إِنَّهُ مِنْ سُلَيْمَانَ وَإِنَّهُ بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

CONTROL OF ADVENTITIOUS ROOT FORMATION IN ARABIDOPSIS

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Abstract

Adventitious or de novo root organogenesis is a process that occurs from wounded or detached plant tissues or organs. In tissue culture experiments, the available hormone concentrations in the medium play significant roles in inducing adventitious roots. However, regeneration from detached organs in natural conditions depends on endogenous hormones. To imitate natural conditions, *Arabidopsis thaliana* Col-0 leaf explants were cultured on B5 medium without any added hormones, in order to investigate the endogenous hormonal signalling and molecular mechanisms that lead to de novo root organogenesis. Use was made of a series of hormone signalling reporter lines in transgenic Arabidopsis, to understand better the roles of auxin, cytokinin, ethylene and gibberellin signalling. Cell proliferation was monitored over a developmental time course, and the expression of a number of genes, and their functional roles through mutant analysis, was also investigated during the regeneration process. It was demonstrated that auxin, gibberellin and cytokinin signalling becomes focused at the wound site in the petiole, associated with the induction of adventitious roots. Auxin signalling-defective mutants such as *axr1*, *axr3* and *pls* were unable to form adventitious roots as well as wild type, reflected in defective expression of auxin pathway genes such as *YUC* family genes and *WOX5*. *pls* and *axr1* were also found to be defective in the expression of the transcription factor gene *NAC1*. Mutants and transgenic overexpression lines for transcriptional regulators RAP2.7, MDF1 and NAC1 showed that the three genes are required for adventitious root formation, and function in an auxin-independent manner to mediate root regeneration. Adventitious root formation from the Arabidopsis leaf therefore requires coordinated expression of a number of transcription factors that work in both an auxin-dependent and -independent manner, and cross talk between auxin and other hormones is important for correct organogenesis.

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Statement of authorship

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

Statement of copyright

List of Abbreviations

ACT2	ACTIN2
AXR1	AUXIN RESISTANCE1
AXR3	AUXIN RESISTANCE3 gene
Col-0	Columbia
CLV3	CLAVATA3
DNA	Deoxyribonucleic acid
DNRR	De novo root regeneration
EDTA	Ethylenediaminetetraacetic acid
EIN2	Ethylene insensitive2
GA	Gibberellic acid
GFP	Green fluorescent protein
GUS	β-glucuronidase
HM	Homozygous
HZ	Heterozygous
IAA	Indol-3-acetic acid
IBA	Indole-3-butyric acid
IPA	Indole-3-propionic acid
KNOX	KNOTTED LIKE HOMEOBOX
LRs	lateral roots
MDF	MERISTEM-DEFECTIVE
MQ	Milli-Q
mRNA	Messenger RNA
MS	Murashige & Skoog
NAA	Naphthalene acetic acid
NASC	Nottingham Arabidopsis Stock Centre
NPA	N-1-naphthylphthalamic acid
PAT	Polar auxin transport
PFA	Paraformaldehyde
PLS	POLARIS
PR	Primary root

QC	Quiescent centre
qRT-PCR	Quantitative real time PCR
RAM	Root apical meristem
RF	Root founder
RP	Root primordium
RNA	Ribonucleic acid
RNA-Seq	RNA Sequencing
RP	Right specific primer
RSA	Root system architecture
RT	Reverse transcription
SAM	Shoot apical meristem
TAIR	The Arabidopsis Information Resource
WOX5	WUSCHEL-LIKE HOMEOBOX5
WT	Wild-type
WUS	WUSCHEL
YUC	YUCCA

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Chapter 1. Introduction:

1-1 Tissue culture in plants

Plants and animals are most commonly both created from sperm and egg cells in the process called fertilization. After the primary fusion of gametes, both plants and animals go through similar processes in which the cells divide and differentiate into specialized cells (Sugimoto et al., 2011). However, some cells stay undifferentiated and they can form different types of new cells following division. In animals, this process is very limited because differentiated cells cannot dedifferentiate to make other cell types or make a whole new animal. However, in plants this is very different, because every cell of the plant, with the exception of anucleate xylem cells, has the ability to form a complete plant and this process is called totipotency. Animal cells do retain pluripotency, i.e. the nuclei retain the genetic material required to specify a new organism, but in practice differentiated animal cells do not regenerate new organisms.

This theory was first tested in 1898 by the German botanist and the father of plant tissue culture, Gottlieb Haberlandt, who first tried to grow excised parts of plants in the lab with an artificial culture medium containing nutrients (Bhojwani and Razdan, 1986). He could not get any cells to divide, but he managed to keep the cells alive in the artificial medium. However, in 1922 Kolte and Robbins were the first to culture plant cells from the root and stem tips and to solve problems of medium contamination (Kolte, 1922; Robbins, 1922). In 1926 indole-3-acetic acid (IAA) was the first natural plant growth regulator to be discovered, by Went (Hussain et al., 2012). Later, other researchers such as Gautheret with White and Nobercourt in 1934–1935 added different components to the medium such as growth regulators (hormones) and vitamins which helped the cells to divide to create an undifferentiated tissue mass (called callus), which was an important step in the creation of differentiated plant tissues (Gautheret, 1939).

During that time plant tissue culture knowledge was developing rapidly, and in 1955 kinetin was discovered, which was considered a 'cell division' hormone. In 1957 Skoog and Miller demonstrated the hormonal control of plant regeneration from callus by adjusting the

balance between auxins and cytokinins in the medium (Skoog and Miller, 1957). A very import technical advance in plant tissue culture was in 1962 when Murashige and Skoog developed what is still known as Murashige and Skoog medium for tobacco cell culture, composed of high concentrations of salts but low in nitrogen, with macro- and micronutrients, sucrose, B vitamins and growth regulators (Murashige and Skoog, 1962).

The traditional hormones that are used in plant tissue culture are auxins, cytokinins and gibberellins (George et al., 2008), but the effects of other hormones on plant development using tissue culture systems include abscisic acid, ethylene, jasmonic acid, brassinosteroids, systemin and oligosaccharides. Auxins are essential in tissue culture for the induction of rooting (Wang and Yao, 2019). Also, auxin plays a major role in inducing adventitious roots in plant cuttings, and in vegetative propagation via tissue culture (Wang and Yao, 2019).

In addition, auxin is essential for cell division and cell elongation. There are a number of natural and synthetic auxins, such as Indole-3-acetic acid (IAA) , Indole-3-butyric acid (IBA), phenylacetic acid (PAA) , Indole-3-propionic acid (IPA) , tryptamine, Naphthalene acetic acid (NAA), 3,6-dichloro-2-methoxybenzoic acid (dicamba), and 4-amino-3,5,6-trichloropiconiolinic acid (picloram). Cytokinins play key roles in cell division and in differentiation (Iwamura, and Koshimizu, 1986). Cytokinins used in tissue culture are commonly kinetin or benzyl aminopurine (BAP), and they have been shown to promote callus induction (Lu, 2020).

Also, cytokinin and gibberellins have been shown to play a part in cell division in callus tissue (Jayaraman, 2015). As an example of tissue culture, Welender et al. (2014) describe the culture of stem cuttings of Arabidopsis on medium containing auxin. On the second day of culture, there was an increase in cytoplasmic density and rounding of the nuclei of the cells. Simultaneously, the first cell division was observed in the lateral bundle parenchyma and neighbouring starch sheath cells, with lower rates of division found in the phloem cap and xylem parenchyma cells. These cells continued to divide until they formed prominent meristematic tissues, obvious on day 3 of culture. Cytological lineages similar to those seen in cells of the root apical meristem indicated the meristematic complexes represent early root primordia. The meristematic complexes progressively developed the shape of root

primordia. From day 4 onward, there was observed a polar organization of the primordia, with cell elongation observed in the proximal part the root. The root tip grew and developed a root cap and strands of procambium, which connected the root tip with the vascular bundle by 6 days of culture. After this, the root grew out through the cortical tissue.

The tissue culture technique has been integral to the production of transgenic plants, with many uses for humanity (Loyola-Vargas and Ochoa-Alejo, 2018). Early example crops include potatoes (Dale and McPartlan, 1992), rapeseed and other crucifers (De Block et al., 1989), but the list is now much larger. Transgenic plant modification has applications in improved crop nutrition, disease resistance, herbicide resistance, growth, and other properties of the edible crop. It is used in industry and in fundamental research. Tissue culture helps to speed up natural processes, such as in propagating plants faster than occurs in the field and for maintaining mutants that may show lethality when grown in soil. Tissue culture for transgenic plant production may involve co-incubation of tissues with Agrobacterium, direct gene transfer into cultured protoplasts, or the biolistic particle delivery system, which involves shooting into cultured cells heavy metal microspheres, such as gold, which are coated with genes of interest (Christou et al. 1990). This technique is very commonly used for the plants that are not readily susceptible to Agrobacterium infection, such as maize or soybean (Christou et al, 1990).

There are many of benefits for using tissue culture technology. According to the United Nations for Food and Agriculture (Food and Agriculture Organization-FAO) in 1994, it was agreed that tissue culture technology provides a valuable method for producing natural foods for citizens. In addition, a report from the International Atomic Energy Agency—Division of Nuclear Techniques in Food and Agriculture - in 2002 stated that tissue culture technology may be a significant method for producing high quality bioactive compounds, with opportunities for economic benefits (Dias et al. 2016). Since tissue culture is carried out in sterile environmental conditions, with controlled light, humidity and temperature, this can overcome problems related to field growth of plants, such as disease and adverse climatic conditions, leading to low yields of secondary metabolites and biomass.

The phenomenon of totipotency means that it is possible to transform any cell from the plant and grow it to form a complete plant containing engineered genes (Wang et al., 1992). There are therefore many important uses of plant tissue culture and understanding the molecular mechanisms behind regeneration is an important objective.

1-2 Arabidopsis as a model experimental organism

1-2-1 Arabidopsis

Arabidopsis thaliana has been used by researchers in plant science for more than 100 years, but it became the model species of choice by the research community in the late 1980s. Its advantages include its small size, rapid lifecycle, small diploid genome and ability to self-fertilize; and its genome was the first plant genome to be sequenced (The Arabidopsis Genome Initiative, 2000). Arabidopsis is small weed, a member of the Brassicaceae family and so is related to many economically important crops even though it is not itself a crop plant. According to the United Nations, Brassicaceae crops are worth $31 billion globally per year (Diaz, 2019). Studies using Arabidopsis are very helpful to understanding many basic biological processes, such as the roles of genes and hormones.

Arabidopsis was first reportedly used by the German botanist Friedrich Laibach (Laibach, 1907), but its wider use was established in the 1940s (Reinholz, 1947) and it is now studied by thousands of researchers around the world who make use of its genome and other genetic resources available online (Diaz, 2019; Pang and Meyerowitz, 1987).

In the work described in this thesis, I studied the formation of adventitious roots from the Arabidopsis leaf *in vitro*, and characterised some genes and hormone signalling pathways involved in the process. This involved the use of mutants as a way to determine whether specific genes are required for the formation of adventitious roots.

1-2-2 The Arabidopsis leaf

The leaf is characterized by multiple cell layers which are highly structured (Schlechter et al. 2019). It represents the main lateral organ of the stem, and growth is across three axes: proximal-distal, medial-lateral, and adaxial-abaxial. In dicotyledonous species, the leaf commonly comprises a blade and a petiole, and consists of several cells types that include epidermal cells (pavement cells, stomatal guard cells, trichomes), palisade cells, spongy meosophyII cells and phloem and xylem cells (Ichihashi & Tsukaya, 2015). Epidermal pavement cells help to determine leaf shape and its microtopography. Leaves have multiple essential functions including photosynthesis, temperature regulation, gas exchange and secondary metabolite secretion (Schlechter et al. 2019).

The leaf petiole supports the leaf blade and can move the position of the blade to the light to maximise photosynthesis (Kozuka et al, 2005). The leaves of Arabidopsis have specialised dorsiventral surfaces, with stomata on the lower (abaxial) surface and trichomes (unicellular hairs) on the upper, adaxial surface. Usually, the shape of Arabidopsis leaves is ovoid but there is natural variation from narrow to wide, related to the genotype (Tsukaya, 2006). The earliest leaves grow in a rosette prior to inflorescence bolting. Usually, the colour of these leaves is dark green with a purple tinge. There are several leaves on the stem (cauline leaves) as well, and these leaves are lighter green in colour and typically without a purple tinge. The rosette leaves have entire margins but sometimes with serration, while the cauline leaves mostly have entire margins.

The leaves of Arabidopsis exhibit a shade avoidance syndrome. When the Arabidopsis plant is exposed to weak light, the leaf petioles elongate and lamina expansion occurs (Reed et al, 1993; Kozuka et al, 2005). In a review, Tsukaya (2006) has discussed how the shape and the size of the Arabidopsis leaf is strongly related to its genotype, and this confirmed by several mutant studies that have revealed a range of shapes, but there is still more research needed to understand better the connection between genotype and phenotype.

1-2-3 Root structure

Roots are essential for sensing and acquiring nutrients and water from soil, although there are challenges to studying roots because their location under the soil and the associated

difficulties in analysing them. But root research is important. There are many studies that show that roots are closely related to the "next green revolution", with importance for food security as the number of citizens significantly increases across the world (Lynch, 2007; Herder et al., 2010). Root system architecture (RSA) is defined as the locative arrangement of the root system and the accurate configuration of the roots axes in the root medium (Lynch, 1995).

Root systems consist of the primary root (PR), lateral roots (LRs), seminal roots, root hairs and adventitious roots (Smith & De Smet, 2012). Roots have the ability to be plastic in their phenotype, which helps the plant change its growth in response to changes in environmental stresses such as reduced water in the soil, reduced nutrients or increased salinity (López-Bucio et al., 2003; Comas et al., 2013; Galvan-Ampudia et al., 2013).
The structure of the Arabidopsis root is extremely ordered (Dolan et al., 1993).

The transverse section of the Arabidopsis root shows radial symmetry with concentric rings of cell files, comprising the epidermis, the cortex and the endodermis, all of these cells surrounding the vascular tissues or stele which are represented by the pericycle, phloem, xylem and procambium (Scheres et al., 1995) (Fig. 1-1). All these different cells type are produced from a group of stem cell initials located at the root tip, which are under the control of, and in contact with, the cells of quiescent centre (QC) which work to maintain the cells in an undifferentiated state (Dolan et al., 1993; van den Berg et al., 1997; Van Berg et al., 1998).

The stem cell niche (SCN) comprises the stem cells and QC. Asymmetric cell division plays a major role in the differentiation process, to create every cell file from a specific stem cell initial and maintains the stem cell through self-renewal while the daughter cell differentiates. In the meristematic zone the cells undergo many cell divisions, after which they begin elongation in the elongation zone, but this may be considered a continuous process - the place between the meristematic zone and the elongation zone is known as the transition zone. The last zone is known as differentiation zone, which contains root hairs, vascular tissues and shows Casparian strip development (Verbelen et al., 2006; Sanz et al., 2009; Petricka et al., 2012).

Thus, many cell divisions and cell elongation contribute to apical root growth and help to push the root tip through the soil (Dolan et al., 1993; Beemster & Baskin, 1998; Petricka et al., 2012). Anatomically, the root can be considered as a cylindrical structure, with outer tissues (the ground and the epidermis tissues) organised as concentric layers around each other. These tissues surround the stele, which includes the vascular tissues (xylem and phloem) surrounded by the pericycle cell layer. The radial symmetry of roots is represented as these concentric tissue layers, which develop co-ordinately with the dual symmetry of tissue in the stele (van Norman 2016).

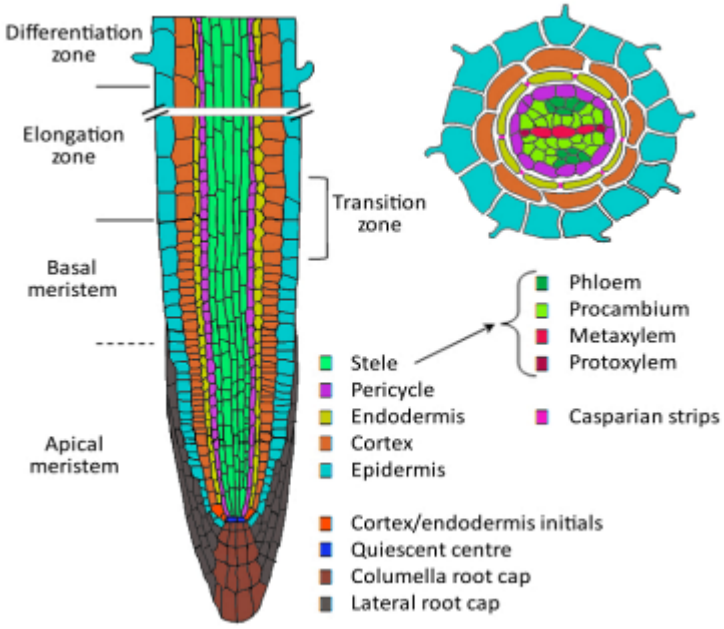


Fig.1-1: Structure of the root in Arabidopsis. Longitudinal section in the root (left), cross section of the root taken from the differentiation zone (right). Different cell types are marked in different colours and developmental zones are indicated. Image adapted from De Smet et al. (2015).

1-3 Leaf formation is under the influence of gene expression and hormonal control

When a seed germinates, it produces a radicle that grows downwards to form the root system, and a plumule that grows upwards to form the aerial parts of the plant. Leaves are one of these aerials parts that are produced from the shoot apical meristem (SAM). The SAM, like the root apical meristem (RAM), contains stem cells in a SCN, and it is responsible for the initiation of new organs (Braybrook and Kuhlemeier, 2010). The number of cells in the SAM is tightly regulated, to ensure correct lateral organ development. Two genes with key roles here are *CLAVATA3* (*CLV3*) and *WUSCHEL* (*WUS*). These two genes together control the number of meristem cells present (Braybrook and Kuhlemeier, 2010).

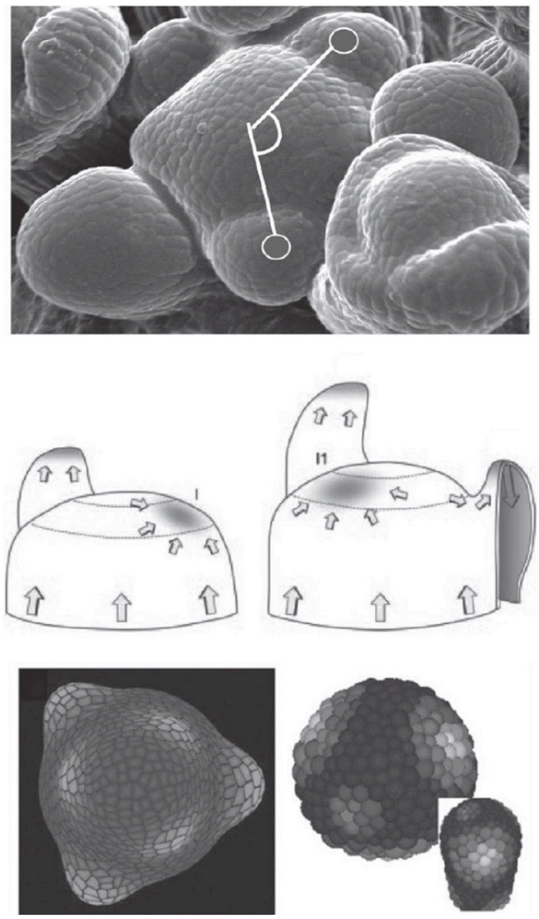


Fig.1-2: Movement of auxin to form leaf primordia (Ha et al., 2010).

The shoot apical meristem has three zones. The first zone is the central zone that contains the cells that maintain their undifferentiated nature; the second zone is the rib zone, located beneath the central zone and gives the rise to central tissue of the stem; and the third zone is the peripheral zone, which contains the cells that divide to form different organs (Braybrook and Kuhlemeier, 2010). The number and location of these cells are kept constant. The stem cell characteristics of the meristem cells are regulated by *KNOTTED LIKE HOMEOBOX* or *KNOX* genes.

Previous studies of mutants of *KNOX* genes in *Arabidopsis* showed that they prevent the stem cells from differentiating, which demonstrates that they play a key role in regulating meristem cell identity (Tucker, et al. 2008). When the plant starts to form new organs (the leaf primoridia), the expression of *KNOX* is repressed (Braybrook and Kuhlemeier, 2010).

There are two kinds of surfaces of the leaf present in most plants. In the first are the light harvesting cells, present on the upper side of the leaf, which carry out photosynthesis; and the second surface, on the lower side of the leaf, is specialised in gas exchange (Efroni et al., 2010). Also we can see the polarity of the leaf in the differential distribution of trichomes and stomata in the leaf (Efroni et al., 2010), and in the positioning of cell types in the vascular bundles (Bustillo-Avendaño et al., 2018). This polarity of the leaf is also known as adaxial (upper) and abaxial (lower; ad-ab) polarity. The adaxial side develops as the ventral side that forms adjacent to the meristem, and the abaxial side, the dorsal side, is the face that forms away from the meristem (Bustillo-Avendaño et al, 2018). The HD-ZIPIII transcription factor family together with ASYMMETRIC LEAVES1/2 AS1/AS2 regulates the formation of the adaxial side, while the KANADIs and the auxin-dependent ARF3/ARF4 proteins, which are Auxin Response Factors, regulate the formation of the abaxial side (Bustillo-Avendaño et al, 2018).

During embryogenesis, the cells of shoot apical meristem (SAM) are created. The leaf development process in Arabidopsis starts post-germination, when the leaf primordium is initiated, followed by establishment of the dorsal and ventral surfaces; and lastly the marginal meristem develops (Efroni et al., 2010). In layer two of the SAM, periclinal cell divisions are responsible for initiation of leaf primordia. In layer one, anticlinal cell divisions

form the upper and lower epidermal cells. The inner cells of the leaves are created as a result of mixing of anticlinal and periclinal cell divisions in layer 3 (Furner and Pumfrey, 1992). In the peripheral zone of the SAM, the transport tissue of the stem starts to form as the procambium cells of leaf, ultimately forming the vascular tissue of the leaf. The meristem cells follow this pattern to form the different parts of the leaf primordium and the three fundamental layers of the leaf (dermal, ground, and vascular tissues). This correlation is for the location of the cells in the meristem but not for cell lineage (Furner and Pumfrey, 1992; Irish and Sussex, 1992).

Previous studies of the anatomy of the SAM in the dry seed of Arabidopsis show that it contains 36-38 cells in each layer. 48 h after seed imbibiiton, the meristem becomes enlarged to form a ridge, and after 96h this develops into two leaf primordia. After 192 h one of the first two leaves becomes larger and at this stage trichome primordia are apparent. In addition, at 192 h the third leaf primordium is visible, and the fourth leaf is beginning to form. The order of leaf formation in *Arabidopsis* is helical and random with respect to clockwise or counter clockwise development (Irish and Sussex, 1992).

The genes of the *KNOTTED 1* class of homeobox have an important role in the maintenance of the SAM versus the initiation step of leaf formation. These genes were first identified in maize (*Zea mays*), through the dominant *KN1* mutation, which results in its enhanced expression. 5,000 genes are known to be regulated by KN1 (Bolduc et al., 2014). The dominant mutation leads to patches of meristematic cells on the leaf blade where it is ectopically expressed, giving rise to the 'knotted' phenotype, and illustrates its important role in determining the identity of meristem cells (Michael et al., 1996). The KN1 homologue in Arabidopsis is called "KNOTTED LIKE ARABIDOPSIS THALIANA" or KNAT and has been shown to have a similar function in the SAM (Lincoln et al, 1994). Loss of function *KNOX* genes in Arabidopsis fail to produce shoot organs after the emergence of the cotyledons (Barton and Poethig, 1993; Long et al., 1996).

The plant hormone auxin plays a major role in controlling leaf organ initiation. Before leaf primordium initiation, the auxin response reaches a maximum at that position in the SAM, due to auxin biosynthesis in the SAM and its transport to the incipient primordium by the

activity of the PIN-FORMED1 (PIN1) auxin transporter (Fig. 1-2). Previous results showed that inhibition or mutation of PIN1 causes a failure of organ initiation, similar to the effects of mutation in the auxin biosynthesis *YUCCA* gene family (Guenot et al, 2012). The initiation of the leaf is linked to the initiation of the midvein, the vascular strand located in the middle of the leaf. The initiation of midvein formation starts when the auxin concentration reaches a high level at the site of leaf initiation, and progressively develops. The high auxin concentration at the site of midvein initiation influences PIN1 polarization, starting at the outermost cell layer L1 (Braybrook and Kuhlemeier, 2010).

Cytokinin also plays a role in leaf initiation through its interaction with auxin signalling. For instance, in Arabidopsis the ARF gene *MONOPTEROS* (*MP*) is negatively regulated by the response regulators ARR7 and ARR15 that are part of the cytokinin signalling pathway (Zhao et al, 2010). Furthermore, it has been shown that mutation of *MP* leads to a high level of cytokinin which causes inhibition of flower initiation (Zhao et al, 2010). This supports the hypothesis that auxin and cytokinin together play a key role in the initiation of the leaf at the Arabidopsis shoot apex, though they have opposite effects in the root.

1-4 Adventitious roots

1-4-1 Introduction to adventitious roots

Roots that are formed on any part of the plant other than the root are called adventitious roots (ARs). They are typically initiated in response to changes in the environment such as flooding, drought, or wounding (Steffens and Rasmussen, 2016). ARs therefore help plants to acclimate to stressful environments. For example, ARs help plants absorb water and gasses in flooding, and they can grow from wound sites as part of a regeneration process (Gonin, et al. 2019). Adventitious roots that develop from a wound site represent de novo organogenesis. This kind of root developmental programme depends on endogenous hormones and arises from procambium or cambium cells, which contain adult stem cells located in the vascular tissues of aerial organs (Chen et al. 2014). According to Chen et al. (2014) there is a simple method to induce the de novo development of root in laboratory conditions, that involves making a wound in the 12 day-old leaf of Arabidopsis, and then

explanting the leaf onto B5 medium lacking any hormones. The leaves are cultured at 22-25°C with 16 hours light, then after 8-10 days after culture (DAC) on B5 medium de novo roots will emerge from the leaf. The creation of ARs on different parts of a plant can be due a natural developmental process or in response to external factors. In cereals the ARs are called crown roots. In rice the crown roots that grow at the same time as a group of nodes, leaf and axillary buds and together with two rows of crown roots, are termed a phytomer (Nemoto et al., 1995).

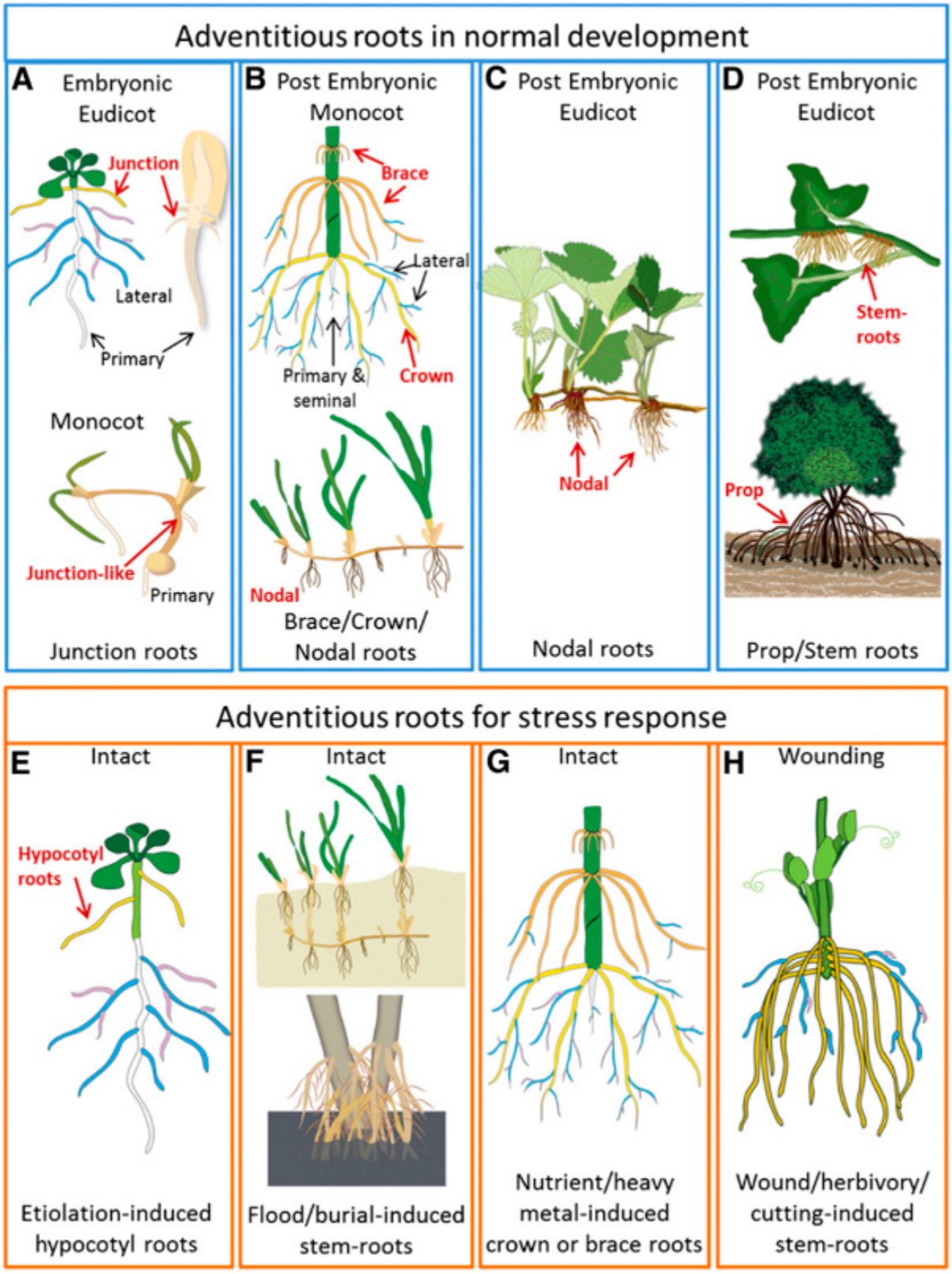


Fig.1-3: Formation of adventitious roots in different conditions (Steffens and Rasmussen, 2016).

Flooding is one external factor that can cause plant stress through deprivation of oxygen. Tomato forms de novo roots when under flooding stress, while other plants such as rice

have root primordia in the stem which allow adventitious root formation in response to flooding (Sauter, 2013; Dawood et al. 2014; Steffens and Rasmussen, 2016). The depth to which the adventitious roots grow depends on the needs of the plant - when the plant needs to avoid an oxygen deficit in wet soil, the roots grow closer to the surface of the soil, but when the plant needs more water, the roots go deeper into the soil (Eysholdt-Derzsó and Sauter, 2019). AR formation is also dependent on other factors. For example in the cereals, crown root formation requires water and nitrogen deficiency, and the crown roots grow deep into the soil to access them (Gaudin, et al., 2011; Saengwilai et al., 2014). Deficiencies of other nutrients such as phosphorus, zinc, iron, calcium, and manganese also play an important role in AR formation to help the plant survive in adverse environments (Li et al., 2009). As indicated above, adventitious root formation is controlled by both internal and external factors (Druege et al., 2018). When adventitious roots are produced from hypocotyls, they are very similar to lateral roots in terms of the organisation of their tissues and development. They grow from a region of the hypocotyl that is similar in function to the root pericycle (Bellini et al., 2014; Verstraeten et al., 2014). Previous work has shown that ARs can be induced in Arabidopsis by certain light conditions (Sorin et al., 2005) or by excision (Sukumar et al., 2013; Chen et al. 2014).

There are two promoting principles involved in damage-related formation of ARs. Firstly, the wound response that is induced during the isolation of the explant tissue from resource supply in the plant. Secondly, the signalling networks that affect organ regeneration post-wounding. ARs can be grown from non-root tissue in isolation in response to signals, including auxin. Detached leaf explants on B5 medium can activate various early signals that include both short- and long-range signals. These early signals are activated at the leaf margin, mesophyII cells and some vascular cells. The short-range wound signals act quickly after cutting the leaf and culturing on B5 medium, and there are two waves of short-range signals. Components of the first wave are both physical and chemical in nature, and involve changes in plasma transmembrane potential, intracellular Ca^{2+} concentration and H_2O_2 generation. The second wave includes plant hormones that include jasmonates and ethylene (Xu, 2018).

Long-range wound signalling starts after 2 days of culture on B5 medium. These signals mostly work near the wound site and have many of functions. One of these functions is to activate expression of the *NAC1* gene (Xu, 2018). NAC1 is required for de novo root formation and repression of *NAC1* expression causes reduced development of adventitious roots. The NAC pathway works independently from the auxin pathway and wounding plays an important role in inducing it in leaf explants of Arabidopsis. In addition, NAC1 may have a role in cell wall metabolism to promote AR development (Chen et al. 2016). It is also suggested that *NAC* genes promote expression of KDEL-tailed Cys endopeptidase (CEP) genes (*CEP1* and *CEP2*). CEP proteins have been found to contribute to programmed cell death, by their secretion to the cell wall to induce inactivation of EXTENSIN (EXT) proteins. EXT proteins are important for cell wall expansion and wound healing, and wound healing may antagonise AR emergence. Therefore, repression of *EXT* genes by NAC proteins may promote AR emergence from leaf explants (Chen et al.,2016). In summary, *NAC1* appears to be working independently of the auxin pathway and may have a role in cell wall metabolism to promote AR development (Chen et al., 2016b).

Another function of long-range wound signals is to induce *YUC4* and *YUC1* expression near the wound region and this helps maintain auxin levels (Xu, 2018). YUC4 and YUC1 mediate auxin biosynthesis and they are important for cell fate transition when de novo organogenesis occurs in explanted leaves (Chen et al. 2016). In addition, the YUC family plays an important role in converting IPA to IAA (Sun et al. 2016). The expression of *YUC* genes reaches a high level within 4 h of culture on B5 medium (Chen et al. 2016a).

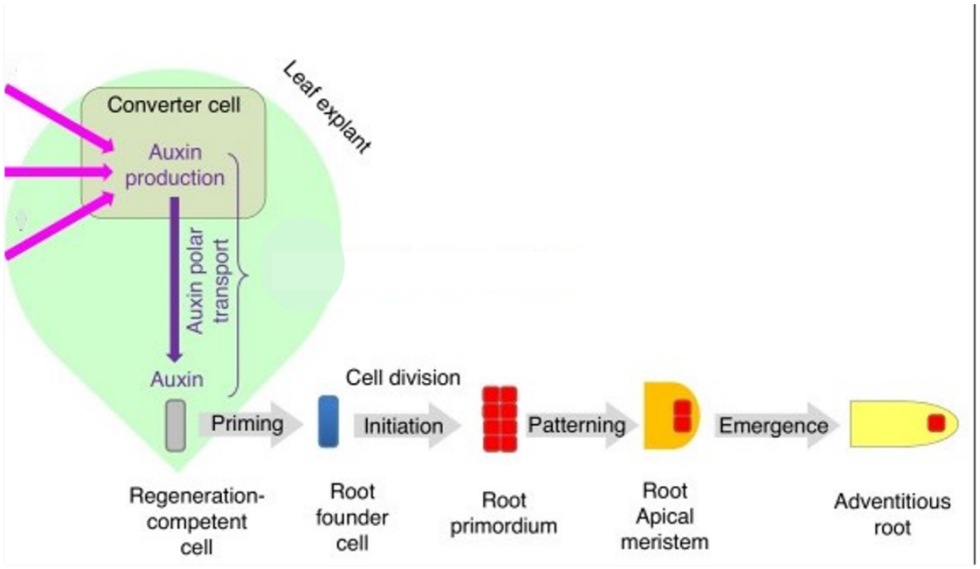


Fig.1-4: Summary of the short-range signalling and gene expression that occurs during the formation of a de novo root (Xu, 2018).

The next important stage in AR development is cell fate transition, at the beginning of which auxin is transferred to competent cells located in the procambium and some parenchyma cells. However, cell fate transition is blocked if auxin signalling is inhibited (Liu et al., 2014; Xu, 2018). The first step of cell fate determination ('Priming') starts when auxin activates the expression of *WUSCHEL RELATED HOMEOBOX11* and *12* (*WOX11/12*). The expression of *WOX11* and *WOX12* assists in the transformation of competent cells to root founder cells at around 1-2 days after leaf culture in B5 medium. In the promoters of *WOX11/12*, there are many auxin response elements (AUXREs). Mutation of some AUXREs or of *WOX11/12* cause partial defects in rooting while the overexpression of *WOX11/12* strongly promotes root formation. The second step of cell fate determination ('Initiation') starts when founder cells become root primordium cells, and at this stage cell division creates cell layers around 2-4 days after leaf culture on B5 medium (Xu, 2018). The expression of these genes was observed during the first cell division at 3 DAC but declined after formation of the dome-shaped root primordium at 4 DAC on B5 medium, and no expression of *WOX11* and *WOX12* was detected once the root apical meristem (RAM) had formed at 5 DAC (Liu et al., 2014). The mechanism controlling founder cell division is still unclear. The homeobox gene *WOX5* is required to start the second step that involves the division of root founder cells to become root primordium cells. In addition, activation of *WOX5* requires auxin, and repression of

auxin causes reduced expression of *WOX5* which in turn reduces de novo root formation (Hu and Xu, 2016).

However, WOX11/12 play a role in activating *WOX5/7* and *LATERAL ORGAN BOUNDARIES DOMAIN16* (*LBD16*). *WOX5/7* and *LBD16* are therefore useful markers of adventitious root primordium cells. In addition, auxin is required for activation of *WOX5/7* and *LBD16* by WOX11/12. *WOX5/7* normally are expressed in the stem cell niche within the root apical meristem. *LBD16* with other genes regulates lateral root formation and cell division in tissue culture. The third step of cell fate determination ('Patterning') starts with the formation of cellular pattern in the root apical meristem, and this occurs by continuous cell division. This step is characterized by the loss of *LBD16* expression and restriction of *WOX5/7* expression to the stem cell niche. In addition, there are many genes involved in meristem formation such as *SHORT ROOT* (*SHR*) and *SCARECROW* (*SCR*). Finally, the fourth step ('Emergence') starts when the de novo root tip grows through the epidermis of the leaf explant, and this occurs after 8 days culture on B5 medium.

1-4-2 Direct and indirect regeneration of de novo roots from the leaf

The cells of the intact plant contain growth regulators, but cultured explants are given these in the medium provided. We can add different growth regulators (hormones) which significantly control the regeneration of different structures such as root or shoot. These growth regulators can initiate callus formation, rooting, shooting and ultimately formation of whole plantlets. There are two types of de novo regeneration: the first type is direct, in which the leaf creates de novo roots directly when cultured on B5 medium; and the second type is indirect, in which the explant or the leaf first creates callus, and then de novo roots develop from the callus (Chen et al., 2014; Liu et al., 2014; Yu et al., 2017; Fig. 1-5). We can consider each in more detail.

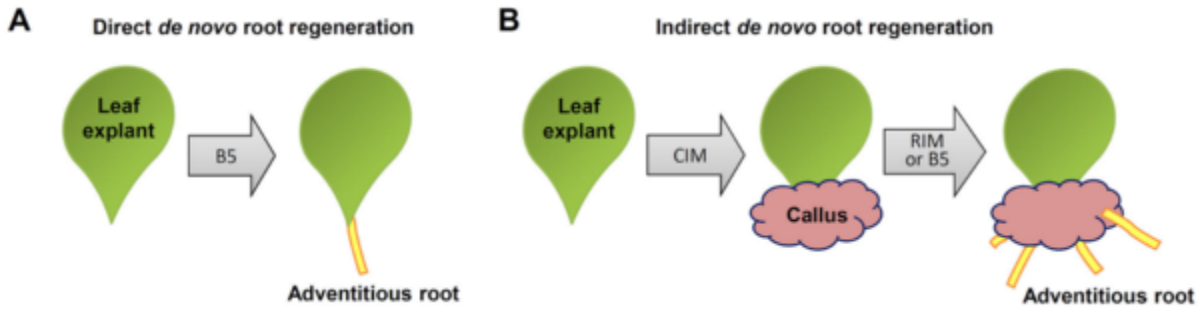


Fig.1-5: Two types of de novo regeneration: (A) direct and (B) indirect. B5: plant tissue culture medium; CIM: callus induction medium; RIM: root induction medium (Yu et al. 2017).

