Uga et al., 2013), it is currently unknown whether the same is true in Arabidopsis however this could potentially allow LAZY4 or the other LAZY proteins to take on the role of the additional AGO controlling element in the model detailed above.

Interestingly, although the higher order *lazy* mutants display in some cases severe gravitropism defects (Yoshihara and Spalding, 2017), the *atlazy4* single mutant has no gravitropism defects. Also, when reoriented, the lateral roots return to their GSA (Personal communication – Suruchi Roychoudhry (Unpublished)). This along with the phenotypic and kinetic data for 80.2.1 MV suggests that the LAZYs (or at least *LAZY4*) are specifically related to the setting of a GSA as opposed to its maintenance as both the knockout and 80.2.1 MV have no gravitropism issues despite their altered lateral root growth angles. Again, this could support a role for LAZY4 in the control of the AGO in the model outlined previously.

The change in R145 of LAZY4 was confirmed as the causal mutation using site directed mutagenesis to reproduce the mutation and complementation of the mutant phenotype when transformed into the *lazy4* knockout. From the whole genome sequencing it was thought that R145 was changed to a Lysine, interestingly however, when *LAZY4* was cloned from 80.2.1 MV cDNA there was an amino acid change in the same position but it was from an arginine to an alanine. This could be due to a number of possibilities including errors in the sequencing and misalignment during mapping to TAIR10. It was decided to make a suite of different mutations at the R145 site to see if amino acids with different properties had different effects, arginine is a large basic amino acid, changes were made to lysine (also basic but smaller and what the whole genome sequencing says is the change in 80.2.1 MV), alanine (small and hydrophobic but introduces less flexibility into the backbone than a glycine, the residue that cloning from 80.2.1 MV cDNA says is the change at position 145) and glutamic acid (a large amino acid but with an acidic side chain to investigate what reversing the charge does to the phenotype). The phenotypes

of the T_1 generation of transformants confirms that a change in R145 of LAZY4 is the cause of the 80.2.1 MV phenotype and that all of the amino acid changes made have the same effect, more vertical lateral roots (Figure 5.16), this suggests that it is the loss of the arginine residue rather than a gain of function from a different residue that is the cause of the phenotype.

Further bioinformatic analysis revealed that R145 is part of a conserved motif found in both LAZY2 and LAZY4 of Arabidopsis but not in the other 4 members of the Arabidopsis LAZY family (Figure 5.15), *LAZY2* is also highly expressed in the root tip (Yoshihara and Spalding, 2017). It also revealed that the motif containing R145 found in LAZY2 and LAZY4 of Arabidopsis is part of an 18 amino acid motif (LPLDRFLNCPSSLEVDRR of which R17 is mutated in 80.2.1 MV) that is widely conserved across a range of species spanning both monocots and dicots including *Brassica rapa, Theobroma cacao, Solanum lycopersicum, Glycine max, Phaseolus vulgaris, Oryza sativa, Zea mays, Sorghum bicolor* and *Triticum aestivum* to name but a few (Figure 5.17), the motif is also loosely conserved (including the double arginine that contains the 80.2.1 MV mutation) in the ancient plant species *Amborella trichopoda* (Figure 5.17) suggesting that this motif appeared early in land plant evolution and is important for protein function. Interestingly, the protein sequence originally published as the rice DRO1 does not contain the 18 amino acid motif (Uga et al., 2013) but other LAZY homologs in rice do suggesting multiple genes may be involved in the determination of root growth angle in rice. The widespread conservation of this motif opens up a number of exciting possibilities for future work. As it appears in a number of crop species it is an ideal candidate to attempt to produce the Steep, Cheap and Deep ideotype detailed by Lynch (Lynch, 2013) into a wide range of crops and the dominant nature of the mutation negates the need for existing knockouts of *LAZY4* to exist in those species and removes the issues surrounding polyploidy detailed earlier. The lack of other deleterious phenotypes (e.g. stunted growth, infertility etc.) also makes it ideal for this purpose.

A number of Arabidopsis ecotypes were found with a polymorphism in the conserved domain of *LAZY4* that contains the 80.2.1 MV mutation that results in

a V143A amino acid change, these also displayed significantly more vertical lateral roots (Figure 5.18). This suggests that multiple residues within that domain have a role in the ability of LAZY4 to control lateral root growth angle, additional mutations could be made in other highly conserved residues within the motif to explore both its function and to determine if any other phenotypes can be produced. Potential sites for additional mutations within the conserved domain could include the cysteine as it has the potential to form disulphide bridges with other conserved cysteines, the proline as it has effects on the shape and flexibility of the amino acid backbone and the two serines as they have the potential to be phosphosites. The arginine adjacent to the arginine that is the site of the causal mutation in 80.2.1 MV (R146 in Arabidopsis) is also a good candidate for mutation as it is conserved in all species except *Dioscorea rotundata* and *Musa acuminata* (Figure 5.17). Further EMS mutagenesis could be carried out upon a homozygous line of 80.2.1 MV with the aim of carrying out a suppressor (or enhancer) screen to determine any interacting partners or genes within the same pathway as *LAZY4*, it is thought that the *LAZYs* are involved in the control of PIN localization in the columella cells (Taniguchi et al., 2017) and could function between statolith sedimentation and the formation of the auxin gradients that drive tropic growth (Yoshihara and Spalding, 2017). It would also be interesting to sequence and map the causal mutation of 71.14.4 MV, it also displays the qualities of an “ideal” mutant as described in the introduction and could give further insight into the mechanisms of lateral root growth angle control.

Alongside those mutants that were found with altered lateral root angle phenotypes, other mutants with interesting root phenotypes were also found. These mutants included 27.3.2 Short (Figure 5.19) which displayed a short root phenotype, this could be as a result of mutations in a number of possible genes. Auxin is known to have an inhibitory effect on cell elongation in roots (and a stimulatory effect on cell elongation in shoots) (Chen et al., 1999, Firn et al., 2000) and mutations that cause defects in either auxin response or auxin levels could affect root length, for example a knockout of the flavin monooxygenase-like protein YUCCA 1that is involved in auxin biosynthesis results in an auxin deficient phenotype and the *yuc1D* mutant in which *YUC1* is overexpressed

using the 35S promoter results in increased auxin levels coupled with high auxin phenotypes such as elongated hypocotyls and increased apical dominance (Mashiguchi et al., 2011). Mutations in the ethylene response pathway that increase ethylene production or response could also result in a shortened root phenotype similar to that given by increased auxin response as elevated ethylene levels also result in shorter, hairier roots (Růžička et al 2007). The *rhd3-1* mutant (also produced using EMS mutagenesis) displays a short root phenotype accompanied by short and wavy root hairs (Wang et al., 1997). RHD3 is a plant member of the dynamin-like Atlastin GTPases, a family of proteins known to be involved in the production of interconnected ER tubules, it is though that the short root phenotype is as a result of impaired vesicle trafficking as secretion of proteins from the ER is not impaired, in *rhd3-1* the golgi stacks aggregate which is known to affect the cell membrane localization of the cellulose synthase CESA6, indeed the accumulation of cellulose in *rhd3* is reduced (Zheng and Chen, 2011), these issues with cellulose synthesis and accumulation could have an inhibitory effect on cell wall expansion resulting in the short root phenotype. Mutations in RHD3 are also known to alter the lateral root GSA, the *rhd3*-1 mutant has more vertical lateral roots in addition to its short root and root hair phenotypes (Mullen and Hangarter, 2003), however 27.3.3 Short does not have more vertical lateral roots making its phenotype unlikely to be a result of a mutation in *RHD3*. Interestingly 27.3.3 Short also displays a jagged edged leaf phenotype (Figure 5.20), the serration of leaf edges is thought to involve two key processes; the regulation of auxin transport by PIN1 and regulation of the activity of the growth repressor CUP-SHAPED COTYLEDON2 (CUC2) by the microRNA miR164, knockout mutants of both these two genes cannot produce leaf serrations. The formation of alternate foci of auxin and CUC2 expression result in the outgrowths and indentations that form the serrated edge (Bilsborough et al., 2011), it is possible that in 27.3.2 Short differences in auxin production or response that could result in the short root phenotype could also result in the serrated leaf phenotype as production of the regulatory microRNA miR164 that post-transcriptionally represses CUC2 is itself regulated by auxin (Bilsborough et al., 2011).

Another group of interesting non-root angle mutants that were found had increased root waving phenotypes, this group consists of the mutants 68.8.5 Wavy Roots and Shoots, 56.23.5 Wavy and 63.23.5 Wavy (Figure 5.19). That Roots exhibit a waving when grown on a flat surface has been known since Darwin and Von Sachs carried out some of the earliest experiments into plant growth responses. Darwin noted that when grown on a smoked glass plate the tracks created by the roots were alternately deep and smooth leading him to hypothesise that roots grew in a three-dimensional spiral, he named this phenomenon circumnutation, the waving of Arabidopsis roots grown on plates can be seen as a flattening of this spiral (Migliaccio and Piconese, 2001). It is thought that root waving on agar plates can be attributed to a combination of a number of factors, these include gravity, circumnutation and gel-root interactions. Inclination of the plate from the vertical results in an increase in root waving, the greater the inclination the greater the amount of waving, this supports the role of gravitropism in root waving (Simmons et al., 1995b, Thompson and Holbrook, 2004). A number of mutants have been described that display alterations in root waving, many of these are related to alterations in root gravitropic response. Increased root waving and sometimes coiling are associated with a reduction or loss of gravitropic response, for example the *roots of reduced gravitropism1* (*rgr1*) mutant, the *aux1-7* mutant and the starchless or reduced starch *adg* and *pgm* mutants all display increased root waving or coiling, the severity of this waving or coiling is linked to the severity of their gravitropic defects (Simmons et al., 1995a). The *wag1wag2* double mutant also exhibits a strong root waving phenotype, the WAGs are protein kinases involved in directing PIN trafficking through phosphorylation and are likely to be involved gravitropic response (Santner and Watson, 2006, Galván-Ampudia and Offringa, 2007). However, as the single mutants *wag1-1* and *wag2-1* do not exhibit strong root waving phenotypes unless grown on inclined plates (Santner and Watson, 2006) and the likelihood of both genes being mutated by the EMS in the wavy lines found in the screen is low it is unlikely that the cause of their increased root waving is due to mutations in the *WAG* genes. Further tests such as primary root gravitropism kinetics (as described for 80.2.1 MV and 71.14.4. MV) will need to be carried out to determine if gravitropism defects are the cause of the root waving phenotypes seen in these mutants. This group of mutants also display a wavy or coiling shoot phenotype with curled or twisted

siliques and petioles (Figure 5.20), a number of mutants have been found that exhibit twisting of the aerial portions of the plant in addition to root twisting or waving phenotypes, these include the *tortifolia* mutants (Migliaccio and Piconese, 2001, Buschmann et al., 2004, Buschmann et al., 2009), the *tornado* mutants (Cnops et al., 2000) and the *spiral* mutants (Furutani et al., 2000). Both the *tortifolia* and *spiral* mutants have been associated with defects in microtubule organization (*spiral 2* has been found to be allelic to *tortifolia 1*) (Cnops et al., 2000, Buschmann et al., 2004), microtubules have been found to be involved in the orientation of the cellulose microfibrils within the cell wall and are therefore crucial to establishing the polarity of cell elongation, application of the microtubule polymerization interfering drugs propyzamide (promotes depolymerisation) and taxol (stabilizes microtubules) to wild type plants results in helical growth of the same handedness despite their opposing effects however the handedness of the helix is opposite to that induced by the *spr* mutants. A model has been proposed that the pitch of the cortical microtubule arrays in the epidermal cells is kept in balance by the actions of the *spr* genes keeping the root in a neutral state as opposed to a left handed or right handed state, the addition of the microtubule interfering drugs pushes this pathway to a left handed state and acts antagonistically to the actions of the *spr* genes (Furutani et al., 2000). The *tornado* mutants have been found to be defective in cell specification throughout the plant including the root apical meristem and root epidermis. However, it is thought that the twisting phenotype is independent of the cell specification phenotype as double mutants of *trn1-1* with a mutant of the epidermal cell fate specification gene *ttg* lack the twisting phenotype, double mutants of *trn1-1* with AXR2 (IAA7) and AXR3 (IAA17) mutants results in an enhanced twisting (Cnops et al., 2000). This suggests the involvement of auxin in the twisting of the epidermal cells and that increased levels of auxin or auxin response could be used to correct the twisting phenotype of *trn1-1*. It is unlikely that the mutation within any of the wavy mutants found within the screen is a mutant in the *TORNADO* genes as the shoot phenotype displayed by the wavy mutants from the screen is not as severe as that of *trn1-1* (Figure 5.20) and the *tornado* mutants display hairier roots than wild type (Cnops et al., 2000) the roots of the wavy mutants found in the screen do not appear visibly hairier (Figure 5.19). It is also unlikely the wavy mutants found within the screen are defective in the *SPIRAL* or *TORTIFOLIA*

genes as the twisted inflorescence phenotype is only seen in dark grown plants, those grown in the light display wild type-like shoots (Furutani et al., 2000), the shoot phenotypes of the mutants found within the screen were observed in light grown plants (Figure 5.20).

5.4 Materials and Methods

5.4.1 EMS mutagenesis of wild type *Arabidopsis thaliana* ecotype Columbia seeds.

Approximately 20,000 seeds of Wt Col-0 were soaked in 40 ml 100 mM Potassium Phosphate buffer pH7.5 overnight at 4°C. Buffer was decanted and replaced with 40 ml fresh 100 mM Potassium Phosphate buffer containing EMS to a concentration of 25 mM. After incubation for 16 hours at room temperature the EMS containing buffer was decanted and 40 ml 100 mM Sodium Thiosulphate was added to neutralize the EMS. After 15 minutes the Sodium Thiosulphate was decanted and a further 40 ml 100 mM Sodium Thiosulphate. After a further 15 minutes the seeds were washed twice with dH_2O for 15 minutes each. Seeds were dried on filter paper overnight before mixing with sand and sowing into trays containing moist compost. Trays were cold treated at 4°C for two days before being placed at 20°C constant 16 Hr days for the plants to grow to maturity. Plants were separated into 80 pools for seed collection.

5.4.2 Screening of the M_2 population for interesting lateral root angle phenotypes.

An aliquot of seeds from each pool was sterilized using chlorine gas. Seeds were placed onto 120 mm square ATS agar plates in sterile conditions. Each plate contained one line of seeds with one wild type seed and five M_2 mutant seeds. One plate was made for each pool per round of the screen. Plates were cold treated for two days at 4°C before being placed vertically at 20°C constant 16 Hr days for 12 days to grow. Plates were photographed using a Sony Cyber-Shot DSC-RX100 and visually inspected for abnormal root phenotypes, specifically those ones relating to the angle of the lateral roots. Plants with

abnormal root phenotypes were placed into soil and allowed to grow at 20°C constant 16 Hr days to produce seeds.

5.4.3 Analysis of lines at M_3 to determine if phenotype is hereditary

Seeds produced by the M_2 plants selected for abnormal root phenotypes were sterilized using chlorine gas. Seeds were placed onto 120 mm square ATS agar plates in one line containing one wild type seed and five seeds of the mutant line. Two plates were made per mutant line. Plates were cold treated and grown as for the M_2 screen. Plates were photographed and the lateral root angle was measured using ImageJ, six 0.5 mm sections were measured per lateral root starting from where it joins the primary, 10 roots were measured per mutant line to determine whether the theoretical mutant phenotype was genuine and hereditary. For non-angle mutants the plants were visually assessed again in the M_3 generation.

5.4.4 Primary root gravitropism kinetics.

Seeds were placed at 1 mm intervals onto 90 mm round ATS agar plates in two lines, half of each line contained Wt Col-0 seeds, the other half of the line contained seeds of the M_3 mutant line to be tested. Plates were cold treated for 2 days at 4°C before being placed at 20°C constant 16 Hr days for 5 days. Plates were placed in the dark for 1hour before being rotated by 90°, an infrared camera (Canon EOS700D) was set up using the image capture application (Apple) to take a photograph every hour, plants were imaged for 6 hours after reorientation. Tip angles were measured using ImageJ, the data was analysed using Microsoft Excel.

5.4.5 Lateral root gravitropism kinetics

Seeds were placed on 120 mm square ATS plates in one line containing 4 wild type seeds and 4 mutant M_3 seeds. Plates were cold treated for 2 days at 4°C before being placed at 20°C constant 16 Hr days for 9 days. Plates were placed in the dark for 1 hour before being rotated by 45°, an infrared camera was used as for the primary root kinetics to take a photograph every 1-hour, plants were

imaged for 6 hours after reorientation. Tip angles were measured using ImageJ, the data was analysed using Microsoft Excel.

5.4.6 Auxin treatment

Seeds were placed on 120 mm square ATS plates in one line around 10 mm apart. Plates were cold treated for 2 days at 4°C before being placed at 20°C constant 16 Hr days for 5 days. Plants were then transferred to plates containing 50 nM IAA or a mock treatment, two mock plates and two IAA containing plates were produced for each line, five plants were transferred onto each plate. Lateral root angles were measured as for the phenotypic analysis of the M_3 lines.

5.4.7 Clinorotation of Arabidopsis roots

Seeds were placed on 120 mm square ATS plates in one line containing four wild type seeds and four seeds of the M_3 line to be tested. Plates were cold treated at 4°C for 2 days before being placed at 20°C constant 16Hr days for 9 days. Plates were photographed and wrapped in aluminium foil to exclude light, after one hour plates were placed on a clinostat set at 1 revolution per minute (RPM) for 6 hours. Plates were then photographed for a second time.

5.4.8 Shoot branch angle phenotyping

Seeds were placed onto moistened compost in 24 cell seed trays, the trays were cold treated at 4°C for two days in darkness before being placed at 20°C constant 16 Hr days for growth. Plants were photographed when at least two cauline branches were over 3 cm long (at around 4 weeks old). Branch angles were measured using ImageJ, starting from where the branch joins the main stem six 0.5 cm sections were drawn and the angle of each section with respect to gravity measured. Ten branches were measured per genotype and the average angle of each section calculated. Data was analysed using Microsoft Excel.

5.4.9 Preparation of 80.2.1 MV for bulk segregant analysis

80.2.1 MV was back-crossed to Wt Col-0 using 80.2.1 MV as the pollen parent and Col-0 as the seed parent. The resulting F_1 seeds were sterilized using chlorine gas and sown onto 120 mm square ATS plates in one line containing one seed of Wt Col-0 and five F_1 seeds. Plates were cold treated for 2 days at 4°C and placed at 20°C constant 16 Hr days for 11 days before photographing and transferring to soil. Plants were then grown at 20°C constant 16 Hr days until they produced seed, seeds produced from all the F_1 plants were pooled into one for the F_2. F_2 seeds were sown onto 120 mm square ATS agar plates, cold treated and grown as for the F_1 seeds, plants were separated into two pools, those that exhibited the mutant phenotype and those that did not. Plants were transferred into soil and grown as for the F_1 until the leaf rosette was mature, a piece of tissue was collected from each plant using a hole punch, tissue from all the plants showing no phenotype was pooled, tissue from all the plants exhibiting the phenotype was collected separately for each plant, all collected tissue was flash frozen in liquid nitrogen. The plants exhibiting the phenotype were then allowed to produce seed. F_3 seeds from each individual F_2 plant that exhibited the phenotype were sown onto 120 mm square ATS agar plate and grown as for the F_1. F_2 tissue from all the plants that were shown to be homozygous for the mutant phenotype by lack of segregation in the F_3 was then pooled. The two pools of tissue were ground into a fine powder using liquid nitrogen and Genomic DNA from the pool of plants with no phenotype and the pool of plants carrying the phenotype was then extracted using the QIAGEN Plant Maxi-Kit as per the manufacturer's instructions, DNA was eluted in 750 µl buffer AE.

5.4.10 Whole genome sequencing of 80.2.1 MV and determination of the causal mutation

The genomic DNA from the Phenotype pool and the no phenotype pool was sequenced using Illumina NextSeq sequencing at the Next Generation Sequencing Facility at St James Hospital, Leeds. Sequences were assembled, mapped to TAIR 10 using BWA-MEM and SNPs were called using GATK as a haplotype caller followed by variant filtration to filter out any SNPs that do not pass GATKs standard variant filtration thresholds. BCFtools was used to look

for overlap in the SNPs and find those that appeared in the Phenotype sequence but not the No Phenotype sequence followed by a variant effect predictor to assess the likely effect of each mutation. Using these predictions the SNPs were filtered out until only those that would cause a missense mutation, 3'UTR variant, promoter variant, 5'UTR variant and a gain of a stop codon. This produced a list of 25 potential candidates, using the IGV genome browser these candidates were assessed visually to choose those with obvious clear changes across multiple reads. Using this information along with phenotypic information on mutants of these genes the most likely candidate was chosen.

5.4.11 Genomic DNA extraction from Arabidopsis

One leaf was placed into a clean 1.5 ml microcentrifuge tube and flash frozen in liquid nitrogen. Tissue was macerated until it became a fine powder before resuspension in 400 µl of extraction buffer (200 nM Tris-HCL pH7.2, 250 mM NaCl, 25 mM EDTA, 0.5% SDS). 400 µl Phenolchloroform Isoamyl alcohol (25:24:1 pH6.7-8.0) was added and the mixture vortexed before centrifugation at 13,000 RPM for 4 minutes. 300 µl of the top layer of the supernatant was transferred to a clean 1.5 ml microcentrifuge tube; 300 µl of isopropanol was added to precipitate the DNA before centrifugation at 13,000 RPM for 5 minutes. The supernatant was removed and the pellet washed with 70% ethanol before resuspension in 20 µl dH_2O.

