Root developmental responses to heterogeneous water and nitrogen supply

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Abstract

Better understanding of the interaction between the soil physical properties determining water and nitrate availability and the root proliferation and gene expression components of nutrient acquisition could contribute to food security, but may have been limited by experimental systems.

A sand rhizotron system was developed to investigate Arabidopsis (*Arabidopsis thaliana*) root responses to altered water and nitrate supply as manipulated by soil physical properties. When this system was compared to agar, root disparities were explained by differences in hydraulic properties, highlighting the importance of the soil physical component. The sand rhizotron system was adopted to quantify root proliferation and gene expression responses to altered water and nitrate availability in wild-type and selected mutant seedlings.

In the sand rhizotron system, primary root length and lateral root density were oppositely regulated by water availability, but similarly independent of nitrate supply. The expression of the nitrate transporter *AtNRT2.1* and the aquaporin *AtPIP2.2* was coordinated across all treatments. Their concentration-dependent hydraulic regulation was confirmed for *AtNRT2.1* by *in situ* imaging of a Green Fluorescent Protein reporter line. *AtNAR2.1* and *AtNRT2.1* expression demonstrated independent responses to water and nitrate availability despite the requirement of *AtNAR2.1* for AtNRT2.1 uptake function. Root proliferation responses to water availability under high (10.0 mM) nitrate were lost in the *atnar2.1* mutant and coincided with altered hormone-associated gene (*AtEIN2*, *AtABI4* and *AtIPT5*) expression. Root proliferation and *AtNAR2.1* responses to water availability under high (10.0 mM) nitrate required *AtPIP2.2*. The coordination of root proliferation and gene expression responses to altered water and nitrate availability is proposed, that includes novel roles for *AtNRT2.1*, *AtNAR2.1* and *AtPIP2.2*.

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Chapter 1. General Introduction

List of Abbreviations and Symbols

A	Cross-sectional area of soil column	LRN	Lateral root length
ABA	Abscisic acid	MIP	Major intrinsic protein
ABI	ABSCISIC ACID INSENSITIVE	N	Nitrogen
ANR	ARABIDOPSIS NITRATE REGULATED	N_2	Di-nitrogen gas
BR	Basal root	NAR	NITRATE ASSIMILATION RELATED
BRL	Basal root length	NH_4^+	Ammonium
cDNA	Complementary DNA	NIP	NOD26-like intrinsic protein
Cl	Solution nutrient concentration	NO_3^-	Nitrate
CLC	Chloride channel	NRT	Nitrate transporter
C_s	Nutrient concentration released from soil store	PCR	Polymerase chain reaction
D	Diffusion coefficient	PEG	Polyethylene glycol
D_e	Effective diffusion coefficient in soil	PIN	PIN-FORMED
D_l	Diffusion coefficient in water	PIP	Plasma membrane intrinsic protein
DNA	Deoxyribonucleic acid	PR	Primary root
EIN	ETHYLENE INSENSITIVE	PRL	Primary root length
f	Impedance factor	Q	Volume of water per unit time
gDNA	Genomic DNA	RNA	Ribonucleic acid
GFP	Green fluorescent protein	SIP	Small basic intrinsic protein
h	Hydraulic head	t	Time
HATS	High affinity transport system	TIP	Tonoplast intrinsic protein
IPT	ADENOSINE PHOSPHATE ISO-PENTENYL-TRANSFERASE	TIR	TRANSPORTER INHIBITOR RESPONSE
k	Permeability	TLRL	Total lateral root length
K_r	Relative hydraulic conductivity	θ	Soil volumetric water content
K_{sat}	Saturated hydraulic conductivity	θ_h	Matric potential volumetric water content
K_{unsat}	Unsaturated hydraulic conductivity	θ_r	Residual volumetric water content
L	Length of soil column	θ_s	Saturated volumetric water content
LATS	Low affinity transport system	Ψ_m	Matric potential
LR	Lateral root	Ψ_o	Osmotic potential
LRD	Lateral root density	Ψ_t	Water potential

Declaration

I declare that no part of this thesis has previously been submitted for a degree awarded by this or any other university. The work in this thesis is entirely my own, unless otherwise stated.

Acknowledgments

> *"L'Agriculture fait sans doute la base du bonheur public, puisqu'elle seule fournit à tous les besoins que la nature a liés à notre existence…"*
>
> *"Agriculture is without doubt the basis of public happiness, since it alone provides all the needs that nature has linked to our existence..."*
>
> Jean-Antoine Claude Chaptal, 'Eléments de Chimie' (1790), Vol. 1, Avertissement, page 3, line 1, J.F. Picot, Montpellier, France.

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individually here, but I'd particularly like to acknowledge my housemates and the football lads for providing welcome distractions from the day-to-day slog of Ph. D. life. I thank them all for many an enjoyable evening spent solving various problems over a pint.

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The completion of this thesis was driven by a passion for science and the natural world, but I could not have hoped to make it this far without the encouragement of the people that I love. I am grateful for the support of my grandparents and wider family which has helped me in innumerable ways to progress this far.

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Dedication

I would like to dedicate this thesis to the memory of my Grandad Norman. One of my earliest recollections of the joy that plants can bring is picking peas with you and Nan in the garden. I have been frequently reminded of you around Rothamsted. Losing you before this was finished affected me more than I anticipated, but I would like to think that I have made you proud.

1. General Introduction

1.1. Global context

"We are at a unique moment in history as diverse factors converge to affect demand, production and distribution of food over the next 20 to 40 years."

Professor Sir John Beddington CMG FRS

Chief Scientific Advisor to Her Majesty's Government and Head of the Government Office for Science (in The Future of Food and Farming, Final Project Report, Foresight, 2011, Government Office for Science, Dept. Business, Innovation and Skills, UK).

1.1.1. The complex challenge of securing food for 9 billion people

The world is presently faced with a complex challenge to the security of its food supply. Crops form the basic diet of the majority of the 7 billion people that currently inhabit the Earth, but just over 1 billion of them already live without enough to eat (St Clair and Lynch 2010). In addition to what is already a major global problem, accepted estimates describe a rise in global population to 9 billion people by 2050 (Foresight 2011). As a result, global agricultural output will need to greatly improve if we are to cope with a 30% increase in global population and secure our food supplies.

An increase in global agricultural activity could match food demand but an increase in global population intensifies the pressure on land. For example, an increased number of people require an increase in the total land used for shelter and energy production. Therefore, it is likely that an increase in agricultural productivity will actually need to be achieved during a reduction in total land used for agriculture as land-use will face alternative pressures from urban population expansion. However, this may be buffered on the global scale by efforts to open up the vast Cerrado region of Brazil for agricultural use (Merten et al. 2010).

The challenge of food security is further complicated by a changing global climate (Adeloye 2010). In particular, fluctuations in local temperature and precipitation patterns can greatly

influence crop yield. This has been evident since the turn of the century with increasingly regular severe weather incidents having catastrophic effects on crop yield, such as drought (e.g. India in 2009) and flooding (e.g. Pakistan in 2010) episodes. At the time of writing, 18.7 million people are affected or at risk across nine countries of the Sahel region of Africa that are facing severe problems of food security related to climate (United Nations Office for the Coordination of Humanitarian Affairs website, 16th July 2012: http://www.unocha.org/crisis/sahel).

Drought events are one of the more agriculturally catastrophic products of a changing global climate. An insufficient supply of water is a fundamental problem for a growing plant as the targeted expansion of organs requires sufficient water pressure within the cells (Tyerman et al. 2002). It also decreases the distance over which nutrients can move towards the plant root, via diffusion or mass-flow, generating nutrient deficiencies, particularly for the less soluble nutrients (Nye and Tinker 1977; Bloom et al. 2002). As the soil dries, its strength increases posing more resistance to the growing root which is already having to forage further to access nutrients that are unable to move towards the root due to decreased water content (Whalley et al. 2006). In addition, the dry soil hampers root-microbe interactions and as a result impairs nitrogen and carbon cycling within the soil and thus the availability of raw materials for building new plant tissues (Gregory et al. 2007). All of these problems result in a decreased capability of the root to acquire vital nutrients from the soil and thus impact upon agricultural yield.

At the opposite end of the precipitation scale, intense rainfall and flooding episodes pose equally ruinous problems for agricultural productivity. Heavy rainfall or fast-moving bodies of water can influence yield through both short and long term changes in the soil composition. The differing mobility of nutrients within the soil profile is dependent on their solubility which in turn determines nutrient deficiencies as a result of leaching from the soil (Goulding 2000). Furthermore, water-logging in the aftermath of a flooding event can reduce the availability of oxygen to the growing plant. This impairs nutrient acquisition as active transport of nutrient ions requires oxygen-dependent adenosine triphosphate (ATP) synthesis (Miller and Cramer 2005; Tavares et al. 2011). Depletion of N within the plant as a result of decreased capability for uptake is further aggravated by the use of soil nitrate by microorganisms as an alternative electron acceptor when oxygen is not freely available (Atwell and Steer 1990).

In terms of nutrient acquisition, an increase in the temperature of the soil can actually help acquisition as root metabolism and ion diffusion and uptake rates increase within the rhizosphere. Under adequate water supply, the surface area for nutrient uptake and water influx is increased as the root grows and temperature-driven increases in transpiration rates help with the acquisition of mobile nutrients through increased mass flow (Nye and Tinker 1977; Jungk 1996; Miller and Cramer 2005). However, if soil drying occurs with an increase in temperature, water pressure differences can actually close stomata thus hampering transpiration and mass flow driven nutrient acquisition (Abbate et al. 2003). In addition, altered temperature can impact on the habitat range of crops and microorganisms. A change in temperature may shift the areas of land within which certain crops are currently agriculturally productive and could alter the capability of microorganisms to cycle vital nutrients within the soil, but could also influence the capability of pest species and diseases to exploit a particular crop as a host (Ingram et al. 2008).

Severe weather episodes can also decrease agricultural productivity via pressure on land-use as large numbers of people are forced to migrate in search of food and shelter (Foresight 2011). Huge areas of land can be irreparably damaged through intense exploitation of local natural resources as people and animals are fed and watered. This often accelerates the deterioration of the soil and vegetation within the region in which they settle and can lead to the desertification of large areas of land, reducing the total potential land available for agricultural activity.

Any potential solution to the complexities of the food security problem will be required to fall within the bounds of sustainable agricultural practice. As our understanding of the long term economic and environmental costs of fertilizer and pesticide use has improved, a sense of international responsibility to improve the sustainability of agricultural output has emerged (Powlson et al. 2011; Sutton et al. 2011). This is a significant point given the central role of fertilizer and pesticides in facilitating the first green revolution. The green revolution of the 1960s saw global agricultural output jump to keep step with a doubling of the global population, via the exploitation of dwarfed high-yield crop varieties in tandem with the widespread adoption and application of chemical fertilizers and pesticides (Borlaug 1992; Hedden 2003; Lynch 2007). It is likely that this time around there will need to be a more intense focus on the selection and

breeding of improved crop varieties, as the liberal use of chemical fertilizers and pesticides is no longer viewed as sustainable.

1.1.2. Enhancing nutrient acquisition can contribute to tackling food security

The complex problem of food security will require a composite agronomic solution, ranging from better access to water and soil management to improved plant breeding and enhanced nutrient acquisition. The improvement of nutrient acquisition by plants represents one promising component of such a solution (Lynch 2007; Garnett et al. 2009; Masclaux-Daubresse et al. 2010; Kraiser et al. 2011). In particular, the improvement of water and N acquisition will become more important in the context of decreased N-fertilizer input and increased flooding and drought events.

Nitrogen and water are fundamental growth-limiting nutrients that partly determine yield and their availability is determined by the root growth environment. Plants require di-nitrogen gas to be fixed as either nitrate or ammonium before nitrogen can be acquired by roots (Sanhueza 1982). The ability of sessile plants to acquire the growth-limiting nutrients water and nitrogen from the soil is largely dependent on root growth and the physical characteristics of the substrate. Nitrate is more mobile in the soil than ammonium and as a result nitrate is often the preferred nitrogen source for plants (Miller and Cramer 2005). Nitrate is delivered to the root surface dissolved in water and its availability is subject to spatial and temporal heterogeneity as a result of microbial activity, soil physical characteristics and altered water availability.

As a result, root physiology demonstrates a high degree of plasticity to cope with altered water and nitrate supply (Malamy 2005; Gifford et al. 2008; Hodge 2009; Ingram and Malamy 2010). By characterizing root physiological responses to altered supply, it may be possible to identify desirable root traits for the improvement of acquisition (Lynch and Brown 2012). This is particularly important for low input agriculture systems where resource availability to the plant may be limited. Better understanding of the root responses to altered nitrate and water availability, and particularly their interaction with the soil physical properties of the root growth environment, could contribute to sustainably securing our food supply.

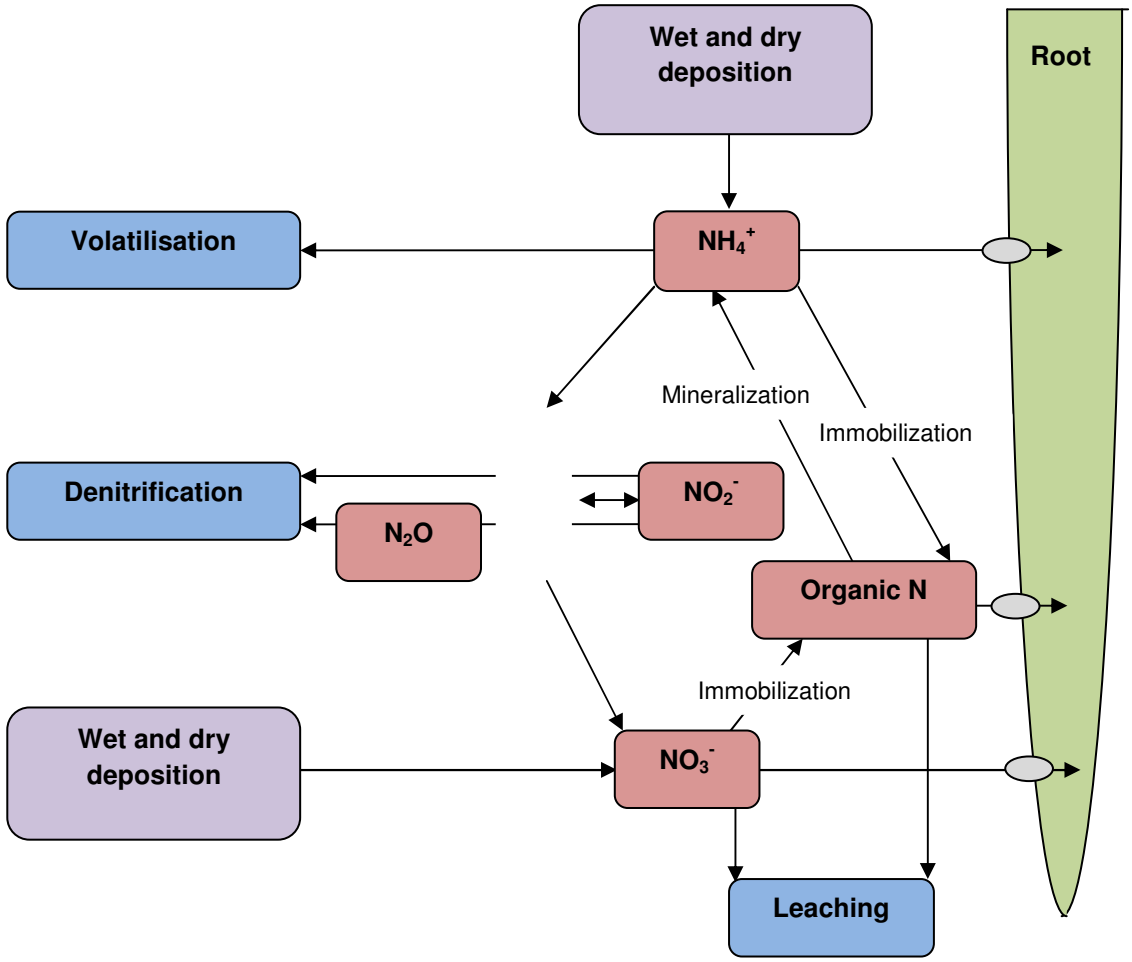
1.2. Root nutrient acquisition

Nutrient acquisition in soils is dependent upon several complex biotic and abiotic processes. While nutrient mobilisation and cycling by root exudates, symbiotic interactions and communities of microorganisms are contributing aspects of root nutrient acquisition (see reviews by Watt et al. 2006; Dennis et al. 2010; Richardson et al. 2011; Croft et al. 2012; Dodd and Ruiz-Lozano 2012), this work focuses on the major soil physical and root physiological components that govern water and nitrate acquisition. The soil physical component determines the availability and delivery of water and nitrate to the root, whereas the root physiological component is mainly comprised of root proliferation and gene expression responses to water and nitrate availability (see reviews by Miller and Cramer 2005; Wang et al. 2006; Kraiser et al. 2011). These components and the complex nature of their interactions are described in detail here. The role of the soil physical properties in the delivery of water and nitrate to the root surface is described, before focus shifts onto the growth of the root itself and how the physiology of the root responds to altered water and nitrate supply.

1.2.1. Soil physical characteristics determine the delivery of water and nitrate to the root surface

While N forms in soil can be converted from one to another (Figure 1.1.) via the action of released exudates or oxygen from the root or microbial activity, ammonium is the major from of inorganic N available to plants in flooded or wetland soils and nitrate is the dominant form in aerobic soils (Xu et al. 2012). The availability of water and nitrate to the root is determined by the physical properties of the soil. The distribution of soil particles defines the soil pore space which can be separated into the saturated and unsaturated zones depending on the extent to which the space is filled with air and water. The geometry of the soil pores largely determines the movement of water through a soil and is described by the water release characteristic (Smith and Mullins 1991), which relates gravimetric water content to tension height and can be measured for a soil column of known volume and weight (see Chapter 2 for details).

Figure 1.1. The cycling between the main nitrogen pools (boxes) and fluxes (arrows) within terrestrial ecosystems. Blue boxes, nitrogen losses; Purple boxes, nitrogen input; Red boxes, main nitrogen forms. NH_4^+, ammonium; NO_3^-, nitrate; N_2O, nitrous oxide; NO_2^-, nitrogen dioxide; N, nitrogen. Di-nitrogen gas fixation and animal input are not included here (redrawn from Chapman and Miller 2011).



The connectivity of pores governs the ease with which water is able to move through the substrate, termed the hydraulic conductivity. Darcy proposed the physical law describing the movement of water through saturated soil (Darcy 1856), demonstrating that the volume of water (Q) flowing per unit time was directly proportional to the cross-sectional area (A) of the soil column and to the difference in hydraulic head (h) causing the flow, and inversely proportional to the length (L) of the soil column. Therefore, the saturated hydraulic conductivity (K_{sat}) can be experimentally determined:

$$K_{sat} = \frac{QL}{Aht} \quad (1)$$

where: K_{sat}, saturated hydraulic conductivity (average permeability, cm/s); Q, discharge volume of water (cm^3); L, length of column (cm); A, area of column (cm^2); h, head difference (cm); t, collection time (s).

There are several methods available for determining saturated hydraulic conductivity and their suitability depends on the nature of the medium (Smith and Mullins 1991). For example, because the pore openings in sand are large and thus exhibit a high permeability ($k > 10^{-4}$ cm/s), the constant head permeability test is performed on sands (see Chapter 2 for details).

In addition to the saturated pore space, the unsaturated pore space contributes to the segregation of water between surface water and ground water in the field (Glinski and Lipiec 1990). The hydraulic properties of the unsaturated zone determine the quantity of water that will infiltrate into the soil from the surface and how much will be lost as run-off. The unsaturated zone also provides oxygen to roots and controls soil strength as a function of water content. Thus it is important to consider both the saturated and unsaturated hydraulic conductivity of soils in assessing the delivery of water and dissolved nutrients to plants.

If the water release characteristic and saturated hydraulic conductivity are known, it is possible to predict the unsaturated hydraulic conductivity of a soil (van Genuchten 1980). The change in relative hydraulic conductivity with matric potential has been broadly predicted from the water release characteristic using the Mualem-van Genuchten approach (van Genuchten 1980; Vogel et al. 2000a; Vogel et al. 2000b; Gregory et al. 2010a; Gregory et al. 2010c; Vereecken et al. 2010; Whitmore et al. 2011). The van Genuchten (1980) function can be fitted to water release characteristic data, assuming porosity does not change:

$$\theta_h = \theta_r + \frac{\theta_s - \theta_r}{[1+(\alpha h)^n]^{1-1/n}} \quad (2)$$

where θ_s, θ_r and θ_h are the saturated, residual and *h* matric potential volumetric water contents (m^3m^{-3}), *h* is the matric potential (kPa), and *α* and *n* are fitted parameters.

Residual water content is restricted to be non-negative and the Mualem constant is applied which is required to obtain the closed-form function for the subsequent hydraulic conductivity predictions (van Genuchten 1980). The relative hydraulic conductivity can then be expressed in terms of the pressure head (van Genuchten 1980):

$$K_r(h) = \frac{\{1-(\alpha h)^{n-1}\,[1+(\alpha h)^n]^{-m}\}^2}{[1+(\alpha h)^n]^{m/2}} \quad (3)$$

where $m = 1 - \frac{1}{n}$

The estimation of unsaturated hydraulic conductivity by this method assumes that porosity remains constant across a range of matric potentials. As a result, there are limitations with the application of this method to soil because the porosity of a soil changes as soils dry and shrinkage occurs (Gregory et al. 2010a; Gregory et al. 2010b). However, the porosity of sand is assumed to remain constant and the application of this method to sand allows for a reasonably accurate determination of changes in relative hydraulic conductivity with matric potential (Hewitt 1966; Smith and Mullins 1991).

Mass flow is driven by the water potential gradient between the atmosphere and bulk soil (Marshall et al. 1996). The water potential in soils is mainly determined by dissolved solutes (osmotic potential) and the capillary pressure of water held between substrate particles (matric potential). While the hydraulic conductivity of a soil can be a limiting step in the delivery of water and dissolved nutrient ions from bulk soil to the root surface (Marshall et al. 1996), differences in water potential between pores are also important. Therefore, the flux of water from soil to plant is determined by differences in water potential, at a given hydraulic conductivity and shoot-transpiration demand.

The total flux of nutrient ions to the root is comprised of a mass flow flux where dissolved nutrient ions are delivered with water moving to the root surface, and a diffusion flux which requires the formation of gradients along which ions can move (Jungk 1996). The mass flow flux is dependent on the water flux into the root and the concentration of the nutrient in the soil solution. Transpirational demand of the plant is governed by plant growth and determines water flux, but the concentration of the nutrient in the soil solution is not necessarily related to plant demand. Therefore, the rate of nutrient ion uptake is often different from the rate of mass flow and it is this disparity which determines the diffusion flux component of total flux. The spontaneous movement of ions or molecules by thermal agitation is termed diffusion and the diffusional flux is described by Fick's first law when considering a cross-sectional area in a homogeneous medium under planar conditions (Fick 1855). The diffusion coefficient of an ion describes its relative mobility along a concentration gradient with net movement towards the lower concentration.

The relative contribution of diffusion and mass flow to nitrogen acquisition is dependent upon the ion source due to their differing solubilities; nitrate (NO_3^-) is a more mobile form (3.26 x 10^{-10} m^2 s^{-1}) than NH_4^+ (2.70 x 10^{-12} m^2 s^{-1}) within soil solution (Owen and Jones 2001). The rate of diffusion for each form is influenced by ion size and charge, water viscosity, temperature, soil moisture, tortuosity and the soil buffer capacity (Miller and Cramer 2005; Cramer et al. 2009). In the field, this is further complicated by the amount of fertilizer applied (Jungk 1996), but also by the structure of the soil which creates obstacles for diffusion (Nye and Tinker 1977).

The diffusion flux in soil is dependent on the physical structure of soil and this led to the introduction of an impedance factor (f) which allowed for the impact on the diffusion coefficient of an ion of the actual distance water must move during diffusion (Nye 1966):

$$D_e = D_l \theta_f \frac{dC_l}{dC_s} \quad (4)$$

This improved the consideration of the influence of the soil physical properties on diffusion of nutrient ions. The effective diffusion coefficient in soil (D_e) is related to the diffusion coefficient in water (D_l) and the volumetric water content of the soil (θ), but also to the total amount of ions in solution (C_l) and those which can be released from the soil store (C_s). For example, the

effective diffusion coefficient in soil of nitrate (1.9 x 10^{-10-11}) is several orders of magnitude lower than the diffusion coefficient in water (1.9 x 10^{-9}) due to the physical factors impeding diffusion within soil (Jungk 1996).

The significance of the mass flow and diffusion for nutrient acquisition in the field should not be underestimated. It was determined that 190 kg of nitrogen per hectare was needed to produce 9500 kg yield of maize (*Zea mays*) grain per hectare, of which 79% was delivered to the root surface by mass flow and 20% by diffusion (Barber 1984; Jungk 1996). Therefore, mass flow and diffusion fluxes of water and dissolved nutrients from bulk soil to the root surface are important physical processes for acquisition by roots. Furthermore, these processes are inextricably linked to the physical properties of the growth substrate and influenced by water and nutrient input into the system. Attempts to improve root water and nitrate acquisition will require a better understanding of how these processes are affected by altered water and nitrate input and how they influence root physiology.

1.2.1.2. The limitations of experimental systems in considering the soil physical component of root nutrient acquisition

The physical properties of the soil represent a significant component of root nutrient acquisition, determining the delivery of water and nitrate to the root and influencing the root physiological component. However, investigating root nutrient acquisition in soil is complex (Chapman et al. 2012). For example, it is difficult to determine what the plant is responding to in a drying soil as water potential becomes increasingly negative, mechanical impedance increases, oxygen availability increases and water flux to the root decreases simultaneously (Passioura 2002; Mittler 2006). As a result, experimental systems with simplified root growth environments were developed to cope with the experimental challenges presented by soil.

These simplified systems enabled the distinct manipulation of treatments and the root responses to be non-destructively analysed under regulated laboratory conditions (Zhu et al. 2011; De Smet et al. 2012). In addition to field studies (e.g. Domenicano et al. 2011; Trachsel et al. 2011), root nutrient acquisition has been recently investigated across a range of experimental systems, including soil (e.g. Dodd et al. 2010; Sengupta et al. 2011), sand (e.g. Clark et al. 2008b; Chapman et al. 2011), vermiculite (Leach et al. 2011), perlite (Choi et al.

2011; Samadi 2011), rubber or glass beads (e.g. Evans 2011), agar or gel culture (e.g. Krouk et al. 2010b; Blouin and Puga-Freitas 2011), and hydroponics (e.g. Knipfer et al. 2011; Yan et al. 2011); with the extent to which these systems consider the important influence of soil physical properties on root nutrient acquisition decreasing through the list.

Sand represents a good alternative to soil because as a porous substrate it retains some of the hydraulic characteristics of soil (Table 1.1.), but removes the influence of background nutrient or microorganisms associated with the experimental use of soil. Sand has been historically used to address root nutrient acquisition responses of larger crop species (Drew and Saker 1975; Whalley et al. 1999; Clark et al. 2002; Brown et al. 2006), but very few studies have combined sand with the superior molecular tools and knowledge associated with Arabidopsis (*Arabidopsis thaliana*) (Chapman et al. 2011). Arabidopsis root growth has been mainly investigated using sterile non-porous laboratory systems, such as agar or hydroponics, although their root growth environments are different from porous substrates such as sand or soil (Table 1.1.). Significant fundamental questions regarding the nutritional and hormonal regulation of root physiology have been addressed using Arabidopsis in agar and hydroponic systems and our understanding of root growth has been considerably advanced as a result. However, these systems may have limited our understanding of the influence of soil physical properties on the root physiological component of nutrient acquisition.

1.2.2. Root growth

The architecture of the root system is defined by continued root development, growth and branching in response to internal and external cues (Ingram and Malamy 2010). Significant understanding of the molecular and physiological mechanisms underpinning root growth has been gained through the widespread adoption of Arabidopsis as the model laboratory plant (Benfey et al. 2010; Smith and De Smet 2012). The sequencing of the Arabidopsis genome and its short generation time, small size and modest growth requirements (Bowman 1994; Pyke 1994), have enabled this model organism to become a powerful research tool (Lavagi et al. 2012), particularly for the investigation of root physiological responses to nutritional cues.