1-4-3 Direct de novo root formation

In direct de novo adventitious root formation, leaf cells pass through the four steps described above in section 1-4-1. In priming, endogenous auxin is transported into procambium and vascular parenchyma cells, which are known to be the regeneration competent cells and are located in the vasculature near the wound site. Auxin activates *WOX11* expression, which promotes the transition of regeneration-competent cells to root founder cells (Liu et al, 2014; Chen et al, 2016 a, b). In the initiation step, *WOX11* and auxin activate *WOX5* and *LBD16* expression, which have a role in transforming root founder cells to root primordia (Liu et al., 2014; Hu and Xu, 2016; Sheng et al., 2017). In this step, *WOX11* expression is reduced but auxin concentration remains high in the root primordium (Liu et al, 2014; Hu and Xu, 2016). In the patterning step the primordium cells start to divide to form a distinct root apical meristem (RAM). At this stage the level of auxin is limited only on to the tip of the meristem to restrict the region of the stem cell niche, while at the same time the level of auxin is reduced in the rest of the primordium (De Klerk et al, 1999; Della Rovere et al, 2013; Druege et al, 2016). Also, *WOX5* is limited to the stem cell niche region while *LBD16* expression is also reduced (Hu and Xu, 2016). During the fourth and final emergence stage, the mature root tip and stem cell niche are formed, and the root tip grows out of the leaf (Chen et al, 2016; Hu and Xu, 2016).

1-4-4 Indirect de novo root formation

Indirect de novo root regeneration occurs when the tissue culture medium contains high levels of auxin and induces callus formation. Previous evidence suggests that the callus is formed through the rooting pathway involving two cell transitions in Arabidopsis (Che et al., 2007; Atta et al., 2009; Sugimoto et al., 2010; Fan et al., 2012; He et al., 2012; Liu et al., 2014). During the priming step, when the transition from competent cell to founder cell occurs, *WOX11* is significantly induced in the founder cell (Liu et al., 2014). During the initiation step, when the transition from founder cells to callus occurs, *WOX11* expression is reduced while *WOX5* expression is increased in the newly formed callus (Liu et al., 2014). *LBD16* expression also occurs in the new callus (Fan et al., 2012). Therefore, the callus expresses genes in a similar way to a group of root primordium cells, under the control of high auxin levels from the callus-inducing medium (CIM). In addition, the callus helps to maintain a root primordium-like cell status under the high level of auxin. However, in tissue culture the distribution of auxin in the callus mass may be disorganized, causing differentiation of some parts of the callus, which helps some cells enter the tissue patterning step, while other cells remain undifferentiated. There are many root meristem genes expressed in the fast dividing and partially differentiated callus mass (Sugimoto et al., 2010; Kareem et al., 2015), but there is not much information about the expression of the *WOX5* and *LBD16* genes in partially differentiated callus. The partially differentiated callus is likely to be of a different status in terms of gene expression compared to root primordia and mature root meristems. Therefore, the callus mass maintains some features of the root primordium while other regions of the callus mass have RAM features due to the effects of supplied auxin which is required for root meristem patterning and growth (Sabatini et al., 1999; Benkova et al., 2003; Okumura et al., 2013; Liu et al., 2014; Hu and Xu, 2016). Once callus containing root primordia is removed from an exogenous auxin supply, endogenous mechanisms induce further root meristem development, during which *WOX5* expression is limited to the stem cell niche of the new RAM, and adventitious root tips may form.

1-4-5 Comparison of the two-rooting types

Based on the information above we can see similarities between direct de novo root regeneration (DNRR) and indirect DNRR on the cell transitions step, whereby they both exhibit priming, initiation, patterning and emergence steps to create a de novo root.

Another similarity is in gene expression, whereby *WOX11* expression is seen in the founder cells in both types, and *WOX5/LBD16* expression is seen in the newly formed callus and root primordium.

Two significant differences between direct DNRR and indirect DNRR have been identified. Firstly, the auxin sources of the two rooting types are different. In direct DNRR, endogenous auxin is the auxin source and is produced from the mesophyll cells, leaf margin cells and some other cells on the leaf, and then is transported to regeneration-competent cells near the wound side (Liu et al., 2014; Chen et al., 2016b). However, in indirect DNRR, exogenous auxin in the medium is the main source, and endogenous auxin may not be important in this process (Chen et al., 2016 a, b).

The second difference between direct and indirect DNRR is that, in direct DNRR, the cell transition is controlled by the (endogenous) auxin concentration focused in a few cells of the root primordium cells, and root formation is limited typically to between 1 to 3 roots. In addition, while the auxin level is high in the root primordium cells, it decreases during the pattering stage. Also, the rooting process does not stop between the initiation and patterning steps. However, in indirect DNRR, the two steps of priming and initiation occur rapidly, and as a result many regeneration-competent cells are induced to form founder cells which then divide to form callus. The exogenous auxin supports abundant callus formation, and many adventitious roots regenerate after removing auxin which reduces endogenous levels - high levels of exogenous auxin inhibit the differentiation of callus cells, and removing the exogenous auxin supply allows the callus cells to start the patterning step. In summary, direct and indirect DNRR share the same cell transition steps but they have different auxin sources and dynamics.

1-5 Hormones signalling and development the root growth

1-5-1 Signalling in the Arabidopsis

As we have seen, auxin is very important for the formation ARs and for root development more generally (Li et al., 2009). The most abundant natural active auxin is indole-3-acetic

acid (IAA), and is required both in the early and later steps of AR formation (de Klerk et al., 1999). The IAA concentration gradient and its accumulation in certain cells (competent cells) are important as early signalling events in AR formation (Ahkami et al., 2013; Lakehal and Bellini, 2019). This process is driven by polar auxin transport (PAT) and local auxin biosynthesis, conjugation, and degradation to control local concentrations of active auxin. PAT plays a key role in establishing the IAA concentration gradient. Accumulation of too high levels of active auxin due to defective homeostasis can cause the spontaneous formation of ARs in Arabidopsis (Boerjan, et al. 1995). Also, the amount of auxin available to a cell plays an important role in the transition of the competent cell to the founder cell during AR formation (Xu, 2018).

Previous research has found that auxin and cytokinin have opposite effects on the formation of ARs, and the synthesis of cytokinin can be inhibited by auxin (Tanaka, et al., 2006; Agulló-Antón, et al., 2013; Gonin et al., 2019). The availability of the auxin and restriction of cytokinin concentrations are important to induce ARs experimentally (Da Costa, et al. 2013). Also, addition of the cytokinin benzyl adenine (BA) and the auxin indole-3-butyric acid (IBA) can induce callogenesis instead of rooting (Klerk, et al. 1995). Jasmonate, nitric oxide and ethylene synthesis are induced in response to wounding during AR formation, and interact with auxin to promote cell division on wounding (de Klerk et al., 1999; Da Costa et al., 2013; Rasmussen et al., 2014). Ethylene plays an important role in AR formation during flooding, by inhibiting auxin transport which causes local accumulation of auxin and increased ethylene production, so that these two hormones act together to induce AR formation (Gonin et al., 2019). Furthermore, ethylene affects the expression *PIN* genes, especially, *PIN3* and *PIN7*, with consequent effects on the transport of auxin. In addition, ethylene controls the regulation of the root during submergence via the transcription factor APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) VII. This transcription factor supports AR growth while inhibiting growth of the main root system (Jackson, 1985; Vidoz et al., 2010; Eysholdt-Derzsó and Sauter, 2017, 2018). There is also evidence that the initiation of ARs is controlled by crosstalk between auxin and jasmonate (Chen et al., 2010; Westfall et al., 2010; Wang et al., 2010). This happens via the AUXIN RESPONSIVE FACTOR (ARF) proteins and the GRETCHEN HAGEN3 (GH3) family. The GH3 proteins play a role in the conjugation of

jasmonate and auxin with amino acids, and as a result of this conjugation, the activation, inactivation, or degradation of these growth regulators.

Nitric oxide also affects the formation of ARs through effects on the conversion of IBA to IAA, and also supports the binding of auxin to its receptor TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX (TIR1/AFB) (Terrile et al., 2012; Fattorini et al., 2017). Salicylic acid and hydrogen peroxide (H_2O_2) also can increase the formation of ARs (Gonin, et al. 2019).

1-5-2 Role and interaction of auxin

The PIN family of auxin efflux carriers mediate the polar transport of auxin (Zazímalová et al, 2010; Ljung, 2013; Adamowski and Friml, 2015). The TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS or TAA family of transaminases help to convert tryptophan to indole-3-pyruvate (Stepanova et al., 2008; Tao et al., 2008) which is then converted to IAA by the YUCCA family flavin monooxygenases. Genes for this enzyme play an important role in the development of the plant - research shows localized expression of these enzymes, suggesting localized synthesis of auxin, and this may an important factor in the distribution of auxin via by this specific pathway (Zhao, 2014). Another pathway for the synthesis of auxin is the Trp-independent pathway (Tivendale et al., 2014).

IBA is converted to IAA by β-oxidation in peroxisomes and is the source of auxin for specific processes (Strader and Bartel, 2011). The expression of the *PIN* gene family and the localization of PIN proteins in cells mediate the distribution of auxin in tissues. Auxin transport into cells is regulated by the AUX/LAX family proteins, which are influx carriers (Swarup and Péret, 2012). Another auxin transport protein family is the MULTIDRUG RESISTANCE p-glycoprotein or MDR/PGP is family, which influences auxin transport under the control of the PIN family proteins. In cells the MDR/PGP family proteins are uniformly localized, while several PIN proteins are localized in polar patterns (Geisler and Murphy, 2005). These protein families work together as IAA influx and efflux carriers, and with localized biosynthesis, they generate auxin asymmetries which in turn helps to regulate cell

divisions and cell differentiation during plant development (Reuille et al., 2006; Grieneisen et al., 2007).

As indicated above, conjugation is another mechanism to regulate active auxin levels in the cells of the plant (Ludwig-Müller, 2011; Ljung, 2013). The conjugation of auxin with sugar or different amino acids inactivates it. The GH3 enzyme family of acyl acid amido synthetases influence the biosynthesis and maintenance of active auxin levels by the conjugation of amino acids to IAA. Some of these amino acid conjugations can be hydrolysed to produce active auxin (Staswick et al, 2005). Another way to regulate auxin levels is by degradation of IAA to 2-oxoindole-3-acetic acid, although the enzyme catalysing this process is still not identified (Kai et al, 2007). A different system for regulating auxin signalling rather than homeostasis involves the Aux/IAA family of transcriptional repressors, the auxin response factor (ARF) transcriptional factors and the TIR1/AFB1-AFB5 F-box components of the SCF complex (Peer, 2013; Salehin et al, 2015). In the presence of low levels of auxin, the Aux/IAA proteins bind with ARFs leading to an inhibition of auxin-responsive gene transcription (Szemenyei et al., 2008). In the presence of high levels of auxin, the Aux/IAA proteins complex with the TIR1/AFB1-AFB5 proteins with IAA in between them, which acts as a 'molecular glue' (Calderon-Villalobos et al, 2010). The formation of this complex promotes ubiquitination and degradation of the Aux/IAA proteins by the 26S proteasome. This helps to relieve the repression of ARFs by the Aux/IAA proteins to allow auxin-mediated transcription (Ulmasov et al, 1999). *Arabidopsis thaliana* has a large number of Aux/IAA, ARF, and TIR genes/proteins, providing a large repertoire of regulatory molecules to modulate cellular responses to auxin. The AUXIN BINDING PROTEIN I has also been considered to be involved in a non-transcriptional auxin signalling pathway involved in cell elongation and cell division, but its role is controversial (Sauer and Kleine-Vehn, 2011; Xu et al., 2014).

1-5-3 Roles and interactions of cytokinin

Cytokinin is considered as a plant hormone involved in signalling to regulate gene expression. There are significant numbers of early transcriptional regulators that are mediated by type-B response regulators, represented as the transcription factors related to

the MYB family. Also, the AP2/ERF transcription factor family are cytokinin response factors. However, current evidence indicates that in Arabidopsis there is only one cytokinin response factor that binds to type-B response regulators, which have the core sequence 5'-GAT(T/C)-3' (Brenner et al., 2012). Cytokinin regulates many development and physiological processes: for example, reproductive behaviour, the activity of shoot and root meristems, leaf senescence and response to light and nutrients (Argueso et al., 2009; Werner and Schmülling, 2009). Previous results show there are many primary response genes that are induced by cytokinin, such as the type B response regulator genes *ARR4* and *ARR5*, which are upregulated within 2h by cytokinin (Brandstatter and Kieber, 1998). Another example is the type A response regulator genes *ARR3*, *ARR6*, *ARR7*, *ARR8*, *ARR9*, *ARR15* and *ARR16* (Imamura et al., 1998; Urao et al., 1998). Cytokinins are synthesised when a prenyl moiety from dimethylallyl diphosphate is added to ATP/ADP (adenosine triphosphate, adenosine diphosphate) forming N6-isopentenyladenine (iP) ribotides, which are acted on by adenosine phosphate isopentenyl transferase (IPT) (Sakakibara, 2006). In *Arabidopsis,* there are nine different genes encoding IPT, named *IPT1* to *IPT9*. Two of these nine gene products play roles in changing the adenine base subset on tRNAs, but the other seven work together in cytokinin synthesis (Kakimoto, 2001; Takei, Sakakibara and Sugiyama, 2001). Another type of cytokinin is zeatin. Trans zeatin (tZ) is made from the iP ribotides by the hydroxylation of the isoprenoid side chain, and this process is controlled by cytochrome P450 enzymes CYP735A1/CYP735A2 (Takei et al., 2004). The cytokinin nucleoside 5'-monophosphate phosphoribohydrolases family *LONELY GUY* (*LOG*) genes release free active cytokinin from the bases. For example, LOG7 is important for the maintenance of shoot apical meristem size and LOG3 and LOG4 may play a role in primary root growth (Kuroha et al, 2009). If cytokinins are conjugated with glucose, they are inactivated and cannot bind cytokinin receptors (Spíchal et al., 2004; Bajguz and Piotrowska, 2009).

Cytokinin oxidases are encoded by a multigene family in most plants. Individual members of this gene family show unique patterns of expression, location inside the cell and enzymatic properties. This gene family is controlled by the levels of cytokinin, which inactivates them. This is achieved by cutting the N6 side chains from tZ- and iP-type cytokinins using copper-dependent amine oxidase enzymes (Schmülling et al, 2003; Werner et al, 2006; Kowalska et al, 2010). When *Arabidopsis* and rice were exposed to exogenous auxin or cytokinins, the

cytokinin oxidase genes were rapidly induced (Tsai et al., 2012; Bhargava et al., 2013; Gao et al., 2014). In *Arabidopsis* and other plants, gene families are connected by cytokinin signalling (Pareek et al., 2006; Du et al., 2007; Pils and Heyl, 2009). In Arabidopsis there are three cytokinin receptors (AHK 2, AHK 3, and CRE 1/ WOL/ AHK 4) with a conserved cytokinin binding CHASE domain, a histidine kinase domain and a receiver domain (Inoue et al., 2001; Higuchi et al., 2004; Nishimura et al., 2004). The role of the AHK gene family is as positive regulators, and double mutants of *ahk2* and *ahk3* in *Arabidopsis* have reduced leaf size and inflorescence stem length (Nishimura et al., 2004). In plants that have AHK members with an endoplasmic reticulum-oriented CHASE domain, most of the cytokinin receptors are found in the membrane of the endoplasmic reticulum (Caesar et al., 2011; Lomin et al., 2011; Wulfetange et al., 2011). This supports the idea that the lumen of the endoplasmic reticulum is the main site for cytokinin binding. There is some evidence that equilibration of nucleoside transporter proteins and purine permeases play roles in cytokinin transport, but there is limited evidence that cytokinin transport occurs through the plasma membrane and in the endoplasmic reticulum (Bürkle et al., 2003; Sun et al., 2005; Hirose et al., 2008). Cytokinin transport from the root to shoot is by ATP binding cassette transporters such as ABCG14 (Ko et al., 2014; Zhang et al., 2014). In addition, ABCG14 has a role in the regulation of shoot growth by delivering cytokinin to the xylem. The cytokinin uptake transporter PUP14 (a purine permease) works antagonistically to ABCG14 by depleting the apoplastic cytokinin ligand that activates the cytokinin receptors (Kang et al., 2017).

1-5-4 Cytokinin and auxin balance in root development

The root apical meristem (RAM) contains the undifferentiated stem cells that are responsible for differentiating and forming root cell files (Schaller et al., 2015). This meristem activity is regulated by auxin and cytokinin. Arabidopsis has a closed meristem. In the closed meristem system, there is a region called the quiescent center (QC), which comprises slowly dividing cells that are surrounded by the initials or stem cells, and together the QC and the stem cells comprise the stem cell niche (SCN; Dolan et al., 1993). The QC prevents the surrounding stem cells from differentiating (van den Berg et al., 1997). The

root apical meristem is formed at the globular stage of embryogenesis. As result of controlled division of the hypophysis at the base of embryo, the stem cell niche is initiated from an upper lens-shaped cell that goes on to form the QC and the lower basal cell going on to form the columella (Laux et al., 2004). Cytokinin is present at high levels in the hypophysis and even after it has divided to form the lens shaped cell, but cytokinin is not present in the basal cell. Interestingly the cytokinin markers ARR7 and ARR15 showed the opposite result, suggesting high levels in the basal cell and lower levels in the lens shaped cell (Müller and Sheen, 2008). The reason for this contradictory result may be that auxin can affect the activities of these genes.

The root apical meristem controls the growth and development of the root after embryogenesis. The mature growing root can be considered as divided into three regions. The first part, near the quiescent center, is the meristematic zone where the cells are dividing actively. Proximal to this is the transition zone (TZ) where cell division slows and expansion begins, and the second region is known as the expansion zone. Proximal to this is the third, the differentiation zone in which expansion has arrested and cell files show clear signs of differentiation. During this process there must be a balance between the number of differentiating cells and dividing cells, so that the meristem can maintain its store of stem cells. Auxin and cytokinin play important roles here, as the balance between them affects the size of the meristem (Beemster and Baskin, 2000). To make this balance happen, these two hormones need to communicate with other. This occurs via a central switch i.e. the Aux/IAA protein SHY2, which maintains the balance by its inactivation by auxin using the SCFTIR1 complex, and activation by cytokinin via activation of the type B ARRs (Tian et al., 2002; Dharmasiri et al., 2003; Dello Ioio et al., 2008; Moubayidin et al., 2010). High cytokinin promotes cell differentiation in the root via SHY2 (Moubayidin et al., 2010).

1-5-5 Signalling systems in adventitious rooting

According to Druege et al. (2016) there are two stimulating principles involved in the formation of ARs, namely 1) the wounding that is caused during the isolation of the explant tissue from resources available in the plant and 2) the signalling networks that affect organ

regeneration post-wounding. ARs can be grown from non-root tissue in response to signals, including auxin. Auxin plays a major role in hormone cross-talk in the control of AR formation. There are complex interactions between auxin and other phytohormones to regulate the process of AR development. There are many kinds of auxin that are used in horticulture and agriculture to promote rooting of cuttings, such as indole-3-butyric acid (IBA), indole-3-acetic acid (IAA) and 1-naphthalene acetic acid (NAA), while IAA is considered to be the most abundant natural auxin (Pacurar et al. 2014). There are other hormones involved in the AR formation process, which have either a positive or negative impact. Firstly, ethylene (ET) is considered as a stimulant of the early induction and late formation phases, while it is considered as an inhibitor in the late induction phase; and ethylene generally is strongly linked to auxin responses through cross-talk mechanisms (Pacurar et al. 2014). For example, the two genes A*SA1/WE12* and *ASB1/WE17* are involved in auxin biosynthesis and are induced by ET signalling. In addition, the *REC1* gene is involved in the regulation of ET and auxin biosynthesis, again suggesting strong linkages between ET and auxin during AR formation (Pacurar et al. 2014).

Cytokinins (CKs) show inhibitory effects on AR development in poplar cuttings and rice, and similarly, strigolactones (SLs) have a negative impact on AR formation in Arabidopsis and pea. Gibberellin (GA) has an inhibitory effect on AR development in poplar cuttings and tomato, while it has a positive impact on AR initiation and elongation in deep-water rice. In addition, jasmonate (JA) has been found have an important role in the regulation of AR formation in tobacco. It is accumulated in early stages of wounding at wound sites in Petunia, and a result of this accumulation is an increased level of cell wall invertases and sink strength. JA also has a role promoting auxin and cytokinin activity in tobacco. However, JA has a negative impact on AR formation in the Arabidopsis hypocotyl (Pacurar et al., 2014). Furthermore, nitric oxide (NO) and H_2O_2 are involved in mediating signalling by auxin action in cuttings, and both NO and H_2O_2 are stimulated by wounding or auxin accumulation during AR formation. Previous studies suggest that NO promotes auxin signalling by S-nitrosylation of the auxin receptor protein TIR1, which helps AUX/IAA protein degradation (Druege et al. 2016). Finally, salicylic acid (SA) has a positive role in AR formation, whereby studies show that mutants in SA biosynthesis (*esds5-1* and *eds5-2* in Arabidopsis) have reduced the AR formation compared with wild type (Pacurar et al. 2014).

Research suggests that the accumulation of auxin in the early stages of adventitious rooting is dependent on polar auxin transport (PAT), which has the double function of controlling the cell cycle and establishing a sink in the rooting zone, by activating sucrolytic enzymes (Druege et al. 2016). As described above, there are specific transporters involved in the regulation of PAT, namely the PIN, AUX/LAX and ABC proteins. The auxin influx carriers have a preferential role during the formation of new meristems and in the differentiation phase (Druege et al. 2016). Interestingly, the expression of four MiAUX is Mangifers is increased on day 4 after rooting induction, and they are located preferentially in the proximal cut region. As result of this, ARs form only from the proximal surface wound site, to where auxin is transported (Pacurar et al., 2014).

Several members of the family of efflux carriers (PIN family) show polar localization to rootward or shootward polar membrane domains, while other PIN family members such as PIN5 have nonpolar localization, and this depends on cell type and developmental context. For instance, PIN2 is located in the shootward plasma membrane domain in epidermal cells, but in the cortex, PIN2 is located in the rootward domain. In addition, PIN2 is nonpolar in the QC and it is located in the rootward polar domain in the endodermis in the meristematic zone, but it is located in the shootward polar domain in elongation zone. Furthermore, PIN3 and PIN7 are non-polar in the columella, but they polarize upon gravistimulation (van Norman, 2016). Moreover, the ABC family also has a role in the regulation of auxin transport. For example, ABCB is expressed above the wound site and contributes to lateral directional auxin transport, promoting auxin accumulation in the pericycle and AR formation from xylem pole pericycle cells (van Norman, 2016). However, repression of auxin transport by using 1-N-napthylphthalamic acid (NPA) blocks AR formation, and mutants in IAA efflux such as *pin1* and *abcb19* show reduced AR formation (Lup et al. 2016).

AR in the rooting zone of explants involves transcriptomic changes that reflect a reduced IAA pool and changes in the control of the auxin transport machinery. In early stages, the two isoforms of IAA-amino acid hydrolase (IAA-AAH) are up-regulated, associated with an early peak of IAA at 2h post excision (hpe), via hydrolysis of amino acid conjugates. Repression of these genes contributes to reduced IAA levels during AR formation. In addition, the GH3 family shows strong induction at 2 hpe, as indicated above (Druege et al.

2016). According to Welander et al. (2014) GH3-3 in hypocotyls is upregulated at 24 h but with a significant increase after 72 h. GH3-3 protein is an auxin-conjugating enzyme, activation of which may be involved in AR-initiation in hypocotyls and stems. The observed increased level of GH3-3 at 72 h may be related to a second stage of auxin effect in which auxin in conjugated to reduce its functionality, associated with root development (Welander et al., 2014).

AR formation from cuttings involves significant transcriptional changes in the auxin-signalling cascade (Druege et al., 2016). The TIR1/AFB- family of F-box proteins are auxin receptors and these proteins are formed from SCF-E3-ubiquitin ligases, which are nuclear regulatory complexes responsible for degradation of the transcriptional repressors AUX IAA proteins (da Costa et al. 2013). The mechanism of auxin signalling via ARFs and TIR is described earlier. ARF6 and ARF8 have a positive effect in inducing AR on hypocotyls, while ARF17 has a negative effect. These three genes encode members of the GH3 family. Auxin plays a role in inducing the activation of ARF-GH3 to reduce jasmonate levels, which have an inhibitory role in AR formation, and so promote AR development (Lup et al. 2016).

1-6 Aim and objectives

The objective of this project is to study auxin signalling changes and responses associated with AR initiation, understand changes in gene expression and link these changes in gene expression and auxin changes to root development in the whole plant. The first aim of my project is to study the development of de novo roots from Arabidopsis Columbia-0 leaf explants, based on the system described above. I will focus on early events of AR initiation, using the first two true leaves harvested from seedlings at 12 d post germination and cultured on B5 medium without added hormones. I will examine the molecular mechanisms that occur during de novo root formation. I will used genotype Col-0 (wild type) and signalling mutants to investigate these mechanisms. In addition, I will analysis and compare gene expression in Col-0 and mutants using qRT-PCR, and use histology to analyse anatomical changes. I also describe the construction of transgenic lines using the Gateway cloning technique to investigate the possible roles of transcriptional regulators that

previously have not been characterized for roles in AR formation. A hypothesis of the project is that the MDF protein, which is a putative splicing factor identified in the lab, is required for auxin signalling and the expression of *NAC*, *WOX5* and *YUC* genes linked to AR development. The project will therefore provide new information of the network of auxin signalling in relation to AR development in Arabidopsis.

Chapter 2. Materials and Methods

2.1 Plant tissue culture

2.1.1 Plant material

Wild type (WT) *Arabidopsis thaliana* Columbia (Col-0) seeds were obtained from lab 1004 stocks, Department of Biosciences, Durham University. All mutant lines were also obtained from lab stocks - *axr1*, *axr3*, *pls* and *ein2*. GUS reporter lines and Salk mutant lines were obtained from the NASC website (http://arabidopsis.info).

2.1.2 Seed sterilisation

To surface sterilize seeds, all the seeds were placed in 1.5 ml Eppendorf tubes and washed with 70% v/v ethanol for 30s. Then, the ethanol was discarded and then the seeds were washed with 15% v/v of bleach (Tesco,UK) that contained 0.1% v/v Tween20 for 15 min. The seeds were then washed 4 times with sterile deionised water and the seeds were left in 1 ml sterile distilled water (sdH2O). Finally, the seeds were stratified for 4 to 7 d in the dark at 4°C which helps synchronise germination.

2.1.3 Culture media

Seedlings were grown *in vitro* on half strength Murashige and Skoog media (1/2MS10) (Murashige & Skoog, 1962) containing 2.2 g/l half strength MS medium (Sigma M5519), including 10g/l of sucrose, adjusted to pH 5.7, and 8 g/l agar. For leaf culture, B5 medium (Sigma G5893) was used (3.2g/l of B5 medium, adjusted to pH5.7, solidified with 8 g/l agar). All the media were sterilized in the autoclave at 121°C, 1.1 bar for 20 min.

2.1.4 Plant growth conditions

Seedlings or isolated leaves were cultured on solidified media and incubated in the growth room or growth cabinets under 16 h light: 8 h dark at 22°C (c. 3000 lux).

Seeds were germinated and grown for 12 d on sterile square Petri dishes (size 10 x 10 cm) containing 50 ml of solid half MS medium. Then, after 12 d the first two leaves were removed with sterilized mini scissors and the leaves were transferred to B5 media using sterilized forceps. For gene expression comparisons at different times, the leaves were collected on 0 h, 3 d, 5 d and 7 d (in the signalling experiment the leaves were collected on 0 h, 24 h, 48 h, 3 d, 5 d, 7 d,) and stored at -80°C prior to RNA extraction.

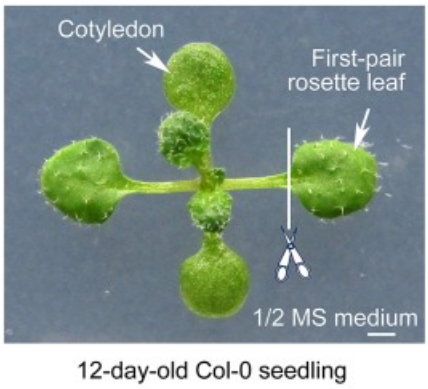


Fig. 2-1: Method and site of leaf cutting after germination on B5 medium. Scale bar 1 mm (after Chen et al., 2014).

2.2 Molecular biology

2.2.1 RNA extraction

An RNA purification kit specific for small amounts of leaf tissue was used (Promega ReliaPrep RNA tissue miniprep system USA, catalogue number Z6111). RNA extraction was performed for every genotype at 0 h, 24 h, 48 h, 72 h, 7 d and 14 d as follows: 5mg fresh weight of leaves was placed in a mortar containing liquid nitrogen and ground with a cold pestle. After that, the ground leaves were transferred to a microfuge tube containing 250 µl LBA (Lithium Acetate Borate) and TG (Tris-Glycine) buffer then the lysis was mixed 7-10 times using a p1000 pipettor and centrifuged for 3 min at 14,000 xg, then the lysis was transferred to a clean tube. Then 85 µl of isopropanol was added and vortex mixed for 5 s. Then the lysis was transferred to a Reliaprep Minicloumn and centrifuged at 12,000 – 14,000 xg for 1 min at 20 – 25°C and the supernatant was discarded. After that 500 µl of

RNA wash solution was added to the Reliaprep Minicolumn and centrifuged at 14,000 xg for 30 s. Then, DNase I solution was prepared as follows: 24 µl of yellow core buffer, 3 µl of 0.09M $MnCl_2$, 3 µl of DNase I enzyme were mixed and transferred directly to the membrane inside the column for 15 min at room temperature to remove DNA. After that, 200 µl of column wash solution was added and centrifuged at 14,000 xg for 15 s. Then was added 500 µl of RNA wash solution and centrifuged at 14,000 xg for 30 s. Once more, 200 µl of RNA wash solution was added and centrifuged at high speed for 2 min. Finally, 15 µl of nuclease free water was added and spun for 1 min at 14,000 xg. A Nanodrop spectrophotometer was used to estimate the concentration of RNA in each sample, and the RNA samples were stored in the freezer at -80 °C until further analysis.

2.2.2 cDNA synthesis

A cDNA synthesis kit from Bioline (UK-BIO-65053) was used. 1 µg of extracted RNA was added to 4 µl of 5X TransAmp Buffer with 1 µl of Reverse Transcriptase and the reaction volume was made up to 20 µl with RNase free water. *ACTIN2* primers were used to test for contamination by DNA (Product length for cDNA: 256bps and Product length for genomic DNA: 342bps). The reaction was run in the PCR machine as follows (Table 2-1):

	Temperature(°C)	Time
Primer annealing	25	10min
Reverse transcription	42	15min
Highly structured RNA	48	15min
Inactivation	85	5min
Refrigerate	4	hold

Table 2-1: PCR programme for cDNA synthesis.

2.2.3 Primer design

Two websites were used to help design the primers for cDNA, Tair and Blast (Table 1). After that, the designed primers were synthesized by the company Eurofins. The list of primers is given in Table 2-2.

Primer name	Sequence	Length
WOX5	(F) CTCTCCTCCCGACATTTCATCAATTCA (R) TGTTCAGATGTAAAGTCCTCAACTGTT	149bp
NAC1	(F) CCTGCTTCTCCAATTTGTCACAGAAC (R) ACAGAATGAGTCGAGGCCTGT	149bp
YUC4	(F) CTTCGACGACCTAAGACCGGAC (R) TTGCCCCGTTCCTCGTTATTTCT	144bp
YUC1	(F) CCCAAAACACTTTTGCCGGT (R) GGAAGTGGGAAGCGTAGGAC	110bp
ACTIN2	(F) GGATCGGTGGTTCCATTCTTGC (R) AGAGTTTGTCACACACAAGTGCA	-
ACTIN2 QPCR	(F) CTTGCACCAAGCAGCATGAA (R) CCGATCCAGACACTGTACTTCCTT	-
UBQ10	(F) GGCCTTGTATAATCCCTGATGAATAAG (R) AAAGAGATAACAGGAACGGAAACATAGT	-
UBC	(F) CTGCGACTCAGGGAATCTTCTAA (R) TTGTGCCATTGAATTGAACCC	-
PIN1	(F) TCAGGGGAATAGTAACGACAACCAG (R) ATCACACTTGTTGGTGGCATCACCT	153bp
PIN3	(F) ATTCCTCTCTACGTGGCCATGATCC (R) AGAGAGGAGAGGGACGGCGAAG	180bp
ARR4	(F) TGAAACTCGCCGACGTGAAACGT (R) ATTCAGTCGAAACAGTCAACGGCGG	180bp
GA3ox2	(F) TTCACATCCCACTCTCAAACCCACC (R) TGGCCACGTGGATATCGGAGAG	180bp
EBS	(F) CCGACAACGACGACGGAGAA (R) CCATCTTCCGTTAGGCTGAGG	180bp
RAP2.7	(F) GTCGACTCAGTACGGTGGTG (R) GTCTTCGTCTCCATCGGCAT	180bp

WOX5	(F) AAGCTTACGTGGCAACAATAACGG (R) AAGATCTAATGGCGGTGGATGTTC	500bp
NAC1	(F) TGGGATGAGGAAGACATTGGTTTT (R) TCAATCTTAGTGAGCTGACTGAGT	500bp
YUC1	(F) TTCATGTGTTGCCAAGGGAGATAC (R) ACCAATTTCGCCAGCGATCTTAAC	500bp
RAP2.7	(F) TAATGGTAGAGAAGCAGTCACGAA (R) TGGATAAAAGTAACCACGTGTTGC	500bp

Table 2-2: List of primers used in RT-PCR and q-PCR experiments, including reference genes.

2.2.4 PCR amplification

PCR amplification was carried out using Bioline Mytaq Red mix polymerase, to check the quality of cDNA and to identify the annealing temperature for each set of primers. The total of the reaction was 20 µl and using 5 µl of 5xmy taq red reaction buffer, 2 µl of CDNA, 1 µl of primers, 0.5 µl of polymerase and 11.5 µl of ddH_2O.

	Temperature(°C)	Time	Number of cycles
Initial denaturation	95	1min	1
Denaturation	95	15s	25_60
Annealing	(variable depending on the primer sites)	15s	
Extension	72	15s	
Final extension	72	1min	1
Refrigerate	4	Hold	1

Table 2-3: PCR amplification programme.

2.2.5 Agrose gel electrophoresis

Agarose gel was prepared as follows: 1.5g of agarose gel was mixed with 100 ml 1x TAE buffer then heated in a microwave oven until the agarose dissolved. On cooling, 7 µl of ethidium bromide solution was added. The agarose gel was poured and covered in 1x TAE buffer. PCR reaction products were loaded in the gel and subjected to electrophoresis at 100 volts for 40 min.

2.2.6 Quantitative RT-PCR (qRT-PCR)

For qRT-PCR I used 2xqPCRBIO SYGreen Blue MIX Lo-ROX from PCR Biosystems (PB20.15) and used three different housekeeping genes (*UBC*, *UBQ10* and *ACTIN2*) as reference genes. The total of the reaction was 20 µl and consisted of 2x 2xqPCRBIO SYGreen Blue MIX buffer, 0.8 µl of each 10 µM forward and reverse primer, 100 ng of cDNA and the reaction volume was made up to 20 µl with ddH2O. Reactions were run on a Rotor-Gene Q Machine (QIAGEN®) as follows (Table 2-4):

	Temperature (°C)	Time
Hold	95	2min
40 x Cycles	95	5s
	60	10s
	72	10s
Melt curve	50 -95	Increasing by 0.2°C every 5s

Table 2-4: Programme conditions used in the Rotor gene Q machine.

2.2.7 RT-PCR amplification

RT-PCR experiments used the One Taq One-Step RT-PCR kit from New England Biolabs (E5315S). Total reaction volume was 50 µl by using One Taq One-Otep reaction mix (2x) 25 µl, One Taq One-Step enzyme mix (25x) 2 µl, gene specific forward primer (10 µM) 2 µl,

gene specific reverse primer (10 µM) 2 µl, total RNA (up to 1 µg) and the reaction volume was made up to 50 µl by using nuclease-free H2O. The PCR reaction used the following conditions (Table 2-5):

	Temperature(°C)	Time	Number of cycles
Reverse Transcription	48	15-30 min	1
Initial Denaturation	94	1 min	1
Denaturation Annealing Extension	94 50-65 68	15 s 30 s 1 min per kb	25-40
Final Extension	68	5 min	1
Refrigerate	4	hold	1

Table 2-5: Programme conditions used for RT-PCR

2.2.8 DNA extraction (Edwards Prep; Edwards et al., 1991)

For genotyping SALK T-DNA insertion lines, a couple of mature leaves were placed in an Eppendorf tube, frozen in liquid nitrogen and ground to a powder using a micropestle. Then was added 400 µl of DNA extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% w/v SDS). The sample with buffer was vortexed for 5 s, then centrifuged at 15000 x g for 4 min. Then 300 µl of the supernatant was transferred to a new tube and was mixed with 300 µl of isopropanol to precipitate the DNA. Then, the sample was allowed to rest for 2 min at room temperature before the sample was centrifuged at 15000 x g for 5 min. The supernatant was removed, and to it was added 200 µl of 70% ethanol to remove salts. The sample was centrifuged again at 15000 x g for 5 min, then the ethanol was removed from the sample and the pellet was left to dry overnight. Finally, the DNA was mixed with 30 µl of sterile water and stored at -20°C until analysis.

Seed line	Locus	Associated gene name	Insertion specific primer	WT band size	Mutant band size	Used
Salk_095200	AT1G56010	NAC1	LBb1.3	1127bp	528-828bp	NO
Salk_135734	AT1G56010	NAC1	LBb1.3	976bp	432-732bp	YES
Salk_058441	AT2G28550	RAP2.7	LBb1.3	1102bp	466-766bp	NO
SALK_058441C	AT2G28550	RAP2.7	LBb1.3	1024bp	480-780bp	YES

Table 2-6: List of SALK lines, gene locus and the predicted T-DNA amplicon size in each mutant.

2.3 Cloning and transformation

2.3.1 RNA extraction

Qiagen Miniprep Kits were used for RNA extraction. 100 mg of mature Arabidopsis leaf was ground in liquid nitrogen then transferred to new 2 ml tubes and 450 µl RTL buffer was added to the tissue, and the sample was incubated at 56°C for 3min. Then the solution was transferred to a QIAShredder spin Column (Lilac) in a 2 ml collection tube and the solution was centrifuged at high speed for 2 min. The solution was transferred to a new microcentrifuge tube without disturbing the cell debris pellet in the collection tube. Then was added 0.5 volume of ethanol (96-100%) and immediately the solution was mixed by pipetting. After that, the liquid (usually 650 µl) was transferred to an RNeasy spin Column with a 2 ml collection tube and was centrifuged for 15 s at ,8000 xg (10,000 rpm), and the solution in the collection tube was discarded. Then, 700 µl of RW1 buffer was added to the RNeasy spin column and centrifuged for 15 s at 8,000 xg (10,000 rpm), and again the solution in the collection tube was discarded. Then, DNase (Promega) was used to remove DNA from the solution, using 24 µl of yellow core buffer with 3 µl of 0.09 M $MnCl_2$ and 3 µl

of DNase I enzyme. The solution was mixed well and placed inside the RNeasy spin column, and incubated at 20°C for 15 min. 500 µl of RPE buffer was then added to the RNeasy Spin Column, the liquid was centrifuged for 15s at 8,000 xg and the solution was discarded from the collection tube. Again, 500 µl of RPE buffer was added to the RNeasy Spin column and then centrifuged for 2 min at 8,000 xg, and the solution in the collection tube was discarded. After that, the RNeasy Spain Column was placed in a new 2 ml collection tube and it was centrifuged at full speed for 1 min to remove any RFP buffer. Then, the RNeasy Spin Column was placed in a new 1.5 ml collection tube and 50 µl of Rnase free water was added and was centrifuged for 1 min at 8,000 xg. Finally, a nanodrop machine was used to determine RNA concentration - 1 µl of Rnase free water was used as a control for comparison with 1 µl of the RNA sample.