5.4.12 RNA extraction and cDNA synthesis from Arabidopsis

Plants of both Wt Col-0 and 80.2.1 MV M_3 were grown on 120 mm square ATS agar plates for 11 days as for the F_1 of the 80.2.1 MV X Wt Col-0 backcross. Whole plants were placed into individual 1.5 ml microcentrifuge tubes and flash frozen in liquid nitrogen. Plants were ground into a fine powder using plastic disposable pestles and the RNA was extracted as per the manufacturer's instructions using the E.Z.N.A Plant RNA Kit (Omega Bio-tek), on column DNase I digestion was carried out on the HiBind RNA Mini Column using the DNase I digestion set (Omega Bio-tek) as per the manufacturer's instructions and the RNA was eluted into 20 µl DEPC water. cDNA was synthesized from

both a Wt Col-0 RNA sample and an 80.2.1 MV RNA sample using the Superscript II Reverse Transcriptase (Invitrogen) and Oligo dT_{12-18} (500 µg/ml) (Invitrogen) as per the manufacturer's instructions, 200ng RNA was used in the initial reaction. RNA was stored at -80°C, cDNA was stored at -20°C.

5.4.13 PCR amplification of *pLAZY4* from Arabidopsis genomic DNA and *LAZY4* from Arabidopsis cDNA

The promoter of *LAZY4* was amplified from Wt Col-0 genomic DNA using Phusion High-Fidelity DNA Polymerase (New England Biolabs) in the following reactions: 5 µl Phusion HF Buffer (10x), 0.5 µl 10 mM dNTPs, 1.25 µl *pLAZY4* ForP1 (10 µM), 1.25 µl *pLAZY4* RevP5r (10 µM), 0.25 µl of a 1:3 dilution of the Wt Col-0 genomic DNA, 0.25 µl Phusion DNA Polymerase, either 1,2,3, or 4 µl 50 mM $MgCl_2$ and 15.5,14.5,13.5 and 12.5 µl of sterile dH_2O respectively to give a total volume of 25 µl per reaction. Cycling conditions can be found in Table 5.3, 5 µl of each reaction was run on a 1% agarose gel containing Gel Red Nucleic Acid Stain (Biotium) to a concentration of 1x for 35 minutes at 85 V. All reactions were successful and the remaining 20 µl of each reaction were pooled. 90 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) was added to 30 µl of the pooled reactions, to this 60 µl 30% PEG8000/30 mM $MgCl_2$ was added. This was mixed using a vortex before centrifugation at 13000 RPM for 15 minutes, the supernatant was removed and the pellet resuspended in 20 µl TE buffer.

LAZY 4 was cloned from both the Wt Col-0 cDNA and the 80.2.1 MV cDNA in the following reaction: 20 µl Phusion HF Buffer (10x), 2 µl 10 mM dNTPs, 5 µl B5r *LAZY4* F (10 µM), 5 µl P2 *LAZY4* R (10 µM), 1 µl Phusion DNA Polymerase, 2 µl cDNA and 65 µl sterile dH_2O to give a total volume of 100 µl per reaction. Cycling conditions can be found in Table 5.3. The total 100 µl of each reaction was run on a 1% agarose gel containing Gel Red Nucleic Acid Stain to a concentration of 1x for 35 minutes at 85 V and the PCR product was extracted from the gel using the QIAEX II Gel Extraction Kit (QIAGEN) following the manufacturer's instructions for agarose gel extraction, DNA was eluted in 20 µl TE Buffer for use in the BP reaction.

All primers used can be found in Table 5.4

Table 5.3 : Cycling conditions for amplification of *pLAZY4* and *LAZY4*

		pLAZY4		***LAZY4***	
Step	**Number of Cycles**	**Temperature (°C)**	**Time**	**Temperature (°C)**	**Time**
Initial Denaturation	1	98	2 Minutes	98	30 Seconds
Denaturation	32	98	30 Seconds	98	10 Seconds
Annealing		61	20 Seconds	54	20 Seconds
Extension		72	3 Minutes	72	1 Minute 20 Seconds
Final Extension	1	72	10 Minutes	72	10 Minutes

Table 5.4: Primers used for cloning and mutagenesis of *LAZY4*		
Used for	**Primer Name**	**Primer Sequence (5'-3')**
Amplification *pLAZY4*	*pLAZY4* For P1	GGGGACAAGTTTGTACAAAAAAGCAGGCTG GACTAGACAATCGACCCAC
	pLAZY4 Rev P5	GGGGACAACTTTTGTATACAAAGTTGTGTC TTAGTGACCCGGAAGAAG
Amplification of *LAZY4*	B5r *LAZY4* F	GGGGACAACTTTGTATACAAAAGTTGTAAT GAAGTTTTTCGGGTGGATGCAG
	p2 *LAZY4* R	TCAGATCTCAAGAACAATGAAATCAGAATCT GGGGGACCACTTTGTACAAGAAAGCTGGG TA
Site Directed Mutagenesis of *LAZY4*	*LAZY4* R145K F	AAGAATCAGTAACGCGCTTTGTGATGAGAA G
	LAZY4 R145K R	TTATCGACCTCAAGACTCGAAGGACAATTC AAG
	LAZY4 R145A R	GCATCGACCTCAAGACTCGAAGGACAATTC AAG
	LAZY4 R145E R	TCATCGACCTCAAGACTCGAAGGACAATTC

5.4.14 BP and LR reactions to produce the *pLZY4::LZY4* construct

The BP reactions for both *pLAZY4* and *LAZY4* were set up as follows: 7 µl PCR product (in TE buffer), 1 µl donor vector 150 ng/µl (pDONR221 p1p5r for *pLAZY4* and pDONR221 p5p2 for *LAZY4*) and 2 µl BP Clonase II Enzyme Mix (Invitrogen). This reaction was placed in a heating block at 25°C for 24 hours, 1 µl Proteinase K (Invitrogen) was added and the reaction incubated at 37°C for 10 minutes. A 30 µl aliquot of DH5α- Select Silver efficiency competent *E.coli*

cells (Bioline) was thawed on ice, 5 µl of the BP reaction was added to this and was kept on ice for 30 minutes. The cells were heat shocked at 42°C for 45 seconds before being placed back on ice for a further 2 minutes. 750 µl LB broth was added and the cells incubated at 37°C 150 RPM for 1 hour before spreading onto LB agar plates containing 40 µg/ml Kanamycin, two plates were spread for each BP reaction, one plate with 100 µl of the transformation and a second with the remainder of the transformation. The plates were placed at 37°C overnight before colonies were picked into 5 ml LB broth containing 40 µg/ml Kanamycin, these cultures were placed at 37°C 150 RPM overnight. Cultures were centrifuged at 3500 RPM for 10 minutes, DNA was extracted using the QIAprep Spin Miniprep Kit (QIAGEN) according to the manufacturer's instructions, DNA was eluted in 30 µl sterile dH_2O.

A restriction enzyme digest was set up for both *pLAZY4* and *LAZY4* using the following reaction: 2.5 µl plasmid DNA, 1 µl 10x Cutsmart Buffer (New England Biolabs), 0.5 µl EcoRV (New England Biolabs), 5.5 µl sterile dH_2O and 0.5 µl XhoI (New England Biolabs) or HincII (New England Biolabs) (for *pLAZY4* and *LAZY4* respectively) per 10 µl reaction. Restriction digests were placed at 37°C for 1 hour before being run on a 1% agarose gel containing Gel Red Nucleic Acid Stain (Biotium) to a concentration of 1x for 35 minutes at 85 V. Those showing correct band sizes were sent off for sequencing using the M13 forward and reverse primers.

An LR reaction was set up using those plasmids that the sequencing showed to be correct, the reaction was set up as follows: *pLAZY4* 10 fmoles, *LAZY4* 10 fmoles, pALLIGATOR V 20 fmoles made up to 8 µl using TE buffer, 2 µl LR Clonase Plus enzyme mix (Invitrogen) was added and the reaction placed at 25°C for 24 hours. 2 µl Proteinase K 2 µg/µl solution was added and the reaction placed at 37°C for 10 minutes before transformation into DH5α- Select Silver efficiency competent *E.coli* cells (Bioline) as above. Transformations were spread onto LB agar plates containing 70 µg/ml Spectinomycin and incubated at 37°C overnight. Colonies were picked into 5 ml cultures of LB broth containing 70 µg/ml Spectinomycin and placed at 37°C 150 RPM overnight. Cultures were centrifuged and plasmid DNA extracted as described above.

A restriction digest was set up for each prep as follows: 1 µl plasmid DNA, 1 µl NEB Buffer 2 (10x), 0.5 µl KpnI (New England Biolabs), 0.5 µl PmeI (New England Biolabs), 1 µl Bovine Serum Albumin (10x) (New England Biolabs), 6 µl sterile dH_2O per 10 µl reaction. Restriction digests were placed at 37°C for 1 hour before being run on a 1% agarose gel containing Gel Red Nucleic Acid Stain (Biotium) to a concentration of 1x for 35 minutes at 85 V. Those showing correct band sizes were sent off for sequencing using the following primers: *pLAZY4* FOR 5'- GGACTAGACAATCGACCCAC-3' and *LAZY4* REV 5'- TCAGATTTCAAGAACAATG-3'. A construct containing *LAZY4* from Wt Col-0 for which the sequence came back as correct was taken forwards for use in the site directed mutagenesis.

5.4.15 Site directed mutagenesis of the *pLZY4::LZY4* construct

Site directed mutagenesis was carried out on the pLZY4::LZY4 construct containing *LAZY4* from Wt Col-0 using the Q5 Site Directed Mutagenesis Kit (New England Biolabs). For each amino acid change to be made the following reaction was set up: 12.5 µl Q5 Hot Start High-Fidelity 2X Master Mix, 1.25 µl 10 µM *LAZY4* R145K F primer, 1.25 µl 10 µM reverse primer containing the correct base changes (see Table 5.4 for primer sequences), 1 µl *pLZY4::LZY4* (25 ng/µl) and 9 µl sterile dH_2O per reaction. Reactions were placed in a PCR machine, for cycling conditions see Table 5.5. A KLD reaction for each amino acid change was set up as follows: 4 µl PCR product, 5 µl 2X KLD buffer and 1 µl KLD enzyme mix, this was incubated at room temperature for 5 minutes. A tube of NEB 5-α competent *E.coli* cells was thawed on ice per amino acid change, 5 µl of the respective KLD reactions was added to each tube of competent cells. The cells were incubated on ice for 30 minutes before heat shock at 42°C for 30 seconds, the cells were then placed back on ice for a further 5 minutes. 750 µl LB broth was added and the cells were placed at 37°C 150 RPM for 1 hour before spreading onto LB agar plates containing 70 µg/ml Spectinomycin, a 100 µl plate and a plate of the remainder of the transformation was spread for each transformation. Plates were incubated at 37°C overnight, colonies were then picked into 5 ml culture of LB broth containing 70 µg/ml Spectinomycin and placed at 37°C 150 RPM for growth overnight. Plasmid DNA

was extracted as described above and sent for sequencing to confirm the correct mutations had been made.

Table 5.5: Cycling conditions for site directed mutagenesis

Step	Number of Cycles	Temperature (°C)	Time
Initial Denaturation	1	98	30 Seconds
Denaturation	25	98	10 Seconds
Annealing		59	20 Seconds
Extension		72	11 Minutes 30 Seconds
Final Extension	1	72	10 Minutes

5.4.16 Preparation of agrobacterium competent cells

A glycerol stock of the Agrobacterium strain “GV3101” was streaked onto LB agar plates containing 100 µg/ml Rifampicin and 25 µg/ml Gentamycin and placed at 28°C for 2 days. A colony was picked into 5 ml LB broth and allowed to grow at 28°C 250 RPM overnight. A 250 ml flask containing 50 ml LB broth was inoculated with 2 ml of the 5 ml overnight agrobacterium culture and placed at 28°C 250 RPM until the optical density reached between 0.5 and 1. The 50 ml culture was placed into a 50 ml falcon tube and centrifuged at 5000 RPM for 20 minutes at 4°C. The supernatant was discarded and the cells resuspended in 1 ml 20 mM $CaCl_2$, this was divided into 100 µl aliquots and flash frozen using liquid nitrogen. Competent agrobacterium cells were stored at -80°C until required for use.

5.4.17 Transformation of agrobacterium

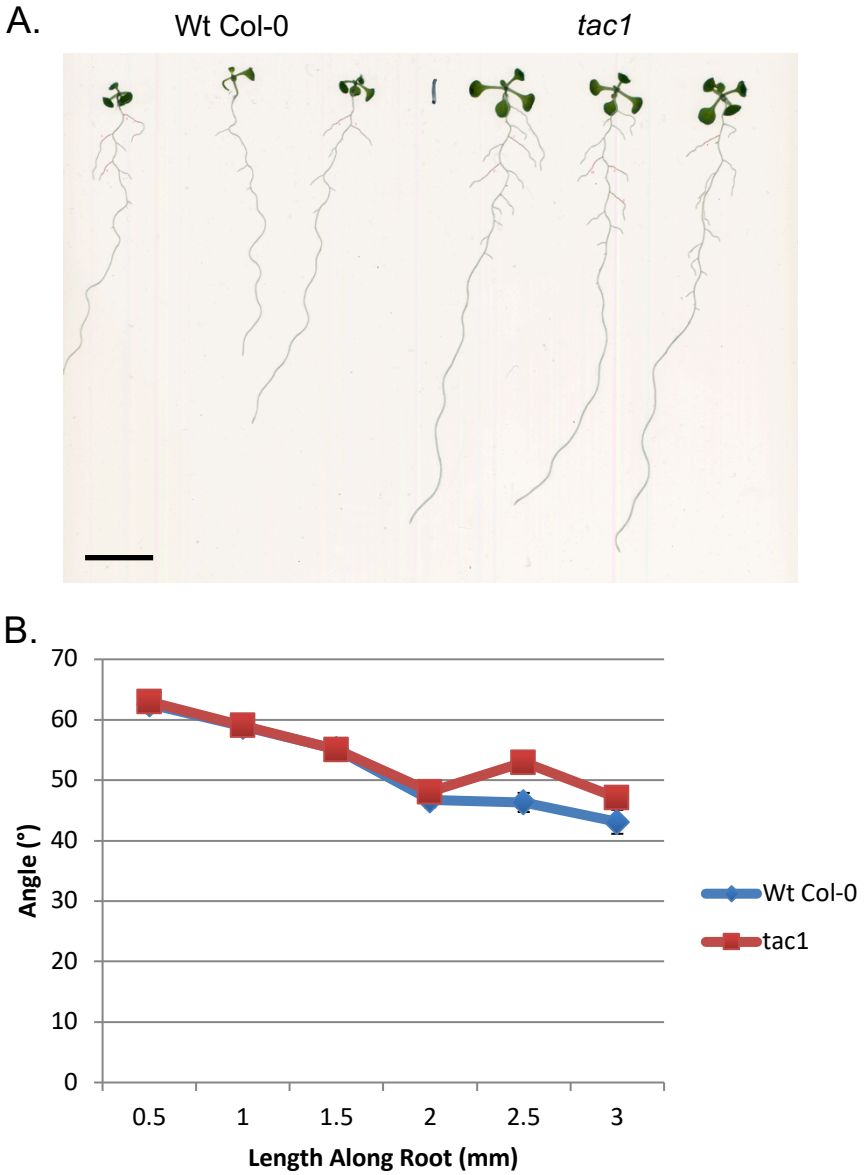
1 µg of plasmid DNA was added to a 100 µl aliquot of agrobacterium competent cells whilst the cells were still frozen. Cells were thawed in a 37°C water bath before the addition of 750 µl LB broth. The cells were placed at 28°C 140 RPM

for 4 hours before spreading onto LB plates containing 100 µg/ml Rifampicin, 25 µg/ml Gentamycin and 70 µg/ml Spectinomycin. Plates were placed at 28°C for 2 days to allow colonies to grow.

5.4.18 Transformation of Arabidopsis using agrobacterium

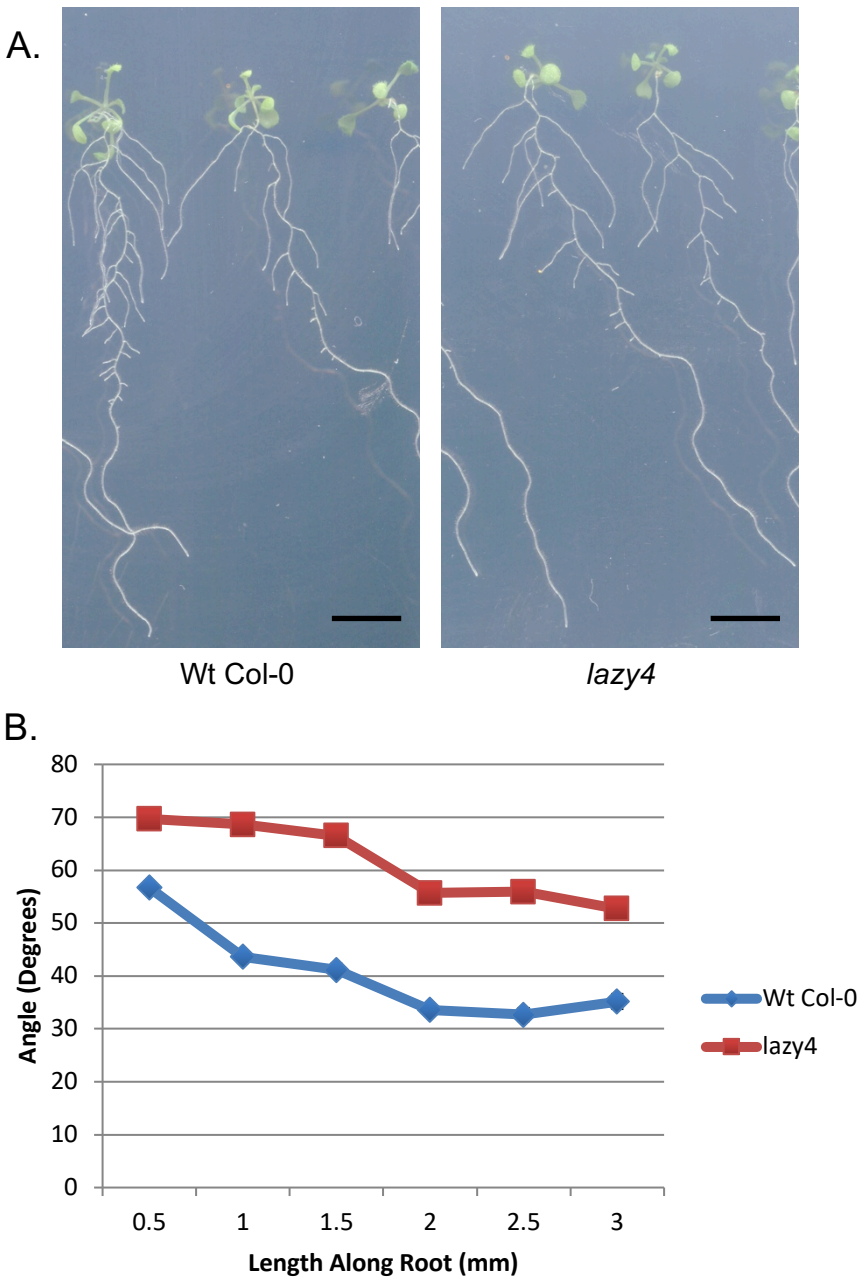
An overnight culture of agrobacterium was set up in a 50 ml falcon tube containing 5 ml LB broth with 100 µg/ml Rifampicin, 25 µg/ml Gentamycin and 70 µg/ml Spectinomycin, this was placed at 28°C 140 RPM to grow. A 2 L conical flask containing 500 ml LB broth was inoculated with 2 ml of the 5 ml overnight culture and placed at 28°C 140 RPM overnight. The 500 ml culture was centrifuged at 6000 g for 12 minutes the supernatant discarded and the cells resuspended in 250 ml 5% sucrose, 20 mM $MgCl_2$. This was poured into a 240 mm petri plate and 25 µl Silwet L-77 was added. Flowering plants of Arabidopsis were dipped into the culture and allowed to sit in it for 5 minutes. After dipping, plants were placed on a tray inside a large, inverted, clear plastic bag with the corners removed to maintain humidity. After 24 hours the entire top of the bag was cut open, the bag was fully removed after 5 days and the plants were allowed to grow until seed was produced. Transformed seed was selected using a GFP microscope to look for seed coat fluorescence, fifteen T_1 seeds were planted for each line and grown and analysed as for the M_3 lateral root phenotyping. After scanning the T_1 plants were transferred into soil and allowed to grow until seed was produced.

5.5 Supplementary figures



Supplementary Figure 5.1: The lateral root angles of *tac1* are the same as Wt Col-0

The lateral root angles of the *tac1* knockout mutant are similar to those of Wt Col-0 (**A**), there is no significant difference (P>0.05, Students T-test) between *tac1* and Wt Col-0 at any point in the first 3 mm of root (n=10) (**B**). Error bars represent SEM, scale bar represents 1 cm.



Supplementary Figure 5.2: The lateral roots of *lazy4* are less vertical than Wt Col-0

The lateral root angles of the *lazy4* knockout mutant are less vertical than those of Wt Col-0 (**A**), there is a significant difference ($P<0.05$, Students T-test) between *lazy4* and Wt Col-0 at all points in the first 3 mm of root (n=10) (**B**). Error bars represent SEM, scale bars represent 1 cm.