The structure of the Arabidopsis root is largely constant due to the genetic limitations of root developmental plasticity (Bowman 1994; Zobel and Waisel 2010). The primary root (PR) is the

first to emerge from the seed and defines the main axis for subsequent root branching. It is comprised of central vascular tissue surrounded by concentric layers of single cells and the root meristem is located just behind the root tip. Within the PR meristem, cell division occurs to produce initial cells which then undergo elongation in the elongation zone located behind the apex. The daughter cells of the initials divide and differentiate into the specific root tissues (Figure 1.2.). Anticlinal divisions maintain each cell file, whilst turgor pressure from water influx into root cells drives expansion of cells. As the root elongates into the growth environment, the root cap prevents the root tip becoming damaged through the release of dead border cells to reduce frictional resistance.

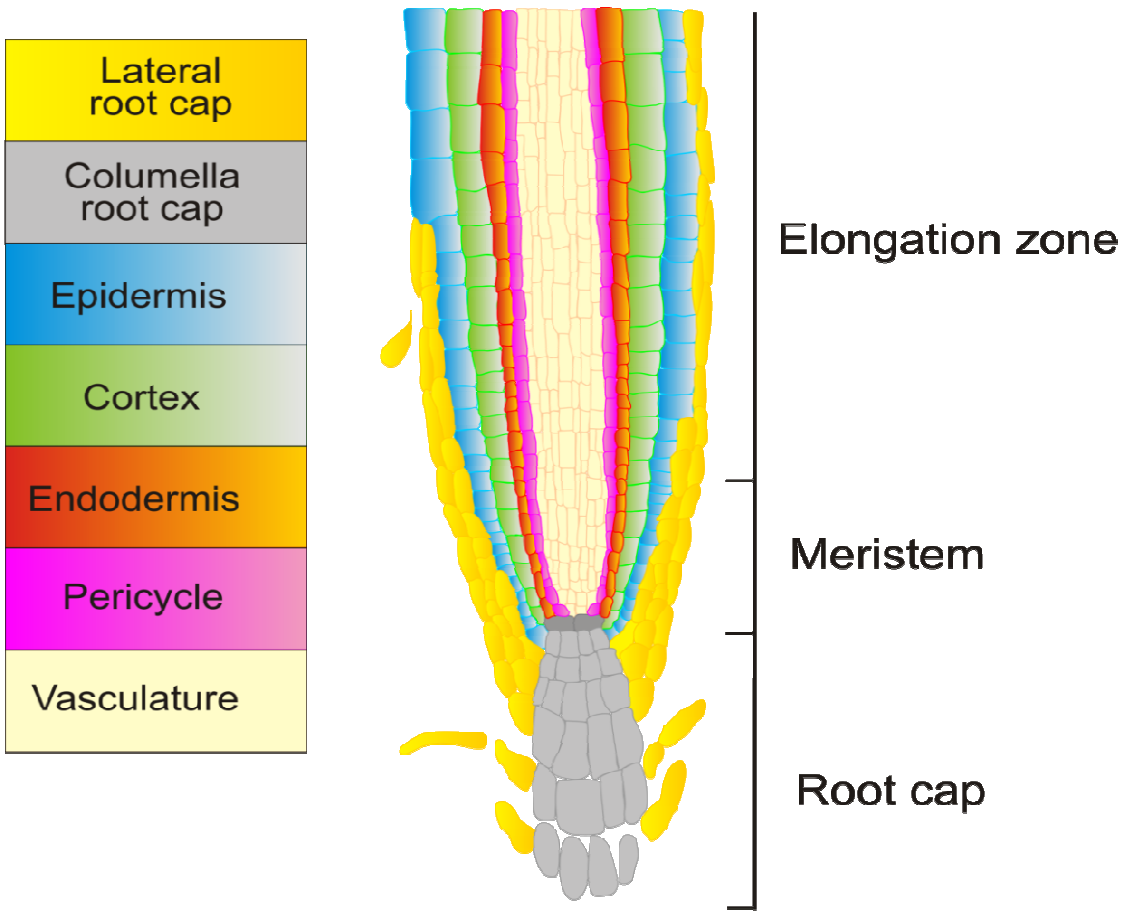
Root branching requires the production of entirely new organs called lateral roots (LRs) and is a significant contributor to the overall root system size (Bowman 1994; Malamy and Benfey 1997; Nibau et al. 2008; Benkova and Bielach 2010; Zobel and Waisel 2010). LRs initiate from founder cells within the pericycle that undergo a series of cellular divisions to generate LR primordia. The growth of LR primordia is maintained until the LR emerges through the adjacent endodermis, cortex and epidermal layers of the PR. It is at this point that a new meristem is established within the LR and is identical to that found in the PR. This becomes the new determinant of continued growth and formation of mature LRs. The acropetal nature of the spatiotemporal positioning of LRs in Arabidopsis is such that new LR primordia are specified on the alternate side of the root, and further along, from the previous LR. Secondary or basal roots (BRs) represent another additional root component which serves to increase the exploratory capacity of the root architecture. A significant component of root architecture (Zobel and Waisel 2010), BRs originate from the hypocotyl which is the organ located between the shoot and PR (Bowman 1994).

Table 1.1. The soil physical parameters that determine root nutrient acquisition vary between experimental systems that use agar, sand or soil (redrawn from Chapman et al. 2012).

Physical parameter	Agar	Sand	Soil
Pore size	Small	Narrow range	Wide Range
Pore distribution	Regular	Approximately normal	Log normal
Pore structure	Limited	Narrow	Complex
Matric potential	Increases with substrate concentration	Function of saturation	Function of saturation
Saturated hydraulic conductivity	Low	Decreases with pore size	Decreases with pore size
Unsaturated hydraulic conductivity	Determined by diffusion gradients	Function of saturation	Function of saturation
Strength	Increases with substrate concentration*	Decreases with increasing saturation by a limited amount, but can be manipulated independently with applied mechanical stress	Decreases greatly with increasing saturation
Nutrient ion distribution	Homogeneous	Heterogeneous over a narrow range of scales	Heterogeneous over a wide range of scales

*However, this cannot be used as an experimental variable (see Clark et al. 1998).

Figure 1.2. Representation of the Arabidopsis root (redrawn from Marchant et al. 1999 by Lynda Castle, Rothamsted Research Visual Communications Unit).



1.2.2.1. Root proliferation responses to water and nitrate supply

The water content of the soil is an important determinant of root proliferation. Decreased water content can increase the strength of a soil and lead to the mechanical impedance of root growth (Whalley et al. 1999). The water content determines the saturation of the soil and thus the hydraulic conductivity. The effect of soil hydraulic conductivity on root development has long been recognized (Passioura 1991), but it has received little attention because it is easier to manipulate water potential than hydraulic conductivity in high-throughput experimental systems such as agar. In the laboratory, the effects of water potential on plant growth have mainly been studied by using an osmoticum, such as polyethylene glycol (PEG), in agar/gel culture or equilibrated vermiculite to manipulate the osmotic component of water potential (Spollen and Sharp 1991; Voetberg and Sharp 1991; Liang et al. 1997; Verslues et al. 1998; Whalley et al. 1998; van der Weele et al. 2000). These approaches have been useful in identifying fundamental responses to decreased water availability, but may underestimate the importance of matric potential and hydraulic conductivity in determining root responses to water and nitrate availability.

The availability of water to roots is decreased at more negative water potentials (Marshall et al. 1996). PR growth increases in response to decreased water availability (Taylor and Ratliff 1969; Sharp et al. 1988; van der Weele et al. 2000; Wiegers et al. 2009; Chapman et al. 2011). Although root elongation has been described even at water potentials as negative as -1.9 MPa (Sharp et al. 1988), -2.0 MPa is generally accepted to be limiting to root growth (Bengough et al. 2006). Increased PR growth in response to decreased water availability may represent a foraging strategy to seek better availability down through the soil profile (Hodge 2009; Croft et al. 2012) and has been targeted as a yield-increasing trait for the improvement of water use by crop plants (Wasson et al. 2012).

Although regulated by water availability, PR growth was shown to be unresponsive to nitrate supply in barley (*Hordeum vulgare*) and maize (Drew et al. 1973; Granato and Raper 1989) and in all, but one (Nossen 0) of the Arabidopsis ecotypes investigated in work using agar (Zhang and Forde 1998; Walch-Liu and Forde 2008). In contrast to Zhang and Forde (1998), Linkohr and colleagues found that 0.01 mM nitrate supply suppressed PR length (PRL) in the Columbia 0 ecotype relative to uniform 1.0 mM supply (see Figure 2A in Linkohr et al. 2002). However, this was attributed to a disparity in harvest time between Linkohr *et al.* (18 days) and Zhang and

Forde (14 days), with the increased growth period perhaps enabling another factor to become limiting for PRL. However, the strong PR response to water availability independent of nitrate supply may reflect the 2-fold economic benefit from investing in roots for water capture relative to N capture (King et al. 2003).

LR growth has also been investigated in response to altered water potential. LR proliferation has been shown to be repressed by decreased water availability in Arabidopsis seedlings (Deak and Malamy 2005; Roycewicz and Malamy 2012) and this response is independent of nitrate (Roycewicz and Malamy 2012). Increasingly negative osmotic potential resulted in altered root and shoot size, whilst nitrate altered root system size with no effect on shoot size when osmotic potential was controlled (Roycewicz and Malamy 2012). At water potentials of less than -0.49 MPa, the regulation of LR growth by water availability is specific to LR proliferation with a negligible effect on LR number (van der Weele et al. 2000; Deak and Malamy 2005). Therefore, it was speculated that LR proliferation is repressed in regions of decreased water availability but maintain the establishment of LR primordia (Deak and Malamy 2005). During the elongation of the root system, this enables LR primordia to be subsequently proliferated into a region of increased water availability when encountered. This repression of LR growth under water deficit is maintained in other species, such as barley and maize (Babé et al. 2012).

Unlike PR growth, LR growth is regulated by water and nitrate. Under uniform supply, Arabidopsis LR proliferation is suppressed by increasing nitrate concentration, despite LR number and PR growth remaining constant across a wide range of concentrations (Zhang and Forde 1998). However, localized patches of high nitrate supply under uniform low nutrient availability has been shown to locally stimulate LR proliferation in barley and Arabidopsis (Drew et al. 1973; Drew 1975; Drew and Saker 1975; Zhang and Forde 1998). The proliferation of LRs towards localised high nitrate patches enables plants to cope with heterogeneous nitrate availability in the field and may represent an adaptive strategy to out-compete neighbouring plants for patchy resources (Robinson 1996; Hodge et al. 1999; Mommer et al. 2012).

Root proliferation responses such as those described above are particularly important for the acquisition of nutrient ions that are only able to move small distances within the soil. This may be due to decreased mobility or physical isolation. It is accepted that root proliferation is an adaptive trait for roots to access less mobile nutrient ions, such as ammonium and phosphate

(Hodge et al. 1999; Robinson et al. 1999; Bayuelo-Jimenez et al. 2011; Trachsel et al. 2011), but there is even a distinct root response to differences in mobility between N forms (Bloom et al. 1993), whereby the less mobile ammonium induces LR initiation while nitrate determines LR proliferation (Lima et al. 2010). The restricted movement of ions due to physical isolation is by nature the result of soil structure. The formation of connected networks of solution is determined by the distribution of pores that can cause the spatial isolation of dissolved nutrients regardless of the ion's mobility. The LR proliferation response to the mobile nitrate ion could indicate a foraging strategy that has evolved to access isolated nutrient pockets under more patchy supply in the field.

1.2.2.2. The hormonal regulation of root proliferation responses to water and nitrate supply

The hormone content of the plant is modified in response to nutrient supply and this can have a significant impact on root proliferation (Forde 2002; Rubio et al. 2009). The root proliferation responses to water and nitrate are partly determined by the hormones auxin, ethylene, abscisic acid (ABA) and cytokinin. Here, the role of these hormones in determining root growth and the function of selected hormone-associated genes in mediating root proliferation responses to nitrate and water supply are described.

The orientation of PR growth is determined by gravity which influences auxin distribution and determines cell elongation on the basal surface so that the root always proliferates towards the gravitational force (Santelia et al. 2008; Rahman et al. 2010). Auxin distribution in the Arabidopsis root is regulated through directional cellular efflux facilitated by eight PIN-FORMED (PIN) proteins (Benkova et al. 2003; Friml 2003; Friml et al. 2003; Blilou et al. 2005). Of particular importance to root architecture are AtPIN1 and AtPIN2. Auxin is made in the shoot and transported to the root via AtPIN1 which is located on the basal surface of the central vascular tissue. The acropetal flow of auxin along the outer cell layers of the root is achieved through AtPIN2, located on the basal surface of cortex cells and on the apical surface of epidermal and root cap cells. Cellular uptake of auxin is achieved through lipophilic diffusion, proton-driven anionic symport via AUXIN/LIKE-AUXIN (AUX/LAX) permeases, and ATP-dependent uptake via a P-glycoprotein (PGP), another subset of which helps to achieve cellular

efflux in addition to the PINs (Feraru and Friml 2008; Mravec et al. 2008). The directed movement of auxin towards, around and away from the root meristem establishes an auxin maximum at this site which maintains meristematic cell identity. Consequently, the decreasing auxin concentration further away from the meristem has been linked to cell differentiation in the mature root.

As well as a role in pattern formation during root development, the shoot-to-root translocation and subsequent oscillation in acropetal redistribution of auxin is required to specify LR growth (Bhalerao et al. 2002; De Smet et al. 2007). Localized auxin distribution was shown to promote LR initiation (Blakely and Evans 1979; Barlier et al. 2000; Fukaki et al. 2005) and auxin accumulates in cells which subsequently become LR primordia, forming a gradient with the maximum concentration located at the tip of the LR primordium (Benkova et al. 2003; Dubrovsky et al. 2008). This accumulation and gradient formation is mediated by the PIN efflux machinery, with AtPIN1 establishing the auxin front at the LR primodrium tip and AtPIN2 recycling some of the auxin along the outer layers, as is the case in the PR (Benkova et al. 2003; Geldner et al. 2004). Polar auxin transport and the perception of auxin by TRANSPORTER INHIBITOR RESPONSE 1 (TIR1) have been shown to be critical for the establishment of a zone of minimum auxin content and auxin response that specifies LR initiation (Ivanchenko et al. 2010; Dubrovsky et al. 2011). The *AtTIR1* gene encodes an auxin receptor that mediates auxin degradation and auxin-regulated transcription (Dharmasiri et al. 2005; Kepinski and Leyser 2005; Tan et al. 2007). BR growth is less well understood in Arabidopsis as BRs are infrequently formed in agar culture, but auxin has been implicated in BR growth in the common bean (*Phaseolus vulgaris*) (Basu et al. 2011) and the mechanism of auxin-transport dependent growth is applicable to each component organ of Arabidopsis root architecture.

Increased auxin translocation from shoot to root under conditions of nitrogen starvation has been demonstrated in Kohlrabi (*Brassica caulorapa*) (Avery Jnr. and Pottorf 1945), wheat (*Triticum aestivum*) (Chen et al. 1998), soybean (*Glycine max*) (Caba et al. 2000) and maize (*Zea mays*) (Guo et al. 2005). This has also been demonstrated in Arabidopsis (Krouk et al. 2010b) and the expression of the auxin transporters *AtPIN1* and *AtPIN2* and the auxin receptor *AtTIR1* are regulated by nitrogen provision (Gutierrez et al. 2007). Therefore, the response of LR growth to nitrate supply may be underpinned by changes in the expression of these auxin-

associated genes. Interestingly, the *AtTIR1* transcript is the target of micro-RNA mediated cleavage to inhibit LR growth in response to osmotic stress as induced by PEG in agar culture (Chen et al. 2012). Given the role of these genes in regulating PR and LR growth, the response of PR and LR growth to water availability could also be mediated by altered expression of these genes but this has not yet been described.

Auxin and ethylene interact to regulate root proliferation, acting synergistically to regulate root elongation and antagonistically to regulate LR growth (reviewed by Muday et al. 2012). Auxin and ethylene have been shown to reduce the rate of cellular expansion in the central elongation zone, which is lost in ethylene-insensitive and auxin-resistant mutants (Rahman et al. 2007; Swarup et al. 2007). In the root elongation zone, elevated ethylene increases auxin response and requires wild-type auxin signalling and transport (Ruzicka et al. 2007; Negi et al. 2008). Auxin stimulates LR formation and proliferation, whilst ethylene decreases LR initiation by regulating auxin transport (Ruzicka et al. 2007; Ivanchenko et al. 2008; Negi et al. 2008; Lewis et al. 2011). The ethylene signal transducer ETHYLENE INSENSITIVE 2 (EIN2) (Alonso et al. 1999) plays an important role in the nitrate regulation of LR length (Tian et al. 2009), with *AtEIN2* modulating stress responses via the ABA signalling pathway (Wang et al. 2007) and acting upstream of *AtTIR1* and *AtPINs* to modify root growth (Kushwah et al. 2011).

ABA has been suggested to be the universal plant stress hormone (Wilkinson and Davies 2002; Verslues and Zhu 2005; Wasilewska et al. 2008), and plays a role in regulating root growth responses to altered water and nitrate supply. The application of external ABA to well-watered plants, inhibits shoot and root growth (Sharp and LeNoble 2002). However, increased endogenous ABA content under decreased water availability, inhibits shoot growth (Zhang and Davies 1990a; Zhang and Davies 1990b; Verslues and Zhu 2005; Dodd et al. 2010). Conversely, maize PR growth is maintained at increased negative water potentials, mediated by the action of increased ABA to limit excess ethylene production (Spollen et al. 2000; Leach et al. 2011).

In addition, ABA signalling inhibits the activation and elongation of LRs under water stress conditions (Deak and Malamy 2005) and ABA and nitrate signalling have been suggested to share common regulatory elements due the impairment of LR responses to nitrate in ABA mutants (Signora et al. 2001; De Smet et al. 2003; Zhang et al. 2007). Specifically, under high

nitrate supply, the ABA related transcription factor ABSCISIC ACID INSENSITIVE 4 (ABI4) has been shown to repress LR growth mediated by decreased AtPIN1 levels, with *AtABI4* expression being repressed by auxin and enhanced by ABA and cytokinin (Signora et al. 2001; Shkolnik-Inbar and Bar-Zvi 2010).

Cytokinin is thought to act antagonistically to auxin in regulating LR development (Fukaki and Tasaka 2009). Mutants with reduced cytokinin levels, perception or signalling exhibit an increase in the number of LRs (Werner et al. 2003; Mason et al. 2005; Riefler et al. 2006), while exogenous cytokinin supply inhibits LR initiation (Laplaze et al. 2007). Cytokinin also induces directional root growth, acting upstream of *AtEIN2* and thus *AtTIR1* and *AtPINs* to determine root elongation (Pernisova et al. 2009; Ruzicka et al. 2009; Kushwah et al. 2011). The limiting step in biosynthesis of cytokinin is encoded by *ADENOSINE PHOSPHATE ISO-PENTENYL-TRANSFERASE 3* (*IPT3*) which modifies root and shoot cytokinin status in response to nitrate (Miyawaki et al. 2004; Takei et al. 2004; Wang et al. 2004; Rahayu et al. 2005). However, less is known about the nitrate response of other root-specific cytokinin biosynthesis genes, although *AtIPT5* is regulated by auxin and cytokinin and responds to nitrate and ammonium under sufficient supply (Takei et al. 2004).

Therefore, complex interactions exist between cytokinin, ABA, ethylene and auxin to modify root growth in response to both altered water and nitrate supply. It seems that auxin is the main driver of these responses, with cytokinin-, ABA- and ethylene-associated genes acting to attenuate the transport, signalling and perception of auxin. Much of the fundamental understanding held for these responses in Arabidopsis has been gained in the agar Petri dish, where understanding of the influence of soil physical properties on these hormonal responses may be limited. For example, the maintenance of root growth under water deficit by ABA has been described above, but barley root growth was unaffected by the application of an auxin-stimulating growth promoter under field conditions despite an increase in ABA at the stem base (Bingham and McCabe 2006). Therefore, a greater understanding of the interplay between these hormone-associated genes in regulating the Arabidopsis root proliferation responses to altered soil physical properties is required.

1.2.3. Root transporters for water and nitrate uptake

Root proliferation in response to altered water and nitrate supply represents one significant aspect of the root physiological component of their acquisition. A second important aspect is represented by root transporter-dependent water and nitrate uptake. Here, the transporters responsible for water and nitrate uptake and their regulation in response to water and nitrate availability are discussed.

1.2.3.1. Root water uptake and movement; the role of aquaporins

Once delivered to the root surface, water enters the root passively or actively predominantly along the region of elongation (Segal et al. 2008). Inside the root, water flows radially through living tissues towards the vascular tissue to be transported throughout the whole plant. Water can flow via the cell wall continuum (i.e. apoplastic pathway), via cytoplasmic continuities (i.e. symplastic pathway), or across cell membranes (i.e. transcellular pathway) via water channels known as aquaporins (Peterson and Cholewa 1998; Steudle 2000; Javot and Maurel 2002; Roose and Fowler 2004; Maurel et al. 2010; Ranathunge and Schreiber 2011).

Although apoplastic barriers can be generated to limit apoplastic water flow (Peterson and Cholewa 1998), there is limited regulation of root water movement when driven by large transpirational fluxes. However, the regulation of aquaporins offers the plant a means to partly control water movement into and throughout the root, particularly when transpiration is decreased (Javot et al. 2003; Da Ines et al. 2010). The cellular localization of many aquaporins is known and their ability to rapidly respond to environmental cues means that changes in their expression are a good indicator of transmembrane pathway activity.

There is a great abundance and diversity in plant aquaporins (Chaumont et al. 2001; Johanson et al. 2001) which demonstrates the need for plants to tightly regulate cellular water content and solute transport (see reviews by Tyerman et al. 2002; Maurel et al. 2008). The aquaporin super-family, or the Major Intrinsic Protein (MIP) super-family, is comprised of hydrophobic proteins that fall within the molecular weight range of 26-34 kDa and exhibit six membrane-spanning alpha-helices (Hove and Bhave 2011). The MIP super-family has been split into 4 constituent groups based on amino-acid sequence comparison and these have different cellular locations

(Maurel et al. 2008): small basic intrinsic proteins (SIPs); NOD26-like intrinsic proteins (NIPs); tonoplast intrinsic proteins (TIPs); and plasma membrane intrinsic proteins (PIPs).

Despite the majority of PIPs being expressed in the leaf, certain PIPs are root-specific and may be the most important aquaporins for the transcellular pathway of root water movement due to their plasma membrane location (Kaldenhoff et al. 1998). Furthermore, the use of antisense-RNA to down-regulate PIPs increased susceptibility to drought or osmotic stress, and led to decreased protoplast osmotic water permeability and an increased root system size (Kaldenhoff et al. 1998; Siefritz et al. 2002). The expression of PIPs responds to nutrient stress (Clarkson et al. 2000) and decreased water-availability has been shown to down-regulate PIP transcripts across several species (Yamada et al. 1995; Li et al. 2000; Alexandersson et al. 2005; Boursiac et al. 2005; Alexandersson et al. 2010; Horie et al. 2011).

Although regulation of PIP-mediated water movement can occur at the protein level (Boursiac et al. 2008; Muries et al. 2011; Hachez et al. 2012), overall protein content, cellular localization and root hydraulic conductance has been shown to correlate with transcript level (Boursiac et al. 2005; Parent et al. 2009; McLean et al. 2011). In fact, it may be better to assess root responses to altered water and nitrate availability at the transcript level rather than indirect measurements such as root hydraulic conductance. The investigation of 13 Arabidopsis accessions found some minor differences in root hydraulic conductance (Sutka et al. 2011), but an independent study comparing aquaporin expression changes across five Arabidopsis accessions that were known to have different water use efficiency found that aquaporin expression responses were largely conserved between accessions (Alexandersson et al. 2010).

Of the *AtPIPs*, *AtPIP1.1* and *AtPIP2.2* demonstrate greatest expression within roots (Alexandersson et al. 2005). Of particular interest for root acquisition responses to altered water and nitrate availability, AtPIP2.2 has been shown to facilitate root water uptake and osmotic water transport under low transpiration conditions (Javot et al. 2003; Da Ines et al. 2010). Located in the cortex, endodermis and stele of elongated root segments, T-DNA knock-out mutants for this aquaporin exhibit a decreased hydraulic conductivity within root cortex cells indicating its importance for osmotic water flow. *AtPIP2.2* expression has been shown to be significantly down-regulated in response to prolonged drought treatment and this down-regulation was rescued upon rehydration (Kawaguchi et al. 2004; Alexandersson et al. 2005;

Alexandersson et al. 2010), highlighting the importance of water availability as a regulator of expression. Although no previous work has reported that *AtPIP2.2* is regulated by nitrate, water channel genes have been shown to be induced by nitrate (Wang et al. 2001) and aquaporin-mediated changes in root hydraulic conductance have also been reported in response to high nitrate supply (Carvajal et al. 1996; Clarkson et al. 2000).

1.2.3.2. Root nitrate uptake

Nitrate transporters (NRTs) that facilitate nitrate movement have been well characterised in Arabidopsis and their roles in nitrate acquisition have been well reviewed (Orsel et al. 2002a; Miller et al. 2007; Tsay et al. 2007; Daniel-Vedele et al. 2009; Miller et al. 2009; Chapman and Miller 2011; Gojon et al. 2011; Kraiser et al. 2011; Miller and Chapman 2011; Xu et al. 2012). In summary, AtNRTs mainly fall into two families based on their transport affinity, with members of the large NRT1/PTR family facilitating low-affinity transport and members of the NRT2 families enabling high-affinity transport. *AtNRT1.1* and *AtNRT2.1* both encode proton symporters, transporting two H^+ ions simultaneously with one nitrate (NO_3^-) ion across the plasma-membrane into the cell, and emerge as two of the most important and interesting nitrate acquisition genes. In addition to their different nitrate transport affinities, these two genes demonstrate regulatory cross-talk with one another, possess transport-independent signalling/sensing roles, regulate root growth and interact with key hormones and hormone-associated genes.

AtNRT1.1 was the first of the 53 members of the AtNRT1/PTR family to be characterized (Doddema et al. 1978). This large family can transport a variety of other substrates in addition to nitrate, including amino acids, auxin, peptides, ABA and glucosinolates (Tsay et al. 2007; Wang et al. 2012). AtNRT1.1 facilitates the proton-coupled transport of nitrate (Tsay et al. 1993) and is located in the epidermal cells of the root-tip (Liu et al. 1999; Remans et al. 2006a). The majority of the AtNRT1s demonstrate low-affinity nitrate transport and have been consequently assigned to the low affinity transport system (LATS) for nitrate (Huang et al. 1999; Chiu et al. 2004; Almagro et al. 2008; Lin et al. 2008; Fan et al. 2009; Li et al. 2010a; Wang and Tsay 2011). However, AtNRT1.1 is unique among the AtNRTs in demonstrating dual-affinity transport function and this capability has been shown to be regulated by the phosphorylation of the T101

threonine residue by CIPK23 kinase (Wang et al. 1998; Liu et al. 1999; Ho et al. 2009). Orthologues in rice (*Oryza sativa*; *Os08g05910* and *Os10g40600*) and oilseed rape (*Brassica napus*; *BnNRT1.2*) have been described (Tsay et al. 2007), and the only other dual-affinity transporter to be isolated (MtNRT1.3) was recently identified in Medicago (*Medicago truncatula*) (Morere-Le Paven et al. 2011).

In contrast to the large NRT1/PTR, there are only 7 genes in the AtNRT2 family which largely determine the high affinity transport system (HATS) in Arabidopsis (Chen et al. 2008). AtNRT2.1 is found at the plasma membrane of cortex and epidermal cells (Nazoa et al. 2003; Chopin et al. 2007) and plays a particularly important role within the inducible HATS (Cerezo et al. 2001; Remans et al. 2006b; Li et al. 2007). Transport function was believed to be achieved by the most abundant monomeric form of the AtNRT2.1 protein, but requires the co-expression of *NITRATE ASSIMILATION RELATED 2.1* (*AtNAR2.1*, also known as *AtNRT3.1*) which targets the AtNRT2.1 monomer to the plasma membrane (Zhou et al. 2000; Okamoto et al. 2006; Orsel et al. 2006; Wirth et al. 2007). More recently however it has been suggested that uptake function is facilitated by the formation of an AtNRT2.1/AtNAR2.1 hetero-oligomer at the plasma membrane (Yong et al. 2010). NRT2.1 genes have been described in wheat (*TaNRT2.1*) and rice (*OsNRT2.1*), with the latter also interacting with *OsNAR2.1* to achieve uptake function (Yin et al. 2007; Yan et al. 2011).