2.3.2 CDNA synthesis and PCR amplification

cDNA synthesis kit from Bioline (UK-BIO-65053) was used. To 1 µg of RNA extract was added 4 µl of 5X TransAmp Buffer with 1 µl of Reverse Transcriptase and the reaction volume was made up to 20 µl with RNase-free water. *ACTIN2* primers were used to test by PCR if there was contamination by DNA (product length for cDNA: 256bps and for genomic DNA: 342bps). The PCR amplification used a My Taq DNA polymerase kit from Bioline (UK-BIO-21105) and a master mix included 5 µl of 5X My Taq Reaction Buffer, template (cDNA) 2 µl, primers (forward and reverse) of *ACTIN2* 1 µl of each primer, My Taq DNA polymerase 0.5 µl and the reaction volume was made up to 20 µl by RNase-free water. The PCR machine set up was as follows: 1 min at 95°C ,15 s at 95°C , 15 s at 55°C, 55 s at 72°C, 10 min at 72°C, 35 cycles.

2.3.3 Agarose gel electrophoresis

Agarose gel was prepared by dissolving 1 g of agarose in 100 ml 1X TAE buffer in a microwave oven. On cooling, 6 µl of propidium iodide was added and the agarose was poured into the tank. After that, 1X TAE buffer was prepared (100 ml of 10X TAE plus 900 ml of H_2O) then the buffer poured on the agarose gel to cover it. Samples were loaded in the

gel with a 1 kbp ladder marker from Bioline, and samples were separated by electrophoresis at 90 volts for 45 min.

2.3.4 Target genes

Two different genes were cloned. The first gene was *RAP2.7* (*AP2.7*; AT2G28550.1; Fig.2-2) and the predicted cDNA sequence length for this gene is 2235 bp. The second gene was *NAC1* (AT1G56010.1; Fig.2-3) and predicted cDNA sequence length for this gene is 2297 bp. The information and sequences of both genes were assembled from the Arabidopsis TAIR website.

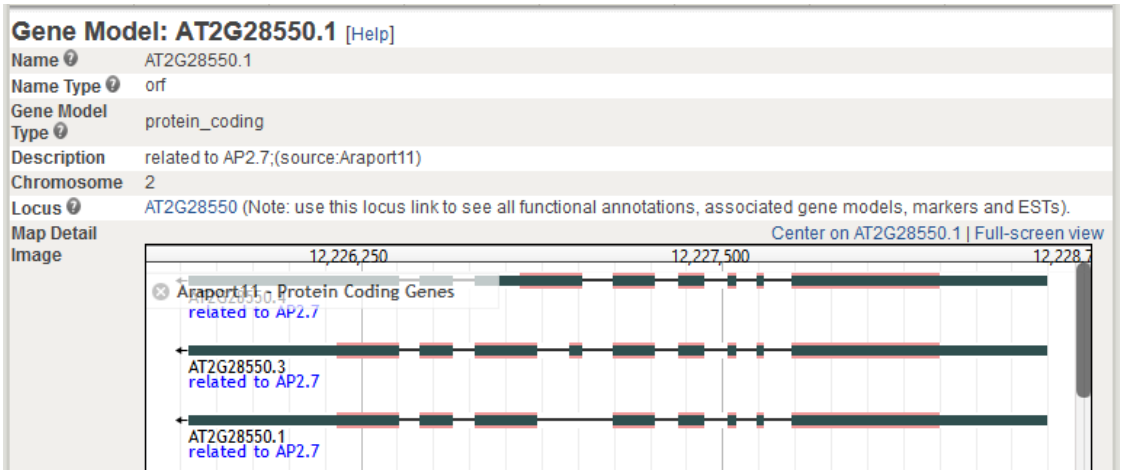


Fig. 2-2: Gene model for *RAP2.7* and the locus detail.

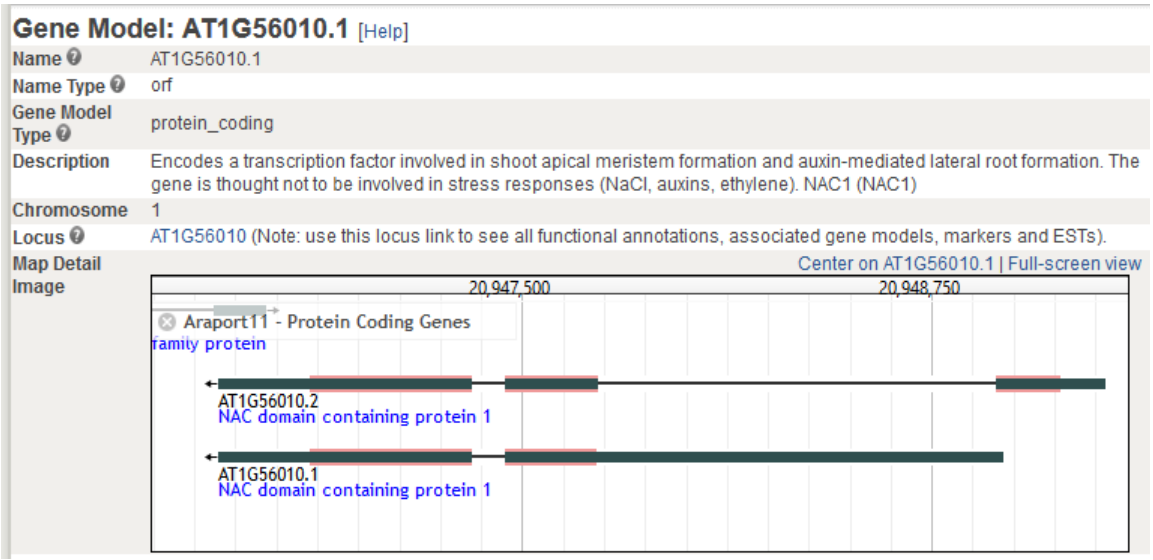


Fig. 2-3: Gene model of NAC1 and the locus detail.

2.3.5 Primer Design and Entry Cloning

The start and end of the *RAP2.7* and *NAC1* genes were determined using the Arabidopsis TAIR website, and primers for cloning were designed. The forward primers have 2 parts: the first part had a forward sequence corresponding to part of vector pDonr 207, and the second part had the forward primer of either the *RAP2* or *NAC1* gene. The forward primer for each gene (*RAP2* or *NAC1*) includes the ATG codon, and the reverse primers include the stop code and include also pDonr 207 reverse primer sequence. The primers for RAP2::GUS and NAC::GUS or ::GFP allowed cloning of the promoter of each gene. All the primers and entry cloning sequences for pDONR207 were designed using the Snapgene programme. In addition, different forward primers at different sites in the *RAP2* and *NAC* genes were designed to confirm entry cloning following PCR cloning and sequencing at the Durham University Genomics facility. The list of primers is presented in Table 2-7.

Gene	Kind of primers	Primer Sequences
RAP2.7 gene	Cloning primers	(F)GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGTTGGATCTTAACCTCAACGCT (R)GGGGACCACTTTGTACAAGAAAGCTGGGTTTTAAGGGTGTGGATAAAAGTAACCACGT
RAP2.7 Pro	Cloning primers	(F)GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGCCGTTGGATCACTGACC (R)GGGGACCACTTTGTACAAGAAAGCTGGGTTCTCAAGCTCTGAAAATTACAAATCTAGCG
RAP2.7 gene	Primer number 1 for sequencing	(F) GGTGACGGAGAAACGAAATTGGTA
	Primer number 2 for sequencing	(F)GTAGACAGAGCACGGGGTTTTCG

	Primer number 3 for sequencing	(F)GGGAGGCTCTTTCACTTCCCTTC
	Primer number 4 for v	(F)CCATTGTTCTCAGTTGCAGCAGC
RAP2.7 pro	Primer number 1 for v	(F)ATACTCGGGACAAAAATGCAACACG
	Primer number 2 for v	(F)ACTCATCACCCACAATTCTACAACA
	Primer number3 for sequencing	(F)TCCCTCACACGTCTCCTTCTATCTT
NAC1 gene	Cloning primers	(F)GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGAGACGGAAGAAGAGATGAAG (R)GGGGACCACTTTGTACAAGAAAGCTGGGTTTCAGCAATTCCAAACAGTGCTTGGA
NAC1 pro	Cloning primers	(F)GGGGACAAGTTTGTACAAAAAAGCAGGCTTATCTATGTCAAGGAAATATCGGAAGGG (R)GGGGACCACTTTGTACAAGAAAGCTGGGTTGATTTGATCCTTTTTTTTTCTAGGT
MDF pro	Cloning primers	(F)GGGGACAAGTTTGTACAAAAAAGCAGGCTTACTCCTAATCTTAGCAACTTTGTCTTCC (R)GGGGACCACTTTGTACAAGAAAGCTGGGTTTTTCAAGAACTTTAGTCAAGACC
NAC1 gene	Primer number 1 for sequencing	(F)ACTAACCGAGCAACGGCCACCGGA
	Primer number 2 for sequencing	(F)ACCTCGAACCTAACCAACTCAGTC

NAC1 pro	Primer number 1 for sequencing	(F)AAGCCTTGATCCGTTAGAACCCTCA
	Primer number 2 for sequencing	(F)ATATGGACCAGCTTCTTCTTCCTCT
	Primer number 3 for sequencing	(F)AGACGGAGATTACAATGCGATACCA
MDF pro	Primer number 1 for sequencing	(F)AAATCGTTCTCCTCTCTTCGCCAC
	Primer number 2 for sequencing	(F)TCCGATGAATTTCGAATCGCTTGAC
pDONR 207		(F)TCGCGTTAACGCTAGCATCTC (R)GTAACATCAGAGAGATTTTGAGACAC
Actin 2		(F) GGATCGGTGGTTCCATTCTTGC (R) AGAGTTTGTCACACACAAGTGCA

Table 2-7: List of primers used for cloning and sequencing.

2.3.6 PCR amplification of *RAP2* and *NAC1*

PCR amplification for *RAP2.7* and *NAC1* genes used the Q5® High-Fidelity 2X Master Mix kit from New England Biolabs. The reaction used 20 µl of cDNA, forward and reverse primers (1 µl of each), Q5 master mix 10 µl and RNase-free water 6 µl (total reaction volume 20 µl). Size separation on agarose gels was carried out to check the band size for both genes. The PCR machine conditions used were: 1:30 min at 95°C ,15 s at 95°C , 30 s at 67°C, 1:30 min at 72°C, 10 min at 72°C, 35 cycles; products were stored at 4°C.

2.3.7 BP reaction and *E. coli* transformation by heat shock

The Bp reaction mix used pDONR207 (109 ng/ µl) 1.5 µl, the PCR product (150 ng, 1 µl), with 1 µl of BP clonase enzyme and 2 µl TE buffer to a total reaction volume of 5.5 µl. The reaction was incubated overnight at room temperature. *E. coli* transformation was done by using 50 µl 1 x DH5aplha ™Competent Cells from Fisher Scientific with 1.5 µl of entry clone. Heat shock of *E. coli* involved incubation for 30 min on ice, 50 s at 42°C, then 2 min on ice, in 220 µl of SOC medium and the reaction was then incubated for 1 h at 37°C with shaking at 220 rpm. Finally, the reaction mix was spread on LB solid medium containing 20 µg/ml Gentamicin and was incubated overnight at 37 °C.

2.3.8 Preparing liquid and solid LB medium for BP colonies

LB medium (1L) contained 10g of tryptone, 5g yeast extract and 10g of sodium chloride. 8g agar in 500ml ddH_2O was added to make the medium solid, and liquid medium lacked agar. The medium was autoclaved at 121°C, 1.1 bar for 20 min. Stock solution of Gentamicin was 50 mg/ml and the working solution contained 20 µg/ml.

2.3.9 PCR Cloning for entry vectors

Ten colonies were selected on LB solid medium for use in a PCR reaction using the 1 x DreamTaq Green DNA Polymerase kit from Thermo Fisher Scientific, with 5 µl of DreamTaq buffer, 0.5 µl of forward primer for the gene and reverse primers of the vector pDONR207, 0.5 µl of MyTaq, 11.5 µl of nuclease-free water. The PCR conditions were as follows: 1 min at 95°C ,15 s at 95°C, 15 s at 55°C, 55 s at 72°C, 10 min at 72°C, with 35 cycles. Agarose gel electrophoresis (1% gel) was used to confirm positive colonies.

Four different colonies were chosen that had a bright band of the correct size on the gel, and these were transferred to 14 ml test tubes containing 7 ml LB liquid medium containing 50 mg/ml gentamicin. The tubes were incubated overnight at 37°C with shaking at 220 rpm. Then, 4 stocks were made from each colony in 250 µl of 50% glycerol plus 250 µl of LB liquid

medium and stored at -80°C . The rest of the medium was centrifuged at 3800 rpm for 12 min for plasmid preparation.

2.3.10 Plasmid extraction and digestion

Plasmid extraction was carried out using the Plasmid Miniprep Kit from ThermoFisher Scientific. After centrifugation, the liquid was removed, leaving the pellet. The pellet was suspended in 250 µl of Resuspension solution, then 250 µl of lysis solution was added and mixed, and 350 µl of the Neutralization solution was immediately added with slow mixing. After centrifuging at 13,000 rpm for 5min, the liquid was transferred to a spin column and again was centrifuged at the same speed for 1 min. After the liquid was discarded from the collection tube, 500 µl of wash solution was added and was centrifuged for 1 min twice at the same speed. The sample was transferred to a test tube and 50 µl of elution buffer was added. Elution buffer was incubated inside the column for 2 min and then centrifuged at 13,000 rpm for 2min. Finally, a nanodrop machine was used to measure plasmid concentration (1 µl of elution buffer as control compared with 1 µl of the plasmid sample) and then stored at -20°C.

The reaction was digested with EcoRV enzyme (plasmid 1 µg/µl, 1 µl of ECORV enzyme, 2 µl of enzyme buffer, made up to 20 µl with ddH_2O). The reaction was incubated for 1h at 37°C and digestion confirmed by gel electrophoresis (1% agarose gel in 100 ml TAE buffer, 6 µl of propidium iodide). The gel was immersed in 1x TAE buffer, and samples loaded with a 1 kB size ladder (Bioline), and separated at 90 volts for 45 min. Digested size predictions were made using the Snap gene programme (two bands of 3081 bp and 1687bp). The samples that showed two bands on the gel, and primers (3.2 µM/ µl), were sent for sequencing at the Durham University Genomics facility - plasmids were diluted to 150 ng/µl and sequences were confirmed using the Snapgene programme.

2.3.11 LR Reaction and *E. coli* transformation by heat shock

LR reactions were carried out for two different destination vectors, one for gene overexpression (pMDC7) and one for for GFP-GUS (pH6NFS7). Plasmid was diluted to 150

ng/µl and 1 µl was mixed with 1 µl of destination vector (150 ng/µl), 1 µl LR reaction enzyme and 3 µl TE buffer. The reaction was incubated at room temperature overnight.
Heat shock transformation of *E. coli* used 50 µl 1 x DH5alpha ™ Competent Cells from Fisher Scientific in a 1.5 µl reaction volume. The reaction mix was incubated for 30 min on ice, followed by 50 s at 42°C and 2 min on ice. Then, 220 µl SOC medium was added and incubated for 1h at 37°C with shaking at 220 rpm. The reaction was spread on LB solid medium containing 50 µg/ml spectinomycin and it incubated overnight at 37 °C.

2.3.12 Preparing liquid and solid LB medium for LR clones

1L LB medium contained 10 g of tryptone, 5g yeast extract and 10 g of sodium chloride. For solid medium, 8 g agar in 500 ml ddH_2O was added, liquid medium lacked agar, and was sterilized by autoclaving as described above. Stock solution of spectinomycin was 50 mg/ml and the working solution was 50 µg/ml.

2.3.13 PCR Cloning for destination vector

5 colonies were selected from LB solid medium and transferred to LB solid medium containing spectinomycin and incubated overnight at 37 °C. PCR was used to amplify sequences using 1 x DreamTaq Green DNA Polymerase kit from Thermo Scientific, using 5 µl of DreamTaq buffer, 0.5 µl of forward primer for the gene and reverse primers for the vector pDONR207, 0.5 µl of MyTaq, 11.5 µl of nuclease -free water and colonies were mixed well with DreamTaq master mix. PCR conditions were as follows: 1 min at 95°C ,15 s at 95°C, 15 s at 55°C, 55 s at 72°C, 10 min at 72°C done, with 35 cycles. Agarose gel electrophoresis (1% gel) was used to confirm positive colonies.

Three different colonies were chosen that had a bright band of the correct size on the gel, and these were transferred to 14 ml test tubes containing 7 ml LB liquid medium containing 50 mg/ml spectinomycin. The tubes were incubated overnight at 37°C with shaking at 220 rpm. Then, 4 stocks were made from each colony in 250 µl of 50% glycerol plus 250 µl of LB liquid medium and stored at -80°C. The rest of the medium was centrifuged at 3,800 rpm for 12 min for plasmid preparation.

2.3.14 Plasmid extraction

This method was as described above (Section 2.3.10).

2.3.15 Making competent cells of Agrobacterium

Sterile flask cultures of agrobacterium were grown in LB medium containing the antibiotic rifampicin (RIF, 100 mg/ml) at 30°C overnight. 5 ml of the culture was added to 100 ml LB medium containing RIF and allowed to grow to OD 0.5 - 1. Samples were kept cool on ice and centrifuged for 5 min at 6,000 rpm at 4°C. The pellets were washed with 30 ml of 0.15 mM sterile NaCl. Again, the pellets were centrifuged for 5 min at 6,000 rpm at 4°C. Then pellets were resuspended in 2 ml of 20 mM $CaCl_2$, transferred to test tubes, frozen in liquid nitrogen, and stored at -80°C.

2.3.16 Agrobacterium transformation

5 µl of plasmid containing the target gene was mixed with 50 µl of competent cells of agrobacterium. Agrobacterium transformation was carried out by incubating the samples for 5 min on ice, 5 min in liquid nitrogen, 5 min at 37°C and then 200 µl of LB medium was added. Finally, the samples were incubated in 28°C for 2h without shaking.

2.3.17 Preparing LB medium liquid and solid for agrobacterium

1L LB medium contained 10 g of tryptone, 5 g yeast extract and 10 g of sodium chloride, with 8 g agar per 500 ml ddH_2O for solid medium and liquid medium lacked agar. Medium was sterilized by autoclaving as described above. Stock solutions were prepared for antibiotics: gentamicin 50 mg/ml with a working solution 20 µg/ml; spectinomycin stock was 50 mg/ml with a working solution 100 µg/ml; rifampicin 50 mg/ml with a working solution 100 µg/ml. Transformed agrobacterium was plated on solid LB medium containing the three antibiotics and incubated at 28 °C for 2 d. Then colonies were transferred by loop to 7 ml liquid LB medium containing the three antibiotics and incubated for 1 d. 4 stocks from each

sample were made in 250 µl of 50% glycerol plus 250 µl of LB liquid medium and stored at -80°C until plant transformation. For larger volumes of agrobacterium culture for immediate plant transformation, the remaining LB medium was mixed with 350 ml of liquid LB medium containing the three antibiotics and incubated at 28°C for 24 h. Then the LB medium was centrifuged at 9,000 rpm for 15 min using 500 ml centrifuge bottles to collect the cell pellet for resuspension and plant transformation by dipping.

2.3.18 Dipping method of Arabidopsis transformation (based on Clough and Bent, 1998)

A 200 ml solution of agrobacterium cells for dipping was prepared containing 10 g of sucrose including 100 µl silwet L-77, and the solution was mixed well by magnetic stirring. After centrifugation as described above (Section 2.3.17) the Agrobacterium cell pellet was transferred to dipping solution and mixed well. Six independent Col-0 Arabidopsis plants per gene for transformation were grown to the flowering stage (ca. 3 weeks). For the first dipping, all the plants were dipped in the solution for 1 min and then covered with a bag overnight. After one week the stocks of Agrobacterium were used for a second dipping - the plants were dipped again in dipping solution for 30 s then covered with a bag overnight. After one and a half months the T1 seeds were collected and sterilised by washing in 70% v/v ethanol for 30 s, 10% hypochlorite for 15 min and washed 4 times in sterile ddH_2O. Then, the seed was germinated on MS medium containing 25 ng/ml of the antibiotic hygromycin. Plates were incubated for 6 h in the light then covered with foil for 3 d and then maintained in the light for 3 d. After that, the lines that showed resistance to the antibiotic were selected, typically about 10 lines for every target gene, and all were transferred to fresh MS medium with antibiotics for another 1 week in a Sanyo cabinet before transfer to soil in the greenhouse for 2 months. After that, the seed from all lines were collected (T2) and then sterilised by ethanol and hypochlorite as described above. Seeds were germinated on MS medium containing 25 ng/ml hygromycin, with the same growth conditions as described for T1 seed. Homozygous and heterozygous mutant and overexpression lines were selected, and for GFP and GUS lines expression levels were determined, with plants showing highest expression levels being used for microscopy. Homozygous lines were transferred into the greenhouse for one and half months and T3

transgenic lines were collected.

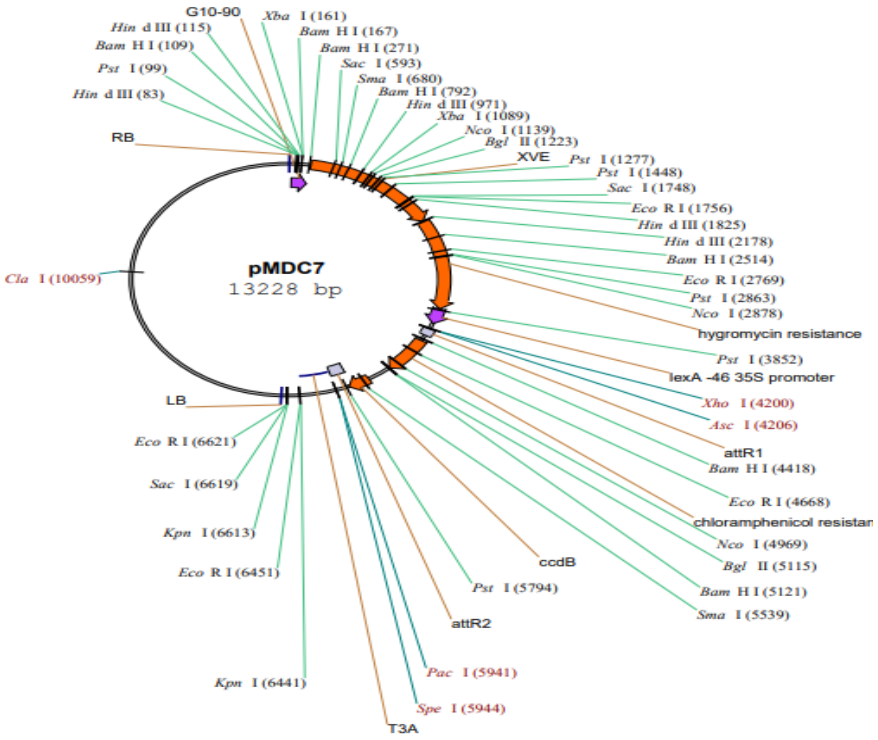


Fig.2-4: Map of the PMDC7 vector used in plant transformation.

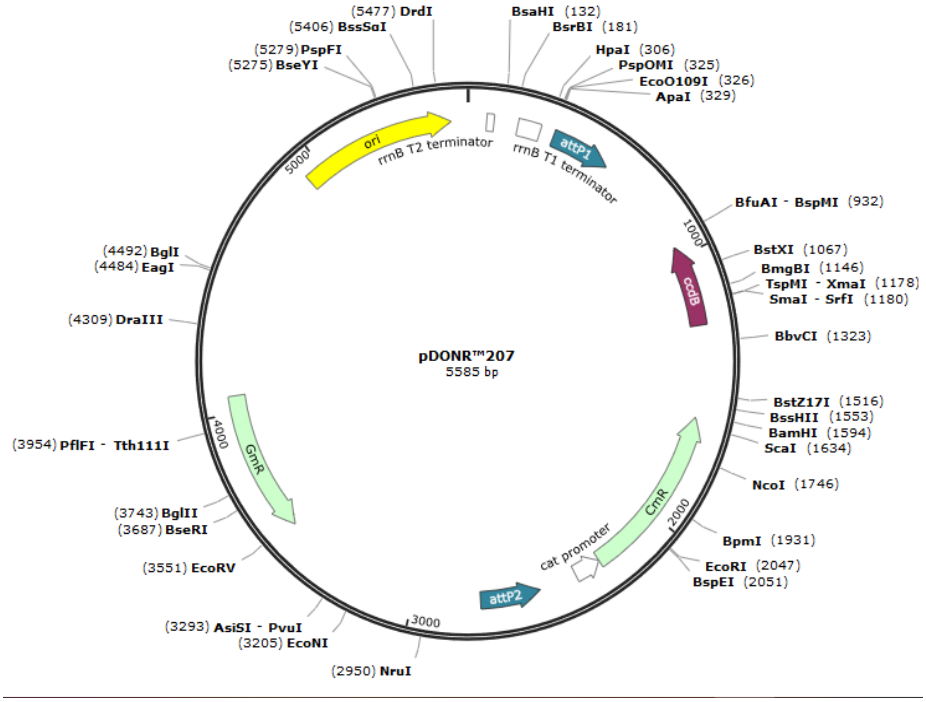


Fig. 2-5: Map of pDONR207 vector used in the BP reaction.

2.4 Histology

2.4.1 Tissue sections

For histology, tissues were embedded in plastic resin using the JB-4 embedding kit (Sigma-Aldrich, 00226) prior to cutting by microtome. Leaves were first fixed by covering with FAA solution (50 ml ethanol, 5 ml glacial acetic acid, 10ml 37% formaldehyde, 35 ml distilled H_2O) for least 1 h, but could be stored in this solution for several months. The second stage was dehydration and JB4 infiltration. JB4 infiltration solution was prepared with 1.25 g of catalyst (benzoyl peroxide, plasticized) mixed with 100 ml of JB4 solution A. The leaves were removed from the FAA solution and replaced with 75% ethanol, then the samples were gently rocked with different percentages of ethanol, from 80% to 95% for least 1 h each. Leaves were then covered with 75% ethanol plus 25% JB4 infiltration for 1 h, 50% ethanol plus 50% JB4 for 1 h, 25% ethanol plus 75% JB4 for 1 h, 100% JB4 for 1 h and finally 100% JB4 infiltration solution overnight. The third stage was the embedding, and embedding

solution was prepared by mixing 25 ml of JB4 infiltration solution with 1 ml of JB4 solution B. Then, the leaves were set up in an embedding mould in embedding solution and covered with Parafilm in a square Petri dish overnight. Longitudinal sectioning (3 µm) was carried out using a microtome and samples were stained with 0.05% toluidine blue for 10s followed by washing with water several times. DPX was added to the sample on the slide and covered by a cover slip and kept on a hot plate at 37°C for 24 h.

2.4.2 GUS buffer and staining

GUS buffer stock contained 100 mM phosphate buffer pH 7.0. To make this, 100 ml each of 0.2 M Na_2HPO_4 and 0.2 M NaH_2PO_4. 0.2 M stock phosphate buffer was made by adding 30.5 ml of 0.2 M Na_2HPO_4 to 19.5 ml of 0.2 M NaH_2PO_4 then diluted 1:1 with water to make 100 mM phosphate buffer. 80 ml phosphate buffer included 10 mM EDTA and 0.1% Triton X-100, made up to 100 ml with water and pH adjusted to 7.0, and stored in a bottle wrapped by foil at 4°C as described by Topping & Lindsey (1997). X-Gluc (5-bromo-4-chloro-3-indolyl glucuronide) was made up as follows: for 20 mM stock: 10.4 mg/ml X-Gluc in N-N-dimethylformamide (aliquoted in fume hood) and stored in 100 µl aliquots at -20°C. GUS staining of leaf used 1 ml of buffer plus 50 µl of X-Gluc and incubated at 37°C. Then the leaf was transferred to 75% v/v alcohol and incubated again at 37°C. Lastly, the leaf was transferred to chloral hydrate solution (200 g chloral hydrate; 20 g glycerol; 50 ml water) and incubated at 65°C for 12h.

2.4.3 ClearSee tissue clearing

All tissues were fixed in 4% paraformaldehyde (PFA) under vacuum (40 g of PFA powder was dissolved in 800 ml of 1xPBS buffer, pH 6.9) for 30 min. After that, the leaves were washed twice with 1xPBS buffer, 1 min for each washing. Then, the ClearSee solution (10 g of 10% Xylitol, 15 g of 15% Na deoxycholate, 25g of urea made up to 100ml with water) was added for 30 min under vacuum and the tissues were then covered with ClearSee and incubated at room temperature for 4 d. Calcofluor white stain 1 µg/ ml was dissolved in ClearSee solution to visualise cell walls.

2.5 Microscopy

2.5.1 Compound light microscopy

Leaf sections were examined using a Zeiss Axioskop compound light microscopy (Carl Zeiss, Cambridge, UK), equipped with a Qlmaging Retiga-2000r camera (Photometrics, Marlow,UK) using a x10 0bjective.

2.5.2 Stereo microscopy

Leaves and seedlings were imaged through the square Petri dish lid to avoid contamination, using a Leica M165 FC Fluorescence Stereo Microscope with Leica DFC 420 camera.

2.5.3 Confocal microscopy

Roots were stained in 1µg/ml of calcofluor white in ClearSee for 1 h under vacuum. The roots were washed with ClearSee for another 1h and the samples were mounted in ClearSee. Roots were then put on slides in sdH_2O, a 1.5 mm cover slip was placed on top, secured by Micropore tape, and imaged using the Leica SP5 TCS confocal microscope (www.leica-microsystems.com) using x40 oil immersion objectives. Excitation of flurophores was performed as follows: GFP 488 nm using the Argon laser, calcofluor white 548 nm.

2.6 Counting cells in histological sections

For counting the cells from petiole sections, images were printed in A0 size and cells counted manually using a pencil to mark each cell.

2.6 Estradiol induction

β estradiol was used for induction of gene expression according to Zuo et al. (2000). For leaf induction treatments, the hormone was a final concentration of 5 µM and was sprayed onto the leaf cultured on B5 medium. Then the leaf was collected after 24 h induction. For 0 h

leaf the seedling on 11 d culture on half MS medium was sprayed with hormone; and after 24 h the leaf was collected after 12 d culture on half MS medium.

Tool	Reference/ Website	Function
Arabidopsis Tair	https://www.arabidopsis.org/	For genetics and molecular biology of plant Arabidopsis.
Web BLAST	https://blast.ncbi.nlm.nih.gov/Blast.cgi	Specific information about the location of genes and the sequences.
Snap gene program	https://www.snapgene.com/	Used for entering cloning of the genes, sequences, and digested enzyme.
Ape program	https://jorgensen.biology.utah.edu/wayned/ape/	Designing primers.
Image j program	https://imagej.nih.gov/ij/download.html	Measure the length of the root, account the number of branches and analysis RTP-CR.
Eurofins website	https://eurofinsgenomics.eu/en/ecom/tools/pcr-primer-design/	Designing primers for qRT-PCR and RT-PCR.

IDT website	https://eu.idtdna.com/pages	Designing primers for cloning.
NASC	http://arabidopsis.info/BasicForm	Used for ordering different lines and mutants.

Table 2-8: List of programmes and websites that support the research.

Chapter 3 Hormone signalling during de novo root formation

3.1 Introduction

Plant hormones and their activities are significant signals in plant root development. Of the hormones, auxin has for many years been known to play a major role in rooting. This has been shown in many studies in different plant species, including Arabidopsis (Ahkami et al. 2013, Atta et al. 2009, Chen et al. 2016, Druege et al. 2016, Gonin et al. 2019). The exogenous application of auxin on seedlings, stem explants or roots can result in the formation and proliferation of new root primordia (Wang and Yao, 2019). Other growth regulators are also involved in the control and regulation of the growth of roots and other structures in the plant, such as callogenesis, germination and shoot development and flowering (Müller and Sheen, 2008, Schaller et al., 2015). The root apical meristem contains undifferentiated stem cell-like cells, responsible for the differentiation of the different root cell types (Schaller et al., 2015). This meristem activity is regulated by a balance between cytokinin and auxin (e.g. Moubayidin et al., 2010). The cells of the root apical meristem undergo cell division to maintain their number under a given set of environmental conditions and expand and differentiate to form the root cell types. Arabidopsis has a closed root apical meristem that controls the growth and development of the root after embryonic cell division. During this process, the number of differentiating cells and multiplication of cells has to be balanced so the newly formed root can grow and at the same time the meristem can maintain its reservoir of stem cells. Auxin and cytokinin play key roles to control this balance, and so they affect the size of meristem (Beemster and Baskin, 2000; Moubayidin et al. 2010).

Adventitious roots can be induced to form directly on tissue explants, such as on the explanted 12 d-old first leaf of Arabidopsis. When cultured on B5 medium the leaf will form adventitious roots after 8 d, without the addition of exogenous hormones (Chen, et al. 2014; Liu, et al. 2014; Yu, et al. 2017). Endogenous auxin is major factor in the control of plant development, involving its directional transport and auxin-specific regulation of gene transcription. An example of directional auxin transport is the distribution of auxin in the

root controlled by the polar auxin transport (PAT) machinery. Compared to other hormones, this polar transport of auxin appears to be unique amongst plant hormones and contributes to the establishment of positional information for development processes. The process of PAT requires the asymmetrical localization of membrane proteins integrated into the plasma membrane (PM) (Rubery & Sheldrake, 1974; Raven, 1975). There are three main families of such proteins: PIN-Formed (PIN), P-Glycoprotein (PGP), and AUX/LAX. The AUX/LAX family members work as influx carriers, transporting auxin into cells (Bennett et al., 1996; Marchant et al., 1999; Yang et al., 2006; Péret et al., 2012). The PIN family has a significant role as auxin efflux carriers (Friml, 2003; Petrášek et al., 2006; Wisniewska et al., 2006). The PGP family members are less effective as transporters for auxin gradient formation, although they work interdependently and work together with the PINs (Mravec et al., 2008).

Auxin is transported through the vasculature of the primary root by AUX1 and PIN1 (Gälweiler et al., 1998). This process is by the positioning of the proteins at the apical and basal sides of the cells respectively, directing polar transport auxin (Swarup et al., 2001). After auxin is transported through the meristem in the root tip it is transported to the root columella cells by PIN4, and this protein is located on the distal side of the cells of the central root meristem (Friml et al., 2002a). Auxin then accumulates in the first layers of the columella cells, and during normal growth is symmetrically distributed laterally by PIN3 and PIN7. Finally, the auxin is transported basipetally to the elongation zone, and also internally to the stem cell niche by AUX1 and PIN2 (Luschnig et al., 1998; Müller et al., 1998; Wolverton et al., 2002). This process is known as the reverse fountain model.

Bioactive gibberellins (GAs) are important hormones in plants and have significant roles in seed germination, leaf expansion, stem elongation and flower and seed development, and also in root development (Fleet & Sun, 2005; Yamaguchi, 2008; Brian, 1959). Regulatory genes/proteins control the concentration of GA in plant tissues and organs during development by a feedback mechanism. For example, GA30x1 in Arabidopsis is subject to negative feedback regulation. In Arabidopsis four GA3ox family members have been identified, namely AtGA3ox1, AtGA3ox2, AtGA3ox3 and AtGA3ox4 (Mitchum et al, 2006).

Gaseous ethylene (C_2H_4) is another major plant hormone that plays roles in the growth and development of plants, seed germination, fruit ripening, organ abscission and senescence (Ju et al., 2015). In the root, ethylene has a negative impact on LR development and root elongation; however, it has a positive impact on root hair formation, and when plants are treated with ethylene they develop characteristic short, fat, hairy roots. Research suggests that ethylene exerts this role in controlling root growth by mediating the regulation of auxin biosynthesis and transport. Ethylene induces the expression of auxin biosynthesis genes as well as auxin transport genes (Swarup et al., 2007; Negi et al., 2008; Stepanova et al., 2008; Lewis et al., 2011). As result of this ethylene stimulates auxin accumulation and suppression of cell expansion in cells exiting the root meristem (Ruzicka et al., 2007; Swarup et al., 2007; Stepanova et al., 2008). In addition, ethylene has a negative impact on cell division in the root meristem (Street et al., 2015), as well as in the regulation of stem cell proliferation and quiescence at the root tip (Ortega-Martínez et al., 2007).

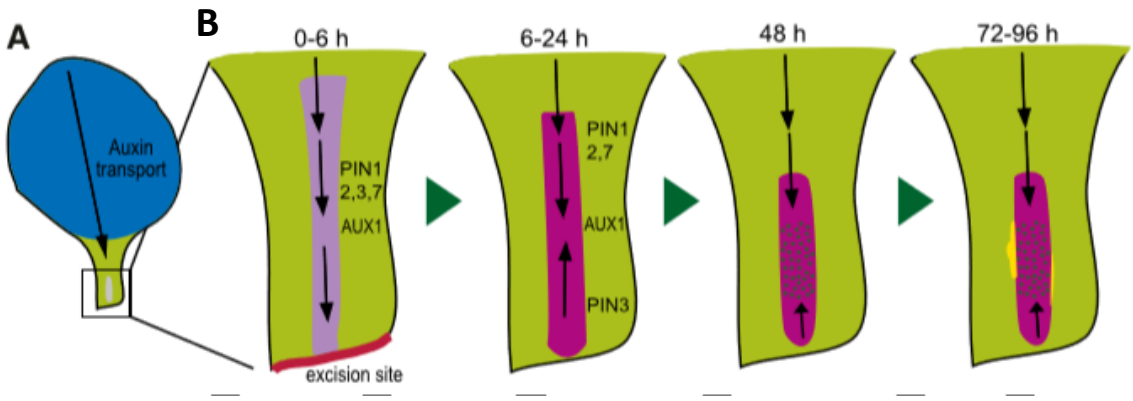


Fig 3-1. De novo root regeneration model in the leaves of Arabidopsis. A & B: Auxin is transported by long distance basipetal transport from the upper leaf down the leaf to defined vascular cells at the base of the petiole, and auxin acts together with cytokinin that is locally produced so as to induce o callus formation: PIN1/2/3/7 and AUX1 regulate auxin transport (Bustillo-Avendaño et al., 2018).

In this chapter I show that vasculature-associated cell proliferation is necessary for de novo root organogenesis and regeneration in Arabidopsis. To investigate links with hormones at

each stage, possible roles of auxin, cytokinin, gibberellin and ethylene were studied by qPCR expression analysis of relevant hormonal pathway genes at the different stages of de novo root regeneration, from three different leaf tissues: leaf blade, petiole and whole leaf. I also used GUS reporter lines to study hormone signalling linked to the process of de novo organogenesis.

To determine the role of auxin, we used the auxin reporters DR5::GUS, PIN1::GUS, PIN3::GUS and PIN7::GUS as a proxy for monitoring changes in auxin accumulation between the upper the leaf (blade leaf) and the petiole. In addition, we used other reporter lines, namely ARR4::GUS (responsive to cytokinin signalling), GA3OX2::GUS (responsive to gibberellin signalling) and EBS::GUS (responsive to ethylene signalling), together with gene expression analysis by qPCR, to monitor changes in these hormone pathways during de novo organogenesis. CYCB1;2::GUS, a marker of cells entering cell division, was also used to monitor the onset of cell division during de novo organogenesis.

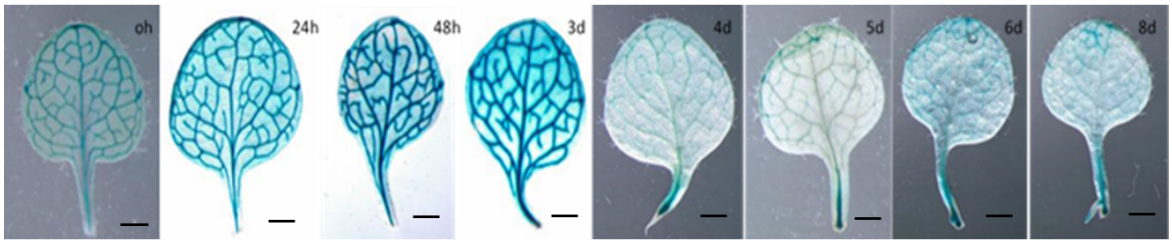
3.2 Results

3.2.1 Auxin signalling

3.2.1.1 PIN1::GUS activity and gene expression analysis in regenerating cultured leaf

PIN1::GUS expression analysis was monitored over an 8 d time course in leaves cultured on B5 medium (Fig. 3-2). At 0 h PIN1::GUS expression was relatively low but seen throughout the leaf vasculature, and expression (GUS activity) increased in the vasculature up to 3 d (Fig. 3-2A). However, by 4d and beyond PIN1::GUS expression was noticeably reduced in the blade of the leaf, but increased in the petiole (Fig. 3-2B). By 6d a new root apical meristem was formed, associated with localized PIN1::GUS activity. By 8d a new de novo root was clearly emerged from the petiole, associated with a reduction in GUS staining in both blade and petiole (Fig 3-2A, B).