Chapter 6 : The Curious Case of Non-Vertical Primaries

6.1 Introduction

In the majority of plants the primary root maintains a GSA that is approximately vertical (0°), although there are some exceptions such as wheat (see chapter 3). The model plant *Arabidopsis thaliana* is an annual weed native to Europe and central Asia and has since become naturalized worldwide, it is tolerant of a wide range of habitats from riverbanks to rocky slopes (Al-Shahbaz and O'Kane, 2002). There are over 750 naturally occurring accessions (ecotypes) that have been collected worldwide and these exhibit a large variation in form, development and physiology (The Arabidopsis Information Resource, 2018), popular laboratory accessions include Columbia (Col-0) and Landsberg (La-0). The first plant reference genome sequence was produced for the ecotype Col-0 (Initiative, 2000) and since then the 1001 genomes project has aimed to sequence many different accessions of Arabidopsis to explore the genetic variation in the species (Weigel and Mott, 2009).

Much of the work on gravitropism in Arabidopsis thus far has focused on the vertically growing primary root however recently some of the focus has shifted to the stably non-vertically growing lateral roots. These non-vertically growing laterals have been shown to maintain GSAs, i.e. the angle that they form with respect to the gravity vector is maintained and if displaced from that angle growth will be induced to return them to that angle, GSAs also occur in the lateral shoots of Arabidopsis. The maintenance of GSAs has been shown to be controlled by auxin, an increase in auxin response or the application of exogenous auxin has been show to result in a more vertical GSA being adopted in lateral roots (Roychoudhry et al., 2013).

The Arabidopsis ecotype Cape Verde Islands (Cvi-0) has been shown to exhibit a "skewed phenotype" in the primary root when grown on solid agar plates, this skew exhibits a handedness and all roots skew to the right hand side when

viewed from the back of the plate (through the agar) (Patel, 2009). This skew is between 45° and 90° from the vertical and begins to develop 6 to 10 days after germination (Patel, 2009, Hijazi, 2013). It has been postulated that the skewed phenotype of the primary root of Cvi-0 is a GSA, previous work has shown that when reoriented or straightened the Cvi-0 primary root will re-establish a non-vertically growing skewed primary root in the same direction as its previous skew (Patel, 2009, Hijazi, 2013). However, further work will be needed to determine if this angle is maintained and controlled in the same way as GSA maintenance in the lateral roots as outlined by Roychoudhry et al (Roychoudhry et al., 2013).

Arabidopsis roots exhibit a waving when grown on inclined agar plates, this has been attributed to a number of factors acting on the plants including gravity, circumnutation and gel-root interactions (Thompson and Holbrook, 2004). Circumnutation is an oscillating movement carried out by plant organs as they grow, the term circumnutation was originally introduced by Darwin as a replacement for Von Sachs earlier term “rotating nutation” (Migliaccio and Piconese, 2001), one of Darwin’s experiments bears a striking similarity to many of the experiments carried out to investigate circumnutation today. He grew plants on a smoked glass plate and noted that the tracks the roots created in the smoke were alternately deep and smooth leading him to the conclusion that the roots grew in a three dimensional spiral, these observations can be linked to the waving pattern observed in Arabidopsis roots grown on an agar plate as a flattening of this spiral (Migliaccio and Piconese, 2001).

The growth movement of the roots can be described as a basic right-handed helix when viewed looking towards the growing tip, although mutants have been described with a left-handed helix (Thompson and Holbrook, 2004, Marinelli et al., 1997). This right handed helical growth can lead to a general skewing to the right on the plate as the roots grow (Thompson and Holbrook, 2004). The roots of plants grown at different inclination pitches exhibit different degrees of waving with greater waving seen at pitches closer to the horizontal, and relatively straight roots observed on Arabidopsis ecotypes such as Col-0 and Ws on plates grown vertically, the growth skew is in addition to the different

magnitudes of waving seen at different pitches of growth surface (Thompson and Holbrook, 2004, Simmons et al., 1995b). This difference in the degree of waving at different inclinations suggests an involvement of gravitropism in root waving, Thompson and Holbrook theorise that root waving is a combination of gravitropism and root impedance against the gel surface and that friction between the root tip and the gel surface impedes the normal movement of the root this, in addition to the growth pressures leads to a bend (Thompson and Holbrook, 2004). At different inclinations the friction is enhanced by gravitropism encouraging the tip to grow against the surface of the gel increasing the magnitude of the bend (Thompson and Holbrook, 2004), it has also been found that increasing the agar concentration results in an increase in root waving further supporting the idea that root waving is a result of root-gel interactions (Santner and Watson, 2006). This link to gravitropism is also supported by the phenotype of the *wag* mutants, the *wag1wag2* double mutant exhibits a strong root waving phenotype on plates grown vertically much like that seen with wild type plants grown on inclined plates. The WAGs are protein kinases closely related to PINOID which is known to be involved in gravitropism due to its role in PIN phosphorylation and trafficking and are likely to also be involved in the gravitropic response, although when grown inside the gel the *wag* mutants display no gravitropic defects (Santner and Watson, 2006, Galván-Ampudia and Offringa, 2007).

Different Arabidopsis accessions are known to exhibit different levels of root skewing when grown on hard agar plates (Vaughn and Masson, 2011). Mutant lines with an enhanced skew often show increased levels of cell file rotations. The handedness of the skew is linked with the direction of the cell file rotations, a left handed cell file rotation is associated with rightward skewing and a right handed cell file rotation is associated with leftward skewing (Vaughn and Masson, 2011). However, differences in cell file rotation to the wild type cannot account for all skew mutant phenotypes, for example the *spr2* mutant has a strong helical growth pattern but its cell file rotation is like that of the wild type (Furutani et al., 2000, Vaughn and Masson, 2011). Many skewing mutants display abnormalities in the organization and polymerization of the cytoskeleton, indeed the phenotype of the *spr1* and *spr2* mutants which results in a reversal

of the direction of the growth helix can be rescued to the wild type direction of the helix in a dose dependent manner by application of the microtubule interaction drugs propyzamide or taxol (Furutani et al., 2000, Vaughn and Masson, 2011). A QTL for root skewing between the accessions Cvi-0 and Ler has been mapped to a region on chromosome 2, though other regions showed potential to be QTLs but the phenotype was not as strong, within this region lines containing different pieces of the Cvi-0 genome introgressed exhibit skewing to different degrees suggesting an additive effect of multiple loci in this region results in some of the skew seen in the Cvi-0 primary root (Vaughn and Masson, 2011).

The primary roots of domesticated tomatoes such as *Solanum lycopersicum* "M82" display a vertically growing primary root (similar to most Arabidopsis ecotypes such as Col-0) however the wild species *Solanum pennellii* exhibits a skewed primary root angle like that of Cvi-0 (Ron et al., 2013). The wild tomato species (comprising section *Lycopersicon* of the genus *Solanum*) such as *Solanum pennellii* grow in a wide variety of habitats in South America ranging from moist Andean uplands to the dry Atacama Desert. *Solanum pennellii* is native to dry environments and as such has been of interest for use in breeding cultivated tomatoes with drought and salt tolerance (Bolger et al., 2014, Easlon and Richards, 2009). It is possible that the shorter and non-vertically growing primary root could be adaptations to the thin and rocky topsoils of its natural habitat on the dry coastal regions of Peru (Ron et al., 2013). It has already been shown that when reoriented the non-vertically growing primary roots of *S. pennellii* return to within 10° of their original angle when reoriented suggesting that they are maintaining a GSA (Ron et al., 2013) but it has yet to be shown if the maintenance of this angle in the *S. pennellii* primary is similar to the maintenance of non-vertical GSAs in Arabidopsis lateral roots and if the lateral roots of tomatoes also adhere to what has been discovered in Arabidopsis.

It is known that auxin controls the GSA of lateral roots in Arabidopsis, it is predicted that it does this by specifying the magnitude of the antigravitropic offset and that mutations in genes relating to auxin response and biosynthesis alter the GSA. For example the *yucca-1D* mutant that has higher levels of auxin

has more vertical lateral roots whereas the lateral roots of the *tir1-1* mutant that has lost some of its ability to respond to auxin are less vertical. The application of exogenous auxins such as IAA and 2,4-D through culture on media containing these auxins results in more vertical lateral root angles (Roychoudhry et al., 2013). The synthetic anti-auxin PEO-IAA is reported to competitively bind into the auxin binding pocket of TIR1 (Hayashi et al., 2012), it would be expected that application of this to Arabidopsis roots would result in the laterals assuming a less vertical GSA much like that of the *tir1-1* mutant.

The aim of this work is to clarify the non-vertical growth phenotype of the primary roots of Cvi-0 and *S. pennellii* and establish the extent to which the mechanisms show features that are common with lateral roots.

6.2 Results

6.2.1 2,4-D corrects the skew of Cvi-0 primary roots but IAA does not

In his thesis, Dr D.V. Patel (Patel, 2009) describes a correction of the skew phenotype of Cvi-0 by the synthetic auxin 2,4-D but not the natural auxin IAA, it was first decided to confirm this. It was found that all concentrations of 2,4-D used (10, 50 and 100 nM) did indeed correct the primary root skew of Cvi-0 under our conditions (10 nM shown in Figure 6.1 B) in concurrence with the findings in Patel's thesis (Patel, 2009) and at higher concentrations than used in the previous work. IAA did not correct the root skew of the Cvi-0 primary from 10 nM (Figure 6.1 C) to higher concentrations than those used in the original experiment (50 nM and 100 nM, Figure 6.2), this suggests there is no threshold concentration of IAA that is able to correct the skew.

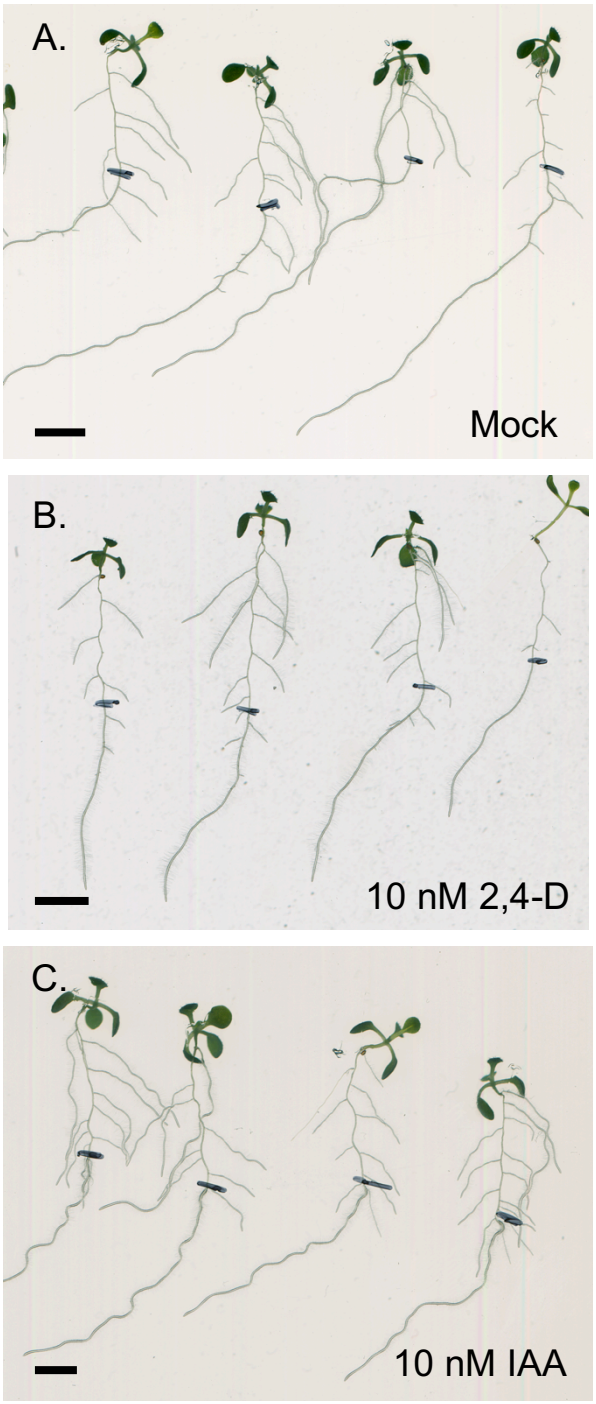


Figure 6.1: 2,4-D corrects the skew of Cvi-0 primary roots whereas IAA does not.

When transferred onto plates containing a mock treatment the primary roots of Cvi-0 reassume their skewed phenotype (**A**). The application of the synthetic auxin 2,4-D corrects the skewing phenotype of the Cvi-0 primary root causing the root tip to re-assume a vertical orientation (**B**). The application of exogenous IAA to the roots of Cvi-0 does not correct the skewing phenotype (**C**). Plants were transferred to hormone containing media after 5 days of growth on ATS agar, plates were scanned after a further 6 days. Scale bars represent 1 cm.

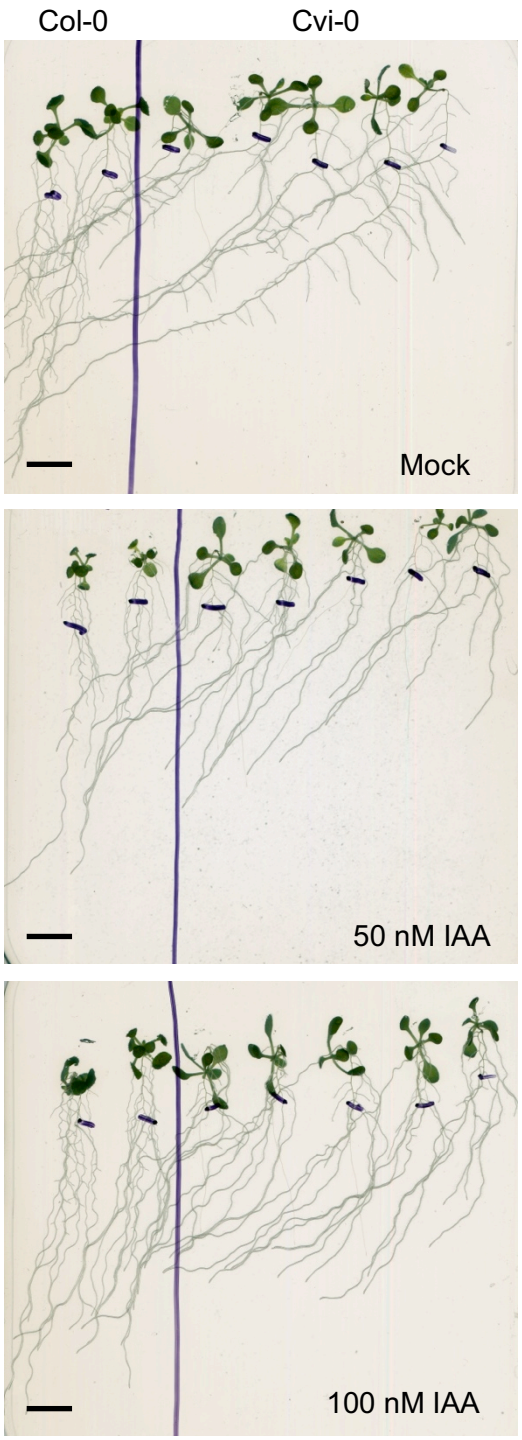


Figure 6.2: High concentrations of IAA do not correct the primary root skew of Cvi-0

The primary root skew phenotype of Cvi-0 is not corrected using higher concentrations of IAA such as 50 nM and 100 nM . Scale bars represent 1 cm.

6.2.2 The application of both IAA and 2,4-D makes the lateral roots of Cvi-0 more vertical

In contrast to their effects on the primary root skew phenotype, IAA and 2,4-D have similar effects on the lateral root GSA phenotype of Cvi-0. The lateral roots become significantly more vertical (Figure 6.3) when treated with both hormones. This effect increases with increasing concentrations of 2,4-D (Figure 6.3 A), on average 10 nM and 50 nM result in an 11° decrease in root angle and 100 nM results in a 22° decrease. In contrast, on average, all concentrations of IAA result in a decrease in root angle of around 16° (Figure 6.3 B), this suggests a difference in the ability to respond or transport IAA and 2,4-D.

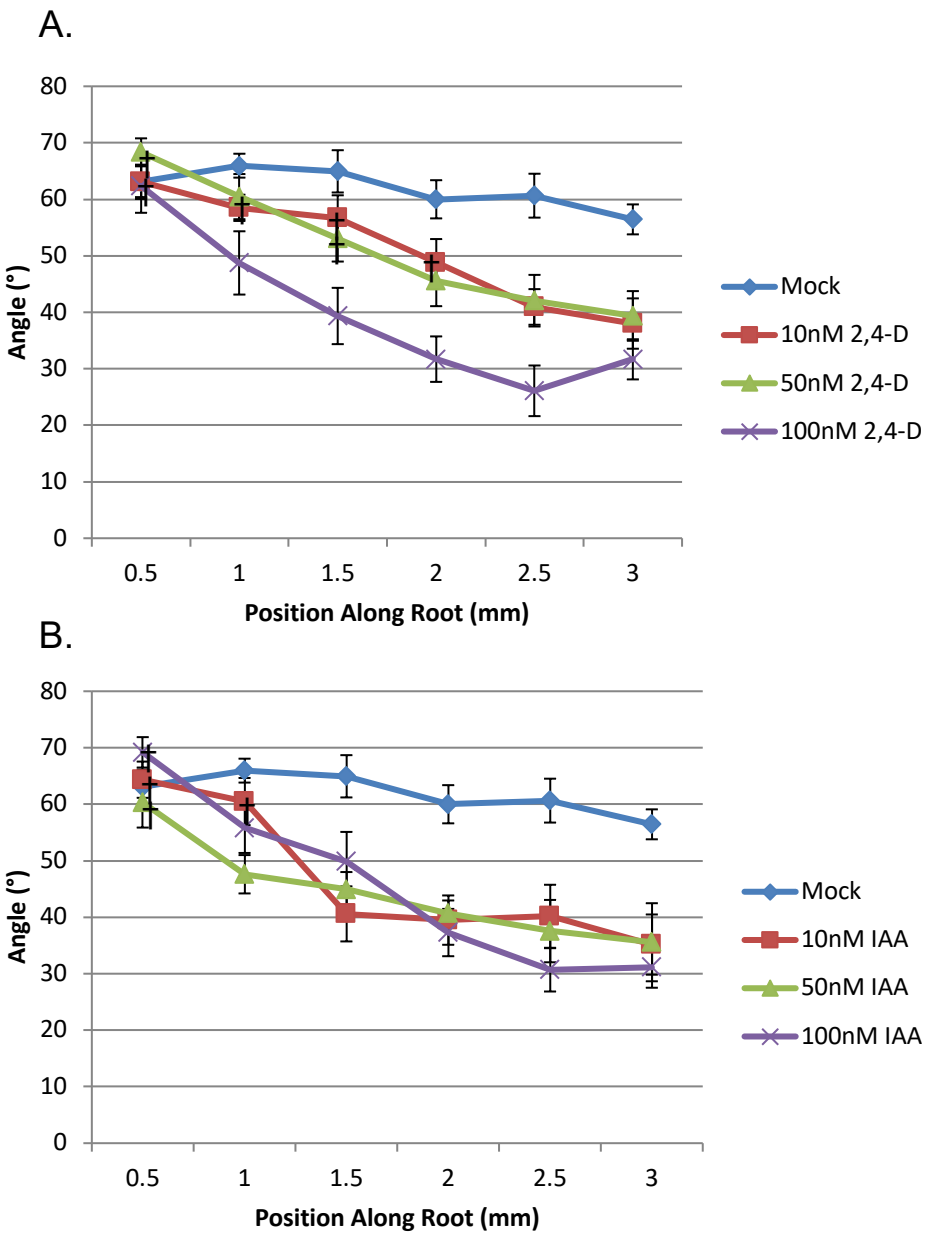


Figure 6.3: The application of both IAA and 2,4-D makes the lateral roots of Cvi-0 more vertical

5 day old plants of Cvi-0 were transferred to ATS agar containing IAA and 2,4-D of differing concentrations. The lateral roots became more vertical on both IAA and 2,4-D, the greater the concentration of hormone the more vertical the roots become. $P<0.05$ at all points when compared with mock treated except those marked with a †, n= 10 roots per treatment. Error bars represent SEM.

6.2.3 The lateral roots of Cvi-0 are less vertical than those of Col-0

It is well documented that there are a number of consistent phenotypic differences between Arabidopsis ecotypes (The Arabidopsis Information Resource, 2018). To investigate the differences in GSA and its control between Cvi-0 and Col-0 (the accession upon which a large amount of previous work has been carried out (Roychoudhry et al., 2013)) first the differences in their lateral root GSA phenotype was examined. The lateral roots of Cvi-0 are less vertical than those of Col-0 (Figure 6.4 C), this could be indicative of a difference in the gravitropic capacity, a change in the magnitude of AGO between the two ecotypes or both.