AtNRT1.1 expression is induced by nitrate and repressed by nitrogen starvation, pH and nitrate metabolites such as nitrite (Tsay et al. 1993; Wang and Crawford 1996; Filleur and Daniel-Vedele 1999; Lejay et al. 1999; Liu et al. 1999; Lejay et al. 2003; Loque et al. 2003; Lejay et al. 2008; Ho et al. 2009). *AtNRT2.1* expression is similarly induced by nitrate, but also by nitrogen starvation, and repressed by high nitrate and nitrate metabolites such as ammonium and glutamate (Zhuo et al. 1999; Filleur et al. 2001; Nazoa et al. 2003; Okamoto et al. 2003; Lejay et al. 2008). Similar phosphorylation mechanisms to that which regulate AtNRT1.1 have been suggested for AtNRT2.1 (Liu and Tsay 2003). Evidence for this is provided by a number of conserved protein kinase C recognition motifs in HvNRT2.1 (Forde 2000). Furthermore, an orthologue of *AtNRT2.1* is known in wheat which acts within the HATS but demonstrates induction by both high and low external nitrate supplies in a similar dual-affinity response to that seen for AtNRT1.1 (Zhao et al. 2004; Yin et al. 2007).

Interestingly, *AtNRT2.1* expression was found to be regulated by AtNRT1.1 itself, as *AtNRT2.1* expression was not repressed in an *nrt1.1* mutant line in response to high nitrate supply (Munos et al. 2004). *AtNRT2.1* was shown to be regulated by the activity of *AtNRT1.1*, rather than simply by its presence, via a nitrogen demand signal as a function of AtNRT1.1-dependent nitrate uptake activity (Krouk et al. 2006). Furthermore, the down-regulation of the AtNRT2.1/AtNAR2.1 functional unit by nitrate itself is triggered by *AtNRT1.1* and this is independent of the negative feedback exerted by downstream nitrogen metabolites (Munos et al. 2004; Krouk et al. 2006). Therefore, the down-regulation of *AtNRT2.1* under high nitrate supply is the response to systemic feedback repression by reduced nitrogen metabolites (Crawford and Glass 1998; Gansel et al. 2001) and also to nitrate-specific AtNRT1.1-dependent local inhibition (Krouk et al. 2006).

Nitrate-responsive genes account for up to 10% of the transcriptome (Krouk et al. 2010c) and nitrate uptake activity specifically correlates with AtNRT transcript abundance which suggests that transcriptional regulation of AtNRTs plays an important role in the plant physiological response to nitrate supply (Zhuo et al. 1999; Okamoto et al. 2003; Wang et al. 2012). Furthermore, the physiological response to nitrate is likely to be a perception of nitrate as the signal because the majority of nitrate-induced effects on gene expression are conserved in mutants that are unable to reduce nitrate (Wang et al. 2004; Alboresi et al. 2005; Krouk et al. 2010a).

Although regulated by nitrate supply, the expression of these key Arabidopsis nitrate acquisition genes has not been investigated in response to altered soil physical properties. However, under low nitrogen supply, soil compaction has been shown to result in decreased N uptake in barley towards the end of a 14 day growth period (Bingham et al. 2010). In addition, soil strength has been shown to regulate rice phosphate and sulphate transporters in a sand column system (Brown et al. 2006). Given the significant influence of soil physical properties on water and nitrate availability, which themselves are known to regulate the expression of *AtPIP2.2*, *AtNRT1.1*, *AtNRT2.1* and *AtNAR2.1*, it would be informative to investigate the influence of altered soil physical properties on these genes.

1.2.3.3. Nitrate transporters and root proliferation

Transport-independent signalling and/or sensing roles have been suggested for *AtNRT1.1* and *AtNRT2.1* in the regulation of root proliferation responses to nitrate supply. A role for *AtNRT1.1* has been identified in regulating PR growth. PR growth is inhibited by glutamate and this is overcome when nitrate is supplied. However, PR growth in *chl1* mutant plants was not inhibited by glutamate (Walch-Liu et al. 2006; Walch-Liu and Forde 2008). When *chl1* mutants were complemented with wild-type *AtNRT1.1*, the inhibition of PR growth by glutamate was restored. However, when complemented with a non-phosphoryable NRT1.1T101A mutant protein then the inhibitory effect of glutamate was not restored. This mutant line still possessed transport function indicating that the ability of *AtNRT1.1* to relieve glutamate-inhibition of PR growth is dependent upon a transport-independent signalling role for the phosphorylated form of AtNRT1.1 (Liu and Tsay 2003). This was confirmed through the use of uptake- and sensing-decoupled mutants with the phosphorylation of T101 shown to be mediated by CIPK23 during high affinity binding (i.e. at low external nitrate supply) and this mechanism was repressed at higher external concentration as low-affinity binding occurred (Ho et al. 2009).

Plants with defective *AtNRT1.1* expression also have a decreased LR elongation response to high nitrate patches (Remans et al. 2006a). This is associated with a dramatic decrease in expression of the MADS-box transcription factor gene *ARABIDOPSIS NITRATE REGULATED 1* (*AtANR1*) which was described to control the LR colonization of high nitrate patches (Zhang and Forde 1998). *atnrt1.1* mutant plants exhibited wild-type nitrate uptake but decreased *AtANR1* expression, suggesting that the high nitrate signal is mediated by *AtANR1* downstream of *AtNRT1.1* and that *AtNRT1.1* regulation of the LR response is independent of its uptake function (Walch-Liu and Forde 2008). AtNRT1.1 transports auxin in the absence of nitrate, but not when nitrate is present, thus controlling the nitrate-promotion of LR elongation by alleviating localised auxin repression of LR growth (Krouk et al. 2010b).

A role for *AtNRT2.1* in sensing nitrate supply or transducing the nitrate signal has been proposed during LR initiation from the study of *atnrt2.1* knock-out mutants (Malamy and Ryan 2001; Orsel et al. 2004; Little et al. 2005; Remans et al. 2006b). Both *AtNRT2.1* and *AtNAR2.1* were found to be positive regulators of LR initiation under limited nitrate or nitrate-free conditions, indicating that *AtNRT2.1/AtNAR2.1* regulation of LR growth is independent of uptake function (Orsel et al. 2006; Remans et al. 2006b). However, this LR regulation by

AtNRT2.1/AtNAR2.1 is specific to the nutrient supply as both became repressors of LR growth under a high sucrose to nitrate ratio in the root growth environment (Little et al. 2005; Orsel et al. 2006). Truncated forms of AtNRT2.1 co-exist with the AtNRT2.1 protein at the plasma membrane and these have been suggested to be possible facilitators of the transport-independent roles described above (Wirth et al. 2007).

1.2.3.4. The hormonal regulation of transporter responses to water and nitrate supply

The expression of key water and nitrate uptake genes is regulated by auxin, ethylene, ABA and cytokinin. The link between drought response and the hormone ABA is well known (see Davies and Zhang 1991; Peleg and Blumwald 2011). As part of this, *AtPIP2.2* expression is increased 2-fold in response to ABA (Jang et al. 2004) and this response is also seen in maize (Parent et al. 2009) and tobacco (*Nicotiana tabacum*) plants (Mahdieh and Mostajeran 2009). However, there is limited evidence for aquaporin regulation by other hormones. In a rare example from rubber trees (*Hevea brasiliensis*), *HbPIP2.1* has been shown to be regulated by auxin, with the promoter region of *HbPIP2.1* possessing an auxin response element (Tungngoen et al. 2011).

Conversely, auxin plays a key role in the regulation of nitrate acquisition genes. The promoter activity of *AtNRT1.1* is induced by increased auxin and this is independent of nitrate supply (Guo et al. 2002). Interestingly, AtNRT1.1 facilitates auxin uptake in the absence of nitrate and this is inhibited in the presence of nitrate. This function is responsible for the nitrate-promotion of LR elongation by alleviating localised auxin repression of LR growth (Krouk et al. 2010b). *AtNRT2.1* expression is rapidly decreased following auxin supply and this requires a shoot-derived signal (Gan et al. 2005).

In addition to the important hormone auxin, the key nitrate uptake genes are also regulated by ethylene, cytokinin and ABA. The ethylene inhibition of LR growth under high nitrate supply is not observed in the *atein2-1* and *atnrt1.1* mutants, while AtNRT1.1 expression is influenced by ethylene signalling, suggesting that AtNRT1.1 has a role in mediating ethylene-regulation of nitrate-dependent LR growth (Tian et al. 2009). In fact, *AtEIN2* controls the nitrogen regulation of *AtNRT1.1* and *AtNRT2.1* themselves (Tian et al. 2009). Genome-wide approaches found that

AtNRT2.1 is also regulated by cytokinin and ABA (Brenner et al. 2005; Nero et al. 2009; Kiba et al. 2011). Microarray data demonstrated that the expression of *AtNRT2.1* and *AtNAR2.1* undergoes a 2-3 fold down-regulation in response to the exogenous application of cytokinin (Kiba et al. 2005; Kiba et al. 2011).

This study also identified nitrate concentration dependent hormone responses for *AtNRT1.1*, *AtNRT2.1* and *AtNAR2.1* expression (Kiba et al. 2011). *AtNRT1.1*, *AtNRT2.1* and *AtNAR2.1* expression was also down-regulated by auxin under high (10.0 mM) nitrate supply, although only *AtNRT1.1* and *AtNRT2.1* retained this response under low (0.1 mM) nitrate supply. In response to ethylene, *AtNRT1.1*, *AtNRT2.1* and *AtNAR2.1* expression was down-regulated under high (10.0 mM) nitrate supply, with *AtNRT1.1* expression up-regulated under low (0.1 mM) nitrate supply. The expression of all three genes was up-regulated by ABA and down-regulated by cytokinin, independent of nitrate supply.

This led to the suggestion that phytohormones are the signals that coordinate root physiological responses to nitrate supply. For example, cytokinin is thought to play a role in both the long-range signal to increase nitrate and carbon metabolism in leaves in response to increased root nitrate supply (Scheible et al. 2004; Brenner et al. 2005), and also in the localised inhibition of AtNRT accumulation in roots supplied with nitrate (Brenner et al. 2005; Kiba et al. 2005). However, there is a great deal yet to understand relating to the interplay between hormones and their influence on the root proliferation and acquisition gene responses to altered water and nitrate supply.

1.3. General conclusions

Enhancing root nutrient acquisition may help to contribute to food security. Soil physical properties are important in regulating the mass flow and diffusional fluxes of water and nitrate to the root, while root proliferation and the expression of important acquisition and hormone-associated genes respond to altered water and nitrate supply (Figure 1.3.). The distribution of soil particles determines the connectivity of pores and thus the ability of water and dissolved nitrate to move towards the root. In some cases the connectivity of pores can be limited and result in the physical isolation of water and nutrients. Uptake of nutrients at the root surface can

create a diffusional gradient along which dissolved ions can move towards the root from the bulk soil.

The interaction between the soil physical and root physiological components represent a significant contribution to water and nitrate acquisition (Figure 1.3.). However, studying root physiological responses to water and nitrate supply in simplified experimental systems may have limited the understanding of the influence of the soil physical component. This may be particularly true for the regulation of selected important genes (Table 1.2.) involved in water and nitrate acquisition and the hormonal regulation of root responses. Therefore, the investigation of root physiological responses to altered water and nitrate availability as manipulated by soil physical properties may provide novel understanding that could help to contribute to sustainably tackling food security by enhancing nutrient acquisition.

Figure 1.3. The delivery of water and nitrate to the root surface is determined by the soil physical properties and root physiology. The distribution of soil particles (brown) determines the connectivity of pores and thus soil hydraulic conductivity. This determines the ability of water (dashed blue arrow) and dissolved nitrate ions (NO_3^-) to move towards the root. In some cases the connectivity of pores can be limited and result in the physical isolation of water and nutrients. Root (green) proliferation and water (blue circle) and nitrate (yellow circle) transporter expression respond to cope with altered availability. Uptake of nutrients at the root surface can create a diffusional gradient (white triangle) along which dissolved ions can move towards the root from the bulk soil.

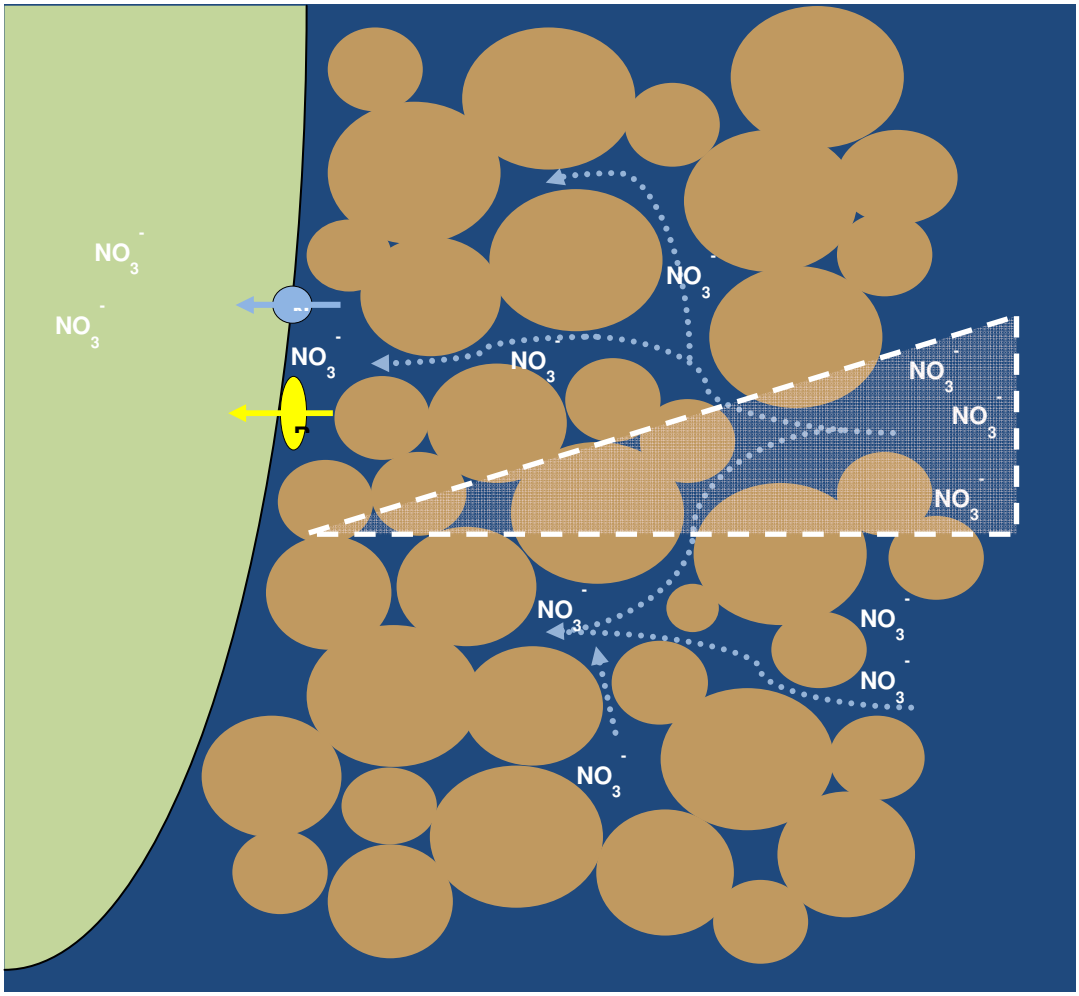


Table 1.2. Selected genes of interest.

Gene	Atg number	Reported function
AtNRT1.1	At1g12110	Dual affinity nitrate transporter and nitrate sensor that controls LR proliferation and can transport auxin in the absence of nitrate
AtNRT2.1	At1g08090	High affinity nitrate transporter and nitrate sensor that controls LR proliferation
AtNAR2.1	At5g50200	Required for AtNRT2.1 nitrate transport function
AtPIP2.2	At2g37170	Osmotic root plasma membrane water transporter
AtPIN1	At1g73590	Basal root auxin transporter in vascular tissue
AtPIN2	At5g57090	Root auxin transporter (basally in cortex cells and apically in epidermal and root cap cells)
AtTIR1	At3g62980	Auxin receptor that mediates auxin degradation and auxin-regulated transcription
AtEIN2	At5g03280	Ethylene signal transducer that plays an important role in the nitrate regulation of LR length
AtABI4	At2g40220	ABA-related transcription factor that represses LR growth under high nitrate supply
AtIPT5	At5g19040	Root-specific cytokinin biosynthesis gene that responds to high nitrate supply

1.4. Thesis outline

Points raised in the General Introduction were tackled in this research by addressing the following project objectives:

- Develop an experimental system to investigate Arabidopsis (*Arabidopsis thaliana*) root physiological responses to altered water and nitrate supply as manipulated by soil physical properties (Chapter 3);
- Quantify root proliferation responses to altered water and nitrate availability within the developed experimental system (Chapter 4);
- Analyse selected water and nitrate acquisition genes (*AtNRT1.1*, *AtNRT2.1*, *AtNAR2.1* and *AtPIP2.2*) in response to altered water and nitrate availability within the developed experimental system (Chapter 5);
- Analyse selected hormone-associated genes (*AtPIN1*, *AtPIN2*, *AtTIR1*, *AtEIN2*, *AtABI4* and *AtIPT5*) in response to altered water and nitrate availability within the developed experimental system (Chapter 5);
- Analyse the root proliferation and gene expression responses of selected reporter and mutant lines to altered water and nitrate availability within the developed experimental system (Chapter 5); and
- Identify novel interactions between root physiological and soil physical components of root nutrient acquisition (Chapter 6).

2. Materials and Methods

2.1. General plant handling methods

2.1.1. Plant material

The Arabidopsis (*Arabidopsis thaliana*) ecotype Wassilewskija (Ws) was used for all wild-type experiments. The *atnar2.1* deletion mutant and *atnar2.1xNpNRT2.1* lines were in the Ws background (Orsel et al. 2006). The *atnrt2.1* insertion mutant was in the Columbia 0 (Col 0) background (NASC ID: N859604) and the *atpip2.2* insertion mutant was in the Landsberg erecta (Ler 0) background (NASC ID: N163821). The green fluorescent protein (GFP) promoter reporter line *proNRT2.1:eGFP* (Kiba et al. 2012) was in the Col 0 background and was a gift to AJM from Takatoshi Kiba (Riken Institute, Japan).

2.1.2. Handling of seed

Seed was sterilised by agitating ~50 seeds in 1.5 ml of 70% ethanol for 8 minutes, before being air-dried on Whatman no 2 filter paper in a Laminar Flow Hood (model HF72, Gelaire Flow Laboratories, UK) until all ethanol was evaporated. Sterilised seed was transferred onto a Whatman no 2 filter paper placed on lint-free paper (Tuddick Mill Ltd., UK.) soaked in distilled water in a 90 mm circular Petri dish (Sterilin Ltd., UK). Petri dishes were sealed with Micropore tape (3M, UK), stored at 4˚C for 48 hours, and germinated under controlled conditions.

2.1.3. Growth conditions

Growth conditions were cycled daily, comprising 16 hrs light (290 µmol m^{-2} s^{-1}) and 8 hrs darkness, at 22˚C and 75% relative humidity. At 5 days post-germination (dpg) seedlings were selected for a primary root length of 3 mm and transferred to the growth substrate (four seedlings per experimental unit). All experiments were positioned 60 cm above the internal base of the Controlled Environment Growth Cabinet (model 228, Weiss Gallenkamp, UK).

2.2. Experimental systems

2.2.1. Nutrient solution

For all physiology experiments a basic nutrient solution was supplied with final concentrations of each component: 0.5 mM $CaSO_4$, 0.5 mM $MgCl_2$, 1.0 mM KH_2PO_4, 10.0 µM $MnSO_47H_2O$, 24.0 µM H_3BO_3, 3.0 µM $ZnSO_47H_2O$, 0.9 µM $CuSO_45H_2O$, 0.04 µM $(NH_4)_6Mo_7O_{24}4H_2O$, 72.0 µM Fe sequestrene (Orsel et al. 2006). Nitrogen was supplied in different forms as 10.0 mM KNO_3 and 5.0 mM NH_4NO_3. For different nitrate concentrations, KNO_3 was added at final concentrations of 0.1, 1.0 and 10.0 mM. In all experiments, K^+ concentration was kept constant by the addition of K_2SO_4. Final volume was achieved by the addition of distilled water. Solutions were autoclaved, adjusted to pH 5.7 and buffered with 1.0 mM 2-(*N*-morpholino)ethanesulfonic acid (MES). All chemicals were supplied by Sigma-Aldrich (UK).

2.2.2. Sand culture

The first aim of the project was to develop a method for the study root nutrient acquisition which facilitated the investigation of plant physiological responses to manipulations of nitrogen and water supply. A novel sand rhizotron system was developed to investigate root developmental responses to altered nitrogen and water supply within a porous growth substrate (see Chapter 3 for details). Acid-washed Redhill T sand (J Wylie and Sons, UK) was used for all sand experiments and sands of a different particle size were obtained by sieving Redhill T into its constituent fractions of <250 µm, 250-425 µm and >425 µm. Little transpiration occurred due to the seedlings being covered with transparent polythene sheeting. One rhizotron represented one experimental unit.

2.2.3. Agar culture

For the comparison between sand and agar culture, seedlings were grown on vertically orientated square agar plates (120 x 120 mm; Fisher Scientific, UK). Agar (Sigma-Aldrich, UK, Cat. Num. A7921) was added to the nutrient solution at a final strength of 2%, autoclaved, adjusted to pH 5.7 and buffered with 1 mM MES. Plants were positioned 2 cm from the upper edge of the plate and orientated so that only roots were in contact with the agar. Plates were

sealed with Micropore tape and set at an angle of 60˚ to encourage growth along the agar surface. One plate represented one experimental unit.

2.3. Quantification of substrate physical characteristics

2.3.1. Substrate water potential

The water potential of growth substrates was measured determined by the chilled-mirror dewpoint technique using a Dewpoint PoteniaMeter (model WP4, Decagon Devices Inc., USA) as instructed in the manual supplied.

2.3.2. Substrate water release characteristic

The water release characteristic for sands of different particle sizes was determined using the burette method (Figure 2.1.). A 60 mm diameter #3 filter funnel (Fisher Scientific, UK) was connected to a burette (double oblique bore class B glass interchangeable PTFE stopcock 10 mL x 0.02 mL, Fisher Scientific, UK) via 3 m of 15 mm diameter PVC tubing. The whole system was saturated with distilled H_2O and the filter funnel was positioned 2 m above the ground. Sand of a known weight and volume was placed in the filter funnel and the top covered to prevent evaporative losses. After overnight stabilisation, the water level of the burette was dropped by 1 ml and repeated every 2 hours until there was no detectable change in burette water height. The sand was weighed, oven dried for 3 days and reweighed. Volume water content (g/100g) was plotted against burette water height (cm).

2.3.3. Substrate hydraulic conductivity

Saturated hydraulic conductivity was measured using the constant head permeability method (Figure 2.2.). A permeameter of known length (L) and area (A) was filled with sand and a funnel positioned at a head (h) of 45 cm. A constant flow of water was established in the funnel and the sand was saturated. The volume of water (Q) discharged from the bottom outlet was measured for (t) 60 s. Saturated hydraulic conductivity (cm/s) was calculated (Equation 1, p 23) and measurements were repeated three times. Using MS Excel (Microsoft Inc., USA), data were

fitted with Mualem van Genuchten models (Equation 2, p24; van Genuchten 1980) to predict unsaturated hydraulic conductivity, enabling direct gravimetric water content to be converted to matric potential for each sand. For absolute hydraulic conductivities, the saturated hydraulic conductivity of the sand was measured at known water content and multiplied by relative hydraulic conductivity to predict unsaturated hydraulic conductivity.

2.4. Nitrate-selective microelectrode measurements

Double-barrelled nitrate-selective microelectrodes were prepared using filamented double-barrelled borosilicate glass (Miller and Zhen 1991). Microelectrodes were mounted on a micromanipulator (model NMN-21, Narashige, Tokyo, Japan). Both microelectrode reference barrels and reference electrodes were backfilled with 200 mM KCl. The experimental unit was secured to the stage of an Olympus SZX9 microscope (UK). Calibration curves were generated using solutions of known nitrate concentrations (0.01, 0.1, 1.0, 10.0, and 100.0 mM). Root surface nitrate activity of intact primary roots (PRs) was measured at the root tip (RT) and 2 mm up from the root tip (RT-2 mm; Figure 2.3.). Only those with comparable calibration and recalibration curves were used for this study. Care was taken to minimise the area of sand exposed for the measurement to avoid drying and steady recordings were obtained indicating that evaporative losses were minimal (Figure 2.4.).

For the analysis of agar, a scalpel was used to cut 0.2 g sections from the plate at the PR surface and 20 mm away. Each was dissolved in 1.8 g of distilled water (1 in 10 dilution) by heating in a microwave oven and microelectrode measurements made on these solutions. A minimum of three replicates was used for all measurements.

Figure 2.1. The burette method used to measure the water release characteristic. The ability of the sand to retain water is calculated using the volume of water lost from the sand at increasing tension (h, cm).

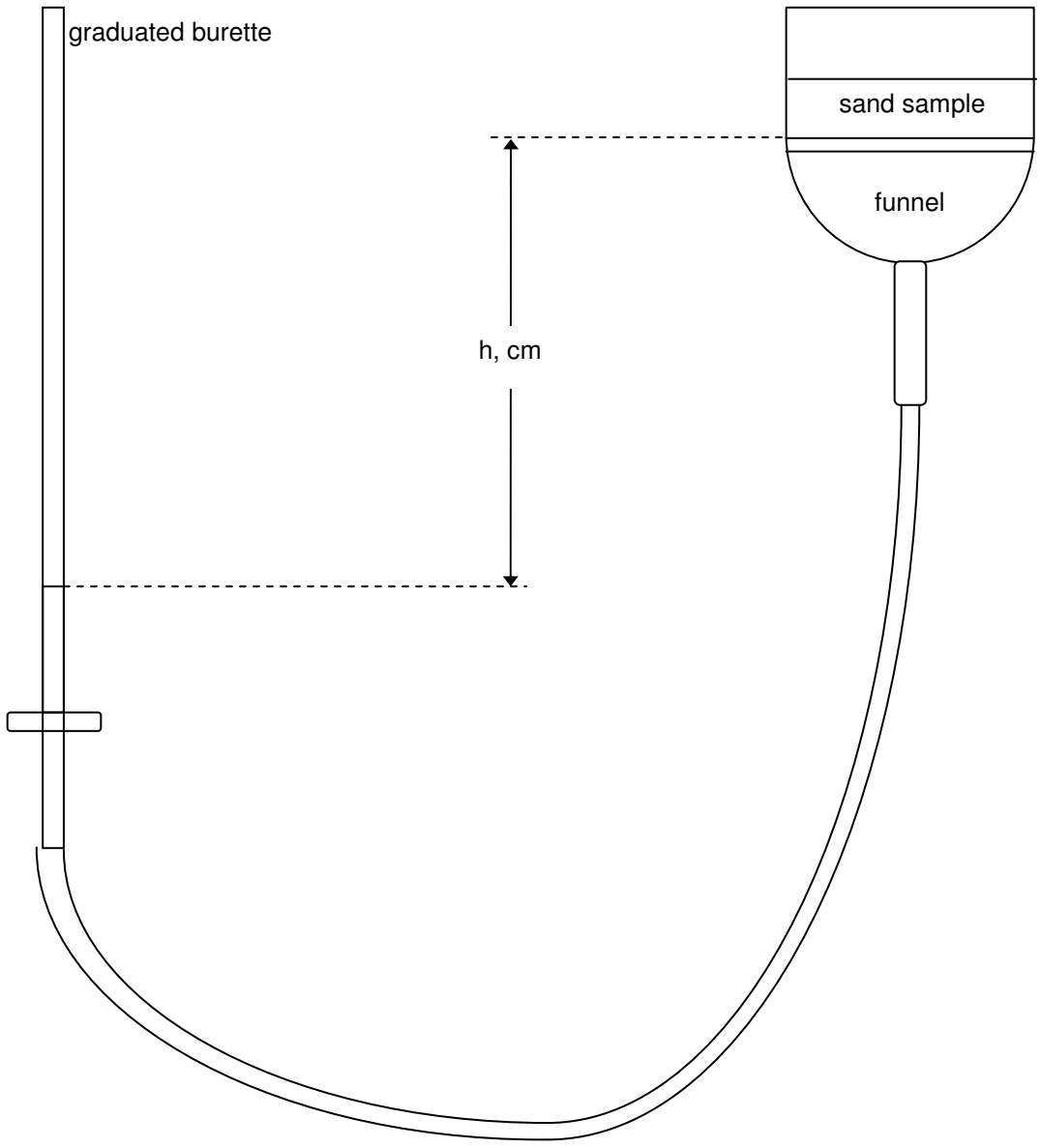


Figure 2.2. The constant head permeability apparatus. The rate of water discharged (Q) is used to determine the saturated hydraulic conductivity of sand in a permeameter of known length (L) at a given pressure head (h).