A



B

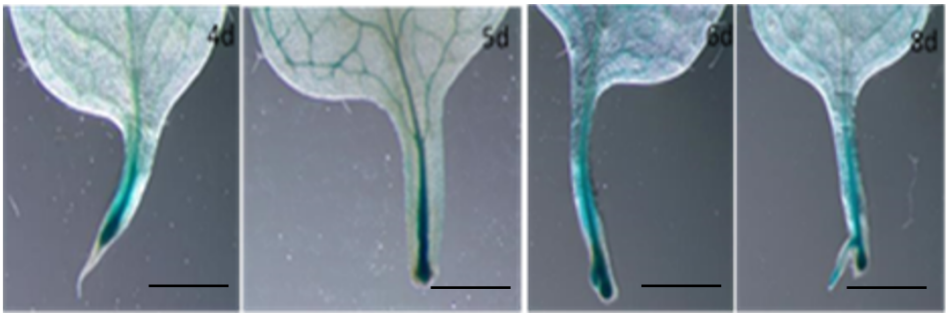


Fig 3-2. (A) PIN1::GUS expression in leaf of Arabidopsis when cultured from 0 h to 8 d, with scale bars = 1 mm. (B) Magnification of images in A, showing PIN1::GUS expression in petiole of leaf of Arabidopsis from 4 d to 8 d, with scale bars = 0.5 mm.

To determine whether the PIN1::GUS activity reflected a change in *PIN1* gene expression, qRT-PCR was carried out. Figure 3-3 shows the result of qRT-PCR analysis of the *PIN1* gene following RNA extraction from three parts of the leaf: whole leaf, leaf blade and petiole when cultured for 0 h, 24 h, 48 h, 3 d, 4 d, 5 d, 6 d and 8 d; *UBC* was used as a reference gene. The results are broadly consistent with the PIN::GUS data. In the leaf blade, *PIN1* expression increased to 48 h, then declined dramatically to lower levels thereafter. Expression in the petiole showed a different pattern, staying relatively low to day 3 and then increasing significantly between days 4 and 6, and then declining at day 8. Expression in the whole leaf followed a similar pattern to the leaf blade, reflecting the large proportion of leaf blade in the whole leaf. These data suggest a redistribution of *PIN1* gene expression between leaf blade and petiole during the adventitious root regeneration process.

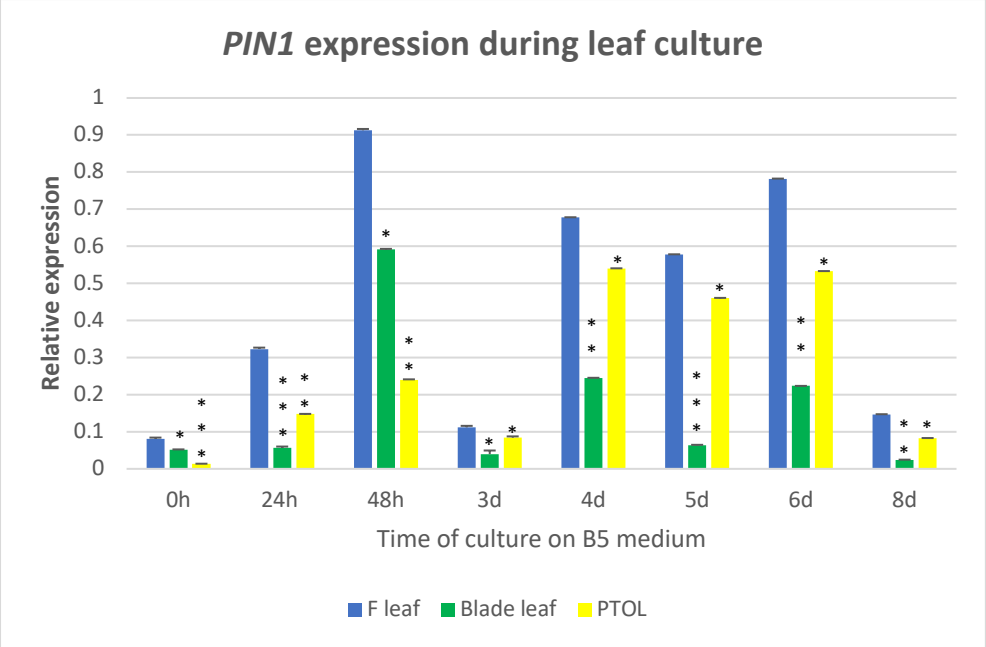
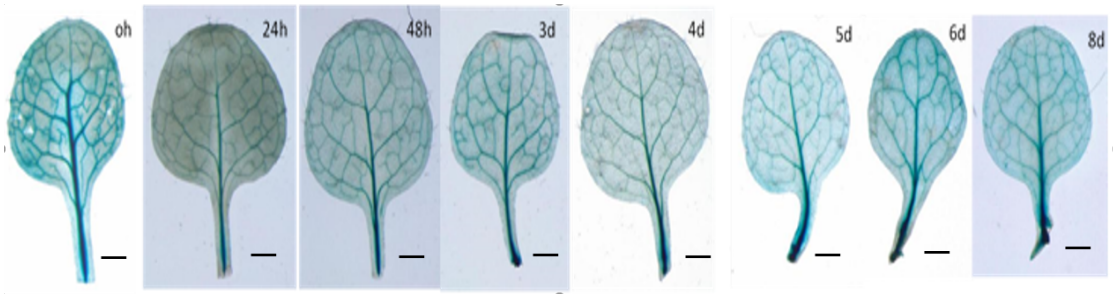


Fig. 3-3. Relative expression of the *PIN1* gene analysed using qRT-PCR in whole leaf (nn, blue bars), leaf blade (green bars) and petiole (yellow bars, PTOL) from 0 h to 8 d by using the *UBC* reference gene. Values represents means and error bars are SEM (n = three biological repeats with three technical repeats). Statistical significance was determined using Student's t-test for independent samples compared to wild type values.

3.2.1.2 PIN3::GUS activity and gene expression analysis in regenerating cultured leaf

PIN3::GUS expression analysis was similarly carried out on cultured leaves over the same 8 d time course. Results showed expression in the leaf vasculature, but there was relatively little detectable change in GUS activity in the leaf blade over the 8 d culture period (Fig. 3-4). By 5d a new root primordium was beginning to emerge, associated with increased and localized PIN3::GUS expression at that position (Fig 3-4B). By 6 d the adventitious root had emerged from the petiole, associated with intense PIN3::GUS activity through to day 8 (Fig. 3-4A,B).

A



B

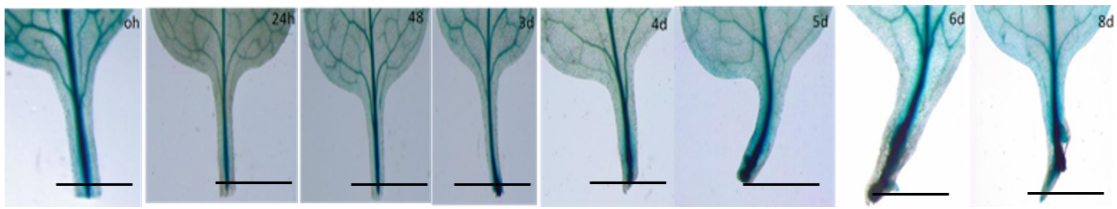


Fig 3-4. (A) PIN3::GUS expression in leaf of Arabidopsis when cultured from 0 h to 8 d, with scale bars = 1mm. (B) Magnification of images in A, showing PIN3::GUS expression in petiole of leaf of Arabidopsis from 0 h to 8 d, with scale bars = 0.5 mm.

Figure 3-5 shows the result of qRT-PCR analysis of *PIN3* gene expression following RNA extraction from three parts of the leaf: whole leaf, leaf blade and petiole when cultured for 0 h, 24 h, 48 h, 3 d, 4 d, 5 d, 6 d and 8 d; *UBC* was used as a reference gene. The results show a general increase in expression in the whole leaf up to day 6, followed at day 8 by a sharp decline. There was a decline in expression between 24 h and 3 d in the leaf blade, followed by an increase until a strong decline at day 8. In the petiole *PIN3* expression was relatively low until 6 d, when there was an increase associated with adventitious root formation, before a decline again by day 8.

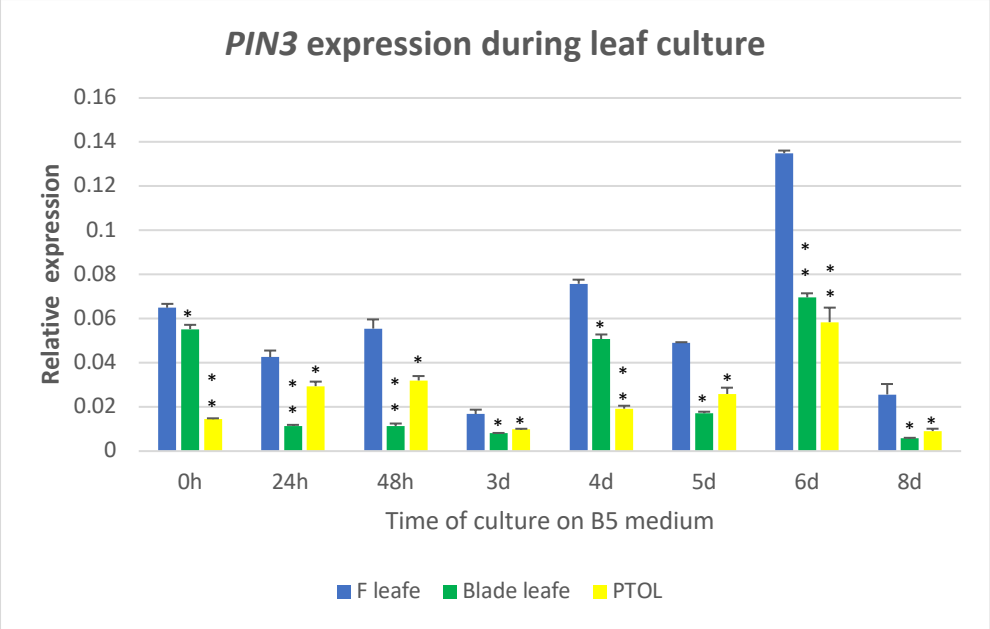
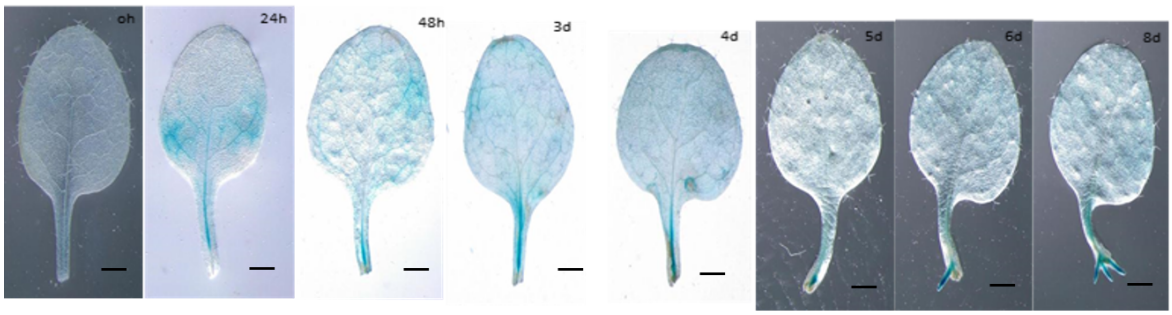


Fig. 3-5. Relative expression of the *PIN3* gene analysed using qRT-PCR in whole leaf (blue bars), leaf blade (green bars) and petiole (yellow bars, PTOL) from 0 h to 8 d by using the *UBC* reference gene. Values represent means and error bars are SEM (n = three biological repeats with three technical repeats). Statistical significance was determined using Student's t-test for independent samples compared to wild type values.

3.2.1.3 PIN7::GUS activity in regenerating cultured leaf

PIN7::GUS showed no significant expression in the 0 h leaf but patchy expression was seen in the leaf blade and petiole by 24 h (Fig. 3-6). This pattern was broadly unchanged to day 4, but by day 5, when the root primordium was initiated, there was an increased intensity of GUS activity at that site. Emerging roots showed relatively strong PIN7::GUS expression (Fig. 3-6B).

A



B

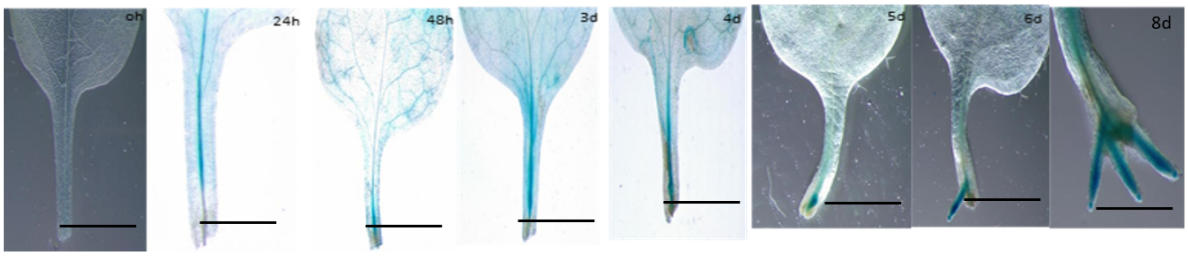
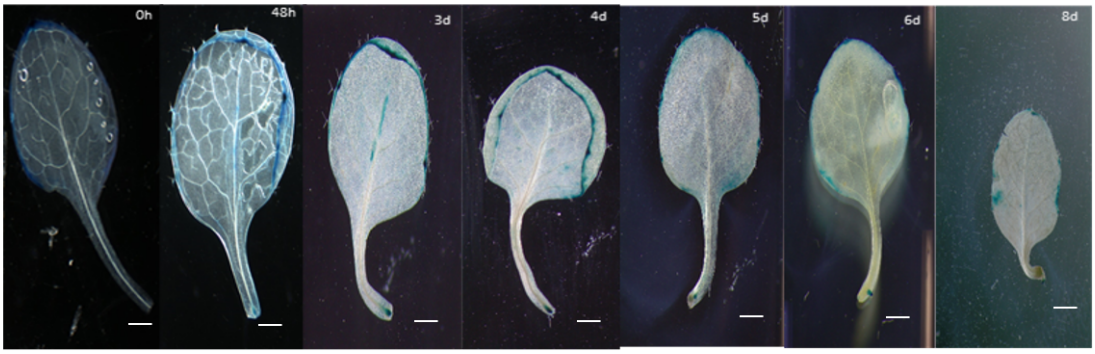


Fig 3-6 (A) PIN7::GUS expression in leaf of Arabidopsis when cultured from 0 h to 8 d, with scale bars = 1 mm. (B) Magnification of images in A, showing PIN7::GUS expression in petiole of leaf of Arabidopsis from 0 h to 8 d, with scale bars = 0.5 mm.

3.2.1.4 DR5::GUS activity in regenerating cultured leaf

The DR5::GUS reporter gene highlights cells with relatively high auxin concentrations or responses (Sabatini et al., 1999), and its expression was monitored during leaf culture over 8 d, as described above. There was little expression of DR5::GUS in the whole blade over the 8 d period, except for some expression at the leaf margins and associated with hydathodes (Fig. 3-7). By 48 h to 3 d expression was detectable in the petiole, and by 5 d became localized to the site of incipient adventitious root meristem formation. As the root emerged from the petiole, DR5::GUS activity was detectable in the new root meristem (Fig. 3-7B), consistent with its known expression in the primary root and lateral root meristems (Sabatini et al., 1999).

A



B

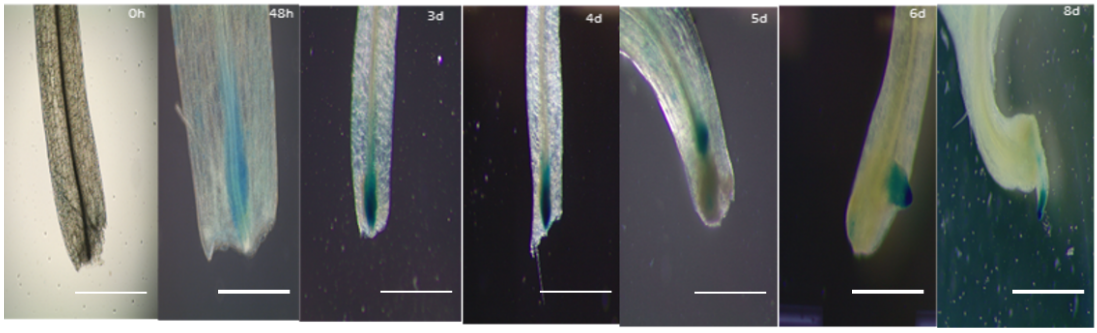


Fig 3-7. (A) DR5::GUS expression in leaf of Arabidopsis when cultured from 0 h to 8 d, with scale bars = 1 mm. (B) Magnification of images in A, showing DR5::GUS expression in petiole of leaf of Arabidopsis from 0 h to 8 d, with scale bars = 0.5 mm.

3.2.2 Cytokinin signalling

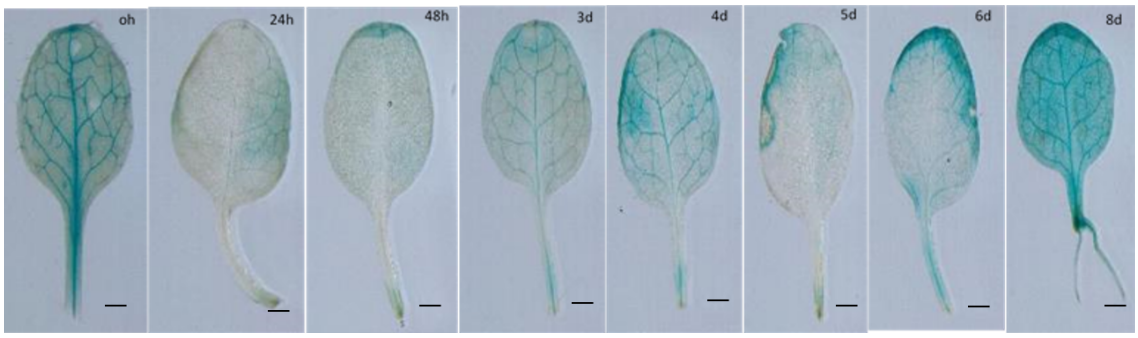
3.2.2.1 ARR4::GUS activity and gene expression analysis in regenerating cultured leaf

The *ARR4* gene of Arabidopsis encodes an A-type response regulator that is transcriptionally induced by cytokinin but negatively regulates cytokinin responses in Arabidopsis (To et al. 2004). The ARR4::GUS construct was therefore used as a reporter of cytokinin responses in the Arabidopsis leaf cultured for 8 d on B5, as described above.

At 0 h, expression was seen mainly in the vascular tissues of the leaf blade and petiole, followed by a general decline in expression over 48 h, then a gradual increase again from 3 d, with relatively strong GUS activity throughout the leaf by 8 d (Fig. 3-8). Expression was

also seen in the emerging adventitious root (Fig. 3-8B). These data indicate an increase in cytokinin concentrations in the leaf by day 8, which are repressed by the induced expression of ARR4.

A



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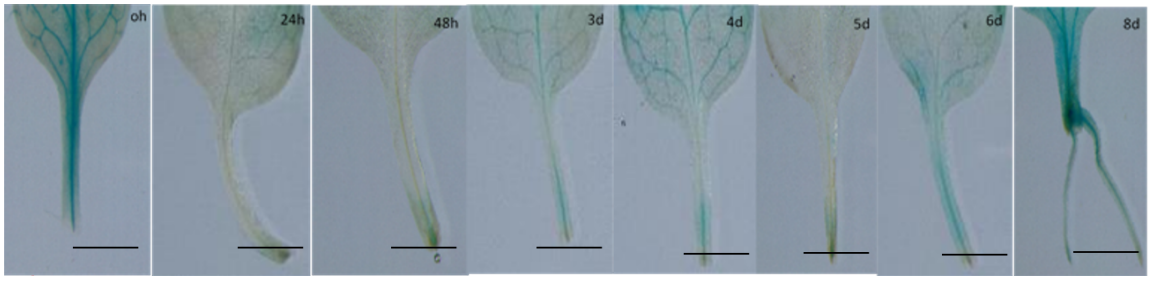


Fig 3-8 ARR4::GUS expression in leaf of Arabidopsis when cultured from 0 h to 8 d, with scale bars = 1 mm. (B) Magnification of images in A, showing ARR4::GUS expression in petiole of leaf of Arabidopsis from 0 h to 8 d, with scale bars = 0.5 mm.

Figure 3-9 shows the result of qRT-PCR analysis of *ARR4* expression following RNA extraction from three parts of the leaf: whole leaf, leaf blade and petiole when cultured for 0 h, 24 h, 48 h, 3 d, 4 d, 5 d, 6 d and 8 d; *UBC* was used as a reference gene. The results show a rapid decline in transcript levels within 24 h of culture, and these levels remain low until 5 d, when increased expression is seen in both leaf blade and petiole, consistent with the ARR4::GUS expression analysis. These results confirm a likely increase in cytokinin concentrations in the leaf after 4 - 5 d of culture, but reduced cytokinin responses (due to the role of the ARR4 response regulator) and mirroring an increase in auxin responses in the leaf during this period.

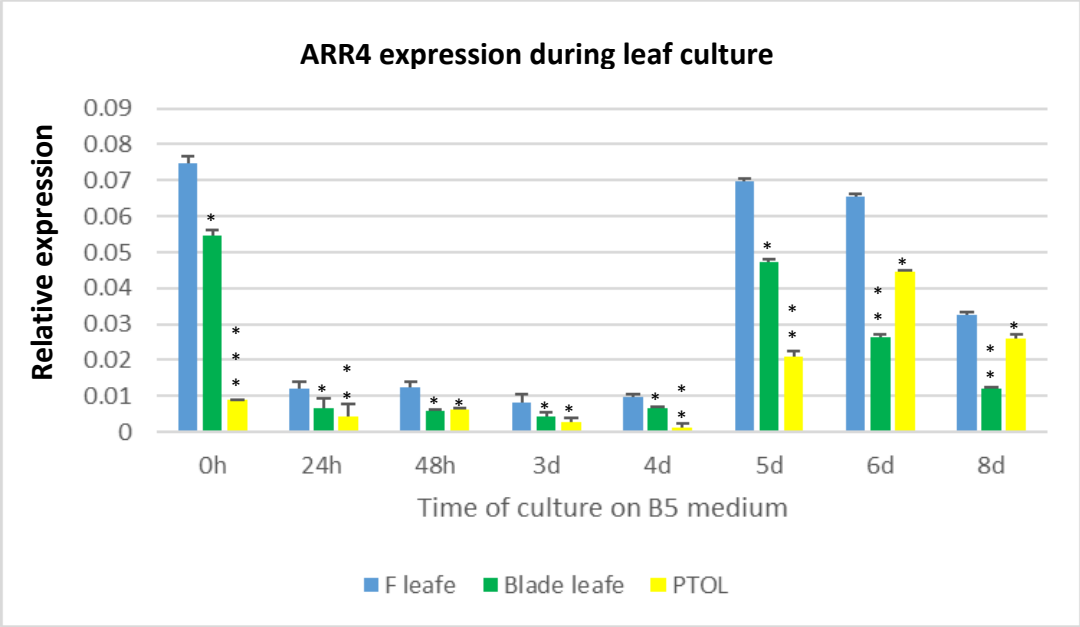


Fig. 3-9. Relative expression of the *ARR4* gene analysed using qRT-PCR in whole leaf (full leaf, blue bars), leaf blade (green bars) and petiole (yellow bars, PTOL) from 0 h to 8 d by using the *UBC* reference gene. Values represents means and error bars are SEM (n = three biological repeats with three technical repeats). Statistical significance was determined using Student's t-test for independent samples compared to wild type values.

3.2.3 Gibberellin biosynthesis

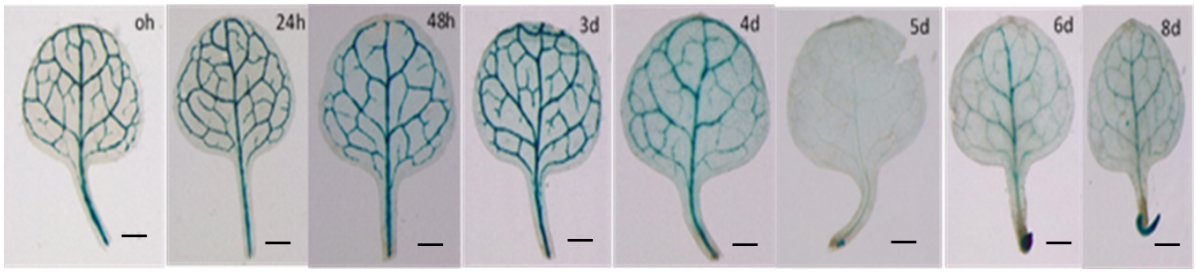
3.2.3.1 GA3ox2::GUS activity and gene expression analysis in regenerating cultured leaf

Gibberellin 3-beta dioxygenase-2 (GA3ox2) is an enzyme that converts inactive gibberellin (GA) precursors GA9 and GA20 to bioactive GAs GA4 and GA1, required for vegetative growth and development in Arabidopsis (Mitchum et al. 2006). GA3ox2::GUS activity was monitored during the 8 d leaf culture time course to determine changes in GA biosynthesis, associated with adventitious root regeneration.

Results show that GA3ox2::GUS activity was strong in the vascular tissues of the leaf blade and petiole (Fig. 3-10), in agreement with previous data (Mitchum et al. 2006). By 5 d the level of GUS activity had declined in the leaf blade but was relatively strong in the developing new root meristem (Fig. 3-10B). This is consistent with previous results that show GA3ox3::GUS activity in the root quiescent centre and columella (Mitchum et al.

2006), and indicative of a role for bioactive GAs in the activity of the adventitious root meristem.

A



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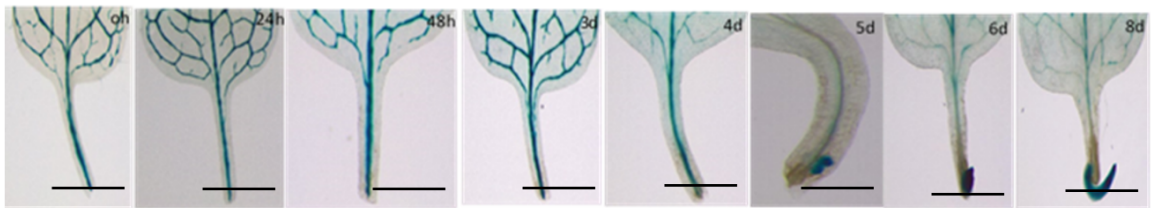


Fig 3-10. (A) GA3ox2::GUS expression in leaf of Arabidopsis when cultured from 0 h to 8 d, with scale bars = 1 mm. (B) Magnification of images in A, showing GA3ox2::GUS expression in petiole of leaf of Arabidopsis from 0 h to 8 d, with scale bars = 0.5 mm.

To confirm the GUS data, the expression of the *GA3ox2* gene was monitored in the cultured leaf over the 8 d time course by qRT-PCR. The results show that expression of the gene gradually increases over the first 3 d of culture in both leaf blade and petiole, declines at 4 d and 5 d and then increases over 6 d and 8 d (Fig. 3-11).

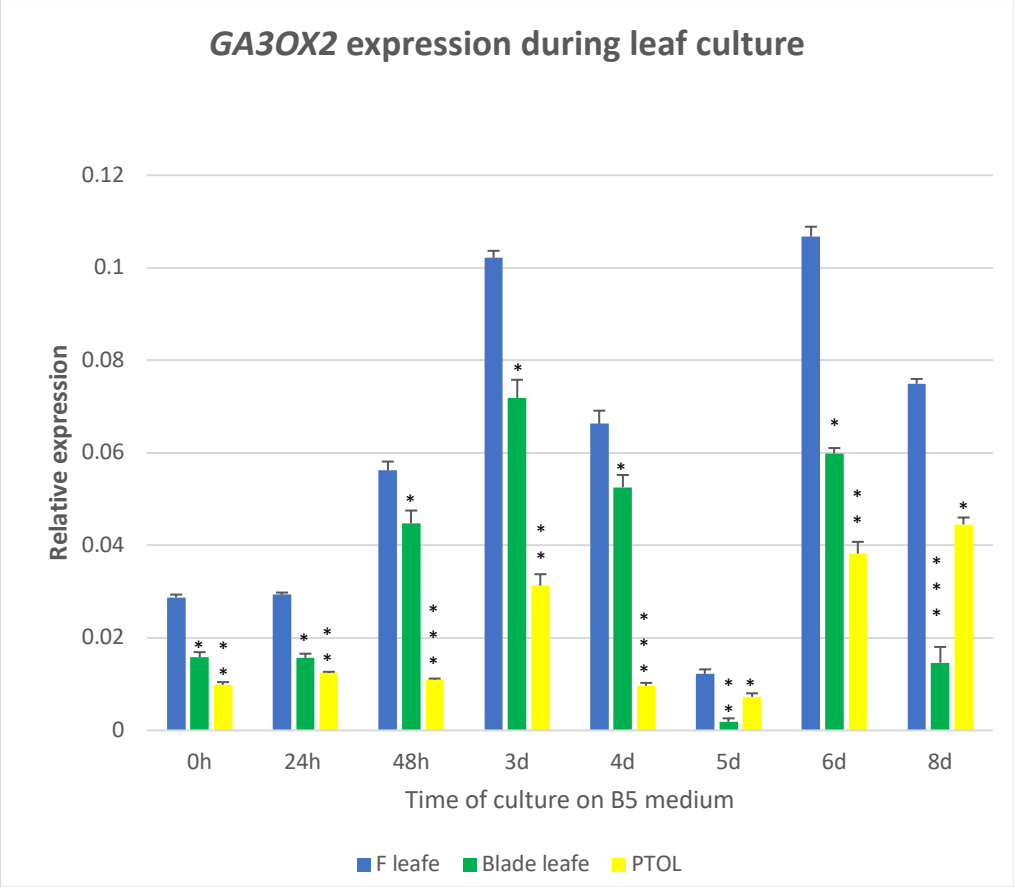


Fig. 3-11. Relative expression of the *GA3ox2* gene analysed using qRT-PCR in whole leaf (full leaf, blue bars), leaf blade (green bars) and petiole (yellow bars, PTOL) from 0 h to 8 d using the *UBC* reference gene. Values represent means and error bars are SEM (n = three biological repeats with three technical repeats). Statistical significance was determined using Student's t-test for independent samples compared to wild type values

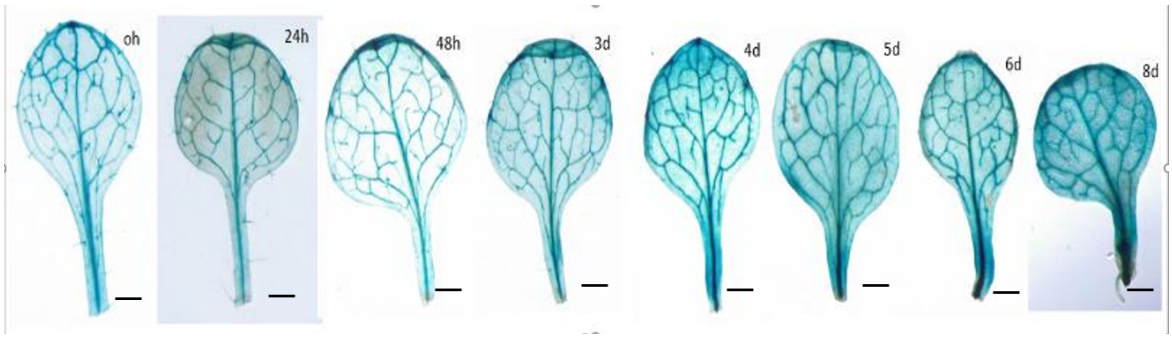
3.2.4 Ethylene signalling

3.2.4.1 EBS::GUS activity analysis in regenerating cultured leaf

The EBS::GUS reporter gene is a synthetic ethylene- (EIN3-) inducible, and can be used to monitor changes in ethylene responses; and has been used to investigate auxin-ethylene interactions during plant development, such as the role of ethylene biosynthesis in the effects of high auxin at the root transition zone, to reduce root elongation; and ethylene may also induce local auxin biosynthesis (Stepanova et al. 2007).

The results presented in Fig. 3-12 show the expression of EBS::GUS in the Arabidopsis leaf cultured on B5 medium for 8 d, as described above. GUS activity is seen predominantly in the vascular tissue of the leaf blade and petiole during the whole time course of leaf culture, but is seen to increase in the petiole from 4-5 d. Most intense expression is seen in the petiole near the wound site where the new adventitious root meristem is formed, at 6-8 d.

A



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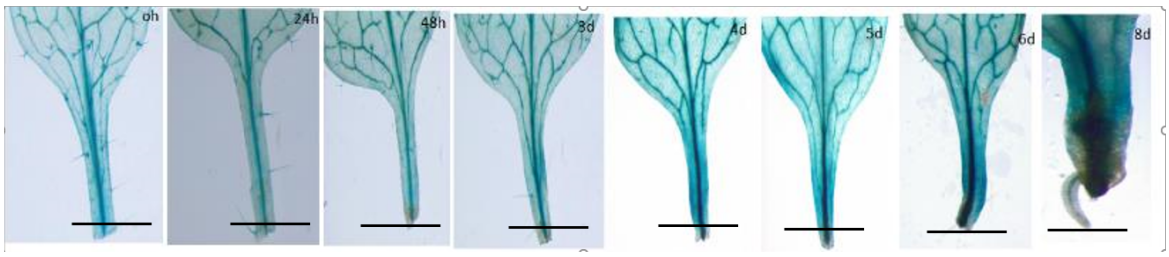


Fig 3-12. (A) EBS::GUS expression in leaf of Arabidopsis when cultured from 0 h to 8 d, with scale bars = 1 mm. (B) Magnification of images in A, showing EBS::GUS expression in petiole of leaf of Arabidopsis from 0 h to 8 d, with scale bars = 0.5 mm.

3.2.5 Cell division activation

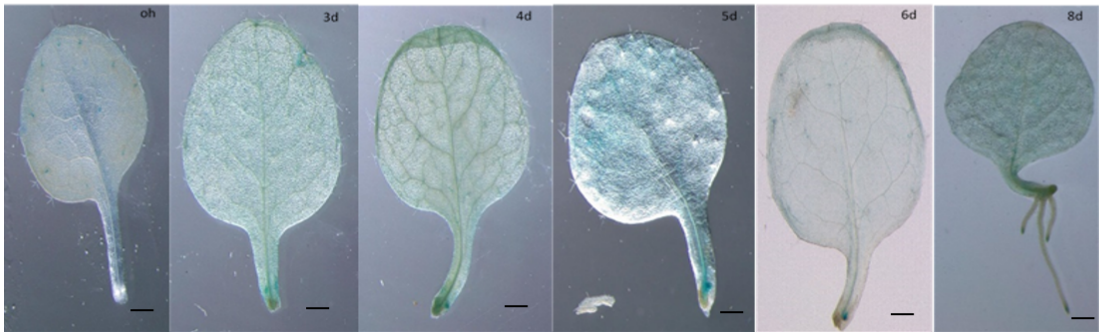
3.2.5.1 CYCB1;2::GUS activity in regenerating cultured leaf

The CYCB1;2::GUS reporter is expressed during the G2-M transition on the plant cell cycle, and has been used to monitor the onset of mitotic cell division in Arabidopsis development,

including the leaf and root (e.g. Hauser and Bauer, 2000; Schnittinger et al. 2002; Casson et al., 2009).

The results show no evidence of cell division in the leaf blade over the 8 d time course (Fig. 3-13). However, by 3 d there is evidence of cell division in the petiole, associated with the initiation of adventitious root formation, which is more evident by 4 d (Fig. 3-13B). Expression is seen in the new emerged root meristem (Fig. 3-13B, 8 h).

A



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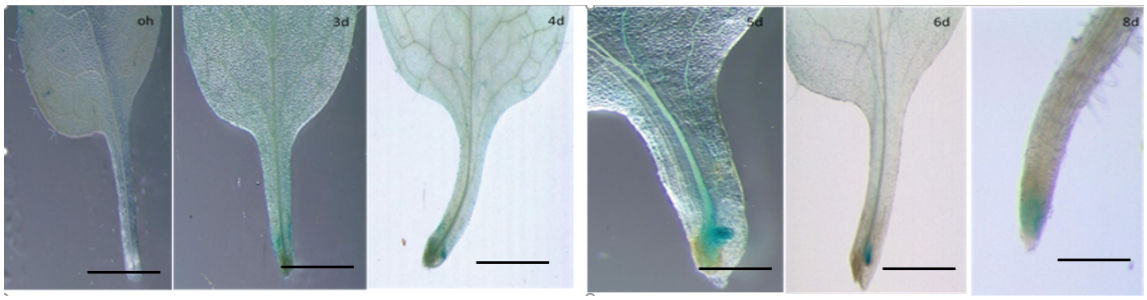


Fig 3-13. (A) CYCB1;2::GUS expression in leaf of Arabidopsis when cultured from 0 h to 8 d, with scale bars = 1 mm. (B) Magnification of images in A, showing CYCB1;2::GUS expression in petiole of leaf of Arabidopsis from 0 h to 8 d, with scale bars = 0.5 mm.

3.3 Summary

Work in this chapter was focused on hormonal signalling during de novo root formation, using GUS reporter lines and qRT-PCR analysis in an in vitro leaf culture system. The results show that auxin pathways (transport proteins, signalling responses) are activated during de

novo root formation, consistent with the known importance of auxin as a regulator of root development. The cytokinin hormone pathway can antagonize auxin responses, and the activation of the cytokinin signalling negative regulator during the root formation process is consistent with a reinforcement of auxin responses. Evidence is provided for an activation of gibberellin synthesis in the developing root, and for ethylene responses, which may be linked to the auxin effects as these hormone pathways exhibit significant crosstalk (e.g. Stepanova 2007). The emergence of the root at around 5-6 d of culture is reflected in an earlier activation of cell division at 3 d, seen as CYCB1;2::GUS expression. The results of this chapter suggest that there is likely cross talk between hormones, and coordination between them during the creation of a de novo root.

Chapter 4. Role of hormone signalling pathways in *de novo* root formation

4.1 Introduction

Unlike animals, plants do not have the ability to move independently and have several mechanisms to survive in a changing environment. De novo organogenesis, such as adventitious root (AR) formation, from wounds or detached plant tissues or organs, is one process of adaptation. This kind of root developmental programme depends on endogenous hormones and arises from procambium or cambium cells, which contain adult stem cells located in the vascular tissues of aerial organs (Chen et al. 2014). The AR development process comprises three successive physiological phases. Firstly, 'induction phase' is the term describing events that happen before any obvious histological events. Secondly, the 'initiation phase' involves the initiation of cell divisions and the formation of internal new root meristems. Finally, the 'expression phase' involves the formation of the typical dome-shaped structures and internal patterning of root primordia, resulting in growth and root emergence (Pacurar et al. 2014).

To investigate hormonal signalling pathways associated with AR formation, a series of hormonal pathway mutants and the expression of root developmental pathway genes were analysed over a time course of AR development using the leaf system previously described. The *POLARIS* gene (*PLS*) was identified in Arabidopsis as a GUS promoter trap transgenic line (Topping et al. 1994). GUS (B-glucuronidase) gene expression driven by the endogenous *PLS* gene promoter appeared mostly in the embryo and seedling root, with some low levels of expression in aerial parts (leaf vascular tissues). The *pls* locus was cloned and the promoter trap T-DNA had integrated into a short open reading frame. RNA gel blot analysis showed that the *PLS* ORF is located within a short transcript (500 nucleotides) and encodes a polypeptide of 36 amino acid residues (Casson et al. 2002). The *PLS* transcript is auxin-inducible. Phenotypically the *pls* mutants show a short root and decreased vascularization of leaves. In addition, *pls* roots are hyperresponsive to exogenous cytokinins and show increased expression of the *ARR5*/*IBC6* gene, which is cytokinin-inducible. Furthermore, mutation of *pls* increases ethylene responses which causes defective auxin transport and

homeostasis. Therefore, PLS is required for correct auxin-cytokinin-ethylene homeostasis to affect root growth and aerial part vascular development (Chilley et al. 2006).