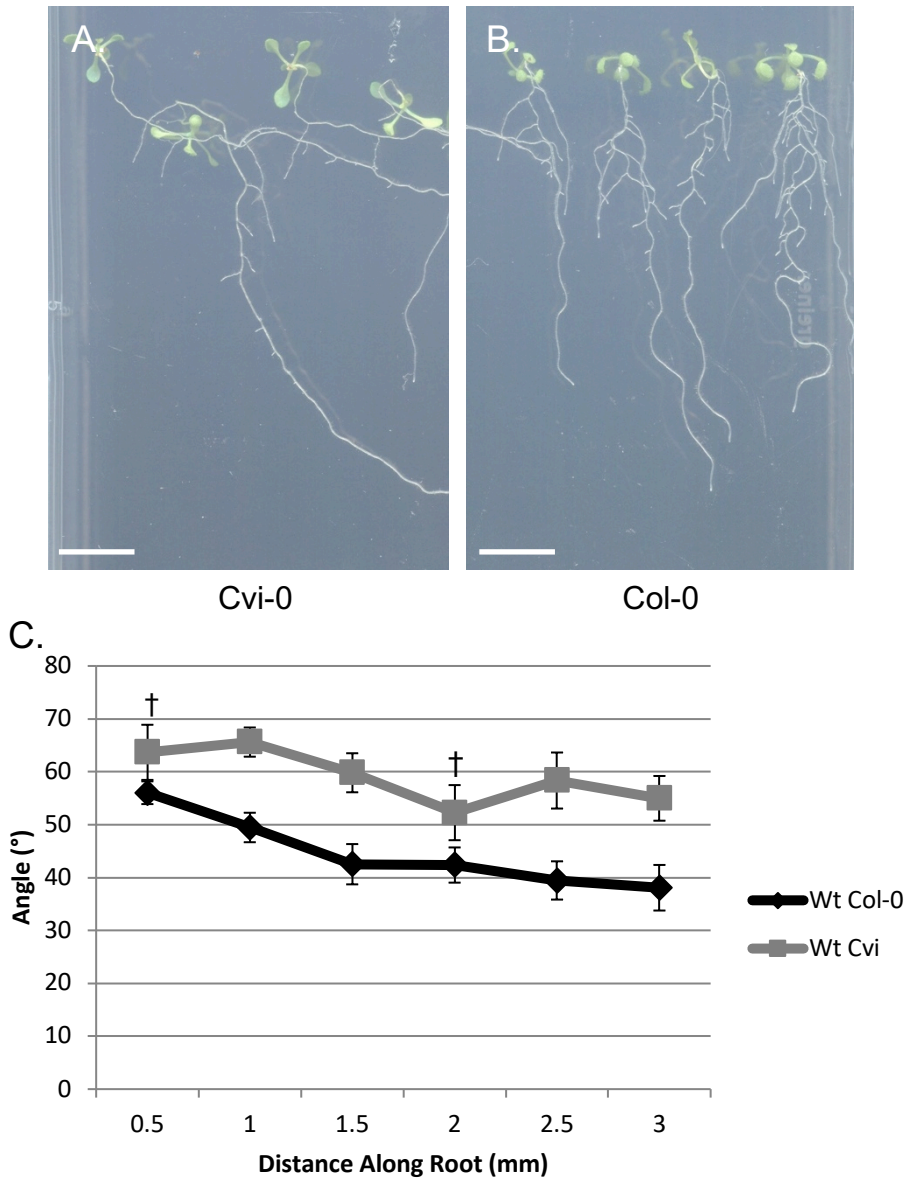


Figure 6.4: The lateral roots of Cvi-0 are less vertical than those of Col-0
The lateral roots of 14 day old plants of Cvi-0 (**A**) and 10 day old plants of Col-0 (**B**). The lateral roots of Cvi-0 are less vertical than those of Col-0 ($P<0.05$, Students T-test, at all points except those marked with a †), they are on average 14° less vertical, n=10 roots per ecotype (**C**). Scale bars represent 5 mm. Error bars represent SEM.

6.2.4 The lateral shoots of Cvi-0 are more vertical than Col-0

To further characterise GSA control in Cvi-0 the lateral shoot GSA phenotype was compared with that of Col-0. The lateral shoots of Cvi-0 are more vertical than those of Col-0 (Figure 6.5), on average the cauline branches of Cvi-0 are 9° more vertical, this could be as a result of a stronger AGO being present in the shoots and roots of Cvi-0 when compared with Col-0.

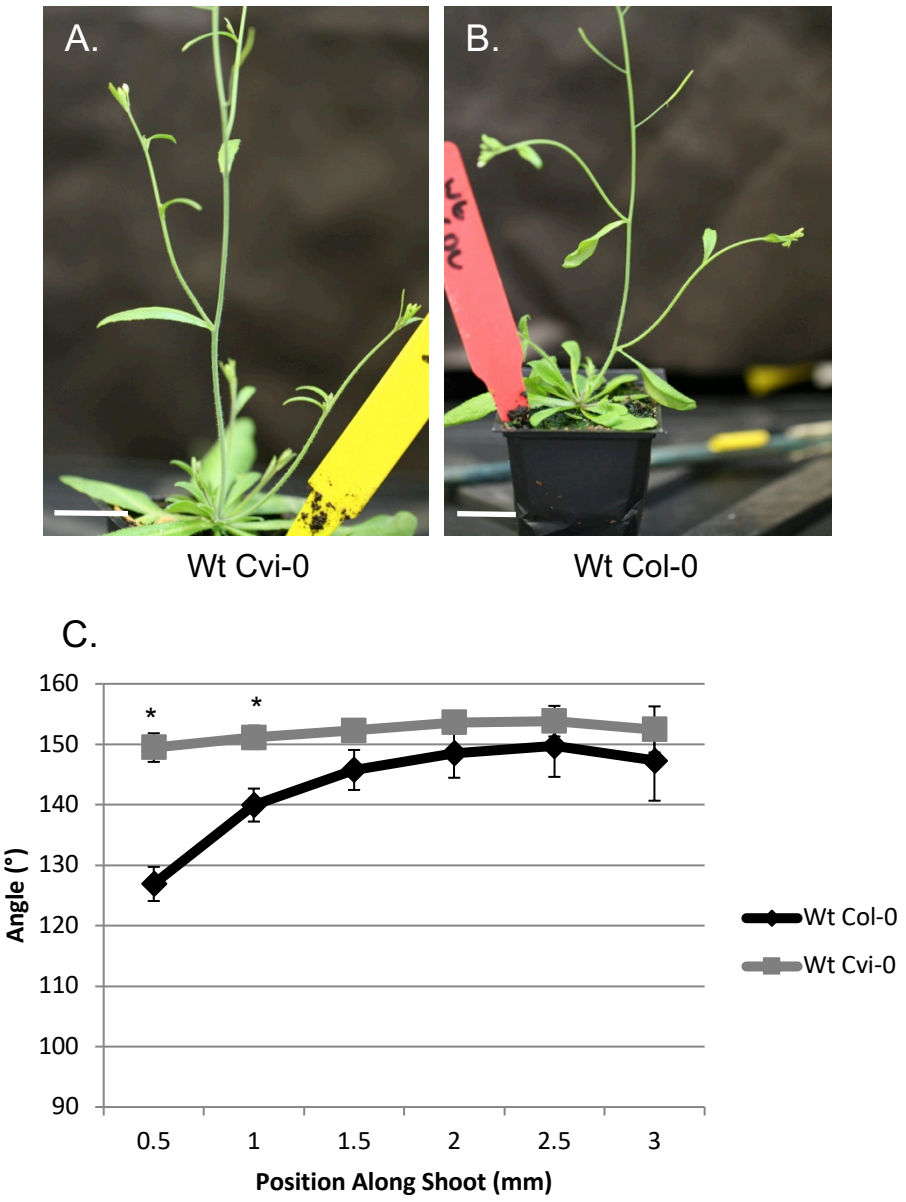


Figure 6.5: The lateral shoots of Cvi-0 are more vertical than Col-0
The lateral shoots of Cvi-0 are more vertical than those of Col-0. **A.** The cauline branches of a 4 week old plant of Cvi-0. **B.** The cauline branches of a 4 week old plant of Col-0. **C.** On average the branches of Cvi-0 are 9° more vertical than Col-0. Scale bars represent 1 cm, 10 branches were measured for both Cvi-0 and Col-0. $P<0.05$ Students T-test for points marked with *. Error bars represent SEM.

6.2.5 Clinorotation of Cvi-0 primary roots

In Col-0 it is thought that an AGO is present in the lateral roots and is either absent or present at a very low level in the primary axis (Roychoudhry et al., 2013). As the primary root of Cvi-0 grows non-vertically and the lateral roots grow less vertically, this could be indicative of a difference in AGO between the two ecotypes with potentially an increased AGO activity in the primary root. Plants placed upon a clinostat are subject to omnilateral gravitational stimulation, this disrupts the plants reference to the gravity vector and results in loss of activity of the gravitropic growth component. In the lateral roots and shoots of Arabidopsis the AGO continues to be active for a time during clinorotation, this results in outwards bending of the lateral branches and lateral root tips (Roychoudhry et al., 2013). To determine if the non-vertically growing primary root of Cvi-0 is a result of an AGO being active in the primary axis, 6 day old Cvi-0 primaries were grown on a 1 RPM clinostat for 6 hours, no outwards bending of the tip of the primary root was observed (Figure 6.6). This indicates that there is no detectable AGO activity in the primary root of Cvi-0 and implies that another mechanism underlies the non-vertical growth phenotype.

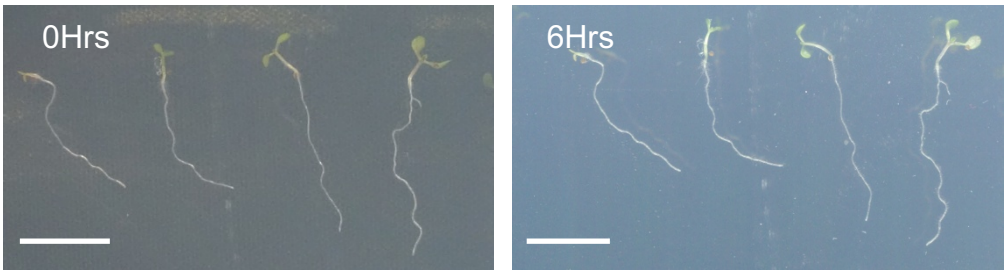


Figure 6.6: The skewed primary roots of Cvi-0 do not bend outwards upon clinorotation

The skewed primary roots of **Cvi-0** were subjected to 6 hours of clinorotation at 1 RPM. Although the roots have visibly grown in that time period they do not show evidence of the outwards bending that would be evident in a lateral root after that time. This suggests that a detectable AGO is not present in the skewed primary roots of **Cvi-0**. Scale bars represent 1cm.

6.2.6 The roots of Cvi-0 return to close to their original angle but always in the same direction as the original skew

When reoriented, an organ that is being maintained at a GSA initiates a growth response to bend either upwards or downwards to return to its original angle (Roychoudhry et al., 2013). 6-day-old plants of Cvi-0 were reoriented by 90° to place the tip of the primary root both above and below its original angle, both upwards and downwards bending Cvi-0 primaries bend back towards their original angle (Figure 6.7 E). However, unlike a GSA being maintained in a lateral root, the bending of the Cvi-0 primary root always returns the root tip to the same direction as the root was originally skewing when bending both downwards (Figure 6.7 A and B) and upwards (Figure 6.7 C and D). This, along with the other results described above, suggests that although a non-vertical angle appears to be maintained in the primary root of Cvi-0 the mechanism by which this maintenance happens is not the same as in the lateral roots of Col-0.

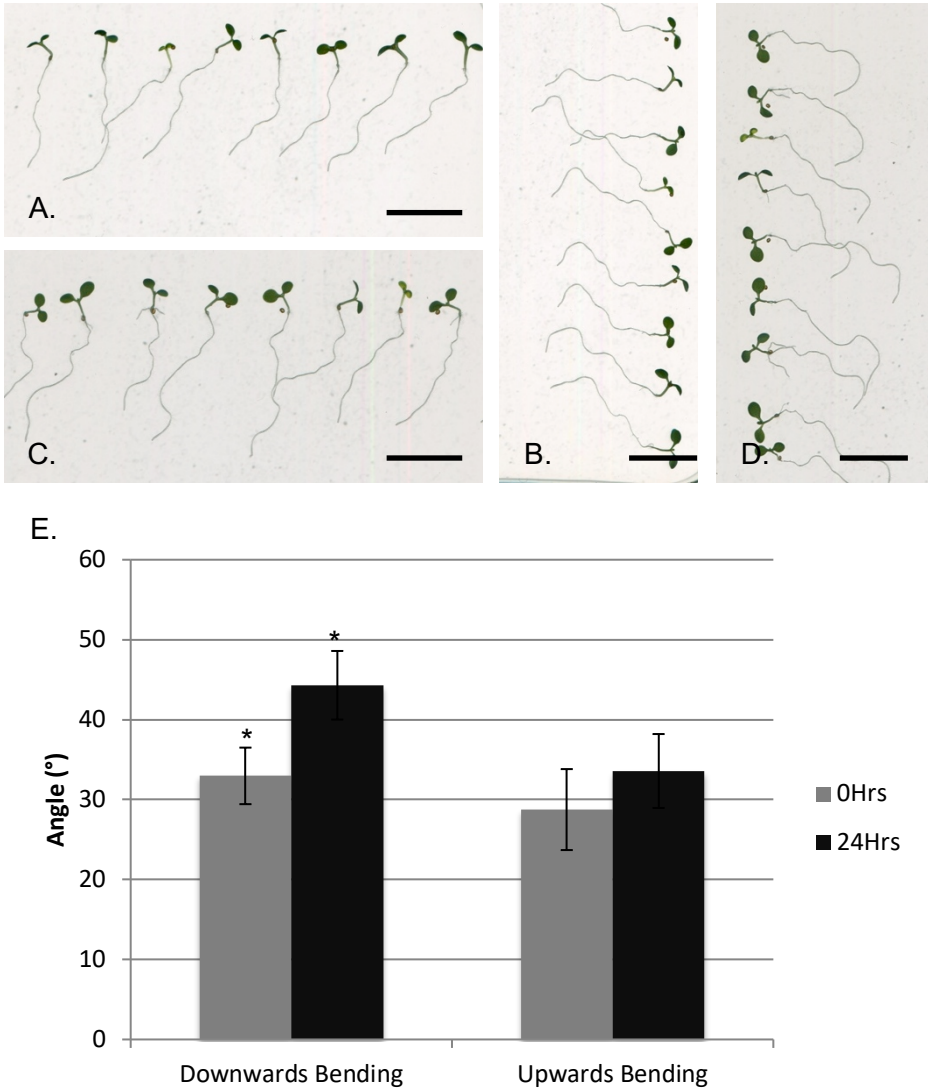


Figure 6.7: The roots of Cvi-0 return to close to their original angle but always in the same direction of the original skew.

When reoriented by 90° so that the primary root tips are both above (**A** and **B**) and below (**C** and **D**) their original angle they bend to return to towards their original angle (**E**) in both directions however they always return to the direction of their original skew. * indicates $P<0.05$ Students T-test. Error bars represent SEM, scale bars represent 1 cm.

6.2.7 2,4-D corrects the skew of the *Solanum pennellii* primary roots whereas IAA does not

A non-vertically growing primary root is also exhibited in the wild tomato relative *Solanum pennellii* (Ron et al., 2013), it was decided to investigate if the non-vertical growth of the *S. pennellii* primary root was controlled by similar mechanisms as those seen in Cvi-0. As the root skew phenotype of Cvi-0 is corrected by the addition of 2,4-D but not IAA, 5-day-old plants of *S. pennellii* were transferred to plates containing 5, 10 and 25 nM of 2,4-D and 5,10, 25, 50 and 100 nM of IAA (25 nM treatment shown in Figure 6.8). For all concentrations of 2,4-D tested the root skew phenotype of *S. pennellii* is corrected but for all concentrations of IAA tested the roots continue to skew. This suggests similar mechanisms are causing the root skew phenotype of *S. pennellii* as those in Cvi-0.

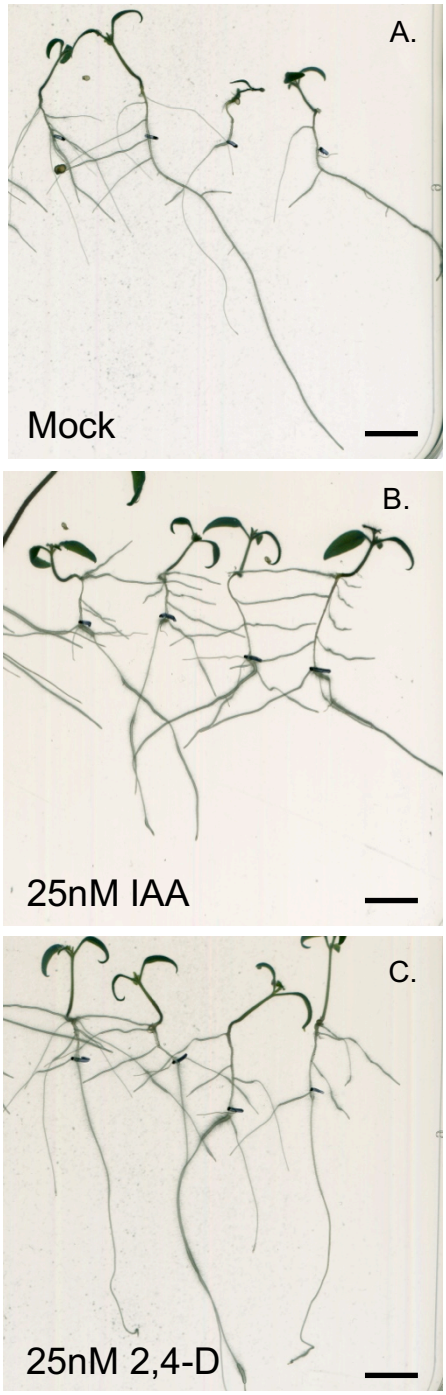


Figure 6.8: 2,4-D corrects the skew of the *Solanum pennellii* primary root whereas IAA does not.

When transferred onto plates containing a mock treatment the primary roots of *S. pennellii* reassume their skewed phenotype (**A**). The application of exogenous IAA to the roots of *S. pennellii* does not correct the skewing phenotype (**B**). Concentrations of up to 100 nM IAA do not correct the skew. The application of the synthetic auxin 2,4-D corrects the skewing phenotype of the *Solanum pennellii* primary root causing the root tip to assume a vertical orientation (**C**). Plants were transferred to hormone containing media after 5 days of growth on ATS agar, plates were scanned after a further 5 days. Scale bars represent 1 cm.

6.2.8 The primary roots of *S. pennellii* return to close to their original angles upon reorientation

If the primary root of *S. pennellii* were behaving like a lateral root such as those of the Arabidopsis ecotype Col-0 it would maintain its non-vertical growth angle as a GSA. To determine if the *S. pennellii* primary root angle is being maintained 5-day-old plants of *S. pennellii* were reoriented by 90° to place the tips of the primary roots both above and below their original angles, plants were then allowed to grow for 24 hours. The angles of the primary root tips were measured before and 24 hours after reorientation. Both upwards and downwards bending primary roots bend back towards their original angle (Figure 6.9 **A** and **B**), on average downwards bending roots return to within 5° and upwards bending roots return to within 9.3° of their original angle (Figure 6.9 **C**).

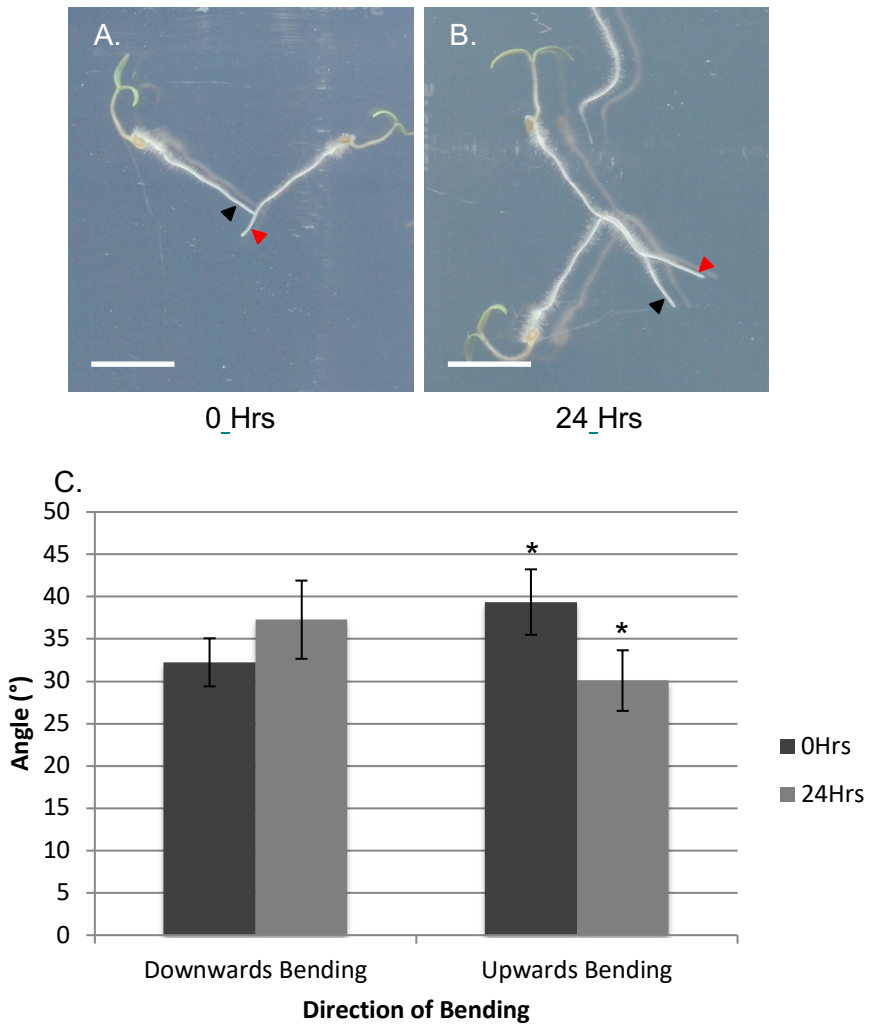


Figure 6.9: The primary roots of *S. pennellii* return to close to their original angles upon reorientation.