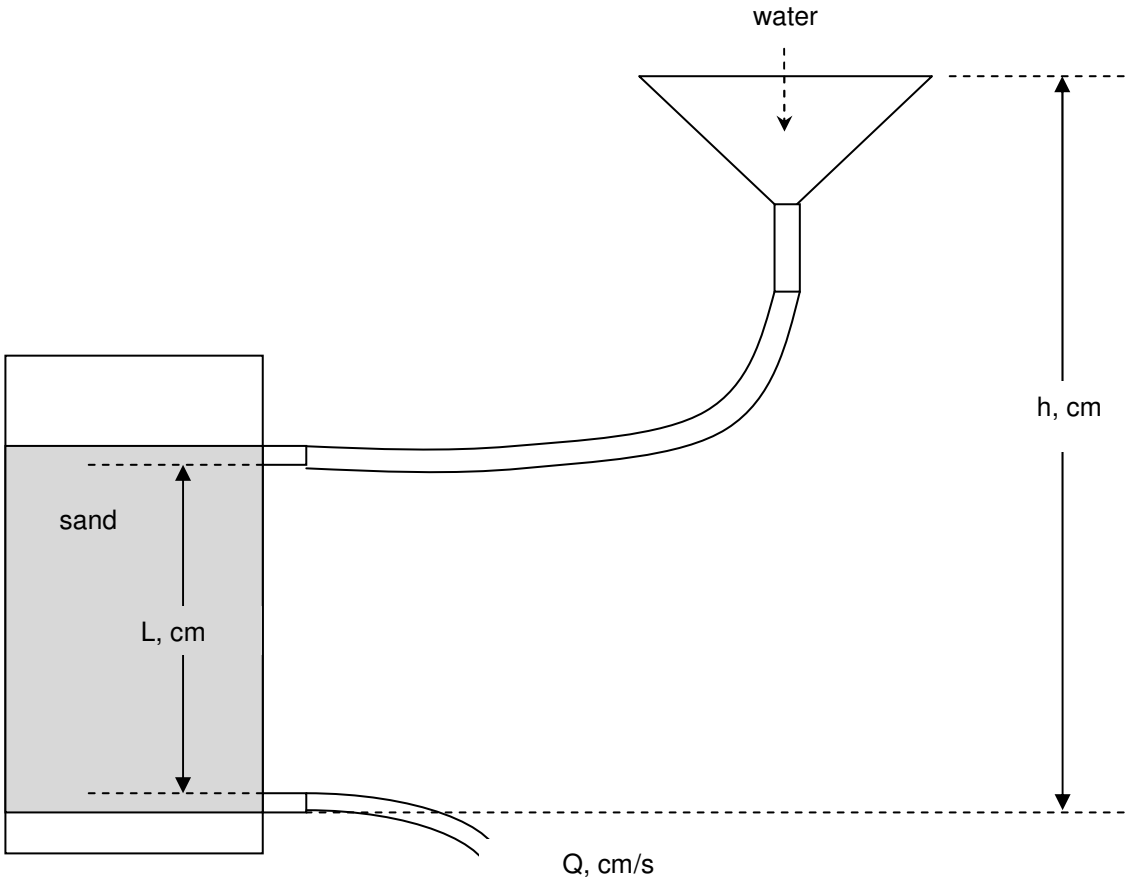


Figure 2.3. Nitrate-selective microelectrodes. A, the experimental unit was secured to the stage of an Olympus SZX9 microscope. B, double-barrelled nitrate-selective microelectrodes were mounted on a micromanipulator. C, nitrate concentration at the surface of intact primary roots was measured at the tip (RT) and 2 mm above the root tip mm (RT-2mm).

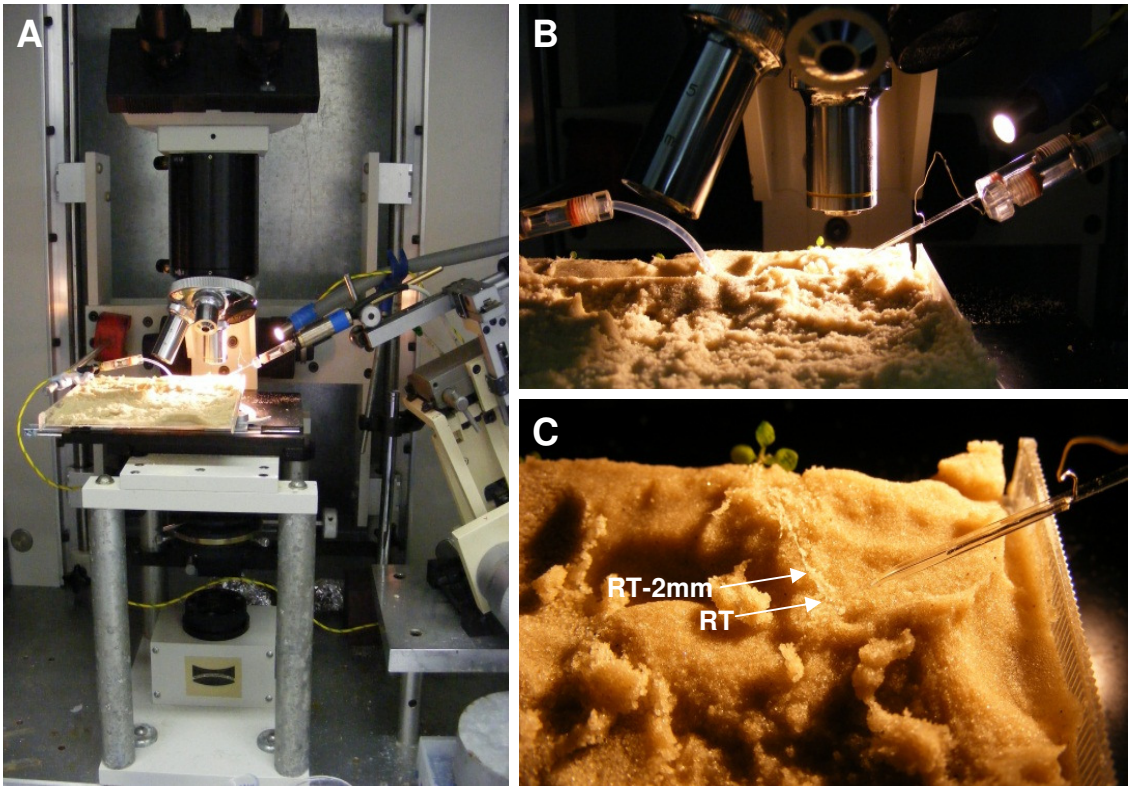
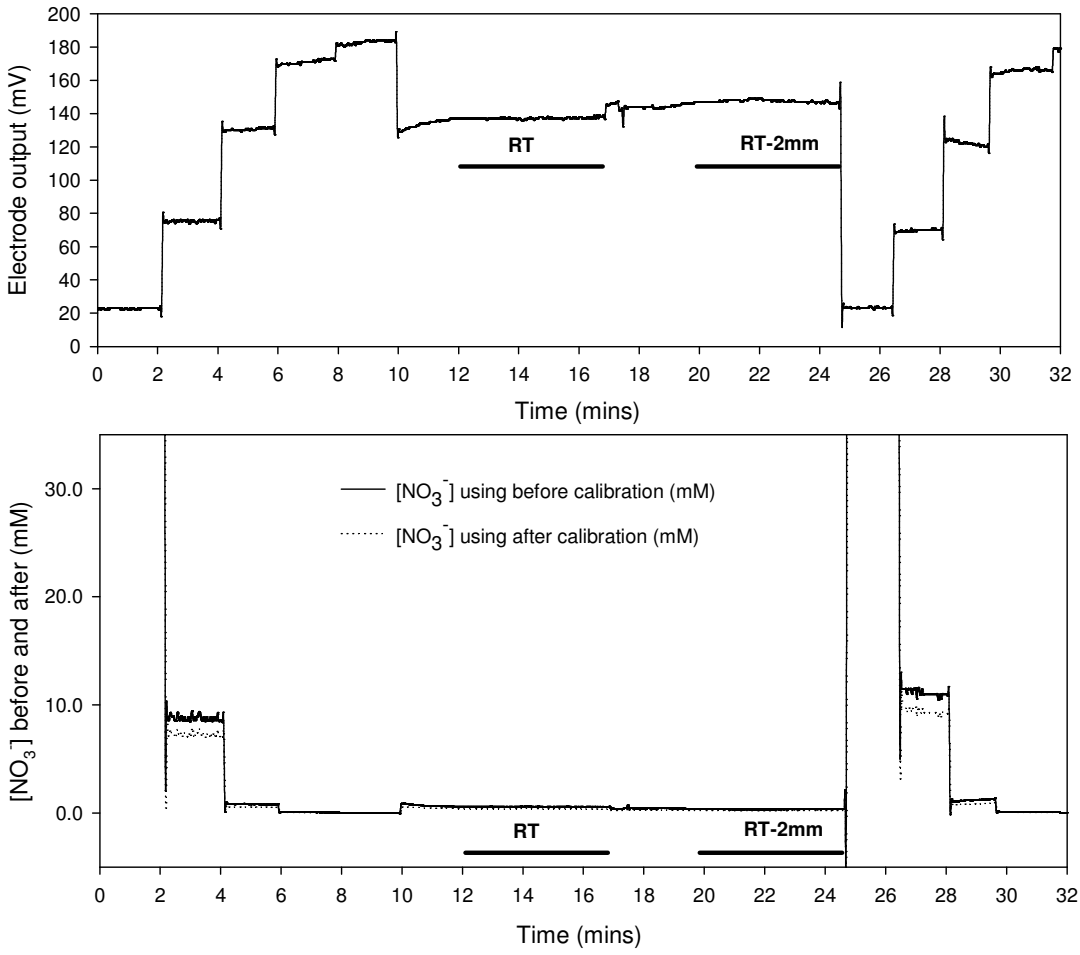


Figure 2.4. A typical nitrate microelectrode recording. Stable measurements were recorded at the root tip (RT) and 2 mm back (RT-2 mm) from the RT of an intact primary root. The recording shows the calibration of the microelectrode before (t=0-10 min) and after (t=25-32 min) the measurement with nitrate solutions of known concentration (0.01 mM, 0.1 mM, 1.0 mM, 10.0 mM and 100.0 mM).



2.5. Characterisation of root proliferation and gene expression

2.5.1. Root proliferation measurements

The second aim of the project was to characterise root proliferation responses to changing nitrate and water supply. At 12 dpg root proliferation characteristics (as described by Zobel and Waisel 2010) were measured and whole root tissue harvested. Primary root length (PRL, mm), basal root length (BRL, mm), lateral root number (LRN, of length ≥ 1mm) and total lateral root length (TLRL, mm) were measured manually using fine-point forceps and a ruler. Lateral root density (LRD, LRN per mm PRL) and total root length (TRL, mm) were calculated from these measurements.

2.5.2. RNA extraction, DNase treatment and cDNA production

RNA was extracted from whole root system tissue samples using the Qiagen RNeasy® Mini Kit (Cat. Num. 74903) following the instructions therein. The concentration of RNA obtained was estimated by measuring the absorbance at 260nm in a NanoDrop Spectrophotometer (Thermo Scientific, UK). RNA was diluted to 10 ng/µl and DNase treatment was carried out using the Promega RQ1 RNase-Free DNase (Cat. Num. M6101) as outlined in the user guidelines. The treated RNA was used as a template to synthesize cDNA (Ambion RETROscript® Kit, Cat. Num. AM1710), and the concentration of each cDNA population was estimated using the Nanodrop Spectrophotometer (Appendix 4). Absence of gDNA was checked by carrying out RT PCR analysis for *ADENINE PHOSPHORIBOSYL TRANSFERASE 1* (*AtAPT1*) expression (the primer pair in Table 2.1. produce a 180 bp band for cDNA and a 320 bp if gDNA is present; Appendix 4).

2.5.3. RT PCR

The third aim of this project was to identify relative changes in expression of key genes for root nitrate acquisition in response to changing nitrate and water supply. This method was used to semi-quantitatively determine the relative abundances of specific transcripts within cDNA populations. Care was taken to standardise each step of the method. Equal amounts of starting RNA were used in the cDNA synthesis reactions (normalised per ng RNA) and the same

concentration of cDNA was used per PCR reaction. Comparison was made between expression of a gene of interest and of the *AtACTIN2* transcript which is constitutively expressed in *Arabidopsis thaliana*. Primer sequences were used from published work (Table 2.2.) and synthesised by Eurofins MWG Operon (Germany). Confirmation that 20 ng cDNA per PCR reaction was sufficient to detect changes in gene expression was determined by serial dilution PCR reactions for the control gene *AtACTIN2* (Appendix 4).

RT PCR was carried out on 20 ng cDNA using REDTaq® ReadyMix™ PCR Reaction Mix (Sigma-Aldrich, UK, Cat. Num. R2523). Reactions were set up as described in the kit instruction manual and the PCR conditions used for all reactions were: an initial denaturation step of 30 seconds at 98ºC, then 35 cycles of 10 seconds at 98ºC, a 30 second annealing step (at 52ºC for *APT1*, 54ºC for *AtEIN2*, 55ºC for *AtPIN1* and *AtPIN2*, 60ºC for *AtTIR1*, 63ºC for *AtACT2*, *AtNRT1.1*, *AtNRT2.1* and *AtPIP2.2*, or 66ºC *AtIPT5*), and an extension step of 72ºC for 30 seconds, all followed by a final extension step of 72ºC for 10 min.

PCR products were separated by electrophoresis on a 1.5% TBE-Agarose gel containing Ethidium Bromide (0.5 µl/100ml) to visualize the DNA under UV light. 4 µl aliquots of each sample were loaded in separate wells and electrophoresed alongside a 4 µl aliquot of Fermentas 100 bp GeneRuler™ (0.5 µg/µl) to size the PCR products. Triplicate samples were electrophoresed at 100V (constant voltage) for 40 minutes and photographed under ultra-violet light in a Syngene Gel Doc using Syngene Gene Snap software (Syngene, UK; Appendix 4). Relative expression raw volumes were calculated using Syngene Gene Tools Software (Syngene, UK), transferred to MS Excel (Microsoft Inc., USA) and raw volumes normalised against the 300 bp band of the DNA ladder.

Table 2.1. Primer pairs used to investigate relative gene expression changes by RT PCR.

Gene	Forward Primer	Product (bp)	Reference
AtACT2 (At3g18780)	F: TCACAGCACTTGCACCAAGCA R: AACGATTCCTGGACCTGCCTCA	161	(KL, unpublished)
AtAPT1 (At1g27450)	F: CGCCTTCTTCTCGACACTGAG R: CAGGTAGCTTCTTGGGCTTC	180 (320)	(Postaire et al. 2010)
AtNRT1.1 (At1g12110)	F: AGACCGAACCAAAAGAACGA R: CCACGATAACCGCAGCAACC	252	(Orsel et al. 2006)
AtNRT2.1 (At1g08090)	F: AGTCGCTTGCACGTTACCTG R: ACCCTCTGACTTGGCGTTCTC	190	(Orsel et al. 2006)
AtNAR2.1 (At5g50200)	F: CCAGAAGATCCTCTTTGCTTCACT R: CCCAATCGAGCTTAGCGTCCA	199	(Orsel et al. 2006)
AtPIP2.2 (At2g37170)	F: GGCAACTTTGCTTGTAAAACTATGC R: AGTACACAAACATTGGCATTGG	102	(Postaire et al. 2010)
AtPIN1 (At1g73590)	F: TATGAGATTTGTCGTTGGACCTGCC R: CGCGATCAACATCCCAAATATCAC	193	(Casson et al. 2009)
AtPIN2 (At5g57090)	F: CATGTGGAAATGGACCAAGACGG R: GACCAAGCAAGGCCAAAGAGAC	209	(Casson et al. 2009)
AtTIR1 (At3g62980)	F: TGCAGGAATCTGAAAGAGCTTG R: GGAAATGGCTAAGCCAGTGG	86	(Shkolnik-Inbar and Bar-Zvi 2010)
AtEIN2 (At5g03280)	F: TATGACAAGTGGACGTGCATGTT R: GCAACTCCCACAACCATGGT	76	(Shkolnik-Inbar and Bar-Zvi 2010)
AtABI4 (At2g40220)	F: TTACCGTGGCGTTCGACAA R: GAGTGCGCTTACGTGGCTCT	71	(Shkolnik-Inbar and Bar-Zvi 2010)
AtIPT5 (At5g19040)	F: AGGATTTTCAGCGTGAAGCAA R: CTATGATCGGGACACGGTCTCT	69	(Takei et al. 2004)

2.5.5. GFP-reporter line microscopy

Sand-grown GFP-reporter line and wild-type seedlings were imaged *in situ* using a stereo light-microscope (M205 FA, Leica, UK) with a GFP2 filter (excitation 480 nm and emission 510 nm) and LAS-AF software (Leica, UK). Rhizotrons were opened like a CD-case and positioned horizontally and intact whole roots imaged within the sand. Channel settings were as follows: Channel 1 (Bright Field), exposure 100ms, gain 1, saturation 50, Zoom 1 (10x), 5 (50x) or 16 (160x), intensity 330; Channel 2 (GFP2), exposure 1s, gain 3, saturation 50, Zoom 1 (10x), 5 (50x) or 16 (160x), intensity 750. Images were produced with two-channels overlaid, then auto-scaled and adjusted to +10% brightness and +10% contrast using MS Word (Microsoft Inc., USA).

2.6. Statistical methods

2.6.1. General ANOVA

All statistical analyses were carried out using GenStat software (13th Edition, VSN International Ltd., UK). For root physiology experiments general analysis of variance (ANOVA) was carried out, followed by comparison of means using the LSD (5%). Data were blocked as 'Experiment/(Situation.Day.Cases)/Seedling' where: 'Experiment' is taken out as a main blocking factor; 'Situation' is funnel or agar location; 'Day' is day of set-up; 'Case' is individual CD case or agar plate; 'Seedling' accounts for technical replication within cases. By combining 'Situation', 'Day' and 'Cases', the analysis accounts for all combinations of each factor. When appropriate, data were transformed onto the log scale (figure legends will indicate). Graphs were made using MS Excel (Microsoft Inc.) or SigmaPlot version 11 (Systat Software Inc., USA) and error bars represent standard deviation.

3. A Novel Sand Rhizotron System

In order to improve our understanding of root nutrient acquisition, it is important to consider both the plant physiological and soil physical components (Chapman et al. 2012). The first aim of this project was to develop an experimental system that facilitated the investigation of Arabidopsis (*Arabidopsis thaliana*) root physiological responses to changes in nutrient supply and that utilised a porous growth substrate so that soil physical characteristics can be quantified and manipulated.

The nutrient acquisition responses of Arabidopsis were investigated because this plant has been widely adopted as a model laboratory organism due to its short generation time, well developed genetics and genomics, small size and modest growth requirements (Bowman 1994; Pyke 1994). Its genome has been sequenced (Kaul et al. 2000), leading to the development of a superior molecular tool-kit for the analysis of gene expression. Furthermore, a large number of mutant and fluorescent reporter line plants with altered expression of genes of interest have been characterised. These research tools have been used to investigate many aspects of root growth, but this work has largely been undertaken in non-porous laboratory systems which have very different physical characteristics than those experienced by a root in soil. The overarching aim of this project was to obtain a greater understanding of root nutrient acquisition by exploiting the superior research tools available for Arabidopsis within an experimental system that facilitates the manipulation of soil hydraulic characteristics and nutrient delivery.

A sand rhizotron system was developed which enabled the control of nutrient supply and the manipulation of soil physical characteristics for the investigation of root responses to changing nitrate and water supply (Figure 3.1.). The system built on aspects of previous work that used rhizotrons to investigate root growth (Futsaether and Oxaal 2002; Whalley et al. 2004b; Devienne-Barret et al. 2006) and other work that utilised sand to manipulate soil physical characteristics within the root growth environment (Whalley et al. 1999; Clark et al. 2002; Brown et al. 2006). Sand was used as the growth substrate because it retains some of the important physical characteristics inherent to soil but, unlike most soils, it has minimal background nutrients and microbial activity which have the potential to influence root growth (Hewitt 1966). Therefore, by using sand, it is possible to quantify and manipulate the soil physical

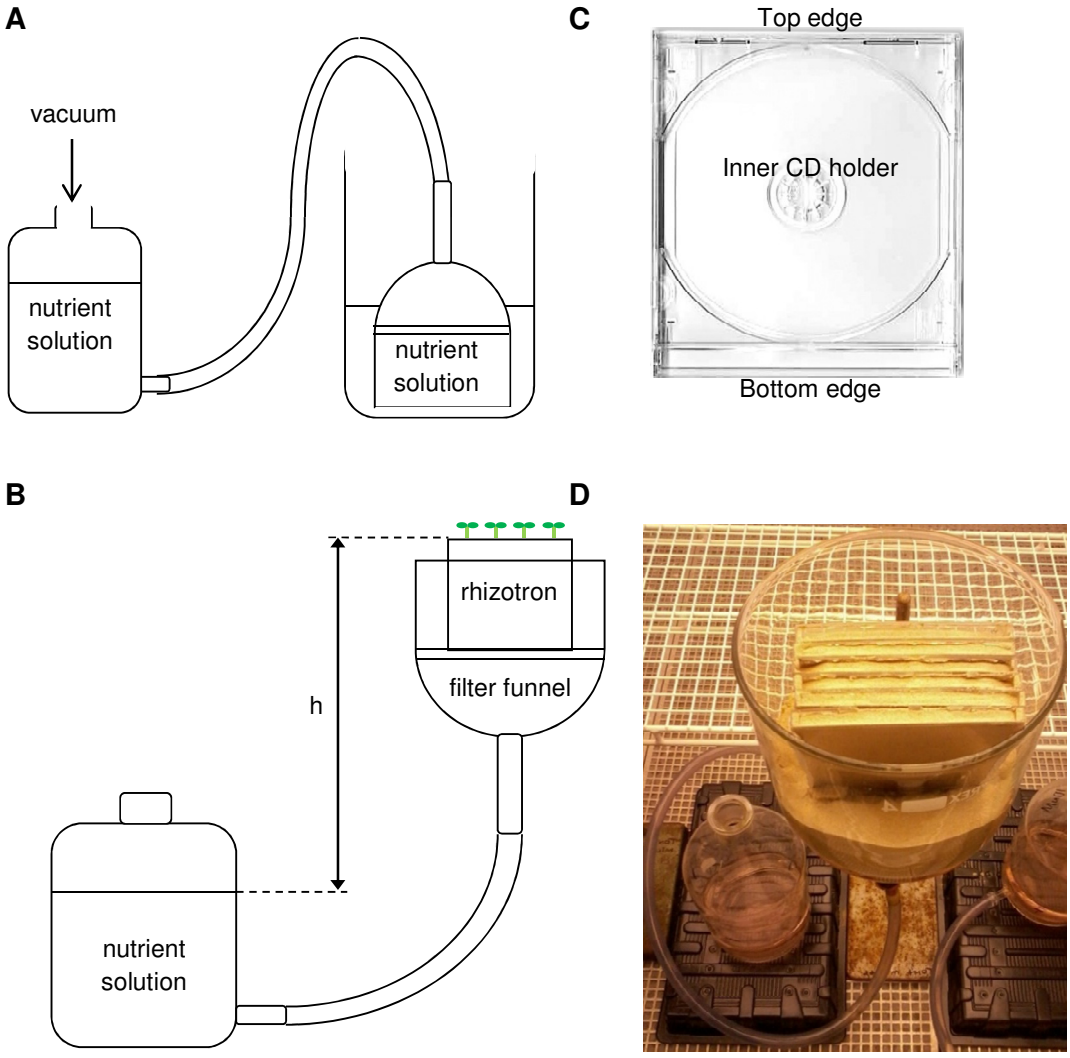
characteristics that influence nutrient availability and minimise the impact on root growth of external factors other than the experimentally manipulated water and nitrate supply.

3.1. The experimental system

The sand rhizotron system (Figure 3.1.) was set-up by vacuum-saturating a 4 L sintered filter funnel (Fisher Scientific, UK) with autoclaved nutrient solution (Figure 3.1.A) in order to support water tensions (h, Figure 3.1.B) across a range of 150-450 mm. Rhizotron experimental units were created by the modification of clear jewel compact-disc (CD) cases (142 x 124 x 10 mm; DVD-and-Media.com, UK) to remove the inner CD holder and top and bottom edges (Figure 3.1.C). Rhizotrons were positioned on an even 30 mm layer of acid-washed Redhill T sand (J Wylie and Sons, UK) within the 4 L sintered filter funnel, filled with acid-washed Redhill T sand (J Wylie and Sons, UK) and saturated with autoclaved nutrient solution (Figure 3.1.D). This facilitated the continuous connection of nutrient solution from the nutrient reservoir (aspirator) to the seedling. Orientation of rhizotrons was at an angle of ~60˚ to encourage the roots to grow near to the surface for easy access at harvest. 5 days after germination (see Chapter 2 for details), four Arabidopsis seedlings were transferred to the growth media on the exposed upper surface before being covered with a clear polythene sheet to minimise evaporative losses. The rest of the system was covered with opaque polythene sheets to restrict exposure to light.

The main purpose of the system was to facilitate independent and combined manipulations of water and nitrogen supply to seedlings within a porous growth substrate. The composition of the nutrient solution was modified to alter the supply of nitrogen to the seedlings. Nitrate concentration (0.1, 1.0 and 10.0 mM) and supply form (KNO_3 and NH_4NO_3) were altered. Water supply was manipulated within the system by the use of sands of a different particle size (<250 µm, 250-425 µm and >425 µm) and by altering the tension height (h, Figure 3.1.B) (150, 300 and 450 mm) between the top of the nutrient reservoir and the seedlings. The hydraulic characteristics for these water treatments were quantified to determine exactly how the water availability to roots is altered within the sand rhizotron system.

Figure 3.1. The sand rhizotron system. A, the sintered filter funnel was vacuum saturated to support tension heights of 150-450 mm; B, rhizotrons were constructed from modified plastic jewel CD cases; C, schematic of the system; D, photograph of the uncovered system.



3.1.1. The hydraulic characteristics of the root growth environment

Robust soil physical methods exist to measure the movement of water through a porous substrate such as sand (Chapter 1, Section 1.2.1.). These were utilised to determine the hydraulic characteristics of each manipulation of water supply within the sand rhizotron system (see Chapter 2 for details). The water release characteristic of each sand used was quantified (Figure 3.2.A) and the saturated substrate hydraulic conductivity was measured (Figure 3.2.B). It was possible to determine relative substrate hydraulic conductivity (Figure 3.2.C) and the data were fitted to the van Genuchten model (using the parameters in Table 3.1.) to estimate the unsaturated substrate hydraulic conductivity (Table 3.2.).

The water potential, osmotic potential, matric potential, and the saturated and unsaturated substrate hydraulic conductivity were characterised for the sands used in this work (Table 3.2.). As expected, the saturated substrate hydraulic conductivity for Redhill T (1.81 $m.d^{-1}$) was within the range of its constituent particle size fractions (1.42-3.23 $m.d^{-1}$) and saturated substrate hydraulic conductivity increased with increasing particle size. Unsaturated substrate hydraulic conductivity decreased from 0.99 to 0.17 $m.d^{-1}$ with increasing particle size and from 1.35 to 0.18 $m.d^{-1}$ with increasingly negative matric potential (Table 3.2.).

Water potential is determined by matric and osmotic potentials and as a result all three parameters became increasingly negative with increasing tension height (Figure 3.1.B, *h*). In the sand rhizotron system, manipulation of tension height served to change water supply by altering the matric potential. Tension heights of 150, 300 and 450 mm correspond to matric potentials of -0.0015, -0.0030 and -0.0045 MPa respectively. Although the matric potential adjacent to the root drops as roots extract water (Carminati et al. 2010), the matric potentials in the sand rhizotron system are a reasonable estimate of the matric potential at the sand-root interface because the hydraulic conductivities are at relatively high bulk matric potential (Whalley et al. 2000). For all sands, water potential decreased by more than could be accounted for by matric potential as the contribution of osmotic potential to water potential is around 100 fold larger than that of the matric potential (Table 3.2.). Therefore, the water potential within the sands was predominantly determined by the osmotic potential of the nutrient solution. The influence of comparatively small changes in matric potential on water potential is minor but can be viewed as a way of manipulating the flux of water through the sand.

