The Role of Ethylene and Auxin in Responses of Roots to Phosphate Supply in White Clover (*Trifolium Repens* L.)

Phuong Dinh Thi Yen

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The Role of Ethylene and Auxin in Responses of Roots to Phosphate Supply in White Clover (*Trifolium Repens* L.)

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Phuong Dinh Thi Yen

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ABSTRACT

Phosphate (P) supply is one of the major determining factors to plant productivity, since the element affects the growth and the development of plants. In response to P-deficiency treatment, plants display alterations in root system architecture caused by changes in primary root (PR) and lateral root (LR) length and LR density. In this thesis, the root growth of the agronomically important legume, white clover (*Trifolium repens* L.) was found to be slightly stimulated in terms of PR length, LR number and total LR length when plants were grown in a P-deficient media (0.01 mM orthophosphate; Pi) when compared with plants grown in a P-sufficient media (1.00 mM Pi) when using a hydroponic growth system.

When plants are grown in a P-sufficient media, treatment with 100 nM exogenous 1-aminocyclopropane-1-carboxylic acid (ACC) and exogenous auxin (5 nM 1-naphthylacetic acid, NAA) resulted in significant increases in white clover PR length, LR number and LR length. However, when ethylene action or auxin transport were inhibited using 300 ppm 1-methylcyclopropene (1-MCP) and 100 nM 1-N-naphthylphthalamic acid (NPA), respectively, root growth was significantly reduced which suggests roles for ethylene and auxin in mediating white clover root growth.

To examine the effects of these hormones on plants grown in P-deficient media, 100 nM ACC treatment significantly enhanced the stimulatory effects of growth on P-deficient media only, while exposure of plants to P-deficiency alone was sufficient to significantly neutralise the inhibitory effects of 1-MCP on root growth. Hence, exposure to P-deficiency is proposed to increase either ethylene biosynthesis or ethylene sensitivity in white clover roots. In contrast, for plants grown in P-deficient media, treatment with 5 nM NAA significantly abolished the stimulation of white clover root growth observed with P-deficiency so it is proposed that exposure to P-deficiency increases either auxin biosynthesis or auxin sensitivity, but the 5nM NAA concentration used was too high to stimulate root growth. Using *DR5p::GUS* transgenic white clover, auxin activity was found in the root tips and root primordia. Using these plants, it is suggested that P-deficient treatment and ACC treatment influenced white clover root growth through an increase in auxin sensitivity.

Overall, ethylene and auxin are found to be essential in mediating white clover root growth in P-sufficiency, and also in mediating root responses to P-deficiency through changes in terms of the biosynthesis and the sensitivity of these two hormones.

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LIST OF ABBREVIATIONS

1-MCP	1-methylcyclopropene
6-BAP	6-Benzylaminopurine
ACC	1-aminocyclopropane-1-carboxylate
ACO	1-aminocyclopropane-1-carboxylate oxidase
ACS	1-aminocyclopropane-1-carboxylate synthase
AVG	Aminoethoxyvinylglycine
BCIP	5 bromo-4-chloro-3-indolyl phosphate
BFA	Brefeldin A
bp	base pair
ca.	approximately
Cef^{300}	Cefotaxime (300 $\mu g.mL^{-1}$)
cm	centimetre
CTR	CONSTITUTE TRIPLE RESPONSE
DAT	day after treatment
DMF	Dimethylformamide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EIN	ETHYLENE INSENSITIVE
ERF	ETHYLENE RESPONSE FACTOR
ERS	ETHYLENE RESPONSE SENSOR
ETR	ETHYLENE TRIPLE RESPONSE
FAA	Formalin acetic acid
g	acceleration due to gravity (9.8 $m.s^{-2}$)
g	gram
GMO	Genetically Modified Organism
GUS	β-glucuronidase
HCl	Hydrochloric acid
IAA	Indole-3-acetic acid
Kan^{150}	Kanamycin sulfate (150 $\mu g.mL^{-1}$)
^{o}C	Degrees in celsius

Kb	kilo base pair
kPa	kilo Pascal
L	Litre
LR(s)	lateral root(s)
M	moles L^{-1}
mg	milligram
$MgSO_4$	Magnesium sulfate
min	minute
mL	millilitre
mM	millimoles L^{-1}
MQ water	water purified by a Milli-purification system
MS	Murashige and Skoog base media
NAA	1-naphthyleneacetic acid
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NBT	Nitro-blue tetrazolium chloride
ng	nanogram
nM	nanomoles L^{-1}
NPA	1-N-naphthylphthalamic acid
P	P value from *t*-test
P	Phosphate
P_{adj}	adjusted P value from Tukey's test
PAT	polar auxin transport
PC2	Physical Containment 2
PCR	Polymerase Chain Reaction
PGU	Plant Growth Unit
pH	- log $[H^+]$
Pi^-	P-deficient treatment
Pi	inorganic phosphate, orthophosphate
Pi^+	P-sufficient treatment
ppm	parts per million
PR(s)	primary root(s)
QC	quiescent centre
RAM	root apical meristem

RH	Relative humidity
RNA	ribonucleic acid
Rnase	Ribonuclease
RO	reverse osmosis
rpm	round per minute
RSA	root system architecture
RT	room temperature
SAM	*S*-adenosyl methionine
SDS	Sodium dodecyl sulfate
SE	standard error of the mean
SSC	Sodium citrate – sodium chloride buffer
TAE	Tris-Acetate-EDTA
TFs	transcription factors
TIBA	2,3,5-triiodobenzoic acid
Tris	Tris(hydroxymethyl)aminomethylamine
Trp	Tryptophan
TY	Tryptone-yeast extract
UV	ultra violet light
V	volts
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight
WT	wild type
X-GLUC	5-Bromo-4-chloro-3-indolyl β-D-glucuronide
µg	microgram
µL	microlitre
µm	micrometre
µM	micromoles L^{-1}

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CHAPTER ONE: INTRODUCTION

1.1. OVERVIEW

Phosphorus (P) is considered as "the most limiting nutrient for crop production" (Schachtman et al., 1998). In response to P-deficiency, plants display a series of biochemical, molecular, physiological and morphological adaptations. For example, it is well established that plants change their root system architecture (RSA) in response to P-deficiency, including adjustment of the primary root (PR) length, in addition to the length and the density of lateral roots (LRs) (Malamy and Ryan, 2001, Williamson et al., 2001, López-Bucio et al., 2003). With these architectural modifications, root systems can explore the soil and establish in areas of higher orthophosphate (Pi; the accessible form of P) concentrations, in order to increase Pi uptake (Borch et al., 1999, Lynch and Brown, 2001).

More recent work has shown that the plant hormone auxin plays a very important role in the regulation of root growth in response to P-deficiency (Williamson et al., 2001, Linkohr et al., 2002, Al-Ghazi et al., 2003, Nacry et al., 2005). Using *Arabidopsis thaliana* as a model, studies have shown that nutrient starvation causes auxin redistribution and a change in auxin responsiveness (López-Bucio et al., 2002, Malamy, 2005, Nacry et al., 2005, Perez-Torres et al., 2008) that leads to root morphological responses. For example, in P-deficient conditions, auxin redistribution and increase in auxin sensitivity results in the reduction of PR length, and an increase in the length and the density of LRs (López-Bucio et al., 2002, López-Bucio et al., 2003, Nacry et al., 2005, Perez-Torres et al., 2008).

As well as auxin, ethylene has also been shown to induce changes in root morphology, in response to P-starvation (Al-Ghazi et al., 2003, Zhang et al., 2003). It has also been shown that a change in ethylene synthesis and sensitivity occurs in plants when they are phosphate deprived (Borch et al., 1999). Furthermore, ethylene has been demonstrated to interact with auxin in the regulation of root growth (Rahman et al., 2001) where ethylene stimulates auxin production and the rate of auxin transport into the roots (Ruzicka et al., 2007, Swarup et al., 2007). Therefore, it has been proposed that the cross-talk between ethylene and auxin is important in regulating responses to P-deficiency (as reviewed in Lynch and Brown, 1997).

Recently, the response of white clover to P-deficiency has been characterised (Roldan, 2008). It has been shown that changes in the pattern of PRs and LRs occur and the induction of root growth by P-stress was accompanied by an increase in expression of genes involved in the ethylene biosynthesis pathway. This indicated that ethylene may be important in root growth adaptation to P-starvation. However, the influences of ethylene, auxin and the interaction between ethylene and auxin on white clover root growth and also on white clover root responses to P-deficiency have not been studied. Thus in this thesis, changes in white clover root morphology were investigated in response to P-deficiency, and to ethylene and auxin treatments.

1.2. PHOSPHORUS IN PLANT GROWTH AND DEVELOPMENT

Phosphorus is one of the major macronutrients essential for plant growth and development (Desnos, 2008). Although phosphorus content may be high in soil, this nutrient is still one of the least available macro elements for plants to take up. This is due to the fact that the majority of phosphorus (80%) is found in insoluble forms and in organic compounds with other cations, such as calcium, iron and aluminium, and these (such as phytate) are inaccessible to plants (Richardson, 1994, Holford, 1997). Thus the accessible form (inorganic form) of this nutrient for plants to absorb, orthophosphate (Pi), occurs in very low abundance in soil (under 10 μM) (Bieleski, 1973, Abel et al., 2002). Moreover, with plant uptake, local areas of P-depleted soil are created around the rhizosphere which are not rapidly replenished, due to the low mobility of Pi (mainly by diffusing at the rate from 10^{-12} to 10^{-15} m^2s^{-1}) (Schachtman et al., 1998, Rausch and Bucher, 2002). This depletion phenomenon limits crop productivity in agricultural systems (around 30% to 40% of arable land contains Pi-limited soil) due to the crucial role of Pi in plant growth and development (Runge-Metzger, 1996, Abel et al., 2002).

1.2.1.The role of Pi in plant growth and development

Pi is one of the essential nutrients for plants because it is not only one of the pivotal components of the plant cell but it is also important for reactants and effectors in plant metabolism (Abel et al., 2002). Thus, in total, Pi contributes approximately 0.2% dry weight of the plant. For example, Pi is a constituent of nucleic acids and phospholipid

molecules that are part of the transcription machinery and of the membranes of plant cells, respectively (Marschner et al., 2005). Pi is also involved in carbon assimilation and the energy balance of cells, through its contribution to photosynthesis and respiration processes (Abel et al., 2002). In addition, Pi is essential for adenosine-5'-triphosphate [ATP, referred to as the "molecular currency" for energy transfer (Knowles, 1980)] synthesis, which is one of the vital processes within living organisms. Pi is also involved in signal transduction cascades and protein phosphorylation, which play essential roles in the regulation of the plant stress responses (Rausch and Bucher, 2002). Therefore, Pi has a vital role in plant growth and development. However, the accessibility of this nutrient is very low and therefore its availability becomes the second most critical factor (after nitrogen) that affects plant productivity. As a result of that, plants need to adapt in order to maintain their survival.

In low Pi conditions, plants express nutrient deficiency symptoms. For example, in *Arabidopsis*, shoots are stunted, leaves are small and dark green, and also anthocyanins and the Pi acquisition enzymes accumulate (Bates and Lynch, 1996). In the roots of *Arabidopsis*, constant P-depletion (1 μM Pi) leads to a decrease in metabolic activity (López-Bucio et al., 2003) which causes the root apical meristem (RAM) to lose the ability to respond to auxin, when compared with roots grown in P-sufficient (1 mM Pi) media. Thus in order to cope with these effects on growth rate and productivity, plants display mechanisms which are part of an adaptation to a Pi limiting status.

1.2.2.The adaptation of plants to Pi limited supply

Because Pi is vital for plant growth and development, and the availability of this mineral in soil is low (less than 10 μM), plants have developed a series of biochemical, molecular, physiological and morphological strategies, in order to access the low levels of soil Pi and also to use these to survive in P-depleted soils (Figure 1.1).

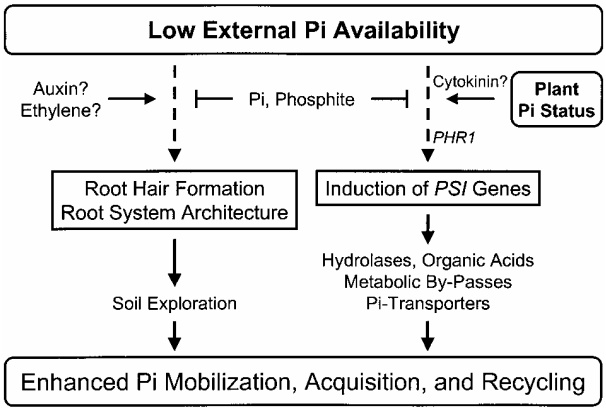


Figure 1.1. An overview of plant responses to phosphate limitation

(adapted from Abel et al., 2002).

1.2.2.1. Biochemical, molecular and physiological responses to P-starvation

In low Pi conditions, plants display a series of biochemical adaptations in order to facilitate the Pi uptake process. One of the adaptive biochemical mechanisms is mineralisation of the rhizosphere with root exudates through the secretion of organic acids (Schachtman et al., 1998, Shen et al., 2005, Jain et al., 2007). These root exudates induce acidification of the rhizhosphere to improve the availability of Pi, so that the root systems can uptake the nutrient (Al-Ghazi et al., 2003).

In addition to these mineralization mechanisms, in P-deficient conditions the plant active Pi transport systems (such as energised transport and co-transport with positively charged ions) also need to be up-regulated to facilitate Pi transport across the membrane and against the Pi concentration gradient. For example, plants actively transport Pi from the soil (lower Pi concentration) to root cells (higher Pi concentration), between cells and between intracellular compartments (Schachtman et al., 1998). As well as improving the uptake efficiency of the root systems, plants have also developed mechanisms to maintain the cytoplasmic Pi concentration that is necessary for the regulation of many endogenous enzymes (Schachtman et al., 1998).

Using microarray analysis, Wu et al. (2003) investigated changes in the expression of approximately 6,172 genes in *Arabidopsis* leaves and roots exposed to P-depleted conditions. They observed that the expression of more than 1,800 genes changed more than two fold (up regulated or down regulated) after three days of treatment. These genes were found to code for proteins that have functions in fundamental cellular processes such as photosynthesis, Pi metabolism, transport mechanism (intercellular and intracellular), cellular division, nucleic acid metabolism and protein synthesis.

Since these changes in gene expression occurred when Pi was withdrawn, they are referred to as the P-starvation responsive genes (Wu et al., 2003). In terms of particular genes, the expression of approximately one hundred genes that encode for transcription factors (TFs), which play very important roles in activating or suppressing the transcription of other functional genes, were also found to be stimulated after 72 hours under P-starvation conditions (Wu et al., 2003). These factors are associated with the down regulation at the transcriptional level of the genes involved in photosynthesis and nitrogen assimilation. The changes in the expression profile of these P-starvation responsive genes is also claimed to differ between different organs (roots versus shoots) and amongst different *Arabidopsis* ecotypes (López-Bucio et al., 2003, Wu et al., 2003). This reflects the divergence of the general plant responses to P-deficiency.

Changes in the TF genes has also been reported by López-Bucio et al. (2003) and Franco-Zorrilla et al. (2004). They found that the *PHOSPHATE RESPONSE REGULATOR1 (PHR1)* (Rubio et al., 2001), *PHOSPHORUS STARVATION RESPONSE1* [*PSR1*, cloned from *Chlamydomonas reinhardtii* (Wykoff et al., 1999)] and the *HOMEODOMAIN-LEUCINE ZIPPER (HD-ZIP)* (Tang et al., 2001) genes were all up regulated in response to P-depletion. In addition, increased expression was reported in genes encoding the Pi transport proteins that facilitate Pi uptake from the root systems to the shoots, where Pi is assimilated (Abel et al., 2002). Furthermore, Abel et al. (2002) stated that the expression of the genes in the P-starvation response system, such as those that code for RNases and phosphatase, were stimulated during P-stress, and the accumulation of the corresponding RNA transcripts for these genes was observed before the plants displayed obvious P-deficient symptoms.

These molecular modifications (in gene expression) lead to changes in the physiological processes in response to P-starvation. For example, P-deprivation induces the expression of the genes encoding phosphatase, phosphohydrolase and ribonucleases, which are proposed to participate in the Pi cycling system (remobilising Pi from older and senescent leaves to younger tissues) (Abel et al., 2002). By studying suspension-cultured cells of black mustard (*Brassica nigra*) and tomato (*Lycopersicon esculentum*) (Goldstein et al., 1988, Plaxton and Carswell, 1999, Baldwin et al., 2001), the enzymes which have roles in Pi cycling, Pi uptake and extracellular RNA degradation, were shown to be induced in response to P-depletion (Köck et al., 1998).

In the laboratory, this response has been used to enhance the ability of transgenic *Arabidopsis* to tolerate P-deficiency by transforming these plants with a purple acid phosphatase gene (encoding one of the phytate catalysing enzymes) (Xiao et al., 2006). The root apoplast of these transgenic *Arabidopsis* showed a four-fold increase in acid phosphatase activity in comparison to the control plants. This resulted in increases in biomass and total Pi content of the transgenic *Arabidopsis,* which was more than twice the control plants after one month grown on media with 2 mM phytate as the sole source of phosphorus (Xiao et al., 2006).

Finally, another mechanism to reduce the high requirement of Pi in plants is that the phospholipid components in cellular membranes are replaced by non-phosphorus containing galactolipids (Härtel and Benning, 2000, Kobayashi et al., 2006). In addition, in order to increase Pi uptake, plants grown in a low Pi concentration media had a higher ratio of root to shoot dry weight, compared with plants grown in P-sufficiency (Bates and Lynch, 2000).

1.2.2.2. Morphological responses to P-starvation

In response to P-deficiency, in addition to acidification of the rhizosphere and changes in gene expression, the division and differentiation of plant cells, particularly root cells, is modulated to induce changes in RSA (Williamson et al., 2001, Linkohr et al., 2002, López-Bucio et al., 2003). Although there is a common point of view that changes in RSA cause an increase in the surface-to-volume ratio under Pi limitation, the mechanisms by which these RSA changes are controlled is still unclear.

In *Arabidopsis* in response to low Pi-supply, changes in LR growth, rather than the PR, occur in order to exploit more available Pi in the surrounding environment when the root cap is in physical contact with the low Pi media (Svistoonoff et al., 2007). Therefore, the PR elongation rate was found to be depressed in P-starved *Arabidopsis* (Williamson et al., 2001, Al-Ghazi et al., 2003, López-Bucio et al., 2005, Sanchez-Calderon et al., 2005, Reymond et al., 2006, Jain et al., 2007, Jiang et al., 2007). A mechanism to explain this inhibition of PR elongation has been proposed by Sanchez-Calderon et al., (2005). Here, the P-deficient signal is perceived at the PR tips, particularly in the quiescent center (QC), the organising region of the root meristem, which then induces a decrease in cell proliferation and cell elongation in the PR. Eventually, the PR tips lose their meristematic activity (Ticconi et al., 2004, Sanchez-Calderon et al., 2005). This may explain the arrest of columella cell development in

Arabidopsis PR tips after 16 days of germination on P-deficient media (Sanchez-Calderon et al., 2005). Moreover, in response to P-deficiency, the inhibition of *Arabidopsis* PR elongation is proposed to be caused by the iron (Fe) toxicity, because the PR elongation in P-deficient media is restored, as in the P-sufficient control, when the iron concentration in the media was decreased (Ward et al., 2008)

In contrast, the length of LRs is reported to be longer in P-deficient than in P-sufficient treatments (Williamson et al., 2001, Al-Ghazi et al., 2003). Moreover, P-deficiency promotes LR formation so that a higher number of LRs were found in P-starved *Arabidopsis*, when compared with plants grown in P-sufficiency (Casimiro et al., 2001, Lynch and Brown, 2001, Ma et al., 2001a, Malamy and Ryan, 2001, López-Bucio et al., 2002, Sanchez-Calderon et al., 2005). An increase in LR formation may be a result of a trigger in the initiation of LR primordia and/or the stimulation of already initiated LR primordia (Nacry et al., 2005). However, as a result of the inhibition of the PR elongation rate and the induction of LR formation, LR density (defined as the number of LRs per one unit of PR length) is also increased in response to P-starvation (Williamson et al., 2001, López-Bucio et al., 2002). The increase in LR formation is helpful for plants to increase the total absorptive surface area and the soil exploration area in response to P-deficiency (Ward et al., 2008). Controversially, the number of *Arabidopsis* LRs was claimed to decrease after ten days on P-depleted media (compared with P-sufficient media) (Al-Ghazi et al., 2003). Similarly, Nacry et al. (2005) found that LR growth of *Arabidopsis* (including length and number parameters) was promoted from day 7 to day 10 in 3 μM Pi media, but was then depressed from day 11 onward.

As well as modulation of RSA, root hair formation is also induced so as to enlarge the absorptive surface area, in response to P-deprivation (Ma et al., 2001a, Ma et al., 2001b, Schmidt and Schikora, 2001, López-Bucio et al., 2005). For example, in 1 μM Pi media, *Arabidopsis* root hair density (defined as the number of root hair per mm of root length) is five times higher than in plants grown in 1 mM Pi media (Ma et al., 2001a). In addition to root hair formation, root anatomy is also modified to increase Pi acquisition as part of the P-starvation responses (Ma et al., 2001a). These changes in root anatomy include an increase in root diameter and an increase in the number of cortical cells and trichoblast files (hair-bearing cells) which also accounts for the increase of root hair formation.

In addition to using *Arabidopsis* as a model for P-stress investigations, root growth patterns in barley (*Hordeum vulgare*), bean (*Phaseolus vulgaris*) and white lupin (*Lupinus albus*), under Pi limited conditions, have also been studied. Drew (1975) found that barley LR development was promoted whilst Bonser et al. (1996) observed changes in the angle of basal roots in bean, in response to P-stress. In contrast to *Arabidopsis*, maize (*Zea mays*), rice (*Oryza sativa*), narrow-leafed lupin (*Lupinus angustifolius* L.) and other 14 monocot and dicot species expand their root systems to explore more soil area in response to P-deficiency, by allocating more resources to the root systems in order to stimulate root elongation (both PRs and LRs) (Narayanan A Reddy, 1982, Mollier and Pellerin, 1999, Shimizu et al., 2004, Wang et al., 2008). Thus changes in root system architecture is a conserved response but it varies between plant species (Hammond and White, 2007).

In addition, white lupin, which is claimed to be tolerant to P-stress, has a special mechanism of root growth modulation: that is, proteoid root formation (Johnson et al., 1994, Shen et al., 2005, Johnson et al., 1996, Gilbert et al., 2000, Zhou et al., 2008). Proteoid roots (also called cluster roots) are tight clusters of rootlets that are usually observed in the Proteaceae family, and they are responsible for exuding organic acids (malate and citrate) and acid phosphatases during P-deficiency, in order to facilitate Pi mobilisation from the soil (Dinkelaker et al., 1995, Watt and Evans, 1999, Gilbert et al., 2000, Schmidt and Schikora, 2001, Lamont, 2003, Shane and Lambers, 2005). Moreover, cluster roots increase the absorptive area for Pi uptake by root systems (Lambers et al., 2006). Another adaptive mechanism to P-deficiency is to accelerate the formation of symbioses with mycorrhiza, commonly arbuscular mycorrhizal (AM) fungi, to increase the absorptive surface by using the hyphal surface (Wittenmyer and Merbach, 2005, Yoneyama et al., 2007). AM fungi have a high capacity to explore and store Pi for later uptake by plants (Harrison et al., 2002). Finally, plants hormones, such as ethylene, auxin and gibberellin, have been shown to be involved in P-deficient responses by plants (López-Bucio et al., 2002, Ma et al., 2003, López-Bucio et al., 2005, Jiang et al., 2007). Particularly, ethylene and auxin responsiveness in root systems has been reported to change in response to P-deprivation and these changes are proposed to play a role in the morphological responses described (Borch et al., 1999, López-Bucio et al., 2002, Ma et al., 2003).

1.3. ETHYLENE

1.3.1.Ethylene biosynthesis and signal transduction pathway

Ethylene is a gaseous hormone that plays a crucial role in a variety of plant developmental processes, such as floral induction, wound responses, flooding resistance, pathogen resistance, sex determination, fruit ripening, leaf senescence and leaf abscission (Bleecker and Kende, 2000). The ethylene biosynthesis pathway (as shown in Figure 1.2) is initiated with the conversion of methionine to *S*-adenosyl methionine (SAM) by the enzyme SAM synthetase. SAM is then converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by the enzyme ACC synthase (ACS) which is the first step in the committed part of the pathway. Finally, ACC is oxidased to form ethylene by the enzyme ACC oxidase (ACO) (Bleecker and Kende, 2000). Thus ethylene synthesis is regulated primarily through two catalysing enzymes, ACS and ACO, in response to environmental or endogenous signals (Kim et al., 1998).

To regulate plant development, ethylene acts *via* a signal transduction pathway (Figure 1.3), which was characterised originally by screening *Arabidopsis* ethylene-response mutants. Firstly, ethylene binds to a family of receptor proteins, including ETHYLENE TRIPLE RESPONSE1/2 (ETR1/ETR2), ETHYLENE RESPONSE SENSOR1/2 (ERS1/ERS2) and ETHYLENE INSENSITIVE4 (EIN4) (Chang et al., 1993, Hua et al., 1995, Hua et al., 1998, Sakai et al., 1998) which causes the inactivation of a Raf-like serine/threonine kinase (CONSTITUTE TRIPLE RESPONSE1; CTR1) which, without ethylene, negatively regulates the downstream transduction pathway components including EIN2, EIN3, EIN5, EIN6 and EIL1 (EIN3-LIKE1) (Kieber et al., 1993, Chao et al., 1997, Hua and Meyerowitz, 1998, Alonso et al., 1999).

Thus the action of ethylene is to relieve this CTR1-mediated repression such that EIN2 is free to signal to EIN3 and the other downstream components (EIN5, EIN6 and EIL1). The ETHYLENE RESPONSE FACTOR1 (ERF1), which is the last component of this pathway, is a TF that regulates the expression of many ethylene-inducible genes (Chao et al., 1997, Hao et al., 1998, Solano et al., 1998)

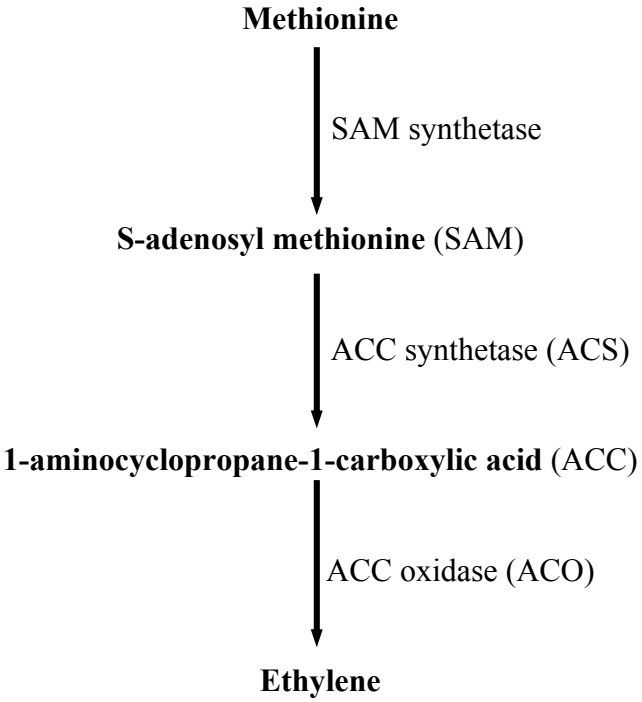


Figure 1.2 An overview of the ethylene biosynthetic pathway

(adapted from Bleecker and Kende, 2000)

Ethylene is synthesised from methionine via SAM and ACC precursor. These steps are catalysed by SAM synthetase, ACS and ACO, respectively, as indicated.

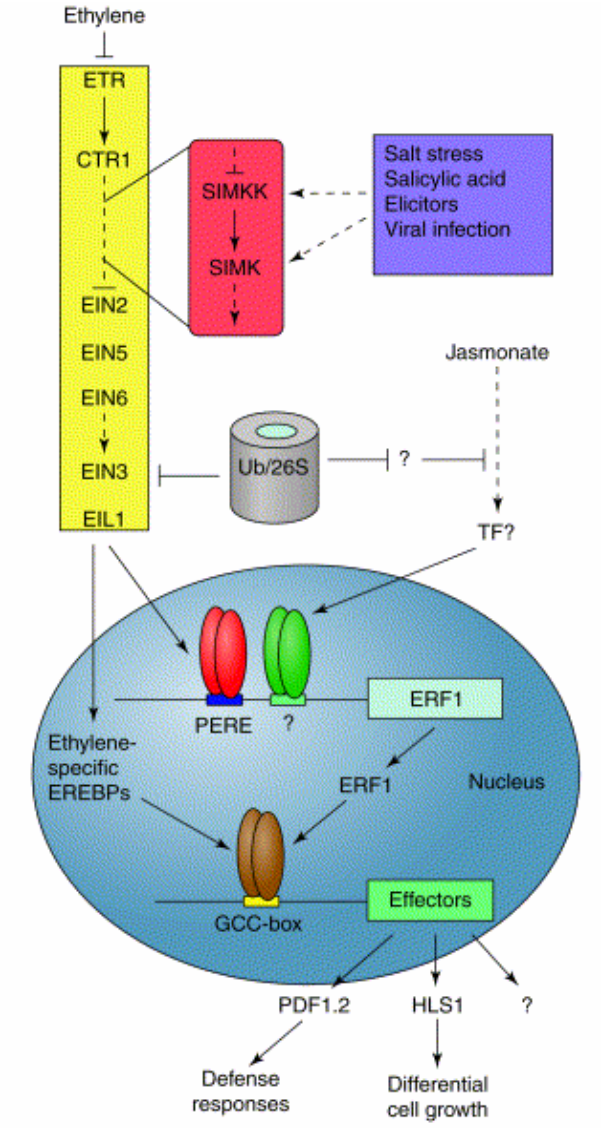


Figure 1.3. A model for the ethylene response pathway in the regulation of gene expression.

(from Guo and Ecker, 2004)

Ethylene is perceived by a family of ETR receptors (ETHYLENE TRIPLE RECEPTOR) to inactivate the constitutive expression of CTR1 (CONSTITUTE TRIPLE RESPONSE1) kinase. CTR1 in turns inhibits EIN2, EIN5 and EIN6 (ETHYLENE INSENSITIVE). Finally, the ethylene signal is transduced to a nuclear protein TF (EIN3/EIL) to regulate the *ERF1* (*ETHYLENE RESPONSE FACTOR1*) transcription, which modulates the expression of many ethylene-induced genes involved in the defence response system and in differential cell growth.

1.3.2.The role of ethylene in root development, particularly in response to P-stress

In response to various environment cues, it has been shown that ethylene plays a critical role in root development, including root primordia formation, root elongation, gravitropism, in addition to the formation of aerenchyma and root hairs (Lin et al., 1990, Tanimoto et al., 1995, Scheres, 1997, Clark et al., 1999, Finlayson et al., 1999, Buer et al., 2003, Buer et al., 2006). Ethylene has been found to be involved in the signalling pathway to regulate cell division in the QC and cell differentiation in the surrounding meristematic stem cells (Ortega-Martinez et al., 2007). In addition, lower concentrations (less than 80 nM) of exogenous ACC has been shown to have stimulatory effects on the initiation of LR primordia. However, higher concentrations of added ACC inhibit LR primordia initiation but promote LR emergence (Ivanchenko et al., 2008).

Particularly in P-deficiency, Lynch and Brown (1997) proposed that changes in ethylene biosynthesis and sensitivity stimulate the responses of roots. In further studies, using common bean (*Phaseolus vulgaris* L.) treated with ethylene and the ethylene synthesis inhibitor aminoethoxyvinylglycine (AVG), the possible regulatory role of ethylene in root responses to Pi supply was proposed to be through an increase in ethylene synthesis and sensitivity (Borch et al., 1999). Furthermore, this research group showed that the role of ethylene in the modulation of *Arabidopsis* root elongation in response to Pi supply, is to alter ethylene responsiveness (Ma et al., 2003). Ethylene was also found regulate the formation of adventitious roots in response to P-deficiency (Kim et al., 2008)

Moreover, ACC treatment of *Arabidopsis* roots could mimic the positive effect of low Pi media on root hair formation (Zhang et al., 2003, He et al., 2005). Also, root hair development was inhibited when the ethylene action inhibitor, 1-methylcyclopropene (1-MCP) and the ethylene biosynthesis inhibitor AVG were added, even under P-deficient conditions. Furthermore, they found that the ethylene insensitive mutants (*ein2-1* and *ein4*) did not show these responses to a limited Pi supply. Therefore, an involvement of ethylene in mediating the root responses to limited Pi supply was demonstrated.

1.4. AUXIN

1.4.1.Auxin biosynthesis and transport

In common with ethylene, auxin has also been shown to play a crucial role in modulating many aspects of plant growth and development, such as cell expansion, cell division and the development of vascular tissue and roots (Ljung et al., 2001). The endogenous auxin, indole-3-acetic acid (IAA), is proposed to be synthesised by two different pathways, a Tryptophan (Trp)-dependent pathway, in which Trp is the precursor of IAA, and a Trp-independent pathway, which bypasses Trp metabolism (Ljung et al., 2005). It is also known that auxin is primarily synthesised in young plant tissues, predominantly in young and expanding leaves, cotyledons, the shoot apex, root primordia and root tips (Ljung et al., 2001, Casimiro et al., 2001, Ljung et al., 2005). In the root tips of *Arabidopsis*, auxin has been found to accumulate in the QC and columella cells using *Arabidopsis* transformed with the *DR5p::GUS* gene construct (an auxin response reporter) to localise auxin responsiveness (Sabatini et al., 1999, López-Bucio et al., 2005, Sanchez-Calderon et al., 2005). In addition, auxin responsiveness was also detected in the *Arabidopsis* LR primordia (López-Bucio et al., 2005).

However, auxin responsiveness can be observed ubiquitously in plants, and in some cases remotely from the synthesis sites, due to auxin (IAA) transport. There are two pathways for IAA transport, "non-directional transport in the phloem" and directional transport (referred as polar auxin transport; PAT) in plant tissues (Friml and Palme, 2002). In PAT, IAA moves from cell to cell through IAA transport proteins comprising the IAA influx (Aux permeases) and the IAA efflux (PIN) proteins (Figure 1.4). In shoots, auxin moves basipetally from the shoot apex to the shoot/root junction (Figure 1.5). In roots, auxin is transported in both directions: the acropetal direction in the central cylinder (from the junction of shoot to root to the root apex) and also in the basipetal direction in the outer root cell layers from the root tip toward the shoot (Muday and DeLong, 2001, Friml and Palme, 2002) (Figure 1.5). This transported auxin has been shown to be involved in the regulation of LR growth and responses to gravity (Muday and DeLong, 2001).

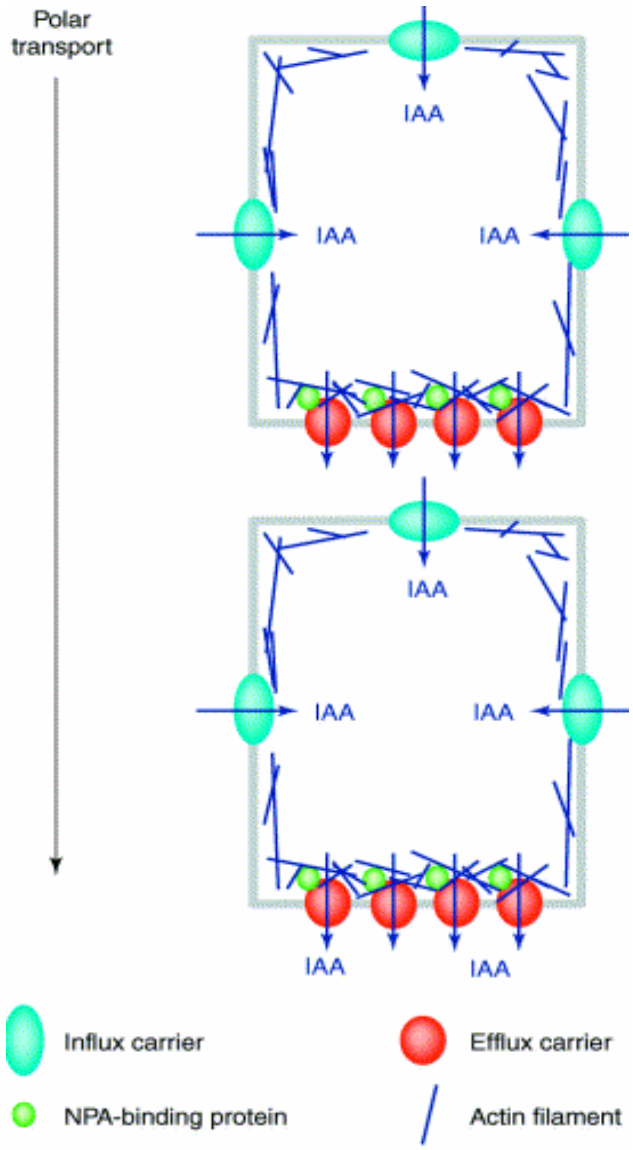


Figure 1.4. Model of the proteins that mediate auxin transport

(from Muday and DeLong, 2001)

Auxin moves from cell to cell through the carrier protein system in which influx carriers transport auxin into cells and efflux carriers (the PIN proteins) transport auxin out of the cells.

(NPA, naphthylphthalamic acid, is an example of an auxin transport inhibitor)

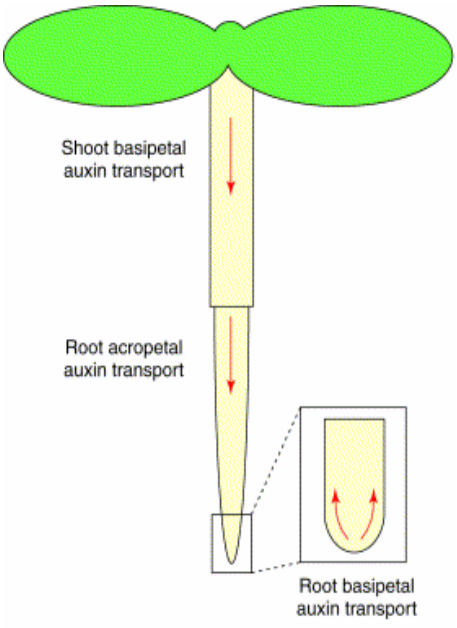


Figure 1.5. Auxin transport is polarity in *Arabidopsis*

Auxin is transported basipetally from the shoot to the junction between the shoot and root. In roots, auxin is transported in both directions, acropetally and basipetally, as shown (Muday and DeLong, 2001)

1.4.2.The role of auxin in root development

The effect of auxin on root growth and development is well known (Aloni et al., 2006). By using the auxin action inhibitor, *p*-Chlorophenoxyisobutyric acid, the auxin transport inhibitors naphthylphthalamic acid (NPA) and 2,3,5-triiodobenzoic acid (TIBA), and also using the auxin signalling defective mutants of *Arabidopsis* (*axr1*, *tir1* and *CSN5tg*), it was found that PR growth and LR production was inhibited and the gravitropic response of the root system were abolished (Casimiro et al., 2001, Malamy and Ryan, 2001, Schwechheimer et al., 2001, Oono et al., 2003). This demonstrates an essential role for auxin, derived from both shoots and roots, in regulating root development (Ljung et al., 2005). In particular, auxin modulates the growth of PRs through regulating the division, differentiation and elongation of cells in the PR apices (Oono et al., 2003).

For LR development, Bhalerao et al. (2002) revealed that in *Arabidopsis*, LR initiation depends on IAA derived from the PR root tip, whereas LR emergence requires leaf-derived IAA before the root system has ability to synthesis auxin. In addition, exogenous auxin (10 μM IAA) treatment induces the formation of LRs in *Arabidopsis* (Laskowski et al., 2006). This observation is consistent with reports elsewhere in which using low concentrations of exogenous auxin or the overproduction of endogenous auxin stimulates LR initiation (Boerjan et al., 1995, Celenza et al., 1995, King et al., 1995, Himanen et al., 2004). Therefore, as expected, blocking auxin transport or using auxin-resistant mutants inhibits LR initiation (Casimiro et al., 2003, Reed et al., 1998). Thus it is well documented that auxin is essential for LR development by inducing LR primordia initiation (Casimiro et al., 2003, De Smet et al., 2006, Lucas et al., 2008). Also, it has been demonstrated that the differentiation of founder cells to form the LR primordia requires auxin as a trigger (Dubrovsky et al., 2008, Swarup et al., 2008). Therefore, auxin plays a key role in root development by altering the growth of PRs and also the initiation and growth of LRs, in order to modulate the RSA, in response to changes in nutrient resources, including Pi supply. It has also been suggested that P-deficiency increases auxin responsiveness which in turn mediates root growth (López-Bucio et al., 2002, Nacry et al., 2005).