5 day old plants of *S. pennellii* were reoriented by 90° and left to grow for 24 hours, the tip angles of the primary roots were measured before and 24 hours after reorientation. It was found that by both upwards and downwards bending on average the roots return to close to their original angles (C). In A and B the black arrows signify a downwards bending root, the red arrows an upwards bending root. Upwards bending n=23, downwards bending n=44, error bars represent SEM, scale bars represent 1 cm, * denotes P <0.05 (Students T test).

6.2.9 There is no outward bending of the *S. pennellii* primary root upon clinorotation

The primary roots of cultivated tomatoes such as “Alicante” grow vertically on ATS agar plates (Figure 6.10), as with the vertically growing primary roots of Arabidopsis ecotypes such as Col-0 we expect there to be no AGO active in the primary axis. As the primary root of *S. pennellii*, similarly to Cvi-0, grows at a non-vertical angle in a lateral root like fashion it was decided to test for the presence of an AGO by subjecting the primary root to omnilateral gravitational stimulation on a clinostat rotating at 1 RPM for 6 hours. As was found for Cvi-0 the primary roots of *S. pennellii* carry on growing in their original direction and there is no evidence of the outwards bending of the tip that would be seen in a lateral root maintaining a GSA by means of an AGO (Figure 6.11). As with Cvi-0 this suggests that there is not an activity with the characteristics of an AGO in the maintenance of the non-vertical growth angle of the *S. pennellii* primary root and that the mechanism by which the angle is maintained is not the same as that seen in lateral roots, a comparison between angle maintenance in the non-vertical primary roots of Cvi-0 and *S. pennellii* and that of Arabidopsis lateral roots can be found in Table 6.1.

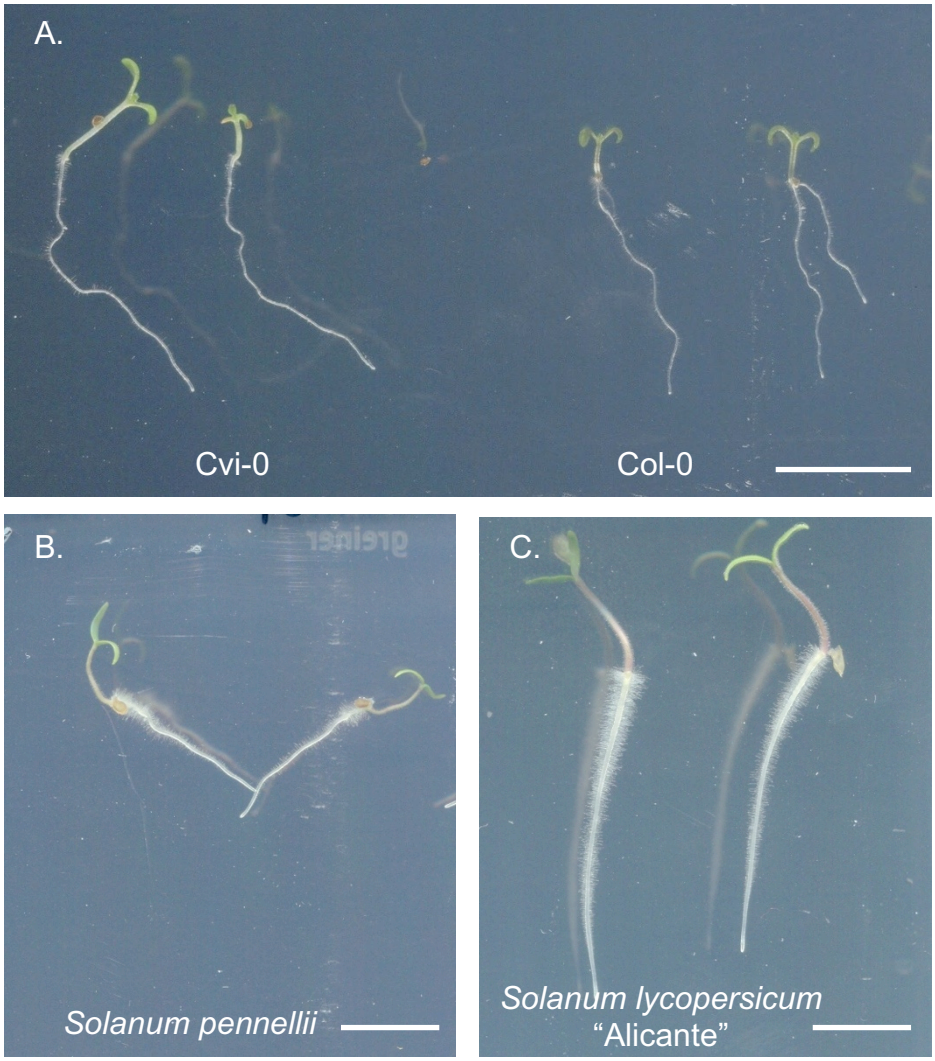


Figure 6.10: The primary roots of both the Arabidopsis ecotype Cape Verdi Islands (Cvi-0) and the tomato *Solanum pennellii* display primary root skew phenotypes.

When compared with the roots of Col-0 the primary root of Cvi-0 displays a right handed skew in the primary root (A). The wild relative of tomatoes *Solanum pennellii* (B) also has a skewed primary root compared to that of cultivated tomatoes such as "Alicante" (C) however unlike Cvi-0 there is no handedness to the direction of its skew. Scale bars represent 1 cm.

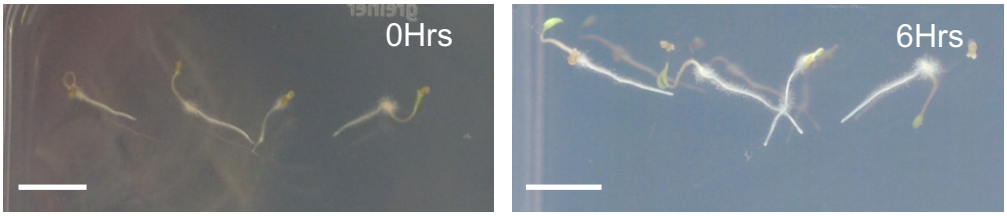


Figure 6.11: The skewed primary roots of *S. pennellii* do not bend outwards upon clinorotation

The skewed primary roots of *S. pennellii* were subjected to 6 hours of clinorotation at 1 RPM. Although the roots have visibly grown in that time period they do not show evidence of the outwards bending that would be evident in an *Arabidopsis* lateral root after that time. This suggests that a detectable AGO is not present in the skewed primary roots of *S. pennellii*. Scale bars represent 1cm.

Table 6.1: A comparison of angle maintenance mechanisms between Arabidopsis lateral roots and the skewed primary roots of Cvi-0 and *S. pennellii*

Arabidopsis Lateral Roots	**Cvi-0**	***Solanum pennellii***
Assume a more vertical angle upon treatment with 2,4-D	2,4-D corrects the skew phenotype of the primary root (essentially becomes more vertical)	2,4-D corrects the skew phenotype of the primary root (essentially becomes more vertical)
Assume a more vertical angle upon treatment with IAA	IAA does not correct the skew of the primary root	IAA does not correct the skew of the primary root
	Primary root skews only in one direction	Primary root skews in both directions
Root tips bend outwards upon clinorotation	Primary roots do not bend outwards upon clinorotation	Primary roots do not bend outwards upon clinorotation
Return to their original angles by bending upwards and downwards, roots will reset to return to their original angles	Primary roots return towards their original angle after reorientation but always in the direction of the original skew	Primary roots will reset to return towards their original angles regardless of the direction of their original skew

6.2.10 The application of exogenous IAA causes the lateral roots of both *Solanum pennellii* and cultivated tomatoes to become less vertical.

In Arabidopsis the application of exogenous IAA results in the lateral roots becoming more vertical (Roychoudhry et al., 2013). In contrast it was found that the application of exogenous IAA to tomatoes causes the lateral roots to become less vertical, results suggest that this is dose responsive as roots treated with 100 nM IAA are less vertical than those treated with 10 nM IAA (Figure 6.12). Roots of the cultivated tomato variety “Alicante” treated with 100 nM IAA on average become 24° less vertical and roots of *S. pennellii* become on average 29.3° less vertical than those of mock treated plants. There also appears to be no upper limit or threshold concentration of IAA where the lateral roots begin to become more vertical than mock treated plants (Figure 6.13 D), roots of “Alicante” treated with 500 nM IAA (Figure 6.13 B) on average become 35.7° less vertical and those treated with 1000 nM IAA (Figure 6.13 C) become 28.9° less vertical than mock treated plants. This could suggest that the mechanism through which root setpoint angle is controlled in tomatoes is different to that of Arabidopsis.

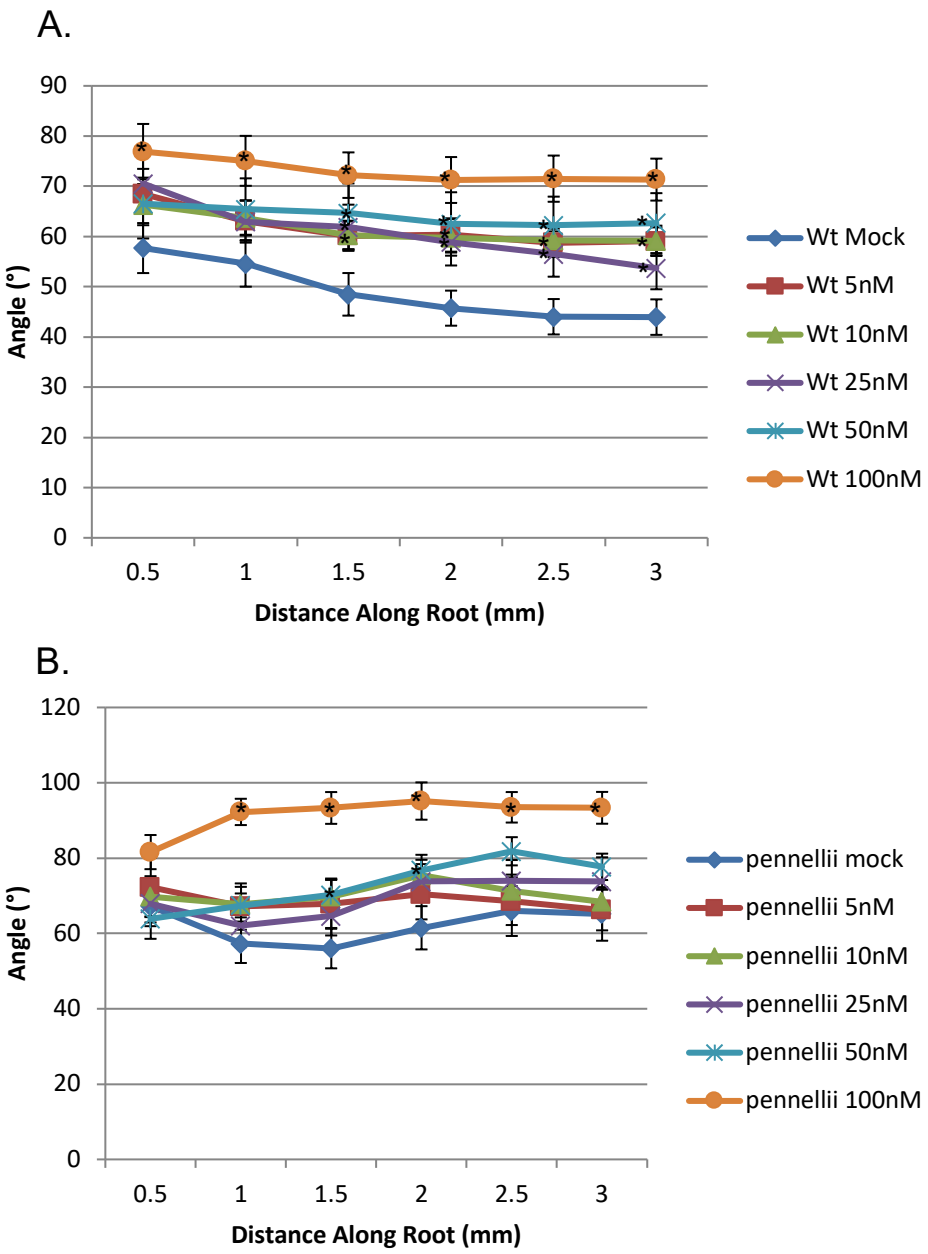


Figure 6.12:The application of exogenous IAA causes the lateral roots of both *Solanum pennellii* and cultivated tomatoes less vertical.

The application of exogenous IAA causes the lateral roots of both *Solanum pennellii* and cultivated tomatoes to become less vertical, the greater the concentration of IAA applied the less vertical they become. 10 roots were measured per treatment for both *S. pennellii* and cultivated tomatoes, error bars represent SEM, * indicates P<0.05 (Students T-test).

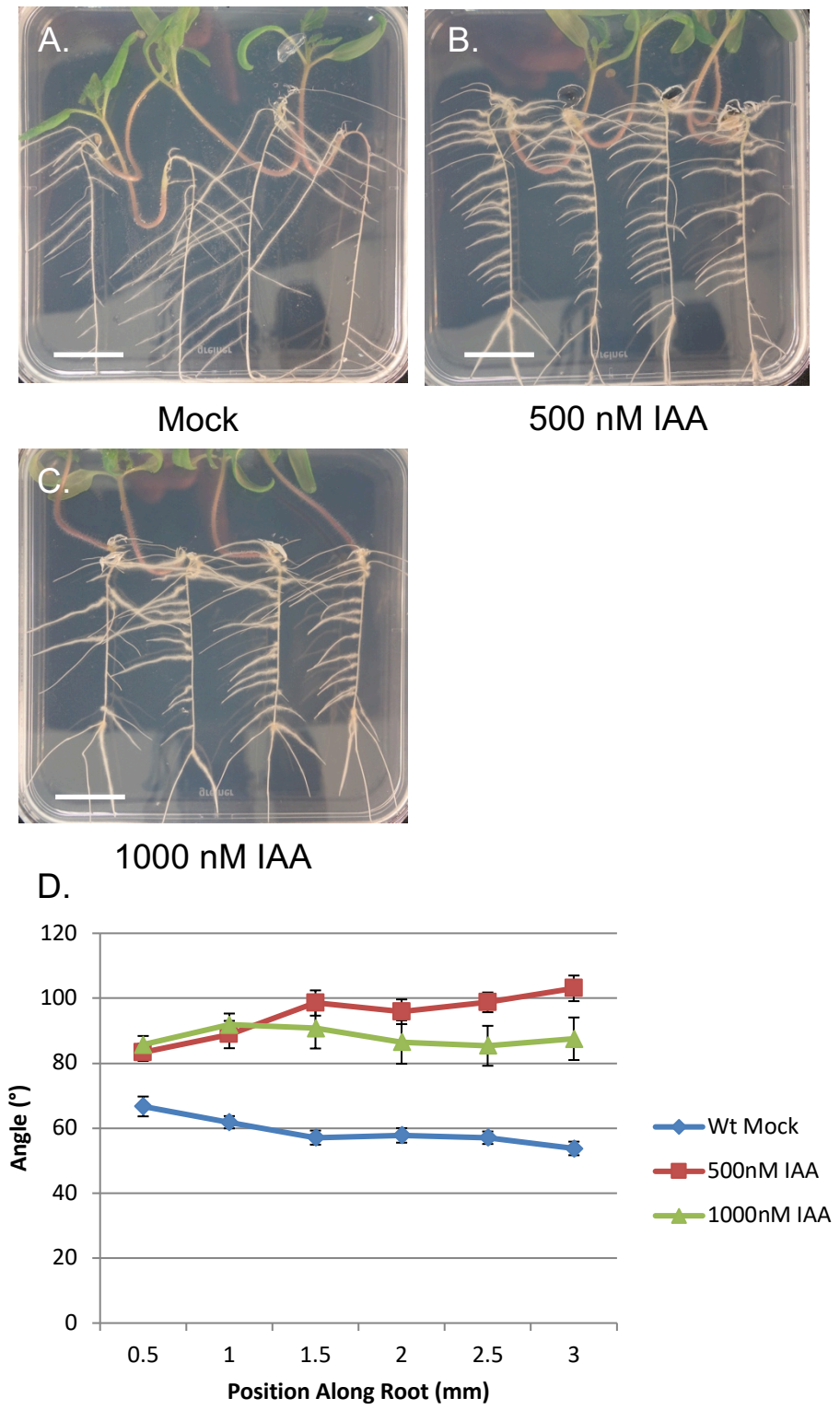


Figure 6.13 : There is no upper limit of auxin concentration to observe the lateral roots of tomatoes becoming less vertical.

The lateral roots of domesticated tomatoes become less vertical upon application of exogenous auxin (See Figure 6.12), treatments with higher concentrations of auxin (**A.** Mock, **B.** 500 nM and **C.** 1000 nM) also result in less vertical lateral roots (**D**) suggesting that there is no threshold concentration where the roots become more vertical, error bars represent SEM, $P<0.05$ for all points (Students T-test), scale bars represent 2 cm, n = 10 roots per treatment.

6.2.11 The TIR1 pathway inhibitor PEO-IAA also makes the lateral roots of tomatoes less vertical.

To further explore the mechanism by which the lateral roots of tomatoes become less vertical in relation to auxin 5-day-old plants of the cultivated tomato “Alicante” were placed on plates containing 5 μM of the antiauxin PEO-IAA, this also results in the lateral roots becoming less vertical, on average they are 11° less vertical than mock treated roots (Figure 6.14 B and D). Co-treatment with 5 μM PEO-IAA and 100 nM IAA has an additive effect on the lateral root phenotype (Figure 6.14 C and D) with lateral roots becoming on average 24° less vertical than mock treated roots and 13.4° less vertical than those roots treated with PEO-IAA alone. This suggests that the mechanism that causes the lateral roots to become less vertical does not act through the TIR1 pathway.

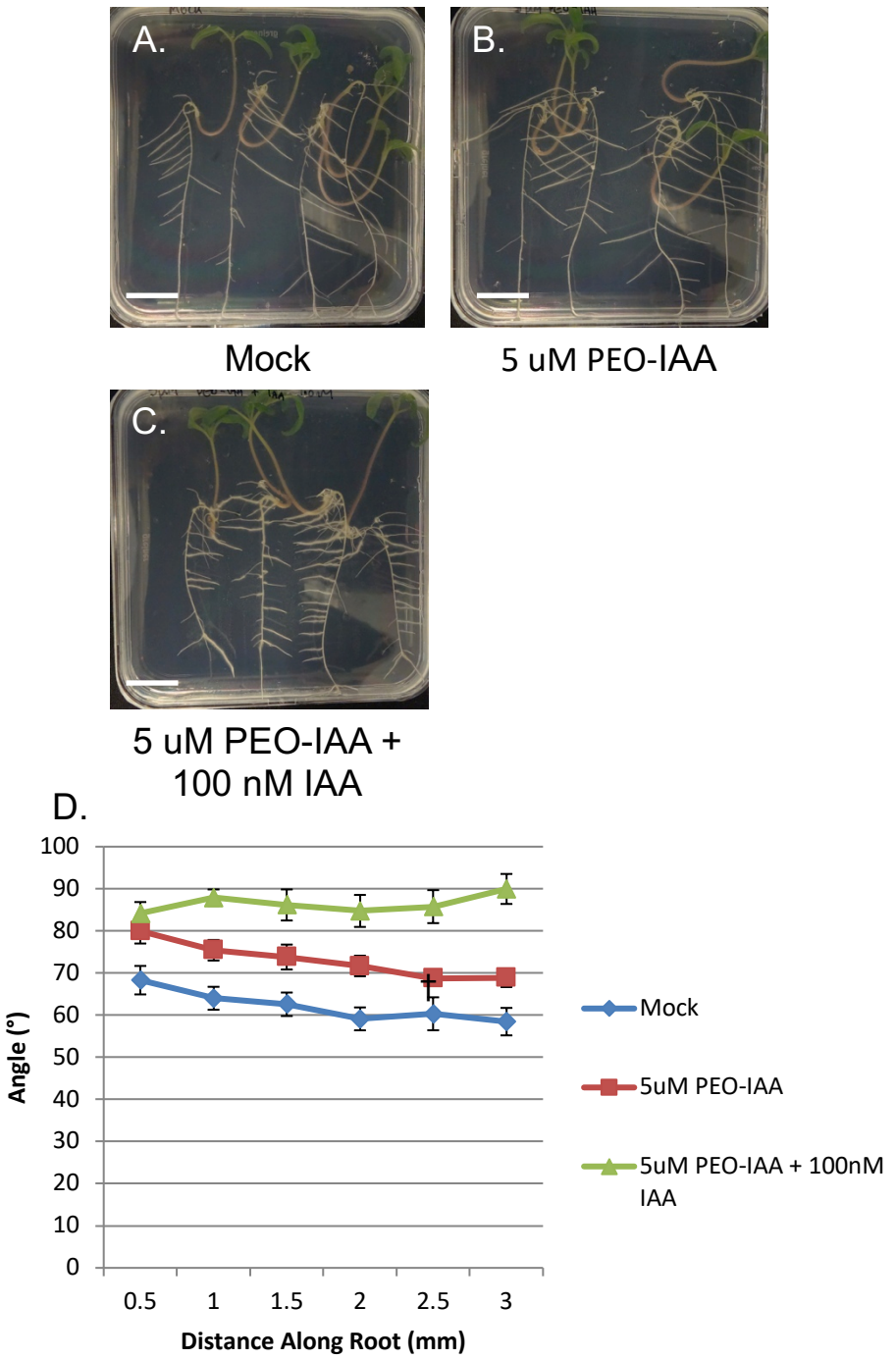


Figure 6.14 : The antiauxin PEO-IAA also makes the lateral roots of tomatoes less vertical.