Table 3.1. van Genuchten parameters for all sands. θ_s and θ_r are the saturated and residual volumetric water contents and α, m and n are fitted parameters.

Sand	θ_s (cm^3/cm^3)	θ_r (cm^3/cm^3)	α (hPa^{-1})	*m*	*n*
Redhill T	0.3972	0.915	0.022	0.768	4.311
<250 µm	0.4490	0.991	0.024	0.839	6.224
250-425 µm	0.4421	1.055	0.032	0.889	8.980
>425 µm	0.4339	1.021	0.034	0.891	9.193

Figure 3.2. Physical characterisation of the sands. A, the water release characteristic (dashed lines indicate the tension heights used); B, saturated hydraulic conductivity (K_{sat}); and C, relative hydraulic conductivity.

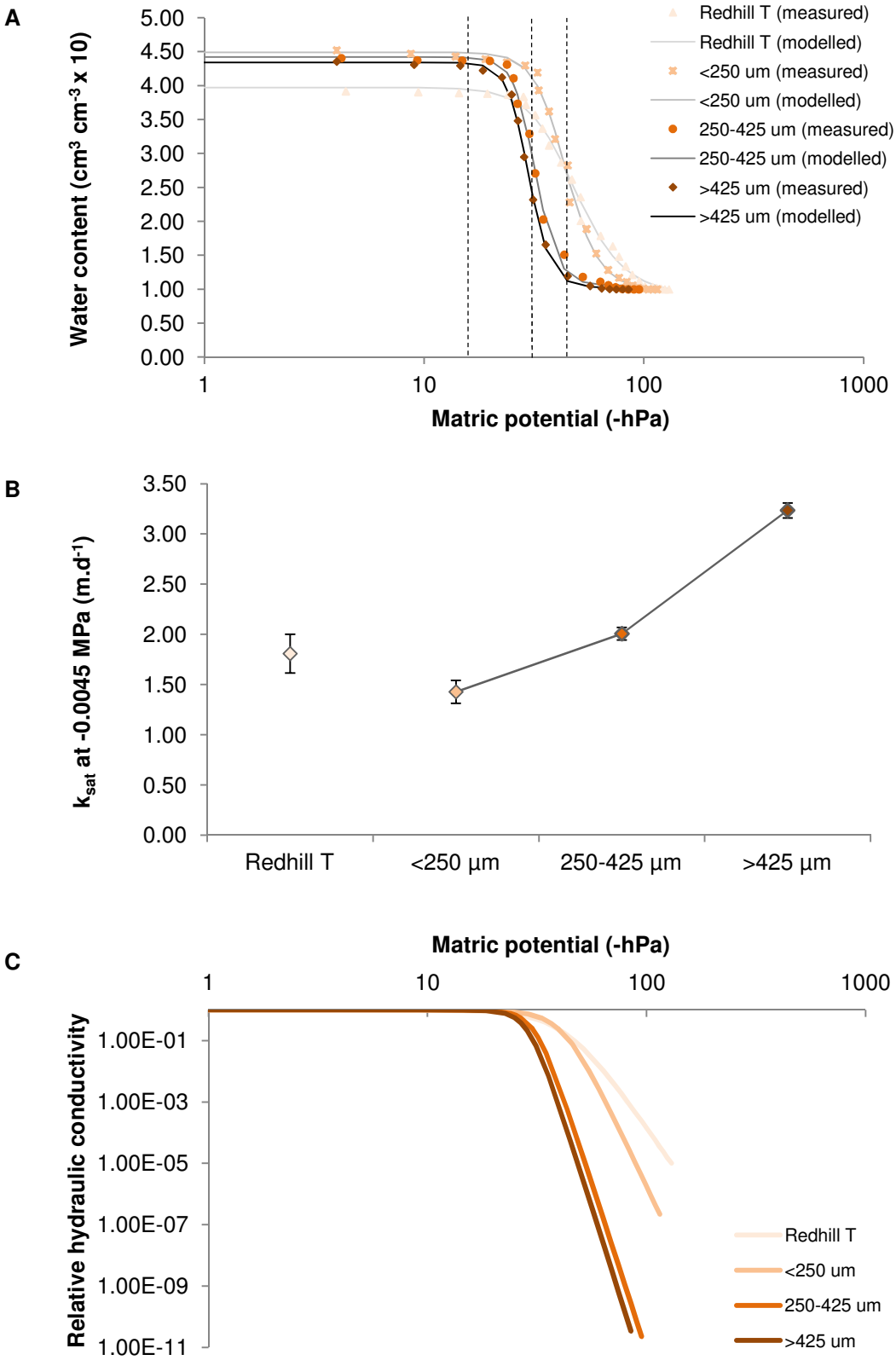


Table 3.2. Summary of sand physical characteristics. Total water potential (Ψ_t), matric potential (Ψ_m), osmotic potential (Ψ_o), saturated hydraulic conductivity (K_{sat}) and unsaturated hydraulic conductivity (K_{unsat}) for all sands. Water potential was altered in Redhill T sands by varying the tension height of the system, which is an approximation to the matric potential seen by the root.

Sand	**Ψ_t (MPa)**	**Ψ_m (MPa)**	**Ψ_o (MPa)**	**K_{sat} ($m.d^{-1}$)**	**K_{unsat} ($m.d^{-1}$)**
Redhill T	-0.1100	-0.0015	-0.1085	-	1.35
Redhill T	-0.3300	-0.0030	-0.3270	1.81	0.79
Redhill T	-0.4000	-0.0045	-0.3955	-	0.18
<250 µm	-0.0600	-0.0030	-0.0570	1.42	0.99
250-425 µm	-0.2600	-0.0030	-0.2570	2.01	0.36
>425 µm	-0.3300	-0.0030	-0.3270	3.23	0.17

In summary, unsaturated substrate hydraulic conductivity decreased as water potential became increasingly negative. Whilst the water potential within the sands was predominantly determined by the osmotic potential of the nutrient solution, manipulation of matric potential can serve to alter sand water flux and represents an estimation of matric potential at the root-soil interface. The effect of manipulating matric potential or the size of sand particles was primarily to change the hydraulic conductivity of the sand to water and hence the flux of water to the root surface. Consequently, these two manipulations alter the availability of water (and dissolved nutrient ions) to roots, but via slightly different combinations of water potential and substrate hydraulic conductivity.

3.2. Comparing the sand rhizotron system to agar culture

Agar culture is a common laboratory system for the investigation of root physiology and has been widely used to determine the influence of nitrate and water on root development. However, the physical characteristics of gel (e.g. agar) and porous (e.g. sand) substrates are different and may alter the availability of water and nitrate to influence root physiology (Spomer and Smith 1996; Zhang et al. 2005; Prunty and Bell 2007; Whalley et al. 2009).

To confirm the suitability of the sand rhizotron system for the investigation of root physiological responses to nutritional supply, a direct comparison of root physiological responses was made with agar culture under identical growth conditions at high nitrate (10.0 mM KNO_3) supply. The comparison was made with Redhill T sand at a matric potential of -3.0 kPa. Arabidopsis ecotype WS seedlings were germinated and grown either on agar plates or sand rhizotrons for 7 days. At 12 dpg, root proliferation parameters were measured and seedlings were harvested. RNA was extracted for RT PCR gene expression analysis (see Chapter 2 for details).

Comparing the sand rhizotron system with agar culture identified some important differences and similarities in the response of root physiological characteristics. The primary root length (PRL) of sand-grown seedlings was significantly longer ($P < 0.01$; d.f. 57) than that of agar-grown plants (Figure 3.3.). Conversely, total lateral root length (TLRL) was significantly longer ($P < 0.05$; d.f. 57) in agar-grown seedlings when compared with those grown in sand. However,

the same relative response of individual characteristics was observed in both systems, i.e. PRL was longer than BRL and BRL was longer than TLRL in both substrates.

The expression of key genes relating to nutrient acquisition was measured using semi quantitative RT PCR. *AtNRT1.1*, *AtNRT2.1* and *AtPIP2.2* expression was unaltered between agar- and sand-grown seedlings (Figure 3.4.). *AtNAR2.1* expression was increased for agar-grown seedlings relative to sand-grown seedlings (Figure 3.4). For the suite of nutrient-regulated hormone genes, no relative change in expression was detected between seedlings grown in agar or sand for *AtPIN1*, *AtPIN2*, *AtTIR1* or *AtEIN2*. However, *AtABI4* and *AtIPT5* were both slightly increased in agar-grown seedlings relative to sand-grown seedlings (Figure 3.5.).

3.3. Why is root physiology different between sand and agar?

When the root growth environments of sand and agar are compared there are several potential factors that could influence root proliferation and gene expression, including differences in sterility, light exposure, gas exchange, and the physical characteristics of the substrates.

It is not possible to state that the sand rhizotron system was as sterile as agar culture. However, the potential influence on root growth of nutrient cycling from microbial activity or growth-promoting bacteria (Kraiser et al. 2011; Jiang et al. 2012) was minimised in the sand rhizotron system through acid-wash treatment of the sand, autoclave treatment of the nutrient solution and by covering the system in opaque sheeting. Thus, it is unlikely that microbial or algal activity is responsible for the differences in root physiology observed between agar and sand.

Phytohormone-mediated light inhibition of root growth has been demonstrated (Adams and Turner 2010) and light exposure to roots is different between agar culture and the sand rhizotron system. Interestingly, *AtABI4* and *AtIPT5* expression was increased in agar-grown seedlings (Figure 3.5.). However, there was no significant difference in total root proliferation between normal agar-grown seedlings and those with limited root light exposure (Figure 3.6.). Hence, the difference in light exposure to roots is unlikely to be driving the altered root proliferation responses between sand and agar.

Figure 3.3. Comparison of root proliferation responses to high nitrate supply between agar and sand. PRL, primary root length; BRL, basal root length; LRN, lateral root number; TLRL, total lateral root length; LRD, lateral root density; TRL, total root length. For each treatment, n = 120; d.f. 57. Significance, ** P < 0.01, * P < 0.1.

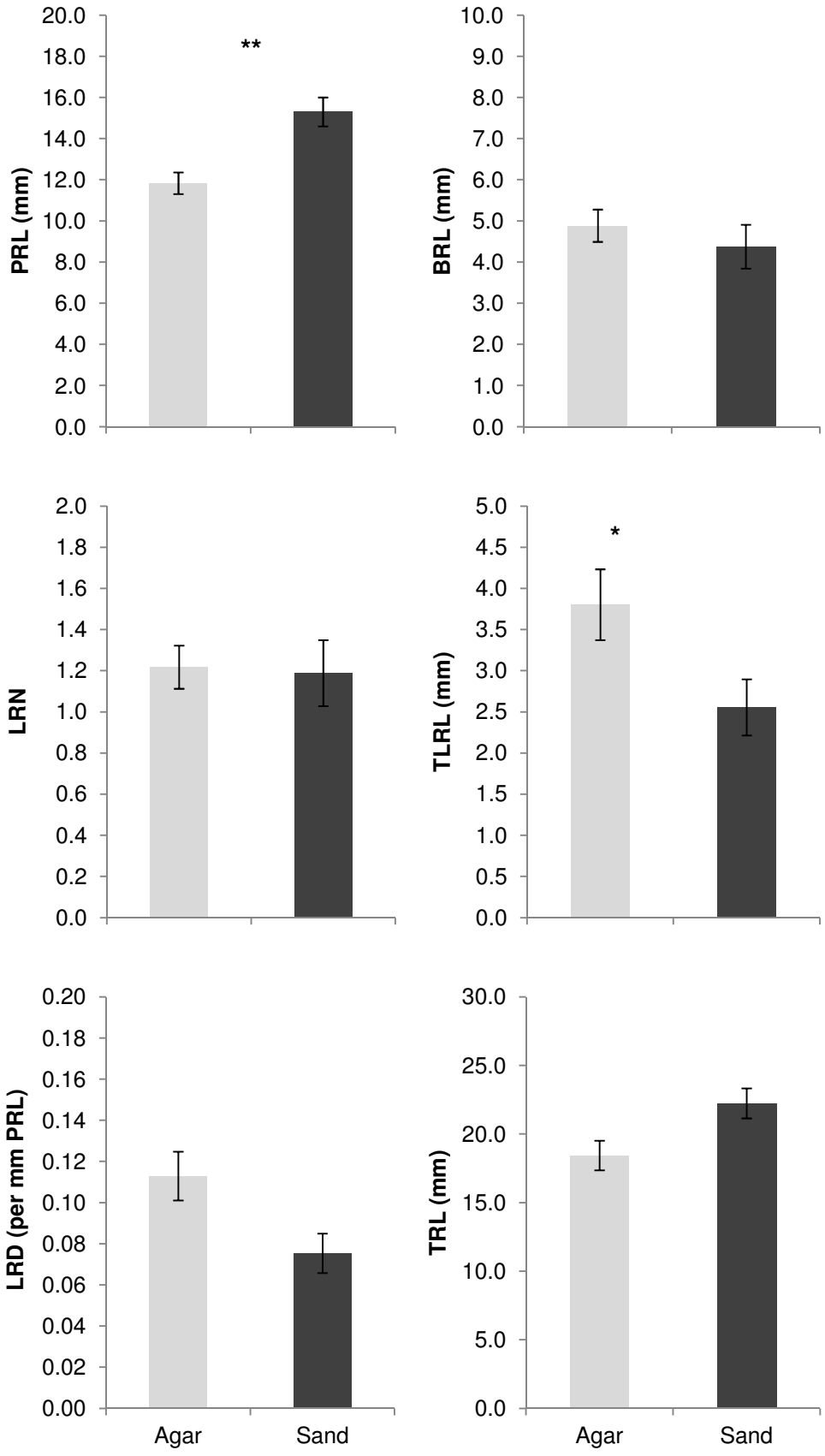
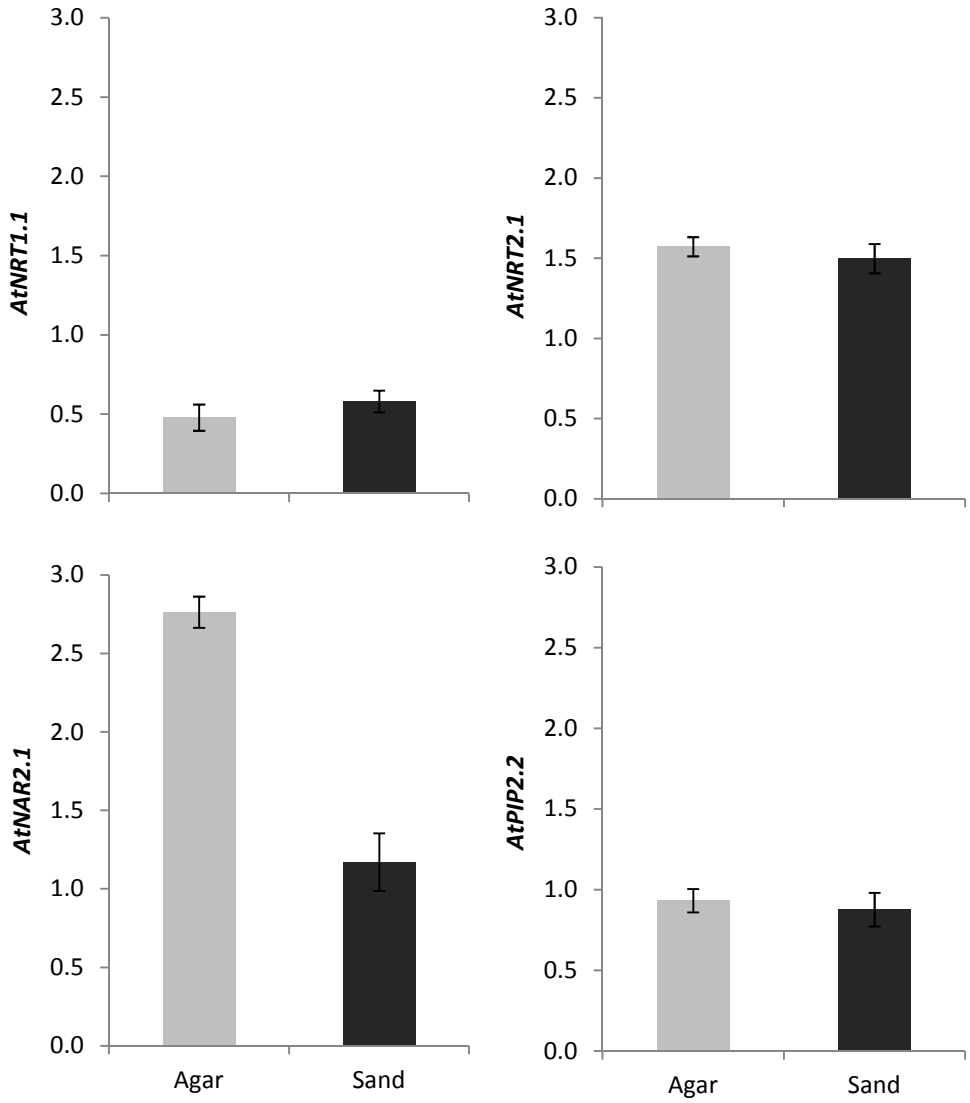


Figure 3.4. Relative expression of important nitrate and water uptake genes in agar and sand systems. For each treatment, n = 120. Level of *AtNRT2.1* expression may be underestimated due to saturation.



Agar and sand may also differ in gas exchange at the root surface which may impact on root growth. Oxygen availability or the supply of gaseous hormones (e.g. ethylene) can influence root growth (Verslues et al. 1998; Ivanchenko et al. 2008). Localised gradients may develop differently for roots in wet sand where there is less opportunity for gas exchange compared to roots on the surface of agar where one half of the root is permanently exposed to air. However, all but one sand treatment (Table 3.2., row 1) subjected roots to unsaturated conditions and previous work concluded that oxygen availability did not limit growth in a similar sand system (Whalley et al. 1999). In addition, Arabidopsis seedlings have a relatively low transpiration demand (Christman et al. 2008) and the seedlings were grown in a covered system. As a result, it is unlikely that significant moisture gradients will develop near the root in the sand rhizotron system. Furthermore, there was no observable difference for the expression of the ethylene signalling gene *AtEIN2* (Figure 3.5.) indicating that external gaseous ethylene concentration was similar between substrates. Therefore, it is reasonable to assume that roots are sufficiently aerated in the sands used in this study and that differences in availability of air or gaseous exchange are not responsible for divergent root physiological responses in agar and sand.

Mechanical impedance in some porous substrates can alter root proliferation and is particularly important for PR growth (Lachno et al. 1982; Bengough and Mullins 1991; Clark et al. 1996; Whalley et al. 2004a; Brown et al. 2006). Physical impedance to root growth is not typical of agar culture because roots grow along the surface of agar. Also, the pore size of sand used in this work is sufficient for fine Arabidopsis roots (typical diameter 150 µm; Bowman 1994) to grow between particles and thus not be impeded. Furthermore, PRL was actually increased in the sand system not decreased as would be expected if the sand was offering mechanical impedance to root growth. Therefore, differences in mechanical impedance between agar and sand cannot explain the root proliferation differences observed in these experiments.

Figure 3.5. Relative expression of hormone-associated genes in agar and sand systems. For each treatment, n = 120.

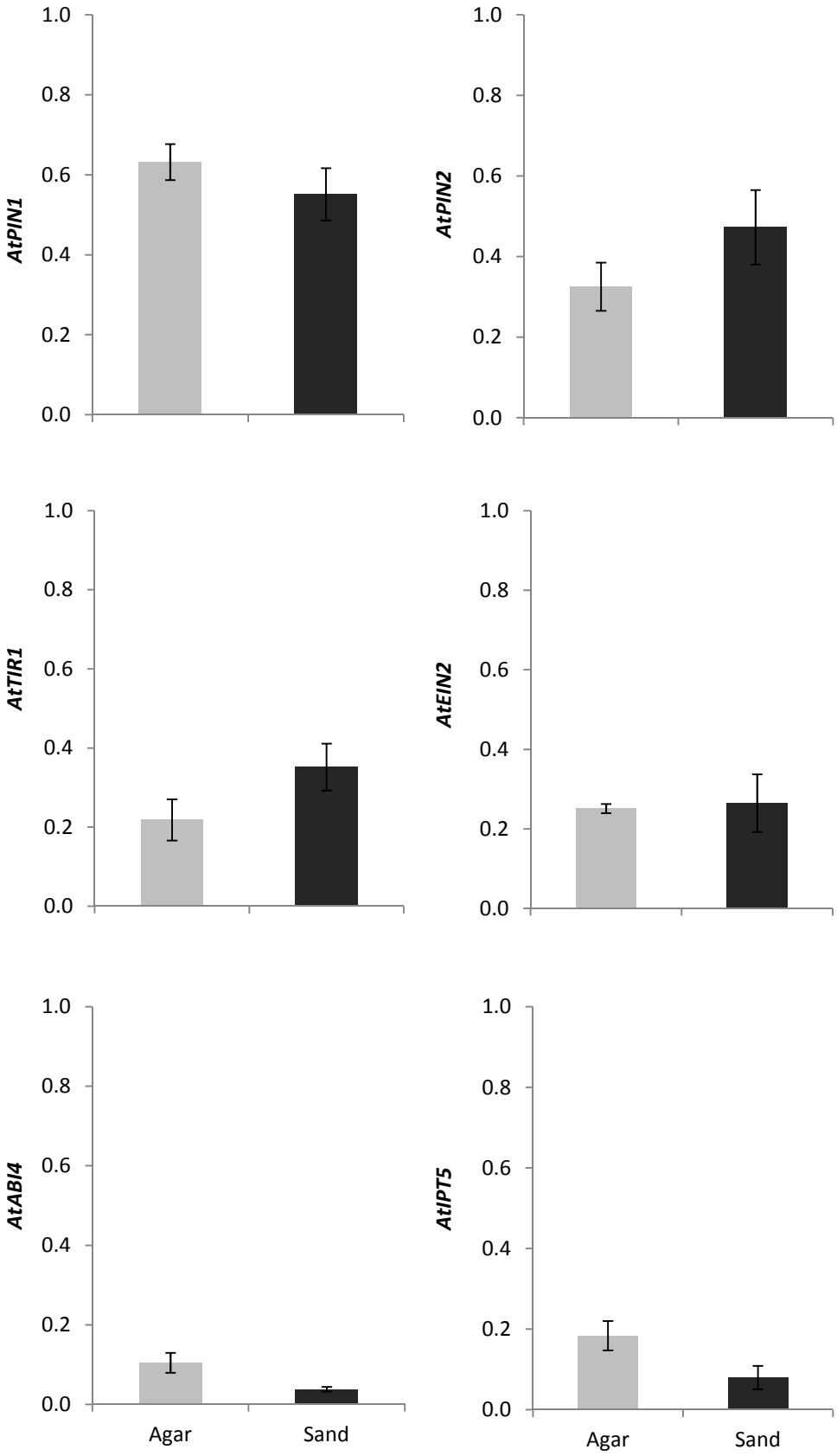


Figure 3.6. Root light exposure had no effect on root length. Seedlings were grown on normal agar plates or on plates with light excluded from the roots by opaque sheeting. There was no significant difference in total root length (TRL, mm). For each treatment, n = 24; d.f. 5.

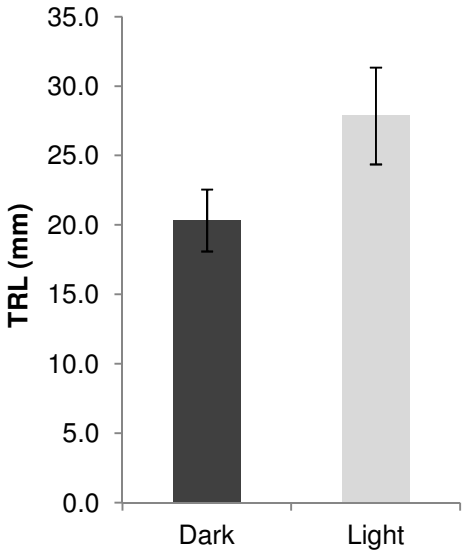
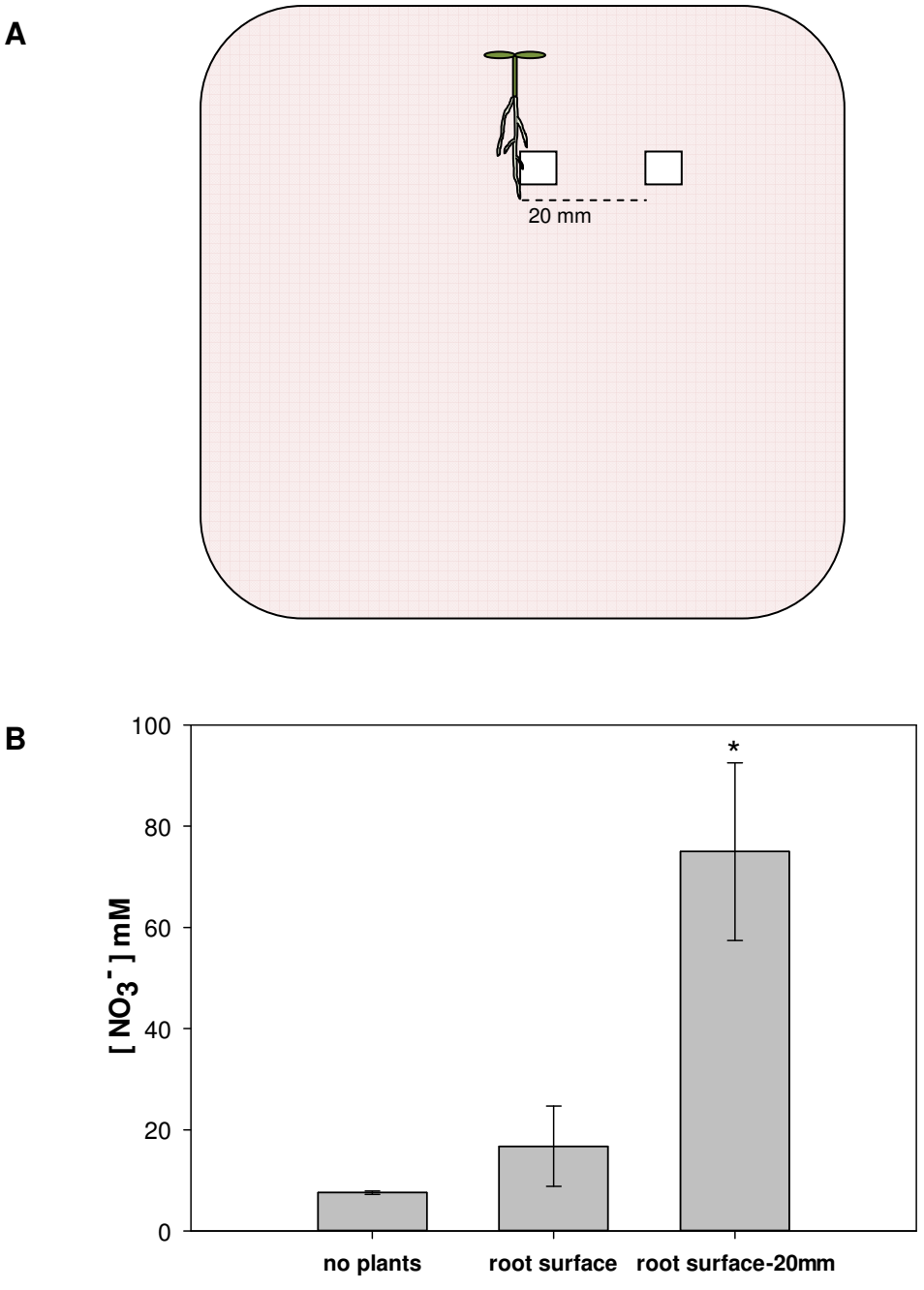


Figure 3.7. Nitrate-selective microelectrodes were used to measure the nitrate concentration of agar. A, samples of agar were removed at the primary root surface and 20 mm away; B, the concentration at 20 mm away from the primary root was significantly greater than at the primary root surface. Agar with no plants was used as a control. For each treatment, n = 3; d.f. 6. Significance: *, P < 0.01.