As mentioned previously, P-deficiency also affects RSA by changing the PR and LR length and also the number and the density of LR roots. Therefore, auxin has been implicated in this response through changes in auxin signalling cascades, auxin

sensitivity and auxin distribution. Firstly, by applying exogenous auxin and auxin antagonists to roots of *Arabidopsis* or analysing the auxin-resistant mutants (*axr1, axr2* and *axr4*), the increase in auxin sensitivity of P-starved roots was shown to relate to changes in the RSA as an adaptation to imposed Pi limiting conditions (López-Bucio et al., 2002). This has been confirmed by the finding of Perez-Torres et al. (2008), in which P-deficiency resulted in an increase in auxin sensitivity resulting in changes to LR formation and emergence. Also, by auxin treatment and the use of auxin-resistant mutants, Al-Ghazi et al. (2003) demonstrated the involvement of auxin signalling in RSA modification, in response to P-starvation. Finally, auxin redistribution, as stimulated by P-deficiency, was proposed to be implicated in RSA changes (Nacry et al., 2005). In these experiments, auxin was observed to over-accumulate in the PR tips, LR tips and LR primordia in response to P-deficiency (Nacry et al., 2005).

As an alternative view, it has been suggested that the responses of *Arabidopsis* roots to Pi supply is affected by shoot phosphate status, not by auxin signalling and sucrose supply (Williamson et al., 2001). To support this, using the auxin-resistant mutants (*axr1, axr4* and *aux1*), Linkohr et al. (2002) suggested an auxin-independent adaptation of *Arabidopsis* RSA to Pi supply. These authors reported that in response to P-stress, LR development and root hair formation were influenced by auxin and sucrose supply, whereas PR growth regulation was independent of auxin and sucrose supply, which is consistent with the result of Williamson's group (2001). This suggests variable roles for sucrose and auxin in the responses in roots to Pi availability.

1.5. CROSS-TALK BETWEEN ETHYLENE AND AUXIN IN ROOT DEVELOPMENT

As mentioned previously, both ethylene and auxin have been shown to play essential roles in the regulation of root development, resulting in an increased interest in the cross-talk of these two hormones in terms of the control of the process. A comparision of the effects on root growth of ethylene and auxin treatment of *A. thaliana* wild-type and ethylene-resistant mutants (*aux1-7* and *eir1-1*, also defined as auxin-transport defective mutants) suggests a positive role for auxin in the regulation of ethylene-inhibited root elongation (Rahman et al., 2001). Ethylene and auxin work in a reciprocal fashion at the PR tip to mediate PR elongation such that ethylene stimulates

auxin synthesis in the root apex which subsequently inhibits root elongation (Swarup et al., 2007, Ivanchenko et al., 2008). This inhibition was dramatically enhanced by treatment with ACC and auxin. Previoulsy, it was shown that the upregulation of auxin biosynthesis by ethylene promotes ethylene-inhibited PR growth (Rahman et al., 2001, Swarup et al., 2007). Thus the action of ethylene is not only to induce auxin biosynthesis, but ethylene also stimulates both acropetal and basipetal auxin transport to inhibit *Arabidopsis* PR cell elongation (Ruzicka et al., 2007, Negi et al., 2008). This demonstrates the requirement for auxin in the regulation of root growth by ethylene.

This cross-talk between ethylene and auxin in *A. thaliana* not only regulates PR elongation but also the formation of LRs (Negi et al., 2008). Here, ACC treatment or the high level of ethylene produced in the ethylene overproducing mutant (*eto1-1*) enhanced IAA transport but inhibited LR formation. In contrast, *etr1-3* and *ein2-5* mutants, in which the ethylene responses are blocked, showed reduction in acropetal IAA transport, but an enhancement in LR formation (Negi et al., 2008). Furthermore, these workers also found that the IAA influx defective mutant (*aux1-7*) had no response to ACC treatment, in terms of LR formation and IAA transport. Therefore, they suggested that ethylene stimulates "long-distance polar IAA transport" (Stepanova et al., 2005) such that in the mutant, a decrease in the IAA local concentration occurred that inhibited LR formation. In addition, lower concentrations of ACC (less than 80 nM) were proposed to enhance auxin biosynthesis and/or responsiveness so as to stimulate LR primordia initiation but higher doses of ACC inhibited LR primordia initiation (Ivanchenko et al., 2008). When ACC was combined with NPA, an auxin transport inhibitor, the inhibitory effects on LR primordia initiation were cumulative (Ivanchenko et al., 2008).

Thus auxin seems to act as a downstream factor of ethylene in the root growth regulated pathway (Ruzicka et al., 2007). However, by comparing the morphological responses to auxin and ethylene, and also changes in gene expression of wild type *Arabidopsis* with the auxin and ethylene mutants in *Arabidopsis*, Stepanova et al. (2007) showed several levels of the interaction between ethylene and auxin. Ethylene and auxin reciprocally influence the biosynthesis of the other hormone, and their respective response pathways (Stepanova et al., 2007). In addition, Lynch and Brown (1997) hypothesised that the cross-talk between ethylene and auxin may be important in inducing responses to P-deficiency. However, no study has directly investigated this interaction.

1.6. PHOSPHATE DEFICIENT RESPONSE IN WHITE CLOVER

White clover (*Trifolium repens* L.) is considered to be an agronomically important legume in pastures for livestock (Baker and Williams, 1987). With a stoloniferous growth habit, white clover can store carbohydrate in the stolons (Chapman and Robson, 1992), and the legume fixes nitrogen, thus providing nitrogen to the pasture ecosystem. However, in P-deficient soils, the competitive ability of white clover is less than the companion grasses with which it is grown (Jouany et al., 2004). In low Pi conditions, tissue phosphate concentrations, nitrogen fixation rates, nodule growth, total leaf area and photosynthetic rate all decline, but the production and the exudation of phytase and phosphatases (APases) acid are induced by the roots (Bowling and Dunlop, 1978, Caradus and Snaydon, 1987b, Caradus and Snaydon, 1987a, Almeida et al., 1999, Hunter and McManus, 1999, Zhang and McManus, 2000, Hogh-Jensen et al., 2002).

To improve phosphorus acquisition, acid phosphatase and phytase genes, which were cloned from *Medicago truncatula,* have been transformed into white clover (Ma et al., 2009). These transgenic white clover lines showed an increase in phytase and acid phosphatase activities and showed a significant improvement in relative biomass productions when maintained on organic phosphorus. In contrast, wild type (non-transformed) white clover lost half of its relative biomass production when maintained on organic phosphorus in comparison with the inorganic phosphorus treatment (control) (Ma et al., 2009). Thus expression of these transgenes can improve the Pi uptake capability of white clover.

In previous experiments with wild type white clover, PR length and LR production were found to increase in P-deficiency (0.01 mM Pi) (Roldan, 2008). Associated with the changes in RSA are changes in the expression of the *TRIFOLIUM REPENS ACO (TR-ACO)* genes, including *TR-ACO1, TR-ACO2* and *TR-ACO3*. Studies have shown an upregulation of *TR-ACO1* transcription and an increase in TR-ACO1 protein accumulation that was significant at 12 hours and 24 hours after treatment (Roldan, 2008). For *TR-ACO2*, there was no significant difference at transcription level but a significant increase in protein accumulation was observed after seven days of treatment. For *TR-ACO3*, its expression pattern did not follow any trend in response to P-deficiency (Roldan, 2008). However, the involvement of auxin and the cross-talk between auxin and ethylene in Pi responses has not yet been studied in white clover.

AIMS OF THIS PROJECT

The key role of auxin, in mediating the observed changes in root architecture in response to Pi supply, has been proposed, based on the results of investigations on the model plant species, *A. thaliana* (López-Bucio et al., 2002, Al-Ghazi et al., 2003, Nacry et al., 2005). In addition, Roldan (2008), using white clover, suggested that ethylene also has a role to play in inducing root growth, particularly LRs, in response to P-depletion. Using these findings as a starting point, influence of the plant hormones, ethylene and auxin, on mediating root growth, particularly in root responses to P-deficiency is to be investigated. In addition, the cross-talk between ethylene and auxin in regulation of white clover root growth will also be studied. Thus the central hypothesis to be tested is that:

The balance between ethylene and auxin is necessary in mediating white clover root growth and that P-deficiency stimulates white clover root growth through elevating ethylene biosynthesis and auxin sensitivity.

In order to address the hypothesis, the project will be conducted through three aims:

- To investigate the influences of ethylene and auxin on white clover root growth and root responses to P-deficiency.
- To characterise the cross-talk between ethylene and auxin in white clover root growth.
- To examine the influences of P-deficiency and exogenous ethylene on auxin responsiveness.

To meet these aims, the following experimental approaches will be used:

- Use the ethylene precursor (ACC), an exogenous auxin (1-naphthylacetic acid – NAA, IAA), an ethylene action inhibitor (1-MCP) and an auxin transport inhibitor (Naphthylphthalamic acid – NPA) to investigate the roles of ethylene and auxin in modulating white clover root growth and root responses to Pi supply.
- To use combinations of ACC with NPA, and NAA with 1-MCP to characterise cross-talk between ethylene and auxin in white clover root growth.
- To use white clover transformed with the *DR5p::GUS* gene, an auxin response marker, to study the changes in auxin responsiveness pattern in response to P-deficiency (0.01 mM Pi) and exogenous ethylene.

CHAPTER TWO: MATERIALS AND METHODS

2.1. PLANT MATERIALS

A single genotype (designated 10F) of white clover (*Trifolium repens* L.) from the Grasslands Challenge cultivar (AgResearch Grasslands, Palmerston North, New Zealand) and seeds from the Grasslands Huia cultivar were used in this study. The Grasslands 10F genotype was used as a model to study the morphological root responses to P-deficiency and phytohormone treatments. The stock plants of this genotype were grown and propagated in potting mix (ODERINGS, Palmerston North, New Zealand) in a glasshouse at the Plant Growth Unit (PGU) at Massey University, Palmerston North. In the glasshouse, under nature light conditions, the temperature was controlled to keep a minimum of 12^{o}C at night and cooled by ventilation during the day which was activated when the temperature inside the glasshouse reached 25^{o}C. The plants were automatically watered twice a day for 5 min, at 10 am and 5 pm.

Seeds of the cultivar, Grasslands Huia were used as the background for transformation with the *DR5p::GUS* construct (described in more detail in Section 2.4). To examine the morphological root responses of this cultivar to P-deficiency, plants derived from seeds of the cultivar were also investigated. The wild type and transformed genotypes of this cultivar were kept and used as stock plants in the Physical Containment Level 2 – Genetically Modified Organism (PC2 – GMO) glasshouse at Massey University, under conditions of 80% relative humidity and 22±2^{o}C and nature lighting with manual watering each day.

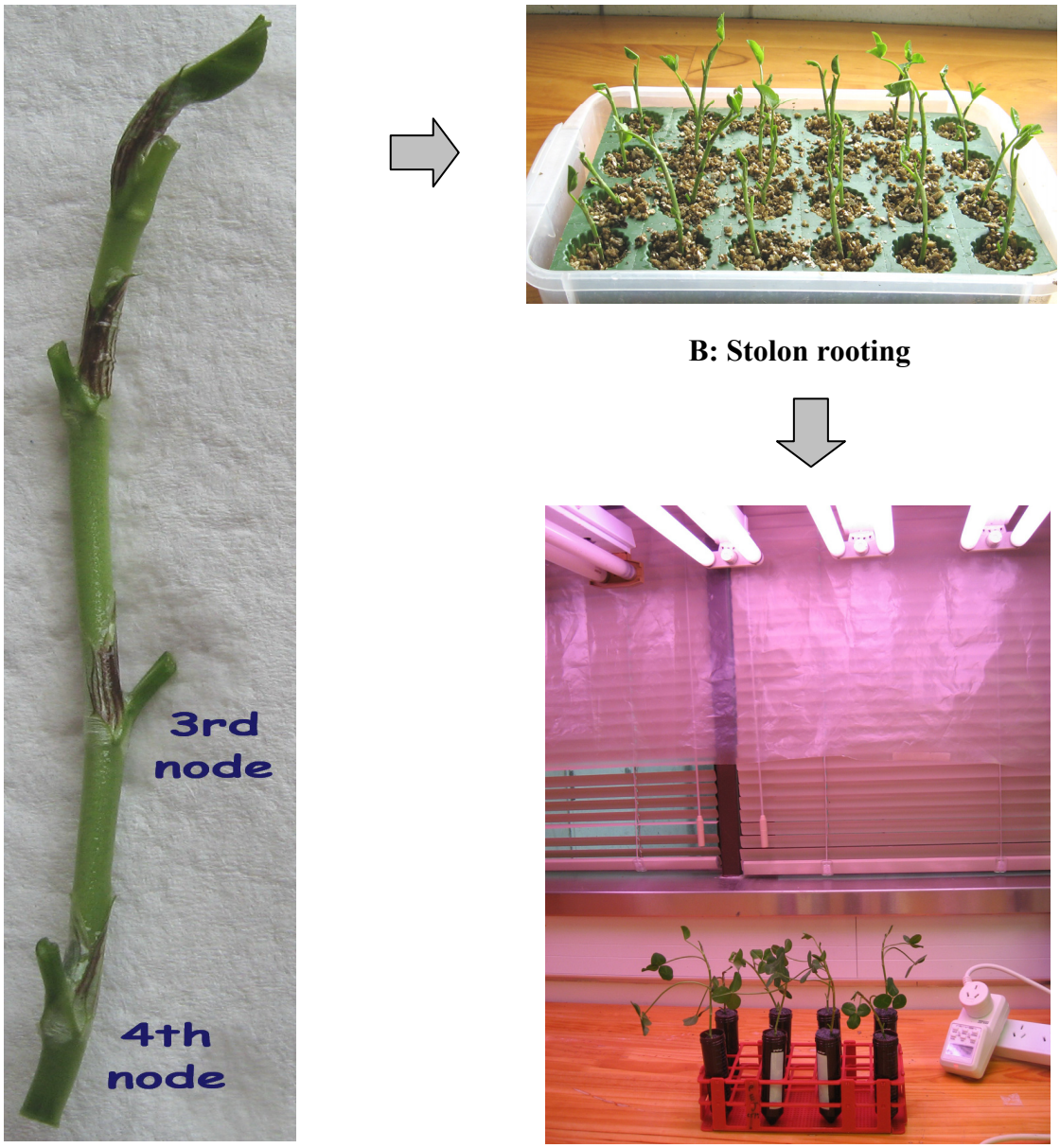
2.2. CHEMICALS

For all the experiments in this thesis, the water used for making solutions or hydroponic media was milli-Q (MQ) water produced by an Ultrapure Water System (Milli-Q, Millipore Crop., Australia) after a reverse-osmosis (RO) process, followed by autoclaving, if necessary. The general chemical agents were obtained from Sigma-Aldrich Chemical Company (St. Louis, Mo., USA), BDH Laboratory Chemicals Division (England), Invitrogen and Merck Ltd., except where specifically stated.

2.3. TREATMENTS

2.3.1.Hydroponic experimental system

In order to conduct experiments, healthy stolons of the wild-type genotype 10F and *cv.* Huia seedlings and transgenic *cv.* Huia lines were excised at the base of the fourth node and then trimmed of all their fully-expanded leaves, as shown in Figure 2.1.A. For the 10F cultivar in the PGU glasshouse, as shown in Figure 2.1.B, both the third and fourth node of these stolons were then buried into vermiculite (2-4 mm, Nuplex Industries, Ltd., Auckland, New Zealand), dampened with a modified Hoagland's solution (Gibeaut et al., 1997) that contained one-third strength macro and full strength micronutrients, as described in Appendices, Table A.1. In the PC2 – GMO glasshouse, both wild type and transgenic lines of the Huia cultivar underwent the same process as for the 10F genotype. After three days rooting in wet vermiculite, healthy stolons were selected with quite uniform roots for transfer to hydroponic media comprising Hoagland's solution and contained in 50 mL black Falcon tubes, to acclimatise for one day (Figure 2.1.C). The concentration of orthophosphate phosphate (Pi) in the Hoagland's solution is 1 mM, so this phosphate concentration is referred to as the control concentration (designated Pi^{+}). From the acclimatisation stage of the experimental periods, the rooted stolons of the 10F genotype were transported from the PGU glasshouse to a light box in the laboratory set at 21±2^{o}C, with 16 h light per day at 65 μmol $m^{-2}s^{-1}$ intensity (Figure 2.1.C). For wild type and transgenic genotypes of the Huia cultivar, the stolons were maintained in the PC2 – GMO glasshouse, using the conditions as described previously.



A: Stolon cutting

B: Stolon rooting

C: Stolons in light box

Figure 2.1. Flow diagram from stolon cutting to the hydroponic experimental system (as shown for the 10F genotype).

White clover stolons were excised at the base of the fourth node, rooted in wet vermiculite for three days and then acclimatised in Hoaglands media for two days in the light box, before being used in experiments.

2.3.2.P-deficient treatments

Following acclimatisation, the stolons were introduced to the P-deficient treatments, in which white clover stolons were also kept in 50 mL Falcon tubes, which contained Hoagland's solution, but the Pi concentration was adjusted to 0.01 mM (Pi^-), as indicated in Table A.1, Appendices. In contrast, in the control (P-sufficient conditions), white clover stolons were kept in normal Hoagland's solution with 1 mM Pi (Pi^+). The solution contained in the tubes was changed (at least) every two days, in order to limit the effects of deficiencies of other nutrient factors.

2.3.3.1-aminocyclopropane-1-carboxylate (ACC) treatments

Using the same system as for the P-deficient treatment, a range of concentrations of 1-aminocyclopropane-1-carboxylate (ACC; 10 μM, 1 μM and 100 nM) were used for the Pi^+ or the Pi^- treatments (as in 2.3.2). To do this, MQ water was used to dissolve ACC to make the 100 mM and 10 mM ACC stock solutions. Then these stock solutions were added into Pi^+ (control) or Pi^- (P-deficiency), in order to obtain final concentrations of 10 μM or 1 μM or 100 nM ACC, depending on the treatments. In these treatments, the Hoagland's solutions were changed every two days, so ACC was re-applied every two days.

Another treatment used was the application of 1-methylcyclopropene (1-MCP): an ethylene action inhibitor. The EthylBloc used had a concentration of 3.8% (w/v) 1-MCP in glucose and so 358 mg of EthylBloc, contained in a 15 mL Falcon tube was dissolved in 10 mL of warm (65^{o}C) water, the chamber sealed quickly and the 1-MCP gas released from the glucose into a 35 L glass chamber to give a final concentration of 300 ppm. The 1-MCP was re-applied after every measuring time point.

Figure 2.2. Experimental system for 1-MCP treatment of hydroponically grown white clover.

The white clover stolons were kept in the totally sealed glass chamber located in a light box set at 21±2^{o}C, with 16 h light per day at 65 μmol m^{-2} s^{-1} intensity that was filled 1-MCP to give a final concentration of 300 ppm. The 1-MCP was re-applied at every measurement time point.

2.3.4.Auxin treatments

For exogenous auxin treatments, various concentrations of 1-naphthylacetic acid (NAA; 100 nM, 50 nM and 5 nM) and indole-3-acetic acid (IAA; 50 nM and 5 nM) were used in the Pi^+ or Pi^- treatments (as mentioned in 2.3.2). To make 10 mM stock solutions, 1.862 mg of NAA (BDH) or 2.079 mg IAA (Sigma Ultra) were dissolved into 100 μL of 1M Sodium hydroxide (NaOH) and then made to 1.0 mL with MQ water and the final concentration in each media was made by the appropriate dilution of each stock solution. The auxins were added to fresh media such that the roots were exposed to the hormone at each media change.

A 100 mM stock solution of the auxin transport inhibitor 1-N-naphthylphthalamic acid (NPA) was prepared by dissolving 2.92 mg of NPA in 200 μL of dimethyl sulfoxide (DMSO) and then made the volume up to 1 mL with MQ water. Dilutions were made, as appropriate, from this 100 mM NPA stock solution, and again the roots were exposed to freshly prepared NPA at each media change.

2.4. ROOT MORPHOLOGY MEASUREMENT

To measure changes in root morphology, the stolons were removed from the Falcon tubes and immersed into a tray, which contained the Pi^+ or Pi^- Hoagland's solution, depending on treatments. Then, the lengths of the primary roots (PRs) and lateral roots (LRs) were measured and the LR numbers were counted as quickly as possible. The stolons were then returned to the treatment media (ethylene, auxin or combined treatments). These measurements were repeated at each time point, during the treatments.

2.4.1.The mean length (cm) of the primary roots

For this thesis, the PRs are defined as the nodal roots, which emerge from the root meristems located at each node of the white clover stolon (Figure 2.3). After cutting, both the third and fourth nodes of white clover stolons were buried into wet vermiculite and hence there were two types of PR — the third-node PR and the fourth-node PR. After measuring the lengths of each type of PR, the mean length of all the third-node and fourth node roots was determined.

2.4.2.The mean of lateral root numbers

Lateral roots are referred to here as the roots which emerge from the PR of white clover stolons (Figure 2.3). The number of LRs per stolon was counted separately for the PRs emerging from both the third and fourth nodes. The mean LR number is calculated from the numbers of the LRs emerging from each PR and is calculated separately for third and fourth-node roots.

2.4.3.The mean of lateral root total lengths (cm)

Lateral root lengths were also measured and summed, to give the total LR length for each PR, and all of the mean LR total length per PR was then calculated. These values were then determined separately for the PRs arising from the third and fourth nodes.

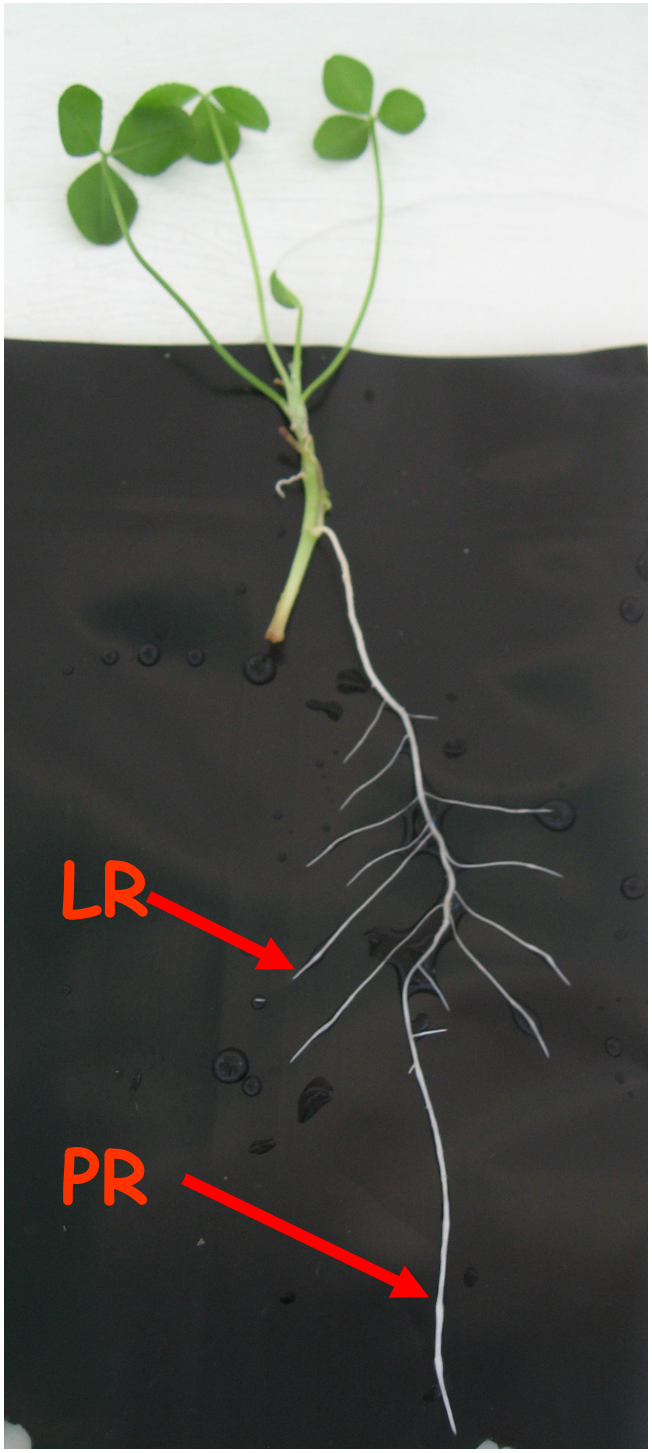


Figure 2.3. Experimental white clover stolon with root system.

PR: The nodal root, which emerged from the node, is defined as the primary root,

LR: Lateral roots were referred as the roots which emerge from the PR.

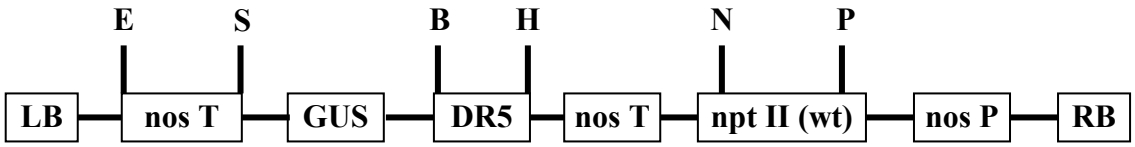
2.5. WHITE CLOVER TRANSFORMATION

Transgenic white clover plants were regenerated from cotyledons, using an *Agrobacterium (A.) tumefaciens*-mediated transformation method (Roldan, 2008). The *A. tumefaciens* strain used was kindly provided by Miss Susanna Leung, Massey University, which is *A. tumefaciens* strain LBA4404 carrying the pRD-410 plasmid (Datla et al., 1992), harbouring the *DR5p::GUS* gene, as described in Figure 2.4. The *DR5p::GUS* construct includes the DR5 promoter which comprises a synthetic auxin response element (Ulmasov et al., 1997), which is fused to the β-glucuronidase (GUS) reporter gene (Huo et al., 2006).

Seeds from the white clover cultivar Grasslands Huia were surface-sterilised with 70% (v/v) ethanol for 1 min and then with 3% (v/v) sodium hypochlorite for 7-8 min (with shaking) and finally rinsed four times with sterile water. Following this, the seeds were covered with sterile water and left to germinate overnight in the dark, at 21^{o}C. The next day, the water covering the germinated seeds was exchanged with 6% (v/v) hydrogen peroxide for 3 min, with gently stirring. The hydrogen peroxide was then withdrawn from the seeds and sterile MQ water used to rinse the seeds five times. After rinsing, the seeds were also immersed in sterile MQ water and then aseptically dissected into two separate cotyledons in a Laminar Flow Cabinet (Clemco, Model CF 43S, Australia), using a dissecting microscope (Zeiss Microscope, Stemi DV4). To do this, the seed coat was removed, the radicle excised and then the two cotyledons were carefully dissected through the plumule. These separated cotyledons were aligned on damp sterile filter paper (3 MM Chr, Whatman® paper) and placed on CR7 media (Appendices, Table A.2) in standard petri dishes. The cotyledons were co-cultivated, by covering with a solution of *A. tumefaciens* harbouring the *DR5p::GUS* gene. A broth of *A. tumefaciens* was prepared by inoculating a single colony of the LBA 4404 strain carrying the pRD-410 plasmid harbouring the *DR5p::GUS* construct into 10 mL of TY^{Kan100} (Appendices, Table A.3) broth, and then incubation for two days at 25^{o}C, with shaking at 180 rpm (round per min). Next, this *A. tumefaciens* broth was centrifuged at 5,000 *x g* (Eppendorf Centrifuge 5702) for 5 min at room temperature and the cell pellet was re-suspended in 0.5 mL of (sterile) 10 mM magnesium sulfate ($MgSO_4$), before using the suspension to cover the dissected cotyledons placed on the CR7 media. After coating the cotyledons with the *A. tumefaciens* cell suspensions, the Petri dishes containing the treated cotyledons were sealed with parafilm® (Pechiney

Plastic, Chicago) and kept in a Plant Growth Cabinet (Model MLR-350H, Sanyo Electric Co., Ltd) at 23^{o}C, 70% relative humidity, with continuous light.

After four days of co-cultivation, these cotyledons were transferred onto selection media comprising CR7 plus 150 μg.mL^{-1} Kanamycin sulfate (Invitrogen) and 300 μg.mL^{-1} Cefotaxime (AFT Pharmaceuticals) (CR7$^{Kan150;\ Cef300}$), with surviving (Kanamycin-resistant) cotyledons transferred onto fresh screening media every fortnight. Any cotyledons which developed shoots were subsequently shifted onto CR5 media (Appendices, Table A.2), containing 100 μg.mL^{-1} Kan and 150 μg.mL^{-1} Cef (CR5$^{Kan100;\ Cef150}$). The developing shoots were moved to fresh CR5 media every fortnight and the antibiotic concentrations decreased to 50 μg.mL^{-1} Kan and 75 μg.mL^{-1} Cef. Following this, any well-grown and healthy shoots were rooted in CR0 media comprising MS media (Appendices, Table A.2), plus 25 μg.mL^{-1} Kan and 50 μg.mL^{-1} Cef (CR0$^{Kan25;\ Cef50}$). When the plantlets were well rooted, they were transferred into autoclaved potting mix (ODERINGS) and maintained in a PC2 – GMO glasshouse, to become stock plants for stolon cuttings.



T-DNA of pRD-410 plasmid

Figure 2.4. Diagrammatic representation of the T-DNA of the pRD-410 vector habouring the *DR5p::GUS* transgene used for *Agrobacterium*-mediated transformation.

LB = left border of T-DNA sequence

nos T = nopaline synthase terminator sequence

GUS = uid A coding sequence

DR5 = auxin induced promoter comprising the highly active synthetic auxin response element

npt II (wt) = wild type neomycin phosphotransferase II sequence (Kanamycin resistant)

nos P = nopaline synthase promoter sequence

RB = right border of T-DNA sequence

E = *Eco* RI

B = *Bam* HI

H = *Hind* III

N = *Nco* I

P = *Pst* I

S= *Sst* I or *Sac* I

2.6. POLYMERASE CHAIN REACTION (PCR) METHOD

2.6.1.DNA extraction (following the protocol from Keb-Llanes et al. (2002))

Transgenic white clover leaves were collected (0.3 g), frozen and ground in liquid nitrogen, to a fine powder. The leaf powder was well mixed with 1.3 mL of extraction buffer (Appendices, Table A.4) in Eppendorf tubes and these tubes were incubated at 65^{o}C for 10 min. Next, 410 µL of ice-cold 5 M potassium acetate was added to the tubes and after mixing, the samples were centrifuged (Eppendorf Centrifuge 5417R) at 15,300 *x g*, 4^{o}C for 15 min. After centrifugation, 1 mL of each supernatant was transferred into a new Eppendorf tube and this was mixed with 400 µL of chloroform: isoamyl alcohol (24 : 1, v/v) with vigorous shaking and the mixture centrifuged at 15,300 *x g*, 4^{o}C for 1 min. The upper layer was removed and mixed with 1 mL of cold isopropanol to precipitate genomic DNA with incubation on ice for 20 min. Following centrifugation at 9,600 *x g*, 4^{o}C for 20 min, the supernatants were discarded and the DNA pellet washed with 500 µL of 70% (v/v) ethanol, before air-drying. The dried DNA pellets were re-suspended in 50 µL of TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA) and used for PCR screening.

2.6.2.PCR

PCR reactions comprised 10 µL of 2x PCR Master mix (Promega, USA), 1 µL of 10 µM DR5rev-pro-F1 primer and 1 µL of 10 µM GUS-seq-R1 primer (Sigma Genosys, as shown below), 2 µL of the extracted genomic DNA and 6 µL of sterile filtered water supplied by the manufacturers. These reactions were carried out following the programme (as shown below) in a Palm-Cycler Thermocycler version 2.2 (R.Corbett Life Sciences, Australia).

- DR5rev-pro-F1: 5'GCAGCACCCGGGGAATTCGTCGACGGTATCG
- GUS-seq-R1: 5'-CGAAACGCAGCACGATAC

The PCR programme comprised one cycle of 94^{o}C for 2 min; 35 cycles of 94^{o}C for 30 sec, then 55^{o}C for 45 sec and 72^{o}C for 40 sec, one cycle of 72^{o}C for 5 min and finally 10^{o}C for 3 min.

2.6.3.Agarose gel electrophoresis

PCR products (μL) were mixed with 3 μL of 10x SUDS loading buffer [0.1 M EDTA, 50% (v/v) glycerol, 1% (w/v) sodium dodecyl sulfate (SDS), 0.025% (w/v) bromophenol blue and 0.025% (v/v) xylene cyanol] and the mixtures and 3 μL of HyperLadder I (BioLine) were loaded separately onto a 7 cm x 10 cm 1% (w/v) agarose (Roche) gels. The agarose gels were horizontally electrophoresed in a gel bed (Bio-Rad Laboratory, Hercules, CA, USA) containing TAE buffer (40 mM Tris, 20 mM glacial acetic acid, 1 mM EDTA, pH 8.0) at 100 V for 1 hour. To visualise the DNA, the gels were stained with ethidium bromide (0.1 $\mu g.mL^{-1}$) for 25 min and after 10 min of destaining, with MQ water, the separated DNA was visualised under UV light (340 nm wavelength in UV transilluminator – UVP inc., San Gabriel, CA, USA) and this was photographed with the Alpha imagerTM 2000 Documentation and Analysis System (Alpha Innotech Corporation, San Leandro, CA, USA).

2.7. GUS STAINING HISTOCHEMICAL ANALYSIS

2.7.1.Staining method adopted from Chen and McManus (2006)

In order to detect the location of GUS activity in the *DR5p::GUS* transformants, excised white clover tissues were immersed immediately into 90% (v/v) cold acetone for 10 min. The tissues were then rinsed twice with 50 mM sodium phosphate buffer, pH 7.2, followed by vacuum infiltration (50 kPa, Air Cadet®, Cole-Parmer Instrument Co., USA) for 15 min with the staining solution [1 mM 5-bromo-4-chloro-3-indolyl-$_D$-glucuronide (Duchefa, Netherlands) in 50 mM sodium phosphate buffer, pH 7.0, containing 0.2% (v/v) Triton X-100, 0.2% (v/v) β-mercaptoethanol]. Finally, the tissues were incubated at 37^oC overnight, in the dark. The following day, tissues were destained, using an increasing concentration of ethanol (15%, 30%, 50%, 70%, 85%, 95% and 100% (v/v)) for an hour at each step. These tissues were then kept in absolute ethanol, before being analysed using a Zeiss Axiophot Compound Light Microscope.

2.7.2.Modified method adopted from Jefferson et al. (1987)

In this method, the fresh tissues were prefixed with 90% (v/v) cold acetone for 10 min, and then rinsed twice with MQ water. The staining solution for this method comprised 1 mM X-GLUC in 50 mM sodium phosphate buffer, pH 7.0, 0.2% (v/v)

Triton X-100, 2 mM ferrocyanide and 2 mM ferricyanide. Thus, after rinsing, the tissues were vacuum infiltrated (50 kPa) with this staining solution for 15 min and then incubated at 37^{o}C, in the dark (the incubation time depended on the experiments). After staining, the tissues were destained with a series of ethanol but at different concentrations (20%, 35%, 50%, 70%, 80%, 90%, 95% and 100% (v/v) for an hour at each step. Finally, the tissues were stored in absolute ethanol, before being analysed under Zeiss Axiophot Compound Light Microscope.

2.8. SOUTHERN BLOT ANALYSIS

2.8.1.Genomic DNA extraction

For Southern analysis, DNA were extracted from 3 g of leaf samples using the method outlined in Section 2.6.1 with the exception that the DNA pellets were re-suspended in 600 μL of TE, instead of 50 μL TE. To this suspension, an extra purification step was conducted, by the addition of 60 μL of (ice-cold) 3 M sodium acetate (pH 5.2) and 360 μL of (ice-cold) absolute isopropanol and then incubation on ice for 20 min. Next, the mixtures were centrifuged at 9,600 *x g*, 4^{o}C for 15 min, in order to collect the DNA and the pellets were then washed with 700 μL of 70% (v/v) ethanol, before air-drying and then resuspension in 50 μL of TE.

2.8.2.Restriction digestion of genomic DNA

After quantification using the NanoDrop ND-1000 spectrophotometer V3.6, 30 μg of genomic DNA for each transgenic line and wild type Huia genotype was digested with *Sac* I (Roche) restriction endonuclease enzymes. Each digest reaction was set up by mixing 30 μg of genomic DNA, 100 units of *Sac* I, 50 μL of 10x Supplement incubation buffer (SuRE/Cut buffer A, Roche) and sufficient sterile MQ water to give a final volume of 500 μL. These digests were then incubated at 37^{o}C for 48 hours, followed by heat inactivation at 65^{o}C for 15 min. After confirming that the genomic DNA samples were totally digested by agarose gel electrophoresis (Section 2.6.3), the reaction products were dried in the DNA Speed Vacuum (Model 110-230, Savant Instruments, USA), in order to concentrate to a volume of *ca.* 30 μL.

2.8.3.Agarose gel electrophoresis

The concentrated restriction digests from Section 2.8.2 containing *ca.* 30 μg of digested genomic DNA were mixed with 5 μL of 10x SUDS loading buffer, before being loaded onto a 0.8% (w/v) agarose gel (15 cm x 15 cm). In order to identify the size of DNA fragments, 5 μL of a HyperLadder I was also loaded and the gel was then horizontally electrophoresed in a gel bed (Bio-Rad Laboratory) containing TAE buffer (as in 2.6.3), at 22 V for 20 h. The gel was then stained with ethidium bromide (0.1 $\mu g.mL^{-1}$) for 25 min and destained in sterile MQ water for 10 min, before the DNA was visualised and photographed, as in Section 2.6.3. The locations of the HyperLadder I bands were recorded, in order to determine the size of DNA fragments after hybridization.

2.8.4.DNA blotting on the Hybond^{TM} – N^{+} membrane

The method, as essentially outlined in the DIG Application Manual for Filter Hybridization (Roche), was essentially followed. After the digested genomic DNA was separated by electrophoresis, stained and destained (Section 2.8.3), the gel was submerged in 250 mM hydrochloric acid (HCl), with shaking for 15 min at room temperature, to depurinate the DNA and then rinsed twice with sterile MQ water. This gel was then submerged in sterile Denaturation solution (0.5 M NaOH, 1.5 M NaCl) incubated at room temperature for 15 min with two changes of solution, and after rinsing with sterile MQ water, the gel was submerged in sterile Neutralization solution (0.5 M Tris-HCl, pH 7.5, 1.5 M NaCl) and incubated at room temperature for 15 min with two changes of solution. The gel was then equilibrated in 20x SSC (saline-sodium citrate buffer containing 3 M sodium chloride, 300 mM sodium citrate, adjusted to pH 7.0 with HCl) for 30 min after which it was subjected to downward capillary transfer using the method of Chomczynski (1992).

To set up the blotting system, three layers of 3MM Whatman® paper (the same size as the gel) were placed on the top of a 10 cm high stack of paper towels, which were cut larger than the gel size. The next layer was the Hybond^{TM} – N^{+} membrane, which was cut to a maximum 3 mm larger than the gel, on each side. The gel (soaking in 20x SSC) was carefully laid over the Hybond membrane, to exclude air bubbles between the gel and membrane and another three layers of 3MM paper (pre-soaked in 20x SSC) placed on the top of the gel. Four strips of 3MM papers (15 cm in width), pre-

soaked in 20x SSC, were then used to form the bridge between the gel stack and the two trays (one tray was placed on each side of the assembly and linked with 3MM paper wick) containing 20x SSC (Figure 2.5). Finally, the whole system was covered with cling wrap, and transfer conducted over 48 h. After that, the Hybond^{TM} – N^{+} membrane was then removed from the gel stack and placed, DNA side-up, onto fresh 3MM paper (which was larger than the membrane and had been pre-soaked in 2x SSC). The membrane was then cross linked at 120 mjoules for 1.2 min using a UV Stratalinker® 2400 (Stratagene, USA) and then briefly rinsed in sterile water. Finally, the membrane was air dried before being stored between two sheets of 3MM Whatman paper in a sealed bag at 4^{o}C, until it was used for hybridisation.

2.8.5.DNA purification from agarose gel

The pRD-410 plasmid, harbouring the *DR5p::GUS* gene, as described in Figure 2.4, was used as a template for a PCR reaction, with DR5rev-pro-F1 and GUS-seq-R1 primers, as in Section 2.6.2. After electrophoresis (Section 2.6.3) of the PCR amplification products, a 556 bp fragment corresponding to the *DR5p::GUS* construct (as described in Appendices Figure A.1) was excised. This agar segment was then put into an Eppendorf tube and 3 volumes of Buffer QG (QIAquick Gel Purification Kit, QIAGEN Pty Ltd, CA., USA) added. The mixture was then incubated at 50^{o}C, with occasional vortexing, until the gel completely dissolved (*ca*. 10 min). This mixture was then transferred to the QIAquick column seated in a 2 mL collection tube and this was centrifuged at 17,000 *x g*, RT for 1 min and the solution in the collection tube discarded. To remove any trace of agarose, 0.5 mL of Buffer QG was added, the centrifugation repeated and the flow-through discarded. Next, 0.75 mL of Buffer PE (QIAGEN) was used to wash the column for *ca*. 5 min, before another 1 min of centrifugation. The washing buffer was discarded from the collection tube and any residual ethanol was removed by an additional centrifugation (also 17,000 *x g*, RT, 1 min). The DNA was then eluted from the membrane of the column, by dropping 50 µL of Buffer EB (QIAGEN) into the centre of the membrane and this was collected in the 1.5 mL Eppendorf tube by a final centrifugation (17,000 *x g*, RT, 1 min).