The *AUXIN RESISTANCE1* (*AXR1*) gene of Arabidopsis was identified as a recessive mutation which showed auxin resistance in root, rosettes, and inflorescences of the plant (Leyser et al., 1993). This gene is located on chromosome 1 and encodes a protein of 540 amino acids, and the protein is related to the ubiquitin-activating enzyme E1 (Leyser et al., 1993; Timpte, 1995). The *axr1* mutant of Arabidopsis has various morphological phenotypes, with disorders in leaf, flower and vascular development, stem and hypocotyl elongation, formation of lateral roots and root gravitropism (Timpte, 1995). There are twenty *axr1* allelic lines that show defective phenotypes in Arabidopsis, such as the mutant line *axr1-12* with strong auxin response defects, and *axr1-3* with less auxin resistance and less severe defects (Lincoln et al. 1990).

ETHYLENE INSENSITIVE (EIN2) is a key regulator of ethylene signalling in Arabidopsis (Roman 1995). The *EIN2* gene encodes a 1294 amino acid protein which includes a membrane-spanning amino terminus and carboxyl terminus (Alonoso 1999). EIN2 function was determined when transiently expressed at the ER membrane in tobacco leaf cells (Bission 2009). Mutation of *EIN2* caused complete insensitivity to ethylene (Roman 1995; Alonso 1999). There are 25 mutant alleles with confirmed insensitivity to ethylene at the morphological, physiological, and molecular level. Also, the *ein2* mutant proved to be resistant to auxin transport inhibitors, cytokinins and abscisic acid (Alonso 1999), confirming the interaction between these hormones and ethylene. The phenotype of *ein2* in *Lotus japonicus*, when supplied with ACC, a precursor of ethylene hormone biosynthesis, featured increased root growth and increased nodulation (Miyata 2013).

The Arabidopsis auxin response gene *AXR3* is located on chromosome 1. Mutation of *AXR3* causes gain of function pleiotropic phenotypes with defects such as reduced root elongation, increased adventitious roots, no root gravitropism, enhanced apical dominance and ectopic expression of auxin-inducible SAUR genes. In addition, *axr3* shows resistance to the hormones ethylene and cytokinin (Leyser et al., 1996).

The aim of the work described in this chapter was to study anatomical changes and molecular mechanisms controlling the development of *de novo* root formation in Arabidopsis Col-0 leaf explants, based on the system described above. I focused on early events of AR initiation, using petioles of the first two true leaves harvested from seedlings of the cultured leaves of wild type (Col-0) and the hormonal mutants *axr1*, *axr3*, *ein2* and *pls* at 12 d post germination and cultured on B5 medium without added hormones. In a preliminary analysis I made a morphological analysis of de novo root regeneration, comparing the length and number of branches of *de novo* roots for each. I then made histological sections to determine the extent of proliferating cells in each. I then analysed the expression of genes *WOX5*, *NAC1*, *YUC1* and *YUC4* using qRT-PCR, to confirm that my experimental system is consistent with previous studies.

4.2 Results

4.2.1 Comparison between wild type and mutant regeneration phenotypes

Figure 4-1 shows illustrative examples of *de novo* root from Col-0, *axr1*, *pls*, *axr3* and *ein2* genotype leaf after germination on B5 medium without added hormones, using vertical plates to reveal root gravitropism. The Col-0 and *ein2* leaves exhibited de novo root formation after 8 d, and the *ein2* root morphology was similar to wild type. The leaf of *axr3* also produced de novo roots by 8 d, but the roots were agravitropic; the size of the leaf was small compared to wild type. However, the leaf of *axr1* and *pls* were unable to produce de novo roots by 8 d, though *pls* was able to regenerate small and abnormally shaped roots after 12 d culture on B5 medium; the leaf of *pls* was smaller in size than wild type, and yellow in colour.

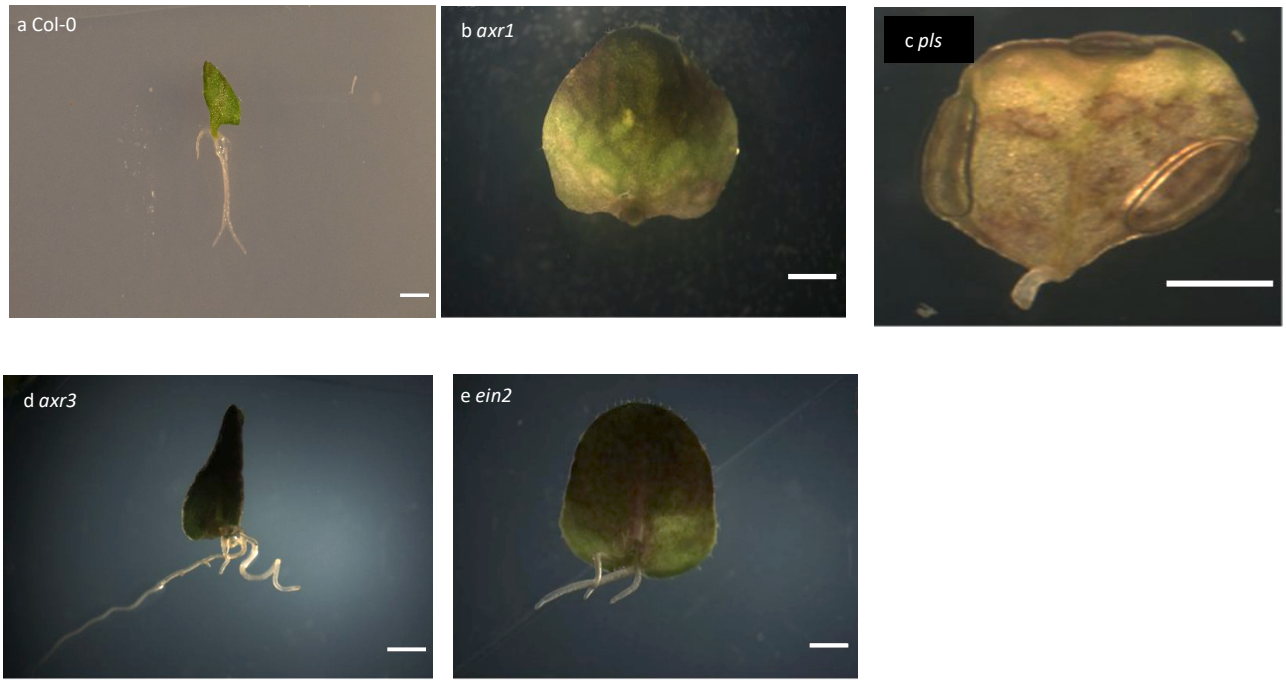


Fig 4-1. Regeneration of de novo roots from *Arabidopsis* leaf germinated on B5 medium at 10 d. (a) Col-0, (b) *axr1* (c) *pls*, (d) *axr3*, (e) *ein2*. scale bar = 1 mm except *pls* = 0.5 mm.

Figure 4-2 shows the mean lengths of *de novo* roots of wild type and mutants after 12, 19 and 26 d of culture. Firstly, the mean lengths of the roots after 12 d for Col-0 was 34.25 mm, for *pls* was 6.51 mm, for axr3 was 11.49 mm, for *ein2* was 20.19 mm and for *axr1* no roots were detected. By 19 d, the wild type root increased to a mean of 52.2 mm, *pls* increased by a much smaller amount to a mean of 7.75 mm, *axr3* roots increased to a mean of 23.17 mm, *ein2* roots were not significantly different to wildtype at a mean of 43.5 mm, while *axr1* was still showing no root formation. This general pattern was retained at 26 da (Col-0 122.41 mm, *pls* 8.30 mm, *axr3* 39.19 mm, *ein2* 117.61 mm, and no roots on *axr1*).

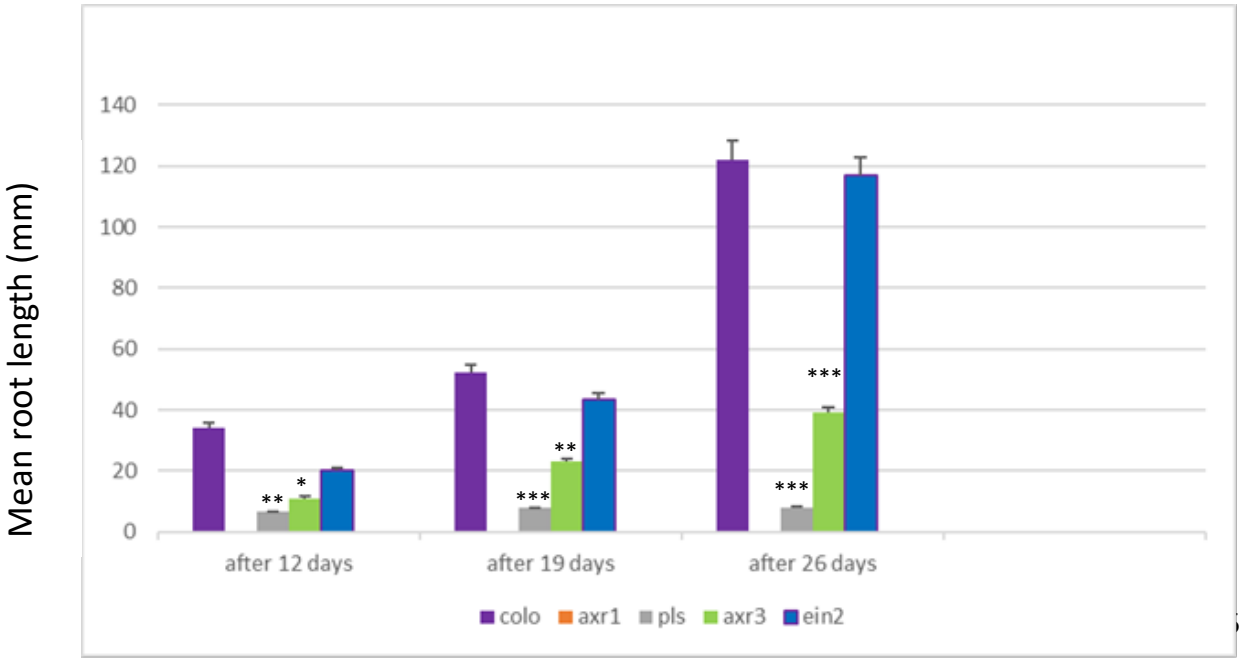


Fig 4-2. De novo root length measurements for wild type and mutants at 12, 19 and 26 d of culture on B5 medium. Values are means of at least 30 samples ± SEM. Statistical significance was determined using Student's t-test for independent samples compared to wild type values, with P-values <0.05 (*), P <0.01 (**), P-value < 0.001 (***).

Figure 4-3 shows the mean numbers of root branches formed on wild type and mutant leaf explants after 12, 19 and 26 d of culture. For wild type, the mean number was 1.4 at 12 d, rising to 36.8 at 26 d. *axr1* was unable to regenerate roots. *pls* produced a mean of 0.9 root branches at 12 d, rising to 3.1 at 26 d. *axr3* produced a mean of 2.1 at 12 d, rising to 13.7 at 26 d. The *ein2* leaf produced a mean of 1.3 branches at 12 d, not significantly different to wild type then or at 19 d, but produced a significantly lower number of branches at 26 d 23.5, P <0.05.

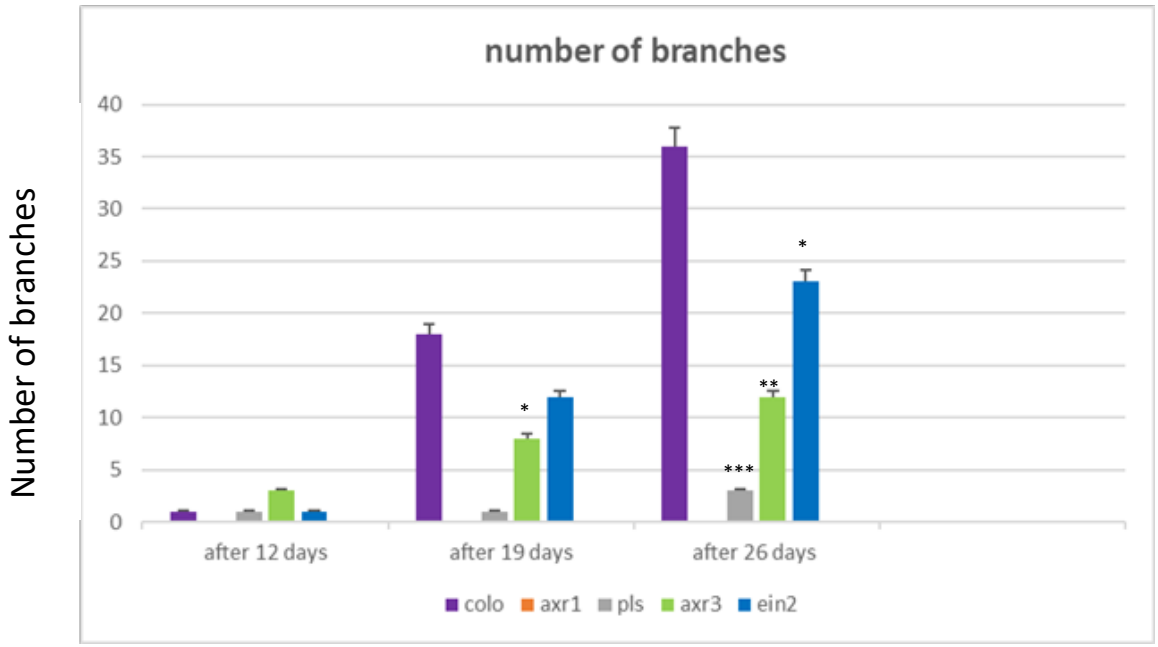


Fig 4-3. Mean numbers of root branches formed on wild type and mutant leaf explants after 12, 19 and 26 d of culture on B5 medium. Statistical significance was determined using Student's t-test for independent samples compared to wild type values, with P-values <0.05 (*), P <0.01 (**), P-value < 0.001 (***).

4.2.2 Histological analysis of de novo root formation in wildtype and mutants

Figure 4-4 shows histological sections of petioles of Col-0, *axr1*, *pls*, *axr3* and *ein2* sampled at 0 h, 3 d, 5 d, 8 d and 16 d after germination on B5 medium lacking exogenous hormones. No cell proliferation was evident at 0 h, when the petiole was placed on the medium, for either the wildtype or mutants. By 3 d on B5 medium both wildtype and mutants showed evidence of cell proliferation in the petiole vasculature and early signs of high cell density associated with future root primordium formation was seen in all except *axr1* by 5 d.

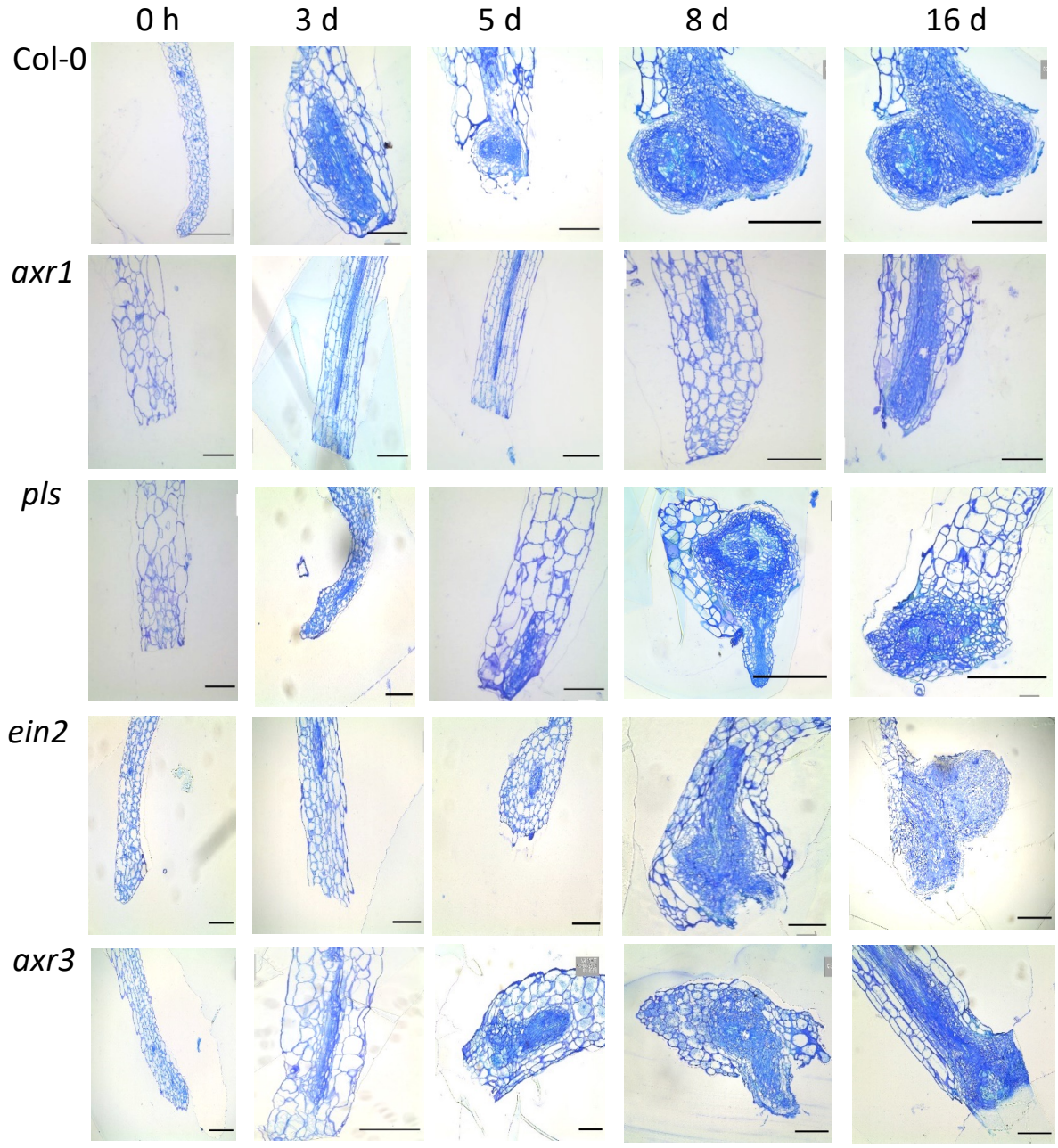


Fig. 4-4. Longitudinal sections of leaf petioles of wild type and mutants sampled at culture periods of 0h, 3d, 5d, 8d and 16 d on B5 medium Scale bar= 100 µm

Figure 4-5 shows the result of the comparison of the number of cells in replicated individual longitudinal sections of petioles of Col-0, *axr1*, *pls*, *ein2* and *axr3* at 3 d, 5 d, 8 d and 16 d of culture. At 3 d the mean number of cells in Col-0 was with 320.36, in *axr1* it was 42.6, in *pls* it was 90.6, in *ein2* it was 130.6 and in *axr3* it was 96 cells. Statistical analysis showed that there were very significant differences between wildtype and all mutants. The numbers of cells increased in all genotypes, such that by 16 d the maximum numbers were obtained, with 1770.6 for Col-0, 313 for axr1, 313 for pls, 1100 for ein2 and 710.6 for axr3 (Fig. 4-5).

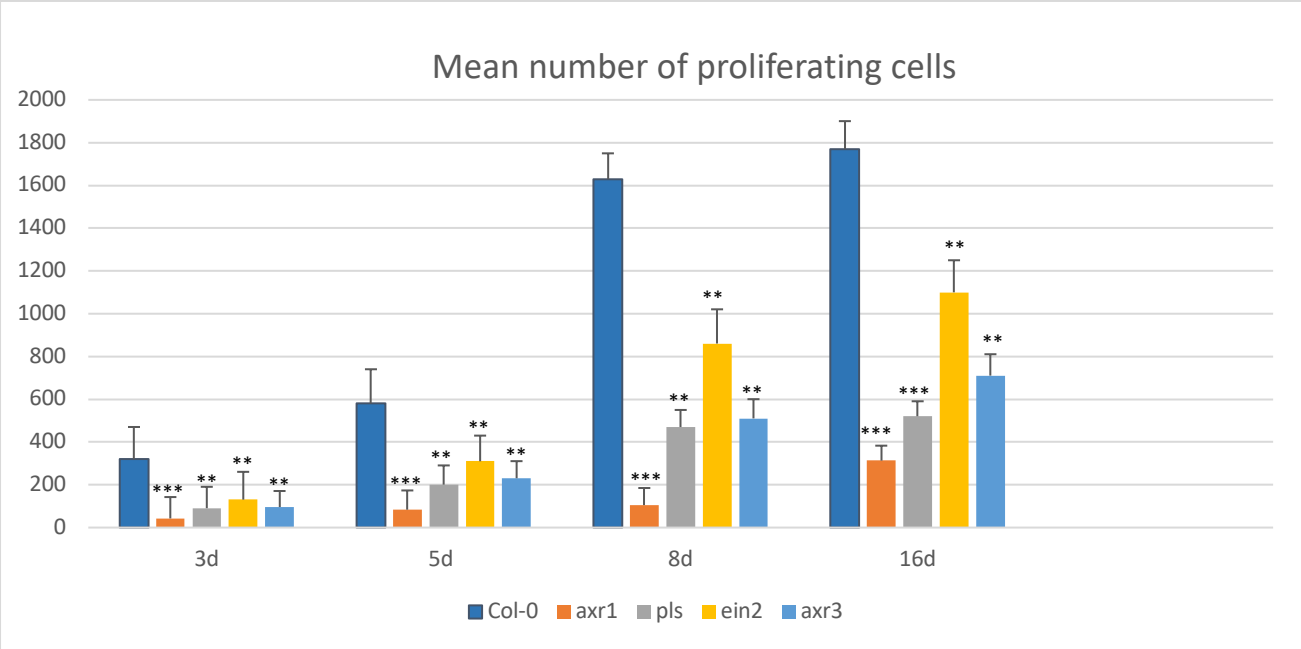


Fig. 4-5. Difference in cell count observed between wildtype and mutants at different times. (a) gives the difference in the count of cells observed between Col-0 and mutants at 3 d. (b) reflects the difference in the number of cells observed between Col-0 and mutants at 5 d. (c) presents the difference in the count of cells at 8 d. (d) represents the difference in the count of cells between Col-0 and mutants at 16 d. Values are means ± SE of three biological repeats. Statistical significance was determined using Student's t-test for independent samples (P-value <0.05, P<0.01, P-value< 0.001).

4.2.3 *NAC1* gene expression during root regeneration in wild type and mutant leaf

Figure 4-6 shows the results of an analysis of *NAC1* gene expression in wild type and mutant leaf (*axr1*, *pls*, *ein2* and *axr3*) during a culture time course of 0 h, 48 h, 3 d, 7 d and 14 d,

using *UBC* as reference gene. The results show a significant increase in *NAC1* expression between 48 h and 3 d, with no significant difference between wild type and mutants during this period. Interestingly, by 14 d the expression of wild type, *ein2* and *axr3* has continued to increase, but has dramatically reduced in both *pls* and *axr1* mutants, associated with leaf yellowing (senescence) in these mutants by this stage.

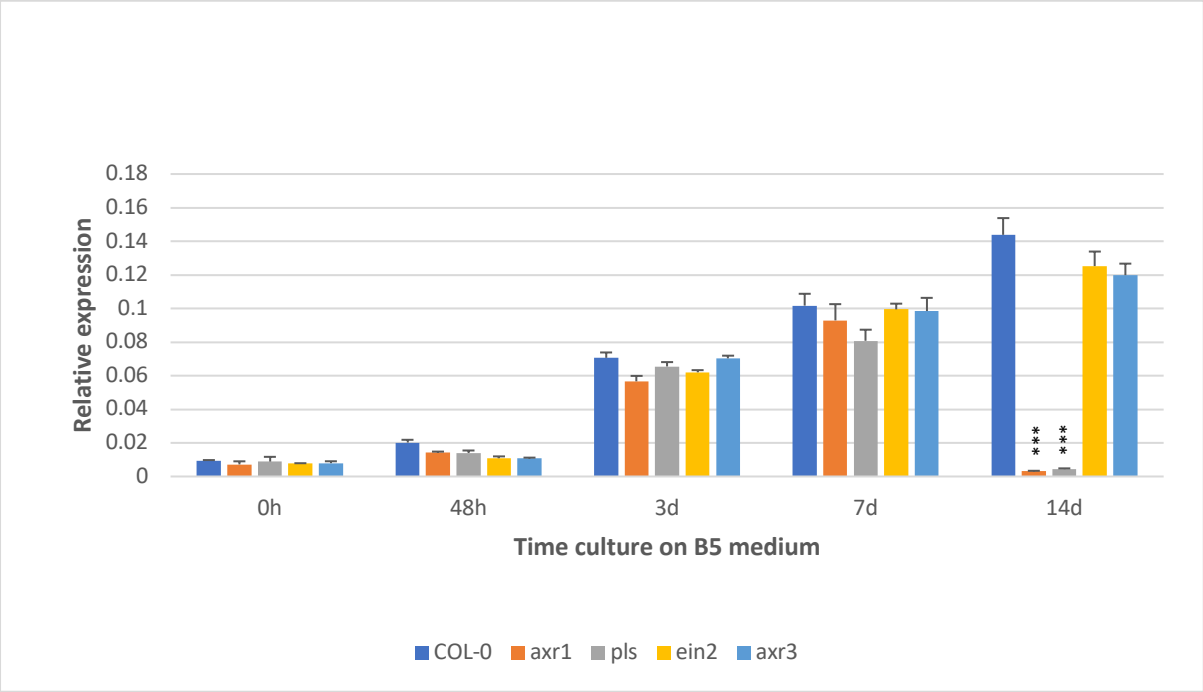


Fig. 4-6 qRT-PCR analysis of *NAC1* gene expression in wild type and mutant leaf (*axr1*, *pls*, *ein2* and *axr3*) during a culture time course of 0 h, 48 h, 3 d, 7 d and 14 d, using *UBC* as reference gene. Values represents means and error bars are SEM (n = three biological repeats with three technical repeats). Statistical significance was determined using Student's t-test for independent samples compared to wild type values; *** = P-value <0.001.

4.2.4 *WOX5* gene expression during root regeneration in wild type and mutant leaf

Figure 4-7 shows the results of an analysis of *NAC1* gene expression in wild type and mutant leaf (*axr1*, *pls*, *ein2* and *axr3*) during a culture time course of 0 h, 48 h, 3 d, 7 d and 14 d, using *UBC* as reference gene. The results show that while *WOX5* expression increased in wild type, *ein2* and *axr3* samples during the culture period, *axr1* and *pls* samples showed

very low levels of expression throughout the 14 d period, and for *axr3*, the expression of *WOX5* was significantly lower than wild type and *ein2* from 3 d onwards, though significantly higher than either *axr1* or *pls*.

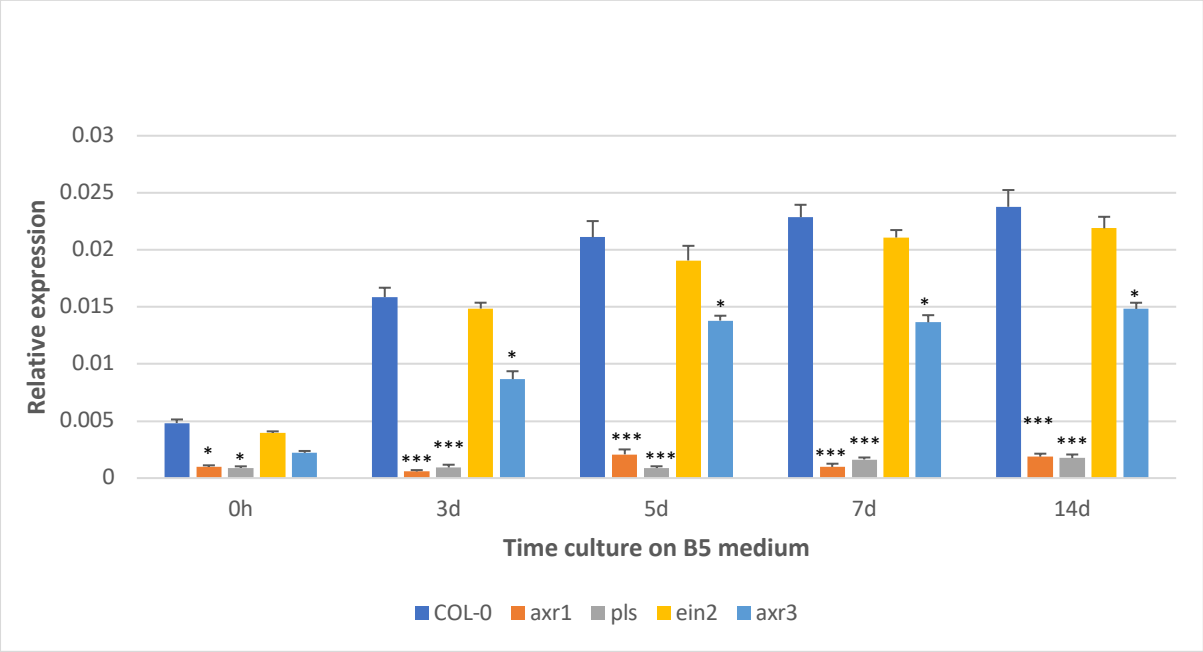


Fig. 4-7. qRT-PCR analysis of *WOX5* gene expression in wild type and mutant leaf (*axr1*, *pls*, *ein2* and *axr3*) during a culture time course of 0 h, 48 h, 3 d, 7 d and 14 d, using *UBC* as reference gene. Values represents means and error bars are SEM (n = three biological repeats with three technical repeats). Statistical significance was determined using Student's t-test for independent samples compared to wild type values; * = P-value <0.05, *** = P-value < 0.001.

4.2.5 *YUC1* gene expression during root regeneration in wild type and mutant leaf

Figure 4-8 shows the results of an analysis of *YUC1* gene expression in wild type and mutant leaf (*axr1*, *pls*, *ein2* and *axr3*) during a culture time course of 0 h, 48 h, 3 d, 7 d and 14 d, using *UBC* as reference gene. The results show that while *YUC1* expression increased in wild type, *ein2* and *axr3* samples during the culture period, *axr1* and *pls* samples showed very low levels of expression throughout the 14 d period, and for *axr3*, the expression of *YUC1* was significantly lower than wild type and *ein2* from 3 d onwards, though significantly

higher than either *axr1* or *pls*. This time course pattern is similar to that of *WOX5* expression.

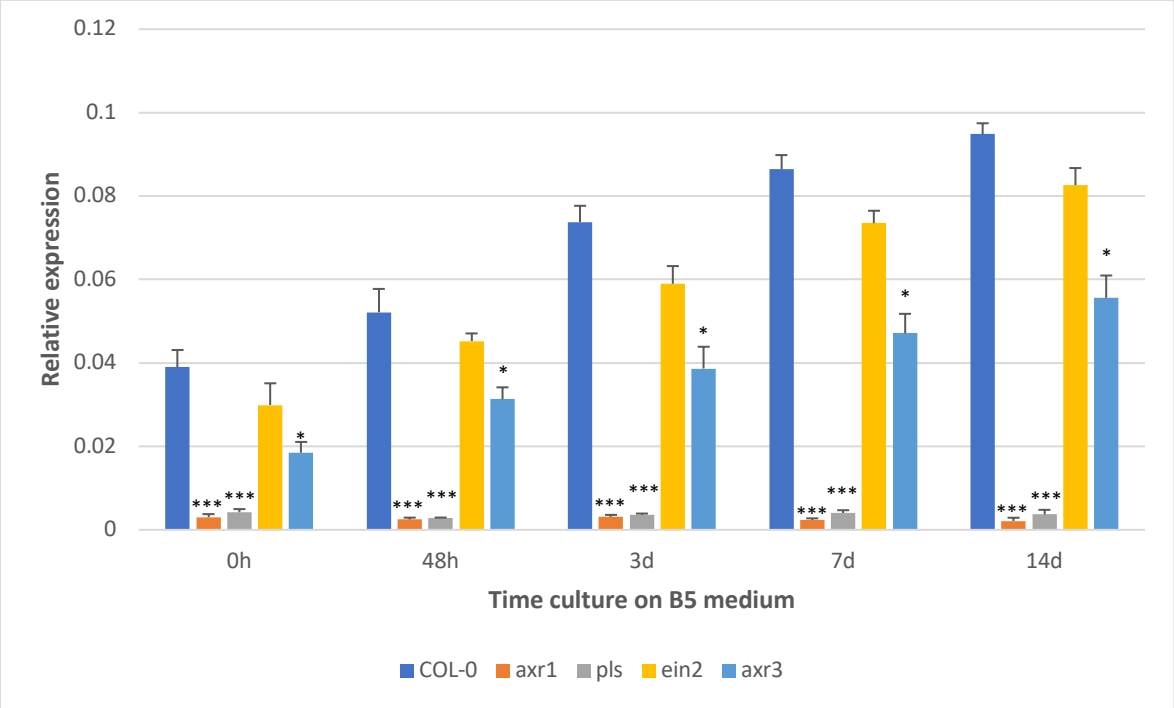


Fig. 4-8. qRT-PCR analysis of *YUC1* gene expression in wild type and mutant leaf (*axr1*, *pls*, *ein2* and *axr3*) during a culture time course of 0 h, 48 h, 3 d, 7 d and 14 d, using *UBC* as reference gene. Values represent means and error bars are SEM (n = three biological repeats with three technical repeats). Statistical significance was determined using Student's t-test for independent samples compared to wild type values; * = P-value <0.05, *** = P-value < 0.001.

4.2.6 *YUC4* gene expression during root regeneration in wild type and mutant leaf

Figure 4-9 shows the results of an analysis of *YUC4* gene expression in wild type and mutant leaf (*axr1*, *pls*, *ein2* and *axr3*) during a culture time course of 0 h, 48 h, 3 d, 7 d and 14 d, using *UBC* as reference gene. The results show that while *YUC4* expression increased in wild type, *ein2* and *axr3* samples during the culture period, *axr1* and *pls* samples showed very low levels of expression throughout the 14 d period, and for *axr3*, the expression of *YUC1* was significantly lower than wild type and *ein2* from 3 d onwards, though significantly

higher than either *axr1* or *pls*. This time course pattern is similar to that of *WOX5* and *YUC4* expression.

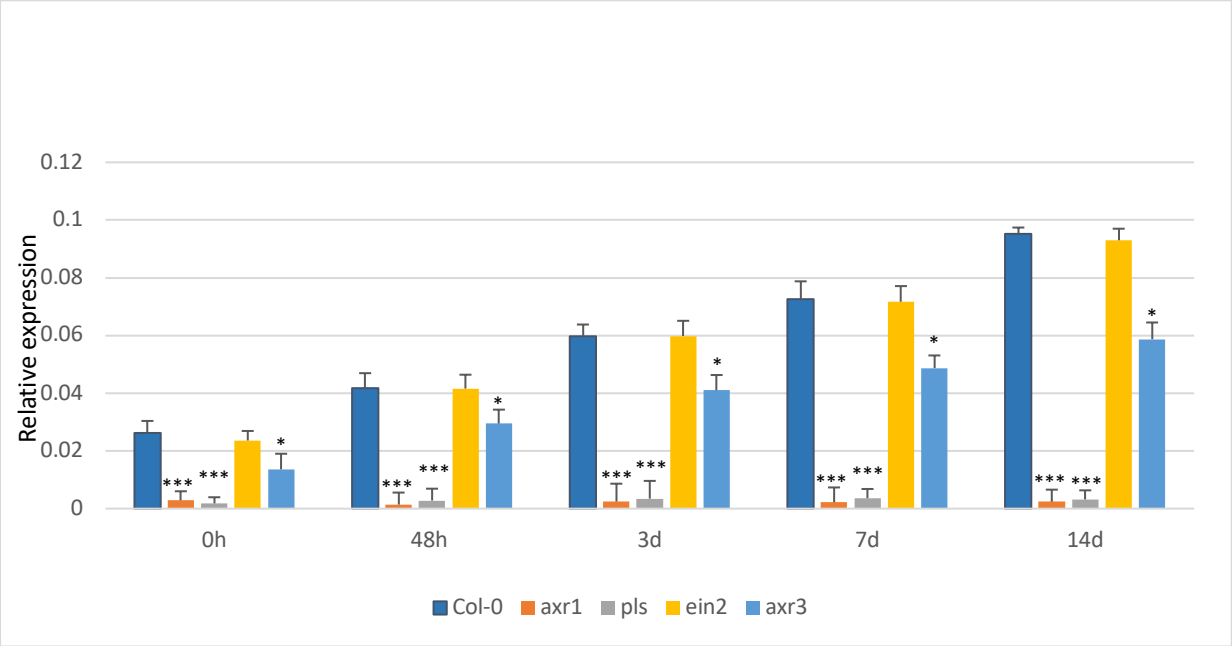


Fig. 4-9. qRT-PCR analysis of *YUC4* gene expression in wild type and mutant leaf (*axr1*, *pls*, *ein2* and *axr3*) during a culture time course of 0 h, 48 h, 3 d, 7 d and 14 d, using *UBC* as reference gene. Values represent means and error bars are SEM (n = three biological repeats with three technical repeats). Statistical significance was determined using Student's t-test for independent samples compared to wild type values; * = P-value <0.05, *** = P-value < 0.001.

4.3 Summary

Work described in this chapter was focused on understanding the requirement of specific hormone signalling pathways for the formation of de novo adventitious roots in Arabidopsis. The approach was to compare the root regeneration responses in specific mutants compared to wild type, and to monitor the expression of genes known to be involved in adventitious rooting, so that the results could be compared with other data in the literature that has used the same adventitious rooting system. The results show that the *axr1* mutant failed to create de novo roots, indicative of a requirement for auxin signalling. Both *pls* and *axr3* mutants formed roots but they were small and distorted compared to wild type,

indicating that the normal function of both genes is necessary for adventitious rooting. On the other hand, the *ein2* mutant developed roots of similar frequency and length as wild type, indicating that, since the *ein2* mutant is ethylene insensitive, ethylene signalling is not required. Expression analysis of the *NAC1*, *WOX5*, *YUC1* and *YUC4* genes showed very low levels of expression in *axr1* and *pls* compared to wild type, associated with reduced auxin responses and increased leaf senescence in both mutants, while *axr3* and *ein2* in particular showed relatively high levels of expression of these genes, associated with a relatively strong adventitious rooting response. The *NAC1* gene is known to function in rooting in a pathway independent from auxin and was not significantly different in wild type vs mutants until there was senescence of the *pls* and *axr1* mutants.

Chapter 5 Investigating the roles of *MDF*, *NAC1* and *RAP2.7* genes in adventitious root formation

5.1 Introduction

In recent years, analysis of the genome sequence of Arabidopsis has progressed quickly. The Arabidopsis genome was the first of higher plants to be sequenced and set the foundations for the functional analysis of almost 30,000 genes (Arabidopsis Genome Initiative, 2000). The current challenge is to determine how these genes work together in cells and how genetic pathways link to phenotype, to ensure adaptability to reproduce and colonize different environments.

The analysis of gene function can be carried out in many ways, and one important approach makes use of transgenics to create loss-of-function or gain-of-function mutants for phenotypic analysis. Gene gain-of-function can be obtained by placing the target gene under the transcriptional control of a constitutive promoter (Wilson et al., 1996; Schaffer et al., 1998). Constitutive misexpression of genes may be useful but sometimes leads to lethality or sterility; and to solve this problem other methods have been developed, such as using a heat shock promoter or chemical induction to activate expression, and which can control expression temporally (Curtis and Grossniklaus, 2003). An example of a chemically induced expression system is the oestrogen (estradiol-)-inducible ectopic gene expression vector. This system provides highly regulated induction, with no adverse effect itself on transgenic plant viability and makes use of the XVE promoter which is sensitive to human oestrogen (Moore et al., 2006). The XVE promoter is available in the PER8 vector for Gateway construction using cassette B to produce the destination vector PMDC7 (Moore et al., 2006; Curtis and Grossniklaus, 2003).

To investigate the mechanisms of adventitious root formation, a number of gain- and loss-of-function mutants of genes were studied for regeneration response in the cultured leaf system. These genes were chosen based on known expression in roots of Arabidopsis, but possible roles in de novo root formation were not known.