3.4. Using nitrate-selective microelectrodes to measure nitrate at the root surface

Differences in physical characteristics between gel (e.g. agar) and porous (e.g. sand) substrates could alter the availability of water and nitrate (Spomer and Smith 1996; Zhang et al. 2005; Prunty and Bell 2007; Whalley et al. 2009). A change in water and nitrate availability influences root proliferation (Sharp et al. 1988; Verslues et al. 1998; Zhang and Forde 1998; Malamy and Ryan 2001; Deak and Malamy 2005) and transporter gene expression (Alexandersson et al. 2005; Krouk et al. 2010a; Bao et al. 2011). Unfortunately, it was not possible to obtain steady water potential readings or to determine substrate hydraulic conductivity for agar using the same methods that were used to characterise the sands. However, *AtNAR2.1*, *AtABI4* and *AtIPT5* expression is known to be responsive to nitrate and differed between agar- and sand-grown seedlings, indicating that nitrate availability may have been altered between the two systems.

It was postulated that an area of localised depletion could occur around the root as nitrate is taken up and that this is not easily replenished in agar relative to sand due to different hydraulic properties. This depletion effect was tested by the use of nitrate-selective microelectrodes to determine the nitrate concentration of agar next to the root and 20 mm away (see Chapter 2 for details; Figure 3.7.A). In agar without plants the nitrate concentration was equal to the original 10.0 mM supplied and nitrate concentration at 20 mm away from the root was around 4 times greater than at the surface of the root ($P < 0.01$; d.f. 6; Figure 3.7.B). The concentration observed at 20 mm away from the root was greater than the initial 10.0 mM added to the agar.

This indicates that rather than a localised depletion of nitrate by root uptake decreasing nitrate availability, water is moving from the edge of the plate towards the root as the plant takes up water from the substrate during growth. This decrease in water content could be due to the finite volume of water within the agar Petri dish system and create the perceived increase in nitrate concentration at 20 mm away from the root.

A relative difference in the volume of water supplied in each system may be important. PRL was increased in the sand rhizotron system compared to agar culture (Figure 3.3.) and increased nitrate concentration at 20 mm away from the root is caused by decreased water content (Figure 3.7.). PRL depends on cellular expansion driven by the influx of water (see review by

Maurel et al. 2008) and has been shown to be unresponsive to nitrate concentration in agar (Forde and Zhang 1998; Linkohr et al. 2002; Deak and Malamy 2005; Walch-Liu and Forde 2008). Consequently, a decreased PRL in agar compared to sand could be explained by a decreased volume of available water to drive cellular expansion.

LR growth is increased in response to patches of high nitrate when initially supplied with low nitrate (Drew 1975; Zhang et al. 1999; Malamy and Ryan 2001) and is repressed in response to decreased water availability (Deak and Malamy 2005). TLRL was increased in agar relative to sand (Figure 3.3.) and the availability of water in agar was decreased compared with the sand rhizotron system. Although initially supplied with high nitrate (10 mM KNO_3), the nitrate concentration at 20 mm away from the root greatly increased (Figure 3.7.) and this was sufficient to induce the LR high nitrate patch response. Thus, an increased TLRL for agar-grown seedlings is likely to be a response to the formation of high-nitrate patches as water content decreases due to the finite volume of water supplied in agar Petri dishes. This response is not seen for sand-grown seedlings at this unsaturated hydraulic conductivity because the greater volume of nutrient solution available and the connectivity of solution mean that patches are less likely to form.

3.5. Summary and conclusions; the sand rhizotron system is fit for purpose

The results obtained indicate that differences in the root physiological response to nitrate supply between agar culture and the sand rhizotron system are caused by differences in the physical characteristics of the substrate. These differences are further compounded by the dissimilarity in volume of nutrients supplied in the respective systems. These data emphasise the need to consider the impact of the soil physical component of root nutrient acquisition on nutrient-regulated root physiology changes.

In addition to some important root physiology differences between the sand and agar systems, there were some conserved responses that highlight the suitability of the sand rhizotron system to investigate nutrient-regulated root physiology changes. The overall response of the root proliferation characteristics was conserved between growth substrates, e.g. PRL was longer than BRL and BRL was longer than TLRL in both substrates (Figure 3.3.). Thus, the sand

rhizotron system detected the same relative response of individual root characteristics that was observed in agar.

The expression of *AtNAR2.1* (Figure 3.4.), and *AtABI4* and *AtIPT5* (Figure 3.5.) was altered between systems (Figure 3.4.). This suggests that differences in hydraulic characteristics that alter nutrient availability can directly influence both root proliferation and gene expression. Therefore, a greater understanding of nutrient acquisition is likely to be gained by investigating root physiological responses to manipulation of the soil physical characteristics that determine nitrate and water availability.

The quantification of the hydraulic characteristics of the sands and the ability to alter the nitrate supply enables the sand rhizotron system to be used for the consideration of both the root physiological and soil physical aspects of nutrient acquisition. The overall nutrient responses observed in agar culture are detected using the sand rhizotron system but there were some important differences driven by disparities in hydraulic characteristics and the volume of nutrients supplied.

The main conclusions of this chapter are summarised below:

- A novel sand rhizotron system was developed to investigate Arabidopsis root nutrient acquisition responses to altered water and nitrate availability as a result of manipulated soil physical characteristics;
- Methods to analyse root proliferation and gene expression components of root nutrient acquisition were developed;
- Disparities in several root proliferation and gene expression parameters between seedlings grown on agar and sand culture may be explained by differences in hydraulic properties of the root growth environment and the volume of nutrients available in each system;
- These results highlighted the importance of considering soil physical properties in root nutrient acquisition studies.

The consideration of root physiological responses in a porous substrate may identify responses that are not observed in agar culture. These may be important in order to better understand root nutrient acquisition in the field as some of the physical characteristics of soil are better retained in sand compared to agar (Chapter 1, Table 1.1., p29). Therefore, the sand rhizotron system was adopted to investigate Arabidopsis root proliferation (Chapter 4) and gene expression (Chapter 5) responses to manipulations of nitrate and water supply.

4. Root Proliferation Responses to Altered Water and Nitrate Supply

The continued growth and proliferation of the root is a significant physiological component of nutrient acquisition (Jungk 1996). In order to better understand the response of this component to altered nitrate and water supply, the root proliferation responses of Arabidopsis (*Arabidopsis thaliana*) seedlings were measured under a series of manipulated nutrient regimes within the sand rhizotron system.

4.1. The influence of different nitrate forms on root proliferation

The influence of nitrogen form on root proliferation in the sand rhizotron system was investigated by comparing nitrogen supplied as potassium nitrate (10.0 mM KNO_3) or as ammonium nitrate (5.0 mM NH_4NO_3; Figure 4.1.). Arabidopsis ecotype WS seedlings were germinated and grown on sand rhizotrons for 7 days. At 12 dpg, primary root length (PRL, mm), basal root length (BRL, mm), lateral root number (LRN, of length ≥ 1mm), total lateral root length (TLRL, mm), lateral root density (LRD, LRN per mm PRL) were measured (see Chapter 2 for details).

When supplied with 10.0 mM KNO_3, PRL, BRL and total root length (TRL, mm; the sum of PRL, BRL and TLRL) were significantly longer than those provided with 5.0 mM NH_4NO_3 ($P < 0.001$; d.f. 72; Figure 4.1.). There was no effect on LR growth (LRN, TLRL or LRD). An increased PRL, and no effect on LRN, was observed when 10.0 mM nitrogen was supplied only as nitrate (10.0 mM KNO_3) relative to a combined supply (5.0 mM NH_4NO_3; Figure 4.1.). LR growth has been previously shown to be unresponsive to the availability of different mineral forms in soil (Bloom et al. 1993).

In a previous agar culture study, no effects were observed for LRN, but PRL was shown to decrease by ~20% for a 20.0 mM increase in ammonium provision relative to potassium (Li et al. 2010b). Despite maintaining a constant potassium supply (via the supply of K_2SO_4), a ~20% decrease in PRL was observed for a 5.0 mM increase in ammonium availability, and a 5.0 mM decrease in nitrate availability, within the sand rhizotron system (Figure 4.1.). As it is not possible to categorically say that the sand rhizotron system is completely sterile, there is the possibility for bacterial conversion of ammonium to nitrate (Miller and Cramer 2005), but this is

probably minimal in acid-washed sand. Moreover, PRL has been shown to be unresponsive to altered nitrate availability (Walch-Liu and Forde 2008), so our data suggest that PRL is responding to an increase in ammonium supply within our system. The aim of the sand rhizotron method is to remove the influence of other factors (i.e. ammonium inhibition) on root proliferation. Therefore, potassium nitrate was adopted as the sole means of nitrate provision for the following experiments that investigated root proliferation responses to altered water and nitrate supply.

4.2. The influence of water supply on root proliferation across a range of nitrate supplies

The influence of altered water supply was investigated at low (0.1 mM), medium (1.0 mM) and high (10.0 mM) nitrate (KNO_3) supply to determine the impact of combined manipulations of water and nitrate supply on root proliferation. Water supply to the root was manipulated via the use of sands of different particle size or sands at different matric potential. These manipulations subjected the seedlings to altered water potentials and substrate hydraulic conductivities (Chapter 3, Table 3.2.). For each treatment, Arabidopsis ecotype WS seedlings were germinated and grown on sand rhizotrons for 7 days. At 12 dpg, root proliferation parameters were measured and seedlings were harvested (see Chapter 2 for details).

At low (0.1 mM) nitrate input, PRL increased with increasing particle size ($P < 0.001$, d.f. 149; Figure 4.2.A) and was increased at the most negative matric potential ($P < 0.001$, d.f. 169; Figure 4.3.A). BRL was decreased at the largest particle size ($P < 0.01$, d.f. 149; Figure 4.2.B) and increased at the least negative matric potential ($P < 0.01$, d.f. 169; Figure 4.3.B). LRN was unresponsive to manipulation of water supply by particle size but was greatly increased at the least negative matric potential ($P < 0.001$, d.f. 169; Figure 4.3.C). TLRL ($P < 0.01$, d.f. 149) and LRD ($P < 0.001$, d.f. 149) decreased at the largest particle size (Figure 4.2.D and E) and both increased at the least negative matric potential ($P < 0.001$, d.f. 169; Figure 4.3.D and E).

Figure 4.1. Root proliferation responses to different forms of nitrate. Significance: * P < 0.001; n = 72. Key: 10.0 mM KNO_3; 5.0 mM NH_4NO_3.

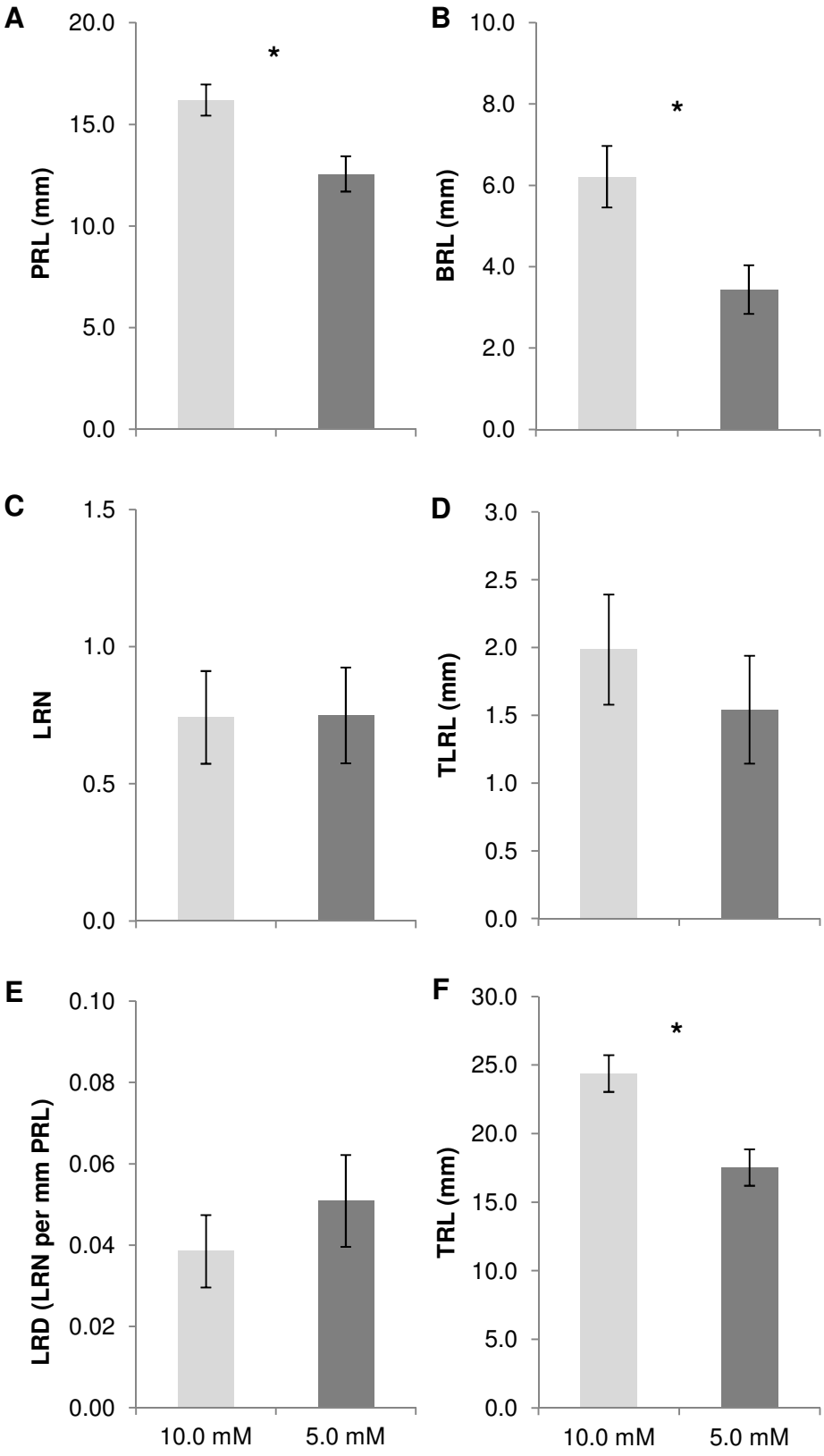


Figure 4.2. Root proliferation responses to manipulation of water supply by altered sand particle size under low (0.1 mM), medium (1.0 mM) and high (10.0 mM) nitrate supply. Significance compared at each nitrate concentration: ***, P < 0.001, **, P < 0.01, *, P < 0.05. D.f. 149. Key: <250 µm; 250-425 µm; >425 µm.

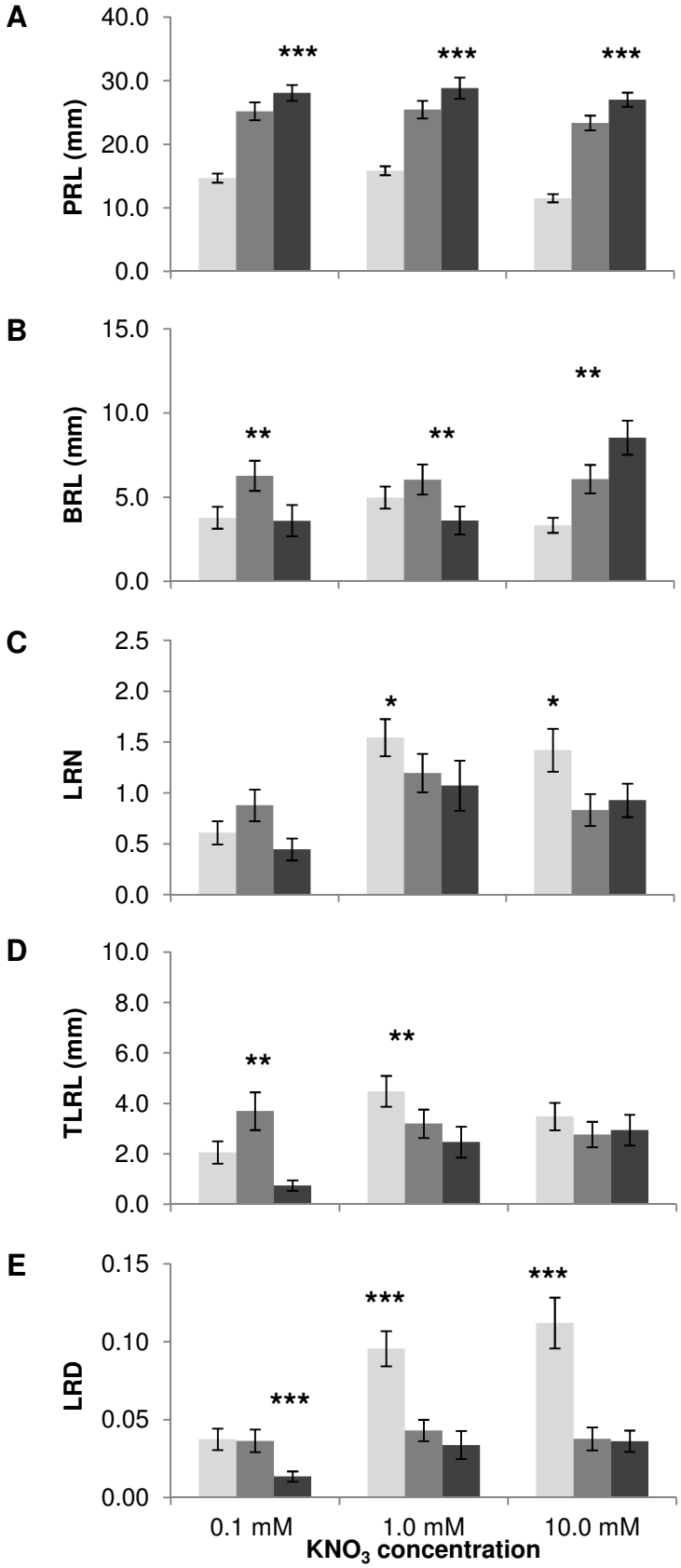


Figure 4.3. Root proliferation responses to manipulation of water supply by altered matric potential under low (0.1 mM), medium (1.0 mM) and high (10.0 mM) nitrate supply. Significance compared at each nitrate concentration: **, P < 0.001, *, P < 0.01. D.f. 169. Key: -1.5 kPa; -3.0 kPa; -4.5 kPa.

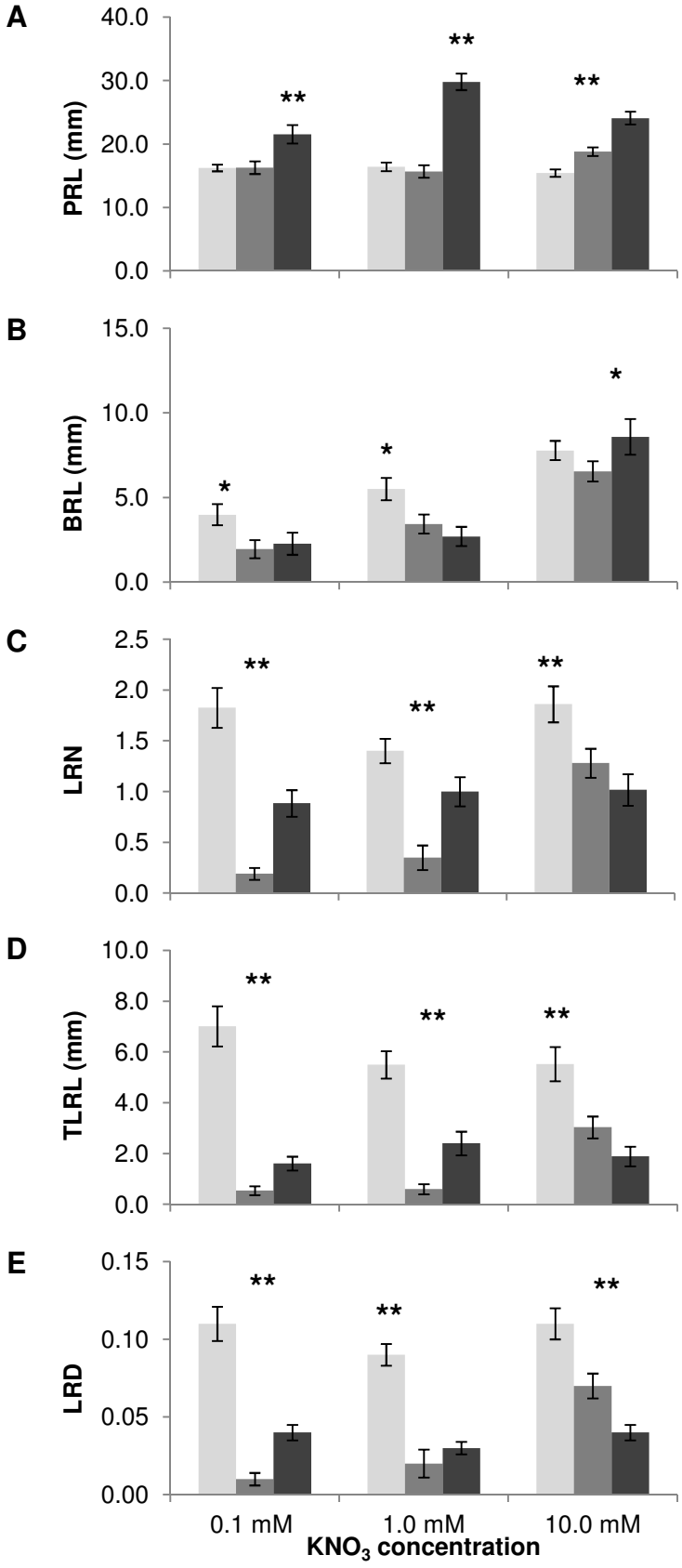
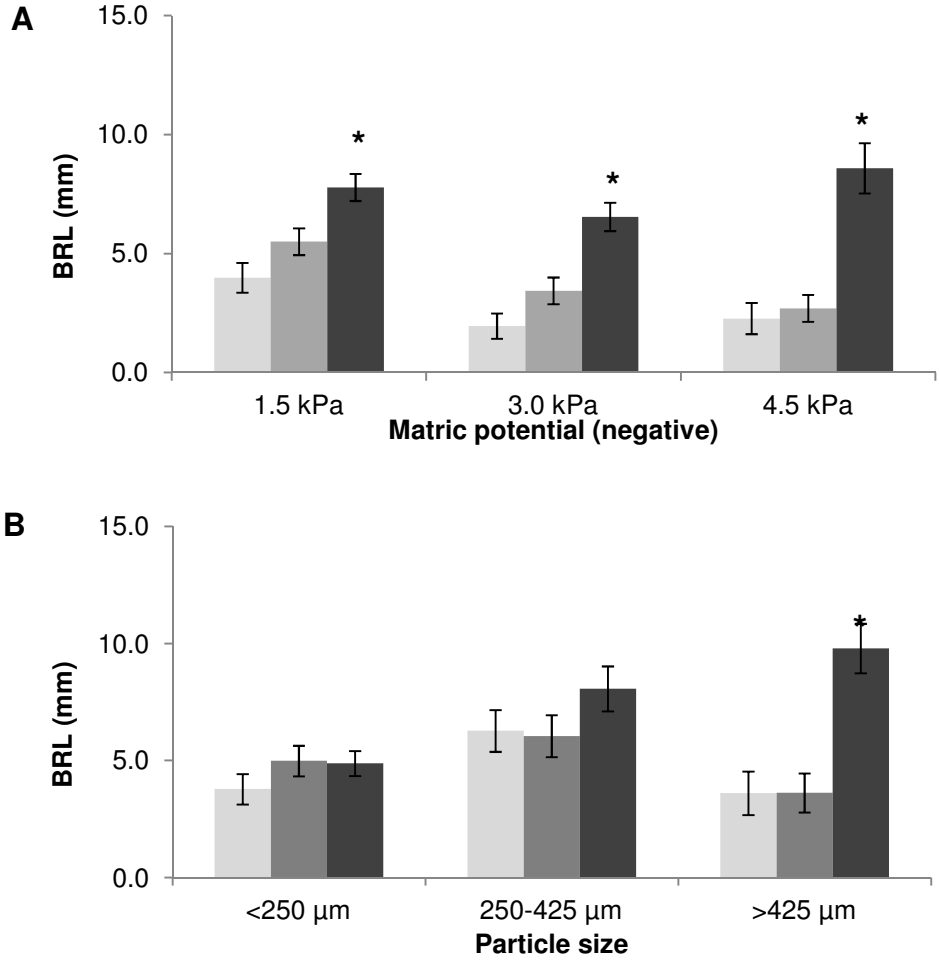


Figure 4.4. Basal root length was stimulated by high nitrate supply when water supply was manipulated by altered matric potential (A) and particle size (B). Significance: *, P < 0.001, A, d.f. 169. B, d.f. 169. Key: 0.1 mM; 1.0 mM; 10.0 mM.



Under medium (1.0 mM) nitrate supply, PRL again increased with increasing particle size ($P < 0.001$, d.f. 149; Figure 4.2.A) and was increased at the most negative matric potential ($P < 0.001$, d.f. 169; Figure 4.3.A). BRL again decreased at the largest particle size ($P < 0.01$, d.f. 149; Figure 4.2.B) and increased at the least negative matric potential ($P < 0.01$, d.f. 169; Figure 4.3.B). LRN was increased at the smallest particle size ($P < 0.05$, d.f. 149; Figure 4.2.C) and the least negative matric potential ($P < 0.001$, d.f. 169; Figure 4.3.C). TLRL was increased at the smallest particle size ($P < 0.01$, d.f. 149; Figure 4.2.D) and the least negative matric potential ($P < 0.001$, d.f. 169; Figure 4.3.D). LRD was also increased at the smallest particles size ($P < 0.001$, d.f. 149; Figure 4.2.E) and the least negative matric potential ($P < 0.001$, d.f. 169; Figure 4.3.E).

At high (10.0 mM) nitrate provision, PRL retains its positive response to increasing particle size ($P < 0.001$, d.f. 149; Figure 4.2.A) and increases with increasingly negative matric potential ($P < 0.001$, d.f. 169; Figure 4.3.A). BRL increases with increasing particle size ($P < 0.01$, d.f. 149; Figure 4.2.B) and is increased at the most negative matric potential ($P < 0.01$, d.f. 169; Figure 4.3.B). LRN was once again increased at the smallest particle size ($P < 0.05$, d.f. 149; Figure 4.2.C) and decreased with increasingly negative matric potential ($P < 0.001$, d.f. 169; Figure 4.3.C). TLRL was unresponsive to changes in particle size but decreased with increasingly negative matric potential ($P < 0.001$, d.f. 169; Figure 4.3.D). Once more, LRD was strongly increased at the smallest particle size ($P < 0.001$, d.f. 149; Figure 4.2.E) and decreased with increasingly negative matric potential ($P < 0.001$, d.f. 169; Figure 4.3.E).

The root proliferation responses to altered water and nitrate supply are summarised in Table 4.1. PRL was negatively regulated by increased water supply and this appeared to be independent of nitrate input (Figures 4.2.A and 4.3.A). By contrast, the response of BRL to water supply was much more dependent on nitrate supply (Figure 4.2.B, 4.3.B and 4.4.). LR growth (LRN, TLRL and LRD) characteristics were all strongly increased in response to water supply as manipulated by altered matric potential. This was the case at each nitrate supply, although LRN, TLRL and LRD were all increased at high nitrate supply under medium water availability (Figure 4.3.C, D and E). Whilst LRN and TLRL responded to increased water supply as manipulated by particle size, only LRD retained a strong positive response to increased water supply that was observed at each nitrate supply (Figures 4.2.E and 4.3.E). At increased water availability LRN, TLRL and LRD were all decreased at low (0.1 mM) nitrate supply.

4.3. Relating root proliferation responses to hydraulic characteristics

PRL has been shown to be stimulated at small negative (-0.1 to -0.2 MPa) water potential, before decreasing with increasingly negative matric potential in a linear fashion (Taylor and Ratliff 1969; Sharp et al. 1988; Verslues et al. 1998; van der Weele et al. 2000; Passioura 2002; Wiegers et al. 2009; Bengough et al. 2011). The water potentials experienced within the sand rhizotron system (-0.06 to -0.40 MPa) exceed the narrow range reported for the stimulation response. However, PRL continued to increase with increasingly negative water potential rather than exhibiting the linear decrease, indicating that PRL may be responding to something other than water potential.