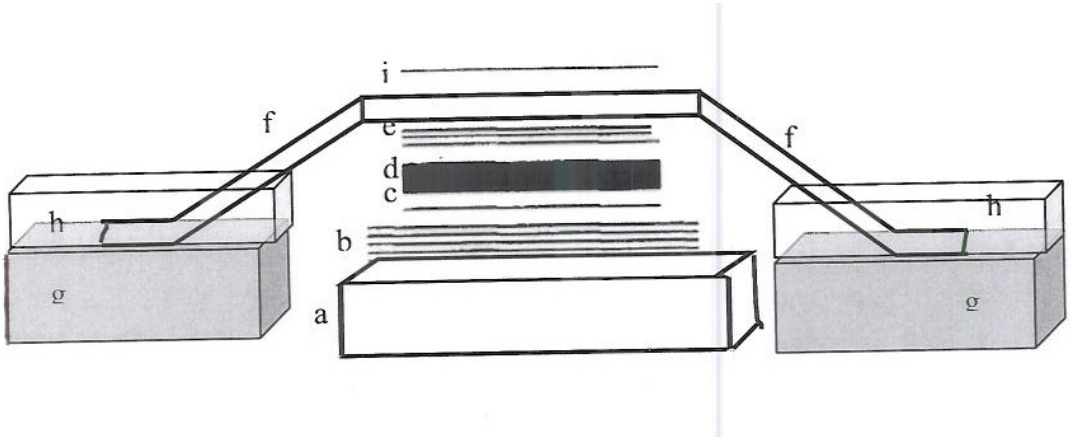


Figure 2.5. Schematics of the blotting assembly used for the downward capillary transfer DNA from gel to Hybond^{TM} – N^{+} membrane

(adopted from Roldan, 2008).

a. Paper towels in a plastic box (ca. 10 cm stack)
b. 3 MM blotting paper (5 layers)
c. Hybond^{TM} – N^{+} membrane
d. DNA agarose gel
e. Moist 3 MM blotting paper (3 layers, damp with 20x SSC)
f. Wick – 3 MM blotting paper – two strips on both sides
g. Support
h. Trays contain 20x SSC
i. Plastic cover

2.8.6.DIG-labelling

Three μg of the purified *DR5p::GUS* fragment (Section 2.8.5), as quantified using a NanoDrop ND-1000 spectrophotometer V3.6, in 16 μL of sterile MQ water was randomly labelled with Digoxigenin(DIG)-11-dUTP, following the instruction manual (version December 2005) accompanying the 'DIG High Prime DNA Labeling and Detection Starter Kit I' (Roche). To do this, the DNA was first denatured by incubation in a boiling water bath for 10 min and then immediate chilling on ice. Then, 4 μL of DIG-High Prime (Roche) was added to the DNA and this mixture was incubated overnight at 37^{o}C. The following day, the reaction tube was heated at 65^{o}C for 10 min in order to stop the reaction, and the labelled probe DNA quantified by the protocol outlined in the kit instruction manual.

2.8.7.Hybridisation

The membrane (225 cm^2), blotted with digested DNA (section 2.8.4), was rolled with the nucleic acid facing inwards, inside a Hybaid^{TM} glass tube (Amerham) containing 20 mL of DIG Easy Hyb (Roche) and was incubated at 43^{o}C to prehybridise. The hybridisation temperature was calculated using the following formula.

$T_m = 49.82 + 0.41$ (% G +C) – (600/l)

$T_{opt} = T_m – 24^{o}C$

in which T_{opt} is the hybridisation temperature, % G + C is 45.14% and l is 556 bp which is the length of the DR5-GUS partial sequence amplified by the DR5rev-pro-F1 and GUS-seq-R1 primers and used as the probe (Appendices, Figure A.2).

The membrane was pre-hybridised at 43^{o}C for 30 min, while the DIG-labelled DNA probes were boiled for 5 min and then quickly chilled on ice, before adding to 7 mL of hybridisation solution, to give a final probe concentration *ca*. 25 ng.mL^{-1}. After 30 min, the prehybridisation solution was replaced by the probe/hybridisation mixture and the membrane incubated at 43^{o}C, for 40 hours.

2.8.8.Washing and detection

After 40 hours, the hybridisation solution was drained off and replaced by the low stringent washing solution, comprising 2x SSC and 0.1% (w/v) SDS. This washing step was conducted as 2 x 5 min washes, under constant agitation at room temperature. The membrane was then washed twice, with 15 min for each wash, in higher stringency conditions comprising 0.5x SSC and 0.1% (w/v) SDS at 60^{o}C, also

under constant agitation. The membrane was then rinsed with Washing buffer (0.1 M maleic acid, pH 7.5, 0.15 M NaCl and 0.3% (v/v) Tween 20) for 2 min before being incubated with Blocking solution (0.1 M maleic acid, pH 7.5, 0.15 M NaCl) for one hour. The membrane was then transferred to the antibody solution (diluted Anti-Digoxigenin-AP in Blocking solution to give a final concentration of 150 mU/mL) for 30 min, followed by two washes, each for 15 min in the Washing buffer, before equilibration in Detection buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl). All these steps, from washing to equilibration, required agitation at room temperature. After *ca*. 5 min in the Detection buffer, the membrane was incubated in freshly prepared colour substrate solution [10 mL of Detection buffer with 400 μL of NBT/BCIP (nitro-blue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate toluidine salt) stock solution included in the kit] without shaking, and in the dark. After two days, when the colour developed, the membrane was washed with sterile MQ water and photographed with the Alpha imagerTM 2000 Documentation and Analysis System (Alpha Innotech Corporation, San Leandro, CA, USA).

2.9. MICROSCOPY METHODS

After GUS staining, the transgenic tissues (apices or roots) were placed on slides with a glass cover (whole-mounts), and the image captured using a Zeiss Microscope (Stemi DV4) for low magnifications or a Zeiss Axiophot Compound Light Microscope with high magnification.

2.10. STATISTICAL ANALYSIS

The root morphology data (the mean PR length, the mean LR number and the mean of total LR length) were analysed by a Microsoft Excel Program, which gave the mean, the standard error of the sample data and the P values (P) using the Student's *t*-test. The P shows the differences between each pair of sample means at each time point. If $P < 0.05$, the difference is significant otherwise it is not significant. For further analyses, Minitab 15 Software (http://www.minitab.com/) was also used to provide a General Linear Model ANOVA analysis, in which the pair-wise comparison was provided by Tukey's test. The Minitab analysis provided the mean values for the pooled data of all the time points during the treatment, and the P values are adjusted (P_{adj}), in order to compare the differences between these means, where $P_{adj} < 0.05$ indicates a significant difference.

CHAPTER THREE: RESULTS

3.1. CHANGES IN ROOT MORPHOLOGY IN WILD TYPE WHITE CLOVER

3.1.1.Changes in root morphology in response to P-deficiency

White clover stolons of genotype 10F of the Grasslands Challenge cultivar were excised and rooted in moist vermiculite for three days. The rooted stolon explants were then transferred to liquid media to acclimatise for one day before the introduction of phosphate (P-) treatments [P-deficiency (Pi^-; 0.01 mM Pi) versus a control of P-sufficiency (Pi^+; 1.00 mM Pi)]. Three aspects of root morphology, the mean length of the primary root (PR), the mean of lateral root (LR) number (per PR) and the mean of total lateral root length were recorded at day 0 (just before the treatments), one day after treatment (DAT) and then at three day intervals to 19 DAT. However, total LR lengths were only able to be recorded until 16 DAT because of a huge biomass of LRs by 19 DAT.

After excisions of the explants, both the third and fourth nodes were buried in damp vermiculite, and so roots emerged from both. However, these two root origins had different responses to P-deficiency (Table A.6 in Appendices). At the beginning of phosphate treatments (day 0), 63% of stolons had roots that had emerged from the third node while 78% of stolons had roots that had emerged from the fourth node.

Figure 3.1.1. A four-node white clover stolon explant excised and trimmed all of the full expanded leaves.

3.1.1.1. Responses of the third – node roots to P-deficiency

Initially, responses to P-deficiency were examined in roots that had emerged from the third node (Figure 3.1.2). In these, no difference was observed until 13 DAT in terms of the mean length of PR (Figure 3.1.2 A). The mean length of the PR was 0.76 cm in the Pi^+ treatment and 1.2 cm in the Pi^- treatment at day 0 and these then grew to 8.66 cm in the Pi^+ treatment and 10.02 cm in the Pi^- treatment by 13 DAT. The difference was not significant statistically even though the mean PR length was 15% higher in the P-deficient media than roots maintained in the P-sufficient media. One reason for this is that root growth varied considerably causing a high standard error and a high P value from the *t*-test for two independent samples. However, at 16 DAT, the mean length of the PRs of stolons maintained in the P-deficient media was statistically different when compared with the P-sufficient treatment (11.8 cm compared with 10.06 cm; $P < 0.1$). Then, at 19 DAT, there is a significant different between the Pi^- and the Pi^+ treatments in which the mean length of the PRs in the Pi^- treatment was 13.52 cm, which is longer than the Pi^+ treated PRs, 11.09 cm ($P < 0.05$; Figure 3.1.2 A). Furthermore, when the data from all of the time points in the treatments is pooled and analysed by Tukey's test, P-deficiency was confirmed to have the stimulatory effect on the PR elongation with $P_{adj} = 0.000$ (the mean PR length in the Pi^- treatment was *ca.* 7.27 cm while in the Pi^+ treatment, the mean value was *ca.* 6.06 cm; Table A.6 in Appendices).

In these experiments, the roots of the stolons which were introduced to the treatments were at a very young stage (around one cm in length and without any LRs), with the LRs only emerging at four days after the treatments (Figure 3.1.2 B). Particularly at 4 DAT, only 56% of the PRs in the P-sufficient treatment had emerged LRs, but 100% of the PRs in the P-deficient treatment had. It was also observed that LR emergence occurred earlier in plants maintained in the Pi^- media when compared with those maintained in the Pi^+ media. In the Pi^+ treatment, the mean LR number increased from 3 at 4 DAT to approximately 42 LRs per PR at 19 DAT, in comparison with 6 at 4 DAT increasing to 51 LRs per PR at 19 DAT in the Pi^- treatment. However, the average number of LRs (per PR per stolon) was not considerably different at the end of the time course and was significantly different only at 7 DAT ($P < 0.05$). At 7 DAT, there were 20 LRs per PR in the Pi^- treatment, which was significantly higher than 12 LRs per PR in the Pi^+ treatment (Figure 3.1.2 B). Also, the mean LR number

of the whole Pi^- treatment (23.78 LRs) was significantly higher than the whole Pi^+ treatment (17.69 LRs) with $P_{adj} = 0.000$ (Table A.6 in Appendices).

The mean of the total LR length in the P-deficient treatment was significant longer than those in the P-sufficient treatment at 7 DAT only (16.21 cm compared to 6.79 cm; Figure 3.1.2 C). After that, the mean lengths in the Pi^- treatment were still longer than those in the Pi^+ treatment (36.65 cm compared with 18.98 cm, 52.5 cm compared with 33.19 cm and 67.36 cm compared with 48.19 cm at 10, 13 and 16 DAT, respectively), but the standard errors (from a *t*-test analysis for two independent samples) were too high to show the difference between two treatments. In contrast, analysis of the whole treatment data by Tukey's test revealed that P-deficiency did have a stimulatory effect on LR elongation. The mean total LR length in the Pi^- treatment was significantly higher than the length in the Pi^+ treatment [*ca.* 25.08 cm compared with *ca.* 15.43 cm; $P_{adj} = 0.006$ (Table A.6 in Appendices)].

Therefore, analysis of the morphological measurements of the roots that emerged from the third node revealed that a greater elongation of the PRs and LRs and also the greater formation of the LRs were observed in the P-deficiency media.

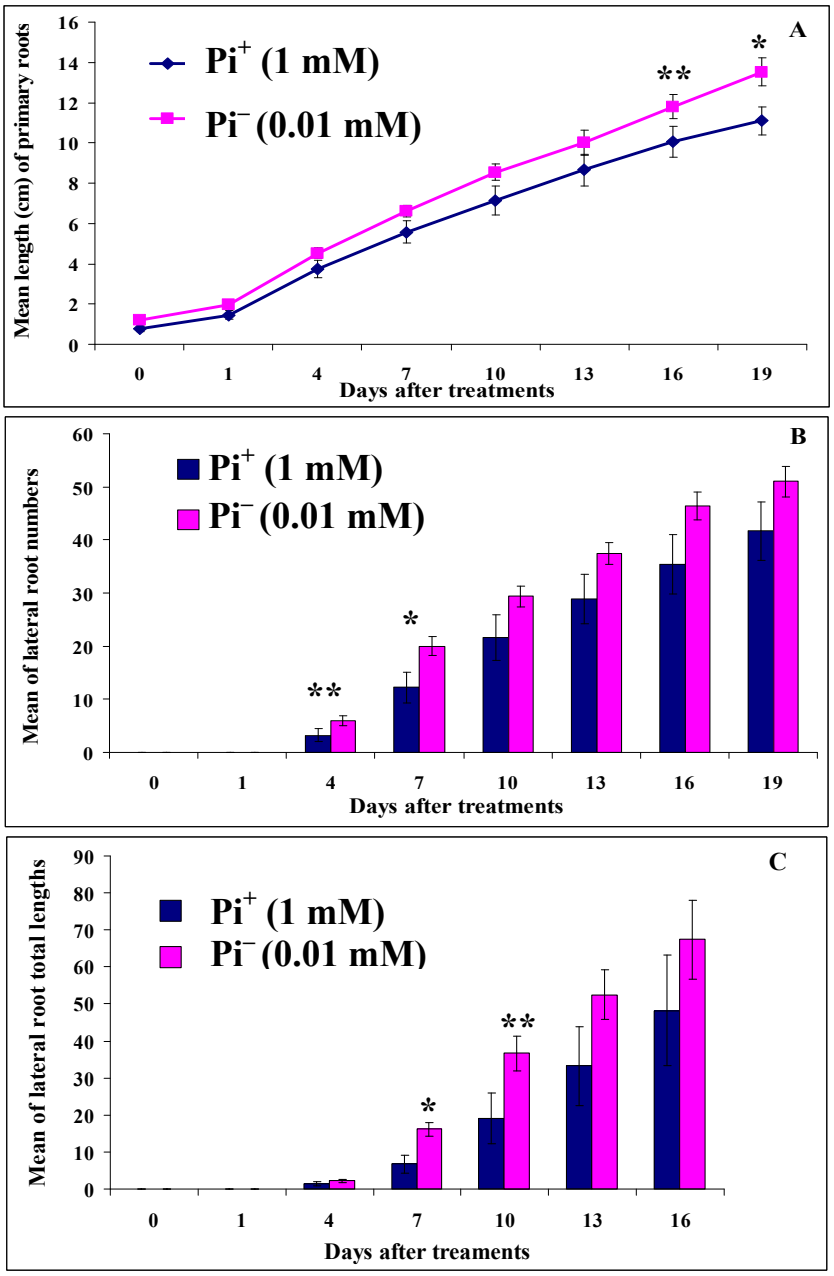


Figure 3.1.2 The effects of P-deficiency on the morphology of roots emerging from the third nodes.

Changes in length of PRs (A), number of LRs (B) and total length of LRs (C) of wild type white clover stolons (genotype 10F) in response to P-deficiency (Pi^-, 0.01 mM Pi) in comparison with P-sufficiency (Pi^+, 1.00 mM Pi).

Values are means of eight individual stolons with standard deviations of the means (represented as error bars).

* or ** indicates the significant difference between the treatments at $P \leq 0.05$ or at $0.05 < P \leq 0.1$, respectively, using *t*-test for two independent samples

This figure is representative of four repeated experiments.

3.1.1.2. Response of roots emerging from the fourth nodes to P-deficiency

In contrast to the roots that emerged from the third node, the roots that emerged from the fourth-node did not respond to the Pi^- treatment in terms of primary root elongation and lateral root development (Figure 3.1.3). In terms of PR length (Figure 3.1.3 A), the fourth-node roots in both the Pi^+ and Pi^- treatments elongated to the same extent as the third-node roots maintained in the Pi^+ media and were shorter than the third-node roots maintained in the Pi^- media. Regardless of the phosphate concentration in the Hoagland's media, the fourth-node roots elongated from *ca*. 1 cm at day 0 to *ca.* 10 cm at 19 DAT.

There was no difference in the number of LRs between the two treatments (Figure 3.1.3 B). For Pi^+ (1.00 mM Pi), 80% of the PRs had LRs at 4 DAT with a mean LR number of 3.22, which after 19 days of treatment, increased to 36.91. Essentially, a similar pattern was observed in the P-deficient treatment where 60% of PRs had LRs at 4 DAT and then the mean LR number increased from 3.5 at 4 DAT to 36.8 at 19 DAT. Similarly, the fourth node roots did not show any response to P-deficiency in terms of total LR length (Figure 3.1.3 C). In both treatments (Pi^+ and Pi^-), the mean of the total LR length increased from around 1.5 cm at 4 DAT to *ca.* 35 cm at 16 DAT.

To summarise, all three characteristics of the fourth-node root morphology (the PR elongation, and the number and the mean total length of the LRs) did not reflect any differences between the Pi^+ and the Pi^- treatments. This was confirmed by analysis of the pooled data of all the time points in the treatment with $P_{adj} > 0.1$ (Table A.6 in Appendices) where the fourth-node roots were found not to respond to P-deficiency in the experimental condition used in this thesis. Therefore, to examine the physiological nature of changes in root architecture in response to P-deficiency, including the role of plant hormones, further studies used roots that had emerged from the third node. Also, the time points used to measure root morphology were shortened to 8 DAT (with six measured time points 0, 1, 2, 4, 6 and 8 DAT) as it was proposed that any change to the roots mediated by plant hormone will occur earlier than changes induced by the nutrient stress.

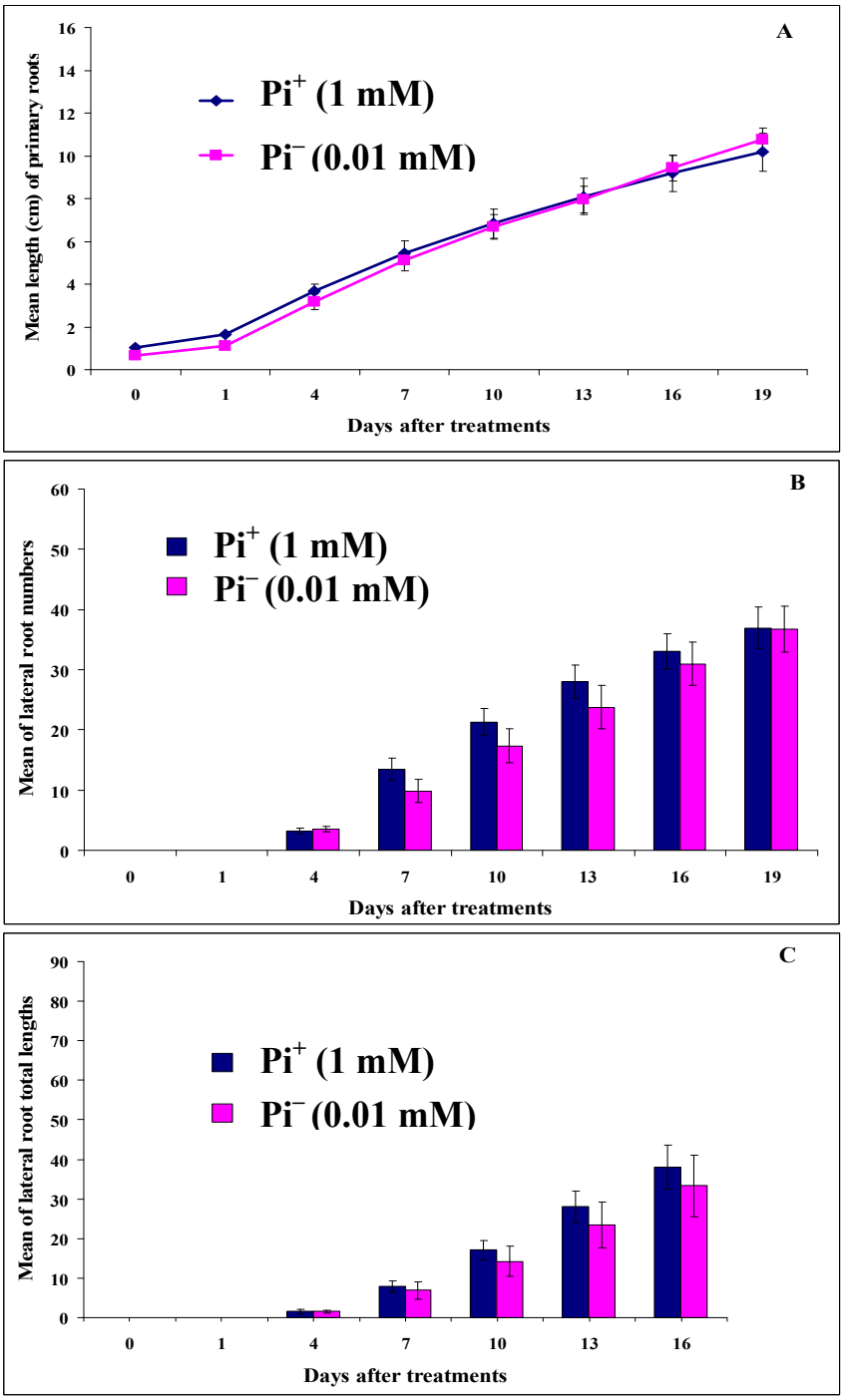


Figure 3.1.3. The effects of P-deficiency on the morphology of roots emerging from the fourth nodes.

Changes in length of PRs (A), number of LRs (B) and total length of LRs (C) of wild type white clover stolons (genotype 10F) in response to P-deficiency (Pi^-, 0.01 mM Pi) in comparison with P-sufficiency (Pi^+, 1.00 mM Pi).

Values are means of ten individual stolons with standard deviations of the means (represented as error bars).

This figure is representative of duplicate experiments.

3.1.2.Effects of plant hormones on root morphology of wild type white clover

3.1.2.1. Effect of ethylene on root morphology

3.1.2.1.1. Initial studies with added 1-aminocyclopropane-1-carboxylate (ACC)

Four-node stolons were excised, rooted and acclimatised for a total of four days in defined media before ethylene treatment. In this experiment, 1-aminocyclopropane-1-carboxylate (ACC) was added to the Hoagland's media to give two concentrations, 1 μM and 10 μM, of ACC study the effect of ethylene on root morphology. Thus two ethylene treatments, Pi^+ + 1 μM ACC and Pi^+ + 10 μM ACC, were compared to a control (Pi^+) treatment.

In these experiments, PR elongation was shown to be extremely inhibited by added ACC. In the control (Pi^+), the mean length of PR was 1.05 cm when the treatments started (day 0) which then elongated to 6.05 cm at 8 DAT. In contrast, growth of the PR in the Pi^+ + 10 μM ACC treatment was severely inhibited such that the mean length was 0.88 cm at day 0 which then increased to only 1.9 cm at 8 DAT. The mean length of the PRs in the Pi^+ + 1 μM ACC treatment elongated *ca.* 4.5 fold (3.75 cm at 8 DAT compared with 0.83 cm at day 0) but the mean was still less than the mean in control treatment. The lengths of the PRs in both the Pi^+ + 10 μM ACC and in the Pi^+ + 1 μM ACC treatments were significantly shorter than the Pi^+ control after just two days and four days after adding ACC, respectively (Figure 3.1.4 A). From these time points, the difference between ACC treatments and the control was amplified until the end of the treatments (8 DAT).

In all three treatments (control and the two ACC treatments), LRs had emerged from the PR at 2 DAT. From this time point, the mean LR number in the Pi^+ treatment increased from 2 to 4.2, then to 11 and to 17.2 at 4, 6 and 8 DAT, respectively (Figure 3.1.4 B). However, when ACC (1 μM) was added, the mean LR number only increased from 1 at 2 DAT then up to 4.2, 7.5 and 10.8 at 4, 6 and 8 DAT, respectively. This inhibitory effect was found to be enhanced as the ACC concentration was increased to 10 μM after which, the mean number of LRs only increased to 8.6 at 8 DAT (Figure 3.1.4 B). Thus at 8 DAT, the two ethylene treatments (1 μM ACC and 10 μM ACC) were significantly different in comparison to the control treatment in terms of LR numbers.

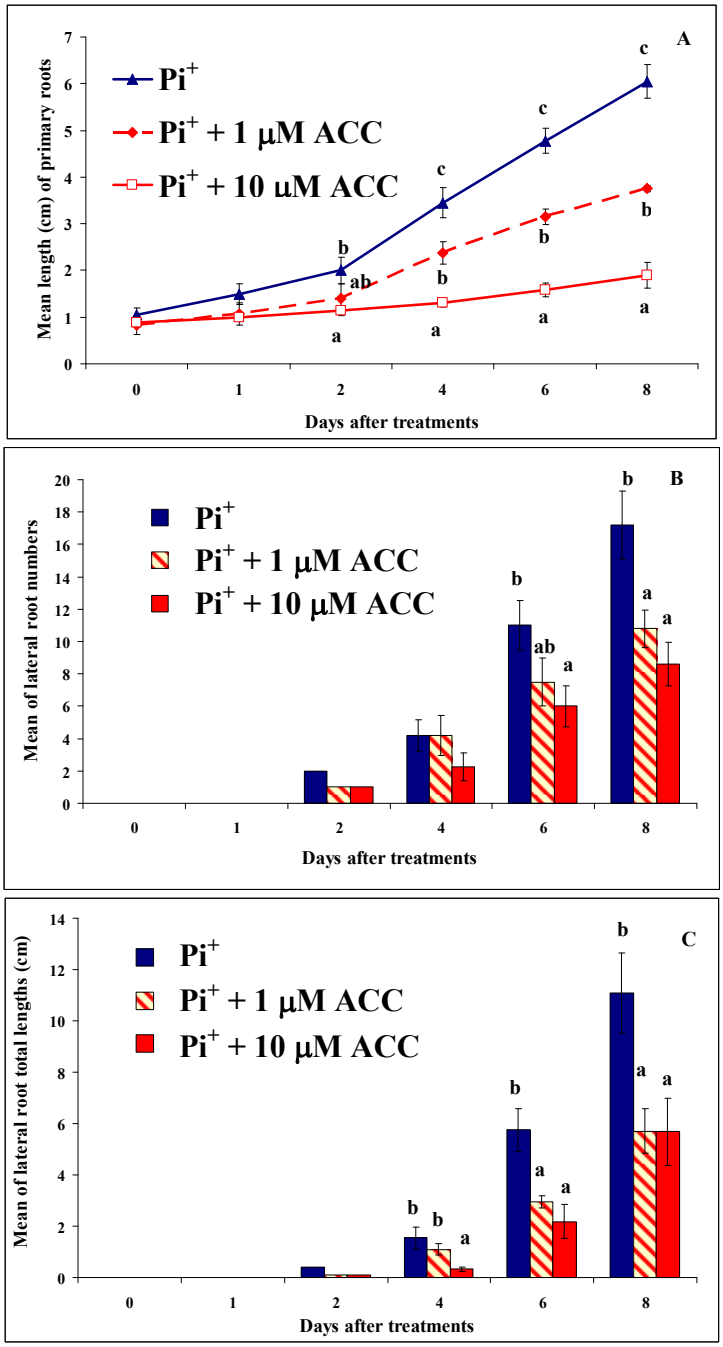


Figure 3.1.4. The effects of added ACC (1 µM and 10 µM) on root morphology. ***Changes in length of PRs (A), number of LRs (B) and total length of LRs (C) of wild type white clover stolons (genotype 10F) in response to ethylene precursor, ACC, (Pi^+ + 1 µM ACC and Pi^+ + 10 µM ACC) in comparison with control (Pi^+) during 6 time points from day 0 to 8 DAT.***

Values are means of five individual stolons with standard deviations of the means (represented as error bars).

Different letters indicate the means in the same time point differ significantly ($P \leq 0.05$), using *t*-test for two independent samples.

As well as decreasing the mean LR number, ACC treatments also resulted in shorter LRs (Figure 3.1.4 C). Both concentrations of added ACC significantly inhibited elongation of LRs at 6 and 8 DAT. For example, at 8 DAT, the mean total LR lengths in both the Pi^+ + 10 μM ACC and the Pi^+ + 1 μM ACC treatments were approximately half of the mean of total LR lengths observed in the control treatment. In addition, many of the ACC-treated roots were observed to grow upward (agravitropically) instead of downward as normal in the hydroponic Hoagland's media.

In summary, both concentrations of ACC used (10 μM and 1 μM) had inhibitory effects not only on PR elongation but also on LR growth. However, it has also been reported that low concentrations of added ACC can stimulate root growth in *Arabidopsis* (Ivanchenko et al., 2008), so a lower concentration of ACC (100 nM) was used to determine if a similar effect on root morphology occurs in white clover.

3.1.2.1.2. Effects of a lower concentration of added ACC on root morphology

The morphology of white clover roots in response to a lower concentration of added ACC (100 nM) in comparison with control (Pi^+) roots was examined (Figure 3.1.5). Even though there was no significant difference between the Pi^+ + 100 nM ACC treatment and the Pi^+ control in terms of the mean PR length, a slight stimulation of PR elongation was observed (Figure 3.1.5 A). Initially, both treatments had a similar mean in terms of PR lengths, with an increase from *ca.* 0.66 cm at day 0 to *ca.* 1.73 cm at 2 DAT for the control roots and from *ca.* 0.76 cm to *ca.* 1.75 cm for the Pi^+ + 100 nM ACC treatment. After that, the mean PR length observed in the Pi^+ + 100 nM ACC treatment was slightly longer than the Pi^+ control at 4 DAT (*ca.* 3.32 cm compared with *ca.* 3.02 cm), then at 6 DAT (*ca.* 4.41 cm compared with *ca.* 4.13 cm) and then at 8 DAT (*ca.* 5.21 cm compared with *ca.* 4.94 cm).

In terms of LR development, the 100 nM ACC treatment caused earlier LR formation in comparison with the control. In this experiment, in the 100 nM ACC treatment, LRs began to emerge from the PRs at 2 DAT, while emergence first occurred from the control PRs at 4 DAT. However, from 4 DAT until the end of the experiment, the mean LR numbers was not significantly different between the 100 nM ACC treatment and the control. A similar pattern was observed in the total LR length, except that the mean in the ACC treated roots was considerably higher than control at 8 DAT ($P < 0.05$).

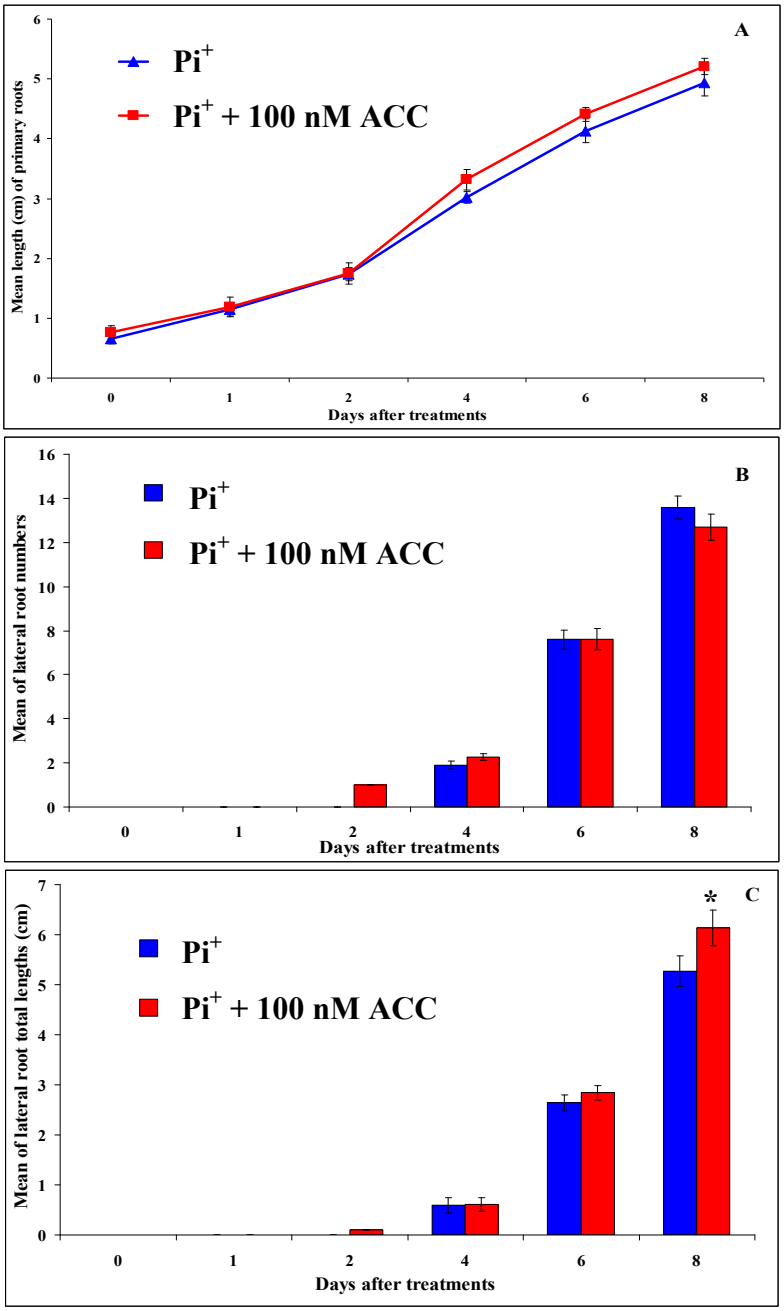


Figure 3.1.5. The effects of added ACC at 100 nM on root morphology.

Changes in length of PRs (A), number of LRs (B) and of total length of LRs (C) of wild type white clover stolons (genotype 10F) in response to ethylene precursor, 100 nM ACC (Pi^+ + 100 nM ACC) in comparison with P-sufficiency as a control (Pi^+) during 6 time points from day 0 to 8 DAT.

Values are means of ten individual stolons with standard deviations of the means (represented as error bars).

* indicates the significant difference between the treatments ($P \leq 0.05$), using *t*-test for two independent samples

This figure is representative of duplicate experiments.

Furthermore, analysis of the whole data set revealed that root growth in the 100 nM ACC treatment was not significantly different from root growth in the Pi^+ control media (Table A.8 in Appendices). Therefore, 100 nM ACC was found to slightly stimulate primary root elongation and to cause earlier LR formation in white clover. As a consequence, 100 nM ACC was chosen to treat white clover stolons in further studies to analyse the effect of ethylene on P-deficiency.

3.1.2.1.3. The effect of added 1-methylcyclopropene (1-MCP) on root morphology

In addition to the study of the effects of added ACC, 1-methylcyclopropene (1-MCP) was used to investigate white clover root growth when the effects of endogenous ethylene are blocked. In terms of mean PR lengths, an increase from *ca.* 0.53 cm at day 0 to *ca.* 4.49 cm at 8 DAT was observed in the Pi^+ control. In the Pi^+ + 300 ppm 1-MCP treatment, the mean PR length increased from *ca.* 0.42 cm at day 0 to *ca.* 4.05 cm at 8 DAT. Particularly at 4 DAT and 6 DAT, the 1-MCP treated PRs had shorter mean lengths than in the control roots ($P < 0.05$; Figure 3.1.6 A). At both these time points, the average length of the 1-MCP treated PRs was around 80% of the control root (1.78 cm and 2.66 cm in comparison with 2.23 cm and 3.36 cm, respectively). Therefore, 300 ppm 1-MCP was found to inhibit the elongation of the PRs.

The inhibition was also found in LR development in terms of the number and the total length (Figure 3.1.6 B and C). As white clover stolons were exposed to 1-MCP, the mean LR number was significantly lower than the control both at 6 DAT and 8 DAT ($P < 0.05$; Figure 3.1.6 B). In contrast, although shorter than the control LR, the mean of the total length of LRs in the 1-MCP treatment was less statistically different, with the difference only at a P value of 0.06 and 0.12 at 6 DAT and 8 DAT, respectively (Figure 3.1.6 C).

In addition, the severe inhibitory effects of 1-MCP on white clover root growth was confirmed by analysis of the data pooled from all of six time points during the treatments (Table A.13 in Appendices). For the whole treatments, the mean PR length in the Pi^+ + 300 ppm 1-MCP (*ca.* 1.81 cm) was significantly lower than the Pi^+ treatment (*ca.* 2.13 cm) with $P_{adj} = 0$. Also, in the Pi^+ + 300 ppm 1-MCP treatment, a mean LR number (*ca*. 0.9 LR) and the mean total length of LR (*ca*. 0.58 cm) was considerably lower than the Pi^+ treatment (*ca* 2.02 LRs with *ca.* 1.04 cm in the mean total length of LRs) with $P_{adj} = 0$ (Table A.13 in Appendices).

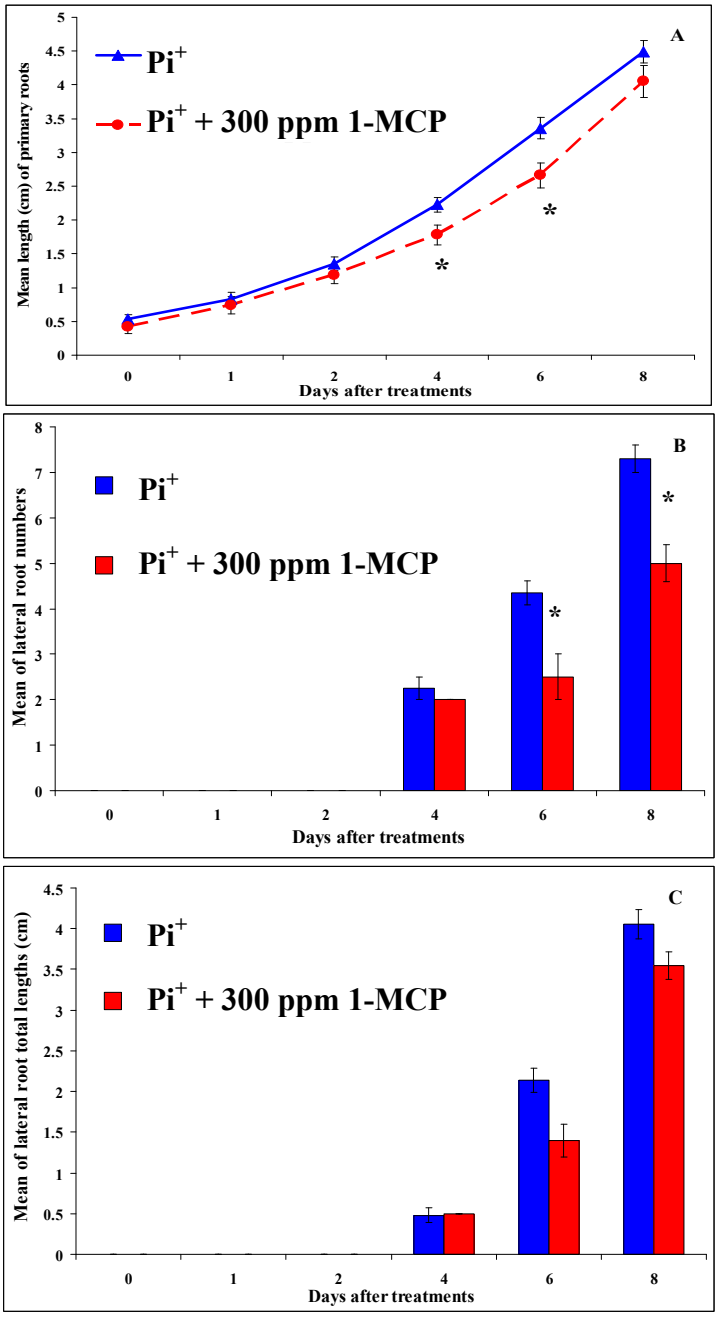


Figure 3.1.6. The effects of added 1-MCP at 300 ppm on root morphology.

Changes in length of PRs (A), number of LRs (B) and of total length of LRs (C) of wild type white clover stolons (genotype 10F) in response to ethylene inhibitor, 1-MCP (Pi^+ + 300 ppm 1-MCP) in comparison with P-sufficiency as a control (Pi^+) during 6 time points from day 0 to 8 DAT.

Values are means of eleven individual stolons for Pi^+ or five stolons for Pi^+ + 300 ppm 1-MCP with standard deviations of the means (represented as error bars).

* indicates the significant difference between the treatments ($P \leq 0.05$), using *t*-test for two independent samples

This figure is representative of duplicate experiments.

3.1.2.2. Effect of auxin on root morphology

3.1.2.2.1. Exogenous auxin treatments

In addition to ethylene, it is generally accepted that auxin is also involved in regulating root system architecture in response to environmental stresses including P-deficiency (Al-Ghazi et al., 2003, Lopez-Bucio et al., 2002, Nacry et al., 2005). Thus 1-naphthylacetic acid (NAA), a synthetic auxin analog, was used to study the changes in white clover roots in response to various exogenous auxin concentrations.

Initially, two concentrations of NAA (50 nM and 100 nM) were used to treat the rooted white clover stolons maintained in hydroponic media. These two concentrations resulted in similar changes in terms of white clover root growth (both for the PRs and LRs) so the results of 100 nM NAA treatment only are presented here.

In the conditions used, the elongation of the PRs was severely inhibited by 100 nM NAA just 4 days after treatment. For the control (Pi^+), after 4 days, the PR elongated more than 4-fold and then after 8 days, more than 8.5-fold in comparison with the measurements at day 0. The mean length of the control PR was 0.53 cm at day 0, then increased to 2.23 cm at 4 DAT and then to 4.49 cm at 8 DAT (Figure 3.1.7 A). In contrast, the PRs in 100 nM NAA treated plants elongated from 0.55 cm at day 0 to only 1.53 cm and then to 3.04 cm at 4 DAT and 8 DAT, respectively. That is, the PR length of plants in the Pi^+ + 100 nM NAA treatment elongated less than 3-fold at 4 DAT and then less than 6-fold at 8 DAT in comparison with day 0. Due to the severe inhibition exerted by the 100 nM NAA treatment, the mean length of the PRs of two treatments (Pi^+ and Pi^+ + 100 nM NAA) were significantly different ($P < 0.001$).