The *MERISTEM DEFECTIVE* (*MDF*) gene of Arabidopsis was discovered in the Lindsey lab as being required for primary root development (Casson et al. 2009). It encodes a putative RS domain protein related to splicing factors. Two alleles were identified: the *mdf-1* allele has a T-DNA insertion within the ninth intron, while the *mdf-2* allele contains an insertion within the ninth exon. Mutation in *MDF* causes a loss of meristematic activity and stem cell identity in the root and vegetative shoot. In addition, the mutant shows decreased levels of *PIN2* and *PIN4* mRNA and this is associated with a reduced auxin maximum in the basal region of the *mdf* embryo and seedling root meristem. Furthermore, seedling roots of *mdf* show reduced expression of *SHORTROOT*, *SCARECROW* and *PLETHORA* genes (Casson et al. 2009). Recent work in the Lindsey lab confirms a role for MDF in RNA splicing control (Helen Thompson et al., unpublished).

NAC family genes/proteins play various roles during plant development. The protein family is characterised by conserved sequences in the N-terminal region. However, sequences at the C-terminal regions have diverged in amino acid sequence and length. In petunia, *NAM* and in Arabidopsis *CUC2* are considered as the originally identified *NAC* genes (Souer et al. 1996; Aida et al. 1997). They were identified as mutants in which shoot apical meristem development was defective. Another Arabidopsis *NAC* gene is *NAP* (Sablowski and Meyerowitz 1998), related to *APETALA3* (*AP3*) and with a key role in controlling cell expansion during floral organ development. In non-meristematic tissue, during senescence, another *NAC* is up-regulated (Sablowski and Meyerowitz 1998). Previous research showed that the *NAC1* gene is involved in de novo root formation (Chen et al. 2016) and in lateral root development mediated by auxin signalling (Xie et al. 2000).

The *RAP2.7* gene is a member of the AP2/ERF family of transcription factors which are involved in regulating the process of flowering and innate immunity, and may be involved in the control of meristematic activity (Tair,2021a). It is known to be expressed in mature leaf and at high levels in the hypocotyl (Tair,2021a). In the Lindsey lab, the gene was found to be a splicing target of MDF (it is mis-spliced in the *mdf* mutant; unpublished data) and has also been identified as being expressed in the stele and endodermis of the Arabidopsis root tip, similar to *MDF* (Birnboim et al 2003). It was therefore interesting to determine whether it might play a role in adventitious root formation.

The aim of the work described in this chapter was to investigate gain- and loss-of-function transgenics of *MDF*, *NAC1* and *RAP2.7*. MDF mutants and transgenic overexpressers (XVE::MDF; unpublished) were already available in the lab. Gateway cloning was used to construct overexpression constructs and transgenic plants for the other two genes, *RAP2.7* and *NAC1*. Loss of function SALK insertion mutants of these genes were also acquired and studied for genotype effects on the development of de novo roots in Arabidopsis, based on the system described earlier. I focused on early events of AR initiation, using the first two true leaves harvested from seedlings at 12 d post germination and cultured on B5 medium without added hormones. In addition, I investigated the expression of the genes *WOX5*, *NAC1* and *YUC1* in the different lines using qRT-PCR, to determine genetic interactions.

5.2 Results

5.2.1 Role of MDF in adventitious root formation

Figure 5-1A shows the length of de novo roots formed from leaf of wild type, MDF transgenic overexpresser (MDF-OV) and the loss-of-function *mdf-1* mutant after 12, 19 and 26 d of estradiol treatment and culture. At 12 d, both Col-0 and MDF-OV leaves produced roots, with no statistically significant difference in size (Fig. 5-1C); but at both 19 d and 26 d the *MDF* overexpresser produced significantly longer roots than wild type. The *mdf* mutant failed to produce any detectable roots over the entire time course, from a relatively small and yellowing leaf (Fig. 5-1A,D).

Figure 5-1B shows the numbers of branches of de novo roots between wildtype, MDF-OV and *mdf-1* after 12, 19 and 26 d. As described above, the *mdf* mutant failed to produce any detectable roots over the entire time course. After 12 d the mean number of branches was 1.66 for Col-0 and 2.56 for MDF-OV, but not statistically significantly different. After 19 d the number of branches increased (mean of 24.2 branches for Col-0, 32.44 branches for MDF-OV) and there was a statistically different difference between Col-0 (mean 40.1 branches) and MDF-OV (mean 74.8 branches) after 26 d. This shows a role for MDF in both

initiation and growth of adventitious roots in this system. Previously it has been shown that *MDF* overexpression could induce ectopic shoot meristems (Casson et al. 2009).

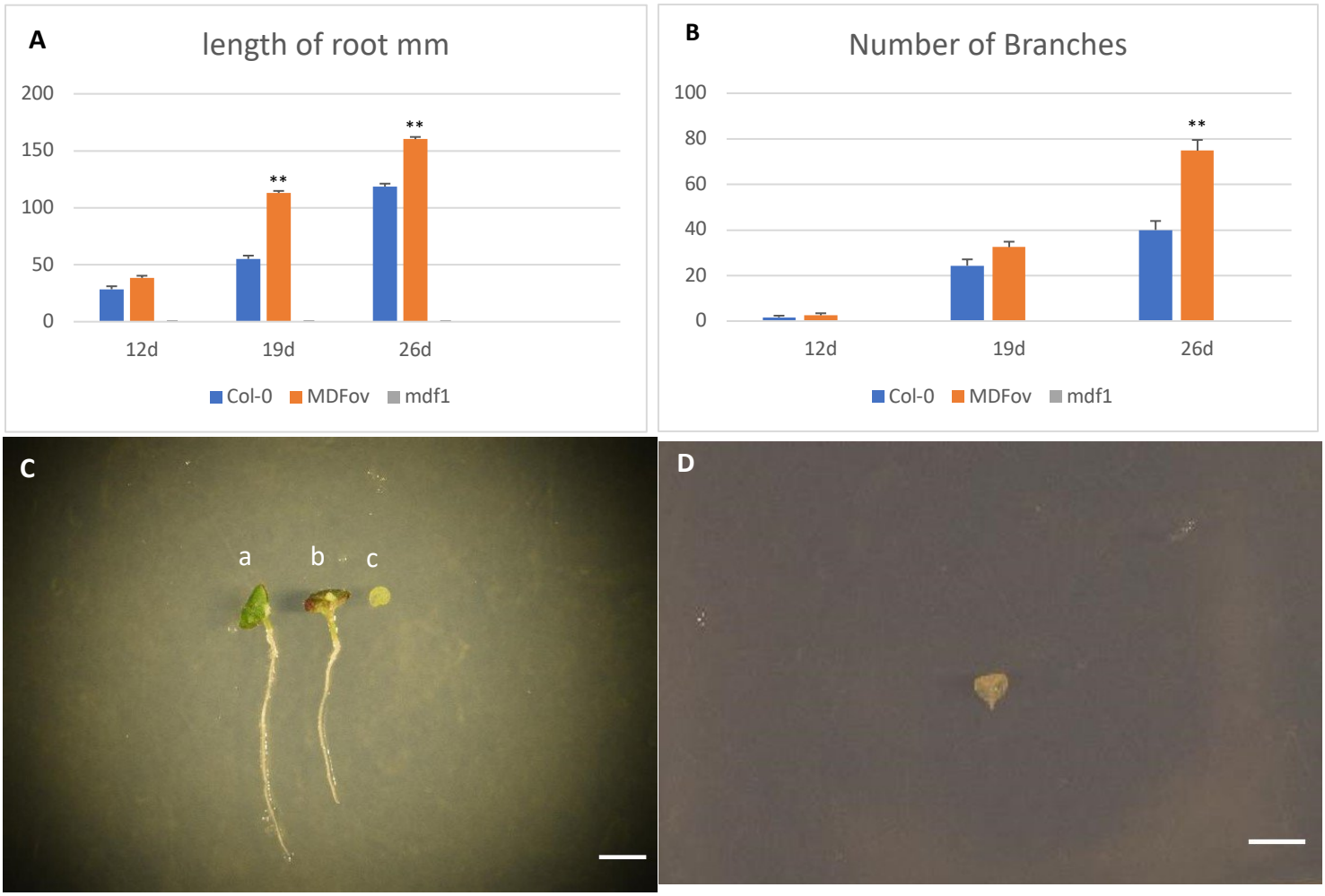


Fig. 5-1. (A) Mean de novo root length in wild type Col-0, MDF-OV and *mdf-1* after estradiol treatment and culture on B5 medium for 12, 19 and 26 d. (B) Mean numbers of root branches in wild type Col-0, MDF-OV and *mdf-1* after estradiol treatment and culture on B5 medium for 12, 19 and 26 d. (C) (a) MDF-OV leaf with de novo root after 12 d; (b) Col-0 leaf with de novo root after 12 d; (c) *mdf-1* leaf after 12 d; (D) *mdf-1* germinated on B5 medium after 19d. Values are averages of at least 30 leaves ± SEM. Statistical significance (A,B) was determined using Student's t-test for independent samples compared to wild type values, with P-values $P<0.01$ (**). Scale bars = 1 cm.

5.2.2 Role of MDF in the regulation of *WOX5* gene expression

To determine whether MDF has a role in the regulation of the *WOX5* gene, which was shown in the previous chapter to be associated with adventitious root regeneration, RNA was extracted from cultured leaf of wild type, *mdf-1* and MDF-OV either treated with oestradiol or untreated over an 8 d time course of culture, and RT-PCR was carried out. Bands were quantified using ImageJ software. Figure 5-2 shows representative gels following RT-PCR to determine *WOX5* gene expression in wildtype, MDF-OV and *mdf-1* samples at different times after germination on B5 medium with and without estradiol treatment, with three technical replicates shown for each sample. Figures 5-3A and B show the quantification of the mean data from three biological replicate experiments, each with three technical replicates.

The results show that, in the absence of estradiol induction, there was no significant difference in *WOX5* expression between wild type (Col-0) and MDF-OV samples, but there was a statistically significantly lower level of expression in the *mdf-1* mutant by 3 d of culture (Figures 5-2, 5-3B). However, when *MDF* expression was induced by estradiol, the MDF-OV samples showed significantly higher levels of *WOX5* expression, showing that MDF is a positive regulator of *WOX5*. This correlated with an increase in both root length and root branch number in MDF-OV samples compared to wild type after longer periods of culture (Figure 5-1), and is consistent with an inductive effect of MDF on adventitious root meristem formation.

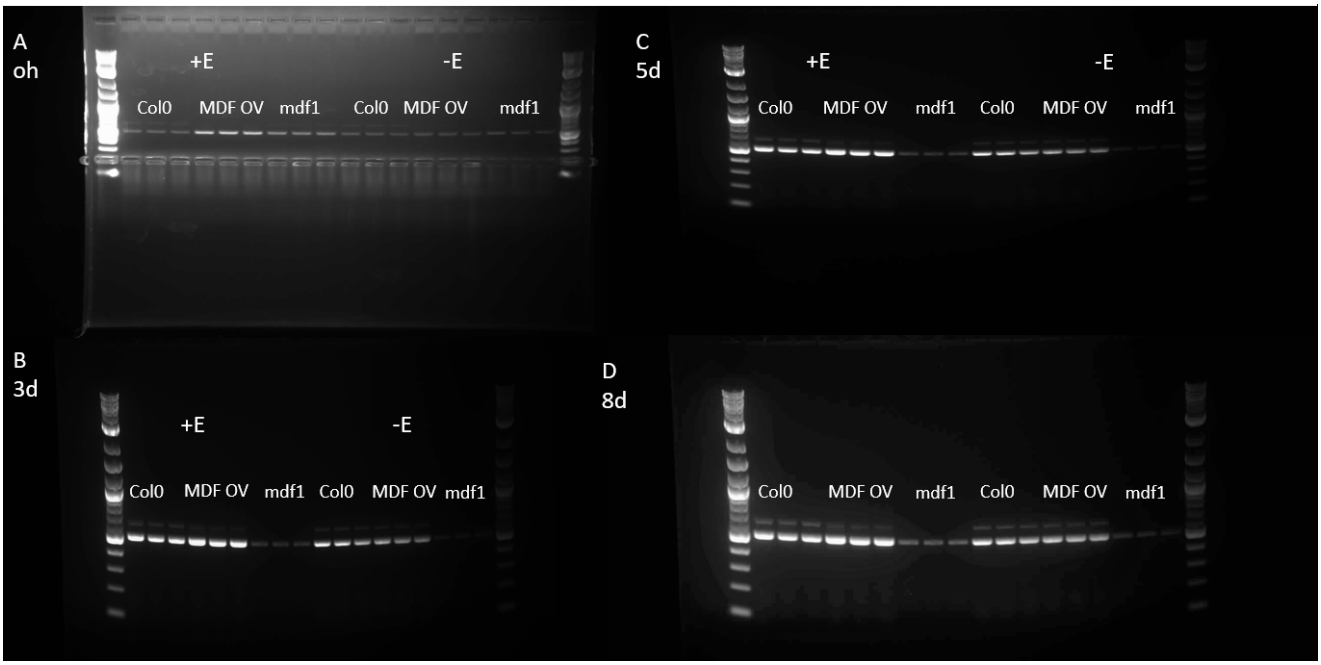


Fig. 5-2. Representative gels following RT-PCR to determine *WOX5* gene expression in wild type, MDF-OV and *mdf-1* samples at (A) 0 h, (B) 3 d, (C) 5 d and (D) 8 d after germination on B5 medium with (+E) and without (-E) estradiol treatment. Three technical replicates are shown for each sample.

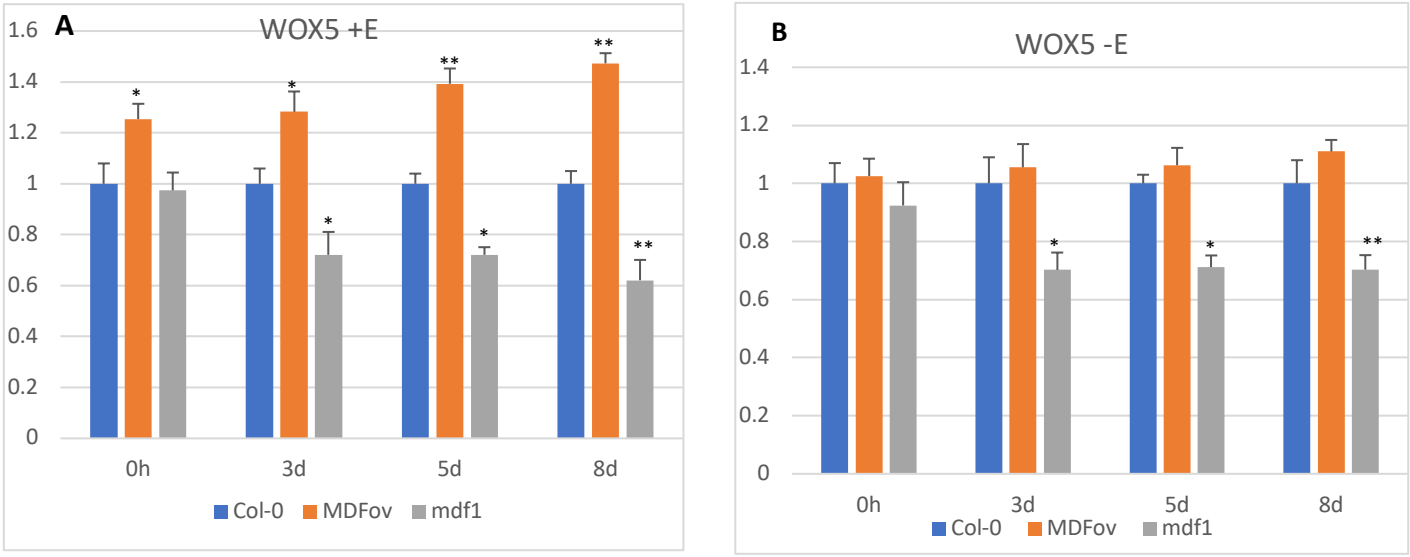


Fig. 5-3. Quantified RT-PCR data for *WOX5* gene expression in wild type, MDF-OV and *mdf-1* samples at 0 h, 3 d, 5d and 8 d after germination on B5 medium (A) with estradiol treatment (+E) and (B) without (-E) estradiol treatment. Gel bands were quantified using ImageJ. Values are means ±SEM for three biological repeats. Statistical significance was determined using Student's t-test for independent samples compared to wild type values, with P-values $P< 0.05$ (*) and $P<0.01$ (**).

5.2.3 Role of MDF in the regulation of *RAP2.7* gene expression

To determine whether MDF has a role in the regulation of the *RAP2.7* gene, RNA was extracted from cultured leaf of wild type, *mdf-1* and MDF-OV either treated with estradiol or untreated over an 8 d time course of culture, and RT-PCR was carried out. Bands were quantified using ImageJ software.

Figure 5-4 shows representative gels following RT-PCR to determine *RAP2.7* gene expression in wildtype, MDF-OV and *mdf-1* samples at different times after germination on B5 medium with and without estradiol treatment, with three technical replicates shown for each sample. Figures 5-5A and B show the quantification of the mean data from three biological replicate experiments, each with three technical replicates.

The results show that, in the absence of estradiol induction, there was no significant difference in *RAP2.7* expression between wild type (Col-0) and MDF-OV samples, and there was no detectable expression in the *mdf-1* mutant in any sample during the experimental time course (Figures 5-4, 5-5). However, when *MDF* expression was induced by estradiol, the MDF-OV samples showed significantly higher levels of *WOX5* expression, showing the MDF is a positive regulator of *WOX5*. This correlated with an increase in both root length and root branch number in MDF-OV samples compared to wild type after longer periods of culture (Figure 5-1), and is consistent with an inductive effect of MDF and RAP2.7 on adventitious root meristem formation.

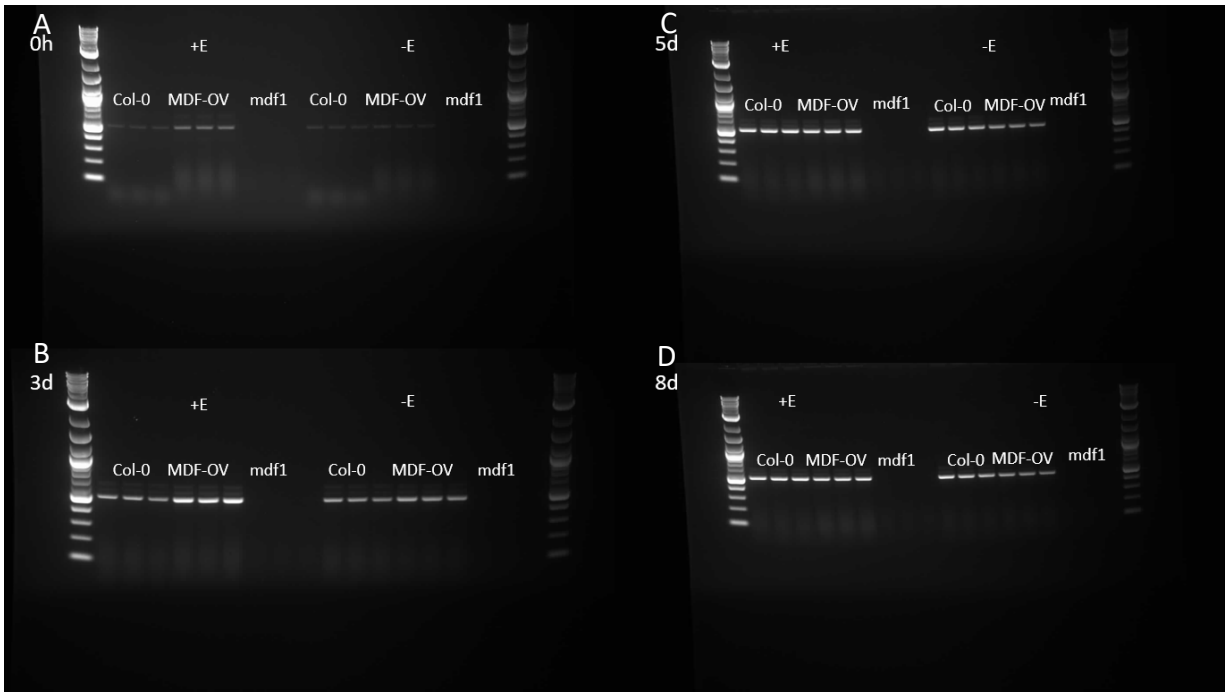


Fig. 5-4. Representative gels following RT-PCR to determine *RAP2.7* gene expression in wild type, MDF-OV and *mdf-1* samples at (A) 0 h, (B) 3 d, (C) 5 d and (D) 8 d after germination on B5 medium with (+E) and without (-E) estradiol treatment. Three technical replicates are shown for each sample.

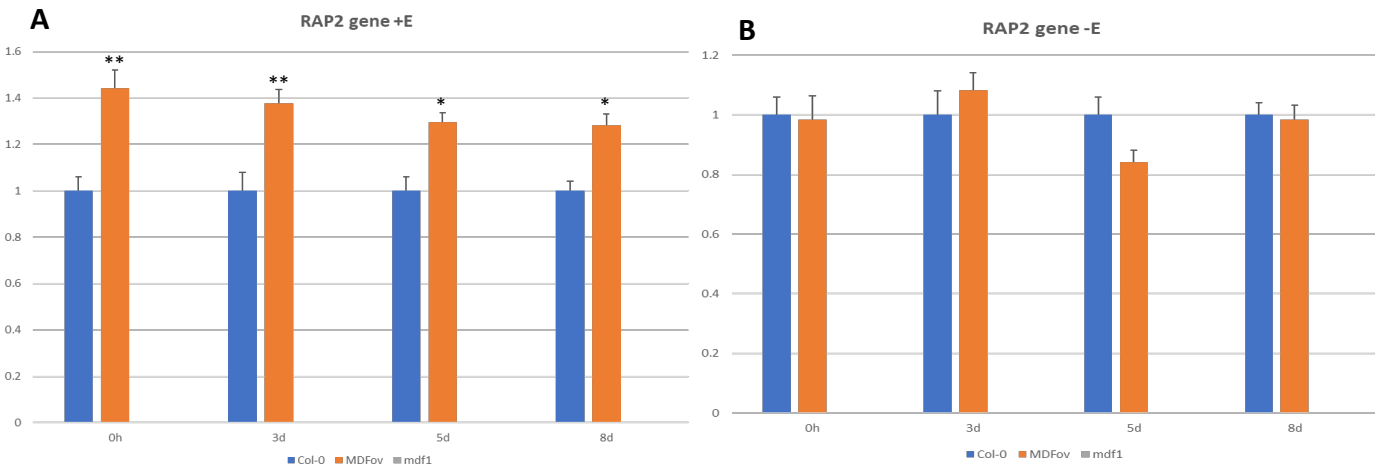


Fig. 5-5. Quantified RT-PCR data for *RAP2.7* gene expression in wild type, MDF-OV and *mdf-1* samples at 0 h, 3 d, 5d and 8 d after germination on B5 medium (A) with estradiol treatment (+E) and (B) without (-E) estradiol treatment. Gel bands were quantified using ImageJ. Values are means ±SEM for three biological repeats. Statistical significance was determined using Student's t-test for independent samples compared to wild type values, with P-values $P< 0.05$ (*) and $P<0.01$ (**).

5.2.4 Role of MDF in the regulation of *NAC1* gene expression

To determine whether MDF has a role in the regulation of the *NAC1* gene, RNA was extracted from cultured leaf of wild type, *mdf-1* and MDF-OV either treated with estradiol or untreated over an 8 d time course of culture, and RT-PCR was carried out. Bands were quantified using ImageJ software.

Figure 5-6 shows representative gels following RT-PCR to determine *NAC1* gene expression in wildtype, MDF-OV and *mdf-1* samples at different times after germination on B5 medium with and without estradiol treatment, with three technical replicates shown for each sample. Figures 5-7A and B show the quantification of the mean data from three biological replicate experiments, each with three technical replicates.

The results show that, either in the absence or presence of estradiol induction, there was no significant difference in *NAC1* expression between wild type (Col-0) and MDF-OV samples at 0 h - although very faint bands can be seen in the gel, this was not detectable to a significant extent by ImageJ software, indicative of very low or negligible levels of expression in leaf at this time point (Figures 5-6A, 5-7). Between 3 d and 8 d in the absence of estradiol treatment, *NAC1* expression was clearly detectable in all samples.

In the absence of estadiol treatment there was no significant difference in *NAC1* expression between wild type and MDF-OV samples, but expression levels in the *mdf-1* mutant were significantly lower than in wild type (Figure 5-7B). When *MDF* expression was induced by estradiol, the MDF-OV samples showed significantly higher levels of *NAC1* expression at 5 d and 8 d, showing that MDF is a positive regulator of *NAC1*. This correlated with an increase in both root length and root branch number in MDF-OV samples compared to wild type after longer periods of culture (Figure 5-1), and is consistent with an inductive effect of MDF and NAC1 on adventitious root meristem formation.

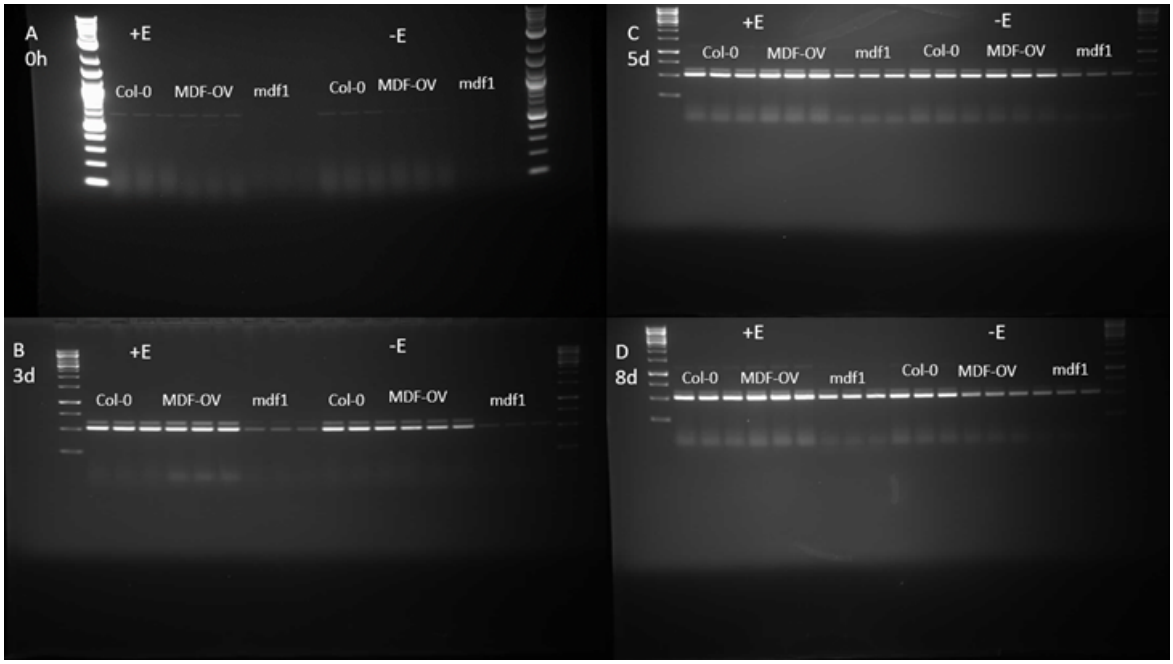


Fig. 5-6. Representative gels following RT-PCR to determine *NAC1* gene expression in wild type, MDF-OV and *mdf-1* samples at (A) 0 h, (B) 3 d, (C) 5 d and (D) 8 d after germination on B5 medium with (+E) and without (-E) estradiol treatment. Three technical replicates are shown for each sample.

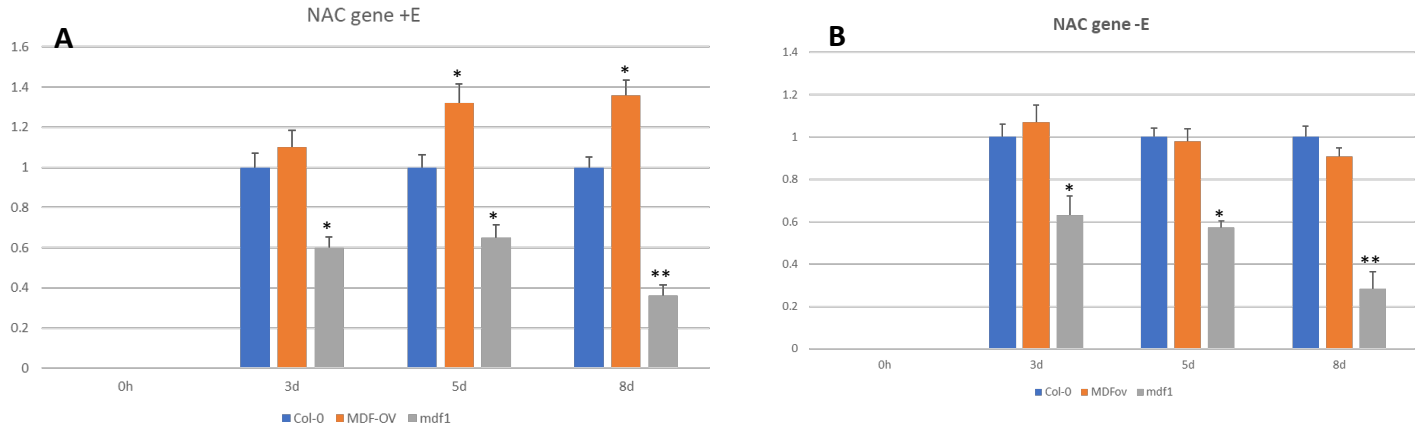


Fig. 5-7. Quantified RT-PCR data for *NAC1* gene expression in wild type, MDF-OV and *mdf-1* samples at 0 h, 3 d, 5d and 8 d after germination on B5 medium (A) with estradiol treatment (+E) and (B) without (-E) estradiol treatment. Gel bands were quantified using ImageJ. Values are means ±SEM for three biological repeats. Statistical significance was determined using Student's t-test for independent samples compared to wild type values, with P-values $P< 0.05$ (*) and $P<0.01$ (**).

5.2.5 Role of RAP2.7 in adventitious root formation

The results above show that the expression of the *RAP2.7* gene is dependent on MDF, and MDF is a positive regulator of adventitious root formation in the leaf system. To determine whether RAP2.7 is itself required for adventitious root formation, loss-of-function and gain-of-function (transgenic overexpressing) plants for the RAP2.7 gene were either obtained from the SALK collection (loss-of-function *rap2.7* mutants) or generated as p35S::RAP2.7 transgenics (overexpressers, RAP-OV). Two independent mutant SALK lines were obtained and genotyped to confirm T-DNA insertion in the *RAP2.7* gene (Materials and Methods), and only one was confirmed as mutant and used in experiments.

Figure 5-8A shows the length of de novo roots formed from leaf of wild type, RAP2.7 transgenic overexpresser (RAP-OV) and the loss-of-function *rap2.7* mutant (SALK RAP) after 12, 19 and 26 d of culture. At 12 d, there was no statistically significant difference in root

length between Col-0 and RAP-OV leaves, but the *rap2.7* mutant produced a significantly shorter root than wild type (Fig. 5-8A,C). At both 19 d and 26 d the *RAP2.7* overexpresser produced significantly longer roots than wild type (Fig. 5-8A,C).

Figure 5-8B shows the numbers of branches of de novo roots between wildtype, RAP2.7-OV and *mdf-1* after 12, 19 and 26 d. After 12 d the mean number of branches was not statistically significantly different between the three genotypes. After 19 d the number of branches increased for both wild type and overexpresser, but there was no significant difference between them; however, the *rap2.7* mutant produced significantly fewer branches than wild type and evidence of senescence (Figure 5-8B). There were statistically more root branches in the RAP2.7 overexpresser, and significantly fewer in the *rap2.7* mutant, than wild type after 26 d. This shows a role for RAP2.7 in both initiation and growth of adventitious roots in this system.

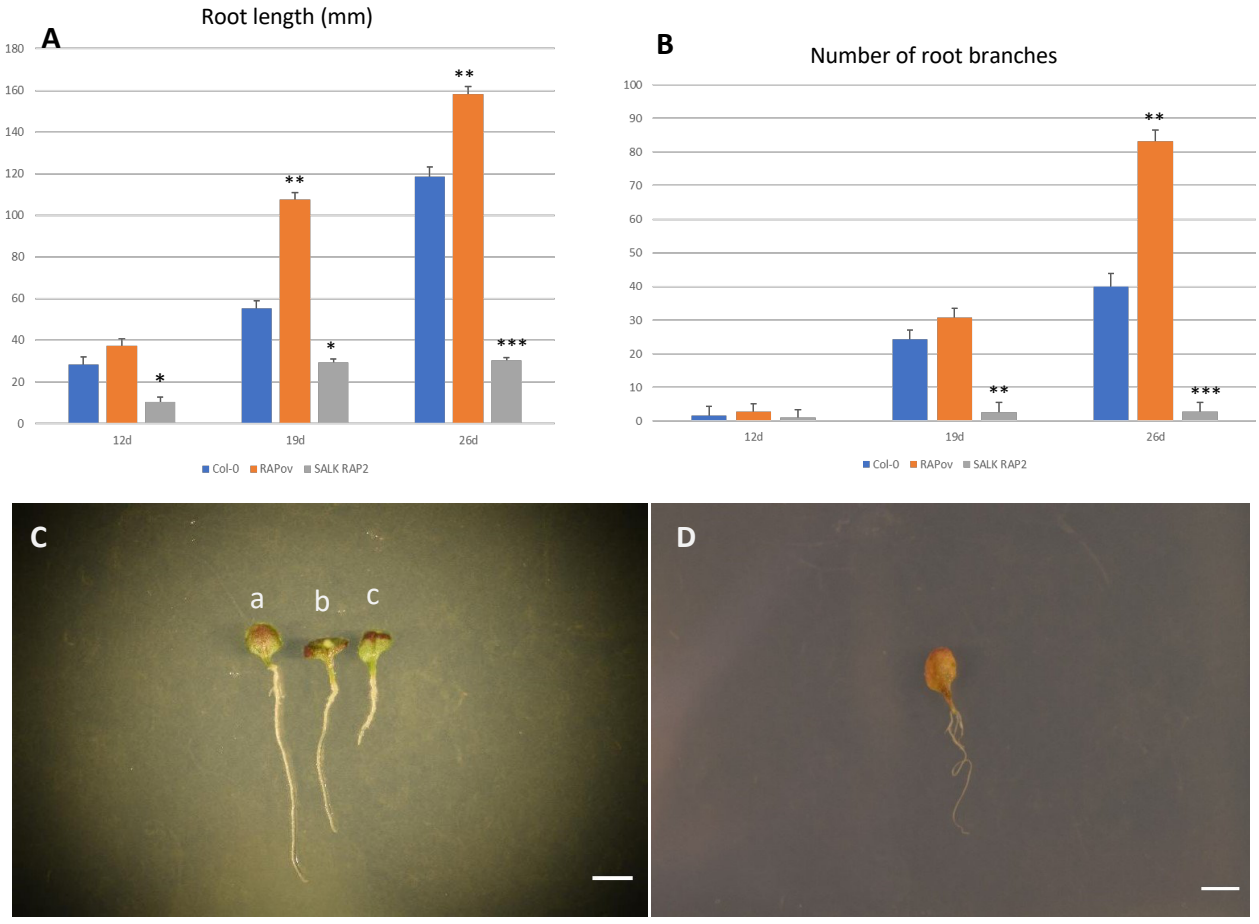


Fig 5-8. (A) Mean de novo root length in wild type Col-0, RAP2.7 transgenic overexpresser (RAP-OV) and loss-of-function *rap2.7* mutant (SALK RAP2) after culture on B5 medium for

12, 19 and 26 d. (B) Mean numbers of root branches in wild type, RAP2-OV and SALK RAP2 after culture on B5 medium for 12, 19 and 26 d. (C) (a) RAP2-OV leaf with de novo root after 12 d; (b) Col-0 leaf with de novo root after 12 d; (c) SALK RAP2 mutant leaf after 12 d; (D) SALK RAP2 mutant leaf germinated on B5 medium after 19 d. Values are averages of at least 30 leaves ± SEM. Statistical significance (A,B) was determined using Student's t-test for independent samples compared to wild type values, with P-values $P<0.05$ (*), $P<0.01$ (**), ($P<0.001$ (***). Scale bars = 1 cm.

5.2.6 Role of RAP2.7 in the regulation of *YUC1* gene expression

In the previous chapter, it was shown that, in wild type, an increase in *YUC1* gene expression occurred during the leaf culture period, correlated with adventitious root regeneration. Since RAP2.7 is required for both initiation and growth of adventitious roots (Section 5.2.5 above), it was investigated whether RAP2.7 might regulate *YUC1* expression, potentially to control auxin signalling during root regeneration. Therefore qRT-PCR was used to determine the level of *YUC1* transcript in the RAP2.7 overexpresser and mutant, compared to wild type, during a 7 d culture period. RNA was extracted and gene expression analysed by PCR using the *UBC* gene as internal standard in regenerating leaf samples at 0 h, 48 h, 3 d and 7 d on B5 medium lacking hormones. The results in Figure 5-9 show that, for all genotypes, the level of *YUC1* expression increased during the experimental time course, but there was no statistically significant difference between mutant and overexpresser of *RAP2.7* compared to wild type. These data show that *YUC1* expression is regulated by a pathway independent of RAP2.7.

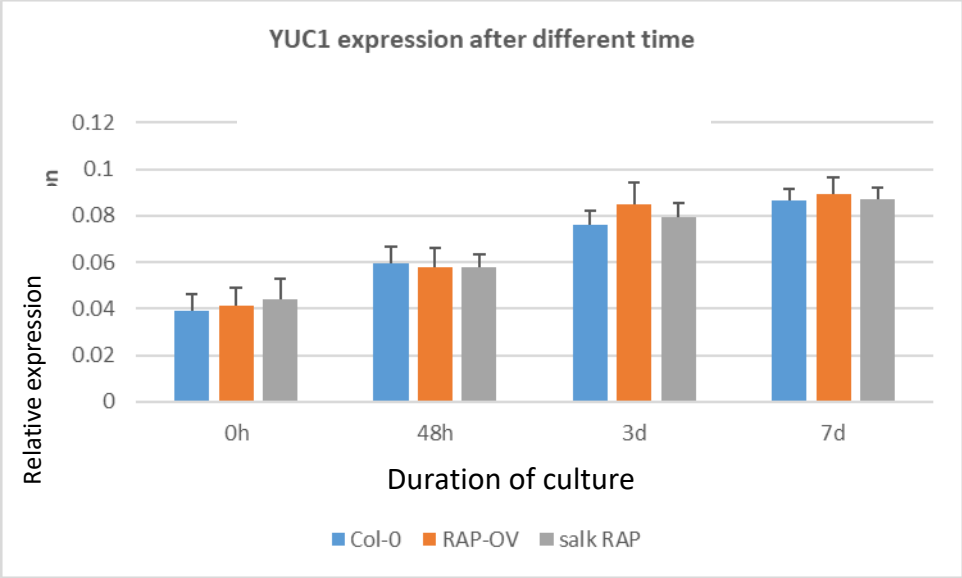


Fig. 5-9. qRT-PCR analysis of *YUC1* gene expression in wild type, RAP2.7 overexpresser (RAP-OV) and *rap2.7* mutant (salk RAP) during a culture time course of 0 h, 48 h, 3 d, and 7 d, using *UBC* as reference gene. Values represent means and error bars are SEM (n = three biological repeats with three technical repeats). Statistical significance was determined using Student's t-test for independent samples compared to wild type values.

5.2.7 **Role of RAP2.7 in the regulation of *NAC1* gene expression**

It was also shown in the previous chapter that, in wild type, an increase in *NAC1* gene expression occurred during the leaf culture period, correlated with adventitious root regeneration. To understand better gene interactions during the regeneration process, a possible role for RAP2.7 in regulating *NAC1* expression was investigated. qRT-PCR was used to determine the level of *NAC1* transcript in the RAP2.7 overexpresser and mutant, compared to wild type, during a 7 d culture period. RNA was extracted and gene expression analysed by PCR using the *UBC* gene as internal standard in regenerating leaf samples at 0 h, 3 d, 5 d and 7 d on B5 medium lacking hormones. The results in Figure 5-10 show that, for all genotypes, the level of *NAC1* expression increased during the experimental time course, but there was no statistically significant difference between mutant and *RAP2.7* overexpresser compared to wild type. These data show that *NAC1* expression is regulated by a pathway independent of RAP2.7.