The contribution of matric potential and osmotic potential to water potential was varied for the two manipulations of water supply, but both manipulations achieve an ~85% change in unsaturated hydraulic conductivity (Chapter 3, Table 3.2.). When PRL data from both manipulations of water supply were pooled, the strongest relationship found was the negative correlation with unsaturated hydraulic conductivity at low (r -0.85, $P < 0.001$; Figure 4.5.A), medium (r -0.88, $P < 0.001$; Figure 4.5.B) and high (r -0.78, $P < 0.001$; Figure 4.5.C) nitrate supplies and in fact the strong regulation of PRL by unsaturated hydraulic conductivity was independent of nitrate supply (r -0.84, $P < 0.001$). Thus, the conserved PRL response between the two manipulations of water supply was a response to altered unsaturated hydraulic conductivity rather than water potential.

BRL showed no clear relationship with water supply manipulated by either particle size or matric potential, but instead was positively regulated by increasing nitrate concentration ($P < 0.001$; Figure 4.4., A d.f. 169, and B d.f. 149). A ~45% increase in BRL was observed when nitrate provision was doubled (from 5.0 mM to 10.0 mM) at constant unsaturated hydraulic conductivity (0.79 $m.d^{-1}$; Chapter 4, Figure 4.1., p86). This doubling of nitrate also coincided with the removal of ammonium supply. Here, a ~65-70% increase was observed for a 10-fold increase (from 1.0 mM to 10.0 mM) in nitrate supply at low unsaturated hydraulic conductivity (0.17-0.18 $m.d^{-1}$; Figure 4.4.). This suggests that BRL is positively regulated by increased nitrate supply at constant unsaturated hydraulic conductivities and could be negatively regulated by ammonium in a similar manner to PRL.

Table 4.1. Summary of root proliferation responses to nitrate supply across two manipulations of water supply.

Root trait	Water manipulation	Low nitrate	Medium nitrate	High nitrate
PRL	Increasing particle size	Increased	Increased	Increased
	Increasing matric potential	Increased	Increased	Increased
BRL*	Increasing particle size	No trend	Decreased	Increased
	Increasing matric potential	Decreased	Decreased	No trend
LRN**	Increasing particle size	Unresponsive	Decreased	Decreased
	Increasing matric potential	Decreased	Decreased	Decreased
TLRL**	Increasing particle size	Decreased	Decreased	Unresponsive
	Increasing matric potential	Decreased	Decreased	Decreased
LRD**	Increasing particle size	Decreased	Decreased	Decreased
	Increasing matric potential	Decreased	Decreased	Decreased

*BRL was positively regulated by nitrate supply rather than water supply.

**LRN, TLRL and LRD were increased at high (10.0 mM) nitrate supply at medium matric potential and decreased at low (0.1 mM) nitrate supply at smallest particle size.

Manipulation of matric potential had a more profound effect on LR growth (Figures 4.2. and 4.3.). LRN, TLRL and LRD all increased at the least negative matric potential ($P < 0.001$, d.f. 169; Figure 4.3.C, D and E) at each nitrate concentration supplied. In fact, when data from both manipulations of water supply were pooled, LRD was negatively correlated with water potential at low (r -0.87, $P < 0.001$; Figure 4.6.A), medium (r -0.89, $P < 0.001$; Figure 4.6.B) and high (r -0.62, $P < 0.05$; Figure 4.6.C) nitrate supplies. However, LRN, TLRL and LRD were increased at high (10.0 mM) nitrate supply at medium matric potential and decreased at low (0.1 mM) nitrate supply at the smallest particle size. Therefore, it is not possible to say that the response of LR growth to water supply was independent of nitrate.

Water potential is mainly determined by the osmotic component in our sand rhizotron system and a similar range of unsaturated hydraulic conductivity is achieved for both manipulations of water supply (Chapter 3, Table 3.2., p71). It has been previously shown that LRD decreased by ~40% in response to a ~25% increase (from -0.514 to -0.6746 MPa) in osmotic potential under constant 10.0 mM nitrate supply (Deak and Malamy 2005). In response to a ~70% increase in osmotic potential (from -0.1085 to -0.3955 MPa) at constant 10.0 mM nitrate supply, a decreased LRD of ~60% was observed (Figure 4.3.E) and a similar change (~65%) was observed for an ~80% increase (from -0.0570 to -0.3270 MPa; Figure 4.2.E). The regulation of LRD by osmotic potential was independent of nitrate supply. LRN demonstrated a similar response (Figures 4.2.C and 4.3.C). This suggests that LRD and LRN are also responding to altered osmotic potential within our system.

In addition to LRN and LRD, TLRL has been shown to decrease by ~60% in response to a ~25% increase (from -0.514 to -0.6746 MPa) in osmotic potential under constant 10.0 mM nitrate supply (Deak and Malamy 2005; Roycewicz and Malamy 2012). A ~65% decrease in TLRL for a ~70% increase (from -0.1085 to -0.3955 MPa) in osmotic potential at constant 10.0 mM supply was found when water flux was manipulated by changing matric potential (Figure 4.3.D). Interestingly, no change in TLRL was observed for an ~80% increase (from -0.0570 to -0.3270 MPa) in osmotic potential under constant 10.0 mM nitrate supply at constant matric potential (Figure 4.2.D). Therefore, it seems that TLRL is responding to small changes in matric potential (from -0.0015 to -0.0045 MPa) independent of changes in osmotic potential and unsaturated hydraulic conductivity. The root proliferation responses to hydraulic characteristics are summarised in Table 4.2..

Figure 4.5. Primary root length (PRL) negatively correlated with unsaturated hydraulic conductivity (K_{unsat}) at low (A), medium (B) and high (C) nitrate supply. Data were plotted for both particle size (white circles) and matric potential (black circles) manipulations of substrate hydraulic conductivity (K_{unsat}). Each point represents the mean value for one replicate of 24 seedlings.

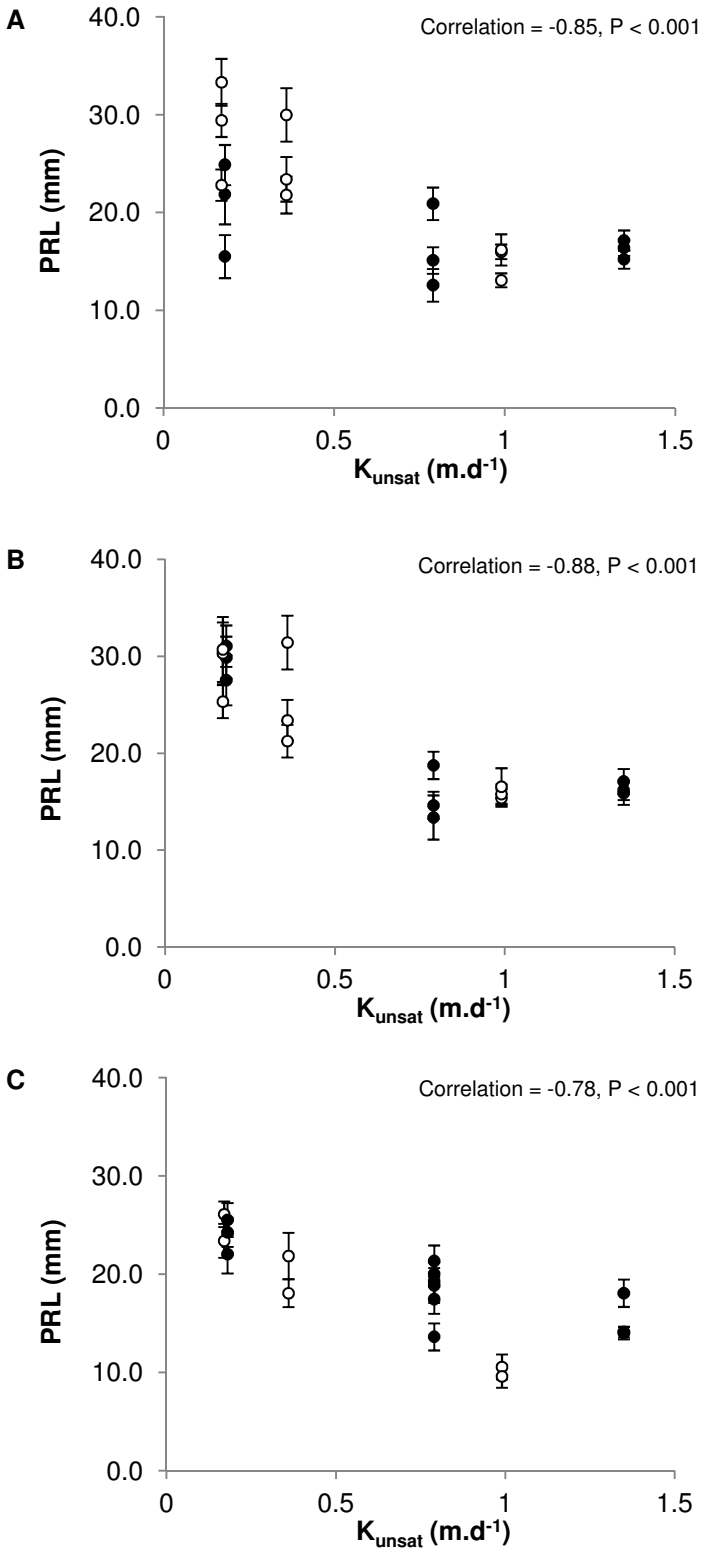


Figure 4.6. Lateral root density (LRD) negatively correlated with water potential (Ψ_t) at low (A), medium (B) and high (C) nitrate supply. Data were plotted for both particle size (white circles) and matric potential (black circles) manipulations of water potential. Each point represents the mean value for one replicate of 24 seedlings.

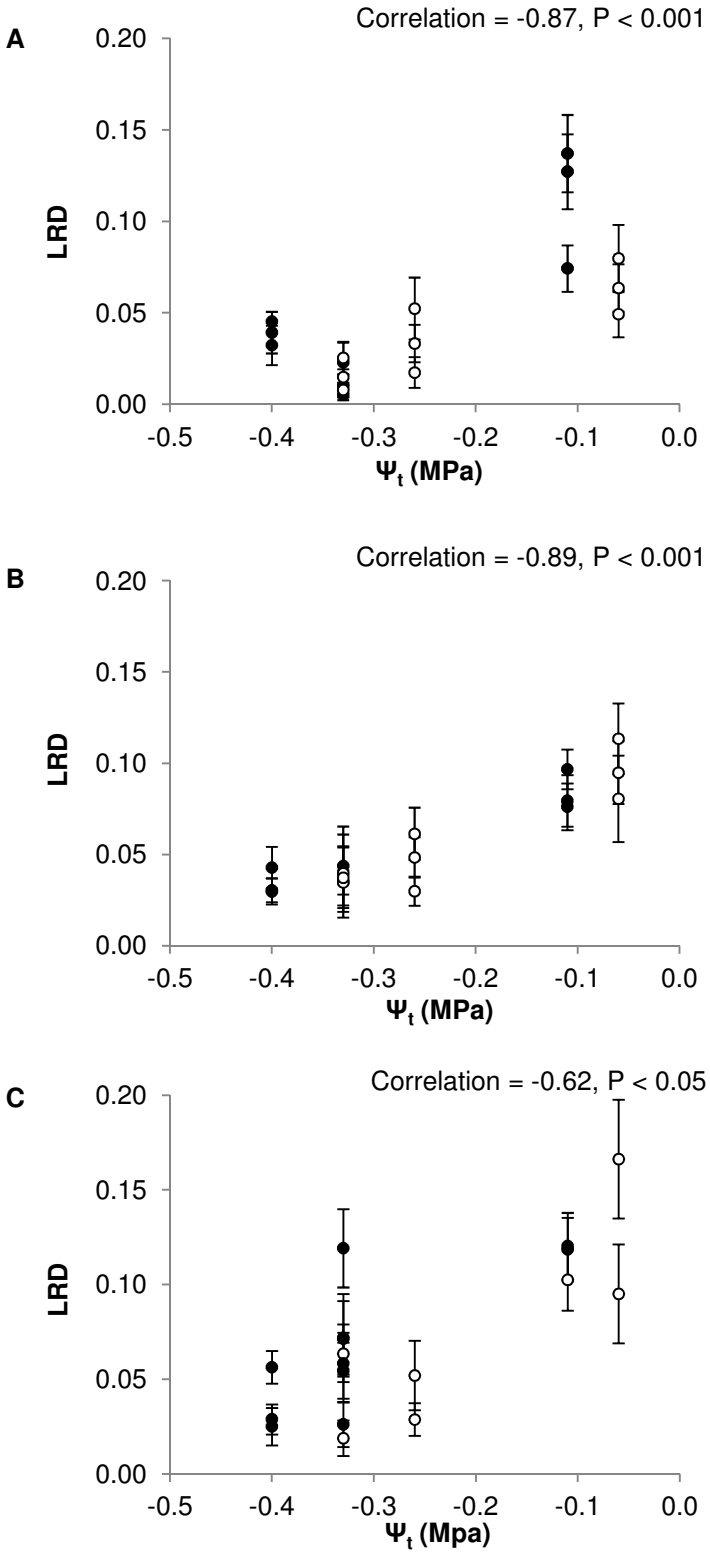


Table 4.2. Summary of root proliferation responses to specific hydraulic characteristics. The strongest relationship for each parameter is presented here.

Parameter	Manipulation of water supply
PRL	Negatively regulated by increasing unsaturated hydraulic conductivity*
BRL	Negatively regulated by increasing unsaturated hydraulic conductivity under high nitrate supply
LRN	Negatively regulated by increasingly negative osmotic potential*
TLRL	Negatively regulated by increasingly negative matric potential*
LRD	Negatively regulated by increasingly negative osmotic potential*

*at each nitrate supply.

4.4. Root proliferation responses to water flux were independent of nitrate availability at the root surface

PRL was regulated by unsaturated hydraulic conductivity and LRD by water potential, at each nitrate supply (Figures 4.5. and 4.6.). Manipulation of hydraulic characteristics influences the delivery of water and dissolved nitrate ions to the root. This makes it difficult to determine whether root proliferation changes are a direct response to water supply or an indirect response to altered nitrate delivery.

Although nitrate has been excluded from PRL regulation in agar (Linkohr et al. 2002; Walch-Liu and Forde 2008), LR growth has been shown to respond to both water availability and high nitrate patches (Drew 1975; Zhang et al. 1999; Ryan et al. 2003; Deak and Malamy 2005). Changes in nitrate availability have been suggested to be sensed by nitrate transporters (NRTs) at the PR tip (AtNRT1.1) and along the PR (AtNRT2.1) which then coordinate root proliferation changes in response to nitrate availability (reviewed by Ho and Tsay 2010).

To determine whether root proliferation was directly responding to water flux or indirectly responding to nitrate availability, nitrate concentrations were determined at the sensing regions of the PR using nitrate-selective microelectrodes for seedlings grown under low (0.1 mM) or high (10.0 mM) nitrate supply at three different unsaturated hydraulic conductivities. For each treatment, Arabidopsis ecotype WS seedlings were germinated and grown on sand rhizotrons for 7 days. At 12 dpg, root proliferation parameters were measured and seedlings were harvested (see Chapter 2 for details).

There was no statistical difference in nitrate concentration at the root surface when compared by general ANOVA (Table 4.3.), despite being subjected to altered unsaturated hydraulic conductivity under both low (0.1 mM) and high (10.0 mM) nitrate supply. However, this could be due the variation in measured nitrate activity and the conversion to nitrate concentration. Evaluation of Table 4.3. reveals that measured nitrate concentrations appeared to be very different at extremes of unsaturated hydraulic conductivity under low (0.1 mM) supply. The difference in nitrate availability at the root surface under low (0.1 mM) nitrate supply may explain why LRN, TLRL and LRD were decreased under low (0.1 mM) supply at increased water availability. Interestingly, the measured nitrate concentrations were much greater than the concentrations originally supplied (Table 4.3.).

Table 4.3. Nitrate concentration at the primary root surface at altered unsaturated hydraulic conductivity (K_{unsat}) manipulated by the use of sands of different particle size. Nitrate-selective microelectrodes were used to measure the nitrate concentration at the root surface of Arabidopsis seedlings supplied with 0.1 and 10.0 mM KNO_3. Values presented are the mean values of 3-5 measurements.

	Nitrate concentration (mM)			
	0.1 mM supply		10.0 mM supply	
K_{unsat} ($m.d^{-1}$)	Root Tip	Root Tip - 2mm	Root Tip	Root Tip - 2mm
0.17	102.1 ± 2.75	69.4 ± 3.13	59.7 ± 7.31	88.6 ± 12.5
0.36	78.8 ± 9.88	81.4 ± 5.06	26.9 ± 8.25	73.1 ± 6.19
0.99	14.4 ± 0.66	32.3 ± 3.90	48.4 ± 6.45	67.2 ± 6.45

4.5. The negative influence of ammonium nitrate is exaggerated in the sand rhizotron system

PRL decreased within the sand rhizotron system by more than would be predicted from agar culture experiments in response to 5.0 mM increase in ammonium provision (Li et al. 2010b). However, larger-than-normal concentrations are required in agar culture to reproduce responses observed in soil or solution culture because of the diffusion limitations of agar (Li and Shi 2007; Barth et al. 2010; Li et al. 2010b). This is likely to be further compounded by differences in the relative mobility of nitrate and ammonium (Owen and Jones 2001), due to a disparity in the effective soil diffusion coefficient (D_e) of around two orders of magnitude between nitrate (3.26×10^{-10} m^2 s^{-1}) and ammonium (2.70×10^{-12} m^2 s^{-1}) (Miller and Cramer 2005). Indeed, variation in root proliferation responses to altered ammonium and nitrate supply have been reported between soil and solution culture (Bloom et al. 1993), presumably due to altered physical characteristics between these substrates that impact on ion delivery to the root. Therefore, a difference in hydraulic characteristics between agar and sand is likely to account for the exaggerated PRL response to ammonium supply observed for sand-grown seedlings, compared to that previously reported for agar (Li et al. 2010b).

There are no reports of nitrate regulation of BRL. The nutritional regulation of BRL can be distinct from the PRL, as is the case for phosphate (Bonser et al. 1996; Liao et al. 2001; Fitter et al. 2002; Zhu et al. 2005; Basu et al. 2011; Péret et al. 2011). Here, the BRL response to ammonium nitrate paralleled that of the PRL (Figure 4.1.) but BRL was regulated by increasing nitrate supply (Figure 4.4.) whilst PRL was not (Figure 4.5.). The decreased BRL under decreased nitrate supply could be a negative response to increased ammonium supply or a response to decreased nitrate supply.

4.6. Why is measured nitrate concentration greater than the original input?

An increase in measured nitrate concentration could be the result of decreased water content, as was suggested for agar nitrate measurements (Chapter 3, Section 3.4., p80). This may indicate that drying of the sand had occurred during measurements, but care was taken to limit the area of sand exposed to the air and steady recordings were obtained suggesting that the evaporative losses were minimal (Chapter 2, Figure 2.4., p55). Furthermore, the duration of

exposure to potential evaporative losses was consistent for all measurements, but the measured nitrate concentrations were similar regardless of nitrate input. This would not be expected if the duration of evaporative losses were equal for 0.1 and 10.0 mM nitrate treatments. Therefore, it is unlikely that the increase in measured nitrate concentration was the result of decreased water content due to evaporative losses.

Alternatively, an increase in measured nitrate concentration could be caused by modified root water or nitrate fluxes (influx and/or efflux). Although nitrate efflux can be high for well supplied and non-stressed plants, it has been shown to remain less than nitrate influx and in fact net nitrate efflux usually only occurs when seedlings are under stress (Aslam et al. 1996; Aslam et al. 1997; Williams and Miller 2001; Segonzac et al. 2007; Wang et al. 2011). Whilst the manipulation of substrate hydraulic characteristics in the sand rhizotron system alters water and nitrate supply, it does not subject the seedlings to stress conditions and so net nitrate efflux is unlikely to contribute to the increased nitrate concentration observed.

An increase in the measured nitrate concentration at the root surface could be generated if the delivery of nitrate ions to the root surface exceeds the net influx of nitrate ions into the root. Interestingly, the exaggerated PRL response to increased ammonium supply in sand (Figure 4.1.) relative to agar (Li et al. 2010b) might also be explained if the delivery of ammonium ions exceeded net influx in a similar manner. However, the rate of accumulation would likely be less than nitrate due to differences in mobility between these ions (Miller and Cramer 2005).

The measured nitrate concentrations at both locations were lower, not higher, at high unsaturated hydraulic conductivity (Table 4.3.). At high unsaturated hydraulic conductivity the connectivity of water is relatively increased and the supply of dissolved nitrate to the root is increased as a result. Therefore, the increased nitrate concentration measured at the root surface could be related to altered water flux rather than the delivery of nitrate to the root surface exceeding the net influx of nitrate.

An increased water influx could result in a localised decrease in water content at the root surface, producing an increase in measured nitrate concentration. The measured nitrate concentrations at both locations were lower at high unsaturated hydraulic conductivity than low unsaturated hydraulic conductivity for both low and high nitrate input (Table 4.3.), which indicates that the greater connectivity of solution at high unsaturated hydraulic conductivity is

sufficient to replenish the water taken up by the root. At low unsaturated hydraulic conductivity, the connectivity is decreased and water taken up is less able to be replenished resulting in an increase in measured nitrate concentration. Thus, the increase in measured nitrate concentration is likely to be due to altered localised water content which is determined by the unsaturated hydraulic conductivity of the sand.

4.7. Summary and conclusions

The negative regulation of PRL by ammonium is exaggerated in the sand rhizotron system (Figure 4.1.) relative to agar (Li and Shi 2007) because of disparities in substrate hydraulic characteristics and because the manipulation of substrate hydraulic characteristics has a greater effect on nitrate delivery than ammonium due to differences in mobility. Two independent manipulations of water supply had large effects on root proliferation characteristics (Figures 4.2. and 4.3.).

PRL and LRD were strongly negatively regulated by unsaturated hydraulic conductivity and water potential respectively at each nitrate input (Figures 4.5. and 4.6.). The response of LRD (and LRN) to water potential was probably a response to osmotic potential (Deak and Malamy 2005), whilst TLRL responded to small changes in matric potential (Deak and Malamy 2005; Roycewicz and Malamy 2012). BRL was regulated by nitrate supply and the difference was particularly large at low unsaturated hydraulic conductivities (Figure 4.4.).

Whilst the manipulation of hydraulic conductivity within the sand rhizotron system did not significantly alter the nitrate availability at the root surface under high (10.0 mM) supply, measured concentrations appeared to be higher at decreased hydraulic conductivity under low (0.1 mM) supply. As a result a difference in nitrate or water availability could be influenced root physiology. An increase in the measured nitrate concentration at the root surface relative to nitrate input was probably due to altered hydraulic conductivity. These results indicate that nitrate concentration at the root surface is kept relatively constant despite changes in water supply, presumably facilitated by modifications to water and/or nitrate influx and related to the high mobility of nitrate.

The main conclusions of this chapter are summarised below:

- The negative regulation of root proliferation by ammonium is exaggerated in the sand rhizotron system;
- In the sand rhizotron system, PRL and LRD were regulated by water availability at each nitrate supply (0.1, 1.0 and 10.0 mM).

Changes in the expression of some nitrate transporter (NRT) and aquaporin genes represent a significant mechanism for the regulation of nitrate and water influx at the root surface (reviewed by Maurel et al. 2008; Wang et al. 2012). As a result, relative changes in gene expression for selected genes were investigated in response to manipulations of water and nitrate supply within the sand rhizotron system (Chapter 5).

5. Gene Expression Responses to Altered Water and Nitrate Supply

Water and nitrate uptake by roots helps to maintain the delivery of dissolved nitrate ions to the root surface by mass flow and diffusion, whereas altered hormone transport and signalling underpin root proliferation changes. The expression of selected nutrient transporters and hormone-associated genes (Chapter 1, Table 1.2., p46) are important root physiological components of nutrient acquisition responses to altered water and nitrate availability. To better understand the responses of these components to altered nitrate and water supply, the expression of selected Arabidopsis (*Arabidopsis thaliana*) acquisition genes (*AtNRT1.1* *AtNRT2.1*, *AtNAR2.1* and *AtPIP2.2*) and hormone-associated genes (*AtPIN1*, *AtPIN2*, *AtTIR1*, *AtEIN2*, *AtABI4* and *AtIPT5*) was analysed (see Chapter 2 for details) for a series of manipulated nutrient regimes within the sand rhizotron system.

5.1. The influence of water supply on the expression of selected nutrient acquisition genes across a range of nitrate supplies

The dual affinity nitrate transporter AtNRT1.1 facilitates nitrate uptake at a wide range of concentrations depending on its phosphorylation status (Walch-Liu and Forde 2008; Ho et al. 2009; Krouk et al. 2010b), whereas the high-affinity nitrate transporter AtNRT2.1 facilitates uptake at lower concentrations (<1.0 mM) and requires *AtNAR2.1* for this function (Nazoa et al. 2003; Little et al. 2005; Orsel et al. 2006). Furthermore, both *AtNRT1.1* and *AtNRT2.1* have been proposed to facilitate nitrate sensing (Ho and Tsay 2010; Gojon et al. 2011). The plasma membrane intrinsic protein (PIP) family aquaporin AtPIP2.2 has been identified as a major contributor to root water uptake and its expression responds to water availability (Javot et al. 2003; Alexandersson et al. 2005; Da Ines et al. 2010).

Gene expression of these important nitrate and water acquisition genes was investigated at different water availabilities in seedlings grown under low (0.1 mM), medium (1.0 mM) and high (10.0 mM) nitrate supply. Arabidopsis ecotype WS seedlings were germinated and grown on sand rhizotrons for 7 days. At 12 dpg, seedlings were harvested and RNA was extracted for semi-quantitative RT PCR gene expression analysis (see Chapter 2 for details).

When nitrate supply was low (0.1 mM), the expression of *AtNAR2.1* was decreased at the largest particle size and *AtPIP2.2* expression was increased at medium matric potential, whereas the expression of *AtNRT1.1* and *AtNRT2.1* were not changed at different particle sizes (Figure 5.1.). The expression of *AtNRT1.1* was increased at the least negative matric potential, whilst *AtNRT2.1* and *AtPIP2.2* was decreased at the intermediate negative matric potential and *AtNAR2.1* expression was unresponsive to altered matric potential (Figure 5.2.).

At medium nitrate supply (1.0 mM), *AtNRT1.1* expression was decreased at the smallest particle size, *AtNRT2.1* expression was slightly decreased at the medium particle size whereas *AtNAR2.1* expression was increased at the largest particle size (Figure 5.1.). AtPIP2.2 expression was apparently unresponsive to particle size (Figure 5.1.). *AtNRT1.1* expression was decreased at the most negative matric potential and *AtNAR2.1* expression was greatly increased at the least negative matric potential (Figure 5.2.). Conversely, the expression of *AtNRT2.1* and *AtPIP2.2* was greatly increased at the most negative matric potential (Figure 5.2.).

Under high nitrate provision (10.0 mM), the expression of *AtNRT1.1* and *AtNAR2.1* was greatly increased compared to low (0.1 mM) and medium (1.0 mM) nitrate supply (Figures 5.1. and 5.2.). The expression of *AtNRT1.1* was increased at medium particle size, whilst *AtNRT2.1* and *AtPIP2.2* expression increased with increasing particle size and *AtNAR2.1* expression was relatively unresponsive to particle size (Figure 5.1.). *AtNRT1.1* expression was greatly increased at the most negative matric potential, whilst *AtNAR2.1* expression was slightly increased at medium matric potential (Figure 5.2.). The expression of *AtNRT2.1* and *AtPIP2.2* decreased with increasingly negative matric potential (Figure 5.2.). The transporter gene expression responses are summarised in Table 5.1.

Figure 5.1. Expression responses of (A) *AtNRT1.1*, (B) *AtNRT2.1*, (C) *AtNAR2.1* and (D) *AtPIP2.2* to manipulation of water supply by altered sand particle size across a range of nitrate concentrations. Treatments: <250 µm; 250-425 µm; >425 µm. Actual level of *AtNRT2.1* expression may be underestimated at due to saturation.