The extreme inhibition of growth was also observed in the patterns of LR development in both numbers and total length of LRs (Figure 3.1.7 B and C). At 6 DAT, the number of LRs in the Pi^+ + 100 nM NAA treatment was approximately half of the LR number observed in the control treatment (Pi^+) and 2 days later, was only two thirds of the LR number in the control. The 100 nM NAA treatment not only reduced the number of LRs, but also delayed the formation and/or emergence of the LRs because these were visible at 4 DAT in the control PRs, but only after 6 DAT in the NAA treated-PRs (Figure 3.1.7 B). The values of the total length of the LRs also reflected the inhibitory effects of the 100 nM NAA treatment. For example, at the end

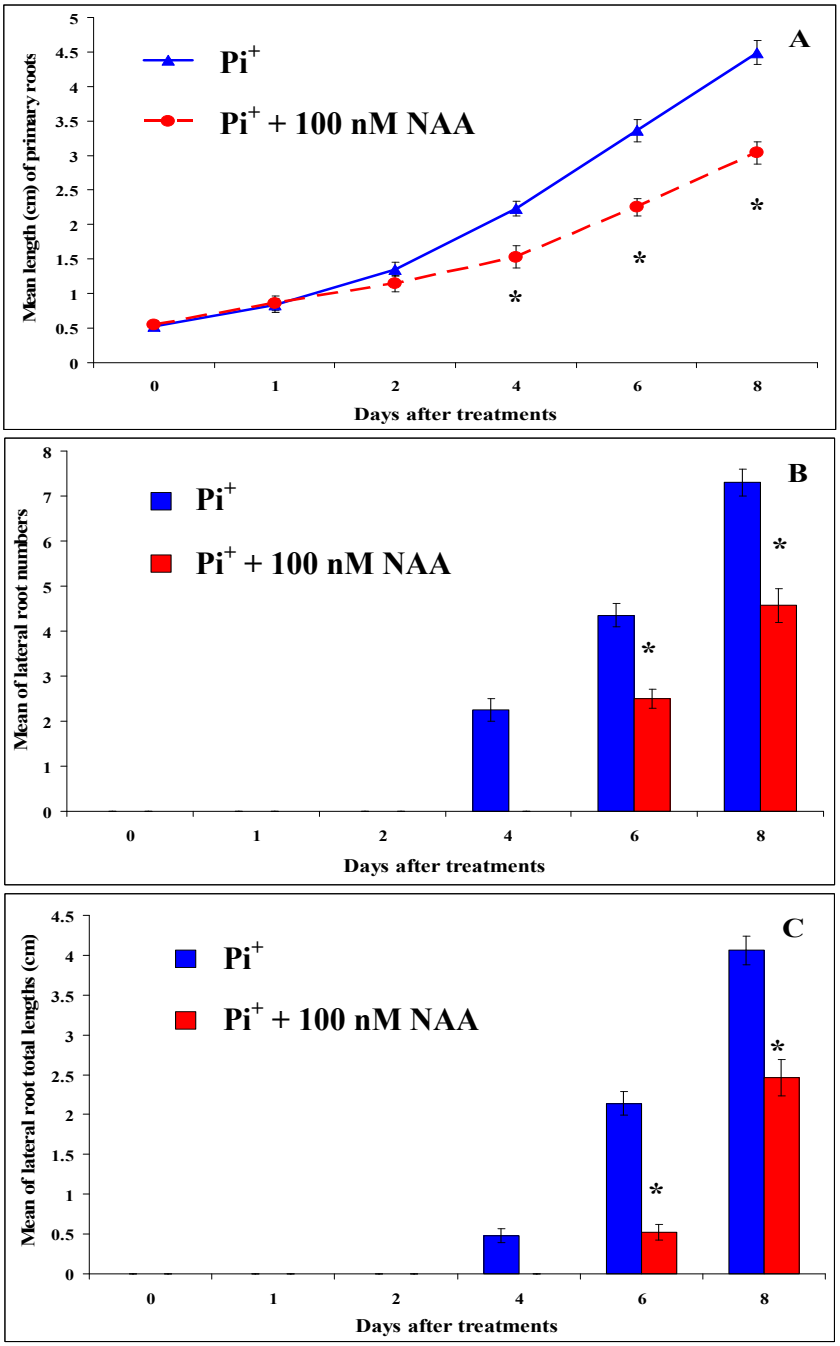


Figure 3.1.7. The effects of added NAA (100 nM) on root morphology.

Changes in length PRs (A), number of LRs (B) and of total length of LRs (C) of wild type white clover stolons (genotype 10F) in response to exogenous auxin, 100 nM NAA (Pi^+ + 100 nM NAA) in comparison with P-sufficiency as a control (Pi^+) during 6 time points from day 0 to 8 DAT.

Values are means of eleven individual stolons for Pi^+ or eight stolons for Pi^+ + 100 nM NAA with standard deviations of the means (represented as error bars).

* indicates the significant difference between the treatments ($P \leq 0.05$), using *t*-test for two independent samples

of the time course (8 DAT), the mean total LR length in the Pi^+ + 100 nM NAA treatment was *ca.* 2.46 cm, compared with *ca.* 4.06 cm in the control (Figure 3.1.7 C).

In other studies, auxin has been observed to have a positive influence on root growth, especially for LR development (eg. Blakely et al., 1988) so these severe inhibitory effects raised the question as that whether the 100 nM NAA concentration is too high to induce white clover root growth. To answer this question, the concentration of NAA was reduced to 5 nM.

In contrast to 100 nM NAA (and 50 nM NAA; data not shown), 5 nM NAA had a positive influence on both PR and LR growth (Figure 3.1.8). Just four days after adding NAA at 5 nM, there was a significant difference in the PR length which was maintained until the last measured time point (8 DAT). To illustrate this, the PR length was 3.88 cm in the control (Pi^+) treatment while it was 4.52 cm in the Pi^+ + 5 nM NAA treatment at 4 DAT. Then, at 8 DAT, the PR length only increased to 5.56 cm in the Pi^+ (control) treatment whereas it increased to 6.51 cm in the Pi^+ + 5 nM NAA treatment. For LR development, in the Pi^+ + 5 nM NAA treatment, LR emergence from the PR was observed at first at 2 DAT and then by 4 DAT, all of the PRs had LRs. For the control, no LRs had emerged at 2 DAT and only 90% of the PRs had LRs at 4 DAT. However, the differences in LR numbers and total LR length was significant only at 6 DAT and 8 DAT with a P value of less than 0.02 from a *t*-test for the numbers of LRs and less than 0.001 for the total lengths of LRs (Figure 3.1.8). At 8 DAT, in the Pi^+ + 5 nM NAA treatment, the LR number was one and a half times the LR number in the Pi^+ control and the total LR length was nearly twice that recorded in the Pi^+ control (10.58 cm compared with 6.22 cm). In addition to the *t*-test analysis, an analysis of the total data set showed that the 5 nM NAA treatment had a stimulatory effect on white clover root growth with significant increases in the PR length, LR number and total LR length (*ca*. 3.67 cm, *ca*. 5.75 and *ca*. 2.86 cm, respectively in the Pi^+ + 5 nM NAA in comparison with the Pi^+, *ca.* 3.20 cm, *ca*. 4.39 and *ca.* 1.82 cm; Table A.17 in Appendices)

The morphological responses of white clover roots to another auxin, indole-3-acetic acid (IAA), was also investigated and it was observed that IAA (added at 50 nM) was also able to stimulate PR and LR growth (Appendices, Figure A.3).

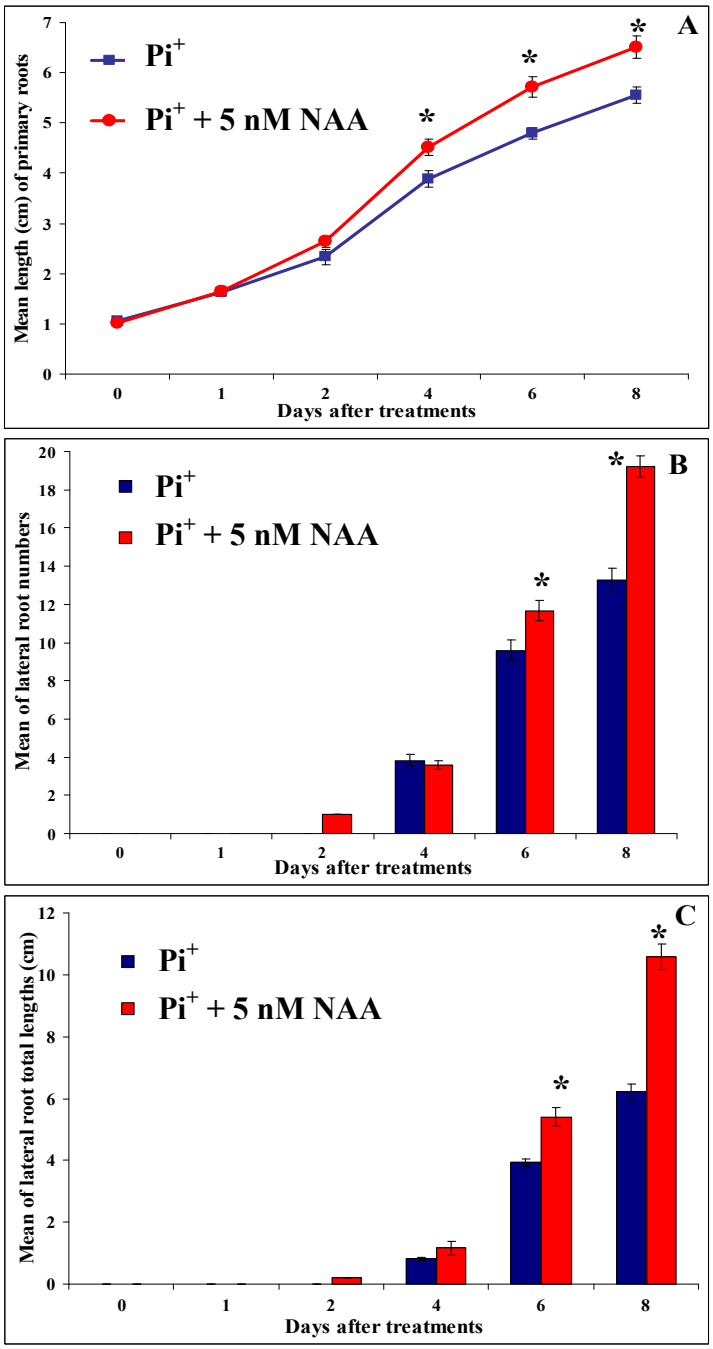


Figure 3.1.8. The effects of NAA added at 5 nM on root morphology.

Changes in length of PRs (A), number of LRs (B) and total length of LRs (C) of wild type white clover stolons (genotype 10F) in response to exogenous auxin, 5 nM NAA (Pi^+ + 5 nM NAA) in comparison with P-sufficiency as a control (Pi^+) during 6 time points from day 0 to 8 DAT.

Values are means of 10 individual stolons for Pi^+ or 17 stolons for Pi^+ + 5 nM NAA with standard deviations of the means (represented as error bars).

* indicates the significant difference between the treatments ($P \leq 0.05$), using *t*-test for two independent samples

To summarise, the effects of exogenous auxin was dependent on the concentration and also on which auxin was used as to whether a negative or positive influence on white clover root growth was observed. A high concentration of NAA (50 or 100 nM) severely inhibited PR elongation and LR formation but a lower concentration (5 nM NAA) had a stimulatory effect on both PR and LR growth. Therefore, 5 nM NAA would be used in further experiments to study the role of NAA in mediating root response to P-deficiency.

3.1.2.2.2. The effects of the inhibition of auxin transport on white clover root morphology

After investigating the role of exogenous auxin, white clover root growth was also examined in response to inhibiting auxin transport using 1-N-naphthylphthalamic acid (NPA). NPA was added to the Hoagland's media to give final concentrations of 10 μM, 1 μM and 100 nM, to treat the white clover stolons. Both the 10 μM and 1 μM treatments totally inhibited root growth – in fact, a degree of necrosis was observed in the root systems, while 100 nM NPA was sufficient to give an inhibitory effect with no obvious damage to the root systems. Thus only the results of the 100 nM NPA treatment are presented (Figure 3.1.9).

The 100 nM NPA treatment inhibited PR elongation, such that the mean length of PR in Pi^+ + 100 nM NPA was approximately one half of the control PRs at 8 DAT (*ca.* 2.6 cm for the NPA treatment compared with *ca*. 4.49 cm for the control PRs). The inhibition was also observed at 4 DAT, where considerable differences in PR lengths were observed (the NPA-treated roots were *ca.* 1.7 cm in comparison with *ca*. 2.23 cm for the control PRs; P from *t*-test less than 0.02). Over the following time course, more severe inhibition on PR growth was observed. Analysis of the whole data for all six time points showed that the mean PR length in the Pi^+ + 100 nM NPA treatment (*ca*. 1.55 cm) was significantly lower than the Pi^+ treatment (*ca*. 2.13 cm) with $P_{adj} = 0$ (Table A.22 in Appendices).

The inhibition of root growth was more pronounced during LR development, including the LR number and the total LR length. It was determined that the control PRs had *ca*. 2, *ca.* 4 and *ca*. 7 emerged LRs at 4, 6 and 8 DAT, respectively, but with 100 nM NPA treatment, only emerged LR numbers of *ca.* 1, *ca.* 1 and *ca.* 1.6 at 4, 6

and 8 DAT, respectively, were observed. Also, the total length of the LRs in the Pi^+ + 100 nM NPA treatment only increased from *ca*. 0.1 cm at 4 DAT to *ca*. 0.86 cm at 8 DAT, which was significantly different from the control LRs with recordings of *ca*. 0.48 cm at 4 DAT to *ca*. 4.06 cm at 8 DAT. The inhibitory effect of 100 nM NPA on the LR development was confirmed by analysis of the whole data set in which plants in the Pi^+ + 100 nM NPA treatment had a mean LR number of *ca*. 0.28 with the mean total LR length of *ca*. 0.13 cm which was significantly lower than the Pi^+ treatment (*ca*. 2.02 and *ca.* 1.04 cm, respectively) with $P_{adj} = 0$ (Table A.22 in Appendices).

Therefore, 100 nM NPA not only inhibited the elongation of the PR but also LR development. It suggests that auxin plays a role in regulating white clover root growth because reduction in auxin concentration using the auxin transport inhibitor (NPA) inhibited root development.

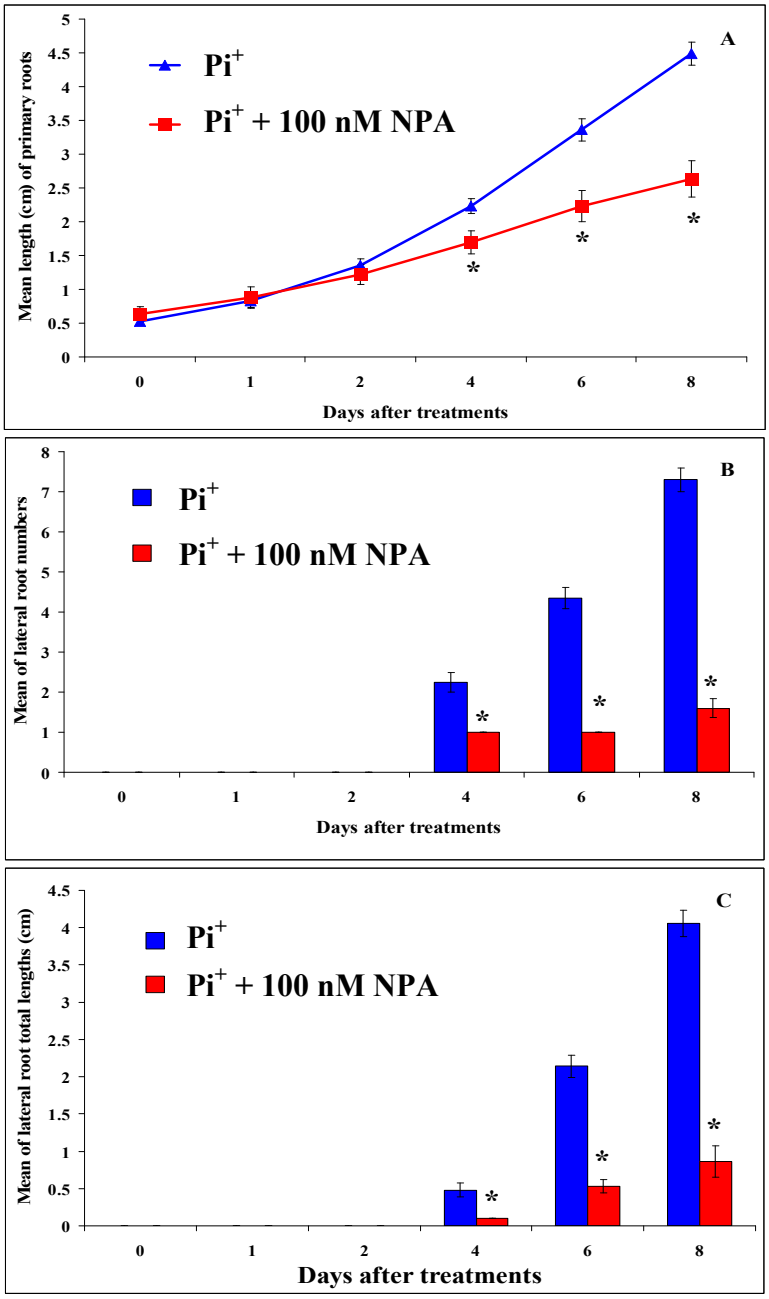


Figure 3.1.9 The effects of NPA on root morphology.

Changes in length of PRs (A), number of LRs (B) and total length of LRs (C) of wild type white clover stolons (genotype 10F) in response to auxin transport inhibitor NPA (Pi^+ + 100 nM NPA) in comparison with P-sufficiency as a control (Pi^+) during 6 time points from day 0 to 8 DAT.

Values are means of 11 individual stolons for Pi^+ or 8 stolons for Pi^+ + 100 nM NPA with standard deviations of the means (represented as error bars).

* indicates the significant difference between the treatments ($P \leq 0.05$), using *t*-test for two independent samples.

This figure is representative of duplicate experiments.

3.1.2.3. Ethylene interacts with auxin

In addition to the study of the influence of ethylene or auxin added separately on white clover root growth, the interaction of ethylene and auxin in mediating root growth was also investigated using a combined application of ACC to elevate ethylene levels and NPA to inhibit auxin transport or, vice versa, a combination of 1-MCP to inhibit ethylene action and added NAA to elevate auxin levels.

3.1.2.3.1. Interaction between ACC and NPA in mediating white clover root growth

It was previously found that 100 nM ACC had a positive effect whereas 100 nM NPA extremely inhibited white clover root growth (3.1.2.1.2 and 3.1.2.2.2). Therefore, 100 nM ACC was added to the NPA treatment (100 nM) in order to determine whether ACC (ethylene) could overcome the inhibitory effects of NPA.

The changes in root morphology (PR length, LR number and total length of LRs) in response to NPA only, to NPA combined with ACC and in comparison with a control (Pi^+) are shown in Figure 3.1.10. For PR growth, the mean length in the NPA combined with ACC treatment was almost the same as in the NPA treatment only, but the lengths in both treatments were shorter than the control (Figure 3.1.10 A). Thus it seems that 100 nM ACC could not overcome the inhibitory effect of NPA on PR elongation.

However, a slight recovery in LR formation was shown after 6 days with the application of ACC at 100 nM to the 100 nM NPA treatment, with the mean LR number twice that observed in the only NPA treatment (Figure 3.1.10 B). Also, at 8 DAT, the mean LR number in the Pi^+ + 100 nM ACC + 100 nM NPA treatment was twice the number in the Pi^+ + 100 nM NPA treatment (3.25 compared with 1.6). However, in terms of the mean total LR length, there was no obvious difference between the two treatments at any time point (Figure 3.1.10 C) even though the total LR length in the Pi^+ 100 nM ACC + 100 NPA treatment was slightly higher than the only NPA treatment. In addition, when values of all the time points were pooled and analysed together by Tukey's test, both of these treatments (the Pi^+ + 100 nM NPA and the Pi^+ + 100 nM ACC + 100 nM NPA) had means of PR length, LR number and total LR length that were significantly lower than the control Pi^+ (Table A.27 in Appendices) so that auxin was found to play an important role in regulating white

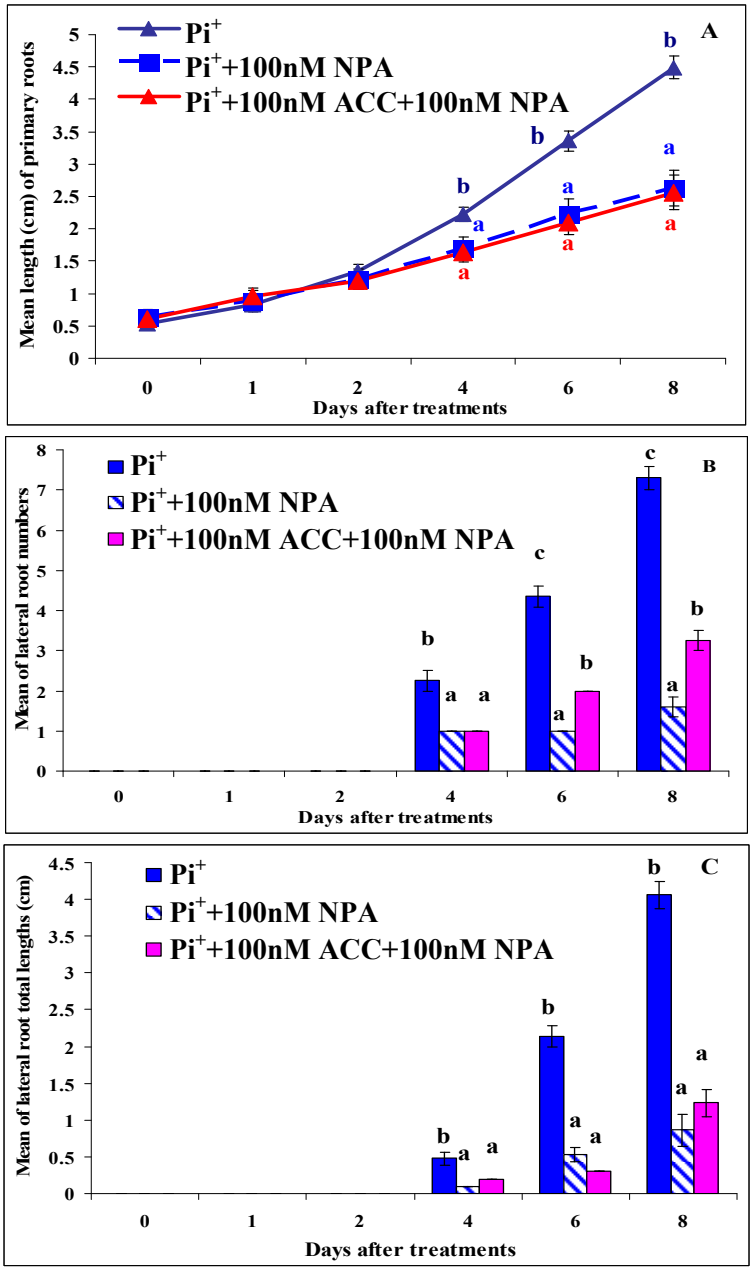


Figure 3.1.10. The interaction between added ACC and NPA in mediating root morphology.

Changes in length of PRs (A), number of LRs (B) and total length of LRs (C) of wild type white clover stolons (genotype 10F) in response to ACC and NPA (Pi^+ + 100 nM ACC + 100 nM NPA) in comparison with P-sufficiency (Pi^+) and only NPA treatment (Pi^+ + 100 nM NPA) during 6 time points from day 0 to 8 DAT.

Values are means of 11 individual stolons for Pi^+ or 8 stolons for Pi^+ + 100 nM NPA or 7 stolons for Pi^+ + 100 nM ACC + 100 nM NPA with standard deviations of the means (represented as error bars). Different letters indicate the significant differences between the treatments in the same time point ($P \leq 0.05$), using *t*-test for two independent samples. This figure is representative of duplicate experiments.

clover root growth and development. It also shows that 100 nM ACC could only partially overcome the inhibitory effects of 100 nM NPA on white clover root growth, particularly in terms of LR formation.

3.1.2.3.2. Interaction between 1-MCP and NAA in mediating white clover root growth

In previous sections (described in 3.1.2.1.3 and 3.1.2.2.1), it was observed that 300 ppm 1-MCP inhibited root development whereas 5 nM NAA stimulated root growth of white clover. Thus NAA was added to the Hoagland's media to a final concentration of 5 nM to treat the white clover stolons which were kept in a totally sealed chamber filled with 300 ppm 1-MCP gas. This treatment was compared with a 300 ppm 1-MCP treatment only (stolons were kept in the chamber without NAA added in the Hoagland's media). These treatments were conducted in P-sufficient conditions so the control for these treatments was Pi^+ (stolons were kept outside the chamber without NAA applied).

In terms of PR elongation, there was no significant difference in PR mean length between the Pi^+ + 300 ppm 1-MCP treatment and the Pi^+ + 300 ppm 1-MCP + 5 nM NAA treatment over all of the time points measured (day 0 to 8 DAT, Figure 3.1.11 A). In both treatments, the mean PR length increased from *ca.* 1 cm to *ca.* 3.7 cm at 4 DAT and then to *ca.* 5.1 cm or *ca*. 4.9 cm at 8 DAT.

In terms of LR numbers, the Pi^+ + 300 ppm 1-MCP treatment and the Pi^+ + 300 ppm 1-MCP + 5 nM NAA treatment did not differ considerably at 6 DAT and 8 DAT but both measurements were lower than the LR number in the Pi^+ control treatment. For example, there was only a mean of *ca.* two LRs formed from PRs if the stolons were introduced to 1-MCP regardless of whether the treatment was with or without NAA whereas in Pi^+, there was *ca.* 9 LRs at 6 DAT and 13 LRs at 8 DAT. Moreover, in the control PRs, a mean of four LRs was observed at 4 DAT, whereas the emergence of LRs was only observed at 6 DAT in the Pi^+ + 300 ppm 1-MCP or Pi^+ + 300 ppm 1-MCP + 5 nM NAA treatments.

In contrast, the mean total LR length observed in the Pi^+ + 300 ppm 1-MCP + 5 nM NAA treatment was significantly lower than the Pi^+ + 300 ppm 1-MCP treatment and lower than the Pi^+ control. The LRs in the Pi^+ + 300 ppm 1-MCP + 5nM NAA treatment had a mean total length of *ca.* 0.3 cm and *ca.* 0.52 cm at 6 and 8 DAT, respectively, whereas the mean total LR length in the Pi^+ + 300 ppm 1-MCP treatment

were *ca.* 1.05 cm and *ca.* 1.2 cm at 6 and 8 DAT, respectively. For the Pi^+ (control), the LRs had a mean total length of *ca*. 4 cm at 6 DAT and 6.22 cm at 8 DAT.

Analysis of the whole data set showed that the mean PR length in the Pi^+ + 300 ppm 1-MCP + 5 nM NAA treatment was not different from the lengths in the Pi^+ control and the Pi^+ + 300 ppm 1-MCP treatment only, but was significantly lower than the length in the Pi^+ + 5 nM NAA treatment (Section 3.1.2.2.1 and Table A.32 in Appendices). For example, the mean total LR length in the Pi^+ + 300 ppm 1-MCP, the Pi^+ + 300 ppm 1-MCP + 5 nM NAA, the Pi^+ and the Pi^+ + 5 nM NAA were *ca*. 3.01 cm, 3.08 cm, 3.20 cm and 3.67 cm, respectively (Table A.32 in Appendices). Interestingly, the effects of NAA on PR elongation in P-deficient treatments were the total reverse of the P-sufficient treatments. For instance, PR elongation was stimulated in the Pi^+ + 5 nM NAA treatment but was inhibited in the Pi^- + 5 nM NAA treatment (in more details in Section 3.1.3.2.1). In contrast, PR elongation was not promoted in the Pi^+ + 300 ppm 1-MCP + 5 nM NAA treatment but was considerably stimulated in the Pi^- + 300 ppm 1-MCP + 5 nM NAA treatment (Table A.32 in Appendices).

For LR development, the Pi^+ + 300 ppm 1-MCP treatment and the Pi^+ + 300 ppm 1-MCP + 5 nM NAA treatment had a mean LR number and also a mean total LR length that was significantly lower than the Pi^+ control. Therefore, not only for PR elongation but also for LR development, the stimulatory effects of 5 nM NAA could not overcome the inhibitory effects of 300 ppm 1-MCP in P-sufficient conditions. On the other hand, 5 nM NAA could partially overcome the inhibitory effects of 300 ppm 1-MCP on LR development in P-deficiency because in the Pi^- + 300 ppm 1-MCP + 5 nM NAA, the LR number and total LR length were significantly higher than in the Pi^- + 300 ppm 1-MCP but still lower than in the Pi^+ control (Table A.32 in Appendices).

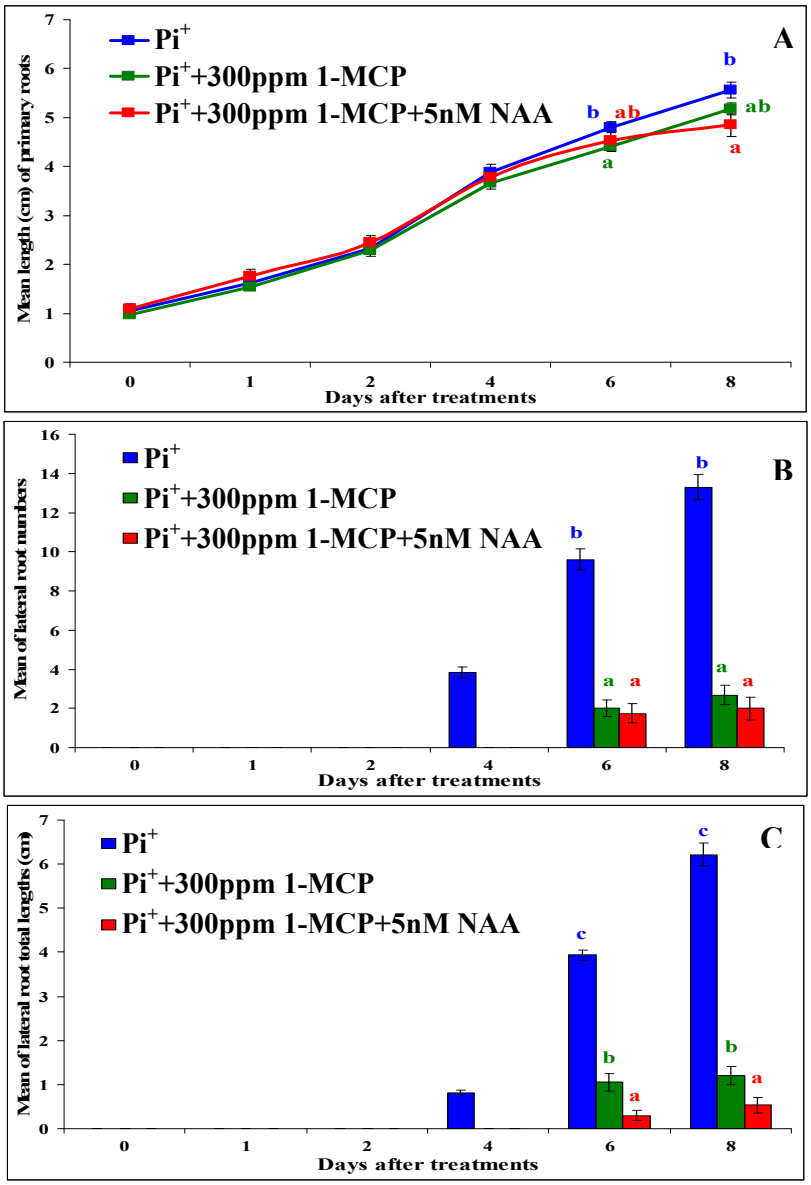


Figure 3.1.11. The interaction between 1-MCP and NAA in mediating root morphology.

Changes in length of PRs (A), number of LRs (B) and total length of LRs (C) of wild type white clover stolons (genotype 10F) in response to the ethylene action inhibitor, 1-MCP and exogenous auxin, NAA (Pi^+ + 5 nM NAA + 300 ppm 1-MCP) in comparison with P-sufficiency as a control (Pi^+) and 1-MCP treatment only (Pi^+ + 300 ppm 1-MCP) during 6 time points from day 0 to 8 DAT.

Values are means of 10 individual stolons or 6 stolons for Pi^+ + 300 ppm 1-MCP or 6 stolons for Pi^+ + 5 nM NAA + 300 ppm 1-MCP with standard deviations of the means (represented as error bars).

Different letters indicate the significant differences between the treatments in the same time point ($P \leq 0.05$), using *t*-test for two independent samples

3.1.3.The effects of plant hormones on root morphology of wild type white clover in response to P-deficiency

The previous results showed that the morphological changes in white clover roots in response to P-deficiency (Section 3.1.1) and the effects of the plant hormones, ethylene and auxin, on P-sufficient roots (Section 3.1.2). In *Arabidopsis*, the cross-talk between the two hormones, ethylene and auxin, is important in regulating root responses to P-deficiency (Lynch and Brown, 1997). Therefore, it is interesting to study the role of ethylene and auxin in root responses to P-deficiency in white clover by examining the combination of P-deficient treatments with these hormones.

3.1.3.1. Ethylene

3.1.3.1.1. The effect of ethylene (as added ACC) on root morphology in roots exposed to P-deficiency

In the previous examination in this thesis (Section 3.1.2.1), it was observed that white clover root growth was seriously inhibited by 1 μM or 10 μM ACC while 100 nM ACC slightly induced both PR and LR growth. Thus to avoid the inhibitory effects of ethylene, 100 nM ACC was used in combination with P-deficiency to study the role of ethylene in response to P-deficiency. The differences in root growth between the Pi^+ (control) and the Pi^+ + 100 nM ACC treatment were described in 3.1.2.1.2 so in this section, the differences between Pi^- (P-deficiency), a Pi^- +100 nM ACC treatment and control Pi^+ were examined.

In these experiments, PR elongation was slightly induced by 100 nM ACC, particularly in the P-deficiency treatment even though the differences were not statistically significant (Figure 3.1.12 A). All four treatments (Pi^+, Pi^-, Pi^+ + 100 nM ACC and Pi^- + 100 nM ACC) are compared together at 8 DAT, where the P-deficiency in combination with ACC treatment had the longest mean PR. The Pi^- and the Pi^+ + 100 nM ACC treatments resulted in the same PR length with roots in both treatments slightly longer than the control (Pi^+) roots. With analysis whole of the data, the mean PR length of the Pi^- + 100 nM ACC (*ca*. 3.04 cm) was not shown to be statistically different from the Pi^- treatment (*ca*. 2.87) but significantly higher than the Pi^+ + 100 nM ACC (*ca*. 2.77 cm) and the Pi^+ treatment (*ca*. 2.61 cm) with $P_{adj} < 0.02$ (Table A.8 and A.9 in Appendices). This suggested that the added ACC may have enhanced the effect of P-deficiency in term of inducing PR elongation.

For LR development, there was a considerable stimulation in the Pi^- + 100 nM ACC treatment which resulted in an earlier formation of LRs when compared with the control treatment (Pi^+). In these, only one day after 100nM ACC was included with the P-deficiency treatment, one out of ten stolons had two LRs emerged from the PRs. At 2 DAT, four out of ten had emerged LRs. In comparison, in the Pi^- or the Pi^+ + 100 nM ACC treatment, LRs were observed from PRs at 2 DAT while the PRs in the control treatment (Pi^+) did not have any emerged LRs until 4 DAT. In addition to earlier LR emergence, the Pi^- + 100 nM ACC treatment produced the highest number of LRs when compared with the other three treatments. The mean LR number in the Pi^- + 100 nM ACC treatment was significantly higher than the other treatments from 4 DAT until the end of the treatments (Figure 3.1.12 B). The stimulatory effect of the P-deficiency and 100 nM ACC treatment on LR formation was proved again when the whole data set was analysed by Tukey's test with the mean LR number of the Pi^- + 100 nM ACC treatment (*ca*. 5.40 LRs) significantly higher than the Pi^- treatment, the Pi^+ treatment and the Pi^+ + 100 nM ACC treatment (*ca*. 4.68, 3.85, 3.70 LRs, respectively) with $P_{adj} = 0$ (Table A.8 and A.10 in Appendices).

The difference between these treatments was amplified in terms of total LR length so that not only were there more LRs, but the Pi^- + 100 nM ACC treatment also promoted LR elongation. During almost all of the time points of these treatments, PRs exposed to the Pi^- + 100 nM ACC treatment had an average total LR length which was longer than the Pi^-, Pi^+ + 100 nM ACC treatments and, in particular, more than twice the control (Pi^+) roots (Figure 3.1.12 C). For example, the mean of the total length of LRs in the Pi^- + 100 nM ACC treatment was *ca*. 1.71, 5.50 and 10.2 cm at 4, 6 and 8 DAT, respectively, while in the Pi^+ treatment, the corresponding measurements were *ca.* 0.59, 2.64 and 5.27 cm. Analysis of the total data set showed that the mean total LR length in the Pi^- + 100 nM ACC treatment (*ca*. 2.94 cm) was considerably higher than in the Pi^- (*ca*. 1.88 cm), in the Pi^+ + 100 nM ACC (*ca*. 1.58 cm) and in the Pi^+ (*ca*. 1.42 cm) treatments (Table A.8 in Appendices).

Moreover, Tukey's test also allows for a separate comparison of the level of the P-treatment only or the ACC treatment only. It was shown that either P-deficiency or 100 nM ACC had a considerably stimulatory effect on the elongation of PRs and LRs and also on LR formation because the mean PR lengths, the mean total lateral root lengths and the mean LR numbers in the Pi^- treatment or in the 100 nM ACC

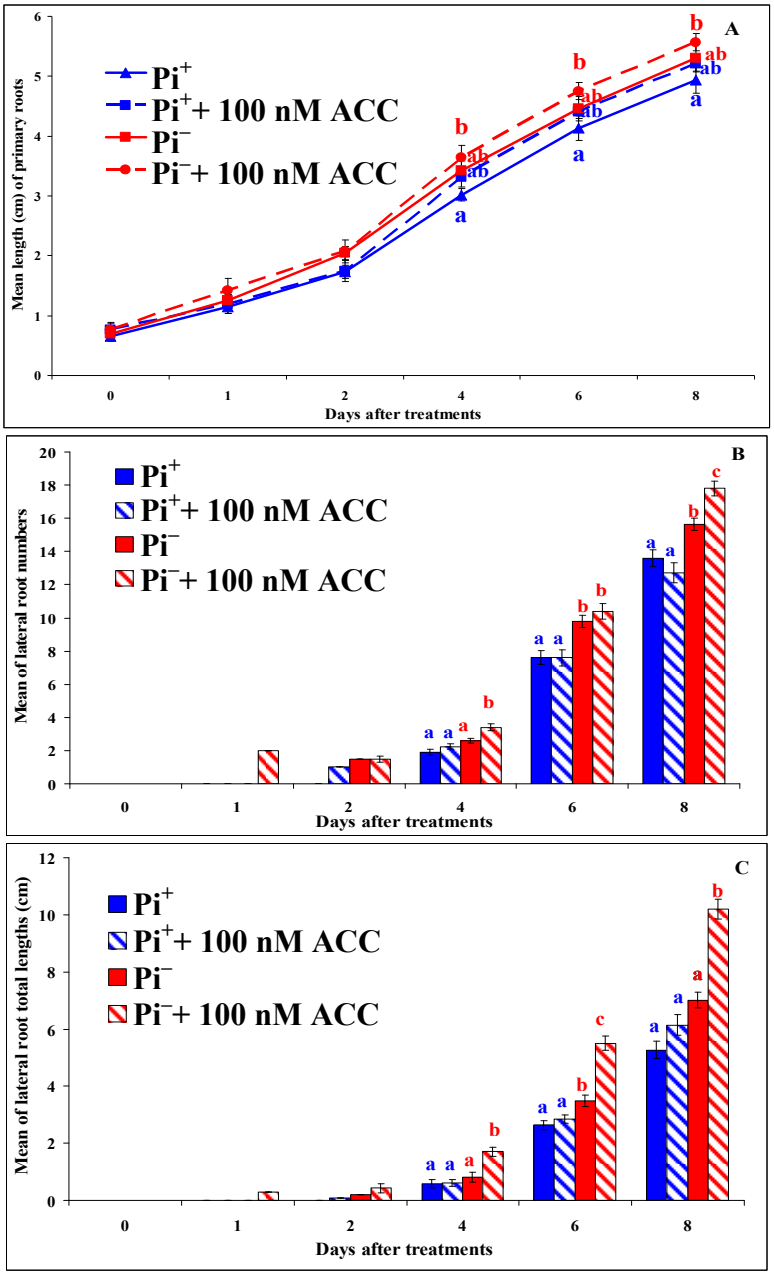


Figure 3.1.12. The effects of ACC in mediating root responses to P-deficiency. ***Changes in length of PRs (A), number of LRs (B) and total length of LRs (C) of wild type white clover stolons (genotype 10F) in response to the ethylene precursor, 100 nM ACC in P-deficient condition (Pi^- + 100 nM ACC) in comparison with P-sufficiency (Pi^+), P-deficiency (Pi^-) and 100 nM ACC in P-sufficiency (Pi^+ + 100 nM ACC) during 6 time points from day 0 to 8 DAT.***

Values are means of 10 individual stolons for each treatment with standard deviations of the means (represented as error bars). This figure is representative of duplicate experiments. Different letters indicate the significant differences between the treatments in the same time point ($P \leq 0.05$), using *t*-test for two independent samples.

treatment were significantly higher than in the Pi^+ control treatment with a $P_{adj} < 0.05$ (Table A.8 in Appendices). So, 100 nM of ACC was shown to have a stimulatory effect on white clover root growth (both PR and LR) in P-deficient conditions. Then, to clarify this proposed role of ethylene on mediating white clover root growth, 300 ppm 1-MCP was also applied to the P-deficient treatments to investigate possible changes in the root systems in response to P-deficiency as ethylene action was inhibited.