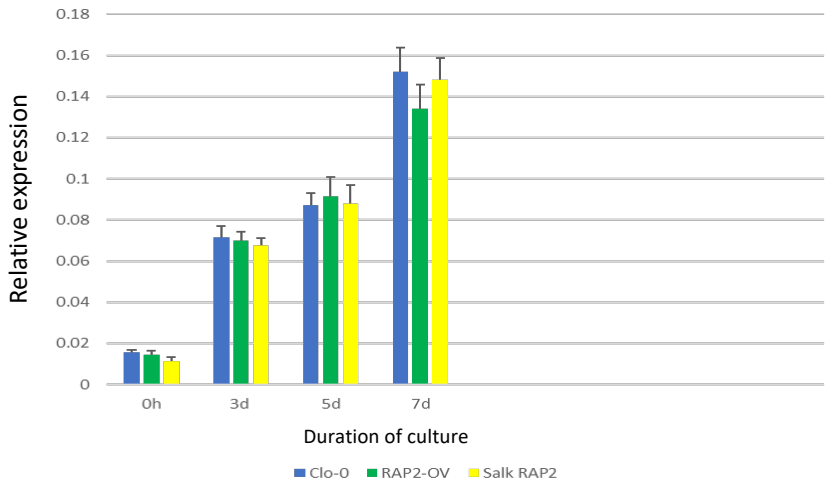


Fig. 5-10. qRT-PCR analysis of *NAC1* gene expression in wild type, RAP2.7 overexpresser (RAP2-OV) and *rap2.7* mutant (salk RAP) during a culture time course of 0 h, 3 d, 5 d and 7 d, using *UBC* as reference gene. Values represent means and error bars are SEM (n = three biological repeats with three technical repeats). Statistical significance was determined using Student's t-test for independent samples compared to wild type values.

5.2.8 **Role of RAP2.7 in the regulation of *WOX5* gene expression**

It was also shown in the previous chapter that, in wild type, an increase in *WOX5* gene expression occurred during the leaf culture period, correlated with adventitious root regeneration. To understand better gene interactions during the regeneration process, a possible role for RAP2.7 in regulating *WOX5* expression was investigated. qRT-PCR was used to determine the level of *WOX5* transcript in the RAP2.7 overexpresser and mutant, compared to wild type, during a 7 d culture period. RNA was extracted and gene expression analysed by PCR using the *UBC* gene as internal standard in regenerating leaf samples at 0 h, 3 d, 5 d and 7 d on B5 medium lacking hormones. The results in Figure 5-11 show that, for all genotypes, the level of *NAC1* expression increased during the experimental time course, but there was no statistically significant difference between mutant and *RAP2.7* overexpresser compared to wild type up to 3 d. However, at both 5 d and 7 d there was found a significant increase compared to wild type in the RAP2.7 transgenic overexpresser,

and a significantly reduced expression in the *rap2.7* mutant. These data show that RAP2.7 is a likely positive regulator of *NAC1* expression in this system.

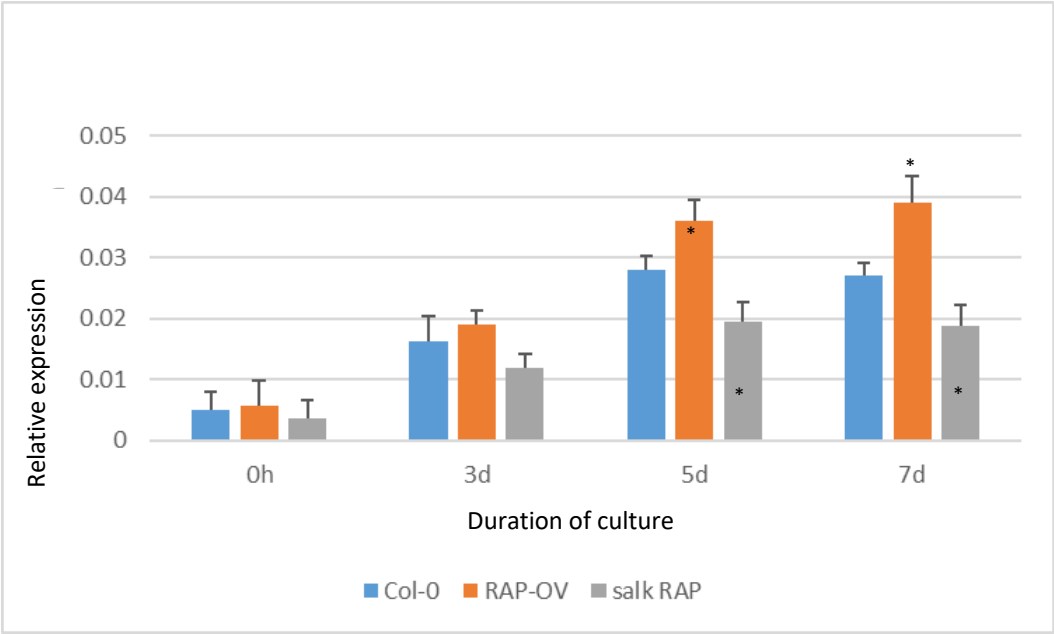


Fig. 5-11. qRT-PCR analysis of *WOX5* gene expression in wild type, RAP2.7 overexpresser (RAP2-OV) and *rap2.7* mutant (SALK RAP) during a culture time course of 0 h, 3 d, 5 d and 7 d, using *UBC* as reference gene. Values represent means and error bars are SEM (n = three biological repeats with three technical repeats). Statistical significance was determined using Student's t-test for independent samples compared to wild type values. P-value <0.05 = (*).

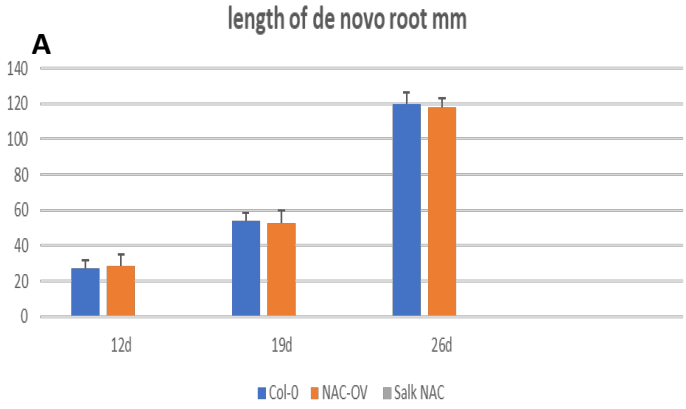
5.2.9 Role of NAC1 in adventitious root formation

The results above show that the expression of the *NAC1* gene is dependent on MDF and RAP2.7, and MDF is a positive regulator of adventitious root formation in the leaf system. To determine whether NAC1 is itself required for adventitious root formation, loss-of-function and gain-of-function (transgenic overexpressing) plants for the *NAC1* gene were either obtained from the SALK collection (loss-of-function *rap2.7* mutants) or generated as p35S::RAP2.7 transgenics (*RAP2.7* overexpresser, RAP-OV). Two independent NAC1 mutant

SALK lines were obtained and genotyped to confirm the T-DNA insertion in the *RAP2.7* gene (Materials and Methods), and only one was confirmed as mutant and used in experiments. Figure 5-12A shows the length of de novo roots formed from leaf of wild type, NAC1 transgenic overexpresser (NAC-OV) and the loss-of-function *nac1* mutant (SALK NAC) after 12, 19 and 26 d of culture. Throughout the entire time course there was no statistically significant difference in root length between Col-0 and NAC-OV genotypes, but the *nac1* mutant failed to produce any adventitious roots (Fig. 5-12A,C).

Figure 5-12B shows the numbers of branches of de novo roots between wildtype, NAC-OV and *nac1* after 12, 19 and 26 d. Throughout the entire time course there was no statistically significant difference in root length between Col-0 and NAC-OV genotypes.

These results show that the *NAC1* gene is essential for adventitious root formation in this leaf system.



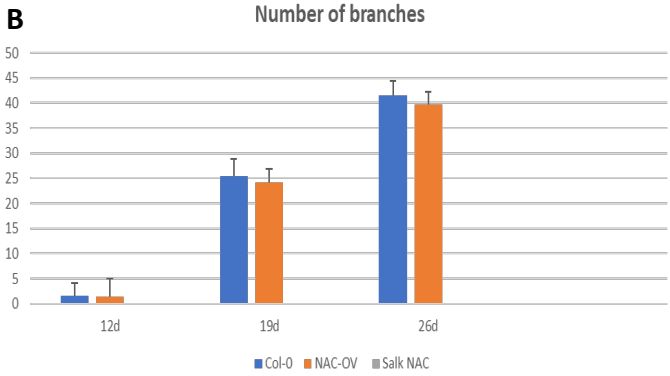


Fig. 5-12. (A) Mean de novo root length in wild type Col-0, NAC1 transgenic overexpresser (NAC-OV) and loss-of-function *nac1* mutant (SALK NAC) after culture on B5 medium for 12, 19 and 26 d. (B) Mean numbers of root branches in wild type, NAC-OV and SALK NAC after culture on B5 medium for 12, 19 and 26 d. (C) (a) Wild type (Col-0) leaf with de novo root after 12 d; (b) NAC OV leaf with de novo root after 12 d; (c) SALK NAC mutant leaf after 12 d. Values are means of at least 30 leaves ± SEM. Statistical significance (A,B) was determined using Student's t-test for independent samples compared to wild type values. Scale bars = 1 cm.

5.2.10 Role of NAC1 in regulating *YUC1* gene expression

To understand better gene interactions during the regeneration process, a possible role for NAC1 in regulating *YUC1* expression was investigated. qRT-PCR was used to determine the level of *YUC1* transcript in the *NAC1* transgenic overexpresser and mutant, compared to wild type, during a 7 d culture period. RNA was extracted and gene expression analysed by PCR using the *UBC* gene as internal standard in regenerating leaf samples at 0 h, 3 d, 5 d and 7 d on B5 medium lacking hormones. The results in Figure 5-13 show that, for all genotypes, the level of *YUC1* expression increased during the experimental time course, but there was no statistically significant difference between mutant and *NAC1* overexpresser compared to wild type. These data show that *YUC1* expression is regulated by a pathway independent of NAC1.

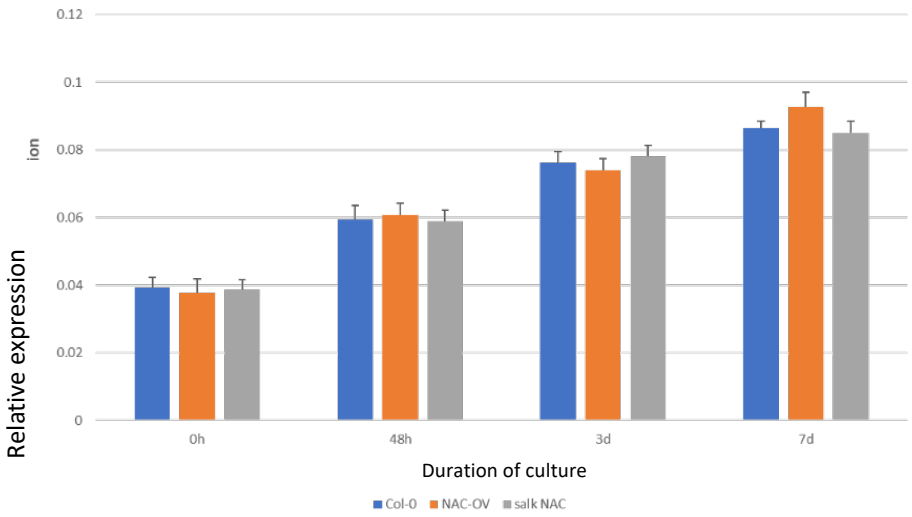


Fig. 5-13. qRT-PCR analysis of *YUC1* gene expression in wild type, NAC1 overexpresser (NAC-OV) and *nac1* mutant (salk NAC) during a culture time course of 0 h, 48 h, 3 d, and 7 d, using *UBC* as reference gene. Values represent means and error bars are SEM (n = three biological repeats with three technical repeats). Statistical significance was determined using Student's t-test for independent samples compared to wild type values.

5.2.11 Role of NAC1 in the regulation of *WOX5* gene expression

A possible role for NAC1 in regulating *WOX5* expression was also investigated. qRT-PCR was used to determine the level of *WOX5* transcript in *NAC1* overexpresser and mutant, compared to wild type, during a 7 d culture period. RNA was extracted and gene expression analysed by PCR using the *UBC* gene as internal standard in regenerating leaf samples at 0 h, 3 d, 5 d and 7 d on B5 medium lacking hormones. The results in Figure 5-14 show that, for all genotypes, the level of *WOX5* expression increased during the experimental time course, but there was no statistically significant difference between mutant and *NAC1* overexpresser compared to wild type. These data show that *WOX5* expression is regulated by a pathway independent of NAC1.

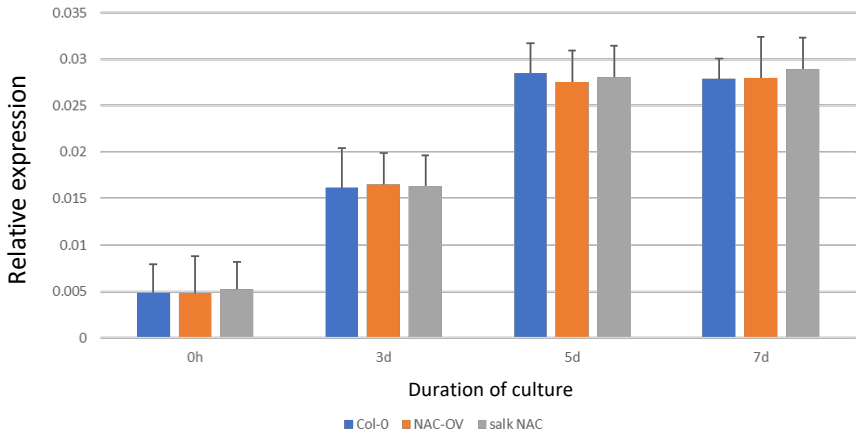


Fig. 5-14. qRT-PCR analysis of *WOX5* gene expression in wild type, NAC1 overexpresser (NAC-OV) and *nac1* mutant (salk NAC) during a culture time course of 0 h, 48 h, 3 d, and 7 d, using *UBC* as reference gene. Values represent means and error bars are SEM (n = three biological repeats with three technical repeats). Statistical significance was determined using Student's t-test for independent samples compared to wild type values.

5.2.12 Role of NAC1 in the regulation of *RAP2.7* gene expression

Finally, a possible role for NAC1 in regulating *RAP2.7* expression was also investigated. qRT-PCR was used to determine the level of *RAP2.7* transcript in *NAC1* overexpresser and mutant, compared to wild type, during a 7 d culture period. RNA was extracted and gene expression analysed by PCR using the *UBC* gene as internal standard in regenerating leaf samples at 0 h, 3 d, 5 d and 7 d on B5 medium lacking hormones. The results in Figure 5-15 show that, for all genotypes, the level of *RAP2.7* expression increased during the experimental time course, but there was no statistically significant difference between mutant and *NAC1* overexpresser compared to wild type. These data show that *RAP2.7* expression is regulated by a pathway independent of NAC1.

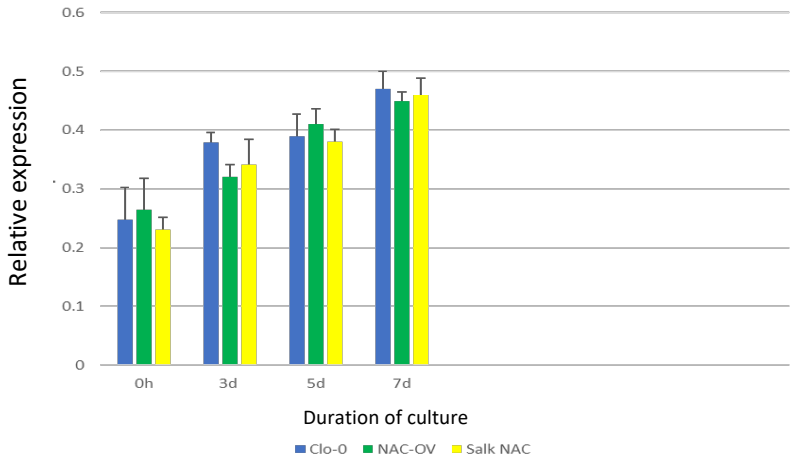


Fig. 5-15. qRT-PCR analysis of *RAP2.7* gene expression in wild type, NAC1 overexpresser (NAC-OV) and *nac1* mutant (salk NAC) during a culture time course of 0 h, 48 h, 3 d, and 7 d, using *UBC* as reference gene. Values represent means and error bars are SEM (n = three biological repeats with three technical repeats). Statistical significance was determined using Student's t-test for independent samples compared to wild type values.

5.3 Summary

Work in this chapter was focused on improving our understanding of the roles of specific known transcriptional regulators in adventitious root initiation and growth; and also, to investigate their roles in signalling pathways potentially involved in the root developmental process. The results showed that MDF, RAP2.7 and NAC1 are each required for normal initiation and growth of adventitious roots, as evidenced by the phenotypes of loss-of-function mutants and transgenic overexpressers. Gene expression analysis showed that MDF is required for regulated expression of *WOX5*, *NAC1* and *RAP2.7* genes; RAP2.7 is required for the correct regulation of *WOX5* but not *NAC1* or *YUC1*; and NAC1 is required for the correct expression of neither *YUC1*, *WOX5* or *RAP2.7*.

Chapter 6. Discussion

6.1 Introduction

Plants have a remarkable regeneration ability. They can readily create new organs from non-embryonic tissue (Hartmann et al., 2010; Chenetal et al., 2014; Liu et al., 2014) and repair damage that occurs upon wounding (Xu et al., 2006; Heyman et al., 2013). For example, when detached leaf tissue of *Arabidopsis thaliana* is placed on B5 medium in the absence of exogenous hormones, the leaf will create adventitious roots at the cut site after 7 to 8 days (Chen et al., 2014). This process is known as de novo root regeneration (DNRR). Wounding plays a major role in inducing the accumulation of the hormone auxin at high concentrations close to the cut surface (Chen et al., 2016). Crosstalk between auxin and cytokinin is an important regulatory mechanism of many aspects of plant development and regeneration (El-Showketal et al., 2013), including in de novo root formation.

There are two established pathways that regulate complete de novo root regeneration from leaf explants, namely the auxin pathway and the NAC1 pathway; and past studies have shown that defects in either of these two pathways causes repression of de novo root development (Chen et al., 2016). During de novo root formation cell division occurs in competent cells in the petiole and transformed these cells to root founder cells, in a process involving the expression *WOX11/12* genes (Liu et al., 2014). The root founder cells then divide to form root primordium cells, associated with expression of *WOX5/7* genes (Hu and Xu, 2016). It has previously been shown that *WOX11/12* gene expression is required to activate *WOX5/7* genes (Hu and Xu, 2016). Finally, the root apical meristem continues to develop from the root primordium cells, leading to de novo root emergence from the leaf explant.

In this Discussion of my results I will highlight important aspects of the research described in this thesis, discuss the wider implications of the work, and suggest ideas for further investigation. In addition, analysis of the results will allow a comparison with the published literature to assess the thesis hypothesis.

6.2 Cell division changes in leaf explants during root regeneration

Increased cell proliferation is essential to create a de novo root. After 2 d of leaf culture, the cells adjacent to the xylem begin replication and create stratified layers from 3 d onwards, which pushes the xylem away and displaces the collenchyma (Bustillo-Avendaño et al, 2018). During this process the petiole becomes thicker because of cell replication in the vasculature and the subsequent formation of primordia. After 4 d of increased cell proliferation, the first primordia are visible and located in the outer layers of the proliferating vasculature. The root primordia become visible with a layered pattern at 5 d of leaf culture. Finally, the new de novo root is formed with a well-organized meristem growing out from the petiole tissues from 7 d onwards. Previous results showed that the reporter J0121-GFP, which highlights pericycle-like cells, is expressed in a pattern limited to a layer around the xylem vessels, but is excluded from procambium, whereas J0661-GFP related to the root pericycle was expressed in cells around the xylem and procambium cells (Bustillo-Avendaño et al., 2018). The number of cells marked with GFP quickly increased for both lines on the first day on B5 medium. However, all proliferating cells were marked with J0661-GFP and some proliferating cells in the J0121 line were not marked with GFP. This indicates that xylem and procambium cells proliferate as part of the reprogramming process (Bustillo-Avendaño et al., 2018).

Cell differentiation is commonly linked to a transition from the mitotic division cycle to the endoreduplication cycle. In previous studies, researchers have used as tools to study cell cycling processes the expression of two genes, *CYCLIN B1;1* and *CYCLIN B1;2*, in unicellular trichomes of Arabidopsis to understand the function of these two mitotic cyclins in endoreduplication. The genes were also fused with the GUS reporter to visualize events during cell differentiation. They found ectopic expression of *CYCLIN B1;2* induces mitotic divisions resulting in multicellular trichomes. The GUS fusion for *CYCLIN B1;2* revealed both nuclear division and cytokinesis (Schnittger et al., 2002). In addition, the expression of another cyclin-GUS fusion, *CYCAT1:CDB:GUS*, was subcellularly localised around the nucleus. Expression started at prophase and began to disappear after the cells formed new cell walls. In metaphase the protein transferred to the cytoplasm, while in telophase it returned to the nucleus (Hauser and Bauer, 2000).

We used *CYCB1;2::GUS* to track cell divisions during de novo root formation. The leaf explant at 0 h showed no GUS activity (Figs. 3-13 A,B), suggesting that the cells in the leaf explant were not dividing at this stage. By 3 days after culture on B5 medium there was weak GUS activity evident in root founder cells in the petiole near the wound site, indicating that cell division had started at this stage, and continued in the developing root primordium and apical meristem. This is broadly consistent with previous observations using a *CYCB1;1-GUS* reporter (Chen et al., 2014). Therefore cell division for de novo root formation starts between 2-3 days after explanation of leaf tissue, resulting in the formation of root founder cells, and continues to create root primordium cells between 4-5 days, followed by root apical meristem formation at 6 days and root emergence from the petiole at 8 days.

As the de novo root emerges from the petiole base of whole leaves, we made longitudinal tissue sections in wildtype and mutants during the regeneration time course, to monitor the mean cell number of cells in replicate longitudinal sections. The wild type underwent cell fate transition (Figure 4-4) with up to a mean of 1770 cells produced by 16 d (Figure 4-5). The auxin resistant *axr1* mutant failed to undergo cell fate transition and failed to reach the root primordium and patterning stages (Figure 4-4), with a maximum of only 313 cells produced by 16 d (Figure 4-5). The low cell proliferation is likely due to a lack of auxin and reduced *WOX5* expression (Figure 4-5) which is essential for cell division, and it is the main gateway for the transition from initiation stage to pattering stage. Thirdly, the *pls* mutant was able form a root primordium and progress to the patterning stage (Figure. 4-4) and while this mutant could produce a de novo root after 12 d, it was small and abnormal. The number of cells produced was up to 480 cells at 16 d. The extent of cell proliferation in *pls* is likely linked to the balance between auxin and cytokinin hormone but may also be related to the associated defective leaf vascularization of the *pls* mutant (Casson et al., 2002).

The *axr3* genotype successfully progressed through cell fate transition (Figure 4-4) and the number of cells increased to maximum mean of 710 cells at 16 d. Although previous results showed that the *axr3* mutation stimulated callus and adventitious root formation from the primary root (Leyser et al., 1996) the data presented in this thesis showed a different effect, whereby *axr3* exhibited a reduced cell proliferation and adventitious root formation, associated with the reduced auxin responsiveness of the mutant. Lastly, the *ein2* mutant

was similar to wild type in de novo root formation, successfully developing primordia and transitioning to the patterning stage (Figure 4-4), though interestingly the cell numbers in sections reached only 1100 cells by 16 d, which was 600 cells less than the wildtype.

	YUC1/4 gene expression	NAC1 gene expression	WOX5 gene expression	Maximum of the number of cell proliferation	Maximum of length root	Maximum of number branches	Root morphology
Col-0	Normal expression	Normal expression	Normal expression	1770 cells	122 mm	36 branches	Normal root
axr1	Weak expression	Normal expression	Weak expression	313 cells	-	-	Block root
pls	Weak expression	Normal expression	Weak expression	480 cells	8 mm	3 branches	Defective and short root
axr3	Middle expression	Normal expression	Middle expression	710 cells	39 mm	13 branches	Defective and no root gravitropism
ein2	Normal expression	Normal expression	Normal expression	1100 cells	117 mm	23 branches	Normal root

Table 6-1. Comparison between wild type and mutant cell division and root morphology during de novo root formation.

6.3 Auxin signalling and responses during de novo root formation

The identities of cells, and a wide range of developmental phenomena, are dependent at least in part on local auxin concentrations and their interaction with other signalling systems. Auxin gradients occur in tissues when auxin moves between cells due to both active transport and diffusion. The PIN family of auxin efflux carriers are indispensable for the regulation of auxin distribution (Omelyanchuk et al., 2016). Their expression, which is itself positively auxin-regulated, can be used to monitor auxin distribution (Omelyanchuk et al., 2016). We used PIN::GUS expression patterns (for PIN1, PIN3 and PIN7) and DR5::GUS

expression, and measured *PIN* and *YUC* transcript levels to observe auxin transport, accumulation and biosynthesis phenomena during de novo root formation from leaf. Early in the leaf culture process, up to 3d, PIN1::GUS activity was seen throughout the leaf blade, particularly in the vascular tissue. However, after this time expression in the leaf blade was reduced and became more apparent in the petiole and developing root primordium (Figure 3-2). The expression of the *PIN1* gene showed a similar pattern across the leaf blade and petiole during this time course (Figure 3-3). PIN3::GUS expression was retained for longer in the leaf vascular tissue in the blade but similarly increased in the petiole at the site of root formation (Figures 3-4, 3-5). PIN7::GUS expression was barely detectable in the leaf blade but was activated during root formation in the petiole (Figure 3-6). These data indicate the activation of the auxin transport system in the petiole during the root development process. Previous studies have shown that the inhibition of auxin transport inhibits de novo root formation (Liu et al., 2014), and the data in this thesis indicate that the expression of PINs 1, 3 and 7 are part of the mechanism. The observed expression of the auxin reporter DR5::GUS (Ulmasov et al., 1997) at the site of adventitious root formation from the leaf (Figure 3-7) supports this view.

PINs 1, 3 and 7 are localized to the plasma membrane and to vesicles that cycle in an action-dependent manner (Friml et al., 2002). In the root, PIN3 is located in columella and it is positioned symmetrically at the plasma membrane, and is affected by gravity stimulation which contributes to changes in auxin distribution as part of the gravitropic response (Friml et al., 2002). Here we also see its recruitment, in terms of transcriptional activity, during de novo root formation.

PIN7 contributes to the auxin maximum in the apical cell of the developing embryo, which helps to create the proembryo and all apical structures of the plant (Xiong et al., 2019). It is also expressed in the columella cells in the root, and it has essential roles in gravitropism (Jiao et al, 2021). Again, we see its activation during de novo root formation from leaf. Previous studies show have shown that mutations in the PIN family cause reductions in gibberellins (Willige et al., 2011) and it may be that gibberellin is important in the primordium development stage of de novo root formation, through crosstalk between auxin and gibberellin, as it is found in this thesis that *GAox2* expression is activated also in

regenerating roots (Figures 3-10, 3-11). In conclusion, our data show that the three members of the PIN family are associated with auxin accumulation at the site of de novo root formation, including at early stages, before root morphogenesis and emergence is complete; and auxin may interact with GA to promote root formation.

Auxin and cytokinin are known to have important roles in cell fate transition during de novo root formation (Skoog and Miller, 1957; Duclercq et al., 2011; Correa Lda et al., 2012). In addition, it was demonstrated that accumulation of auxin near the wound region during the early stages of root formation is important to induce the *WOX11/12* genes and start the initiation stage, which is the first stage of cell fate transition. This leads to root founder cell formation, and auxin is involved by activating *WOX5/7* genes (Figure 6-1) (Liu et al., 2014).

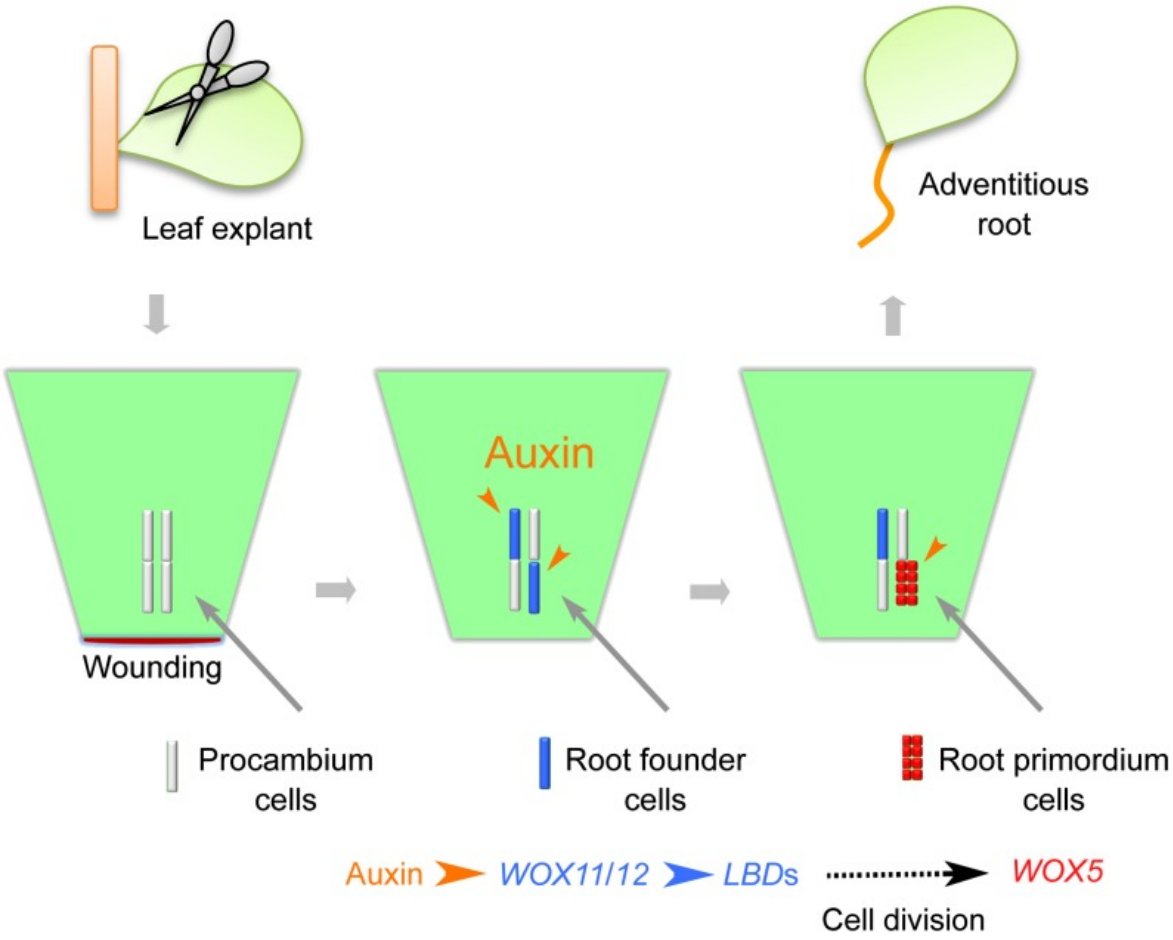


Fig 6-1. A model of organogenesis of de novo root from explant leaf Arabidopsis, showing cellular and molecular events. There are two steps of cell fate cell transition occurring during de novo root formation (Liu et al., 2014).

De novo root regeneration usually happens in nature when plant organs are wounded or detached, and both auxin biosynthesis and transport are believed to play essential roles in this process (Greenwood et al., 2001; De Klerk, 2002; Ahkami et al., 2009; Correa Lda et al., 2012; Chen et al., 2014; Liu et al., 2014; Xu and Huang, 2014). The Arabidopsis genome encodes at least 11 *YUC* genes which are involved in auxin biosynthesis (Cheng et al., 2006). Both *YUC1* and *YUC4* are induced by wounding, while *YUC2* and *YUC6* gene expression is not inducing by wounding, and they have role in maintaining the basal auxin level in the leaf (Chen et al., 2016b). *YUC1* and *YUC4* expression patterns suggest they have a dual role. In the first role they produce auxin in mesophyll cells of the leaf within and after 4 hours after wounding, and then the polar auxin transport system transfers auxin to the competent cells near the wound region (Chen et al., 2016 b). A second role is in producing auxin in the competent cells near the wound site within 1-2 days after culture on B5 medium, in order to maintain a high auxin level in those competent cells (Chen et al., 2016 b). *YUC1* and *YUC4* genes respond to both light and dark conditions but *YUC5*, *8* and *9* genes only respond to darkness (Fig. 6-2) (Chen et al., 2016 b).

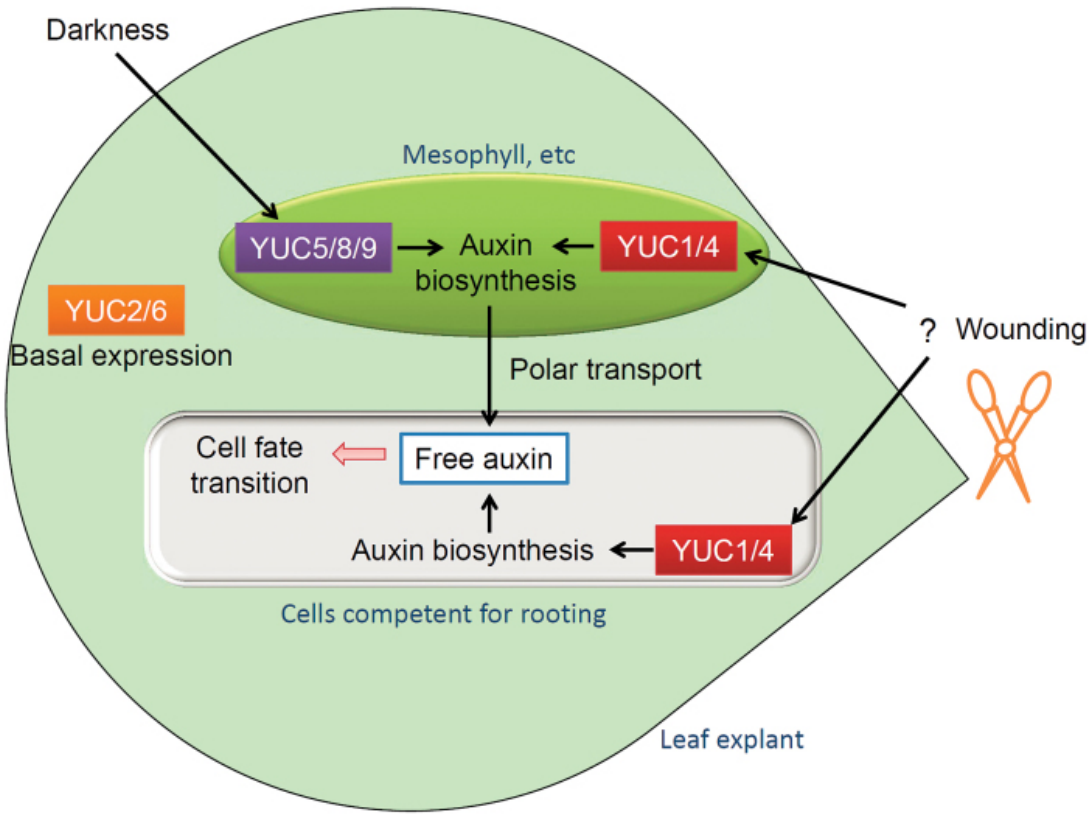


Fig. 6-2. YUC-mediated auxin biogenesis in a leaf explant. YUC1- and YUC4-mediated auxin biogenesis is in response to wound signalling principally in both mesophyll cells and competent cells. YUC5/8/9- mediated auxin biogenesis is in darkness in mesophyll cells. YUC2- and YUC6-mediated auxin biogenesis supports the basal auxin concentration in the leaf, important for regeneration (Chen et al., 2016b).

Previous research showed that when the auxin biosynthesis inhibitor yucasin was used to block YUC protein function, the regeneration of the root was also blocked (Chen et al., 2016b). Similarly, the double mutant *yuc1 yuc4* showed partially blocked root regeneration, while the quadruple mutant *yuc1 yuc2 yuc4 yuc6* caused severe inhibition of de novo root formation, confirming the essential requirement for auxin biosynthesis via *YUC* genes in this process.

The data presented in this thesis show that the mutants *axr1*, *axr3* and *pls*, which each exhibit auxin signalling defects, exhibited reduced expression of *YUC1* and *YUC4*, while the mutant *ein2*, which is insensitive to ethylene, expresses *YUC1* and *YUC4* at wildtype levels (Figures 4-8, 4-9). This reduced *YUC1* and *YUC4* expression was associated with reduced de novo root formation in *axr1* and reduced length of the root and number of root branches in both *axr3* and *pls* genotypes (Figure 4-1). This is consistent with previous studies that show that AXR1 is essential for auxin-mediated gene transcription in plant development, and mutation in this gene causes reduced plant high, hypocotyl elongation, root gravitropism and fertility (Lincoln et al., 1990). In addition, the *axr1* mutant shows reduced auxin-induced accumulation of *SAUR-AC1* mRNA in seedlings, rosette leaves and mature roots (Timpte et al., 1995). This indicates that AXR1 is required for de novo root formation from leaf explant at least in part through effects on *YUC* gene expression.

axr1 was unable to form de novo roots (Figure 4-1b). In addition, *pls* formed small and defective roots with a low number of the number of branches (Figure 4-1c), consistent with previous results which showed the length of primary root of *pls* was 50% that of wild type (Casson et al., 2002). The *axr3* genotype produced more de novo roots compared to *axr1* and *pls*. The *axr3* de novo root phenotype was short in length, with a reduced number of branches and defective in root gravitropism. This is consistent with past results that show

the root system of *axr3* is significantly different from the wildtype, being short and slow growing; after 16 d the *axr3-1* root iss 7% the length of wild type, lacks root hairs and is defective in root gravitropism. The *axr3-1* mutation is semi-dominant, and induces enhanced auxin signalling that limits root growth (Leyser et al., 1996).

Casson et al. (2002) showed that the *PLS* gene of Arabidopsis is essential for the correct balance of auxin and cytokinin which in turn is required for correct root growth and vascular patterning. Mutation in this gene causes low auxin and high cytokinin accumulation, with a short root and reduced vascular tissue in leaves. This consistent with low expression of *YUC1* and *4* in the *pls* mutant, with defective de novo root formation (Figure 4-1c). qPCR analysis showed that the *axr3* genotype has less impact on *YUC1* and *YUC4* expression than *axr1* and *pls* genotypes (Figures 4-8, 4-9). This is consistent with previous results that show the expression of the *SAUR-AC1* gene was not different between wild type and *axr3* (Leyser et al., 1996).

Lastly, de novo root formation from leaf of the ethylene-insensitive *ein2* genotype was similar to wildtype. This is consistent with previous results suggesting that the *ein3* mutant, also ethylene-insensitive, was able to form de novo roots more than wild type, and it was proposed that the ethylene hormone is a negative regulator of de novo root formation (Li et al., 2020). While there is good evidence that ethylene inhibits root development (e.g. Casson et al., 2002), the results in this thesis for *ein2* are not completely in agreement with the data for *ein3*, as *ein2* does not regenerate more or longer roots than wildtype, showing that de novo root formation is not promoted by ethylene insensitivity (Fig. 4-2); and indeed there may be fewer root branches from the *ein2* leaf than for wildtype at 26 d of culture, suggesting some ethylene sensitivity is required for root branching over a long culture period (Figure 4-3).

qPCR analysis showed the *ein2* genotype has no effect on *YUC1* and *YUC4* expression during leaf explant culture (Figures 4-8, 4-9). This indicates that ethylene insensitivity does not affect the auxin pathway during de novo root formation. We used the ethylene reporter EBS::GUS to monitor ethylene responses during de novo root formation (Chapter 3). The synthetic EBS promoter includes fives copies of the EIN3 binding domain fused with the 35S

promoter and fused to the GUS gene (Stepanova et al., 2007). Our data showed that EBS::GUS was expressed in the leaf blade up to 8 d, and in the petiole but not root primordium at 5 d- 6 d (Figure 3-12). However, after 8 d GUS expression occurred in the emerging de novo root, suggesting ethylene responses are not obviously important in early stages of primordium development. Previous results have shown that the hormone ethylene is involved in the plant wound response and ageing (Figure 6-3A) (Li et al., 2020); and both wounding and ageing signalling influence de novo root formation (Chen et al., 2014). Wound signalling is a prerequisite for de novo root initiation, by inducing auxin synthesis and accumulation near the wound site, while ageing negatively influences the rate of de novo root formation (Chen et al., 2014).