When compared with mock treated roots (**A**),treatment with the antiauxin PEO-IAA also makes the roots of tomatoes less vertical (**B**), treatment with IAA in addition to PEO-IAA has an additive effect causing the lateral roots to become less vertical than with just PEO-IAA alone (**C**,**D**). This suggests that the mechanism that causes the lateral roots to become less vertical does not act through the TIR1 pathway. n=10 roots per treatment, error bars represent SEM, $P<0.05$ (Students T-test) for all points except those marked with †. Scale bars represent 2 cm.

6.2.12 Application of NPA also makes the lateral roots of tomatoes less vertical

To investigate if the difference in the angle change response of tomato lateral roots to auxin was due to differences in auxin transport. 5- day old plants of the cultivated tomato “Alicante” were placed onto plates 0.5 μM of the auxin transport inhibitor NPA (Figure 6.15 B), this caused the lateral roots to become less vertical when compared with mock treated plants (Figures 6.14 and 6.15 C). On average the lateral roots of plants treated with 0.5 μM NPA are 41.5° less vertical than mock treated plants. This suggests that differences in the movement of auxin in tomato roots when compared with Arabidopsis roots are not responsible for the opposing changes in lateral root angle in response to exogenous auxin.

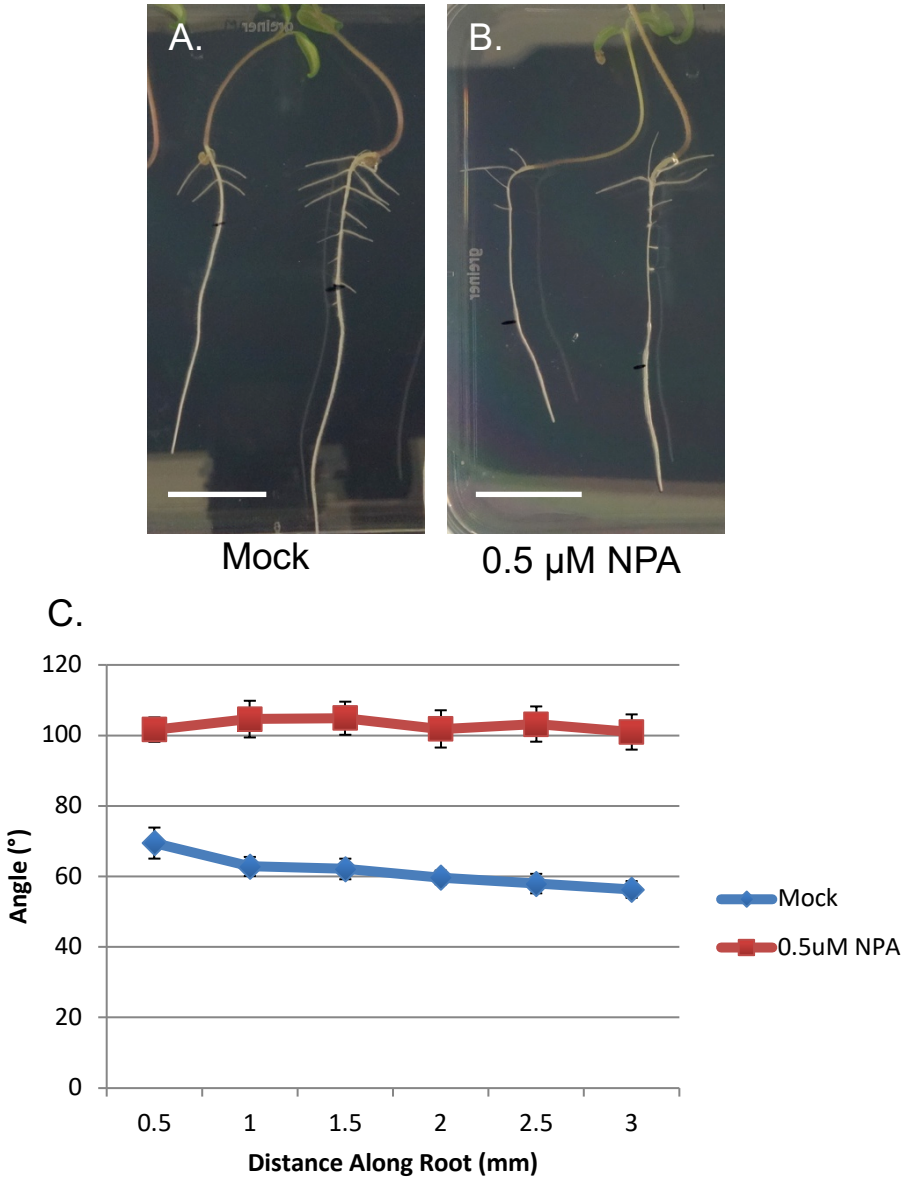


Figure 6.15: Application of NPA also makes the lateral roots of tomatoes less vertical

Compared with a mock treatment (**A**), application of the auxin transport inhibitor NPA (**B**) also makes the roots of tomatoes less vertical (**C**), this suggests that changes in auxin transport are not involved in the mechanism by which the roots of tomatoes become less vertical. Error bars represent SEM, $P<0.05$ (Students T-test) for all points. n=10 roots per treatment, scale bars represent 2 cm.

6.2.13 IAA also makes the lateral roots of Aubergine less vertical but not those of Chilli peppers or *Nicotiana benthamiana*

The Solanaceae family contains a number of economically important plants including tomatoes, potatoes, tobacco and peppers. It was decided to investigate if the effects of auxin on lateral root angle in tomatoes extended to other members of this large plant family. The lateral roots of both Chilli peppers (*Capsicum annuum* “Jalapeno”) and *Nicotiana benthamiana* become more vertical upon the application of 50 nM IAA (Figure 6.16 A and B), on average the lateral roots of chill peppers become 15.7° more vertical when compared with mock treated plants, lateral roots of *Nicotiana* are on average 9.6° more vertical than mock. However, in a similar manner to tomatoes (*Solanum* lycopersicum), the lateral roots of aubergine (*Solanum melongena*) become slightly less vertical when treated with 50 nM IAA (Figure 6.16 C). On average the lateral roots of aubergine treated with 50 nM IAA are 5.3° less vertical than mock treated roots. This suggests that whilst the lateral roots becoming less vertical upon application of exogenous IAA may not be family wide within the Solanaceae it may be consistent within the genus *Solanum*.

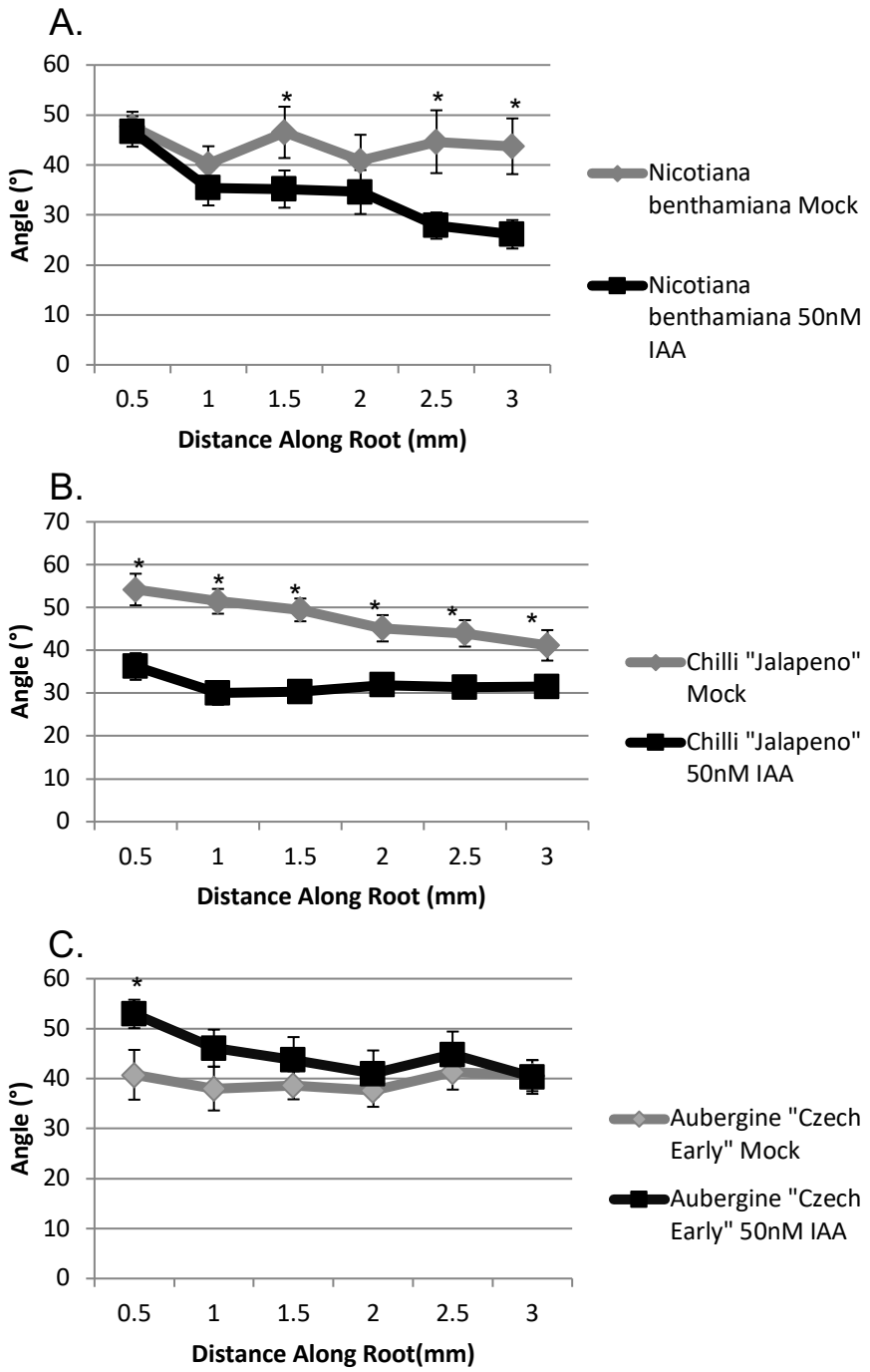


Figure 6.16: IAA also makes the lateral roots of Aubergine less vertical but not those of Chilli peppers or *Nicotiana benthamiana*

Application of exogenous IAA makes the lateral roots of *Nicotiana benthamiana* (**A**) and the chilli pepper variety "Jalapeno" (**B**) more vertical in a similar manner to that seen in Arabidopsis. Application of exogenous IAA to makes the lateral roots of Aubergine (**C**) less vertical in a similar manner to tomatoes. n=10 roots measured per treatment, $P<0.05$ (Students T-test) at all points marked with *, error bars represent SEM.

6.3 Discussion

Much more is known about gravitropism in the primary root and shoot than in the lateral roots and shoots. The work described in this thesis thus far has focused on gaining a better understanding of the mechanisms behind non-vertical growth in the lateral organs across a number of species of plants. Primary roots in the majority of cases grow vertically or almost vertically downwards (the primary roots of both wheat and rice do not always grow vertically downwards in a 3D system, see Chapter 3). However, cases exist of non-vertically growing primary roots and investigating how these non-vertical primary roots grow and if their angles are maintained as GSAs has been much of the focus of this chapter.

The primary roots of both the Arabidopsis ecotype Cape Verdi Islands (Cvi-0) and the wild tomato *Solanum pennellii* exhibit a skewing (Figure 6.10). It was hoped that by determining if they are maintained at GSAs in a similar manner to that of lateral roots, that comparative analysis between these examples and variants with a vertically growing primary root, such as the Arabidopsis ecotype Col-0 and the cultivated tomato *Solanum lycopersicum,* would shed some light on what distinguishes a vertically growing primary root from a non-vertically growing lateral root, in other words what makes a lateral, lateral.

Previous work on the primary root skew of Cvi-0 found that the addition of the synthetic auxin 2,4-Dichlorophenoxyacetic acid (2,4-D) but not the natural auxin IAA could correct the root skew phenotype (Patel, 2009). 2,4-D was developed as a herbicide during the second world war as a means to selectively control dicot weeds in fields of monocot crops such as wheat, rice and maize. It is structurally very similar to IAA and although it is unable to be metabolised by plants it shares many of the molecular mechanisms associated with the transport of, and response to, IAA (Song, 2014). For example, it is transported into cells by the auxin influx transporter AUX1, this was shown to be the case as root growth of the *aux1* mutant is not inhibited by the presence of both IAA and 2,4-D (Marchant et al., 1999). It is also recognised by the auxin receptor TIR1; it is thought that the structure and size of the binding pockets of the different AFBs may confer selectivity to the different auxinic herbicides, for example the

afb5 mutant is resistant to picloram (Song, 2014, Walsh et al., 2006). It has been shown that the *tir1-1* mutant is resistant to 2,4-D (Song, 2014, Parry et al., 2009) and, as with IAA, the binding pocket of TIR1 can accommodate 2,4-D allowing it to act as the "molecular glue" between TIR1 and the Aux/IAAs (Song, 2014, Calderon-Villalobos et al., 2010, Tan et al., 2007). However, 2,4-D is a poor auxin at the receptor level requiring concentrations tenfold greater than IAA to activate the auxin receptor TIR1 to the same level (Kepinski and Leyser, 2005). It is also a poor substrate for the auxin efflux carriers when compared with IAA and NAA (Delbarre et al., 1996) meaning that it accumulates within the cell due to the import activity of AUX1 (Marchant et al., 1999). 2,4-D has also been reported to specifically promote cell division as opposed to cell elongation (both are responses to addition of exogenous auxin) and that this promotion can be inhibited by the addition of the G-protein inhibitor Pertussis toxin (Campanoni and Nick, 2005), this suggests that 2,4-D may also act through additional pathways to TIR1 mediated auxin signalling. A key reason for differences in response between IAA and 2,4-D could be the plants inability to metabolise 2,4-D (Song, 2014) allowing further accumulation of the compound within the plants cells. To confirm Patel's observation and to determine if there was a threshold concentration at which IAA would correct the root skew Cvi-0 was treated with multiple concentrations of IAA and 2,4-D. It was found that in concurrence with Patel's work 2,4-D corrected the root skew but IAA did not (Patel, 2009) and that at IAA concentrations higher than those previously used the skew was still not corrected suggesting that there is no threshold at which IAA is able to correct the root skew (Figure 6.2). A similar experiment was carried out on *S. pennellii* and it was found that for all concentrations tested 2,4-D corrected the root skew phenotype whereas IAA did not (Figure 6.9), this suggests that similar mechanisms are at work in both examples of non-vertically growing primaries. It is possible that differences in response between IAA and 2,4D in both Cvi-0 and *S. pennellii* could be due to changes in the binding pockets of their AFBs relative to their counterparts with vertically growing primaries, for example another of the AFBs may have gained the ability to bind 2,4-D. Another more likely possibility is that 2,4-D is affecting a different pathway to the traditional auxin response pathway through TIR1 and the AFBs for example through a G-protein mediated signalling pathway (Campanoni and Nick, 2005). To further explore the mechanism by which 2,4-D corrects the skew of the Cvi-0 and *S.*

pennellii primary roots, an EMS mutagenesis based screen could be carried out to look for mutants resistant to the corrective effect of 2,4-D on the root skew. Genotyping of a 2,4-D resistant mutant of either Cvi-0 or *S. pennellii* could help determine the pathway through which 2,4-D has its effect on the primary root skew.

In contrast to their opposing effects on the primary root of Cvi-0, IAA and 2,4-D have similar effects on the lateral roots; both induce more vertical growth of the lateral roots in Arabidopsis. There appears to be a difference in the dose response between IAA and 2,4-D, increasing concentrations of 2,4-D result in increasingly more vertical roots whereas all concentrations of IAA used make the lateral roots more vertical to a similar degree, although the highest concentration of both treatments resulted in a similar angle change (Figure 6.3). Previous work comparing the effects of 2,4-D and IAA found that in the case of the inhibition of root growth the dose/response curves are very similar between the two auxins (Maher and Martindale, 1980), however this work was carried out on primary roots and the difference in lateral root angle change could be indicative of differing responses to both auxins between the primary roots and the lateral roots. The difference in response between the primary root and the lateral roots could perhaps be explained by differences in expression of for example a modified AFB or begin to be indicative of differing mechanisms of non-vertical growth angle control between the non-vertically growing primaries and the laterals.

Upon reorientation both Cvi-0 and *S. pennellii* induced growth in both upwards and downwards directions to return their non-vertical primaries back towards their original angles (Figures 6.7 and 6.9). However the primary root of Cvi-0 exhibits a “handedness” in its skew (the *S. pennellii* primary does not) (Figure 6.10), when reoriented the primary root will always grow back towards the direction of its original skew whether bending upwards or downwards (Figure 6.7). The growth movements of roots on plates have been described as a right-handed helix (although left handed mutants exist (Marinelli et al., 1997, Thompson and Holbrook, 2004)), this can lead to a skewing to the right as the roots grow, Thompson and Holbrook have postulated that this root skewing is

as a result of both gravitropism and impedance of the root against the gel surface as it circumnutates (Thompson and Holbrook, 2004). The primary root of Cvi-0 also consistently skews to the right, even upon reorientation (Figure 6.7), so it is possible that the non-vertically growing primary is as a result of a "Gravi-circumnutational equilibrium" in which the magnitudes of gravitropism and circumnutation act in balance (rather like that of gravitropism and the AGO in lateral roots (Roychoudhry et al., 2013)) to produce non-vertical growth in the direction of the growth helix. The primary roots of *S. pennellii* do not exhibit this handedness as the primary roots of different plants skew in different directions (Figure 6.10B), therefore it is unlikely that the skew in the primary is due to circumnutation unless the direction of the growth helix is not consistent between plants. Further experiments would need to be carried out to determine if the direction of the growth helix correlates with the direction of the skew in *S. pennellii* primary roots, this could be done using a microscope to examine the direction of the cell file rotations of plants skewing in either direction. Whole genome sequencing of pools of plants that skew either left or right could be used to determine if any polymorphisms existed only in plants that skewed in a particular direction to determine if there is a genetic control mechanism behind the direction of the skew.

In lateral roots the maintenance of non-vertical GSAs is controlled by a balance of two auxin fluxes, a gravitropic auxin flux and an antigravitropic offset (AGO), it has been proposed that the magnitude of the AGO regulates the GSA. An antigravitropic offset is dependent on auxin transport for it to effect a change on root growth as it has been shown that changes to auxin signalling in the gravity sensing cells alone have the ability to modify the GSA (Roychoudhry et al., 2013). Perhaps the differing effects of IAA and 2,4-D on the non-vertical primary roots could be explained by changes in auxin transport brought about by changes in the concentration of auxin, it has been shown that an increase in auxin concentration both results in a decrease in the expression of the PIN efflux proteins (Vieten et al., 2005) and an increase in the expression of the protein kinase PINOID which is known to control apical to basal trafficking of the PIN proteins (Friml et al., 2004). It is possible that whatever element of auxin signalling causes the difference in response to IAA and 2,4-D could trigger

changes in auxin efflux by the PIN proteins either through their expression or targeting and that differences in PIN polarity between the Cvi-0 and Col-0 primaries could lead to a non-vertical primary. The lateral roots of Cvi-0 are less vertical than those of Col-0 (Figure 6.4) and the lateral shoots of Cvi-0 are more vertical than those of Col-0 (Figure 6.5), this could be indicative of a stronger AGO being present throughout the Cvi-0 plant opening up the potential for there to be an AGO present in the non-vertical primary. When the lateral roots and shoots of Arabidopsis are subject to omnidirectional gravitational stimulation upon a clinostat the plants reference to the gravity vector is lost and therefore the gravitropic auxin flux is also lost, however the AGO continues to act for a longer period after the loss of the gravitropic auxin flux resulting in an outwards bending of both roots and shoots (Roychoudhry et al., 2013). To test for the presence of an AGO in both the Cvi-0 and *S. pennellii* primaries, plants were placed upon a clinostat for 6 hours at 1 RPM, neither Cvi-0 nor *S. pennellii* display any outwards bending as would be expected in a lateral root and both continue to grow in the direction they were growing before clinorotation (Figure 6.6). This could be explained by a difference in kinetics between the loss of the gravitropic auxin flux and the AGO between lateral roots and the skewed primary roots, the AGO could be lost earlier in the skewed primary root than in a lateral root with this preventing the manifestation of an outward bend due to the speed of the growth response. This suggests that there is no AGO active in the primaries of both Cvi-0 and *S. pennellii* and along with the previous data indicates that the mechanism by which a non-vertical growth angle is generated and maintained is not the same as in the lateral roots.

Another potential explanation for the non-vertical primaries of both Cvi-0 and *S. pennellii* is that of alterations to the system of microtubules within the cell when compared with their vertically primary-rooted counterparts. The *spiral* and *lefty* mutants of Arabidopsis both display root skewing to either the right in the case of *spiral* or the left in the case of *lefty*. Both sets of mutations interfere with the formation of cortical microtubules in the correct orientation, it is thought that the orientation of the cortical microtubules specifies the orientation of the cellulose microfibrils that will make up the cell wall and therefore they specify the direction of cell elongation (Thitamadee et al., 2002, Furutani et al., 2000).