3.1.3.1.2. The effect of the ethylene action inhibitor (1-MCP) on root morphology in plants maintained in P-deficiency

In a previous Section (3.1.2.1.3), 1-MCP was shown to have a quite severe inhibitory effect on both PR and LR growth in P-sufficient conditions, and here these impacts are considered under P-deficient conditions (Figure 3.1.13). In these experiments, the inhibitory effect of 1-MCP on PR elongation, which was observed in P-sufficient roots, was eliminated in the P-deficient treatment. In addition, there is no statistically significant difference between the Pi^- and the Pi^- + 300 ppm 1-MCP treatments in terms of PR length, which gradually increased from *ca.* 0.51 cm at the beginning of the treatments (day 0) to *ca.* 2.50 cm at 4 DAT and *ca.* 5.03 cm at 8 DAT in the Pi^- treatment, and from *ca.* 0.6 cm at day 0 to *ca.* 2.54 cm at 4 DAT and *ca.* 5.24 cm at 8 DAT, in the Pi^- + 300 ppm 1-MCP treatment. However, the mean PR length in the Pi^- + 300 ppm 1-MCP treatment was significantly different ($P \leq 0.05$) with the length in the Pi^+ treatment at 6 and 8 DAT and with PR length in the Pi^+ + 300 ppm 1-MCP treatment at 4, 6 and 8 DAT. These results suggest that the concentration of 1-MCP (300 ppm) was enough to inhibit PR elongation in P-sufficient roots, but not in those grown in P-deficiency due to either an increased level of ethylene or increased ethylene sensitivity.

The presumption that an increased level of ethylene in P-deficient roots caused an elimination of the inhibitory effect of 1-MCP in terms of PR elongation was consolidated by the evidence provided when examining changes of LR development. As ethylene action was supposed to be prevented by 1-MCP, LR development (both in terms of the number of formed LRs and LR elongation) was clearly inhibited in P-sufficiency (as described previously in 3.1.2.1.3) from 6 DAT and in P-deficiency (Figure 3.1.13 B, C) from 4 DAT. Interestingly, in both Figure 3.1.13 B and C, it is

noticeable that the LR development pattern in the Pi^- + 300 ppm 1-MCP treatment was similar to the Pi^+ (control) treatment. For instance, at 6 DAT, roots in the Pi^+ treatment had an average 4.35 LRs with a total length of *ca.* 2.14 cm, while roots in the Pi^- + 300 ppm 1-MCP treatment had a mean number of *ca*. 4.67 LRs with an average total length of 2.17 cm. At 8 DAT, the number of LRs increased to *ca*. 7.3 LRs with a mean total length of c*a*. 4.06 cm in the Pi^+ treatment and increased to *ca*. 6.8 LRs with a total length of *ca*. 4.14 cm in the Pi^- + 300 ppm 1-MCP treatment. Thus P-deficiency was assumed to neutralise the inhibitory effects of 300 ppm 1-MCP on the LR growth providing further evidence for an increase in ethylene production in response to P-deficiency.

When the data from all six time points were pooled together for analysis, the mean PR length in the Pi^- + 300 ppm 1-MCP treatment (*ca.* 2.49 cm) was significantly higher than the length in the Pi^+ treatment (*ca*. 2.13 cm) and in the Pi^+ + 300 ppm 1-MCP treatment (*ca*. 1.80 cm) ($P_{adj} < 0.001$) but not significantly higher than in the Pi^- treatment (*ca*. 2.36 cm) (Table A.13 and Table A.14 in Appendices). For LR development, the mean LR number and the total LR length in the Pi^- + 300 ppm 1-MCP treatment were similar to the Pi^+ control and were considerably higher than in the Pi^+ + 300 ppm 1-MCP treatment, but significantly lower than in the Pi^- treatment ($P_{adj} < 0.05$, Table A.13, Table A.15 and Table A.16 in Appendices). These analyses confirmed that P-deficiency resulted in a lessening of the inhibitory effects of 300 ppm 1-MCP on the white clover root growth.

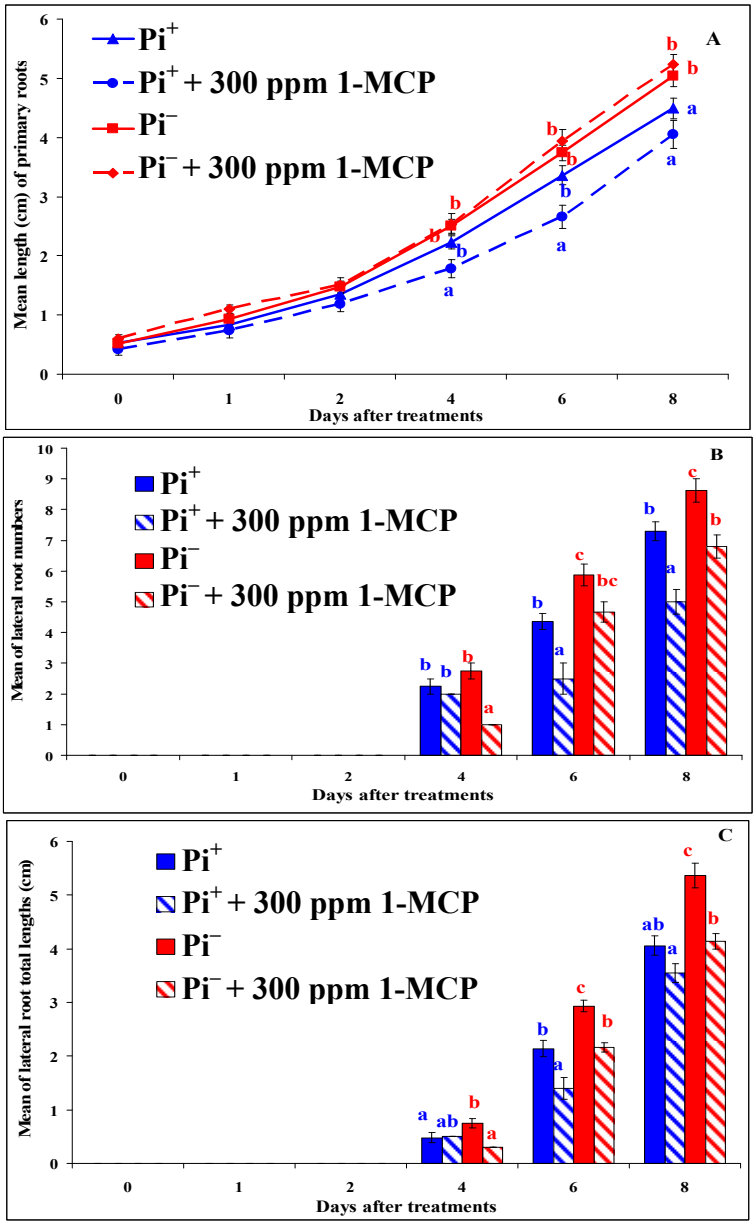


Figure 3.1.13. The effects of 1-MCP in mediating root responses to P-deficiency. ***Changes in length of PRs (A), number of LRs (B) and total length of LRs (C) of wild type white clover stolons (genotype 10F) in response to the ethylene action inhibitor, 300 ppm 1-MCP in P-deficient condition (Pi$^-$ + 300 ppm 1-MCP) in comparison with P-sufficiency (Pi$^+$), P-deficiency (Pi$^-$) and 300 ppm 1-MCP in P-sufficiency (Pi$^+$ + 300 ppm 1-MCP) during 6 time points from day 0 to 8 DAT.***

Values are means of 11 individual stolons for Pi$^+$ or 8 stolons for Pi$^-$ or 5 stolons for each 1-MCP treatment with standard deviations of the means (represented as error bars).

Different letters indicate the significant differences between the treatments in the same time point ($P \leq 0.05$), using *t*-test for two independent samples.

This figure is representative of duplicate experiments.

3.1.3.2. Auxin

3.1.3.2.1. The effect of exogenous auxin treatment on root morphology in roots exposed to P-deficiency

In a previous Section (3.1.2.2.1), the stimulatory role of 5 nM NAA was observed in both PR growth and LR development in P-sufficient conditions. Now, the influence of this low concentration of NAA on white clover roots grown in P-deficient conditions was examined.

A contrary trend of the effect of 5 nM NAA on PR elongation was found at 4 DAT (Figure 3.1.14 A). Instead of inducing PR elongation (as observed in P-sufficiency; Section 3.1.2.2.1), 5 nM NAA neutralised the stimulatory effects of P-deficiency. For instance, the mean PR length in the Pi^- + 5 nM NAA treatment was shorter than in the Pi^- treatment and was almost the same as in the Pi^+ treatment (control) over all of the time points examined. Hence, the mean PR length in the Pi^- + 5 nM NAA treatment was significantly lower than in the Pi^+ + 5 nM NAA treatment ($P < 0.06$) from 4 DAT until 8 DAT.

In common with PR elongation, 5 nM NAA treatment in P-deficient or P-sufficient media had converse effects on LR development (Figure 3.1.14 B, C). Both P-deficiency and 5 nM NAA caused an earlier emergence of LRs at 2 DAT but at 4 DAT, there was no difference in the number of LRs and in the total LR lengths in comparison with the control (Pi^+) treatment. However, in terms of LR numbers and total LR lengths, at 6 DAT, a reduction was recorded in the Pi^- + 5 nM NAA treatment when compared with the Pi^- treatment whilst a stimulation was found in the Pi^+ + 5 nM NAA treatment (as discussed in 3.1.2.2.1). At 8 DAT, in the Pi^- + 5 nM NAA treatment, a LR developmental pattern was observed that was similar to the control treatment (Pi^+). This also means that instead of stimulation of LR development as in P-sufficiency, 5 nM NAA neutralised the stimulatory effects of P-deficiency on white clover root growth.

When the data from all six time points was pooled for analysis, the mean PR length of plants exposed to the Pi^- + 5 nM NAA treatment was significantly lower than the length in the Pi^+ + 5 nM NAA treatment ($P_{adj} = 0$) but was not different with the Pi^+ and the Pi^- treatments (Table A.17 and Table A.18 in Appendices). For LR development, the Pi^- + 5 nM NAA treatment was not different from the Pi^+ treatment

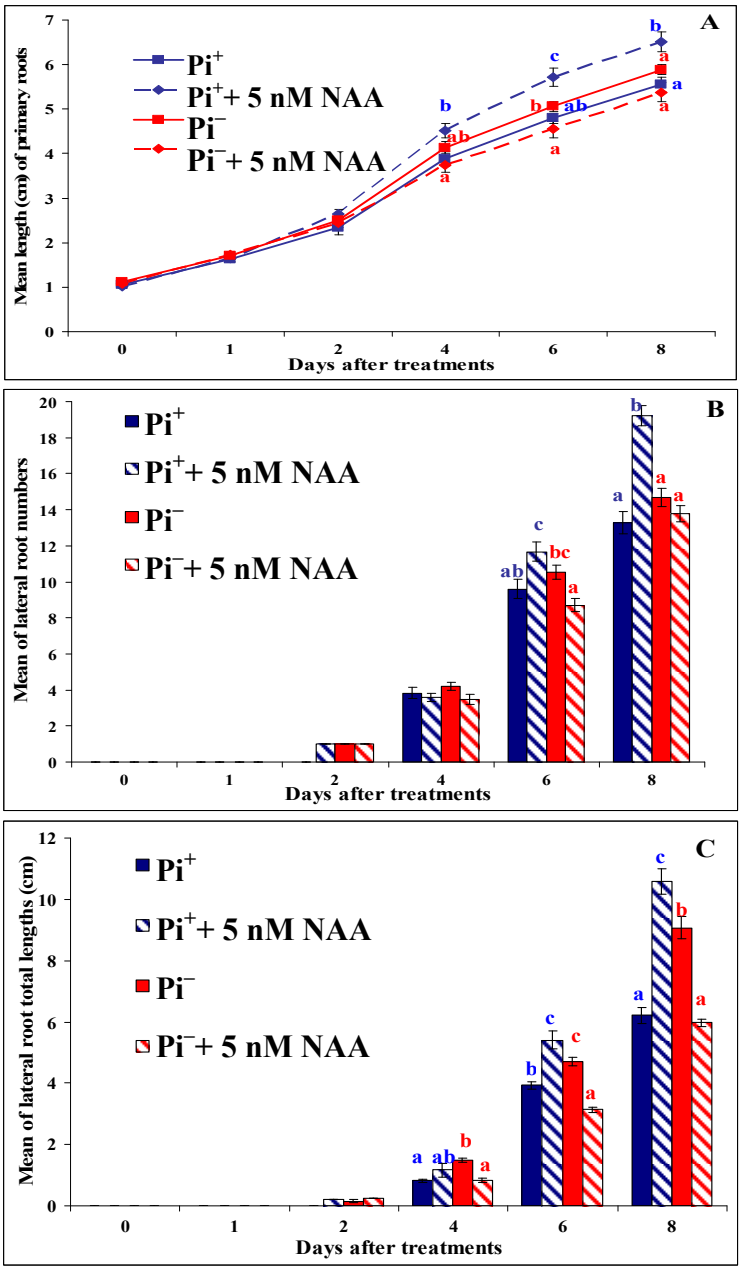


Figure 3.1.14. The effects of NAA in mediating root responses to P-deficiency. ***Changes in length of PRs (A), number of LRs (B) and total length of LRs (C) of wild type white clover stolons (genotype 10F) in response to exogenous auxin, 5 nM NAA in P-deficient condition (Pi^- + 5 nM NAA) in comparison with P-sufficiency (Pi^+), P-deficiency (Pi^-) and 5 nM NAA in P-sufficiency (Pi^+ + 5 nM NAA) during 6 time points from day 0 to 8 DAT.***

Values are means of 10 individual stolons for Pi^+ and Pi^- or 17 stolons for Pi^+ + 5 nM NAA and 16 stolons for Pi^- + 5 nM NAA with standard deviations of the means (represented as error bars).

Different letters indicate the significant differences between the treatments in the same time point ($P \leq 0.05$), using *t*-test for two independent samples.

but was significantly lower in terms of LR number and total LR length than the Pi^+ + 5 nM NAA and the Pi^- treatments ($P_{adj} < 0.05$, Table A.17, A.19 and A.20 in Appendices). Therefore, these results again showed that 5 nM NAA had converse effects on white clover root growth when exposed to P-deficiency and P-sufficiency, and that 5 nM NAA could overcome the P-deficient influences.

3.1.3.2.2. The effect of the auxin transport inhibitor (NPA) on root morphology in plants exposed to P-deficiency

In contrast to the observation that exogenous auxin when added at low concentration (5 nM NAA) to P-deficient plants eliminated the P-deficiency responses in terms of white clover root development, 100 nM NPA always prevented the growth of white clover roots regardless of whether they were maintained in P-sufficient or P-deficient media. By *t*-test analyses, it was observed that in terms of PR length, LR number and total LR length, over all of the time points from day 0 to 8 DAT, the inhibitory effects of 100 nM NPA on the roots kept in P-deficient media were similar to those kept in P-sufficient media (which are described in more detail in 3.1.2.2.2).

Supporting the *t*-test, analysis on pooled data by Tukey's test also showed that the mean PR length, the mean LR number and the mean total LR length in the Pi^- + 100 nM NPA treatment was not different from the Pi^+ + 100 nM NPA but significantly lower than the Pi^+ and Pi^- treatments ($P_{adj} < 0.05$, Table A.22, A.23, A.24 and A.25 in Appendices). Therefore, white clover root systems were proposed to lose the capability to respond to P-deficiency when auxin transport was inhibited by NPA. Another explanation may be that the concentration of NPA was too high to mediate differences in root responses to phosphate availability.

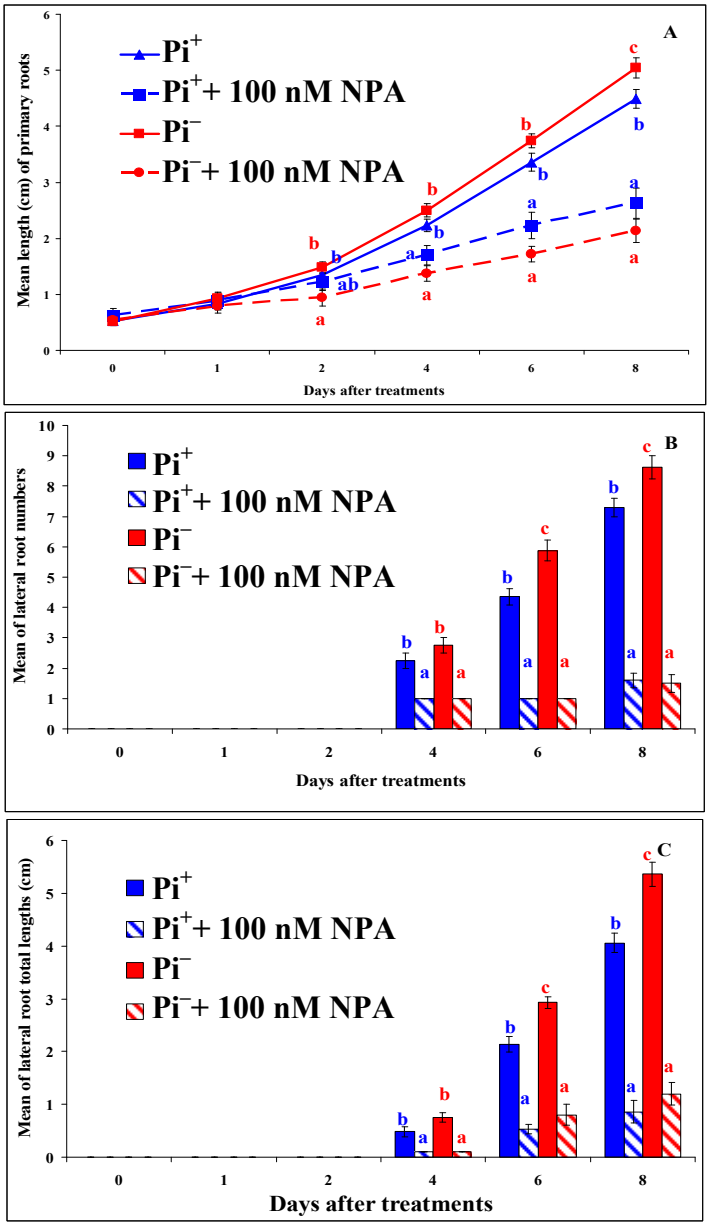


Figure 3.1.15. The effects of NPA in mediating root responses to P-deficiency. ***Changes in length of PRs (A), number of LRs (B) and total length of LRs (C) of wild type white clover stolons (genotype 10F) in response to the auxin transport inhibitor, 100 nM NPA in P-deficient condition (Pi^- + 100 nM NPA) in comparison with P-sufficiency (Pi^+), P-deficiency (Pi^-) and 100 nM NPA in P-sufficiency (Pi^+ + 100 nM NPA) during 6 time points from day 0 to 8 DAT.***

Values are means of 11 individual stolons for Pi^+, or 9 stolons for Pi^- + 100 nM NPA or 8 stolons for Pi^- and Pi^+ + 100 nM NPA with standard deviations of the means (represented as error bars). Different letters indicate the significant differences between the treatments in the same time point ($P \leq 0.05$), using *t*-test for two independent samples. This figure is representative of duplicate experiments.

3.2. TRANSGENIC WHITE CLOVER

3.2.1.Transformation and regeneration of white clover lines putatively transformed with the *DR5p::GUS* construct

A total of 2,500 seeds of white clover *cv.* Grasslands Huia were dissected and co-cultivated with *A. tumefaciens* for four days and then *ca.* 4,500 green cotyledons were selected for placement onto the $CR7^{Kan150;\ Cef300}$ media. After 28 days, only *ca.* 700 cotyledons survived, and these were continuously screened on the $CR5^{Kan100;\ Cef150}$ media and then on the $CR5^{Kan50;\ Cef100}$ media. Some of these cotyledons formed little shoots, while others were still at the callus stage before shifting onto the CR5 media. After 28 days on $CR5^{Kan50;\ Cef100}$ selection media, shoots regenerating from *ca.* 100 cotyledons developed to form clumps of plantlets. These clumps of plantlets were subdivided to make up individual transformed white clover lines before shifting onto the CR0 $^{Kan25;\ Cef50}$ media to induce rooting. Up to this stage, the surviving lines were coded in succession from TR1 to TR72. After 30 days on CR0, there were only 55 lines large enough for transfer to autoclaved potting mix to become stock plants in the Physical Containment Level 2 GMO glasshouse.

3.2.2.Screening putative transgenic *DR5p::GUS* lines of white clover

3.2.2.1. The use of the Polymerase Chain Reaction (PCR) for screening

When the 55 transformed white clover lines were established in the GMO glasshouse, young leaves (the first to third full-expanded leaves) were collected to extract genomic DNA (Section 2.6.1) which was used as a template for PCR. In PCR, the plasmid DNA harbouring the *DR5p::GUS* construct and the genomic DNA of wild-type white clover were used as positive and negative controls, respectively. By using the DR5rev-pro-F1 and GUS-seq-R1 primers (Section 2.6.2), PCR products of *ca.* 600 bp were deemed to be positive. That is, the genomic DNA harboured the *DR5p::GUS* insert (Figure 3.2.1). The reason for expecting that a *ca.* 600 bp band indicates a positive result is that the sequence between these two primers in the *DR5p::GUS* construct is 556 base-pair in length (Figure A.2 in Appendices). By the PCR screening method, 55 transformed lines are recorded in Table 3.2.1 with 37 lines positive and 18 lines negative.

Lane 1 2 3 4 5

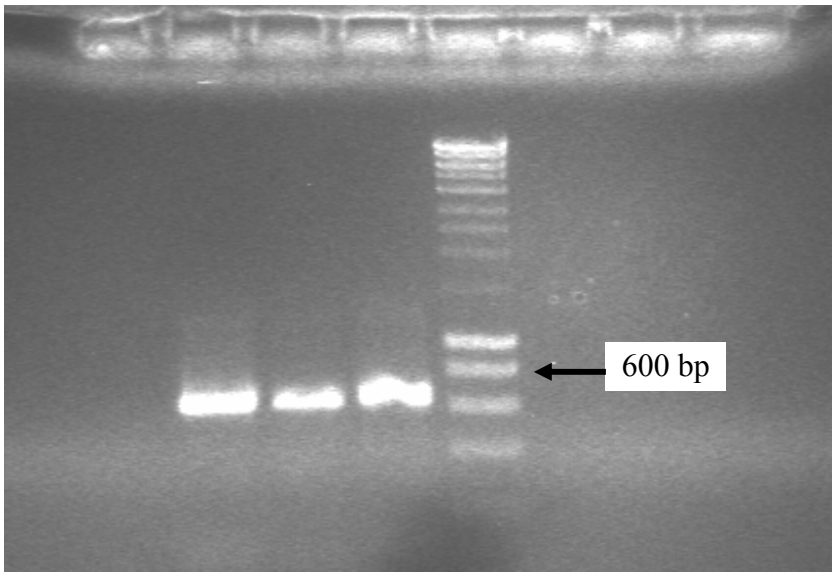


Figure 3.2.1. Example of screening of putative *DR5p::GUS* white clover transformants using PCR.

Extracted DNA from different putatively transformed white clover lines were screened by PCR using the DR5-rev-pro-F1 and GUS-seq-R1 primers and the PCR products were separated by electrophoresis on a 1% (w/v) agarose gel, and the amplification products visualized by ethidium bromide staining.

Lane 1: wild type white clover (Grasslands Huia)

Lane 2: TR2

Lane 3: TR29

Lane 4: TR4

Lane 5: HyperLadderI

3.2.2.2. Screening of putative transformants using GUS staining

Tissues for GUS staining analysis were also collected when the plantlets were established in the GMO glasshouse. The young shoot apices and the nodal root primordia were chosen for GUS analysis because it has been shown previously from studies in *Arabidopsis* that both of these tissues have high expression of *DR5p::GUS* (Aloni et al., 2003). Using GUS staining (Section 2.7.1), the white clover lines were screened to identify those expressing the GUS transgene. After incubating with X-GLUC at 37^{o}C overnight and destaining through a series of increasing ethanol concentrations, 26 putative transgenic lines displayed GUS staining both in the shoot apices and in the nodal root primordia even though the location and intensity of staining differed. For example, one line (TR6) displayed GUS staining only in the apices whereas TR1 and TR17 displayed GUS staining only in the nodal root primordia (Table 3.2.1). An example of the range of GUS staining intensities is shown as Figure 3.2.2. There were 14 lines (TR1, TR3, TR4, TR9, TR16, TR17, TR22, TR26, TR34, TR36, TR38, TR47, TR60 and TR63) that had stronger staining in the nodal root primordia when compared with the apices (Table 3.2.1). In contrast, GUS staining in 6 lines (TR2, TR6, TR54, TR64, TR68 and TR72) was more intense in the apices when compared with the nodal root primordia. Other lines (TR5, TR15, TR24, TR29, TR30, TR37, TR44, TR65 and TR66) had the same level of staining in the apices and the nodal root primordia (Table 3.2.1), while 26 lines did not display any GUS staining (Table 3.2.1).

If the PCR results were compared, there were 28 transgenic white clover lines that were positive for both PCR and GUS staining. Twenty one of these displayed intense GUS staining and another six lines showed faint staining in the root primordia. In the other line, TR6, GUS activity was only found in the apices but not in the root primordia. In contrast, nine lines were positive by PCR but negative by GUS-analysis, while one line, TR1, was negative by PCR but positive by GUS analysis.

According to Ding et al. (2003), the transformation frequency is defined as "number of independent confirmed transgenic plants produced per one hundred treated cotyledons". Thus, the transformation frequency achieved in this thesis was 0.56% (28 individual transgenic lines were confirmed as harbouring the *DR5p::GUS* construct by PCR and GUS staining that was regenerated from 5000 cotyledons), which belong to the range reported in white clover transformation (Ding et al., 2003)

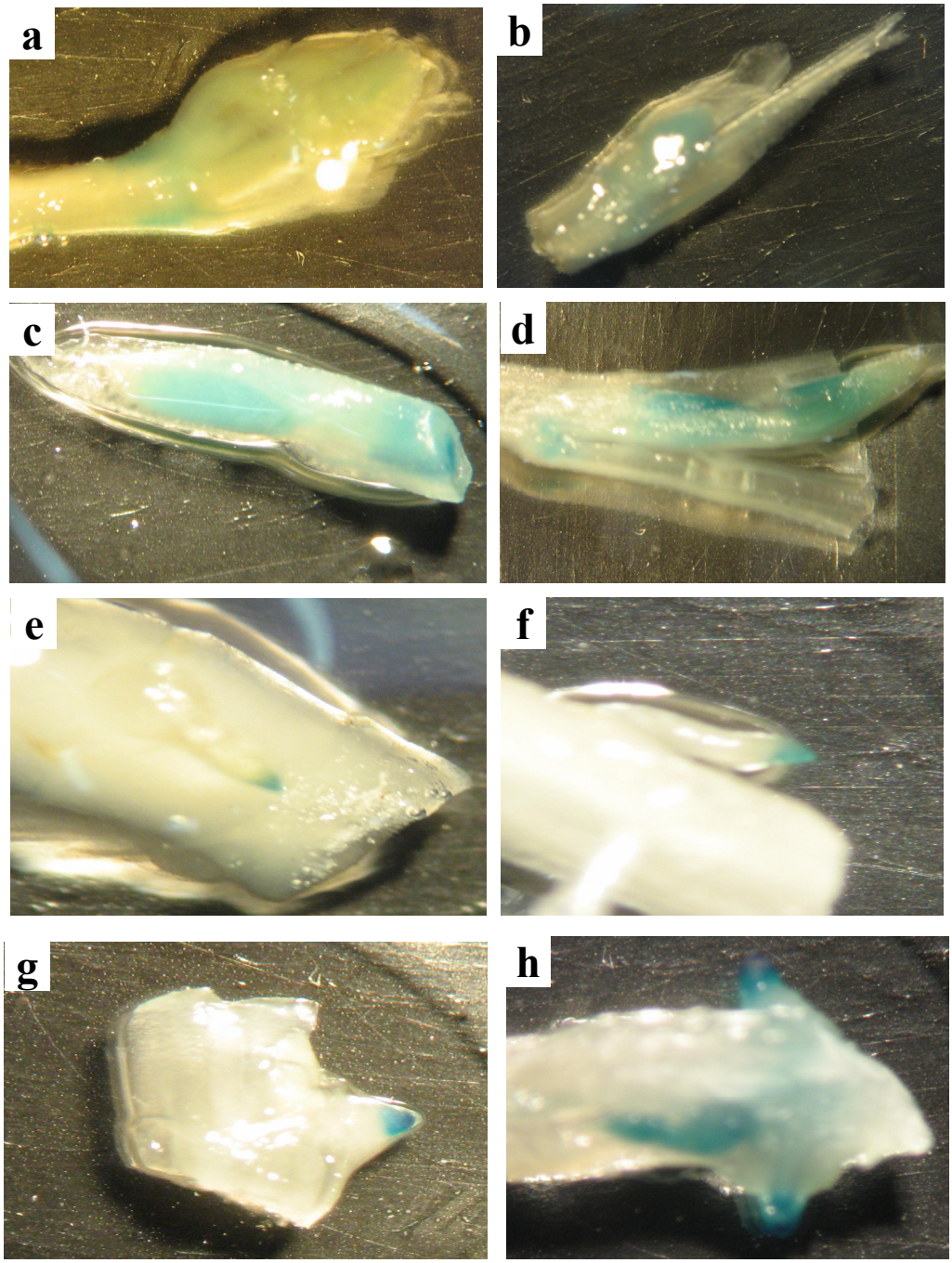


Figure 3.2.2. GUS staining intensity in *DR5p::GUS* transformed white clover

Apices and nodal root meristems were collected from different *DR5p::GUS* transgenic white clover lines and were stained with X-GLUC solution overnight at 37^{o}C after vacuum infiltration at *ca.* 35 kPa for 15 min.These stained samples went through the destaining process with a series of different concentrations of ethanol, were examined using a Zeiss (Stemi DV4) Microscope at 10x magnification and were captured using Canon PowerShot A550 digital camera (the size in the images is 10x actual size).

a, b, c and d: stained apices

e, f, g and h: stained nodal root meristem

a and e: faint staining in TR29 (F)

b and f: light staining in TR44 (+)

c and g: intense staining in TR5 (++)

d and h: very intense staining in TR15 (+++)

Table 3.2.1. **Summary of results of screening of putative *DR5p::GUS* transgenic white clover lines using PCR and GUS staining analysis, as indicated**

Line	PCR	GUS exposure pattern		Line	PCR	GUS exposure pattern	
		Apices	Root primordia			Apices	Root primordia
TR1	–	–	+	TR38	+	+	+++
TR2	+	++	F	TR40	–	–	–
TR3	+	++	+++	TR41	+	–	–
TR4	+	F	++	TR43	–	–	–
TR5	+	++	++	TR44	+	+	+
TR6	+	+	–	TR47	+	+	++
TR7	–	–	–	TR48	+	–	–
TR8	–	–	–	TR50	–	–	–
TR9	+	F	+	TR51	+	–	–
TR10	–	–	–	TR54	+	++	+
TR11	–	–	–	TR55	–	–	–
TR12	–	–	–	TR56	+	–	–
TR15	+	+++	+++	TR57	–	–	–
TR16	+	+	+++	TR58	–	–	–
TR17	+	–	+	TR59	–	–	–
TR19	–	–	–	TR60	+	+	++
TR21	–	–	–	TR61	+	–	–
TR22	+	F	++	TR62	+	–	–
TR24	+	F	F	TR63	+	+	+++
TR25	–	–	–	TR64	+	+++	++
TR26	+	++	+++	TR65	+	+++	+++
TR29	+	F	F	TR66	+	F	F
TR30	+	F	F	TR67	–	–	–
TR31	+	–	–	TR68	+	++	+
TR32	+	–	–	TR69	+	–	–
TR34	+	+	++	TR70	–	–	–
TR36	+	+	++	TR72	+	++	+
TR37	+	F	F				

PCR results:
– : negative
+ : positive

GUS staining intensity:
– : non-staining
F : faint-staining
+ : light staining
++ : intense staining
+++ : very intense staining

3.2.2.3. Southern blot analysis

After screening by both PCR and GUS staining, there were 28 lines confirmed as harbouring the *DR5p::GUS* construct, but during the planting and propagating processes in the GMO glasshouse, only eight of these lines provided enough stolons for further experiments (TR2, TR4, TR24, TR29, TR34, TR44, TR48 and TR66). Therefore, the genomic DNA of these eight lines were used to analyse the copy number of the transgene (*DR5p::GUS*) insert into the Huia genome using Southern analysis (Section 2.8). For this, genomic DNA was digested using *Sac* I because it will cleave the transgenic white clover genomic DNA randomly, but there is also a single site in the inserted T-DNA within the nopaline synthase terminator sequence, nos T (but not in the DNA sequence incorporated by the two primers DR5rev-pro-F1 and GUS-seq-R1; see Figure 2.4 for a map of the pRD-410 vector). Thus *Sac* I digested the genomic DNA of putative transformants at least within every nos T sequence.

Southern analysis revealed that the *DR5p::GUS* probe hybridised to a single band in TR44 and TR29 (*ca.* 8,000 bp band for TR44 and *ca.* 10,000 bp band for TR29), and two bands of *ca.* 6,000 bp and more than 10,000 bp in TR66 (Figure 3.2.3). In TR 34, the probe again hybridised to two bands of more than 10,000 bp. The probe hybridised to three bands (one less than 10,000 bp and the other two more than 10,000 bp) in TR48 and four bands appeared on the membrane (*ca*. 6,000 bp, 8,000 bp, 10,000 bp and more than 10,000 bp) in TR24 (Figure 3.2.3). In contrast, the probe did not hybridise to any band in TR2 and TR4 although both were positive by PCR and GUS screening. That may be explained by the loss of genomic DNA during preparation for electrophoresis or the concentration of the DNA loaded was not enough to be detected by the DIG-labelling (nonradio-active labelling) Southern analysis method.

The genomic DNA which was used in the Southern analysis was supposed to be totally digested by *Sac* I because there is no genomic DNA band in the gel shown in Panel A, Figure 3.2.3. In addition, as mentioned above, *Sac* I digested the genomic DNA at least within every nos T sequence. Thus Southern analysis can indicate the minimum copy number of inserted transgenes into each transgenic white clover lines. TR24 had at least four copies, TR48 had three copies, TR66 and TR34 had two copies, and TR44 as well as TR29 are proposed to have a single copy. The two other, putative negative lines, TR2 and TR4 need to be analysed further by using the more sensitive radio-labelled probes.

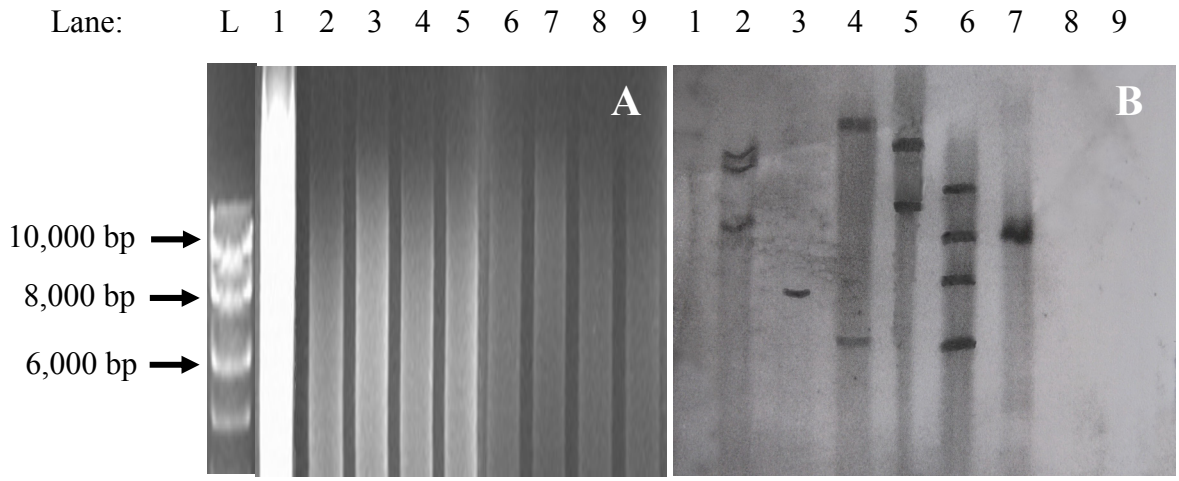


Figure 3.2.3. Southern analysis of putative *DR5p::GUS* white clover transformants.

Digested genomic DNA with *Sac* I was separated on a 0.8% agarose gel (15 cm x 15 cm) at 22 V for 20 hours (Panel A) and transferred to the Hybond – N^+ membrane by the downward capillary transfer method. The membrane was hybridised with a DIG-labelled *DR5p::GUS* probe and hybridisation revealed using recognition by an Anti-Digoxigenin-AP antibody and NBT/BCIP substrate (Panel B).

In both Panel A and B:

Lane L: HyperLadderI

Lane 1: digested genomic DNA of wild type white clover (Grasslands Huia)

Lane 2: digested genomic DNA of TR48

Lane 3: digested genomic DNA of TR44

Lane 4: digested genomic DNA of TR66

Lane 5: digested genomic DNA of TR34

Lane 6: digested genomic DNA of TR24

Lane 7: digested genomic DNA of TR29

Lane 8: digested genomic DNA of TR2

Lane 9: digested genomic DNA of TR4

3.2.3.Morphological root responses of transgenic white clover to P-deficiency

3.2.3.1. Morphological root responses of Grasslands Huia to P-deficiency

Due to the fact that Grasslands Huia was used as the background for *DR5p::GUS* transformations, the morphological root responses of this wild type white clover cultivar were investigated to act as a control for the root responses of the transgenic lines. Using a similar hydroponic system as described for the treatments of the 10F genotype of white clover, Grasslands Huia stolons were excised and rooted for three days, followed by acclimation in Hoagland's media for one days and then they were introduced to either a P-deficient (Pi^-; 0.01 mM Pi) treatment or P-sufficient (Pi^+; 1.00 mM Pi) treatment for eight days. Specific changes in root morphology were measured at 6 time points including day 0, and 1, 2, 4, 6 and 8 DAT.

A stimulatory effect of P-deficiency in terms of both PR elongation (Figure 3.2.4 A) and LR formation (Figure 3.2.4 B) was observed. At day 0, because of randomly distributing the stolons into treatments, the mean PR length in the Pi^+ treatment was *ca.* 1.38 cm which is slightly shorter (but not significantly) than in the Pi^- treatment (*ca.* 1.63 cm). However, later on, from 1 DAT to 4 DAT, the mean PR length in the Pi^- treatment was significantly higher than the Pi^+ treatment with a P value of less than 0.05 from a *t*-test (Figure 3.2.4 A). After that, there was no significant difference between Pi^+ and Pi^- in terms of PR length from 6 DAT until 8 DAT. However, analysis of the whole data set over all of the time points of these treatments showed that the difference in PR length was still significant between the Pi^+ treatment and the Pi^- treatment (P_{adj} = 0.045, Table A.37 and A.40 in Appendices). These results confirmed the promotion in PR elongation in P-deficient media.

Stimulation was also observed in the number of LRs in response to P-deficiency (Figure 3.2.4, Panel B). The mean LR number in the Pi^- treatment was higher than the Pi^+ treatment, especially at 8 DAT. Plants in the Pi^- treatment had a mean number of *ca.* 18.6 LRs while the Pi^+ treatment only had 14 LRs (with a difference of P from a *t*-test of less than 0.05). Moreover, the other response to P-deficiency was that the LRs emerged just one day after the treatment, which was earlier than in the control (LRs were first observed at 4 DAT in the Pi^+ treatment).