In addition, previous results showed that the ethylene-stabilized transcription factor EIN3 directly repressed the expression of both *WOX11* and *WOX5*, which are key cell fate-determining genes, providing a possible mechanism for ethylene-mediated inhibition of de novo root formation (Figure 6-3B) (Li et al., 2020). Furthermore, ethylene can reduce auxin accumulation to cause reduced root regeneration (Fig 6-3B) (Li et al., 2020). This idea is in agreement with our result which showed EBS::GUS expression is not seen in early root primordium development; i.e., ethylene responses are repressed at this stage to allow root formation to initiate. Past studies have shown that there is crosstalk between ethylene and auxin in the root, whereby ethylene induces two Trp biosynthetic genes, *WEI2/ASA1/TIR7* and *WEI7/ASB1*, which leads to increased auxin concentrations in the root tip and elongation zone, which is inhibitory to growth (Stepanova et al., 2007). However, our data showed that auxin signalling was not affected in *ein2*. It can be proposed that excess ethylene is inhibitory to the root regeneration process, but ethylene insensitivity has little adverse effect, and needs to be maintained to allow adventitious root formation to occur.

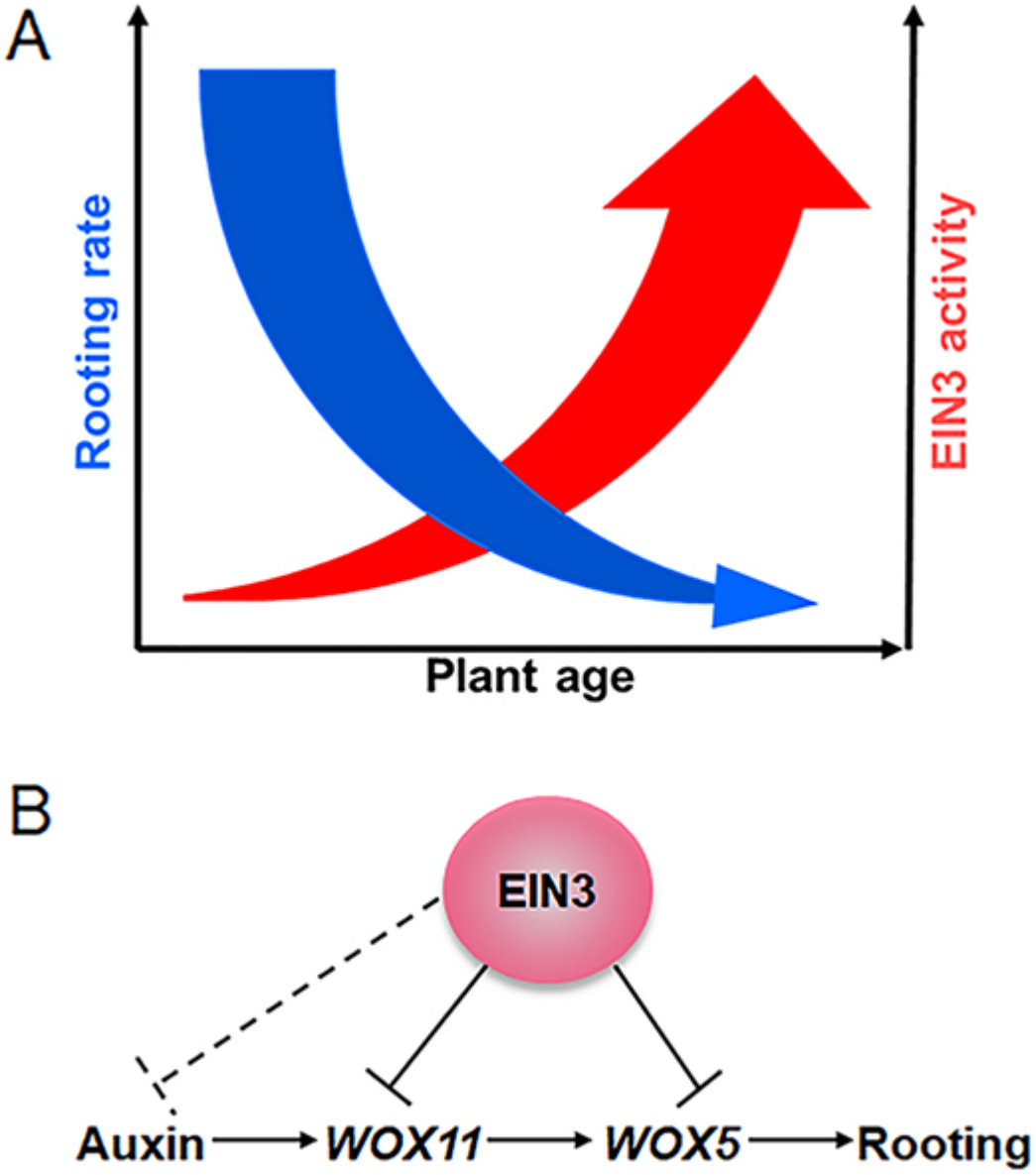


Fig. 6-3(A). EIN3 activity is gradually induced with increasing plant age, causing the rate of de novo root formation to decline. (B) Auxin induces the cell fate transition genes *WOX11* and *WOX5* to initiate root formation in detached leaf explants. EIN3 inhibits the transcription of *WOX11* and *WOX5* to suppress root regeneration. Ethylene can also inhibit auxin accumulation, most likely through EIN3 (as depicted by the dotted lines). Blunt ends mean suppression; arrowheads indicate advancement (Li et al., 2020).

6.4 Cytokinin signalling and response during de novo root formation

Cytokinins are N6-substituted adenine derivatives that are involved in many processes such as plant growth and development, including shoot initiation and development, cell division, light response and leaf senescence (Mok and Mok, 2001b). The ARR protein family are type A *Arabidopsis thaliana* response regulators involved in cytokinin signalling, and there are 10 genes that are rapidly induced by the hormone cytokinin. These genes have overlapping functions and they work as negative regulators for cytokinin signalling (To et al., 2004). *ARR4* is the one of the 10 genes and the reporter line ARR4::GUS shows expression in the vasculature of both shoots and roots, with strong expression in the shoot (To et al., 2004). I used the ARR4::GUS reporter, and qPCR analysis of *ARR4* expression, to investigate the role of cytokinin signalling during de novo root formation.

Results showed that both *ARR4::GUS* and *ARR4* gene expression was strong in the whole leaf at 0h, but declined over the next 3-4 d (Fig 3-8). After this point, expression started to increase again, from 5 d during de novo root formation and then emergence by 8 d. This result is consistent with previous observations for *ARR5*, which suggested that cytokinin concentrations were relatively low, and therefore signalling high (since the ARRs repress cytokinin responses), in leaf explants in a subset of vascular-associated cells near the petiole after 2 days culture, associated with proliferation of vasculature (Bustillo-Avendaño et al., 2021). Thereafter, expression increased at 5 d, during the formation of root primordia (Bustillo-Avendaño et al,. 2021). For callus formation there is needed a confluence of auxin and cytokinin response in the same cells (Gordon et al., 2007). This idea is agreement with our results, which shows specific expression of both *ARR4::GUS* (Figure 3-8) and auxin *DR5::GUS* (Figure 3-7) in the vasculature. This indicates there is specific regulation of auxin and cytokinin signalling in the petiole to allow de novo root formation, with *ARR* expression dampening cytokinin responses to promote auxin effects, and this fails in mutants with disruptive auxin or cytokinin responses, such as *pls* or *axr* mutants (Fig 4-1C).

6.5 Gibberellin signalling and respond during de novo root formation

Previous studies have demonstrated that Arabidopsis has a small family of GA3ox enzymes which consists of four members, GA3ox1, GA3ox2, GA3ox3 and GA3ox4, required for the biosynthesis of active GAs (Mitchum et al., 2006). It was shown that each gene is expressed

in specific organs in Arabidopsis, suggesting they have different roles in plant growth and development. For example, the *GA3ox1* gene was found to be expressed at high levels in all organs examined (Mitchum et al,. 2006). However, the expression of the other three genes was limited to specific organs or developmental stages. Both *GA3ox1* and *GA3ox2* genes are important for germination and vegetative growth, and *GA3ox1*, *GA3ox3* and G*A3ox4* are major genes required for the development of reproductive organs (Mitchum et al,. 2006). In this thesis expression of the *GA3ox2::GUS* reporter and of the native gene was used to investigate the possible role of GA in de novo root formation.

The expression of *GA3ox2::GUS* was strong in the vascular tissues of the leaf blade and petiole of explanted leaf, but became more focused in the developing adventitious root by 5 d and beyond, a pattern reflected in the expression of the native *GA3ox2* gene (Figures 3-10, 3-11). Previous studies showed that *GA3ox2* is expressed in specific cell types, i.e cortex and endodermis in roots of germinating seedlings, and in the root tip in the elongation zone, quiescent centre (QC) and columella cells of older roots (Mitchum et al., 2006). Although single mutants of *ga3ox1* and *ga3ox2* are not affected in the phenotype of plant, the *ga3ox1 ga3ox2* double mutant shows severe defects in seed germination (Mitchum et al., 2006). It seems likely that GA has a role in the patterning stage during de novo root formation, particularly at the root primordium and root apical meristem root stages. Future work could examine the effect of the double mutant on adventitious root formation.

6.6 Role of the *NAC1* gene during de novo root formation

6.6.1 *NAC1* gene expression is essential for de novo root formation:

The wound site provides many signals that are responsible for cellular responses to damage. An early major response pathway is auxin-mediated cell fate transition, which occurs in the competent cells of the vasculature at that site (Chen et al., 2016). A second important pathway is the auxin-independent activation of NAC transcription factors that regulate the cellular environment in both competent cells and mesophyll within the wound region to support root tip emergence (Figure 6-4) (Chen et al., 2016).

Figure 5-12 shows that the loss-of-function *nac1* mutant was unable to regenerate de novo roots from leaf even after 26 days culture. This demonstrates an essential role for NAC1 in the formation of de novo roots. qPCR analysis (Figure 4-6) showed an increase in *NAC1* expression in the wildtype and auxin defective mutants *axr1*, *axr3* and *pls* and the ethylene-insensitive *ein2* up to 7 d of culture, but expression declined in the *axr1* and *pls* mutants thereafter, and these mutants failed to regenerate normal roots. The fact that *NAC1* expression remained high in the *axr3* mutant and was also seen in *axr1* up to 7 d, indicates its regulation is independent of AXR1- and AXR3-dependent auxin signalling.

These observations are consistent with previous studies in which NAC1 protein was fused with a SRDX repression domain to create a non-functional NAC1 protein, which led to repression of root regeneration in transgenic plants (Chen et al., 2016). Further evidence for a role for NAC1 in de novo root formation is the expression of the *NAC1::GUS* reporter gene at the wound region after 2 d of leaf culture; GUS activity also increased in many regions of the leaf, such as mesophyII cells and competent cells, after 4 d culture (Chen et al., 2016). Therefore, NAC1 is essential for the formation of de novo roots from leaf explants in Arabidopsis.

6.6.2 *NAC1* gene is activated independently of auxin and ethylene during cell fate transition

The results above show that the expression of the *NAC1* gene is not affected by lack of auxin and ethylene signalling (i.e. in the *axr1*, *axr3* and *ein2* mutants) during the root regeneration process (Figure 4-6). This is consistent with previous studies that show that treatment of cultured leaf with a polar auxin transport inhibitor (NPA) prevents auxin accumulation near the wound site, but the *NAC1* gene was expressed normally in that tissue (Chen et al., 2016). Furthermore, treatment of cultured leaf with the synthetic auxin NAA was found to induce expression of the auxin response gene *GH3.2* within 2 h, but *NAC1* was not induced (Staswick et al., 2005; He et al., 2012; Chen et al., 2016), and is in agreement with the observation that *NAC1* expression is independent of the mutants *axr1*, *axr3* and *pls* which, like *ein2*, also has ethylene signalling defects (though ethylene hyper-signalling for *pls* and ethylene insensitivity for *ein2*; Chilley et al., 2006).

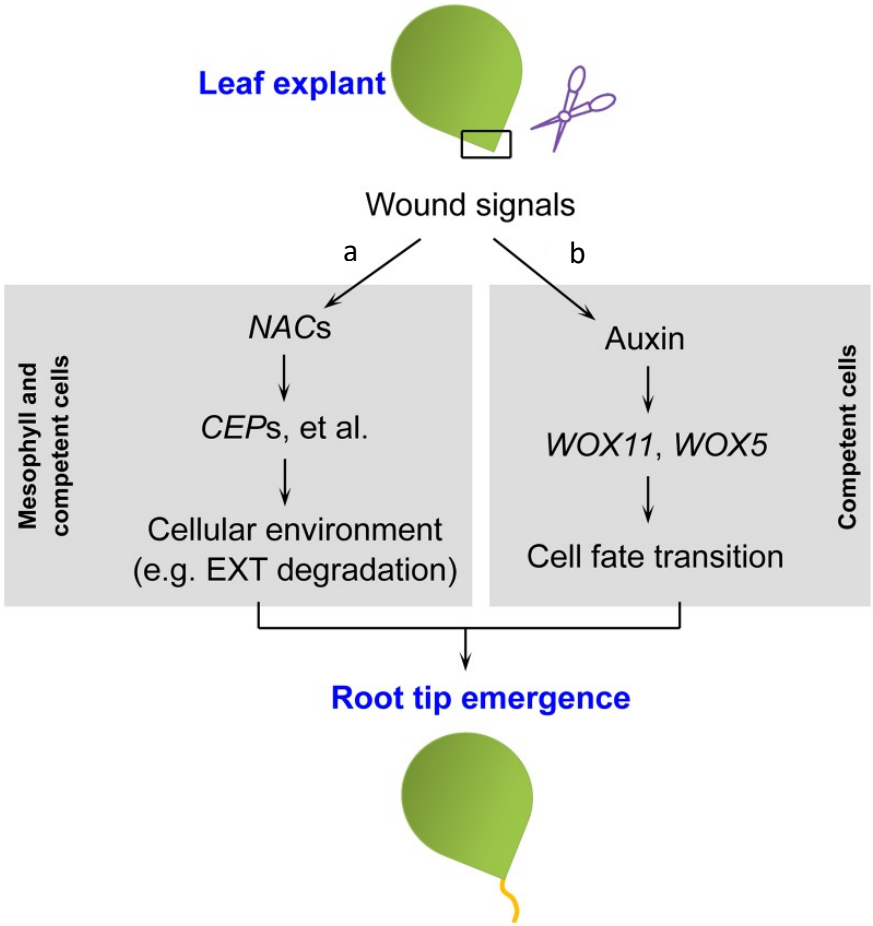


Fig 6-4. Wound signalling inducing two signalling pathways during de novo root regeneration. (a) NAC pathway working independently from auxin. (b) Auxin pathway mediating cell fate transition (Chen et al., 2016).

6.7 *WOX5* expression in wild type and mutants

Plants have a strong ability for root organogenesis from differentiated tissues (Xu et al., 2006; Sena et al., 2009; Liu et al., 2014; Efroni et al., 2016). De novo root regeneration from leaf explants requires a cell fate transition step to create an adventitious root from leaf cells (Hu and Xu, 2016; Fig. 6-5). The first step of this process is *priming*, which is characterized by the formation of adventitious root founder cells and activation of *WOX11/12* expression in the founder cells (Hu and Xu. 2016). The second step of cell fate transition is *initiation*, which results in the formation of the dome-shaped root primordium through cell division; the expression of *WOX11/12* declines and is replaced by *WOX5/7* expression, which helps to transition the root founder cell to a root primordium cell (Hu and Xu, 2016). The third step is

patterning, which results in the formation of the primary root apical meristem (RAM) and, in this step, *WOX5/7* expression is progressively limited to the stem cell niche, suggesting the stem cell niche is created at this stage. The last step is *emergence* which results in the growth of the new root tip from the leaf explant. In this step the RAM is mature, with a well-organized stem cell niche, and undergoes rapid cell division, elongation and cell differentiation (Figure 6-5) (Hu and Xu, 2016).

Auxin is a key hormone controlling cell fate transition during the formation of de novo roots, required for the expression of the *WOX5* gene during the primordium stage, and its expression is repressed by the polar auxin transport inhibitor NPA, associated with the inhibition of adventitious root regeneration (Hu and Xu, 2016). In addition, the *WOX5* gene is important for creating the root primordium - WOX5::GUS activity is seen in the midvein of the leaf explant near from wound site at 4 d, and the *wox5* mutant shows reduced adventitious rooting. The *wox5 wox7* double mutation shows a further reduction in de novo root formation, showing that these two genes work synergistically in de novo root development (Hu and Xu, 2016).

These observations are consistent with data presented in this thesis showing that both *axr1* and *pls* mutants, which have low auxin responses, exhibit low expression of *WOX5* (Figure 4-7), inhibiting de novo root formation. However, the *axr3* mutant is semi-dominant, and so expression of *WOX5* is higher than in *axr1* and *pls*, so allowing de novo root formation similar to wildtype. Similarly, *WOX5* expression in *ein2* was no different to wildtype (Figure 4-7). This is consistent with previous research that showed EIN3 suppresses both *WOX11* and *WOX5* transcription; and ethylene reduces auxin accumulation, so repressing rooting (Li et al., 2020). Moreover, both *ein2-5* and *ein3 eil1* showed a higher rate of de novo root formation than wildtype (Li et al., 2020), though in this thesis no such effect was seen for *ein2*. Nevertheless, this indicates that enhanced ethylene concentrations or signalling inhibit de novo root formation.

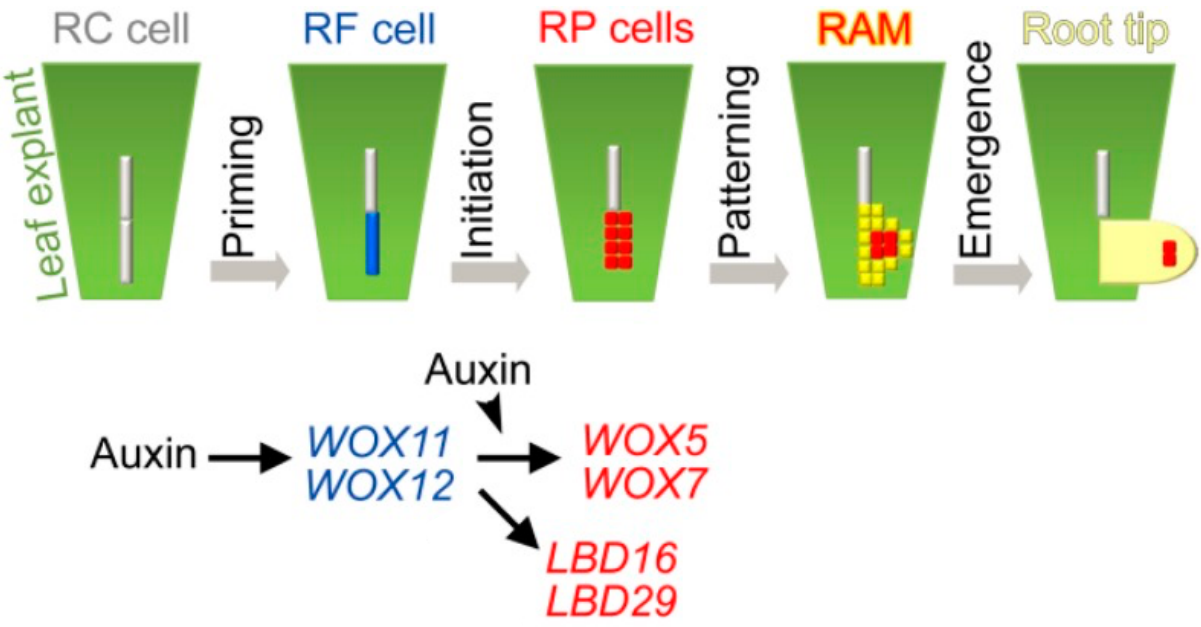


Fig 6-5. De novo root organogenesis comprises priming, initiation, patterning, and emergence steps. Auxin promotes founder-cell-specific *WOX11/12* gene expression which activates downstream expression of *WOX5/7* and *LBD* genes which are required for root primordium formation. RC cell, regeneration-competent cell; RF cell, root founder cell; RP, root primordium (Hu and Xu. 2016).

6.8 Role of MDF in de novo root regeneration

Previous results showed the *MDF* gene is required for meristem development and growth of the Arabidopsis root, with the *mdf1* mutant having a significantly shorter primary root than the wild type seedling (3.4 ± 0.2 mm versus 21.6 ± 1.2 mm at 7 d post germination; Casson et al. 2009). In this thesis it was found that *mdf-1* was unable to form de novo roots by 8 d, and the leaf yellowed over this time period. On the other hand, overexpression of MDF led to the production of longer de novo roots than wild type, with very significant differences at 19 d and 26 d. The transgenic MDF overexpresser also produced a higher number of root branches, with a very significant difference by 26 d. This indicates an essential role for MDF in de novo root formation.

The result of RT-PCR analysis showed that the expression of the *WOX5* gene was high in the *MDF-1* overexpresser compared to wild type, with significant differences at 0 h, and 3 h, and with very significant differences at 5 d and 8 d. Consistent with this, the *mdf-1* mutant showed low expression of *WOX5* with significant differences at 3 d and 5 d and with a very significant difference at 8 d. According to Casson et al (2009), the *MDF* gene is first expressed early in embryogenesis, and MDF regulates PIN transporter proteins and meristem transcription factor gene expression (e.g. *PLT1*, *PLT2*), with a role in regulating correct auxin distribution. This explains the low expression of *WOX5* in the mutant, essential for progression of root regeneration.

The *mdf-1* mutant showed no expression of *RAP2.7* at any time in the leaf culture process. On the other hand, *RAP2.7* gene expression was higher than in wild type when MDF was overexpressed.

Similarly, the expression of the *NAC1* gene was significantly reduced in the *mdf-1* mutant compared to wild type, and significantly higher in the MDF overexpressing line. Previous studies show that the regulation of the *NAC1* pathway is poorly understood (Chen et al. 2016), but the results in this thesis showed that MDF positively regulates *NAC1* expression which, along with its effects on the expression of *WOX5* and *RAP2.7*, explains the positive regulatory effects on de novo root formation. MDF therefore appears to be a key regulator of many transcription factor and auxin-related genes, presumably associated with its role as a likely global regulator of RNA splicing and transcription in meristems (Casson et al., 2009 and unpublished data, Lindsey lab).

6.9 Role of RAP2.7 in de novo root regeneration

RAP2.7 is a member of the AP2/ERF family of transcription factors which are involved in the control of meristematic activity. It was identified in the Lindsey lab as a splicing target of MDF. The *rap2.7* mutant was able to form de novo roots by 8 d of leaf culture, but by 19 d the leaf started to yellow and the root stopped growing, with reduced numbers of root branches compared to wild type. On the other hand, the RAP2.7 transgenic overexpresser

produced longer roots and more root branches compared to wild type (Figure 5-8). This indicates these both *MDF* and *RAP2.7* genes may work together in the origination of the root meristem, both in the seedling primary root and in adventitious roots. Our results also showed that *YUC1* gene expression was not affected in both the *RAP2.7* overexpresser or mutant, indicating that RAP2.7 functions independently of auxin synthesis or signalling. *NAC1* expression was also not affected in either *RAP2.7* overexpresser or mutant. According to Chen et al (2016), the NAC pathway works independently of the auxin pathway, with the *nac1* mutant unaffected in *WOX11* and *WOX5* gene expression. The result suggests that RAP2.7 works independently of both the NAC1 pathway and the auxin pathway. MDF itself is also not regulated by auxin (Casson et al., 2009), though it regulates some aspects of auxin biology such as *PIN* gene expression. Our results also showed that *WOX5* gene expression, which is auxin-regulated, was not affected in either *RAP2.7* overexpresser or mutant early in the root regeneration process (at 0 h and 3 d), but in later stages (5d and 7d) *WOX5* expression was higher in the overexpresser and lower in the mutant than in wild type (Fig. 5-11). *RAP2.7* expression in the meristem may therefore have a role in regulating *WOX5* and therefore de novo root formation, though in an auxin-independent and MDF-dependent manner.

Previous studies showed that the *NAC1* gene is required for de novo root formation and defects in this gene block root regeneration (Chen et al., 2016). Similarly, it was found in this thesis that the *nac1* mutant was unable to create de novo roots, while the overexpresser produced similar length and numbers of roots as wild type. We showed that the *YUC1* gene expression was not affected in either *nac1* mutant or overexpresser, suggesting that NAC1 does not have a role in producing auxin, or in auxin signalling. This is consistent with previous results which suggest that NAC1 works independently of auxin in de novo root formation (Chen et al., 2016).

Similarly, *WOX5* gene expression was not affected in either *NAC1* overexpresser or mutant, further supporting the view that NAC1 has no role in the auxin pathway responsible for cell fate transition. *RAP2.7* gene expression was also not affected in either *NAC1* overexpresser or mutant, suggesting that the *RAP2.7* gene works independently of the NAC1 pathway. These results are summarised in the model represented in Figure 6-6.

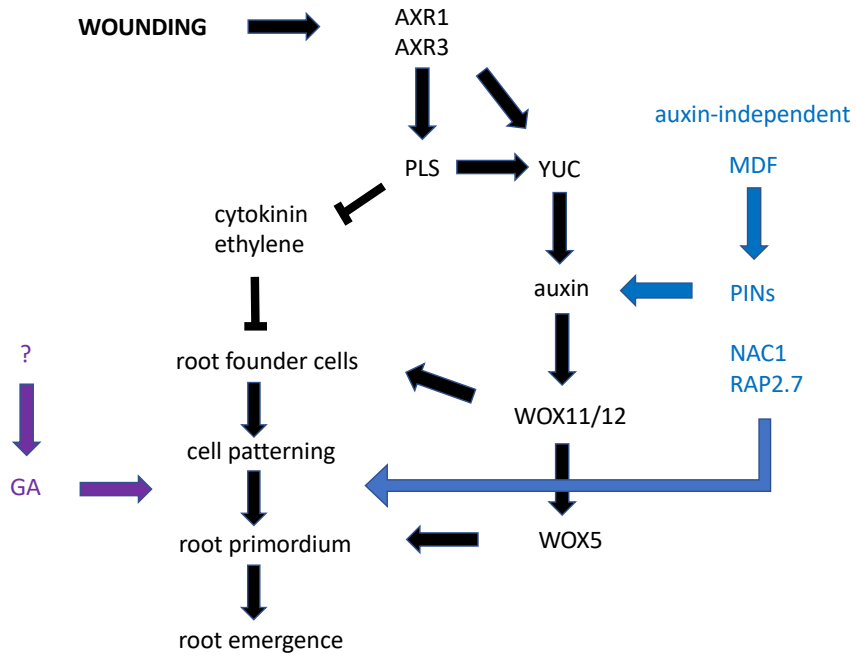


Fig. 6-6. Summary network model for data produced in this thesis, integrated with known information on gene-hormone interactions. Wounding induces the AXR/auxin-dependent and MDF/auxin-independent pathways. These interact at the interface of PINs, which require MDF activity and regulate auxin transport produced by the YUC pathway. Auxin activates the *WOX* genes and MDF activates the independently regulated NAC1 and RAP2.7 pathways. It is not known how GA is regulated in the developing adventitious root but is likely required for root cell expansion. PLS is positively regulated by auxin and is a negative regulator of both ethylene and cytokinin, to suppress the repressive effects of these hormones on root development.

7. Conclusions and future work

Hormone signalling is important for creating de novo roots from leaf explants of *Arabidopsis*. The hormone auxin is particularly important in both early and late stages of de novo root formation. The PIN family of auxin transporters move auxin from the blade leaf to the petiole and the wound site, where cell proliferation is activated. The hormone cytokinin is also important through its interaction with auxin, and initiation of cell division in the petiole. Results in this thesis also suggest that gibberellin may play a role in later stages of regeneration, at the primordium stage. The absence of correct auxin signalling causes defects in de novo root formation, as seen in *axr1* and *pls*, while the role of ethylene signalling is less obvious, with ethylene-insensitive mutants such as *ein2* showing little if any effect on root regeneration, though high ethylene responses may have adverse effects (*pls* has high ethylene signalling, linked to its low auxin and high cytokinin endogenous concentrations). The *MDF* and *RAP2.7* genes work via an independent pathway, though are clearly required for root regeneration from the leaf explant.

There are several areas that could be considered for future work. This includes a better understanding of the possible role of gibberellin at the root primordium stage, and mutants in gibberellin synthesis or signalling could be used to study effects on de novo root formation. Furthermore, as the results above showed that *MDF* and *RAP2.7* genes work via an auxin- independent pathway but the nature of this pathway is not clear, RNA sequencing coukld help to identify the genes involved in this pathway. Other hormones not yet investigated for possible roles root regeneration include abscisic acid and jasmonic acid.

Finally, there are some limitations with the cultured leaf system. Because of the tissue density of the leaf, I found that the use of GFP reporters were not feasible, through lack of laser penetration. This is why GUS reporters were used, but they have a reduced dynamic compared to GFP. However, the system does provide a valuable system for monitoring cellular and molecular processes in tissue regeneration and illustrates well the plasticity that characterizes plant development.

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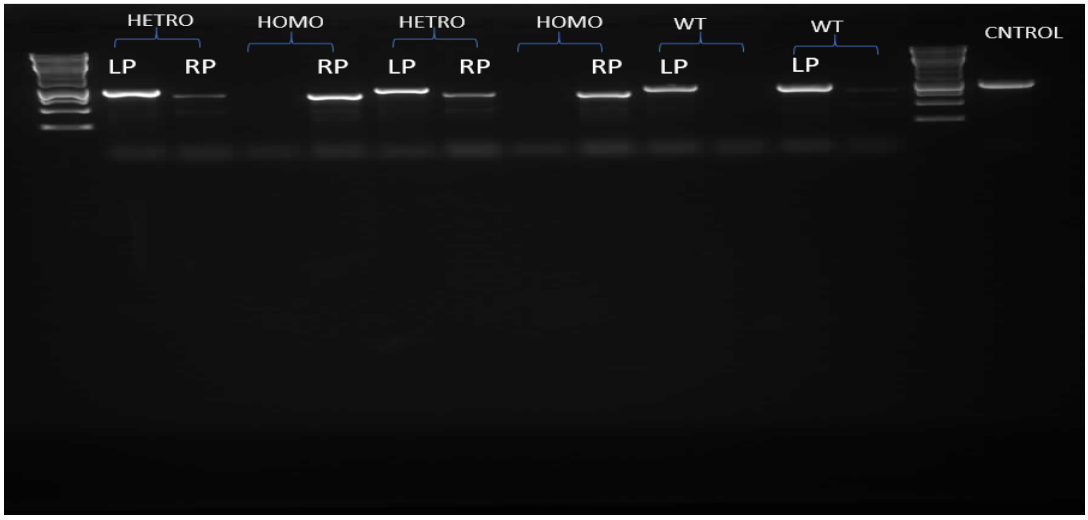
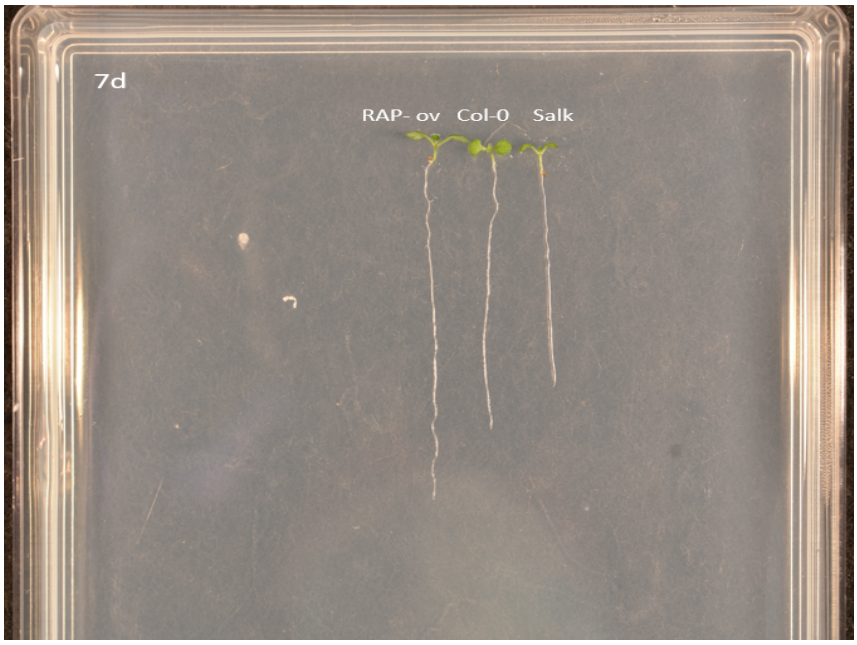
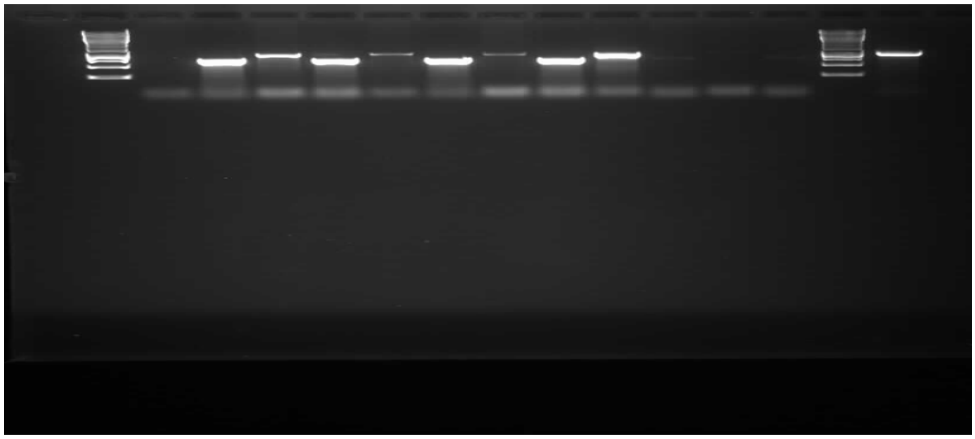
9. Appendices:



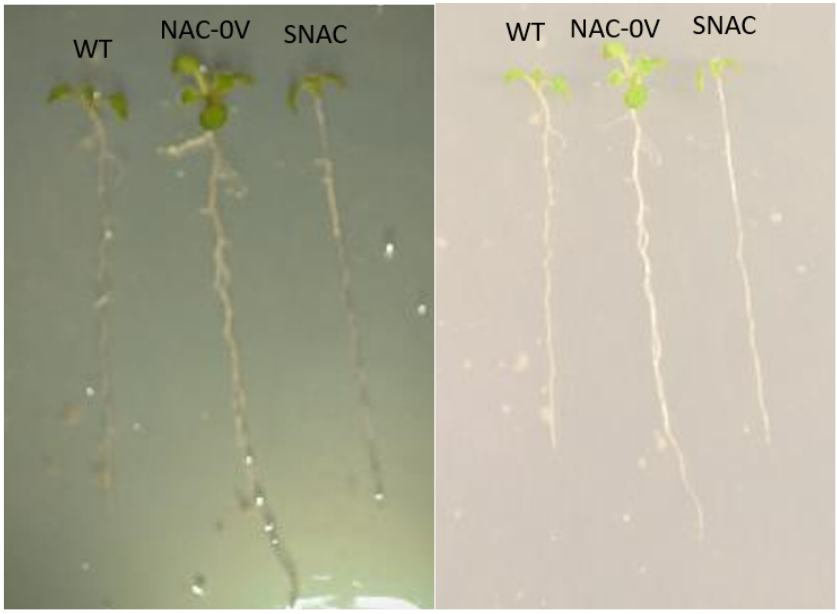
Appendix I: Genotyping of SALK mutant *rap2.7* to determined homozygote, heterozygous and wild type lines.



Appendix II: Root length of wild type, RAP-OV and SALK rap2.7 at 7 d post germination on MS medium.



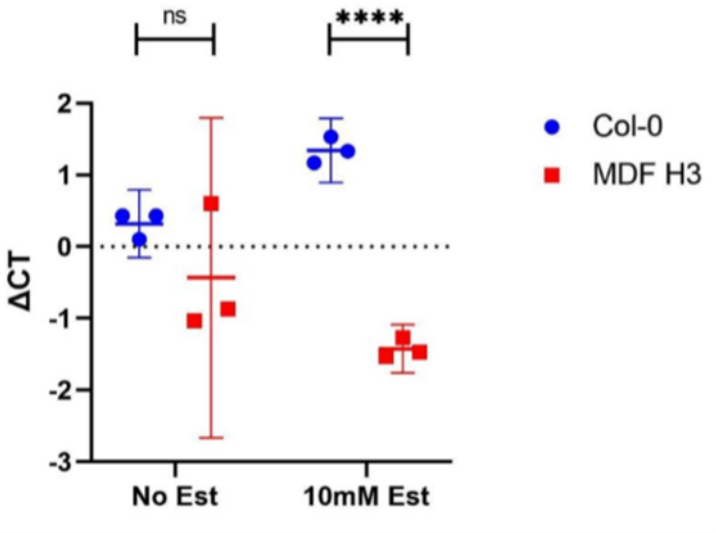
Appendix III: Genotyping of SALK *nac1* mutant to identify homozygote, heterozygous and wild type lines.



Appendix IV: Root length of wild type, NAC1-OV and salk nac1 at 7 d post germination on MS medium.

	a	b	c	d	e	f	g	h	i	
1	cola yuc4	Colb yuc4	Colc yuc4	axr1a yuc4	axr1b yuc4	axr1c yuc4	plsa yuc4	plsb yuc4	plsc yuc4	
2	cola yuc4	Colb yuc4	Colc yuc4	axr1a yuc4	axr1b yuc4	axr1c yuc4	plsa yuc4	plsb yuc4	plsc yuc4	
3	cola yuc4	Colb yuc4	Colc yuc4	axr1a yuc4	axr1b yuc4	axr1c yuc4	plsa yuc4	plsb yuc4	plsc yuc4	
4	eina UBC1	einb UBC1	einc UBC1	axr3a UBC1	axr3b UBC1	axr3c UBC1	water	water	water	
5	eina UBC2	einb UBC2	einc UBC2	axr3a UBC2	axr3b UBC2	axr3c UBC2	water	water	water	
6	eina UBC3	einb UBC3	einc UBC3	axr3a UBC3	axr3b UBC3	axr3c UBC3	water	water	water	
7										
8										
	a	b	c	d	e	f	g	h	i	
1	Cola UBQ103	Colb UBQ103	Colc UBQ103	axr1a UBQ103	axr1b UBQ103	axr1c UBQ103	plsa UBQ103	plsb UBQ103	plsc UBQ103	
2	eina wox5	einb wox5	einc wox5	axr3a wox5	axr3b wox5	axr3c wox5	eina UBQ101	einb UBQ101	einc UBQ101	
3	eina wox5	einb wox5	einc wox5	axr3a wox5	axr3b wox5	axr3c wox5	eina UBQ102	einb UBQ102	einc UBQ102	
4	eina wox5	einb wox5	einc wox5	axr3a wox5	axr3b wox5	axr3c wox5	eina UBQ103	einb UBQ103	einc UBQ103	
5	eina UBC1	einb UBC1	einc UBC1	axr3a UBC1	axr3b UBC1	axr3c UBC1	axr3a UBQ101	axr3b UBQ101	axr3c UBQ101	
6	eina UBC2	einb UBC2	einc UBC2	axr3a UBC2	axr3b UBC2	axr3c UBC2	axr3a UBQ102	axr3b UBQ102	axr3c UBQ102	
7	eina UBC3	einb UBC3	einc UBC3	axr3a UBC3	axr3b UBC3	axr3c UBC3	axr3a UBQ103	axr3b UBQ103	axr3c UBQ103	
8	water	water	water	water	water	water	water	water	water	

Appendix V: Example of how qPCR samples were organized prior to amplification.



Appendix VI: qRT-PCR analysis of *MDF* gene expression in wild type and MDF-OV. Using UBC reference gene was quantified without treatment and after treatment with 10 mM estradiol. t-test with no treatment (p = 0.28, df: 2.180); 10 mM estradiol (****p= 0.000051, df 3.720) (Rodrigo Matus thesis, Durham University, 2021).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