Indeed, the *lefty* mutations are within two α tubulin genes *TUA6* and *TUA4*, both are dominant negative mutations and their null mutants are phenotypically the same as wild type, there are 6 expressed α-tubulin genes in Arabidopsis and it is thought that other α-tubulin isoforms can compensate for the loss of function mutants (Thitamadee et al., 2002). It is possible that Cvi-0 and *S. pennellii* contain mutations or polymorphisms in their microtubule network machinery that can account for the skewing observed in their primary root, however the root skewing phenotype of *lefty* is not rescued by the application of 2,4-D (Patel, 2009). Auxins are known to have an effect on another component of the cytoskeleton, the actin filaments (Nick, 2010, Rahman et al., 2007). IAA has little effect on the extent of the actin cytoskeleton whereas 2,4-D removes actin and slows down cytoplasmic streaming (Rahman et al., 2007), this differing effect on the cytoskeleton could explain the difference in response of the skewed primary roots to the two compounds

In the case of *S. pennellii* there are numerous morphological differences between the roots of *S. lycopersicum* whos primary roots grow vertically downwards and *S. pennellii* these include alterations to the number of layers in the vascular cylinder and the root length (Ron et al 2013). Using a number of introgression lines with the *S. lycopersicum* cultivar M82 Ron et al (2013) found that the root angle phenotype of *S. pennellii* is a polygenetic trait controlled by a number of loci, it is possible that one of these loci could contain the possible upstream element that could control the magnitude of the AGO as discussed in Chapter 5 and although there is no outwards bending on the clinostat and therefore no evidence of an AGO being present in the *S. pennellii* primary root (Figure 6.6) the regulation or expression of the upstream element could be different in the non-vertical primary when compared to the laterals. Potentially, loss of a gravistimulus in the primary root could also result in changes to the upstream element that explains the lack of evidence of an AGO in the clinorotated *S. pennellii* primary.

The treatment of the roots of both *S. pennellii* and *S. lycopersicum* with IAA and 2,4-D resulted in an interesting observation regarding the effects of auxin on the lateral root GSA of tomatoes. In Arabidopsis, treatment with exogenous auxin

results in the lateral roots assuming a more vertical GSA (Roychoudhry et al., 2013), it has also been found that application of exogenous IAA results in more vertical seminal and crown roots in the cereal crops wheat and rice respectively (see Chapter 3). However, upon treatment with IAA and 2,4-D the lateral roots of both tomato species became less vertical (Figure 6.12 shows data for treatment with IAA) and results suggest that this is dose responsive with higher concentrations of IAA resulting in a greater modification of the GSA. A similar response has been reported in the non-vertically growing basal roots of bean where treatment with lower concentrations of auxin (50-70 nM) resulted in a less vertical GSA (Roychoudhry et al., 2017). However, higher concentrations (90-100 nM) shifted the GSA to a more vertical orientation suggesting that the dose dependent relationship between auxin and GSA control in bean is more complex than seen in Arabidopsis and differs between root types, the true lateral roots of bean become more vertical upon treatment with auxin like those of Arabidopsis (Roychoudhry et al., 2017). It has been postulated that this may be related to the differing cell elongation responses to auxin in roots and shoots, it may be significant that the basal roots are adventitious and therefore arise from shoot tissue and their auxin response may mirror that of the shoots (Roychoudhry et al., 2017). It was decided to determine if this differing response at higher concentrations of auxin was also present in tomatoes and if a threshold concentration existed at which the change in GSA would switch to a more vertical one. It appears that there is no threshold or upper limit at which the lateral roots become more vertical as both 500 nM and 1000 nM of IAA resulted in the roots becoming less vertical (Figure 6.13) suggesting that the mechanism by which auxin controls lateral root angle in tomatoes is different to that seen in Arabidopsis.

In order to further explore the mechanism by which the tomato lateral roots become less vertical, a number of chemical treatments were applied that are known to affect auxin response and transport in Arabidopsis. The α-alkyl-IAA, PEO-IAA is an auxin antagonist that specifically binds into the auxin binding pocket of the receptor TIR1 and blocks its function, PEO-IAA is known to affect gravitropic curvature of maize coleoptiles (Nishimura et al., 2009). Treatment with PEO-IAA also resulted in the lateral roots of tomatoes becoming less

vertical (Figure 6.14) suggesting that the mechanism by which the lateral roots become less vertical is not through the TIR1 auxin response pathway. Indeed there is an additive effect, when treated with IAA and PEO-IAA the lateral roots become less vertical than those treated with PEO-IAA alone (Figure 6.14), it is possible that the mechanism by which auxin controls root angle in tomatoes is mediated by a different AFB as the selectivity of different auxinic herbicides is thought to be dictated by the size and shape of the binding pockets of the different AFBs (Song, 2014). It was also decided to test whether changes in auxin transport were possible mechanisms for making the lateral roots less vertical. 1-N-napthylphthalamic acid (NPA) is a known inhibitor of polar auxin transport (although its mechanism of action is unclear it has been postulated that there is an NPA binding protein) (Friml and Palme, 2002), previous work has shown it to have an effect on GSA control in Arabidopsis as when treated with NPA there is a reduced outwards bending upon clinorotation of both lateral roots and shoots (Roychoudhry et al., 2013). Upon treatment with NPA the lateral roots of tomatoes become less vertical (Figure 6.15) suggesting that whilst auxin transport is important for the maintenance of GSAs in the lateral shoots of Arabidopsis it is not involved in the mechanism by which the lateral roots of tomatoes become less vertical upon auxin treatment. Auxin is thought to inhibit internalisation of the PIN auxin efflux carrier proteins by a mechanism independent of TIR1 mediated signalling; PEO-IAA in addition to blocking TIR1 related signalling, also inhibits the internalisation of PIN1 and PIN2 in plants treated with the vesicle trafficking inhibitor Brefeldin A (BFA) (Robert et al., 2010), as the lateral roots of tomatoes become less vertical upon treatment with PEO-IAA (Figure 6.14) this also supports the a lack of changes in auxin transport in the mechanism by which tomato lateral roots become less vertical. It is worth noting that in treatments with IAA and 2,4-D the other known phenotypes associated with an increase in auxin, increased root hair length and decreased root elongation (Overvoorde et al., 2010) are also observed (Figures 6.12, 6.13 and 6.14) suggesting that the root angle altering mechanism is independent of the auxin dependent mechanisms controlling root and root hair elongation.

Tomatoes are part of a large family of plants called the Solanaceae which contains many important crops including potatoes, chilli and sweet peppers, aubergine and tobacco and the model plants petunia and *Nicotiana benthamiana*. Given that the lateral roots in many plants (Arabidopsis (Roychoudhry et al., 2013), Bean (Roychoudhry et al., 2017), wheat and rice (see Chapter 3)) become more vertical when treated with exogenous auxin, other members of the Solanaceae were treated with auxin to determine if the less vertical lateral roots were restricted to only tomatoes or the wider plant family. The lateral roots both chilli pepper (*Capsicum annuum*) and *Nicotiana benthamiana* become more vertical when treated with auxin however the roots of aubergine (*Solanum melongena)* become slightly less vertical (Figure 6.16). It is possible that this effect of auxin on lateral root GSA is confined to the genus *Solanum*, *Solanum* is a large genus found across the globe, it contains over 1400 species and can be split into twelve clades. Tomato and aubergine are contained within two separate clades (Bohs, 2005) this suggests that this reaction to auxin could have originated before the clades began to split and be across the whole genus. Additional work could confirm the response of aubergine to auxin and compare how similar it is to that of tomato before determining how widespread the response is across the genus.

To conclude, examples of non-vertically growing primary roots were tested to determine if they were maintained in the same manner as that of lateral roots. It was found that neither the primary roots of Cvi-0 or *S. pennellii* maintain their non-vertical primaries using the same mechanism as the lateral roots of Arabidopsis although in both cases the angles are maintained, this could be due to a number of possible mechanisms including interactions between gravitropism and circumnutation and alterations to the cytoskeleton between non-vertically growing primaries and vertically growing primaries. In addition to this, a novel response of the lateral roots of tomatoes to auxin was found. The lateral roots of tomatoes becoming less vertical upon application of exogenous auxin appears to be independent of TIR1 auxin signalling and auxin transport. This response could potentially be common to members of the genus *Solanum* of which a number are important food crops.

6.4 Materials and Methods

6.4.1 Cvi-0 reorientations

Seeds of *Arabidopsis thaliana* ecotype Cvi-0 (Cape Verde Islands) were sterilized using chlorine gas. Seeds were placed onto 120 mm square ATS agar plates and cold treated at 4˚C for two days. Plants were grown at 20˚C constant, 16 Hr photoperiod for 6 days. Plants were photographed, reoriented by 90˚ and allowed to grow for 24 Hrs before being photographed again. Root angles were analysed using ImageJ.

6.4.2 Cvi-0 hormone treatments

Plants were placed onto 120 mm square ATS agar plates and cold treated as for the reorientations. Plants were grown at 20˚C constant, 16 Hr photoperiod for 5 days before being transferred to 120 mm square ATS agar plates containing the relevant hormone, the root tip of each plant was marked on the plate. Plants were allowed to grow on the hormone containing media for 7 days before being photographed. Root angles were measured using ImageJ.

6.4.3 Shoot branch angle phenotype of Col-0 and Cvi-0

Seeds of Arabidopsis ecotypes Col-0 and Cvi-0 were placed onto moistened compost and cold treated for 2 days at 4˚C. Plants were grown at 20˚C constant, 16 Hr photoperiod, 60% RH until they bolted and two cauline branches of around 3-5 cm in length were produced from the primary shoot (at around 4 weeks old). Plants were photographed and shoot angles of six 0.5 cm sections were measured from the base of the cauline branches using ImageJ. Ten branches were measured per ecotype.

6.4.4 Solanum pennellii and tomato reorientations

To sterilize both *Solanum pennellii* and Wt tomato seeds, seeds were placed in a syringe, a solution of 50% household bleach was then drawn into the syringe and the seeds were left in the solution for 30 minutes. The bleach solution was ejected from the syringe and numerous washes of clean deionised water were

used to remove the bleach from the seeds. Seeds were placed onto 120 mm square ATS agar plates and placed at 4˚C for two days. Plants were grown at 20˚C constant, 16-hour photoperiod for 5 days for primary reorientations, for lateral reorientations plants were then transferred to plates containing either a mock treatment or 10 nM IAA and allowed to grow for a further 2 days before reorientation. In both cases plants were photographed before being reoriented, 90˚ for primary roots and 45˚ for lateral roots. Plants were allowed to grow for 24 hours before being photographed a second time. Root angles before and after reorientation were measured using ImageJ.

6.4.5 *S. pennellii* and Cvi-0 clinorotation

Plants of *S. pennellii* and Cvi-0 were grown on ATS agar plates as detailed above for their respective reorientation experiments. Plates were photographed before being wrapped in aluminium foil to exclude light and being left to rest for 1 hour. Plates were attached to a variable speed clinostat set at 1 RPM and placed perpendicular to the plants original direction of the gravity vector. Plants were clinorotated for 6 hours at room temperature before being imaged for a second time, images were compared to determine if an outward bending had taken place in the primary root.

6.4.6 Tomato hormone treatments

Seeds were sterilized, placed onto 120 mm square ATS agar plates and cold treated as for the reorientations. Plants were grown at 20˚C constant, 16-hour photoperiod for 5 days before being transferred to 120 mm square ATS agar plates containing the relevant concentration of hormone (IAA, 2,4-D, PEO-IAA and NPA). Hormones were added to the liquid ATS medium to the correct concentration before the plates were poured; see Chapter 2 for stock concentrations and solvents. Plants were allowed to grow on the IAA containing plates for a further 5 days before being photographed. Root angles were measured using ImageJ.

6.4.7 Aubergine, Chilli and Nicotiana auxin treatments

Seeds of Aubergine “Czech Early” and Chilli pepper “Jalapeno” were sterilized as for tomatoes using bleach, seeds of *Nicotiana benthamiana* were sterilized using chlorine gas. Seeds were placed onto 120 mm square petri dishes of ATS agar and cold treated at 4°C for 48 hours. Plates were placed at 20°C constant 16 hour days for 5-10 days (depending on growth rate of species) before plants were transferred to 120 mm square ATS agar plates containing 50 nM IAA. Plates were placed back to grow until sufficient laterals were produced for analysis, plates were scanned and root angles analysed using ImageJ as described previously.

Chapter 7 : General Discussion

For over two hundred years the mechanisms behind how plants respond to gravity have both puzzled and fascinated scientists, from the early plant anatomist Nehemiah Grew, to the gentleman scientist Thomas Knight and the great Charles Darwin (Meidner, 1985, Knight, 1806, Darwin, 1880), to this day these mechanisms are not fully understood (Baldwin et al., 2013). Response to gravity is a key aspect that is central to the final shape of a plant as it is the only external stimulus that is constantly acting upon it. The green revolution of the mid 20th century introduced dwarf cultivars of rice and wheat, this reduced lodging (the prostration of the tillers due to events such as storms) and increased yield (Lynch, 2007). This emphasises how the overall architecture of a plant can be a major factor in its success as a crop and how by modifying this architecture we can improve crop yields, a key issue affecting humanity as we move through the 21st century, as an increasing global population puts more pressure on the land to satisfy its growing food requirements (Lynch, 2013).

Lateral growth at a non-vertical angle away from the primary axis is a key adaptation to optimise the plants ability to capture resources such as light, by careful positioning of shoots and leaves, and water and nutrients by regulating the distribution of the root surface area within the soil profile. Much of the work thus far carried out on gravitropism has focused on the primary axis (i.e. roots that grow vertically downwards and shoots that grow vertically upwards) and only recently have the mechanisms behind non-vertical growth begun to be studied. One key aspect of non-vertical growth is gravitropic setpoint angles (GSAs), angles that are maintained with respect to the direction of the gravity vector regardless of whether the plant is moved from its original orientation (Roychoudhry et al., 2013). It is thought that the maintenance of these angles is controlled by two opposing fluxes (both a gravitropic and an antigravitropic flux) of the phytohormone auxin acting in balance to produce stable, non-vertical growth (Roychoudhry et al., 2013). Whilst the maintenance of GSAs is beginning to be understood, the processes involved in the setting of GSAs, and

the differences between the GSA maintaining non-vertically growing lateral organs and the vertically growing primary root and shoot are still virtually unknown.

The initial work carried out on GSAs and their maintenance was done in the model plant *Arabidopsis thaliana* (Roychoudhry et al., 2013), however their existence (and, if confirmed, the mechanisms of their maintenance) in other plants was unknown. It was decided to study the cereal crops wheat and rice to determine if their non-vertically growing organs maintained GSAs, and, if they did, how similar were the mechanisms behind how these GSAs were maintained to Arabidopsis. It was found that both the non-vertically growing seminal roots of wheat (Figures 3.2 and 3.3), the crown roots of rice (Figure 3.2 and 3.4) and the tillers of both wheat and rice (Figure 3.8 and 3.9) induced growth to return towards their original angles when reoriented, this demonstrates active maintenance of these angles and suggests wheat and rice are maintaining GSAs. This was further demonstrated by clinorotation, this removes the plants reference to gravity, in the absence of a gravitropic stimulus, and therefore a gravitropic auxin flux, the antigravitropic flux continues to act resulting in outwards bending of the non-vertically growing organs, the outwards bending is seen in both Arabidopsis (Roychoudhry et al., 2013) and rice and wheat roots and tillers (Figure 3.1 and 3.7). Final tests were carried out to demonstrate the involvement of auxin in GSA maintenance in wheat and rice, the lateral roots of Arabidopsis become more vertical when treated with exogenous auxin (Roychoudhry et al., 2013), this was also seen in the seminal roots of wheat and the crown roots of rice (Figure 3.6). Overall it was found that GSAs are maintained in the cereal crops wheat and rice and that they are maintained by similar mechanisms to those seen in Arabidopsis. This demonstrates a cross species conservation of these angle control mechanisms and how research into GSA maintenance in Arabidopsis and other species can translate into crop species. This can then be used for the improvement of architecture to give greater yields or increased tolerance to suboptimal conditions. It also allows application of the understanding of GSA maintenance mechanisms to research involving a number of existing mutants and genes within cereal crops that are known to affect architecture such as the wide tiller

angles of the *lazy1* mutation (Yoshihara and Iino, 2007), the narrow tiller angles of the *tac1* mutant (Yu et al., 2007) and the deeper roots resulting from overexpression of *DEEPER ROOTING 1* (Uga et al., 2013).

The variation in form across the plant kingdom is vast with many structures existing in one subset of plants but not another, for example the brace roots seen in maize are not found in common beans (*Phaseolus vulgaris*). As is seen in beans, within the same plant the class of an organ has a bearing on its response and angle control, the non-vertically basal roots of bean become less vertical upon treatment with lower concentrations (50-70 nM) of auxin only becoming more vertical using higher concentrations (90-100 nM) whereas the true lateral roots become more vertical upon all auxin treatments in a manner similar to that of Arabidopsis (Roychoudhry et al., 2017). Further work could be done to determine which structures within plants are being maintained at GSAs, for example the horizontally growing rhizomes of ginger (*Zingiber officinale*) and the runners of bamboo (e.g. *Sasa palmata*), and if a GSA is being maintained, what aspects of that maintenance are common with Arabidopsis, rice and wheat. This could be used to establish a universal model of GSA maintenance for all plant organs.

Wheat is one of the world's most important crops and provides 20% of all calories consumed by humans worldwide (Dubcovsky and Dvorak, 2007). A screen was carried out on TILLING lines with the aim of identifying genes involved in the control of root angle in wheat, root angle is an important element in the architecture of the root system. Root architecture is known to have a great effect on wheat yield in water limiting environments (Manschadi et al., 2006) and mutants with altered root growth GSAs could be used to tailor wheat root architectures to different soil types. For example, a deep, long root system could be advantageous for the uptake of nitrates and water from the lower layers of the soil whereas a shallower root system, although vulnerable to drought, could be of advantage for topsoil foraging of phosphates (Lynch, 2013). In order to search for wheat mutants with altered architectures, 397 TILLING lines were screened in a 2D, germination pouch based system, a wide range of root architectures were found amongst the different lines. A

number of different architecture traits contribute to the overall hull area encompassed by the root system, with the length and root angle of the non-vertically growing seminal roots providing a greater contribution than primary root traits, highlighting the importance of the non-vertically growing roots in resource capture (Figure 4.6). This, along with the ability of nutrient deficiency to modulate the root GSA of a number of plants (Roychoudhry et al., 2017), further demonstrates the need to understand the mechanisms of GSA control across a number of different species in order to produce more efficient root systems for resource capture, and to increase yield from lower input farming and sub-optimal soil conditions such as the phosphate poor soils of Africa, Asia and Latin America (Lynch, 2013).

Lines were divided into four archetypes for further testing (Figure 4.8), these included long and more vertical, long and less vertical, short and more vertical and short and less vertical, it was found that the length and angle traits segregated within spikes (Figure 4.9) confirming that the traits of length and angle are not linked (Figure 4.4). Changing multiple traits is not required to improve crop performance, it has been shown that changing a single trait such as angle can create an advantageous phenotype e.g. drought tolerance without the need to modify other factors (Uga et al., 2013). However, whilst modifications to a single trait may result in improvements to crop performance to achieve true “ideotype” root systems a greater understanding of the control of a number of traits will be needed. Root architecture in cereal crops is complex and is a combination of a number of factors including length and angle of a number of different root types generated at different vegetative stages (Araki et al., 2002). A field trial was carried out on lines with only altered root angle, those with more vertical and those with less vertical lateral roots than the majority of the other lines screened, to determine if the traits seen in the 2D system applied to the 3D, heterogeneous environment of the soil. Of the four lines tested it was found that two lines, one more vertical and one less vertical, displayed an altered angle phenotype than that of the wild type with a larger percentage of their roots being either at a more vertical angle or a less vertical angle (Figure 4.12). Whilst 2D screening is higher throughput and logistically easier in terms of maintaining constant conditions and measurement of root traits this highlights

the importance of field-testing or at least growth in the 3D environment of the soil to validate phenotypes found in 2D screening systems. This ensures that the root architecture phenotypes of lines such as the ones found in this work translate into the soil and have the potential for use to incorporate genes into elite breeding lines to be used in agriculture. The traits gained from these lines could be used towards the creation of ideotype root systems, such as the "Steep, Cheap and Deep" architecture proposed by Lynch to optimise water and nitrate uptake in maize root systems, which has been hypothesised is applicable to a number of other cereal crops including wheat (Lynch, 2013).

Beyond the "Steep, Cheap and Deep" ideotype, the contribution of different traits to encourage shallower and deeper rooting could be explored further and exploited to tailor individual wheat cultivar root systems to different soil types, for example: a shallower root system may be of advantage in the clay soils of Yorkshire as although clay soils hold onto nutrients well they are also prone to waterlogging, a shallower root system would raise a larger proportion of the roots above the high water table. Whereas a deeper root system may be of advantage on the sandy soils of Norfolk to allow the plant to access reserves of water and nitrates from lower layers of the soil as sandy soils are prone to drought and nutrient leeching. By combining individual traits, forms of the same cultivar (e.g. Cadenza) with distinct root architectures e.g. steep and deep vs wide and shallow could be produced to improve performance on different soil types whilst maintaining all the other advantageous traits of that cultivar such as tiller number and grain size.