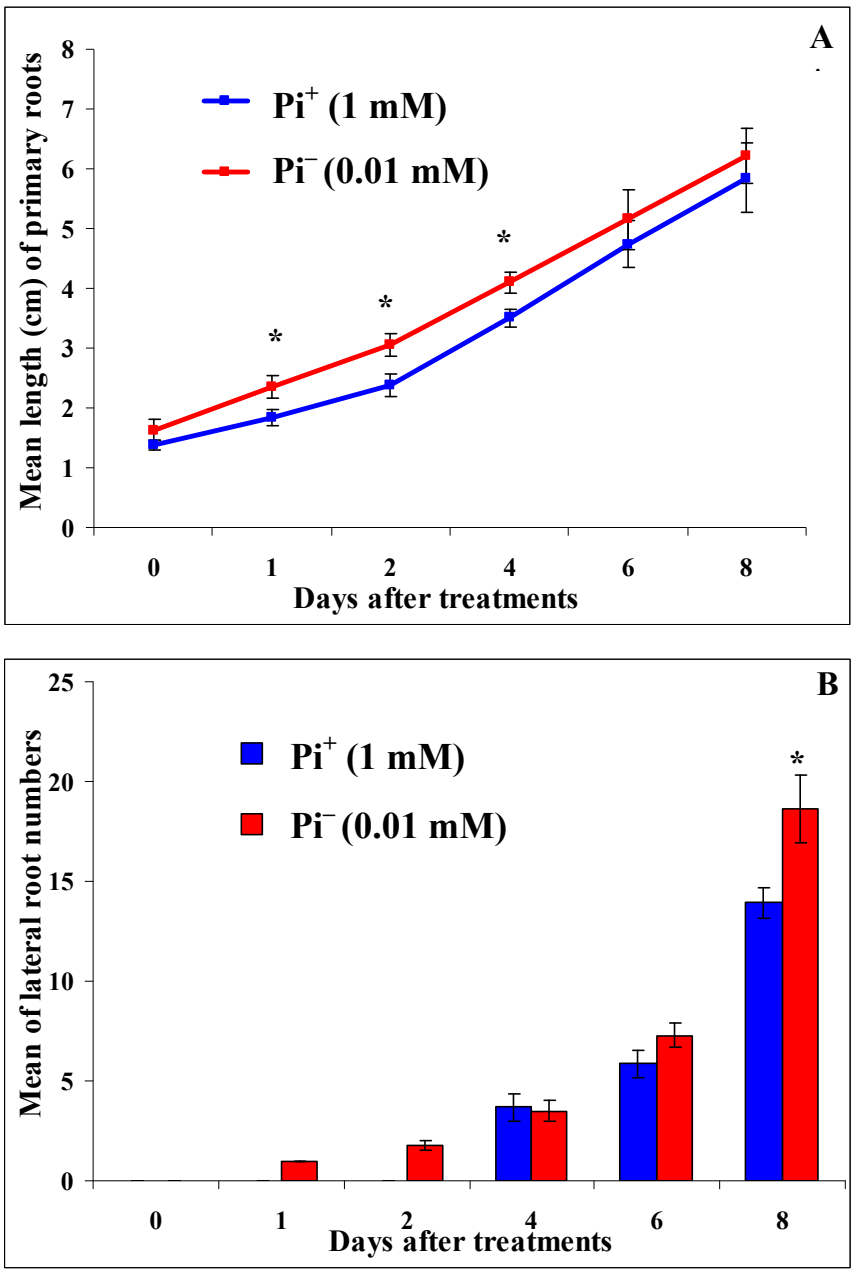


Figure 3.2.4. The effects of P-deficiency on specific aspects of root morphology of wild type white clover (Grasslands Huia)

Changes in length of PRs (A) and number of LRs (B) of wild type white clover stolons (Grasslands Huia) in response to P-deficiency (Pi^-, 0.01 mM Pi) in comparison with P-sufficiency (Pi^+, 1.00 mM Pi).

Values are means of six individual stolons with standard deviations of the means (represented as error bars).

* indicates the significant difference between the treatments ($P \leq 0.05$), using *t*-test for two independent samples

This figure is representative of duplicate experiments.

3.2.3.2. Morphological root response of TR 29 to P-deficiency

P-deficiency treatments were also conducted on the transgenic *DR5p::GUS* lines. In these trials it was determined that among the eight transgenic lines, even though the root growth and development were not exactly the same, these lines had broadly similar patterns in terms of response to P-deficiency (data not shown). Moreover, by using Southern analysis, TR29 was shown to be harbouring a single copy of the *DR5p::GUS* construct and so the root morphological responses of the TR29 line to P-deficiency are shown as Figure 3.2.5.

In common with the wild type Huia and the 10F genotype, a stimulatory effect of P-deficiency on PR elongation and LR formation was observed in TR29. From day 0 to 4 DAT, the same mean PR length was measured in both the Pi^+ and the Pi^- treatments with an increase from *ca.* 2.5 cm to *ca.* 5.16 cm in the Pi^+ treatment and to *ca.* 5.45 cm in the Pi^- treatment. After that, the PRs in the Pi^- treatment elongated considerably more than the PRs in the Pi^+ treatment, particularly at 8 DAT (mean value of *ca.* 8.54 cm compared to *ca.* 7.1 cm). However, as the data from the whole time points were pooled together and analysed, the mean PR length of these two treatments was slightly different, but not statistically significant ($P_{adj} = 0.093$, Table A.37 and Table A.40 in Appendices).

For the number of LRs, stimulation was also observed from 6 to 8 DAT. Particularly at 8 DAT, the difference in LR number between roots in the Pi^+ and Pi^- treatments was significant (Pi^+ roots had *ca.* 20 LRs whereas Pi^- roots had *ca*. 27 LRs; $P < 0.02$ by *t*-test). Before that time point, a similar number of LRs was recorded in each treatment. However, when data from all of the time points of the whole treatment were pooled and analysed, the mean LR number was still significantly different in the P-deficient treatment ($P_{adj} = 0$, Table A.37 and Table A.41 in Appendices) in comparison with the P-sufficient treatment.

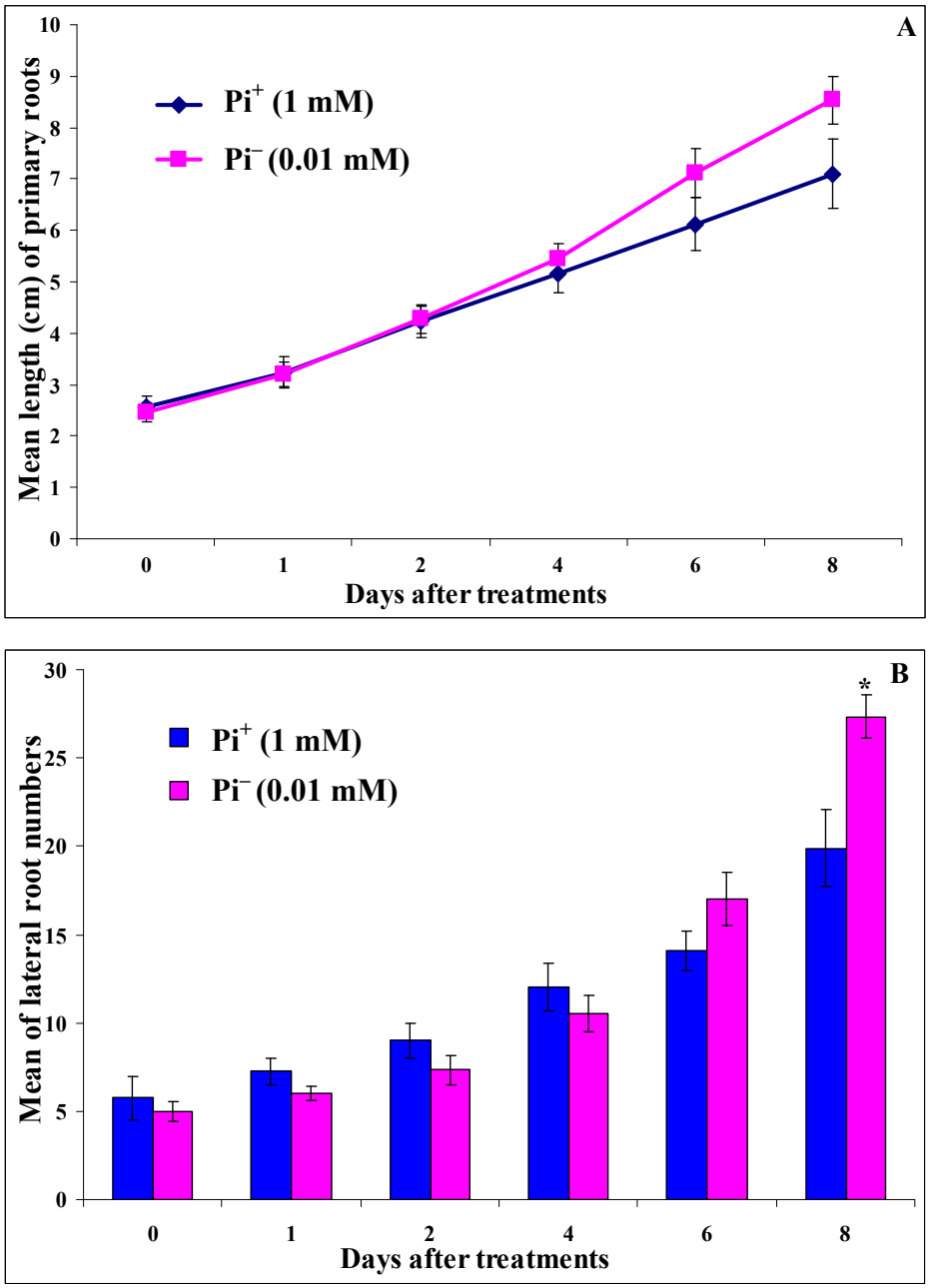


Figure 3.2.5. The effects of P-deficiency on specific aspects of root morphology of the TR29 white clover transgenic line

Changes in length of PRs (A) and number of LRs (B) of TR29, DR5p::GUS transgenic white clover stolons in response to P-deficiency (Pi^-, 0.01 mM Pi) in comparison with P-sufficiency (Pi^+, 1.00 mM Pi)

Values are means of six individual stolons with standard deviations of the means (represented as error bars).

* indicates the significant difference between the treatments ($P \leq 0.05$), using *t*-test for two independent samples

This figure is representative of duplicate experiments

3.2.3.3. Differences in root morphology between genotype of white clover in response to P-deficiency

All of the data of the three white clover genotypes (10F cultivar, Huia cultivar and the transgenic TR29 line) in the P-treatments were pooled, and it was determined that different genotypes had significantly different PR lengths and LR numbers ($P_{adj} < 0.005$. Table A.37, A.38 and A.39 in Appendices). The transgenic TR29 line had the fastest PR growth when compared with Grasslands Huia and Grasslands 10F (the mean PR length of TR29, Huia and 10F were *ca.* 4.95 cm, 3.52 cm and 2.74 cm, respectively, Table A.37 in Appendices). Also, the TR29 white clover line had more emerged LRs (*ca.* 10.29) than Huia and 10F (a mean value of *ca.* 4.26, Table A.37 in Appendices).

Even though the root growth of these three genotypes was different, all of them still showed the stimulatory responses in terms of PR length and number of emerged LRs when grown in P-deficient media. In the P-deficient media, the mean PR length of the three genotypes was *ca.* 3.93 cm which was significantly higher than in the Pi^+ treatment (*ca.* 3.54 cm; $P_{adj} = 0$, Table A.37 in Appendices). The mean LR number in the Pi^- treatment (*ca*. 7.02 LRs) was also considerably higher than the Pi^+ treatment (*ca.* 5.53 LRs; $P_{adj} = 0$, Table A.37 in Appendices).

3.2.4.GUS expression patterns in *DR5p::GUS* white clover transformants in response to P-deficiency and to hormone treatment

Among eight surviving white clover transgenic lines which were genetically analysed by Southern blots (as mentioned in Section 3.2.2.3), the TR29 transgenic white clover line was also chosen to investigate the GUS expression patterns in response to P-deficiency and to hormone treatment. The reasons for this choice are that TR29 was proposed to harbour a single copy of *DR5p::GUS* transgene and that the GUS activity in TR29 white clover is not too intense to distinguish the changes of auxin responsiveness to different environmental conditions, including P-deficiency and hormone treatment.

3.2.4.1. The effects of P-deficiency and ACC on GUS expression in PR tips

As mentioned above, TR29 was chosen to analyse the GUS expression. For the trial P-deficient treatment, the root systems were harvested at 1 DAT to conduct GUS staining (Section 2.7.1). After incubating with staining solution overnight, the roots which were maintained in both P-deficient and P-sufficient media had a similar GUS expression pattern in terms of staining intensity and localisation. For instance, GUS staining was found to be very intense in PR tips, LR tips and LR primordia of these roots (Figure 3.2.6). This GUS staining pattern was also observed in the other transgenic lines at the same locations (root tips and root primordia) as in TR29 but with various GUS intensity (data not shown) caused by the variation in the transgenetic background (as mentioned in Section 3.2.2.3). Due to the similar intensity of GUS staining in both the P-deficient and P-sufficient treatments, it is difficult to investigate the involving of auxin responsiveness in mediating root response to P-deficiency.

Therefore, a second staining method was used (as described in Section 2.7.2) which involved the addition of 2 mM ferrocyanide and 2 mM ferricyanide in the staining solution and a shorter incubation period (four hours). Using this method, it was possible to distinguish differences in GUS staining patterns in roots grown in P-deficient and P-sufficient treatments. Therefore, this method was used to stain TR29 roots which were harvested one day after four treatments: Pi^+ (P-sufficiency as a control), Pi^- (P-deficiency), Pi^+ + 1 μM ACC (increased ethylene level in P-sufficiency) and Pi^- + 1 μM ACC (increased ethylene level in P-deficiency).

However, due to the short incubation time, GUS activity could not be detected in the LR primordia but could be detected consistently in the root tips (similar in PR roots and LR roots). Hence, GUS-staining results for the PR tips of TR29 are represented here to show the differences in GUS expression in response to P-deficiency and also in response to ACC treatment (Figure 3.2.7).

After staining for only four hours, GUS activity could not be detected in the PR tips of the control treatment (Pi^+, Figure 3.2.7 A) but was detectable in the PR tips of roots exposed to the ACC treatment in both the P-deficient and P-sufficient media. Also, in the P-deficient (Pi^-) treatment only, staining was unevenly distributed in the columella cells and quiescent centre (QC) (Figure 3.2.7 B). In the ACC treated roots in both Pi^- and Pi^+ media, GUS expression was detected evenly in the same cell types (the columella cells and the QC) (Figure 3.2.7 C and D). Therefore, it was observed that both P-deficiency and ACC treatment could intensify the activity of GUS in the PR tips in comparison with the Pi^+ control treatment. In other words, the auxin responsiveness was found to be enhanced in the PR tips by the P-deficient treatment and/or by the elevation of ethylene level. Further experiments, therefore, were undertaken to determine whether the changes in GUS activity in the PR tip of TR29 in response to P-deficiency and ACC treatment were caused by either an increase in the free-auxin levels or by a change in auxin sensitivity.

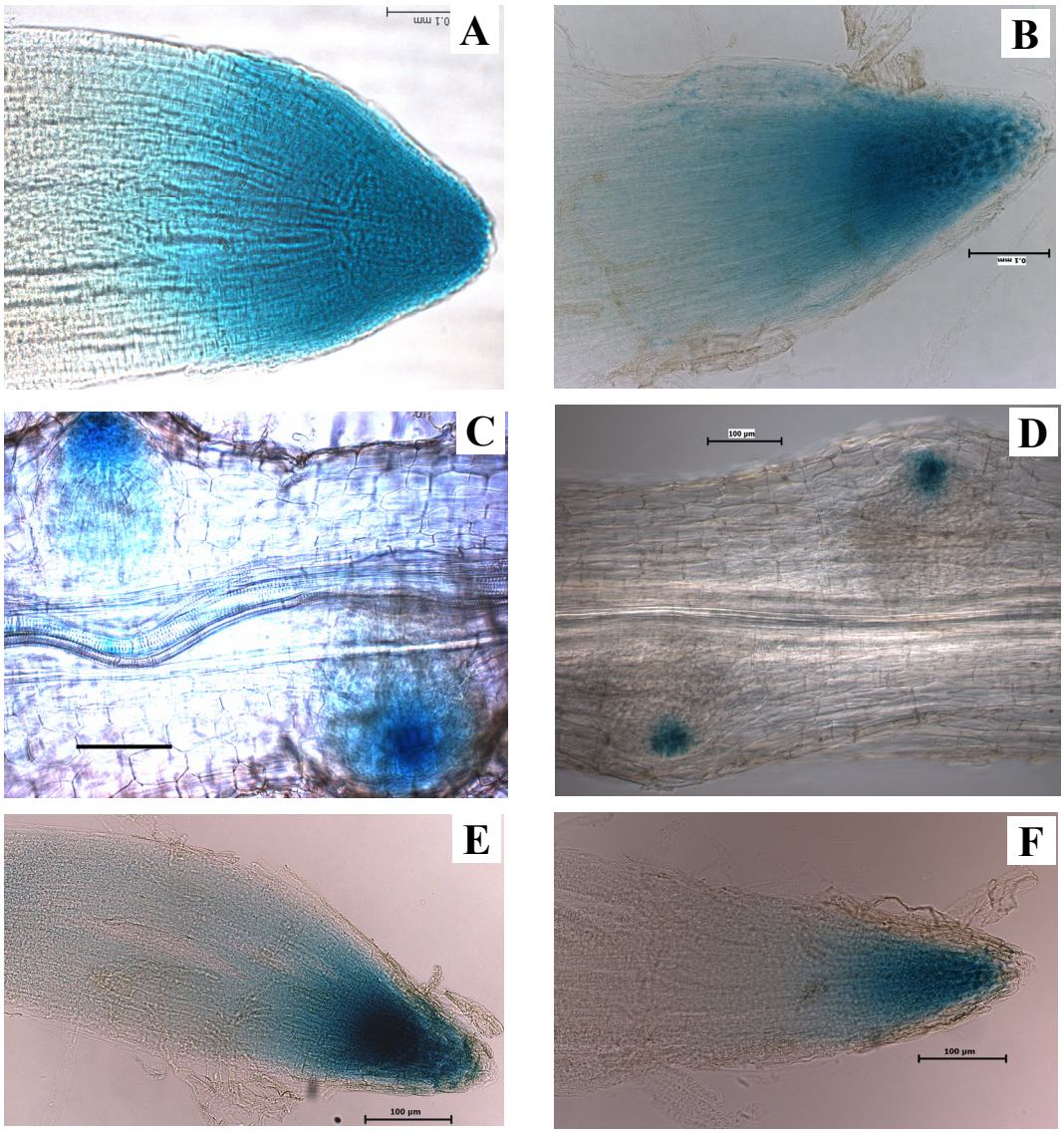


Figure 3.2.6. GUS staining patterns of *DR5p::GUS* transformed TR29 white clover in response to a P-deficient treatment.

Root systems were harvested from TR29 stolons after being maintained in the P-sufficent media, Pi^{+}, (A, C, E) or P-deficient media, Pi^{-}, (B, D, F). These samples were then stained with X-GLUC solution overnight at 37^{o}C after vacuum infiltration at *ca.* 35 kPa for 15 min. Samples were then destained through an ethanol series and the images were captured using a Zeiss Axiophot Compound Light Microscope at 20x magnification. The bar represents 100 µM.

A and B: primary root tips

C and D: lateral root tips

E and F: lateral root primordia.

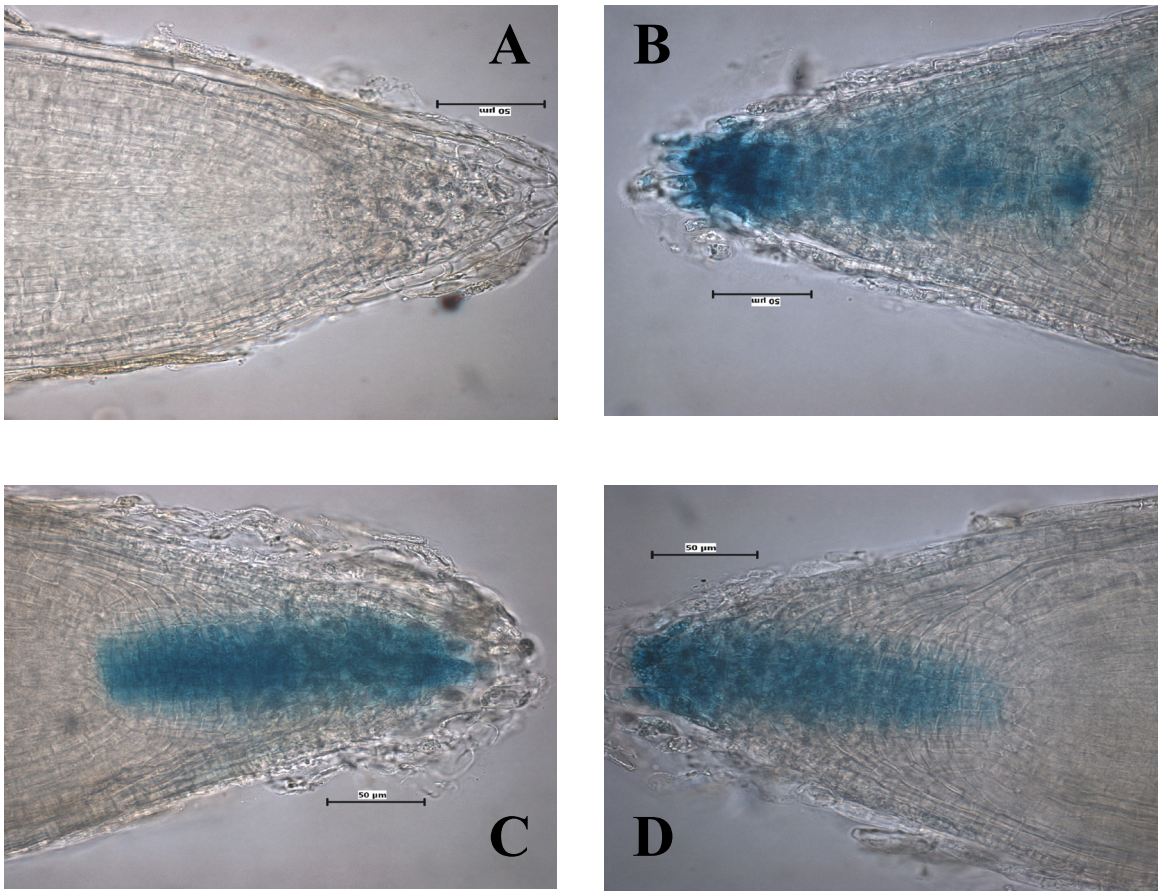


Figure 3.2.7. GUS staining of the primary root tips of the *DR5p::GUS* transformed TR29 line in response to P-deficiency and ACC treatment.

Primary roots were collected from treated TR29 stolons at 1 DAT

A: P-sufficient treatment (Pi^+), B: P-deficient treatment (Pi^-),

C: Pi^+ + 1 µM ACC and D: Pi^- + 1 µM ACC.

These samples were then stained with X-GLUC solution (including ferrocyanide and ferricyanide) for four hours at 37°C after vacuum infiltration at *ca.* 35 kPa for 15 min. Samples were then destained through an ethanol series and the images were captured using a Zeiss Axiophot Compound Light Microscope at 40x magnification. The bar represents 50 µM.

3.2.4.2. The effects of P-deficiency, NPA in combination with either NAA or ACC on GUS expression in PR tips.

To investigate whether the differences in GUS staining in response to Pi^- and ACC treatments is caused by either an increase in the free-auxin levels or a change in auxin sensitivity, NPA was used to inhibit auxin transport in TR29 stolons. A concentration of 100 nM NPA was used because it was shown previously that this concentration is sufficient to inhibit auxin transport and cause severe inhibition of root growth in the 10F genotype (Section 3.1.2.2.2). In addition, 5 nM NAA and 100 nM ACC (both of these concentrations were shown previously to influence root growth; Section 3.1.3.2.1 and 3.1.3.1.1) were also used in combination with 100 nM NPA. To ensure that there was enough time for NPA, NAA and ACC to cause any effects, TR29 stolons were maintained for three days in Pi^+/Pi^-, Pi^+/Pi^- + 100 nM NPA; Pi^+/Pi^- + 100 nM NPA + 5 nM NAA; Pi^+/Pi^- + 100 nM NPA + 100 nM ACC and finally Pi^+/Pi^- + 100 nM NPA + 5 nM NAA + 100 nM ACC media, after an initial period of one days to acclimatise to the hydroponic system. Then, the root systems of these treated stolons were collected to conduct the GUS-staining at 37^{o}C for four hours (as described in Section 2.7.2). The changes of GUS staining patterns were recorded in the PR tips and these patterns are presented as in Figure 3.2.8.

First of all, without hormone treatment, it was found consistently, and in agreement with Section 3.2.4.1, that GUS staining in the P-deficient PR tips was stronger than that observed in the P-sufficient roots (Figure 3.2.8 A and B). There was no staining in the PR tips of plants exposed to the Pi^+ treatment but staining could be observed in the Pi^- treatment. Moreover, when NPA was applied, the PR tips exposed to both the Pi^+ + 100 nM NPA and Pi^- + 100 nM NPA treatments did not display any evidence of staining (Figure 3.2.8 C and D). Thus it was confirmed again that NPA did reduce the free auxin level in PR tips which caused elimination of GUS staining. In contrast, NAA was postulated to intensify GUS staining such that in the Pi^- + 100 nM NPA + 5 nM NAA treatment, PR tips had greater staining in the columella and the QC cells (Figure 3.2.8 F). However, the same concentration of NAA, in the Pi^+ + 100 nM NPA + 5 nM NAA treatment, did not restore the GUS staining which was eliminated by NPA in P-sufficiency (Figure 3.2.8 E). This suggests that 5 nM NAA could not compensate for the reduction in auxin level induced by NPA treatment. Therefore, it is

proposed that this concentration of exogenous NAA could trigger GUS expression in the PR tips due to the increase in auxin sensitivity induced by P-deficiency.

Furthermore, 100 nM ACC was also expected to increase the sensitivity to auxin in the PR tips. When ACC was applied, GUS staining was observed in the columella cells and the QC in the Pi^- + 100 nM NPA + 100 nM ACC treatment (Figure 3.2.8 H) while without ACC, the Pi^- + 100 nM NPA treatment did not display any GUS staining. In contrast, the Pi^+ + 100 nM NPA + 100 nM ACC treatment did not show any GUS staining (Figure 3.2.8 G). This may be the result of an association of both P-deficiency and ACC in changing auxin sensitivity. Furthermore, 5 nM NAA was also used in combination with 100 nM NPA and 100 nM ACC and the GUS staining was successfully induced in the columella cells and the QC in the P-deficiency as shown in the Pi^- + 100 nM NPA + 5 nM NAA + 100 nM ACC (Figure 3.2.8 J). In the P-sufficient treatment, GUS staining could be observed but was very faint (Figure 3.2.8 I). These results confirm that both ACC and P-deficiency increases the auxin sensitivity in white clover roots.

Overall, from the patterns of GUS staining shown in Figure 3.2.8, the effects of NPA and NPA with either NAA or ACC were observed on the white clover PR tips. In the conditions of these treatments, in white clover primary roots, 100 nM NPA was found to reduce the free auxin level which could not be compensated for by the addition of 5 nM NAA in the Pi^+ treatment. However, 100 nM ACC and/or 5 nM NAA in P-deficient media could trigger the GUS expression which was eliminated by 100 nM NPA in the PR tips, presumably by an increase in auxin sensitivity.

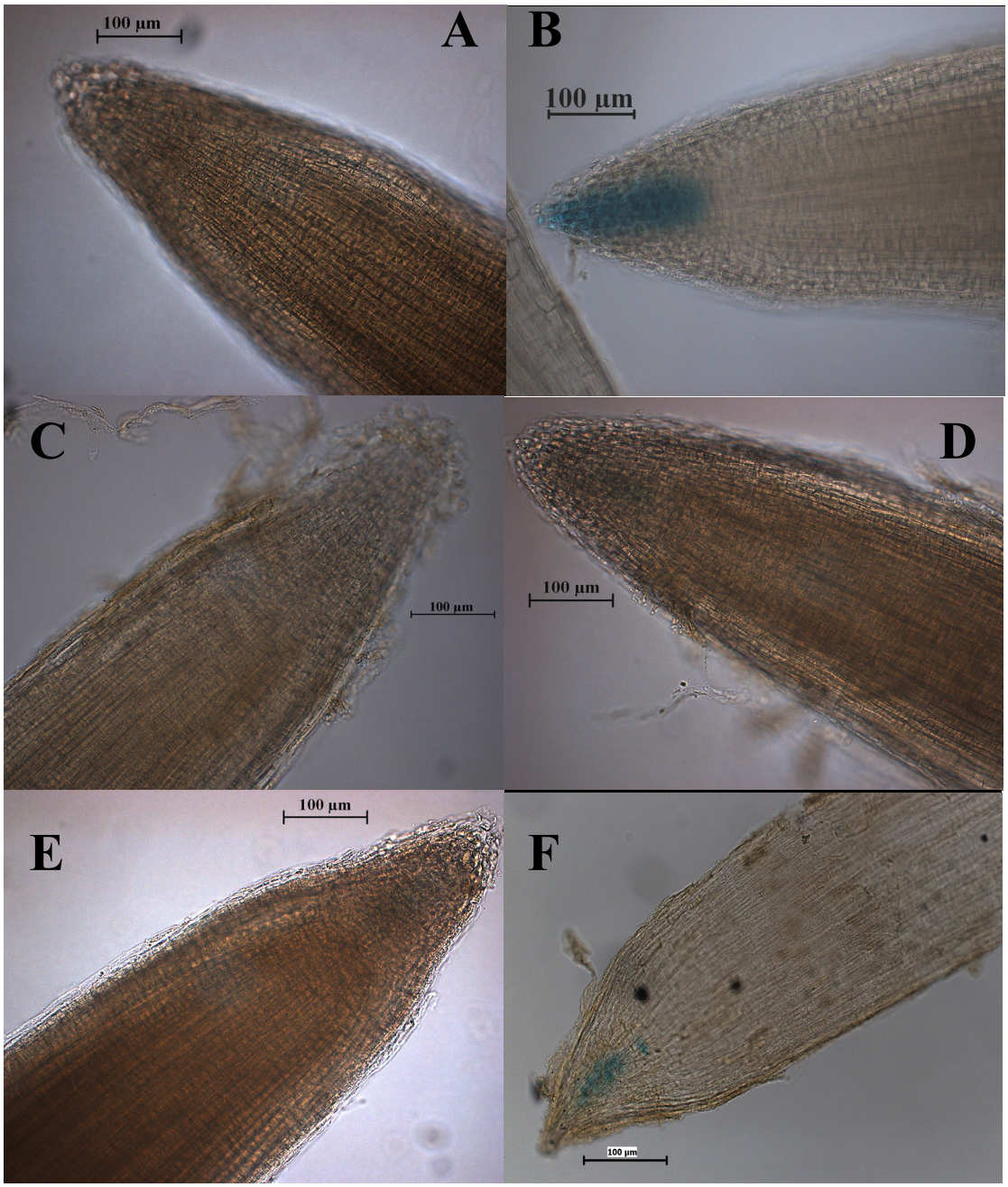


Figure 3.2.8.a. GUS staining of the primary root tips of the TR29 line of *DR5p::GUS* transformed white clover in response to Pi^- deficiency and hormone treatments

Primary roots were collected from treated TR29 stolons treated as indicated

A: P-sufficient treatment (Pi^+),
B: P-deficient treatment (Pi^-),
C: Pi^+ + 100 nM NPA,
D: Pi^- + 100 nm NPA,
E: Pi^++100nM NPA+5nM NAA,
F: Pi^-+ 100 nM NPA + 5 nM NAA,

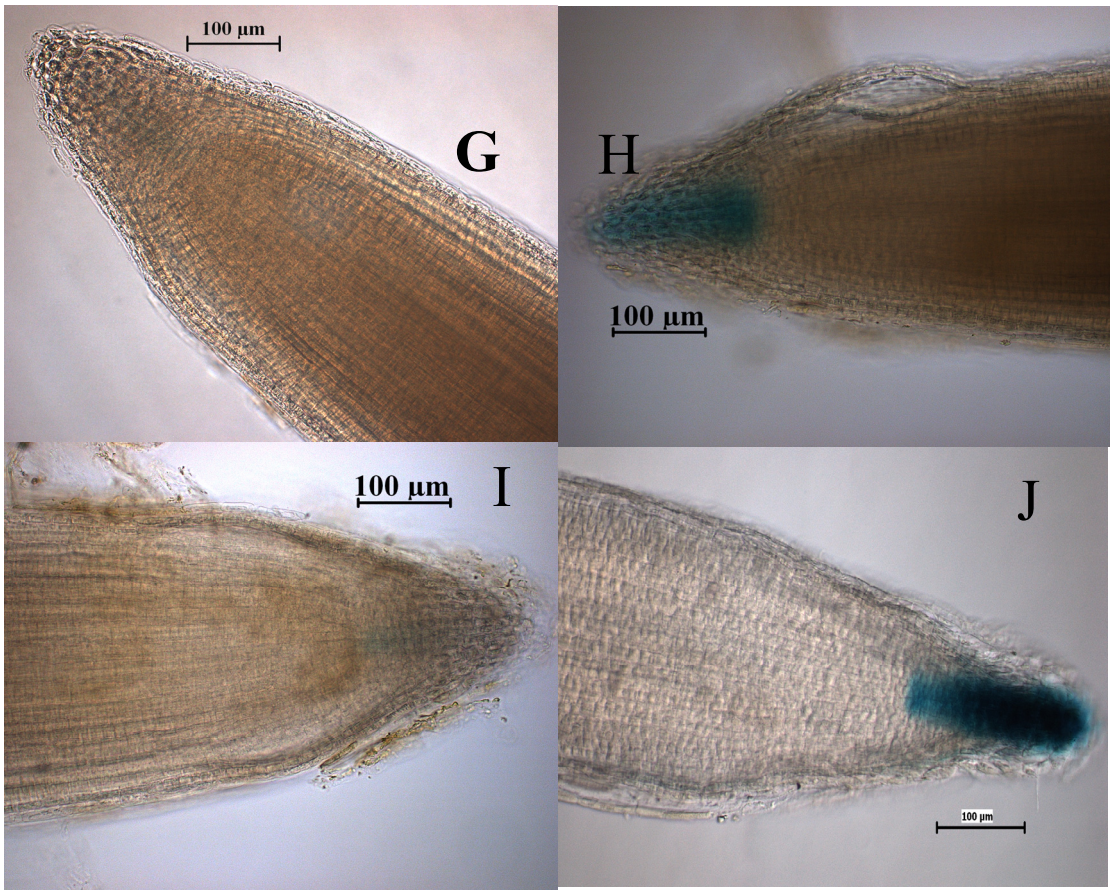


Figure 3.2.8.b. GUS staining of the primary root tips of the TR29 line of *DR5p::GUS* transformed white clover in response to Pi^- deficiency and hormone treatments

Primary roots were collected from treated TR29 stolons treated as indicated

G: Pi^+ + 100 nM NPA + 100 nM ACC,

H: Pi^- + 100 nM NPA + 100 nM ACC,

I: Pi^+ + 100 nM NPA + 5 nM NAA + 100 nM ACC,

J: Pi^- + 100 nM NPA + 5 nM NAA + 100 nM ACC.

PR samples were then stained with X-GLUC solution (including ferrocyanide and ferricyanide) for four hours at 37°C after vacuum infiltration at *ca.* 35 kPa for 15 min, then destained through an ethanol series. The images are captured using a Zeiss Axiophot Compound Light Microscope at 20x magnification. The bar represents 100 µM.

CHAPTER FOUR: DISCUSSION

4.1. WHITE CLOVER ROOT MORPHOLOGY IN RESPONSE TO P-DEFICIENCY

Phosphate (P) supply is one of the major determining factors in plant productivity, and there is much research interest in the influence of P availability on plant growth and development. Therefore, in this thesis, the morphological responses of root systems of the agronomically important legume, white clover (*Trifolium repens* L.), was examined in response to P supply, in particular P limitation.

The concentration of P, supplied as orthophosphate (Pi) at 0.01 mM in Hoagland's media has been shown previously to result in negative effects on white clover growth, such as a decline in leaf phosphate status and the induction of root acid phosphatase activity (Roldan, 2008). Therefore, in this thesis, 0.01 mM Pi was chosen as the concentration for the P-deficient treatments applied to the three genotypes of white clover (Grasslands 10F, Grasslands Huia and a transgenic line, designated TR29) used. In comparison, a concentration of 1.00 mM Pi was used as the P-sufficient control. A preliminary study of the morphology of the roots which emerged from the third and fourth nodes of white clover stolon explants (Figure 3.1.1) showed that the third-node roots responded to the P-deficiency, but the fourth-node roots did not. An explanation for this occurrence is still unknown, but it may be that as the point of excision (wounding effect), proper development of roots did not occur from the fourth-node. Adventitious roots commonly arose from just above the wound site, the development of which has been shown to be regulated by ethylene in other plant species (Lin et al., 1990), and so interaction may interfere with response of the nodal roots. Therefore, in this thesis, the morphology of roots emerging from the third-node as true nodal roots was used to investigate responses to Pi supply.

It has been shown that the three genotypes had different growth rates in terms of primary root (PR) elongation and lateral root (LR) numbers, but they had similar responses to P-deficiency (Section 3.2.3.3). Statistical analysis revealed that the transgenic TR29 line showed the highest growth rate in terms of PRs and LRs, followed by Grasslands *cv.* Huia and finally Grasslands *cv.* 10F (from Section 3.2.3.3, the mean PR length of TR29, Huia and 10F were *ca.* 4.95 cm, 3.52 cm and 2.74 cm, respectively; and the mean LR number of TR29, Huia and 10F were *ca.* 10.29, *ca*.

4.26 and *ca*. 4.26, respectively). The difference in root growth rates may be due to genotypic variation between the Grasslands *cv.* Huia and *cv.* 10F and the effects of transgene (*DR5p::GUS*) inserted into the Huia genome. It is known that white clover germplasm does vary considerably in terms of morphological responses to added Pi (Caradus et al., 1992).

Differences in root morphology have also been observed across four different *Arabidopsis* ecotypes (Col-0, Nossen, RLd and Ws). Although after 12 days of germination on media with 1 μM Pi (P-deficient media), all four ecotypes showed more LRs and shorter PRs than those grown in P-sufficient media with 1 mM Pi, the LR densities of these ecotypes were significantly different (Nob ≈ Col-0 > Rld > Ws) (López-Bucio et al., 2002). However, another factor which could have resulted in root morphological differences in the white clover genotypes observed in this thesis is that the P-supply treatments were not conducted at the same time of year and it is known that day length can influence root growth (Zimmerman and Hitchcock, 1929) and at the same location, light intensity and temperature have also been shown to influence root growth (Lambers and Posthumus, 1980, Davidson, 1969).

However, these three genotypes showed similar overall responses to P-deficiency, such as an increase in PR elongation and also in LR formation which may be part of a mechanism to increase soil exploration by white clover to increase the efficiency of Pi uptake. In the P-deficient media, the mean PR length of each genotype was significantly higher than the P-sufficient controls (Section 3.1.1.1 and 3.2.3). This result confirms the findings of Roldan (2008) in terms of white clover root responses to P-deficiency. The stimulation of PR elongation in white clover is also in common with the Pi-stress responses of most other plants, such as maize (*Zea mays*), rice (*Oryza sativa*), narrow-leafed lupin (*Lupinus angustifolius* L.) and fourteen other monocots and dicots (Narayanan and Reddy, 1982, Mollier and Pellerin, 1999, Shimizu et al., 2004, Wang et al., 2008), which expand their root systems, in order to explore more soil area in response to P-deficiency. This will occur by allocating more resources to the root systems, in order to stimulate root elongation.

However, the changes in root systems, in response to P-deficiency, is not universal and it varies between plant species (Hammond and White, 2007). In contrast to the stimulatory effect of P-deficiency on PR elongation in white clover, when the Pi supply was less than 0.1 mM, PR elongation is inhibited in *Arabidopsis* (Williamson

et al., 2001, Al-Ghazi et al., 2003, López-Bucio et al., 2003, López-Bucio et al., 2005, Jiang et al., 2007) and in common bean (*Phaseolus vulgaris* L.) (Lynch and Brown, 2001). The inhibition in PR is proposed to be through a decrease in meristematic activity (Ticconi et al., 2004, Sanchez-Calderon et al., 2005). In particular, after 16 days of germination on the P-deficient media (0.001 mM Pi), formation of new columella cells in *Arabidopsis* PR tips were arrested, because of the loss of meristematic activity (Sanchez-Calderon et al., 2005). Moreover, in response to P-deficiency, inhibition of PR elongation in *Arabidopsis* is proposed to be actually related to iron (Fe) toxicity, because the PR elongation in P-deficient media was recovered, as in the P-sufficient control, when the iron concentration in the media was decreased (Ward et al., 2008). This P-Fe interaction was not examined in this thesis.

Exposure of all three genotypes of white clover (*cv.* 10F, *cv.* Huia and TR29) to a P-deficient media not only resulted in an increase in PR growth but also stimulated LR formation (Section 3.1.1.1 and 3.2.3), which also enhances the exploratory capacity of the root system. An increase in the number of white clover LRs in the P-deficient media is consistent with the observations of Roldan (2008). In *Arabidopsis*, the stimulatory effects of P-deficiency on LR formation has also been observed (Williamson et al., 2001, Linkohr et al., 2002, López-Bucio et al., 2002, Al-Ghazi et al., 2003, López-Bucio et al., 2003, López-Bucio et al., 2005, Nacry et al., 2005, Sanchez-Calderon et al., 2005, Jain et al., 2007, Jiang et al., 2007). In contrast, Linkohr et al. (2002) found negative effects of P-deficiency, such that a decreased number of initiated LRs was observed in *Arabidopsis*.