Using sequence information about the mutations in each TILLING line and a subset of lines with a similar phenotype e.g. more vertical roots, it is possible that a number of mutations will be common to that phenotype and could be used to establish a QTL for that trait. Using these QTLs and existing knowledge of the pathways involved in GSA setting and maintenance from Arabidopsis could enable the exploration of GSA maintenance mechanisms in wheat on a molecular level, and could again work towards establishing a universal cross-species model of GSA maintenance.

As the similarity between GSA maintenance in Arabidopsis and crops such as rice and wheat had been demonstrated, an EMS mutagenesis based screen was carried out in Arabidopsis to find mutants with altered root GSAs to help explore the mechanisms behind GSA maintenance and the setting of GSAs. A number of mutants were found, some with interesting phenotypes not related to the lateral root GSA (Figure 5.19). Two mutants were found with strong, consistently more vertical lateral root GSA phenotypes (Figure 5.2 and 5.9), neither mutant demonstrated a shoot angle phenotype (Figure 5.3 and 5.10) or any reduction in gravitropic capacity (Figure 5.4 and 5.11) suggesting that genes carrying the causal mutations were involved in the setting of the GSAs as opposed to their maintenance, in the roots alone. One mutant (71.14.4 MV) carried a recessive mutation (Figure 5.13) whereas the other mutant (80.2.1 MV) carried a dominant mutation (Figure 5.7). As it had the stronger phenotype it was decided to genotype the dominant mutant, 80.2.1 MV and it was found that the causal mutation was a single amino acid change in the gene *AtLAZY4* (Figure 5.16). *AtLAZY4* is a member of the *LAZY* family, a group of 6 genes known to be involved in the control of lateral branch angle in Arabidopsis, *AtLAZY4* is highly expressed in the root tip (Yoshihara and Spalding, 2017) and is known to alter the root GSA as the knockout mutant, *atlazy4*, displays a less vertical lateral root GSA (Supplementary Figure 5.2) and no gravitropic defects (although triple mutant knockout, *atlazy1 atlazy2 atlazy4,* has lateral roots that grow upwards (Ge and Chen, 2016)). The mutation within 80.2.1 MV is a change at position 145 of the amino acid sequence, in the wild type version this residue is an arginine but in the mutant it is an alanine, this was confirmed by site directed mutagenesis and, interestingly, it does not appear to matter what the amino acid at this position is changed to as multiple different mutations resulted in the same phenotype (Figure 5.16). This suggests that it is the lack of the arginine at this position rather than a gain of function from another amino acid that results in the phenotype. It was found that this mutation is contained within a motif that is conserved in the Arabidopsis LAZY2, and the LAZY4 and LAZY2 homologs of a number of other species including crops such as Wheat, Rice (interestingly, the protein originally thought to be the LAZY4 homolog in rice, DRO1, does not contain this motif and is very different in sequence to the Arabidopsis LAZY4 (Uga et al., 2013) although another protein in rice does contain the motif and is close to AtLAZY4 in sequence), soybean, tomato and

cabbage (Figure 5.17). Ecotypes with a naturally occurring polymorphism in this motif (V143A) also display more vertical lateral roots than Wt Col-0 (Figure 5.18) suggesting that other residues in the motif are important to its function, further experiments using site directed mutagenesis could be carried out to delineate the conserved residues involved in angle control and further probe the mechanism of action by which LAZY4 is involved in the setting of GSAs.

Due to the almost universal conservation of this motif across plant species it is possible that the mutation resulting in more vertical lateral roots in Arabidopsis could be replicated in a number of different species, for example, in elite crop lines, as a means of introducing steeper rooting without having deleterious effects on other aspects of the plant making it an ideal candidate to attempt to introduce the “Steep, Cheap and Deep” ideotype proposed by Lynch (Lynch, 2013). The dominant nature of the mutation also works in its favour for use in crops as it would be able to easily introduce the steeper rooting phenotype into polyploid crops such as wheat where the effects of a recessive mutation would be easily masked by other copies of the gene (Uauy et al., 2009, Lawrence and Pikaard, 2003). Using this mutation and a detailed study of the function of the other highly conserved residues in this region, perhaps by further site directed mutagenesis, it is possible that through simple, single amino acid changes we could modulate the non-vertical growth angles of a number of crop species allowing us to produce ideotype root systems such as “Steep, Cheap and Deep” (Lynch, 2013) and beyond, to potentially tailoring the root angles of the different kinds of roots through differential expression e.g. more vertical seminal roots for better nitrogen and water acquisition but less vertical crown roots for better topsoil foraging for phosphorus. This would allow us to achieve the levels of crop yields required whilst lowering the input from artificial fertilisers as the plant would be more capable of gathering resources for itself, this would reduce the impact of the use of artificial fertilisers on the environment and reduce the cost for farmers to produce large quantities of crops, thus lowering their price.

The application of exogenous auxin induces a more vertical lateral root phenotype in Wt Col-0 (Roychoudhry et al., 2013), the pARL2::axr3-1 mutant contains a stabilised form of IAA17 and should therefore have a less vertical

phenotype as the Aux/IAA cannot be degraded via the TIR1 auxin response pathway resulting in transcriptional repression of auxin responsive genes . However, the pARL2::axr3-1 mutant shows a more vertical lateral root phenotype similar to that of 80.2.1 MV, it also has a similar gravitropism kinetics phenotype (Figure 7.1). The knockout mutant of *atlazy4* has a less vertical lateral root phenotype (Supplementary Figure 5.2) suggesting that AtLAZY4 is required to induce vertical growth in the lateral roots. There are a number of ways that the mechanisms of action of *LAZY4* and *AXR3* could be linked for mutations within them to produce such similar phenotypes. The simplest explanation would be that *AXR3* directly transcriptionally regulates the production of *LAZY4*. It has already been shown that auxin negatively regulates the expression of the rice homolog of *LAZY4* (*OsDRO1*)(Uga et al., 2013) and though it has not been proven, if this is the case in Arabidopsis, the reduction in auxin signalling in pARL2::axr3-1 due to the stabilisation of the Aux/IAA could result in increased expression of *LAZY4* and a more vertical root phenotype. qPCR could be used to investigate the expression levels of *LAZY4* and the other *LAZY* proteins in the lateral roots of the pARL2::axr3-1 mutant and determine if the *LAZYs* are linked to *AXR3* via transcription.

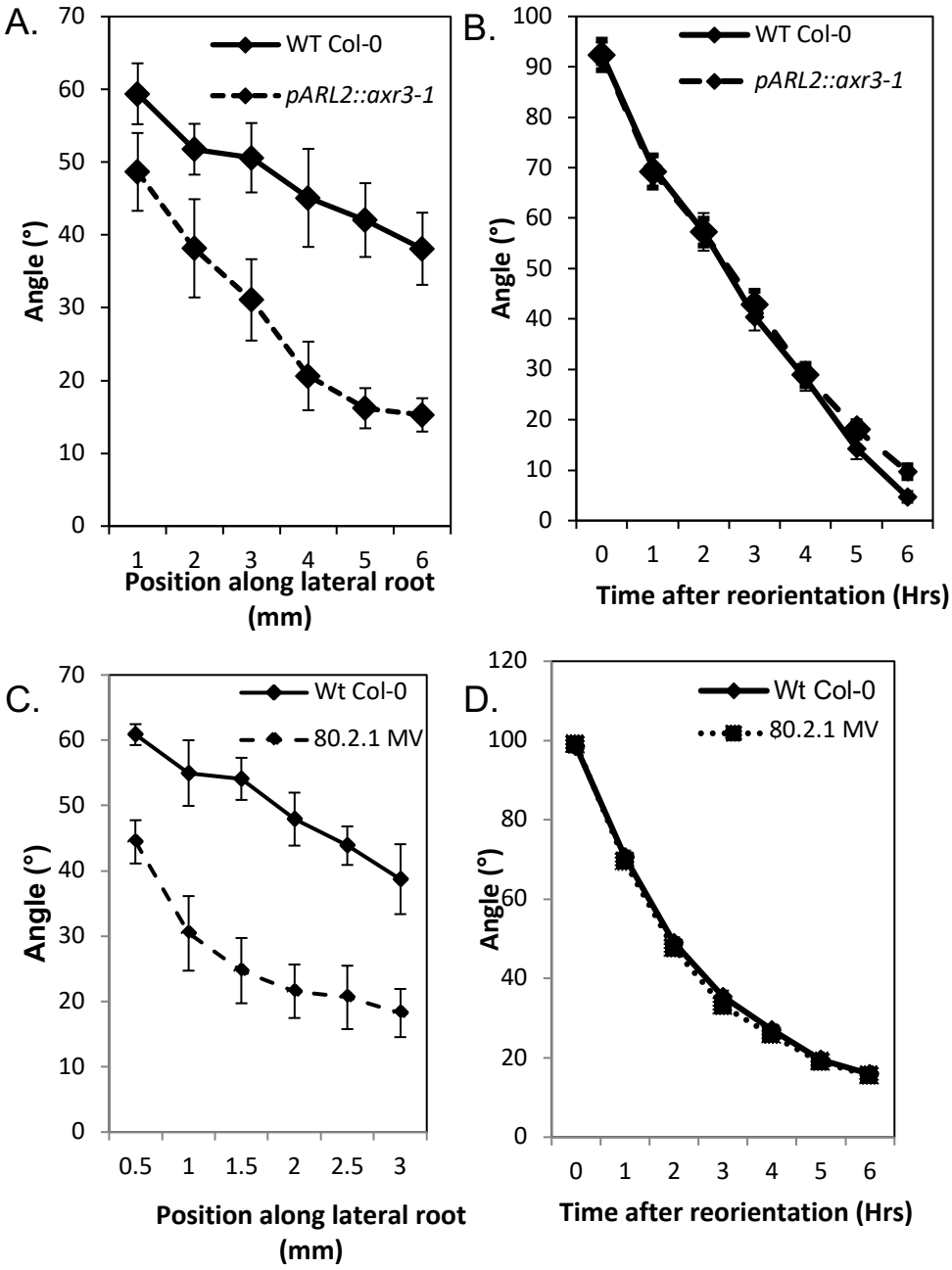


Figure 7.1: Lateral root angle phenotype and gravitropism kinetics of pARL2::axr3-1 and 80.2.1 MV

pARL2::axr3-1 has more vertical lateral roots than Wt Col-0 (**A**) and has no defects in its gravitropism kinetics (**B**). Data courtesy of Dr Suruchi Roychoudhry and Dr Katelyn Sageman-Furnas. 80.2.1 MV has a similarly more vertical lateral root phenotype (**C**) and no gravitropism defects (**D**). Data as seen in Figures 5.2 and 5.4.

Another possibility is that AXR3 controls the activity, trafficking or degradation of *LAZY4* post-transcriptionally via the actions of another protein and that the axr3-1 mutation results in an increase in *LAZY4* activity and more vertical lateral roots. *GFP-LAZY4* fusion proteins expressed in the pARL2::axr3-1 line could be used to determine if protein levels of localisation are affected by the stabilised Aux/IAA, changes in protein levels could also be tested using western blotting with antibodies for either *LAZY4* or the GFP fusion protein.

A third possibility is that *AXR3* is downstream of *LAZY4*, however, it has been reported that *AtLAZY1* localises to both the nucleus and cell periphery, most likely the plasma membrane, but the nuclear pool is not required for its function in shoot gravitropism (Yoshihara and Spalding, 2017, Yoshihara et al., 2013). It is possible that the same is true for *LAZY4* making it unlikely that *LAZY4* is directly interacting with *AXR3* and affecting its function although there is the possibility that another protein could act as an intermediate. To investigate this the pARL2::axr3-1 construct could be transformed into the *atlazy4* knockout mutant, if the phenotype of the pARL2::axr3-1 mutant was produced via a AtLAZY4 dependent pathway the phenotype would not be complemented.

The phenotypes of both mutants could be explained by changes in the transcription, trafficking and activity of the PIN proteins. In the case of AXR3, the PIN proteins are known to be regulated by auxin both transcriptionally and through their activity and stability on the plasma membrane in a feedback dependent manner (Vieten et al., 2005, Adamowski and Friml, 2015), in the pARL2::axr3-1 mutant the enhanced transcriptional repression could lead to either a loss of PIN protein at the plasma membrane or a loss of a protein that controls their cycling or localisation such as GNOM or the WAG kinases (Kleine-Vehn et al., 2008, Adamowski and Friml, 2015) resulting in more vertical lateral roots.

In the case of 80.2.1 MV, given the likely plasma membrane localisation of AtLAZY1 (Yoshihara and Spalding, 2017, Yoshihara et al., 2013). and the involvement of AtLAZY4 in the localisation of PIN3, a possible mechanism for

LAZY mediated control of growth angle could be via the control of PIN cycling and distribution on the plasma membrane. This would therefore control the amount of auxin flow on each side of the organ and the magnitude of the antigravitropic and gravitropic auxin fluxes. Crossing of the 80.2.1 MV mutant and the *atlazy4* knockout mutant with GFP marker lines for PIN3, PIN4 and PIN7, known to control auxin flow out of the columella cells (Rosquete et al., 2013), could be used to determine if control of PIN localisation is the mechanism by which LAZY4 controls root angle, and, if so, how the mutation at R145 affects PIN distribution. For example, the mutation could result in an internalisation of PINs thus resulting in a reduction of auxin efflux; this could explain the mild effect of exogenous auxin on the lateral root angles of 80.2.1 MV (Figure 5.6).

Within the Arabidopsis LAZY family the motif carrying the 80.2.1 MV mutation is only found in AtLAZY2 and AtLAZY4, both of which are expressed in the root and not the shoots, the phenotypes of the knockout mutants support this as they only show altered growth angles in the roots (Yoshihara and Spalding, 2017, Taniguchi et al., 2017). AtLAZY1 has a dramatic shoot angle phenotype and is highly expressed in the shoots but has no root phenotype (Taniguchi et al., 2017, Yoshihara et al., 2013, Yoshihara and Spalding, 2017). As AtLAZY1 does not contain the conserved motif from LAZY4 (Figure 5.15) it is likely that its angle control function is mediated via a different mechanism than that of the root LAZYs (AtLAZY4 and AtLAZY2), this could indicate different angle control mechanisms in roots and shoots. The extent to which the systems are different would be dependent upon how central the role of the LAZYs is in angle control and how much can be explained by the differing responses to auxin of the roots and shoot (Chen et al., 1999). This could be explored by expression of a mutagenized AtLAZY4 e.g. LAZY4 R145A under a shoot specific promoter to determine if the more vertical phenotype seen in roots due to this mutation also manifests in the shoots.

To attempt to uncover what differentiates a non-vertically growing lateral from a vertically growing primary (i.e. what induces the maintenance of non-vertical growth, in essence what makes a lateral, lateral) comparisons were made

between lateral roots and examples of non-vertically growing primary roots. The primary roots of the Arabidopsis ecotype Cvi-0 and the tomato species *Solanum pennellii* both exhibit a skew in the growth of their primary roots making the tip of the root grow at a non-vertical angle (Figure 6.10). Interestingly, in both Cvi-0 and *S. pennellii* the lateral root skew phenotype can be corrected to a vertically growing one by application of the synthetic auxin 2,4-D but not the natural auxin IAA (Figures 6.1, 6.2 and 6.8). There are a number of differences between the way that plants transport and respond to IAA and 2,4-D. 2,4-D has a much weaker ability to activate the auxin receptor TIR1 making it a poor auxin at receptor level (Kepinski and Leyser, 2005), however this can be countered by high concentrations caused by accumulation within the plant cells. 2,4-D can be transported into cells by the auxin importer AUX1 (Marchant et al., 1999) but is a poor substrate for the efflux transporters (Delbarre et al., 1996) and is not metabolised by the plant (Song, 2014) resulting in a build up. A likely explanation for the differing abilities of IAA and 2,4-D to correct the root skews of Cvi-0 and *S. pennellii* is through their actions on the cytoskeleton, a number of mutants exhibiting root skew phenotypes contain alterations to the components of their cytoskeletons (Furutani et al., 2000, Thitamadee et al., 2002), 2,4-D removes actin and slows down cytoplasmic streaming whereas IAA has little effect on the extent of the actin cytoskeleton (Rahman et al., 2007). It is possible that it is the ability of 2,4-D to modify the cytoskeleton that is key to correcting the root skew phenotype of Cvi-0 and *S. pennellii,* this also suggests that the root skew phenotypes could be due to changes in the cytoskeleton as opposed to maintenance of a GSA as in the lateral roots.

A number of gravitropism based assays such as reorientations (Figures 6.7 and 6.9) and clinorotation (Figures 6.6 and 6.11) were carried out on Cvi-0 and *S. pennellii,* and although both non-vertical primaries exhibited angle maintenance when reoriented, neither displayed outwards bending of the primary root tip on the clinostat suggesting that these non-vertical primaries are not maintained by the same mechanism as in lateral roots. The non-vertical primaries are more likely to be a result of an equilibrium between gravitropism, the skewing of the root tip due to circumnutation, and interactions with the gel surface upon which they were grown (Thompson and Holbrook, 2004). Circumnutation is caused by

uneven growth on opposing sides of the root and can be linked to rotation of the cell files in the arabidopsis root as it grows (Migliaccio et al., 2013). Skewing mutants such as those that exhibit cytoskeletal abnormalities also exhibit alterations to their circumnutation (Migliaccio et al., 2013, Furutani et al., 2000). This, combined with the differing effects of IAA and 2,4-D, supports the theory that the non-vertically growing primaries of Cvi-0 and *S.* pennellii are due to an equilibrium between gravitropism and circumnutation. Although this meant that these examples of non-vertically growing primaries could not provide any insight into what induces the non-vertical growth in laterals, as a result of auxin treatments on these plants another interesting observation was made relating to the lateral roots of tomatoes. The application of auxin, in both tomato and Arabidopsis, inhibits cell elongation. In Arabidopsis, the *tir1-1* mutant is less sensitive to the growth inhibitory effects of auxin than wild type (Ruegger et al., 1998). In Arabidopsis lateral roots, the addition of exogenous auxin induces a more vertical GSA, and the *tir1-1* mutant displays a less vertical GSA (Roychoudhry et al., 2013). These evidences suggest that the pathways via which auxin controls root elongation and growth angles are TIR1-dependent. However, in tomatoes, treatment with exogenous auxin results in the lateral roots assuming a less vertical angle (Figure 6.12). Further, treatments with auxin signalling and transport inhibitors suggest that the mechanism by which auxin regulates GSA in tomato is independent of both TIR1 auxin signalling and auxin transport, and could act through an alternative pathway. Whether the TIR1-dependent pathway is responsible for the inhibition of root growth is also still to be demonstrated. Further studies with knockout mutants of the tomato homologs of the AFBs and TIR1 could be used to determine through which pathway auxin affects lateral root growth and angle in tomato.

The lateral roots of aubergine (*Solanum melongena*) also become slightly less vertical upon the application of exogenous auxin (Figure 6.16), although this is only significantly so adjacent to the junction of the primary and lateral roots, this could suggest that this is a feature of the genus *Solanum.* This genus also contains the crop species, potatoes (*Solanum tuberosum*), tamarillo (*Solanum betaceum*) and wonderberry (*Solanum retroflexum*). It would be interesting to see how this difference in root angle response to auxin relates to *LAZY4* as the

rice homolog of *DRO1* has been reported to be negatively regulated by auxin (Uga et al., 2013), although it is currently unknown whether the same is true in Arabidopsis, to which the tomato LAZY4 is more closely related in sequence. Perhaps the mutation at the conserved arginine that induces more vertical lateral roots in Arabidopsis would have an opposing effect in tomatoes and result in less vertical lateral roots. It is possible that by studying the difference in lateral root response to auxin between tomatoes and Arabidopsis could provide new insight into the mechanisms that set and control the maintenance of GSAs. Further studies could be carried out using transgenic lines based upon tomato homologs of auxin transport and response genes (e.g. a stabilised version of tomato homolog of IAA17 such as in the Arabidopsis *axr3-1* mutant) to dissect the pathway through which the unusual root angle response to auxin is mediated and how auxin is involved in root angle control in tomatoes.

To conclude, unlocking the mechanisms behind non-vertical growth in plants could provide a key turning point in our efforts to improve plants for our own benefit. Non-vertical growth is the main way in which plants shape themselves for maximum resource capture, understanding what is behind it, and what controls it, gives the potential for it to be modified so that resource capture can be improved in specific ways to suit specific purposes, be it fastigiate fruit trees to allow more trees to be grown in a given area, or deeper roots to allow crops to be grown on drought prone soils. Gravity could be considered the “baseline” stimulus for the establishment of plant architecture as it is the only growth shaping force that is constantly acting upon the plant (as opposed to light or touch for example), therefore an understanding of how gravity relates to non-vertical growth through the gravitropic setpoint angle is critical to allow us to make these plant architectural changes. This work has established that gravitropic setpoint angles exist in economically important plants, explored the potential for different plant architectures in a single species and proved that those architectures can translated from a lab based screen into a “real world’ soil context. Explored the potential for common mechanisms in non-vertical growth in lateral branches with the unusual phenomenon of non-vertical primaries, looked at the differing effects auxin has on lateral root angle in two different species and found a point mutation that could not only helps towards

our understanding of GSA setting and maintenance but could also have the potential to be applied to crops in order to change their root architecture for our benefit. Hopefully this has gone some way to contribute to our understanding of the mechanisms of non-vertical growth in plants and with further work could provide us a means with which to modify plants to alleviate both the economic and environmental pressures facing the planet through the rest of this century and beyond.

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