Furthermore, in this thesis, the mean total LR length of Grasslands 10F was also observed to be markedly increased in response to P-deficiency (Section 3.1.1.1). In *Arabidopsis*, Al-Ghazi et al. (2003) also found an increase in LR length in response to P-starvation (media without Pi). However, depending on the time of P-deficient treatment (at 3 μM Pi), responses in *Arabidopsis* varied. For example, LR development was induced from 7 to 11 DAT, but was inhibited after 11 days. Also, the P-deficient effects varied at different LR developmental stages. For instance, P-deficiency activated already initiated root primordia, but inhibited the initiation of new primordia (Nacry et al., 2005).

Overall, using the experimental conditions as described in this thesis, exposure to P-deficiency resulted in the stimulation of PR elongation, LR formation and LR

elongation in white clover, and is a proposed mechanism to enhance soil exploration and Pi uptake ability by the root systems. The stimulation in PR elongation is in common with rice, maize and lupin but in contrast with *Arabidopsis* and common bean. However, the stimulation of LR formation and elongation is in common with almost all other species in terms of responses to P-deficiency.

4.2. THE EFFECTS OF PLANT HORMONES ON ROOT MORPHOLOGY OF WILD TYPE WHITE CLOVER WHEN SUBJECTED TO P-DEFICIENCY

4.2.1.Ethylene

Ethylene has been shown to play an important role in root development, including root primordia formation, root elongation and root gravitropism (Clark et al., 1999, Buer et al., 2003, Buer et al., 2006). In particular, root system responses to P-deficiency have been shown to occur through changes in ethylene biosynthesis and sensitivity (Lynch and Brown, 1997). Thus the role of ethylene in root growth, in addition to the root responses to P-deficiency of white clover, was studied.

A range of concentrations of 1-aminocyclopropane-1-carboxylate (10 μM, 1 μM and 100 nM ACC), an ethylene precursor, were used initially to study white clover root morphological responses to ethylene. Both 10 μM and 1 μM ACC treatment caused severe inhibitory effects on PR elongation and LR growth in P-sufficient media (Section 3.1.2.1.1). After eight days in the Hoagland's media containing 1 μM ACC, the root parameters were approximately half of the control roots and more drastically, in the Hoagland's media containing 10 μM ACC, PR elongation had almost ceased. In addition, it was observed that many of these treated roots (PRs and LRs) lost the gravitropic response.

In *Arabidopsis*, it was found that 10 μM, 1 μM and 100 nM ACC treatment caused inhibitory effects, not only on PR elongation but also on LR formation, in plants grown in both P-sufficient and P-deficient media (López-Bucio et al., 2002). For example, *Arabidopsis* germinated on media with 1 μM ACC developed shorter PRs than those germinated on the control media without ACC (Ruzicka et al., 2007). The inhibitory effects on root growth were assumed to be a consequence of rapid down regulation of cell elongation and division, caused by the ethylene evolved from the ACC (Le et al., 2001). In addition to decreased growth, *Arabidopsis* roots also lost the

gravitropic response in a 2.5 μM ACC treatment (Buer et al., 2006). Recently, Ivanchenko et al. (2008) showed that a high concentration of ethylene (using more than 100 nM ACC as the exogenous ethylene treatment) inhibited PR elongation, LR initiation and LR emergence in *Arabidopsis*.

On the other hand, in *Arabidopsis*, very low concentrations of ethylene (40 nM or 80 nM added ACC) did not alter PR elongation but did promote LR initiation (through a proposed influence on the activity of cells in the pericycle) and LR emergence (Ivanchenko et al., 2008). Previous research has shown that ACC oxidase is expressed in root tissue of white clover and is induced in response to P-deficiency (Roldan, 2008). Therefore, white clover root growth was also studied in response to a lower concentration of ACC (100 nM) in the media. In P-sufficient media, 100 nM ACC did slightly stimulate the elongation of white clover PRs and LRs even though the differences between the 100 nM ACC treatment and the control were not statistically significant (Section 3.1.2.1.2). However, it was found that the 100 nM ACC treatment enhanced the stimulatory effects of P-deficiency on white clover root growth. This included PR elongation and more markedly LR formation and elongation (Section 3.1.3.1.1). The mean LR number and the mean total LR length in the P-deficient treatment combined with 100 nM ACC were significantly higher than those in the P-deficient treatment without ACC, which in turn were slightly higher than the P-sufficient treatment, regardless of whether ACC was added or not. Thus low level of ethylene is hypothesised to play an active role in mediating root growth by increasing the extent of the root system to increase Pi uptake capability in P-deficient conditions.

The positive influence of 100 nM ACC treatment on white clover root growth is in common with *Arabidopsis*. As mentioned above, 40 nM or 80 nM ACC concentrations could increase LR initiation and LR emergence in *Arabidopsis* (Ivanchenko et al., 2008). Furthermore, under P-deficiency, the stimulatory effects of ethylene on root elongation were observed not only in *Arabidopsis* (Ma et al., 2003) but also in common bean (*Phaseolus vulgaris* L.) (Borch et al., 1999). It has been shown that ethylene has opposite influences on root systems depending on whether the roots are exposed to P-sufficient or P-deficient (less that 0.05 mM Pi) media such that ethylene is involved in P-deficient root responses by promoting root growth (López-Bucio et al., 2002, Ma et al., 2003, Zhang et al., 2003). However, the signalling

pathway through which ethylene mediates root responses is still unidentified (Yuan and Liu, 2008)

In addition to the ACC treatment, white clover was also treated with 300 ppm 1-methylcyclopropene (1-MCP) to inhibit the action of ethylene in both P-deficient and P-sufficient media. In P-sufficient media, plants exposed to 300 ppm 1-MCP showed severe inhibition of PR elongation and LR growth (Section 3.1.2.1.3). For instance, in the P-sufficient treatment exposed to 300 ppm 1-MCP, the mean PR length (*ca.* 1.80 cm), the mean LR number (*ca*. 0.9 LR) and the mean total LR length (*ca*. 0.58 cm) were significantly lower than in the P-sufficient control (*ca.* 2.13 cm as the mean PR length, *ca* 2.02 LRs with *ca.* 1.04 cm as the mean total LR length). It shows that ethylene not only plays an active role, but also an essential role in regulating root growth. In contrast, exposure of plants to the P-deficient media resulted in some reversal of the inhibitory effects of 300 ppm 1-MCP (Section 3.1.3.1.2). For example, the mean PR length in the Pi^{-} + 300 ppm 1-MCP treatment was significantly higher than in the Pi^{+} control and the Pi^{+} + 300 ppm 1-MCP treatment. Also, the mean LR number and the mean total LR length in the Pi^{-} + 300 ppm 1-MCP treatment were similar to the Pi^{+} control which were considerably higher than those in the Pi^{+} + 300 ppm 1-MCP treatment, but they were significantly lower than those in the Pi^{-} treatment. These results suggest that in white clover, the concentration of 1-MCP (300 ppm) was sufficient to totally inhibit PR elongation and LR development in P-sufficient roots, but it only partially inhibited root growth in plants exposed to P-deficiency. This suggests that P-deficiency may cause an increase of endogenous ethylene or an increase of ethylene sensitivity.

In contrast, when ethylene synthesis or action is inhibited in common bean (Borch et al., 1999) or in *Arabidopsis* (Ma et al., 2003), root elongation was observed to be stimulated under P-sufficiency but inhibited in P-deficiency. In addition, in the P-deficient media, root hairs were initiated nearer to the root tips while in response to P-sufficiency, the root hairs were initiated farther from the root tips (Borch et al., 1999, Ma et al., 2003). In the common bean, when ethylene synthesis was inhibited by 1.3 μM amino-ethoxyvinylglycine (AVG), the LR density increased when grown in P-deficient soil, but it decreased when grown in P-sufficient soil (Borch et al., 1999). In addition, less than 0.1 ppm of exogenous ethylene was able to reverse the influence of AVG, in which the LR density decreased in P-deficiency and increased in P-

sufficiency. Borch et al. (1999) also suggested that the influences of ethylene on common bean root growth depended on phosphate availability due to the fact of P-deficiency was proposed to change ethylene responsiveness and synthesis.

In addition, by using ethylene inhibitors or an ethylene precursor (ACC), in *Arabidopsis*, ethylene was found not to be involved in the initiation of LRs, but it played an important role in inhibiting PR elongation and stimulating LR elongation in response to phosphorus stress (López-Bucio et al., 2002, Ma et al., 2003). As in *Arabidopsis*, in white clover the use of an ethylene inhibitor (1-MCP) or the ethylene precursor ACC demonstrated the important roles of ethylene in mediating root growth in normal conditions or in response to P-stress. Depending on the exogenous ethylene concentration added, the positive effects of low concentrations of ACC or the negative effects of high concentrations ACC on root growth were observed in white clover here and in *Arabidopsis* (Ivanchenko et al., 2008). Therefore, regulation of the ethylene level is necessary for plant growth and development. Moreover, in agreement with the other studies in *Arabidopsis* and in common bean (Borch et al., 1999, Ma et al., 2003, Zhang et al., 2003), the influences of ethylene on root growth depend on P-availability. The presumption that P-deficiency increases the level of ethylene, or increases the ethylene sensitivity of white clover root systems, might explain the elimination of the inhibitory effect of 1-MCP in P-deficiency, in terms of PR elongation and LR development. However, it has not been verified yet whether an increase in ethylene biosynthesis or change in ethylene sensitivity determines this response.

4.2.2.Auxin

In common with ethylene, auxin also plays a critical role in mediating PR growth, LR production and the gravitropic responses of the root system (Casimiro et al., 2001, Malamy and Ryan, 2001, Oono et al., 2003). Auxin is considered to be the key signal in mediating LR formation (Casimiro et al., 2003, De Smet et al., 2006), including the initiation of LR primordia (Benková et al., 2003, Casimiro et al., 2003) and the emergence of LR primordia (Laskowski et al., 2006). Thus in this study, a range of concentrations of exogenous auxins (1-naphthylacetic acid, NAA and indole-3-acetic acid, IAA) were applied, in order to study white clover root responses both in P-sufficient and P-deficient media.

In P-sufficient media, exposure to both 50 nM NAA (data not shown) and 100 nM NAA caused extremely inhibitory effects on PR elongation and also on LR development (LR numbers and total LR lengths). After eight days in the Hoagland's media with 100 nM NAA, the mean PR length was only two thirds of the length of roots in the P-sufficient control and the mean LR number (and the mean total LR length) was only half of the values measured in the control treatment (Section 3.1.2.2.1). Similarly, *Arabidopsis* which have been germinated on media supplemented with 200 nM NAA, had a shorter PR than in the control (Ruzicka et al., 2007). In addition, exogenous auxin added as 2,4-D (from 5 nM to 50 nM) was found to severely inhibit PR elongation in plants grown in P-sufficient conditions (López-Bucio et al., 2005).

In contrast, a stimulatory influence of exogenous auxin in white clover root growth was found in plants grown on P-sufficient media when the concentration of NAA was reduced to 5 nM, or the concentration of IAA was reduced to 50 nM and 5 nM. For example, 5 nM NAA treatment resulted in a significant increase in PR length, LR number and total LR length (*ca*. 3.67 cm, *ca*. 5.75 and *ca*. 2.86 cm, respectively), in comparison with the P-sufficient control (*ca.* 3.20 cm, *ca*. 4.39 and *ca.* 1.82 cm, respectively, Section 3.1.2.2.1). Thus the effects of exogenous auxin were dependent on the auxin concentration, and also dependent on which auxin was used, as to whether there was negative or positive influence on root growth. It was also discovered in *Arabidopsis* that exogenous auxin (less than 100 nM NAA) reduced PR elongation (Casimiro et al., 2001), but it stimulated LR formation (Casimiro et al., 2001), while in radish (*Raphanus sativus*), a low concentration of exogenous auxin increased LR production (Blakely et al., 1988).

As mentioned previously, exogenous auxin can mimic the effects of P-deficiency on *Arabidopsis* root growth in terms of the inhibition of PR elongation and the stimulation of LR formation (López-Bucio et al., 2002, Nacry et al., 2005). This suggests that either an increase in synthesis or increase in sensitivity of auxin plays an important role in responses to P-deficiency (López-Bucio et al., 2002). However, in *Arabidopsis*, the role of auxin in regulating root responses to P-deficiency is not consistent. For instance, Williamson et al. (2001) revealed that root responses to P-deficiency were independent of auxin signalling, but they were dependent on the shoot phosphate status. In particular, the PR response to P-deficiency (from 0.01 mM to

0.05 mM Pi) was found to be auxin-independent, because the PR responses of auxin-resistant mutants (*axr1, axr2* and *axr4*) to P-deficiency were similar to the responses of wild types (Williamson et al., 2001, Lopez-Bucio et al., 2002, Al-Ghazi et al., 2003). In contrast, the LR response to P-deficiency (0.02 mM Pi) was declared to be auxin-dependent in *Arabidopsis* (Jain et al., 2007). Therefore, the role of exogenous auxin (5 nM NAA) was investigated in terms of root responses of white clover to P-deficiency (0.01 mM Pi).

In this thesis, treatment of roots in the P-deficient media with 5 nM NAA resulted in the mean PR length being significantly lower than that measured in white clover PRs exposed to a 5 nM NAA treatment in P-sufficient media, but it was not different to the length in the P-sufficient or the P-deficient treatments with no NAA added. This means that 5 nM NAA stimulated PR elongation under P-sufficiency, but not in white clover plants exposed to P-deficiency. In contrast, 5 nM 2,4-D treatment reduced PR elongation in *Arabidopsis* grown on both P-sufficient and P-deficient media (López-Bucio et al., 2005).

For LR development in white clover, the 5 nM NAA treatment in combination with P-deficiency did not differ from the P-sufficient control. However, LR number and total LR length in the 5 nM NAA treatment under P-deficiency were significantly lower than those in the P-deficient treatment with no added NAA and were markedly lower than the 5 nM NAA treatment under P-sufficiency. Therefore, 5 nM NAA inhibited LR growth under P-deficiency, but it stimulated root growth under P-sufficiency. These results again suggest that low concentrations of auxin (5 nM NAA) applied to plants grown under P-deficiency reversed the effect of P-sufficiency on white clover root growth. In contrast, treatment with less than 10 nM of exogenous 2,4-D was found to have a greater stimulatory effect on LR development in *Arabidopsis* when maintained in P-deficient media when compared with plants maintained in P-sufficient media. Possibly, these two species (white clover and *Arabidopsis*) show different responses to different exogenous auxin (2,4-D or NAA) treatments. Presumably, P-deficiency increases either auxin biosynthesis or auxin sensitivity in white clover and so 5 nM exogenous NAA could induce “overdose” auxin symptoms in root systems which are similar to applying high concentrations of exogenous auxin to a P-deficient root.

However, in *Arabidopsis*, it was observed that NAA, when added at 100 nM, amplified the effects of P-starvation (no Pi supply) on PR elongation, but it abolished the effects of P-starvation on LR elongation (Al-Ghazi et al., 2003). It was also suggested that auxin signalling was involved in the P-deprivation responses (Al-Ghazi et al., 2003). In addition, in *Arabidopsis* grown in the P-deficient media (0.02 mM Pi), the pattern of auxin accumulation was assumed to be redistributed, such as a relocation of free auxin from the PRs to the LRs (Jain et al., 2007). A redistribution of auxin was also observed in *Arabidopsis* grown in the nutrient media containing only 0.003 mM Pi. For example, auxin over-accumulated in the apex of PRs and LRs, and also in already formed LR primordial which resulted in a reduced PR elongation rate, a reduced primordia abortion and earlier LR emergence (Nacry et al., 2005). However, the auxin level was found to decrease in PR zone where the LR primordia were induced to initiate from the PR pericycle cells (Nacry et al., 2005). That resulted in a decrease of primordia density. Also, low P availability (0.003 mM Pi) was found to result more in the mediation of auxin transport to redistribute the auxin, rather than increasing auxin synthesis (Nacry et al., 2005).

In addition to the redistribution of the auxin, P-starvation has been shown to increase the sensitivity of *Arabidopsis* roots, PR tips and LR primordia to auxin (López-Bucio et al., 2002, Nacry et al., 2005), whilst exposure to high Pi in the media decreases auxin sensitivity (López-Bucio et al., 2002, Sanchez-Calderon et al., 2005). An increase in auxin sensitivity in P-deficiency could explain the adverse effects of 5 nM NAA treatment on white clover root growth when grown under P-deficiency. That is, 5nM NAA was sufficient to cause auxin toxicity (similar to the effects of high auxin concentration) in plants grown under P-deficiency, but not so in those grown in P-sufficiency.

In *Arabidopsis*, a change in auxin transport is involved in the regulation of the initiation of LR primordia in response to P-deficiency (0.001 mM to 0.003 mM Pi) (López-Bucio et al., 2005, Nacry et al., 2005). Thus the white clover explants were not only treated with exogenous auxin but they were also treated with the auxin transport inhibitor (1-N-naphthylphthalamic acid, NPA), in order to investigate root growth and root responses when the auxin level was reduced. A range of NPA concentrations (10 μM to 1 μM and 100 nM) in the Hoagland's media were used to treat the white clover stolons but both the 10 μM and 1 μM treatments had an extreme effect on the root

systems, such as halting root growth and inducing root necrosis. The 100 nM NPA treatment was sufficient to cause an inhibitory effect on PR elongation and also LR development (LR number and total LR length) but with no obvious damage to the root systems (Section 3.1.2.2.2). It was shown that the mean PR length, the mean LR number and the mean total LR length in the 100 nM NPA treatments, regardless of exposure to P-sufficient or P-deficient conditions, were significantly lower than the Pi^{+} and Pi^{-} treatments without added NPA (Section 3.1.3.2.2).

Consistently, it has been found that auxin transport inhibitors reduce PR elongation (Ivanchenko et al., 2008) and inhibit LR formation in *Arabidopsis*, both in high and low (0.001 mM) Pi media (Reed et al., 1998, López-Bucio et al., 2002). NPA is proposed to prevent LR development by "blocking the first transverse divisions" of root cells by causing an accumulation of endogenous IAA in the root apex, and a reduction of the IAA level in the basal tissues, which are critical for LR initiation (Casimiro et al., 2001). However, this inhibitory effect of NPA can be restored by exogenous IAA in *Arabidopsis* (Reed et al., 1998).

More recently, it has been observed that the application of other auxin transport inhibitors, such as brefeldin A (BFA) or 2,3,5-triiodobenzoic acid (TIBA) to *Arabidopsis* inhibited LR formation in both P-sufficienct and P-deficient treatments but the effect was more marked in plants grown in P-sufficient media (López-Bucio et al., 2002, Nacry et al., 2005, López-Bucio et al., 2005). Furthermore, Perez-Torres et al. (2008) found that *Arabidopsis* grown on P-deficient media (0.01 mM Pi) comprising 1 μM NPA could form LR primordia, whilst no primordia formed in the P-sufficient media supplied with NPA. These seedlings, both on P-deficiency and P-sufficiency were then supplied with a low concentration of NAA (10 nM), which subsequently resulted in LR production in seedlings maintained in the P-deficient media but not in those maintained in the P-sufficient media. Therefore, it could be confirmed that "P deprivation heightens the root system's sensitivity to auxin" (Perez-Torres et al., 2008). Moreover, 20 μM BFA not only inhibited LR formation, but it also abolished gravitropic responses (López-Bucio et al., 2005).

Overall, it has been suggested that auxin plays a role in regulating root growth in *Arabidopsis* and white clover, because a reduction in auxin level mediated by the auxin transport inhibitor (NPA), inhibited root development. Also, auxin transport plays a fundamental role in activating the pericycle cells to form LR primordia

(López-Bucio et al., 2005). In addition, the phosphate response pathways act through auxin signalling and P-deficiency increases auxin sensitivity (Malamy, 2005). Particularly in white clover, the competence of a response by the root system to P-deficiency was lost when auxin transport was inhibited by 100 nM NPA. It is possible that 100 nM of NPA was too high to mediate the root responses to phosphate availability.

4.3. THE INTERACTION BETWEEN ETHYLENE AND AUXIN IN MEDIATING WHITE CLOVER ROOT GROWTH

It was noted previously that treatment with 100 nM ACC had a positive effect on white clover root growth whereas 100 nM NPA extremely inhibited this growth (3.1.2.1.2 and 3.1.2.2.2). However, the positive role (on root growth) of 100 nM ACC treatment could not overcome the inhibitory effect of NPA on PR elongation. When white clover stolons were treated with 100 nM ACC in combination with 100 nM NPA, the PR length was almost the same as with the NPA treatment only and it was significantly shorter than that measured in plants grown in the P-sufficient control media (3.1.2.3.1). In common with white clover, in *Arabidopsis*, PR elongation in the combination treatment of ACC (1 μM and 100 nM) and NPA (also 1 μM and 100 nM) is consistently significantly lower than those in the control treatment without ACC and NPA (Ruzicka et al., 2007).

However, in white clover a slight recovery in LR formation was shown at 6 DAT and 8 DAT, where the mean LR number in the Pi^+ + 100 nM ACC + 100 nM NPA treatment was twice that observed in the Pi^+ + 100 nM NPA treatment. In addition, all values (including PR length, LR number and total LR length) in both treatments were still significantly lower than the control (Pi^+, plants grown in the P-sufficient media only) so that treatment with 100 nM ACC could only partially restore white clover root growth from the inhibitory effects of the 100 nM NPA treatment, particularly in terms of LR formation. In contrast, 1 μM NPA and 200 nM ACC show cumulatively adverse effects on *Arabidopsis* LR initiation, but not in cell length or cell production rate (Ivanchenko et al., 2008). These workers suggested that "either the promoting effect of low ACC concentrations on lateral root initiation is mediated through auxin signaling, or that the absence of auxin signaling is limiting, and that ethylene is insufficient to override the absence of the auxin signal". Therefore, when auxin

transport was inhibited by NPA, the effects of exogenous ethylene (or applied ACC) was eliminated. This could explain the inefficiency of 100 nM ACC to restore growth in the presence of the inhibitory effects of 100 nM NPA in white clover. In *Arabidopsis* mutants that are affected in auxin perception or basipetal auxin transport, ethylene could not stimulate the auxin response, nor mediate root growth (Ruzicka et al., 2007).

It was also noted previously that the 300 ppm 1-MCP treatment inhibited root development, whereas treatment with 5 nM NAA stimulated root growth of white clover (Section 3.1.2.1.3 and 3.1.2.2.1). When 5 nM NAA was combined with 300 ppm 1-MCP as a treatment, the mean PR length was no different from that measured in the P-sufficient control and the 300 ppm 1-MCP treatment only, but was significantly lower than the lengths in the Pi^+ + 5 nM NAA treatment only. Thus the 5 nM NAA treatment could only partially overcome the inhibitory effect of 1-MCP in terms of PR elongation. In contrast, for LR development, added 5 nM NAA could not overcome the inhibitory effect of 1-MCP (Section 3.1.2.3.2). Therefore, the stimulatory effects of treatment with 5 nM NAA could not totally overcome the inhibitory effects of the 300 ppm 1-MCP treatment on PR elongation and LR formation. These results suggest that ethylene is essential, not only in mediating root growth and root responses to P-deficiency, but also for responses to auxin. This is in contrast with the report from Stepanova et al. (2007) in which auxin-mediated responses in *Arabidopsis* roots did not require ethylene.

Ethylene has been proposed to mediate root growth, particularly PR elongation, through an increase in auxin biosynthesis and through stimulation of basipetal auxin transport towards the root elongation zone, where a local auxin response is activated to inhibit cell elongation (Ruzicka et al., 2007). In agreement with Ruzicka et al. (2007), ethylene was found to induce auxin biosynthesis in the root apex and both of these hormones together inhibited root elongation (Swarup et al., 2007). The effect of ethylene on cell expansion in *Arabidopsis* roots depends on auxin transport and auxin responses in the elongation zone. In addition, Rahman et al. (2001) and Swarup et al. (2007) revealed that exogenous auxin heightened the inhibitory effects of high concentrations of ACC on root cell elongation in *Arabidopsis*. On the other hand, 1 μM IAA could override the negative effects of 1 μM ACC to induce LR formation (Negi et al., 2008). It was also found that the inhibitory effects of ethylene required

"normal levels of auxin biosynthesis, transport, and/or response" but auxin-mediated responses did not require ethylene to inhibit root growth (Stepanova et al., 2007). To avoid the severe effects of ACC and IAA, Ivanchenko et al. (2008) reduced the concentration of IAA to 50 nM and ACC to 200 nM, in a combination treatment. They then found that auxin and ethylene acted synergistically to inhibit LR formation in *Arabidopsis* roots (Ivanchenko et al., 2008). In addition, in this thesis, a lower concentration of added ACC (100 nM) could not overcome the inhibitory effect of added 100 nM NPA on PR elongation but could slightly overcome the inhibitory effect on LR formation in white clover. In white clover, added 100 nM NPA may still be high to cause the severely adverse influences which could not be overridden by the stimulatory effects of added 100 nM ACC on root growth. Even though the interaction between auxin and ethylene in mediating white clover root growth is not clear, it is known in *Arabidopsis* that auxin interacts with ethylene in mediating the root meristem by regulating cell expansion (Swarup et al., 2007, Ruzicka et al., 2007).

4.4. GUS EXPRESSION PATTERNS IN *DR5p::GUS* WHITE CLOVER TRANSFORMANTS IN RESPONSE TO P-DEFICIENCY AND TO HORMONE MANIPULATED TREATMENTS

4.4.1. GUS expression levels in different transgenic lines

The young shoot apices and the nodal root primordia were chosen as tissues for GUS analysis, because it has been shown previously that both of these tissues have high GUS expression driven by the DR5 promoter (Aloni et al., 2003). Using GUS staining (Section 2.7.1), the transgenic white clover lines were screened to identify those expressing the GUS transgene. From the 55 *DR5p::GUS* transgenic lines examined, there were 26 putative transgenic lines displaying GUS staining, both in the shoot apices and in the nodal root primordia. The intensities of these stains could differ, such as staining in the shoot apices, which were stronger than those in the nodal root primordia and *vice versa*. One line (TR6) displayed GUS staining only in the apices, whilst TR1 and TR17 displayed GUS staining only in the nodal root primordia, and the other 26 lines did not display any GUS staining (Table 3.2.1). Variations in the localisation of GUS expression among the different transgenic lines may be caused by the random insertion of the *DR5p::GUS* construct into the genome, as has been observed previously with the insertion of a *GH3p::GUS* construct (Larkin et al.,

1996). Also, differences in the insert position, or in the number of *DR5p::GUS* copies (because of random insertion), could cause the various levels of GUS staining observed, which produced intense GUS staining in some lines, whereas there was very faint staining in others (Figure 3.2.2 and Table 3.2.1).

If the PCR results were compared, there were 28 transgenic white clover lines that were positive for both PCR and GUS staining. In contrast, nine lines were positive by PCR, but negative by GUS-analysis. This could be due to gene silencing of the inserted constructs which can be influenced by the number of transgene copies (usually high) through the regulatory mechanisms of transgene transcription or post-transcriptional silencing, or by transgene methylation (Fagard and Vaucheret, 2000). On the other hand, one line, TR1, was negative by PCR, but positive by GUS analysis. It is possible that TR1 was generated from the already differentiated epicotyl and hypocotyl not going through the callus stage, after co-cultivation with the *Agrobacterium tumefaciens* carrying the *DR5p::GUS* construct and so may be a chimera. Therefore, different parts of TR1, such as the root primordia may be carrying the *DR5p::GUS* construct but the leaves (used as the material for PCR analysis) and the shoot apices are not transformed. At the end of the transformation process, the achieved transformation frequency was 0.56% which belongs to the range reported by Ding et al. (2003) (from 0.3% to 6% for white clover transformation).

During the planting and multiplication processes in the GMO glasshouse, only eight of these lines had the capacity to provide sufficient stolons for further experiments (TR2, TR4, TR24, TR29, TR34, TR44, TR48 and TR66). By Southern analysis, it was assumed that TR24 had at least four copies; TR48 three copies; and TR66 and TR34 had two copies of *DR5p::GUS*. Two other lines, TR44 and TR29 are proposed to have a single copy of *DR5p::GUS* (Section 3.2.2.3). In contrast, the probe did not hybridise to any band in TR2 and TR4, which were positive by PCR and GUS screening. This may be explained by the loss of genomic DNA during DNA extraction, the DNA degradation which damaged the *DR5p::GUS* constructs or the concentration of the loaded DNA was not sufficient to detect by DIG-labelling (nonradio-active labelling) Southern analysis method. Thus TR2 and TR4 could be analysed further by the use of more sensitive radio-labelled probes.

4.4.2.The effects of P-deficiency, ethylene and auxin on GUS expression in PR tips

As mentioned previously, it is proposed that P-deficiency and ethylene may alter the sensitivity of the root to auxin. Therefore, changes in auxin responsiveness in white clover root system in response to P-deficiency and hormone treatment were assessed with the *DR5p::GUS* transformants. After overnight staining, GUS activity was found to be very intense in the PR tips, LR tips and LR primordia of all the *DR5p::GUS* transgenic lines examined even though the intensity varied in different lines. Again, this may have been caused by the random insertion of the *DR5p::GUS* construct into the genome as has been observed for other transgene inserts in white clover (Larkin et al., 1996). However, PR tips were the focus of staining as it has been claimed that the root cap, particularly the quiescent centre (QC), was involved in sensing nutrient deficiency (Sanchez-Calderon et al., 2005, Svistoonoff et al., 2007). In addition, the QC plays an important role in root responses including mediating root system architecture (Sanchez-Calderon et al., 2005).

Therefore, the focus of any change in auxin responsiveness in response to P-deficiency and hormone treatment was investigated in the PR tips of TR29. Among the *DR5p::GUS* transgenic lines, TR29 was chosen to represent the GUS expression patterns because in TR29, harbouring a single copy, GUS activity was found to stably expresse. After staining overnight, GUS activity was located mainly in the columella cells and the QC of the root tips which were exposed to both P-sufficiency and P-deficiency. This is in common with *Arabidopsis*, in which GUS activity driven by the DR5 promoter was also found in the columella and the QC cells of PRs in 10 day-old seedlings maintained in P-sufficient media (Sabatini et al., 1999, López-Bucio et al., 2005, Sanchez-Calderon et al., 2005).

However, it was difficult to compare the intensity of GUS staining between treatments (Pi^+ and Pi^-) in the same lines. Therefore, the staining time was shortened to four hours to make it possible to detect differences in GUS activity in response to P-availability and hormone treatment. In TR29, intense GUS staining was observed only in the PR tips of plants maintained in the P-deficient media and in both P-deficient and P-sufficient media when combined with treatment with 100 nM ACC, but it was not observed in the P-sufficient control without added ACC (Section 3.2.4.1) at 1 DAT. Therefore, GUS activity was enhanced in plants exposed to P-deficiency and

also by the addition of 1 μM ACC (regardless of exposure to P-sufficiency or P-deficiency) just one day after treatment. In other words, auxin responsiveness was found to be enhanced in the PR tips by P-deficiency, or a treatment to elevate ethylene production. Enhancement of auxin responsiveness could be caused by the increase of either auxin accumulation or auxin sensitivity in the PR tips during P-deficiency or during ethylene treatment as has been observed in *Arabidopsis* (Lopez-Bucio et al., 2002, Nacry et al., 2005, Stepanova et al., 2005, Stepanova et al., 2007, Swarup et al., 2007, Ivanchenko et al., 2008). Moreover, Ivanchenko et al., (2008) revealed that ethylene influences LR initiation, through its effect on the PR tips, particularly in the QC (Ortega-Martinez et al., 2007).

In contrast, it is claimed that P-deficiency reduced GUS expression (under control of DR5 promoter) in the *Arabidopsis* PR tips (López-Bucio et al., 2005, Sanchez-Calderon et al., 2005). This reduction of auxin responsiveness could be a consequence of the redistribution of auxin, which preferentially accumulated in the LR primordia rather than in the PR tips (López-Bucio et al., 2005, Sanchez-Calderon et al., 2005). Therefore, the GUS expression, under control of DR5 promoter, was found to be enhanced in the LR primordia when plants were grown in P-deficient media (López-Bucio et al., 2005).

Furthermore, in this thesis, 100 nM NPA was applied in order to determine whether auxin responsiveness or auxin biosynthesis changed in response to P-deficiency and ethylene treatment. It was found that 100 nM NPA treatment suppressed GUS expression in the PR tips in plants grown in both P-sufficient and P-deficient media (Section 3.2.4.2) and it has been previously shown that NPA treatment resulted in the severe inhibition of white clover root growth (Section 3.1.2.2.2). Thus 100 nM NPA was confirmed as being sufficient to reduce the free auxin level in PR. Ivanchenko et al. (2008) also found NPA decreased the GUS expression (driven by DR5 promoter) in the columella and QC cells.

In addition, in white clover, 5 nM NAA treatment could intensify GUS staining in the columella and in the QC of the PR tips from plants maintained in the P-deficient media with NPA treatment, but it could not intensify staining in the NPA treatment in P-sufficient media. Therefore, under P-deficiency, 5 nM NAA treatment could compensate for the reduction in the auxin level induced by NPA treatment, but not under P-sufficiency. Thus it is proposed that this concentration of exogenous NAA

could trigger GUS expression in the PR tips due to an increase in auxin sensitivity induced by the P-deficiency.

Moreover, it is also proposed that the 100 nM ACC treatment increases the sensitivity to auxin in the PR tips, because GUS staining was observed in white clover PR tips when ACC was applied to the NPA treatment under P-deficiency. However, in P-sufficiency, added ACC could not trigger GUS expression in the NPA treated roots. This may be the result of an association of both P-deficiency and ACC to change auxin sensitivity.

Finally, added 5 nM NAA was also used in combination with added 100 nM ACC in the 100 nM NPA treatment and both of these factors successfully resulted in GUS staining under P-deficiency, but not under P-sufficiency (Section 3.2.4.2). These results suggest that both added ACC and P-deficiency treatment increase the auxin sensitivity of white clover roots and this is consistent with the results found in *Arabidopsis*. That is, added ACC and IAA, separately, could increase the expression of GUS reporter gene driven by the DR5 promoter in the *Arabidopsis* root apex and both of these factors act synergistically to further enhance GUS expression (Ivanchenko et al., 2008). Also, Ivanchenko et al. (2008) revealed that exogenous ethylene or auxin, or a combination of both, could reverse the induction of GUS expression, in the columella and QC cells of *Arabidopsis* roots by NPA. This effect of exogenous ethylene and auxin was also observed in this study of the white clover root system.

CONCLUSIONS

This thesis has examined the changes in white clover RSA in response to P-deficiency and the role of ethylene and auxin in mediating these changes. Under the conditions used in this thesis, growth of plants in a P-deficient (0.01 mM Pi) media stimulated white clover root growth in terms of primary root (PR) elongation, lateral root emergence and lateral root length.

For plants grown in P-sufficient media, and when ethylene action was putatively inhibited using 300 ppm 1-methylcyclopropene (1-MCP), white clover root growth was severely reduced which suggests an important role for ethylene in mediating this growth. Moreover, low concentrations of exogenous 1-aminocyclopropane-1-carboxylic acid (100 nM ACC) stimulated white clover root growth but higher concentrations of ACC (1 μM and 10 μM) inhibited the root growth. Thus the level of ethylene is critical for the fine control of white clover root growth.

In common with ethylene, auxin is also vital for root growth in plants grown in P-sufficient media because this growth was almost abolished when auxin transport was (putatively) inhibited using 100 nM 1-N-naphthylphthalamic acid (NPA). Furthermore, treatment with a low concentration of exogenous auxin (5 nM 1-naphthylacetic acid, NAA) significantly increased PR length, LR number and total LR length in comparison with the control without NAA treatment. In contrast, higher concentrations of NAA (50 nM and 100 nM) significantly reduced white clover root growth in terms of PR length, LR number and total LR length. Hence, the appropriate concentration of auxin is also critical for the regulation white clover root growth.

After determining the essential role of ethylene and auxin in mediating white clover root growth, the interaction between these two hormones was also investigated. For plants grown in a P-sufficient media, treatment with 100 nM ACC could only partially overcome the extremely inhibitory effects of added 100 nM NPA on white clover root growth, particularly in terms of LR formation. In white clover, 100 nM NPA treatment induces severe adverse effects on root growth and at this concentration, added 100 nM ACC is not sufficient to restore normal root growth. Also, the stimulatory effects of 5 nM NAA in P-sufficient conditions could not totally overcome the inhibitory effects of 300 ppm 1-MCP on PR elongation and LR formation. These results confirm that both auxin and ethylene are essential in mediating white clover root growth although the nature of interaction between these two hormones has not been shown clearly. It is

still not clear in white clover whether auxin, for example, can influence ethylene biosynthesis or sensitivity and/or whether ethylene influences auxin biosynthesis or sensitivity. Moreover, a study of the morphological patterns of root growth only in response to added hormone can only suggest how auxin and ethylene interact.

Ethylene and auxin are not only important in mediating root growth in P-sufficient conditions but also may play a role in the regulation of root responses in plants exposed to P-deficiency. Treatment with ACC (100 nM) enhanced the stimulatory effects on white clover root growth observed for plants grown in P-deficient media in terms of PR length, LR number and total LR length. In support of this, growth on the P-deficient media neutralised the inhibitory effects of 300 ppm 1-MCP observed on root growth. Thus P-deficiency is presumed to elevate ethylene biosynthesis or ethylene sensitivity in the white clover root system. In contrast, 5nM NAA abolished the effects of P-deficiency on white clover root growth and P-supply (both 0.01 mM and 1 mM Pi) did not alter the extremely inhibitory effects of 100 nM NPA on root growth. Thus exposure to P-deficiency is also proposed to increase either auxin biosynthesis or auxin sensitivity such that the 5 nM NAA treatment is sufficient to abolish the stimulatory effects of P-deficiency, but not the effects of 100 nM NPA.

Finally, auxin activity has been found in the root tips (both PRs and LRs) and root primordia as determined by a GUS reporter gene driven by an auxin responsive promoter (*DR5p::GUS*). Moreover, it was found that the P-deficient treatment and the ACC treatment enhanced the GUS activity in PR tips in comparison with the P-sufficient control treatment. This suggests that exposure to P-deficiency and exogenous ACC treatment increases auxin responsiveness which is caused by either an increase in auxin sensitivity or an elevation in the auxin level. Furthermore, the combinations of NPA with ACC, NPA with NAA, and NPA with both ACC and NAA applied in P-deficient media resulted in an increase in GUS activity in PR tips in comparison with these combinations applied in P-sufficient media. These results suggest that either exogenous ACC and/or P-deficiency increases auxin sensitivity.

In this thesis, it was found that exposure to P-deficiency did stimulate white clover root growth and that both ethylene and auxin play important roles in white clover root growth in both P-sufficient and P-deficient conditions. In addition, exogenous ACC did elevate auxin sensitivity in white clover root systems. However, ethylene biosynthesis is only expected to be elevated by P-deficiency but the level of ethylene in white clover roots was not measured.

FUTURE WORK

In this thesis, the stimulatory effects of P-deficiency on white clover root growth and the role of ethylene and auxin in mediating white clover root growth in P-sufficient and P-deficient conditions have been characterized. However, further work is required to define this interaction more accurately.

The concentration of NPA (auxin transport inhibitor) should be reduced to investigate whether exogenous ethylene or auxin can override the inhibitory effects of NPA observed on the white clover root system or not. In the combination treatments to characterize the cross-talk between ethylene and auxin, the concentrations of the combined exogenous hormones or hormone inhibitors should be further adjusted (for example, hormone inhibitors should be reduced) to be able to reveal more clearly the interaction and whether auxin acts up-stream or down-stream of ethylene pathway.

In support of this, it would be interesting to quantify changes in the levels of auxin and ethylene in the P-deficient treatment and in hormone treatments so that more evidence might be found to prove whether changes in ethylene and auxin biosynthesis or changes in the sensitivity to these hormones plays a major role in mediating white clover root responses. As well, if using 1-MCP as a control for ethylene action, then the effectiveness of this compound should be further verified perhaps by looking at the effect of the treatment on an ethylene-induced gene, eg. chitinase. Likewise, it needs to be confirmed that added NPA does inhibit IAA transport in white clover.

The GUS staining method should be optimized in terms of the staining time (increase the incubation time from four to six or eight hours, for example) so that the changes of GUS activity in the LR primordia might be visualized to help in understanding more about the role of auxin responsiveness in LR production.

Furthermore, other aspects of root system architecture should be examined such as angle between PR and LR, distance between the first emerged LR and the PR tip, number of LR primordia and number of emerging LR primordia in terms of white clover root responses to P-deficiency and hormone (ethylene and auxin) treatments. Also, the interaction effects between P-supply and iron (Fe) level in the regulation root growth should also be considered to clarify whether Fe level plays a role in P-deficient responses or not. These studies may contribute to understanding more about the ability of root systems to adjust changes in the surrounding environment.

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APPENDICES

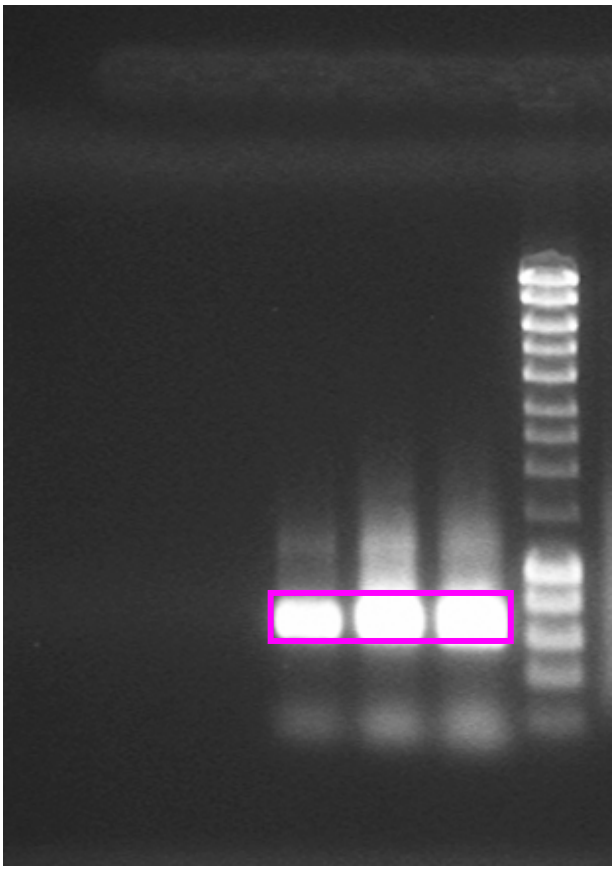

Figure A.1. The DNA fragments excised to provide the template for DIG-labelling

(Section 2.8.5 and 2.8.6)

The partial *DR5p::GUS* sequence (556 bp) amplified by the DR5rev-pro-F1 and GUS-seq-R1 primers using PCR. The PCR products were separated using agarose gel electrophoresis and visualised under UV light. The bands which were enclosed in a purple frame were excised out to purify the DNA to prepare the DIG-labelled probe.

GAATTCGTCGACGGTATCGCAGCCCAGGGAGACAAAAGGGAGACAAAAG
GGAGACAAAAGGGAGACAAAAGGGAGACAAAAGGGAGACAAAAGGGAG
ACAAAAGGGAGACAAAAGGGAGACAAAAGGGGGCAGGCCTCGATAAGCT
TGATATCGAATTAATTCCTGCAGCCCCGCAAGACCCTTCCTCTATATAAGG
AAGTTCATTTCATTTGGAGAGGTATTTTTACAACAATTACCAACAACAACA
AACAACAAACAACATTACAATTACTATTTACAATTACAATTACAGGGGAT
CGATCCAAGGAGATATAACAGGATCCCCGGGTGGTCAGTCCCTTATGTTA
CGTCCTGTAGAAACCCCAACCCGTGAAATCAAAAAACTCGACGGCCTGTG
GGCATTCAGTCTGGATCGCGAAAACTGTGGAATTGATCAGCGTTGGTGGG
AAAGCGCGTTACAAGAAAGCCGGGCAATTGGCTGTSCAGGCAGTTTTAAC
GATCAGTTCSCGATGCAGATATTCGTAATTATGCGGGCAACGTCTGGTATC
TAGCTGT

Figure A.2. The 556 bp sequence of the *DR5p::GUS* fragment amplified as described in Figure A.1. (sequencing by Miss Susanna Leung, Massey University).

A: Adenine

C: Cytosine

G: Guanine

T: Thymine

S: Guanine or Cytosine

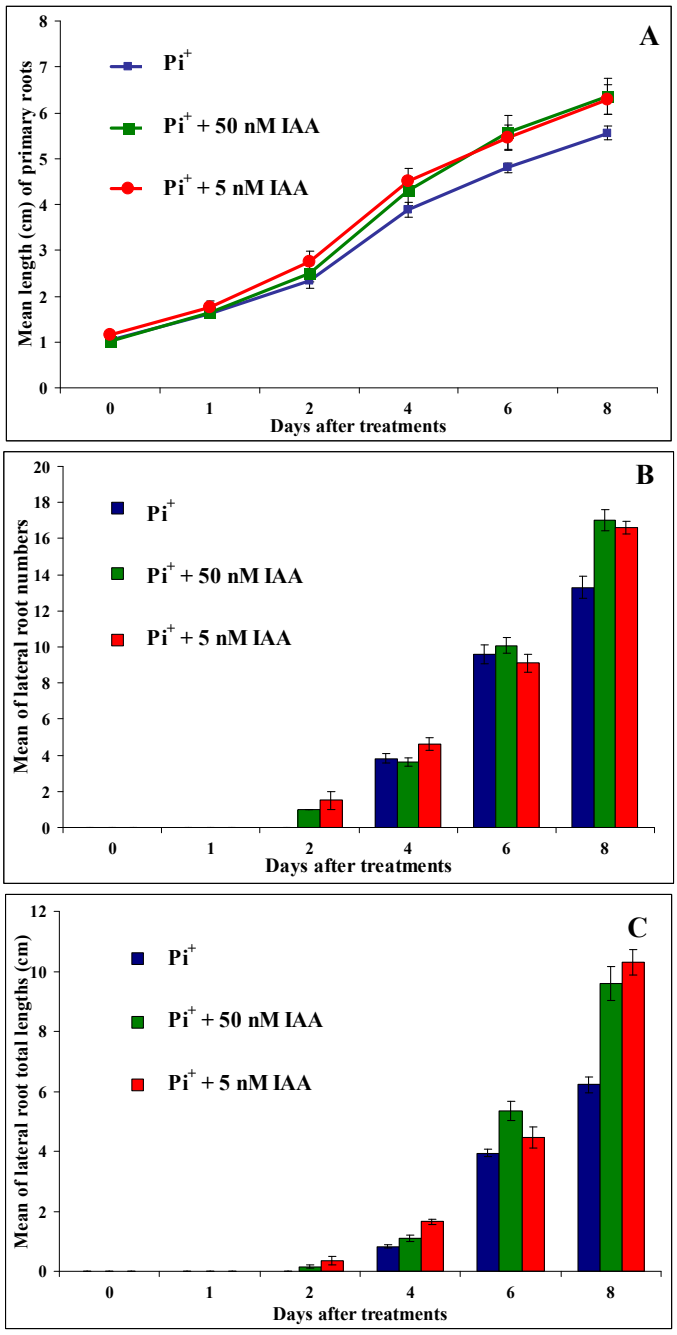

Figure A.3. The effects of added IAA on root morphology.

Changes in length of PRs (A), number of LRs (B) and total length of LRs (C) of wild type white clover stolons (genotype 10F) in response to exogenous auxin, 5 nM IAA (Pi^+ + 5 nM IAA) and 50 nM IAA (Pi^+ + 50 nM IAA) in comparison with P-sufficiency as a control (Pi^+) measured over 6 time points from day 0 to 8 DAT.

Values are means of 10 individual stolons for Pi^+ or 14 stolons for Pi^+ + 50 nM NAA or 15 stolons for Pi^+ + 50 nM NAA with standard deviations of the means (represented as error bars).

Table A.1. Composition of the one-third strength macro and full strength micronutrients Hoagland solution (Gibeaut et al., 1997)

Macronutrients	Working concentration (mM)	Weight (g) per litre for 10X stock solution
KNO_3	1.25	1.264
$Ca(NO_3)_2$	1.50	3.543
$MgSO_4$	0.75	1.849
KH_2PO_4	1.00	1.360

Micronutrients	Working concentration (μM)	Weight (mg) per litre for 100X stock solution
KCl	50.0	372.8
H_3BO_3	50.0	309.2
$MnSO_4$	10.0	151.0
$ZnSO_4$	2.0	57.5
$CuSO_4$	1.5	37.5
$(NH_4)_6Mo_7O_{24}$	0.075	9.3
Na_2O_3Si	0.1	2.1
FeNa EDTA	72.0	2642.8

The Hoagland's media used in this thesis is a combination of the one-third strength macro and full strength micronutrients (1X). For the P-deficient treatment, the concentration of KNO_3 is increased to 2.24 mM and the concentration of KH_2PO_4 is reduced to 0.01 mM.

Table A.2. Composition of the Murashige and Skoog (MS) basal salt mixture

Macro stock	Final concentration (mg/L)	20 x Stock (g/L)
NH_4NO_3	150	33
KNO_3	1900	33
$CaCl_2.2H_2O$	440	8.8
KH_2PO_4	170	3.4
$MgSO_4.7H_2O$	370	7.4

Micro stock	Final concentration (mg/L)	200 x Stock (g/L)
H_3BO_3	6.2	1.24
$MnSO_4.4H_2O$	22.2	4.46
$ZnSO_4.7H_2O$	8.6	1.72
KI	0.83	0.166
$Na_2MoO_4.2H_2O$	0.25	0.05
$CuSO_4.5H_2O$	0.025	0.005
$CoCl_2.6H_2O$	0.025	0.005

Vitamins	Final concentration (mg/L)	100 x Stock (g/100mL)
Nicotinic Acid	10	0.1
Thiamine.HCl	100	1.0
Pyridoxine.HCl	10	0.1

FeNaEDTA	Final concentration (mg/L)	100 x Stock (mg/100mL)
$NaEDTA.2H_2O$	3.73	37.3
$FeSO_4.7H_2O$	2.78	27.8

MS media composition, pH 5.7	Amount per Litre
Macro stock	50 mL
Micro stock	5 mL
Vitamins	10 mL
FeNaEDTA	10 mL
Myo-Inositol	100 mg
Sucrose	30 g
Phytoagar (Duchefa Biochemie)	8 g

	MS media	1-naphthylacetic acid	6-Benzylaminopurine
CR0	1 Litre	0 $mg.L^{-1}$	0 $mg.L^{-1}$
CR5	1 Litre	0.05 $mg.L^{-1}$	0.1 $mg.L^{-1}$
CR7	1 Litre	0.05 $mg.L^{-1}$	1 $mg.L^{-1}$

Table A.3. Composition of the TY^{Kan100}

TY^{Kan100} broth	Concentration (w/v)
Tryptone	5 g. L^{-1}
Yeast extract	3 g. L^{-1}
$CaCl_2.6H_2O$	1.3 g. L^{-1}
Kanamycin sulphate	100 mg.L^{-1}

Table A.4. Genomic DNA Extraction Buffer

Extraction Buffer A	Final concentration
Hexadecyltrimethylammonium bromide (CTAB)	2% (w/v)
Tris-HCl (pH 8.0)	100 mM
EDTA	20 mM
NaCl	1.4 M
Polyvinylpyrrolidone (PVP)	4% (w/v)
Ascorbic acid	0.1% (w/v)
2-mercaptoethanol (BME)	10 mM

Extraction Buffer B	Final concentration
Tris-HCl (pH 8.0)	100 mM
EDTA	50 mM
NaCl	100 mM
β-mercaptoethanol (BME)	10 mM

Extraction Buffer	Amount per sample (1.3 mL)
Extraction buffer A	300 μL
Extraction buffer B	900 μL
20% (w/v) sodium dodecyl sulfate (SDS)	100 μL

RESULTS OF MINITAB ANALYSIS USING THE GENERAL LINEAR MODEL

Table A.5. Analysis of variance for phosphate treatments, using Adjusted SS for Tests

Source	P values		
	PR length	LR number	Total LR length
Nodes	0.000	0.000	0.000
P-treatments	0.008	0.025	0.046
Node*P-treatments	0.001	0.000	0.003

Table A.6. Summary of Least squares means for root morphological patterns [primary root length (cm), lateral root number and total lateral root length (cm)] of white clover *cv.* 10F in phosphate treatments.

Factors	PR length		LR number		Total LR length	
	Mean (cm)	P_{adj}	Mean	P_{adj}	Mean (cm)	P_{adj}
Nodes						
3rd	6.67 ± 0.15 b	0.000	20.74 ± 0.70 b	0.000	20.25 ± 1.45 b	0.000
4th	5.68 ± 0.13 a		16.00 ± 0.63 a		12.20 ± 1.29 a	
Phosphate treatments						
Pi^+	5.91 ± 0.14 a	0.007	17.30 ± 0.65 a	0.024	14.28 ± 1.32 a	0.046
Pi^-	6.44 ± 0.14 b		19.44 ± 0.69 b		18.16 ± 1.42 b	
Node*Phosphate treatments						
3rd*Pi^+	6.06 ± 0.20 a	0.000	17.69 ± 0.96 a	0.000	15.43 ± 1.92 a	0.006
3rd*Pi^-	7.27 ± 0.21 b		23.78 ± 1.02 b		25.08 ± 2.17 b	
4th*Pi^+	5.76 ± 0.18 a	> 0.1	16.91 ± 0.87 a	> 0.1	13.14 ± 1.82 a	> 0.1
4th*Pi^-	5.60 ± 0.19 a		15.09 ± 0.92 a		11.24 ± 1.82 a	

Values are the mean ± SE

P_{adj}: adjusted P-value from Tukey's test for independent samples.

Different letters indicate the significant difference among the mean values at 95% confidence ($P_{adj} < 0.05$).

Pi^+: P-sufficient treatment (1 mM Pi)

Pi^-: P-deficient treatment (0.01 mM Pi)

Table A.7. Analysis of variance for ACC and phosphate treatments, using Adjusted SS for Tests

Source	P values		
	PR length	LR number	Total LR length
P-treatments	0.000	0.000	0.000
ACC treatments	0.010	0.023	0.000
P-treatments*ACC treatments	0.990	0.001	0.000

Table A.8. Summary of Least squares means for root morphological patterns [primary root length (cm), lateral root number and total lateral root length (cm)] of white clover *cv.* 10F in ACC treatments combined with phosphate treatments.

Factors	PR length		LR number		Total LR length	
	Mean (cm)	P_{adj}	Mean	P_{adj}	Mean (cm)	P_{adj}
Phosphate treatments						
Pi^+	2.69 ± 0.05 a	0.000	3.78 ± 0.09 a	0.000	1.50 ± 0.05 a	0.000
Pi^-	2.95 ± 0.05 b		5.04 ± 0.09 b		2.41 ± 0.05 b	
ACC treatments						
0 nM	2.74 ± 0.05 a	0.010	4.27 ± 0.09 a	0.023	1.65 ± 0.05 a	0.000
100 nM	2.90 ± 0.05 b		4.55 ± 0.09 b		2.26 ± 0.05 b	
Phosphate treatments*ACC treatments						
Pi^+*0 nM	2.61 ± 0.06 a	Table A.9	3.85 ± 0.12 a	Table A.10	1.42 ± 0.07 a	Table A.11
Pi^+*100 nM	2.77 ± 0.06 ab		3.70 ± 0.12 a		1.58 ± 0.07 a	
Pi^-*0 nM	2.87 ± 0.06 bc		4.68 ± 0.12 b		1.88 ± 0.07 b	
Pi^-*100 nM	3.04 ± 0.06 c		5.40 ± 0.12 c		2.94 ± 0.07 c	

P_{adj}: adjusted P-value from Tukey's test for independent samples.

Different letters indicate the significant difference among the mean values at 95% confidence ($P_{adj} < 0.05$).

Pi^+: P-sufficient treatment (1 mM Pi)

Pi^-: P-deficient treatment (0.01 mM Pi)

ACC: 1-aminocyclopropane-1-carboxylate, an ethylene precursor.

Table A.9. Adjusted P values for pair-wise comparison of primary root length by Tukey's test in ACC treatments combined with phosphate treatments.

	Pi^+*0 nM	Pi^+*100 nM	Pi^-*0 nM	Pi^-*100 nM
Pi^+*0 nM	1			
Pi^+*100 nM	0.261	1		
Pi^-*0 nM	0.021	0.709	1	
Pi^-*100 nM	0.000	0.020	0.253	1

Table A.10. Adjusted P values for pair-wise comparison of lateral root number by Tukey's test in ACC treatments combined with phosphate treatments.

	Pi^+*0 nM	Pi^+*100 nM	Pi^-*0 nM	Pi^-*100 nM
Pi^+*0 nM	1			
Pi^+*100 nM	0.826	1		
Pi^-*0 nM	0.000	0.000	1	
Pi^-*100 nM	0.000	0.000	0.000	1

Table A.11. Adjusted P values for pair-wise comparison of total lateral root length by Tukey's test in ACC treatments combined with phosphate treatments.

	Pi^+*0 nM	Pi^+*100 nM	Pi^-*0 nM	Pi^-*100 nM
Pi^+*0 nM	1			
Pi^+*100 nM	0.339	1		
Pi^-*0 nM	0.000	0.013	1	
Pi^-*100 nM	0.000	0.000	0.000	1

Table A.12. Analysis of variance for 1-MCP and phosphate treatments, using Adjusted SS for Tests

Source	P values		
	PR length	LR number	Total LR length
P-treatments	0.000	0.000	0.000
1-MCP treatments	0.093	0.000	0.000
P-treatments*1-MCP treatments	0.000	0.722	0.624

Table A.13. Summary of Least squares means for root morphological patterns [primary root length (cm), lateral root number and total lateral root length (cm)] of white clover *cv.* 10F in 1-MCP treatments combined with phosphate treatments.

Factors	PR length		LR number		Total LR length	
	Mean (cm)	P_{adj}	Mean	P_{adj}	Mean (cm)	P_{adj}
Phosphate treatments						
Pi^+	1.97 ± 0.04 a	0.000	1.46 ± 0.11 a	0.000	0.81± 0.05 a	0.000
Pi^-	2.43 ± 0.04 b		2.14 ± 0.11 b		1.18 ± 0.06 b	
1-MCP treatments						
0 ppm	2.25 ± 0.03 a	0.093	2.33 ± 0.09 b	0.000	1.24 ± 0.05 b	0.000
300 ppm	2.15 ± 0.05 a		1.27 ± 0.12 a		0.75 ± 0.06 a	
Phosphate treatments*1-MCP treatments						
Pi^+*0 ppm	2.13 ± 0.05 b	Table A.14	2.02 ± 0.12 b	Table A.15	1.04 ± 0.06 b	Table A.16
Pi^+*300 ppm	1.81 ± 0.07 a		0.90 ± 0.18 a		0.58 ± 0.09 a	
Pi^-*0 ppm	2.36 ± 0.05 c		2.65 ± 0.14 c		1.44 ± 0.07 c	
Pi^-*300 ppm	2.49 ± 0.07 c		1.63 ± 0.18 b		0.92 ± 0.09 b	

P_{adj}: adjusted P-value from Tukey's test for independent samples.

Different letters indicate the significant difference among the mean values at 95% confidence ($P_{adj} < 0.05$).

Pi^+: P-sufficient treatment (1 mM Pi)

Pi^-: P-deficient treatment (0.01 mM Pi)

1-MCP: 1-methylcyclopropene, an ethylene action inhibitor

Table A.14. Adjusted P values for pair-wise comparison of primary root length by Tukey's test in 1-MCP treatments combined with phosphate treatments.

	Pi^+*0 ppm	Pi^+*300 ppm	Pi^-*0 ppm	Pi^-*300 ppm
Pi^+*0 ppm	1			
Pi^+*300 ppm	0.000	1		
Pi^-*0 ppm	0.005	0.000	1	
Pi^-*300 ppm	0.000	0.000	0.487	1

Table A.15. Adjusted P values for pair-wise comparison of lateral root number by Tukey's test in 1-MCP treatments combined with phosphate treatments.

	Pi^+*0 ppm	Pi^+*300 ppm	Pi^-*0 ppm	Pi^-*300 ppm
Pi^+*0 ppm	1			
Pi^+*300 ppm	0.000	1		
Pi^-*0 ppm	0.005	0.000	1	
Pi^-*300 ppm	0.262	0.020	0.000	1

Table A.16. Adjusted P values for pair-wise comparison of total lateral root length by Tukey's test in 1-MCP treatments combined with phosphate treatments.

	Pi^+*0 ppm	Pi^+*300 ppm	Pi^-*0 ppm	Pi^-*300 ppm
Pi^+*0 ppm	1			
Pi^+*300 ppm	0.000	1		
Pi^-*0 ppm	0.000	0.000	1	
Pi^-*300 ppm	0.667	0.037	0.000	1

Table A.17. Summary of Least squares means for root morphological patterns [primary root length (cm), lateral root number and total lateral root length (cm)] of white clover *cv.* 10F in NAA treatments combined with phosphate treatments.

Factors	PR length		LR number		Total LR length	
	Mean (cm)	P_{adj}	Mean	P_{adj}	Mean (cm)	P_{adj}
Phosphate treatments						
Pi^+	3.44 ± 0.04 b	0.033	5.07 ± 0.11 b	0.010	2.34 ± 0.06 b	0.029
Pi^-	3.27 ± 0.04 a		4.57 ± 0.12 a		2.09 ± 0.06 a	
NAA treatments						
0 nM	3.30 ± 0.05 a	0.231	4.67 ± 0.13 a	0.229	2.18 ± 0.07 a	0.912
5 nM	3.41 ± 0.04 a		4.97 ± 0.10 a		2.24 ± 0.06 a	
Phosphate treatments*NAA treatments						
Pi^+*0 nM	3.20 ± 0.07 a	Table A.18	4.39 ± 0.18 ab	Table A.19	1.82 ± 0.10 a	Table A.20
Pi^+*5 nM	3.67 ± 0.05 b		5.75 ± 0.14 c		2.86 ± 0.08 b	
Pi^-*0 nM	3.40 ± 0.07 a		4.94 ± 0.18 b		2.55 ± 0.10 b	
Pi^-*5 nM	3.15 ± 0.05 a		4.19 ± 0.14 a		1.62 ± 0.08 a	

P_{adj}: adjusted P-value from Tukey's test for independent samples.

Different letters indicate the significant difference among the mean values at 95% confidence ($P_{adj} < 0.05$).

Pi^+: P-sufficient treatment (1 mM Pi)

Pi^-: P-deficient treatment (0.01 mM Pi)

NAA: 1-naphthylacetic acid (auxin)

Table A.18. Adjusted P values for pair-wise comparison of primary root length by Tukey's test in NAA treatments combined with phosphate treatments.

	Pi^+*0 nM	Pi^+*5 nM	Pi^-*0 nM	Pi^-*5 nM
Pi^+*0 nM	1			
Pi^+*5 nM	0.000	1		
Pi^-*0 nM	0.428	0.023	1	
Pi^-*5 nM	0.999	0.000	0.074	1

Table A.19. Adjusted P values for pair-wise comparison of lateral root number by Tukey's test in NAA treatments combined with phosphate treatments.

	Pi^+*0 nM	Pi^+*5 nM	Pi^-*0 nM	Pi^-*5 nM
Pi^+*0 nM	1			
Pi^+*5 nM	0.000	1		
Pi^-*0 nM	0.385	0.009	1	
Pi^-*5 nM	0.990	0.000	0.027	1

Table A.20. Adjusted P values for pair-wise comparison of total lateral root length by Tukey's test in NAA treatments combined with phosphate treatments.

	Pi^+*0 nM	Pi^+*5 nM	Pi^-*0 nM	Pi^-*5 nM
Pi^+*0 nM	1			
Pi^+*5 nM	0.000	1		
Pi^-*0 nM	0.000	0.224	1	
Pi^-*5 nM	0.818	0.000	0.000	1

Table A.21. Analysis of variance for NPA and phosphate treatments, using Adjusted SS for Tests

Source	P values		
	PR length	LR number	Total LR length
P-treatments	0.604	0.006	0.000
NPA treatments	0.000	0.000	0.000
P-treatments*NPA treatments	0.000	0.000	0.000

Table A.22. Summary of Least squares means for root morphological patterns [primary root length (cm), lateral root number and total lateral root length (cm)] of white clover *cv.* 10F in NPA treatments combined with phosphate treatments.

Factors	PR length		LR number		Total LR length	
	Mean (cm)	P_{adj}	Mean	P_{adj}	Mean (cm)	P_{adj}
Phosphate treatments						
Pi^+	1.84 ± 0.04 a	0.604	1.15 ± 0.07 a	0.006	0.58 ± 0.03 a	0.000
Pi^-	1.81 ± 0.04 a		1.42 ± 0.07 b		0.78 ± 0.03 b	
NPA treatments						
0 nM	2.25 ± 0.04 b	0.000	2.33 ± 0.07 b	0.000	1.24 ± 0.03 b	0.000
100 nM	1.40 ± 0.04 a		0.23 ± 0.07 a		0.12 ± 0.03 a	
Phosphate treatments*NPA treatments						
Pi^+*0 nM	2.13 ± 0.05 c	Table A.23	2.02 ± 0.09 b	Table A.24	1.04 ± 0.04 b	Table A.25
Pi^+*100 nM	1.55 ± 0.06 b		0.28 ± 0.10 a		0.13 ± 0.05 a	
Pi^-*0 nM	2.36 ± 0.06 d		2.65 ± 0.10 c		1.44 ± 0.05 c	
Pi^-*100 nM	1.25 ± 0.06 a		0.19 ± 0.10 a		0.12 ± 0.05 a	

P_{adj}: adjusted P-value from Tukey's test for independent samples.

Different letters indicate the significant difference among the mean values at 95% confidence ($P_{adj} < 0.05$).

Pi^+: P-sufficient treatment (1 mM Pi)

Pi^-: P-deficient treatment (0.01 mM Pi)

NPA: 1-N-naphthylphthalamic acid, an auxin transport inhibitor.

Table A.23. Adjusted P values for pair-wise comparison of primary root length by Tukey's test in NPA treatments combined with phosphate treatments.

	Pi^+*0 nM	Pi^+*100 nM	Pi^-*0 nM	Pi^-*100 nM
Pi^+*0 nM	1			
Pi^+*100 nM	0.000	1		
Pi^-*0 nM	0.025	0.000	1	
Pi^-*100 nM	0.000	0.004	0.000	1

Table A.24. Adjusted P values for pair-wise comparison of lateral root number by Tukey's test in NPA treatments combined with phosphate treatments.

	Pi^+*0 nM	Pi^+*100 nM	Pi^-*0 nM	Pi^-*100 nM
Pi^+*0 nM	1			
Pi^+*100 nM	0.000	1		
Pi^-*0 nM	0.000	0.000	1	
Pi^-*100 nM	0.000	0.913	0.000	1

Table A.25. Adjusted P values for pair-wise comparison of total lateral root length by Tukey's test in NPA treatments combined with phosphate treatments.

	Pi^+*0 nM	Pi^+*100 nM	Pi^-*0 nM	Pi^-*100 nM
Pi^+*0 nM	1			
Pi^+*100 nM	0.000	1		
Pi^-*0 nM	0.000	0.000	1	
Pi^-*100 nM	0.000	0.999	0.000	1

Table A.26. Analysis of variance for ACC and NPA combined treatments, using Adjusted SS for Tests

Source	P values		
	PR length	LR number	Total LR length
ACC treatments	0.000	0.000	0.000
NPA treatments	0.000	0.000	0.000
ACC*NPA treatments	0.000	0.000	0.000

Table A.27. Summary of Least squares means for root morphological patterns [primary root length (cm), lateral root number and total lateral root length (cm)] of white clover *cv.* 10F in ACC and NPA combined treatments.

Factors	PR length		LR number		Total LR length	
	Mean (cm)	P_{adj}	Mean	P_{adj}	Mean (cm)	P_{adj}
ACC treatments						
0 nM	1.84 ± 0.04 a	0.000	1.15 ± 0.08 a	0.000	0.58 ± 0.04 a	0.000
100 nM	2.14 ± 0.05 b		2.06 ± 0.09 b		0.86 ± 0.04 b	
NPA treatments						
0 nM	2.45 ± 0.04 b	0.000	2.86 ± 0.08 b	0.000	1.31 ± 0.04 b	0.000
100 nM	1.53 ± 0.05 a		0.35 ± 0.09 a		0.13 ± 0.04 a	
ACC*NPA treatments						
0nM*0nM	2.13 ± 0.06 b	Table A.28	2.02 ± 0.11 b	Table A.29	1.04 ± 0.05 b	Table A.30
0nM*100nM	1.55 ± 0.07 a		0.28 ± 0.12 a		0.13 ± 0.06 a	
100nM*0nM	2.77 ± 0.06 c		3.70 ± 0.11 c		1.58 ± 0.05 c	
100nM*100nM	1.51 ± 0.07 a		0.43 ± 0.13 a		0.14 ± 0.06 a	

P_{adj}: adjusted P-value from Tukey's test for independent samples.

Different letters indicate the significant difference among the mean values at 95% confidence ($P_{adj} < 0.05$).

Pi^{+}: P-sufficient treatment (1 mM Pi)

Pi^{-}: P-deficient treatment (0.01 mM Pi)

ACC: 1-aminocyclopropane-1-carboxylate, an ethylene precursor

NPA: 1-N-naphthylphthalamic acid, an auxin transport inhibitor.

Table A.28. Adjusted P values for pair-wise comparison of primary root length by Tukey's test in ACC and NPA combined treatments.

ACC*NPA	0 nM*0 nM	0 nM*100 nM	100 nM*0 nM	100 nM*100 nM
0 nM*0 nM	1			
0 nM*100 nM	0.000	1		
100 nM*0 nM	0.000	0.000	1	
100 nM*100 nM	0.000	0.975	0.000	1

Table A.29. Adjusted P values for pair-wise comparison of lateral root number by Tukey's test in ACC and NPA combined treatments.

ACC*NPA	0 nM*0 nM	0 nM*100 nM	100 nM*0 nM	100 nM*100 nM
0 nM*0 nM	1			
0 nM*100 nM	0.000	1		
100 nM*0 nM	0.000	0.000	1	
100 nM*100 nM	0.000	0.840	0.000	1

Table A.30. Adjusted P values for pair-wise comparison of total lateral root length by Tukey's test in ACC and NPA combined treatments.

ACC*NPA	0 nM*0 nM	0 nM*100 nM	100 nM*0 nM	100 nM*100 nM
0 nM*0 nM	1			
0 nM*100 nM	0.000	1		
100 nM*0 nM	0.000	0.000	1	
100 nM*100 nM	0.000	1	0.000	1

Table A.31. Analysis of variance for phosphate treatments in combined with 5 nM NAA and 300 ppm 1-MCP, using Adjusted SS for Tests

Source	P values		
	PR length	LR number	Total LR length
P-treatments	0.001	0.831	0.812
NAA treatments	0.026	0.001	0.087
1-MCP treatments	0.240	0.000	0.000
P-treatments*NAA treatments	0.003	0.627	0.001
P-treatments*1-MCP treatments	0.000	0.001	0.004
NAA treatments*1-MCP treatments	0.935	0.209	0.336
P-treatments*NAA treatments*1-MCP treatments	0.000	0.000	0.000

NAA: 1-naphthylacetic acid (auxin)

1-MCP: 1-methylcyclopropene, an ethylene action inhibitor

Table A.32. Summary of Least squares means for root morphological patterns [primary root length (cm), lateral root number and total lateral root length (cm)] of white clover *cv.* 10F in phosphate treatments combined with NAA treatments and 1-MCP treatments.

Factors	PR length		LR number		Total LR length	
	Mean (cm)	P_{adj}	Mean	P_{adj}	Mean (cm)	P_{adj}
Phosphate treatments						
Pi^+	3.24 ± 0.04 a	0.001	2.81 ± 0.10 a	0.831	1.27± 0.06 a	0.812
Pi^-	3.41 ± 0.04 b		2.78 ± 0.10 a		1.25 ± 0.06 a	
NAA treatments						
0 nM	3.27 ± 0.04 a	0.026	2.55 ± 0.10 a	0.001	1.19 ± 0.06 a	0.087
5 nM	3.39 ± 0.04 b		3.04 ± 0.10 b		1.33 ± 0.05 a	
1-MCP treatments						
0 ppm	3.36 ± 0.03 a	0.240	4.82 ± 0.08 b	0.000	2.21 ± 0.05 b	0.000
300 ppm	3.30 ± 0.04 a		0.76 ± 0.12 a		0.31 ± 0.07 a	
Phosphate treatments*NAA treatments						
Pi^+*0 nM	3.11 ± 0.05 a	Data are not shown	2.53 ± 0.15 a	Data are not shown	1.07 ± 0.08 a	Data are not shown
Pi^+*5 nM	3.38 ± 0.05 b		3.09 ± 0.14 b		1.48 ± 0.08 b	
Pi^-*0 nM	3.43 ± 0.05 b		2.57 ± 0.15 a		1.32 ± 0.08 ab	
Pi^-*5 nM	3.40 ± 0.05 b		2.99 ± 0.14 ab		1.18 ± 0.08 a	
Phosphate treatments*1-MCP treatments						
Pi^+*0 ppm	3.44 ± 0.04 c	Data are not shown	5.07 ± 0.11 c	Data are not shown	2.34 ± 0.06 c	Data are not shown
Pi^+*300 ppm	3.04 ± 0.06 a		0.54 ± 0.17 a		0.20 ± 0.09 a	
Pi^-*0 ppm	3.28 ± 0.04 b		4.57 ± 0.16 b		2.09 ± 0.06 b	
Pi^-*300 ppm	3.55 ± 0.06 c		0.99 ± 0.17 a		0.42 ± 0.09 a	
NAA treatments*1-MCP treatments						
0 nM*0 ppm	3.30 ± 0.05 a	Data are not shown	4.67 ± 0.13 c	Data are not shown	2.18 ± 0.07 b	Data are not shown
0 nM*300 ppm	3.24 ± 0.06 a		0.43 ± 0.17 a		0.20 ± 0.09 a	
5 nM*0 ppm	3.41 ± 0.04 a		4.97 ± 0.10 c		2.24 ± 0.06 b	
5 nM*300 ppm	3.36 ± 0.06 a		1.10 ± 0.17 b		0.42 ± 0.09 a	

Table A.32 continued

Factors	PR length		LR number		Total LR length	
	Mean (cm)	P_{adj}	Mean	P_{adj}	Mean (cm)	P_{adj}
Phosphate treatments*NAA treatments*1-MCP treatments						
Pi^+*0 nM*0 ppm	3.20±0.1 abc		4.39±0.2 cd		1.82±0.1 c	
Pi^+*0 nM*300 ppm	3.01±0.1 a		0.67±0.2 a		0.32±0.1 ab	
Pi^+*5 nM*0 ppm	3.67±0.1 e		5.75±0.1 e		2.86±0.1 d	
Pi^+*5 nM*300 ppm	3.08±0.1 ab	Table 33	0.42±0.2 a	Table 34	0.09±0.1 a	Table 35
Pi^-*0 nM*0 ppm	3.40±0.1 bcd		4.94±0.2 d		2.55±0.1 d	
Pi^-*0 nM*300 ppm	3.46±0.1 cde		0.19±0.2 a		0.09±0.1 a	
Pi^-*5 nM*0 ppm	3.15±0.1 ab		4.19±0.1 c		1.62±0.1 c	
Pi^-*5 nM*300 ppm	3.64 ± 0.1 de		1.78±0.2 b		0.74±0.1 b	

P_{adj}: adjusted P-value from Tukey's test for independent samples.

Different letters indicate the significant difference among the mean values at 95% confidence ($P_{adj} < 0.05$).

Pi^+: P-sufficient treatment (1 mM Pi)

Pi^-: P-deficient treatment (0.01 mM Pi)

NAA: 1-naphthylacetic acid (auxin)

1-MCP: 1-methylcyclopropene, an ethylene action inhibitor

Pi*NAA(nM)*1-MCP(ppm)	Pi^+*0*0	Pi^+*0*300	Pi^+*5*0	Pi^+*5*300	Pi^-*0*0	Pi^-*0*300	Pi^-*5*0	Pi^-*5*300
Pi^+*0*0	1							
Pi^+*0*300	0.615	1						
Pi^+*5*0	0.000	0.000	1					
Pi^+*5*300	0.944	0.999	0.000	1				
Pi^-*0*0	0.428	0.007	0.023	0.062	1			
Pi^-*0*300	0.243	0.004	0.410	0.032	0.999	1		
Pi^-*5*0	0.999	0.829	0.000	0.995	0.074	0.044	1	
Pi^-*5*300	0.002	0.000	0.999	0.000	0.363	0.850	0.000	1

Table A.33. Adjusted P values for pair-wise comparison of primary root length by Tukey's test in phosphate treatments combined with NAA treatments and 1-MCP treatments.

Pi*NAA(nM)*1-MCP(ppm)	Pi^{+}*0*0	Pi^{+}*0*300	Pi^{+}*5*0	Pi^{+}*5*300	Pi^{-}*0*0	Pi^{-}*0*300	Pi^{-}*5*0	Pi^{-}*5*300
Pi^{+}*0*0	1							
Pi^{+}*0*300	0.000	1						
Pi^{+}*5*0	0.000	0.000	1					
Pi^{+}*5*300	0.000	0.995	0.000	1				
Pi^{-}*0*0	0.385	0.000	0.009	0.000	1			
Pi^{-}*0*300	0.000	0.845	0.000	0.998	0.000	1		
Pi^{-}*5*0	0.990	0.000	0.000	0.000	0.027	0.000	1	
Pi^{-}*5*300	0.000	0.018	0.000	0.001	0.000	0.000	0.000	1

Table A.34. Adjusted P values for pair-wise comparison of lateral root number by Tukey's test in phosphate treatments combined with NAA treatments and 1-MCP treatments.

Pi*NAA(nM)*1-MCP(ppm)	Pi^{+}*0*0	Pi^{+}*0*300	Pi^{+}*5*0	Pi^{+}*5*300	Pi^{-}*0*0	Pi^{-}*0*300	Pi^{-}*5*0	Pi^{-}*5*300
Pi^{+}*0*0	1							
Pi^{+}*0*300	0.000	1						
Pi^{+}*5*0	0.000	0.000	1					
Pi^{+}*5*300	0.000	0.927	0.000	1				
Pi^{-}*0*0	0.000	0.000	0.224	0.000	1			
Pi^{-}*0*300	0.000	0.927	0.000	1	0.000	1		
Pi^{-}*5*0	0.818	0.000	0.000	0.000	0.000	0.000	1	
Pi^{-}*5*300	0.000	0.290	0.000	0.010	0.000	0.010	0.000	1

Table A.35. Adjusted P values for pair-wise comparison of total lateral root length by Tukey's test in phosphate treatments combined with NAA treatments and 1-MCP treatments.

Table A.36. Analysis of variance for phosphate treatments in different white clover cultivars, using Adjusted SS for Tests

Source	P values	
	PR length	LR number
Cultivars	0.000	0.000
P-treatments	0.000	0.000
Cultivars*P-treatments	0.525	0.055

Table A.37. Summary of Least squares means for root morphological patterns [primary root length (cm) and lateral root number] of the three white clover cultivars in phosphate treatments.

Factors	PR length		LR number	
	Mean (cm)	P_{adj}	Mean	P_{adj}
10F	2.74 ± 0.06 a		4.27 ± 0.18 a	
Huia	3.52 ± 0.08 b	Table 38	4.26 ± 0.23 a	Table 39
TR29	4.95 ± 0.08 c		10.29 ± 0.25 b	
Pi^+	3.54 ± 0.06 a	0.000	5.53 ± 0.18 a	0.000
Pi^-	3.93 ± 0.06 b		7.02 ± 0.18 b	
10F*Pi^+	2.61 ± 0.09 a		3.85 ± 0.26 a	
10F*Pi^-	2.87 ± 0.09 a		4.68 ± 1.26 a	
Huia*Pi^+	3.28 ± 0.11 b	Table 40	3.60 ± 0.33 a	Table 41
Huia*Pi^-	3.75 ± 0.11 c		4.92 ± 0.33 a	
TR29*Pi^+	4.73 ± 0.12 d		9.13 ± 0.36 b	
TR29*Pi^-	5.18 ± 0.11 d		11.44 ± 0.33 c	

P_{adj}: adjusted P-value from Tukey's test for independent samples.

Different letters indicate the significant difference among the mean values at 95% confidence ($P_{adj} < 0.05$).

Pi^+: P-sufficient treatment (1 mM Pi)

Pi^-: P-deficient treatment (0.01 mM Pi)

10F and Huia are two genotypes of Grassland white clover, and TR29 is the *DR5p::GUS* transgenic white clover.

Table A.38. Adjusted P values for pair-wise comparison of primary root length of different white clover cultivars by Tukey's test.

	10F	Huia	TR29
10F	1		
Huia	0.000	1	
TR29	0.000	0.000	1

Table A.39. Adjusted P values for pair-wise comparison of lateral root numbers of different white clover cultivars by Tukey's test.

	10F	Huia	TR29
10F	1		
Huia	1	1	
TR29	0.000	0.000	1

Table A.40. Adjusted P values for pair-wise comparison of primary root length by Tukey's test in phosphate treatments in different white clover cultivars.

	10F*Pi$^+$	**10F*Pi$^-$**	**Huia*Pi$^+$**	**Huia*Pi$^-$**	**TR29*Pi$^+$**	**TR29*Pi$^-$**
10F*Pi$^+$	1					
10F*Pi$^-$	0.281	1				
Huia*Pi$^+$	0.000	0.046	1			
Huia*Pi$^-$	0.000	0.000	0.045	1		
TR29*Pi$^+$	0.000	0.000	0.000	0.000	1	
TR29*Pi$^-$	0.000	0.000	0.000	0.000	0.093	1

Table A.41. Adjusted P values for pair-wise comparison of lateral root number by Tukey's test in phosphate treatments in different white clover cultivars.

	10F*Pi$^+$	**10F*Pi$^-$**	**Huia*Pi$^+$**	**Huia*Pi$^-$**	**TR29*Pi$^+$**	**TR29*Pi$^-$**
10F*Pi$^+$	1					
10F*Pi$^-$	0.198	1				
Huia*Pi$^+$	0.991	0.105	1			
Huia*Pi$^-$	0.11	0.993	0.057	1		
TR29*Pi$^+$	0.000	0.000	0.000	0.000	1	
TR29*Pi$^-$	0.000	0.000	0.000	0.000	0.000	1