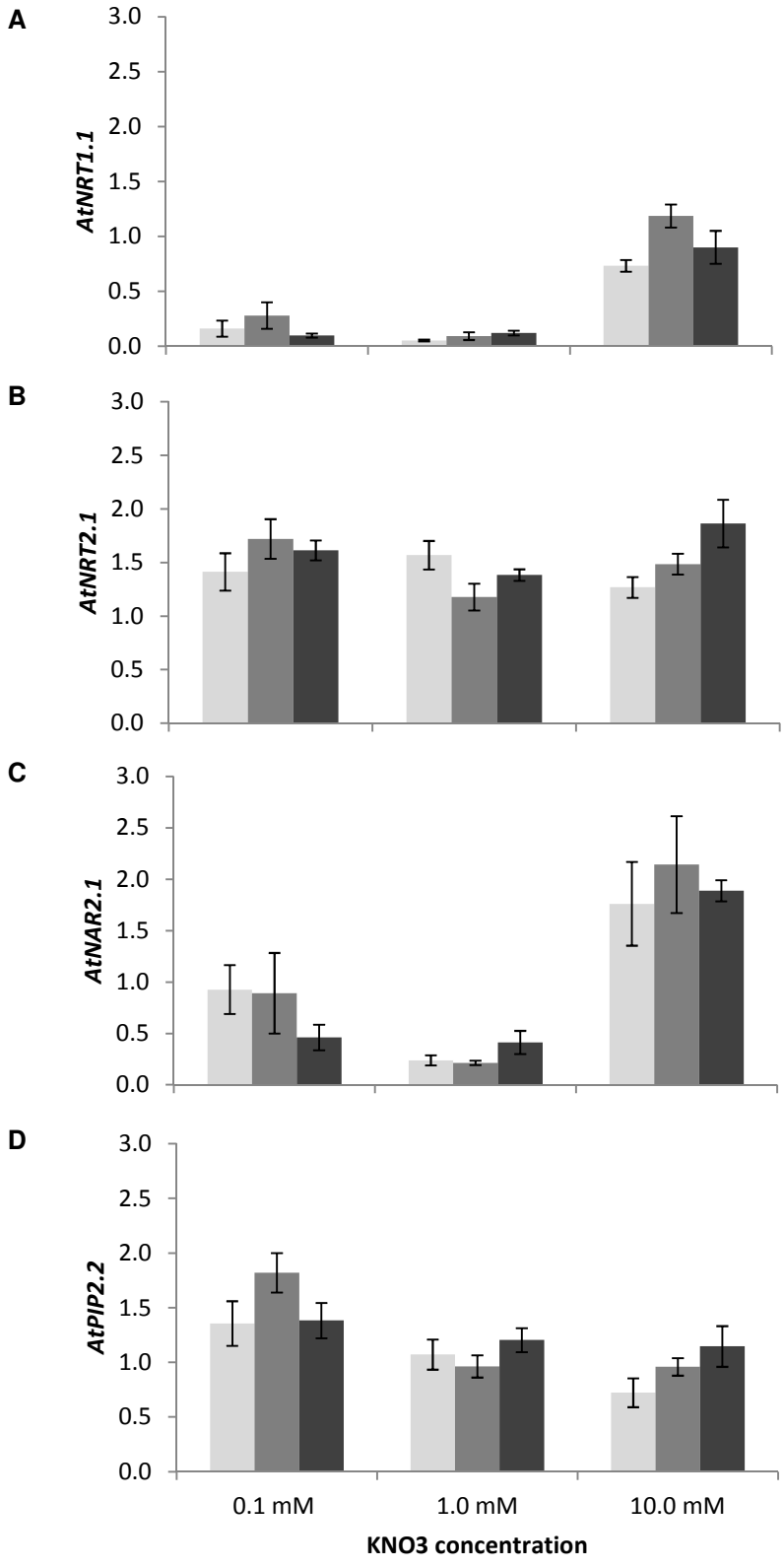


Figure 5.2. Expression responses of (A) *AtNRT1.1*, (B) *AtNRT2.1*, (C) *AtNAR2.1* and (D) *AtPIP2.2* to manipulation of water supply by altered matric potential across a range of nitrate concentrations. Treatments: -1.5 kPa; -3.0 kPa; -4.5 kPa. Actual level of *AtNRT2.1* expression may be underestimated due to saturation.

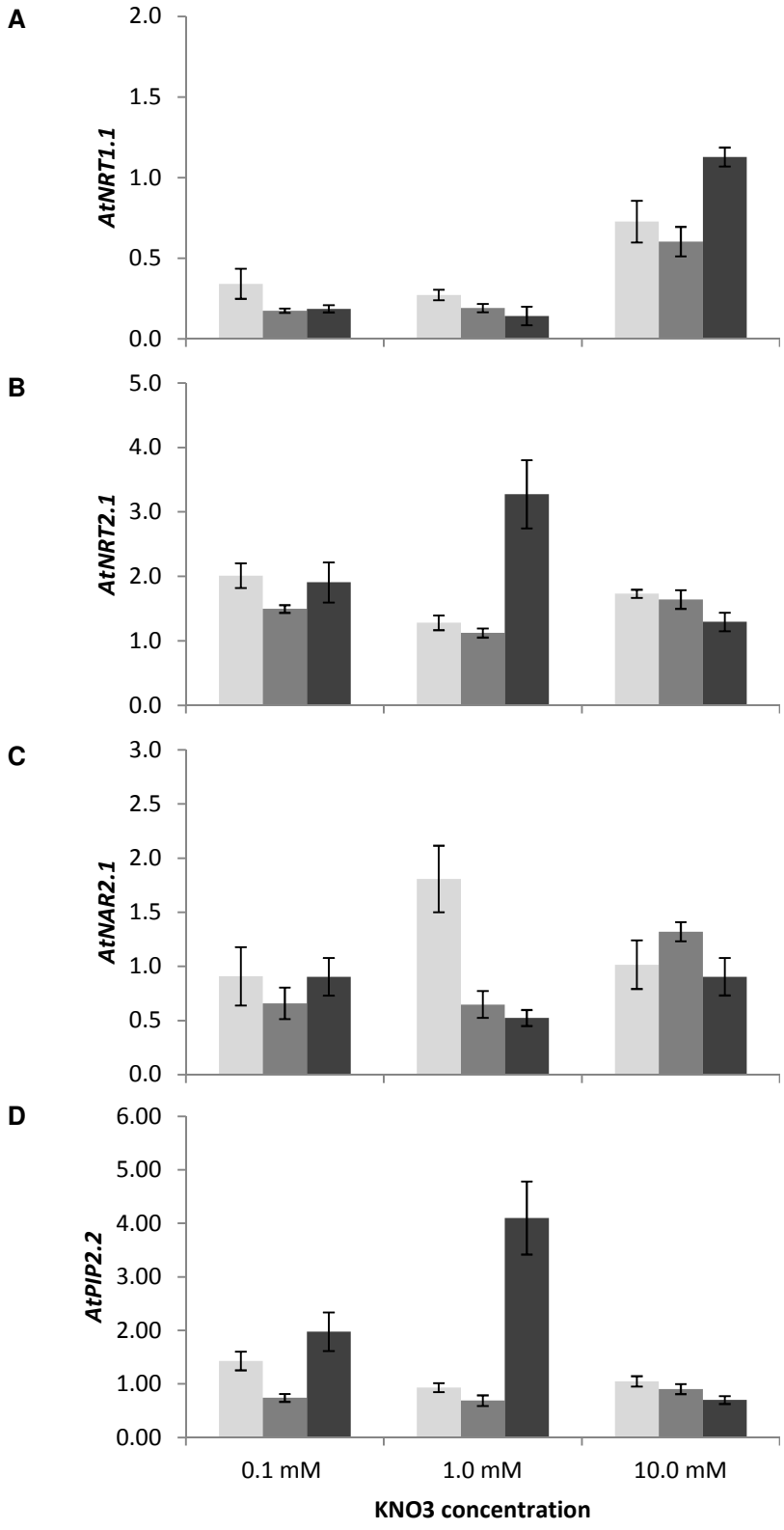


Table 5.1. Summary of wild-type nutrient acquisition gene expression responses to nitrate supply across two manipulations of water supply. Overall trends where found are presented here.

Gene	Water manipulation	Low nitrate	Medium nitrate	High nitrate
AtNRT1.1	Increasing particle size	No trend	Increased	Increased
	Increasing matric potential	Decreased	Decreased	Increased
AtNRT2.1	Increasing particle size	No trend	No trend	Increased
	Increasing matric potential	No trend	Increased	Decreased
AtNAR2.1	Increasing particle size	Decreased	Increased	No trend
	Increasing matric potential	No trend	Increased	No trend
AtPIP2.2	Increasing particle size	No trend	No trend	Increased
	Increasing matric potential	No trend	Increased	Decreased

5.2. The response of nutrient acquisition gene expression to substrate hydraulic characteristics

Sands of a different particle size were used to subject seedlings to a range of altered water potentials and unsaturated hydraulic conductivities at a constant matric potential (Chapter 3, Table 3.2., p71).

Overall *AtNRT1.1* expression was increased at high (10.0 mM) nitrate supply when water availability was manipulated by altered particle size (Figure 5.1.). When water supply was manipulated by altered matric potential, *AtNRT1.1* expression decreased with increasingly negative matric potential at low (0.1 mM) and medium (1.0 mM) nitrate supply, but increased with increasingly negative matric potential at high (10.0 mM) nitrate supply (Figure 5.2.).

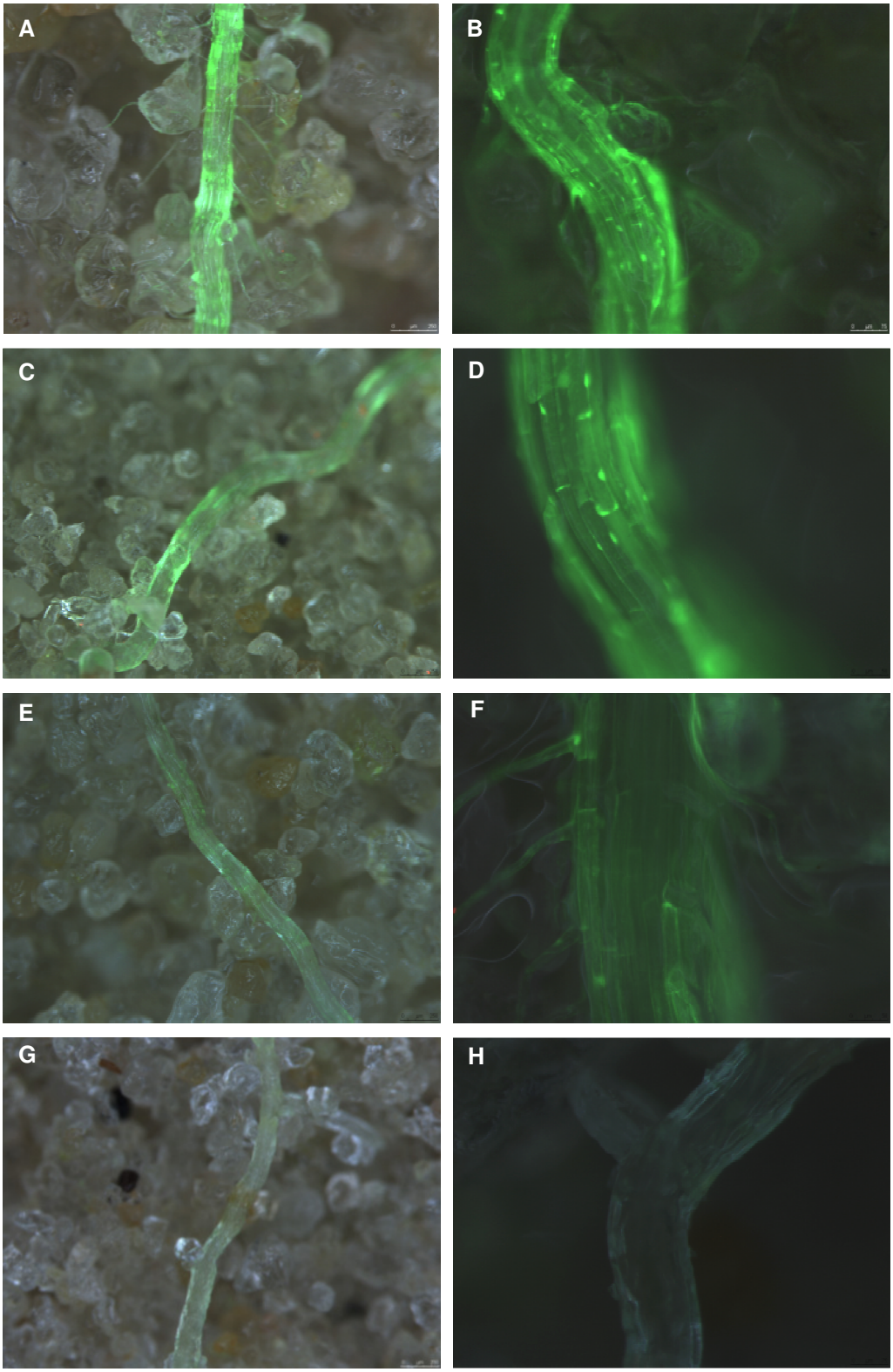
AtNAR2.1 expression was increased at high (10.0 mM) nitrate supply when water availability was manipulated by altered particle size (Figure 5.1.). When water supply was altered by manipulation of matric potential, *AtNAR2.1* expression was not regulated by water availability at low (0.1 mM) or high (10.0 mM) nitrate supply, but was increased at the least negative matric potential under medium (1.0 mM) nitrate supply.

Across all treatments, the expression of *AtNRT2.1* and *AtPIP2.2* was coordinated (comparing Figures 5.1. and 5.2.). When water availability was manipulated by particle size of the sand, expression of both genes demonstrated no overall trend at low (0.1 mM) or medium (1.0 mM) nitrate supply (Figure 5.1.). When water availability was manipulated by altered matric potential, the expression of both genes showed no trend at low (0.1 mM) nitrate supply but increased at the most negative matric potential under medium (1.0 mM) nitrate supply (Figure 5.2.). Under high (10.0 mM) nitrate supply, the expression of *AtNRT2.1* and *AtPIP2.2* was decreased at the largest particle size (Figure 5.1.) and increased at the most negative matric potential (Figure 5.2.). Therefore, it seems that expression of these two genes was unregulated by water availability at low (0.1 mM) nitrate supply, increased at relatively decreased water availability under medium (1.0 mM) nitrate supply, but becomes oppositely regulated by water availability at high (10.0 mM) nitrate supply.

In order to confirm whether expression was actually regulated by water availability under high (10.0 mM) nitrate supply, a *proNRT2.1:eGFP* reporter line (Kiba et al. 2012) was imaged *in situ* in sand rhizotrons at 12 dpg at extremes of unsaturated hydraulic conductivity under high (10.0

mM) nitrate supply (Figure 5.3.). *AtNRT2.1* expression was decreased at low unsaturated hydraulic conductivity relative to high unsaturated hydraulic conductivity under high (10.0 mM) nitrate supply. Expression decreased from unsaturated hydraulic conductivity of 1.35 $m.d^{-1}$ (Figure 5.3.A and B), to 0.99 $m.d^{-1}$ (Figure 5.3.C and D) and again to 0.17 $m.d^{-1}$ (Figure 5.3.E and F). Thus, *AtNRT2.1* expression was regulated by water availability at high (10.0 mM) nitrate supply. The responses of these key nutrient acquisition genes to water availability was apparently dependent on nitrate supply.

Figure 5.3. *In situ* imaging of a *proNRT2.1:eGFP* line confirmed that *AtNRT2.1* expression decreased with decreasing unsaturated hydraulic conductivity (K_{unsat}) at high nitrate supply. *proNRT2.1:eGFP* seedlings were grown under high (10.0 mM) nitrate supply at K_{unsat} of 1.35 (A and B), 0.99 (C and D) and 0.17 (E and F) $m.d^{-1}$. Wild-type (Col 0) images are presented for comparison at K_{unsat} of 1.35 (G) and 0.17 (H) $m.d^{-1}$. Images captured at 80x magnification (A, C, E and G) and 256x magnification (B, D, F and H).



5.3. The influence of water supply on the expression of hormone-associated genes across a range of nitrate supplies

The directional transport of auxin by the efflux transporters AtPIN1 and AtPIN2 and the perception by the receptor AtTIR1 contribute to specific root proliferation changes (Blilou et al. 2005; Dharmasiri et al. 2005; Gutierrez et al. 2007; Vidal et al. 2010). The ethylene signal transducer *AtEIN2* controls the nitrogen regulation of LR length (Tian et al. 2009). Cytokinin biosynthesis in roots is partly achieved by *AtIPT5* which responds to nitrate and ammonium under sufficient supply and is regulated by auxin and cytokinin (Takei et al. 2004). The ABA-related transcription factor AtABI4 is involved in the repression of LR growth under high nitrate supply (Signora et al. 2001; Shkolnik-Inbar and Bar-Zvi 2010). Gene expression of these important hormone-associated genes was investigated at different water availabilities in seedlings grown under low (0.1 mM), medium (1.0 mM) and high (10.0 mM) nitrate supply. Arabidopsis ecotype WS seedlings were germinated and grown on sand rhizotrons for 7 days. At 12 dpg, seedlings were harvested and RNA was extracted for RT PCR gene expression analysis (see Chapter 2 for details).

Under low (0.1 mM) nitrate supply, the expression of *AtPIN2* decreased, whilst *AtABI4* expression increased, with increasing particle size (Figure 5.4.). The expression of all other hormone-related genes was unresponsive to water availability as manipulated by altered particle size at low (0.1 mM) nitrate supply (Figure 5.4). When water availability was manipulated by matric potential, the expression of *AtTIR1* and *AtABI4* was decreased at the most negative matric potential (Figure 5.5.). The expression of all other hormone-related genes was unresponsive to water availability as manipulated by altered matric potential at low (0.1 mM) nitrate supply (Figure 5.5.).

At medium (1.0 mM) nitrate supply, the expression of *AtTIR1* was decreased at the smallest particle size, whilst *AtEIN2*, *AtABI4* and *AtIPT5* expression was increased at the intermediate particle size, and all other hormone-related genes were unresponsive to particle size (Figure 5.4.). The expression of *AtPIN1* and *AtTIR1* was increased, whilst *AtEIN2* expression was decreased, at the most negative matric potential (Figure 5.5.). *AtIPT5* expression decreased with increasingly negative matric potential, whilst all other hormone-related genes were unresponsive to matric potential (Figure 5.5.).

Under high (10.0 mM) nitrate supply, *AtPIN2*, *AtABI4* and *AtIPT5* expression was increased at the intermediate particle size, whereas *AtTIR1* expression was decreased at the smallest particle size (Figure 5.4.). The expression of *AtPIN2* increased with increasingly negative matric potential, while the expression of all other hormone-associated genes was unresponsive to matric potential at this nitrate concentration (Figure 5.5.).

The response of several hormone-related genes to water supply was dependent on nitrate supply. At the smallest particle size, *AtTIR1* expression decreased, while *AtEIN2* expression increased, with increasing nitrate supply (Figure 5.4.). *AtIPT5* expression increased with increasing nitrate supply at the intermediate particle size (Figure 5.4.). *AtPIN2* expression increased, whilst *AtABI4* expression decreased, with increasing nitrate concentration at the largest particle size (Figure 5.4.).

AtPIN1 expression decreased, whilst *AtABI4* expression increased, with increasing nitrate supply at the least negative matric potential (Figure 5.5.). At the intermediate matric potential, *AtEIN2* expression was decreased at low nitrate supply (Figure 5.5.). At the most negative matric potential, *AtPIN1* expression was increased at medium nitrate supply, while *AtTIR1*, *AtEIN2*, *AtABI4* and *AtIPT5* expression increased with increasing nitrate supply (Figure 5.5.).

Therefore, the expression of several hormone-associated genes was regulated by water and nitrate supply, with the expression of certain genes coordinated in their responses (Table 5.2.).

Figure 5.4. Expression responses of (A) *AtPIN1*, (B) *AtPIN2*, (C) *AtTIR1*, (D) *AtEIN2*, (E) *AtABI4* and (F) *AtIPT5* to manipulation of water supply by altered particle size at a range of nitrate concentrations. Treatments: <250 µm; 250-425 µm; >425 µm.

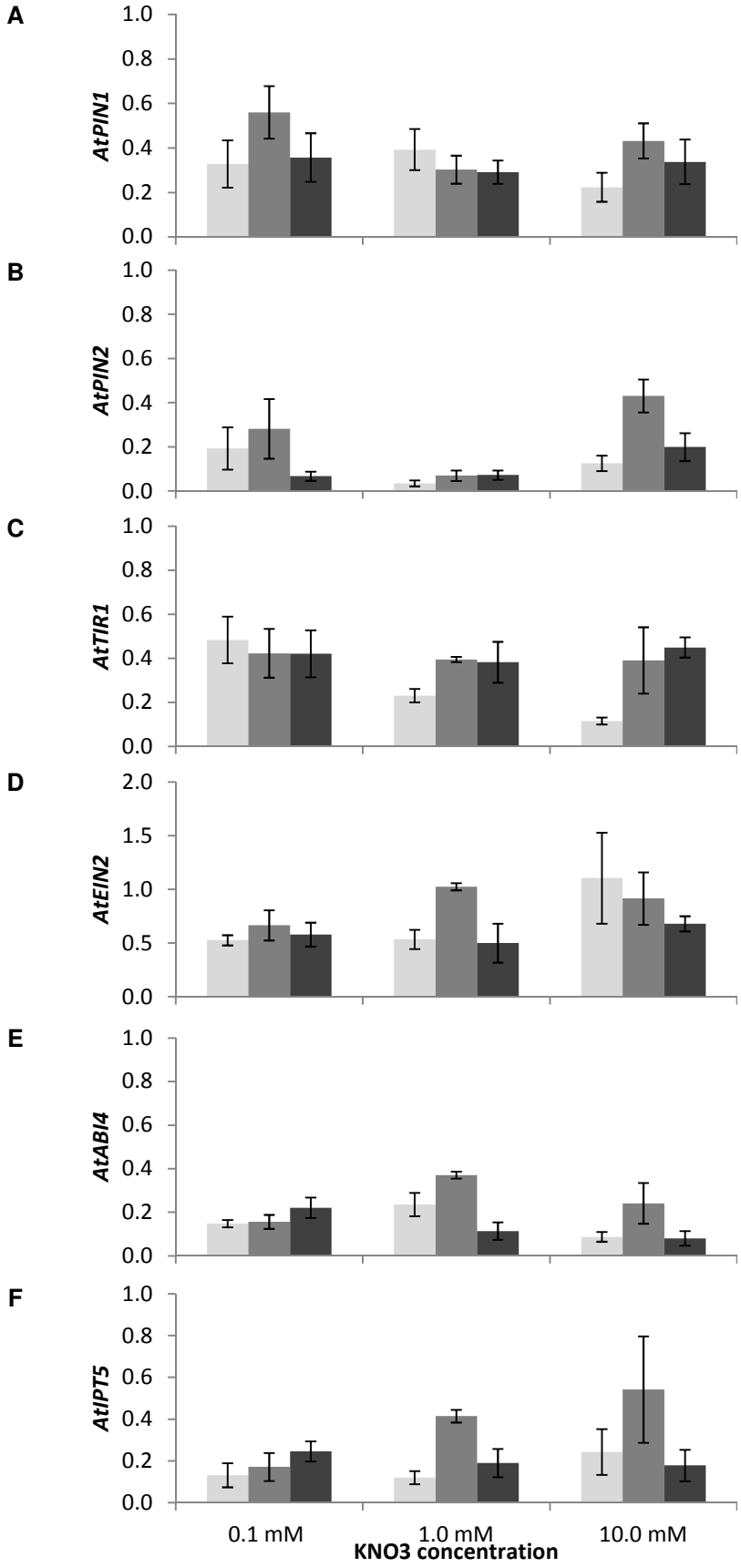


Figure 5.5. Expression responses of (A) *AtPIN1*, (B) *AtPIN2*, (C) *AtTIR1*, (D) *AtEIN2*, (E) *AtABI4* and (F) *AtIPT5* to manipulation of water supply by altered matric potential across a range of nitrate concentrations. Treatments: -1.5 kPa; -3.0 kPa; -4.5 kPa.

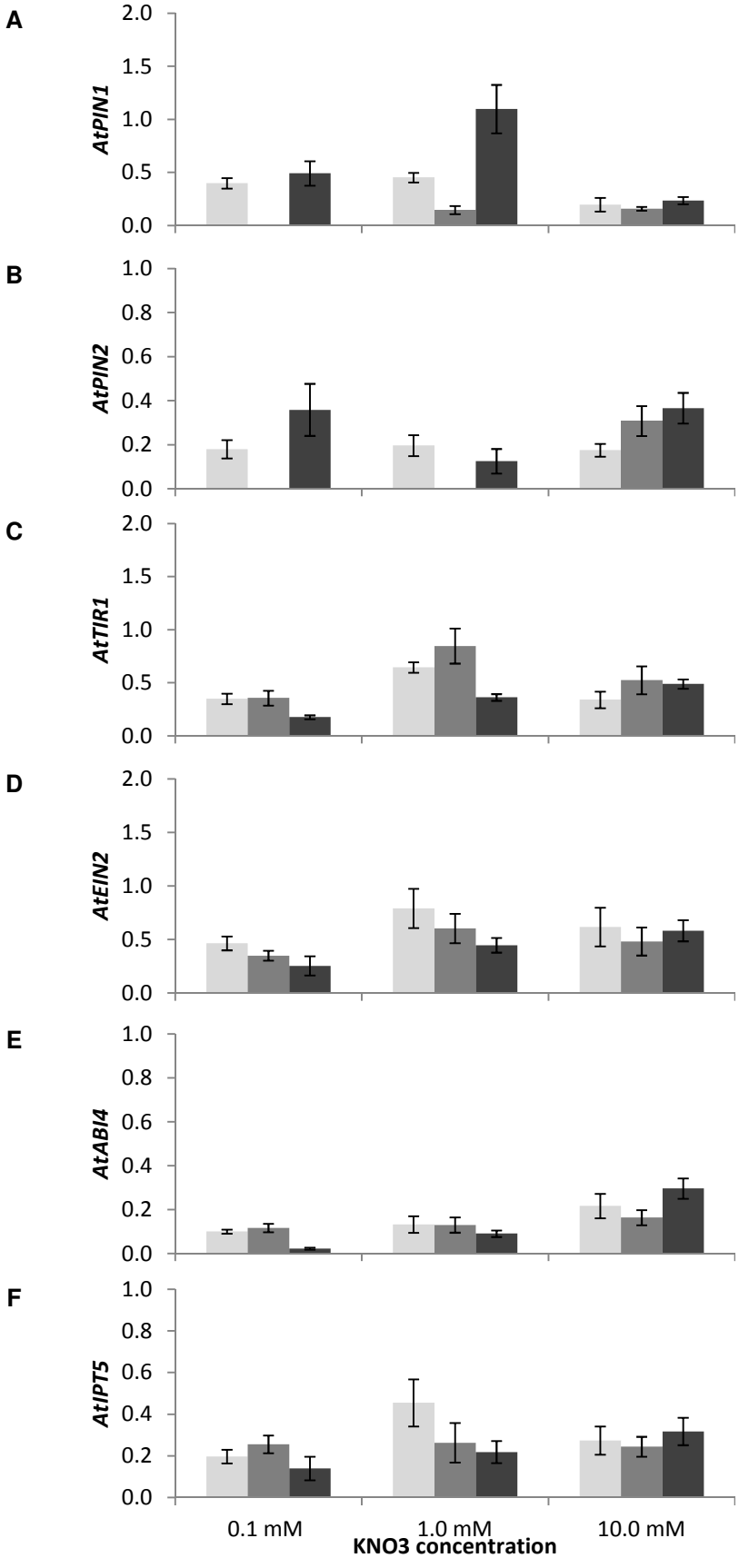


Table 5.2. Summary of wild-type hormone-associated gene expression responses to nitrate supply across two manipulations of water supply.

Gene	Water manipulation	Low nitrate	Medium nitrate	High nitrate
AtPIN1	Increasing particle size	No trend	No trend	No trend
	Increasing matric potential	No trend	Increased	No trend
AtPIN2	Increasing particle size	Decreased	No trend	Increased
	Increasing matric potential	No trend	No trend	Increased
AtTIR1	Increasing particle size	No trend	Increased	Increased
	Increasing matric potential	No trend	Increased	No trend
AtEIN2	Increasing particle size	No trend	No trend	No trend
	Increasing matric potential	Decreased	Decreased	No trend
AtABI4	Increasing particle size	Increased	No trend	No trend
	Increasing matric potential	Decreased	No trend	No trend
AtIPT5	Increasing particle size	No trend	No trend	No trend
	Increasing matric potential	No trend	Decreased	No trend

5.4. Relating the gene expression and root proliferation responses to nitrate and water availability

When water supply was manipulated via altered particle size at low (0.1 mM) nitrate supply, the decrease in the expression of *AtNAR2.1* (Figure 5.1.C) and *AtPIN2* (Figure 5.4.B) coincided with an increase in PRL (Figure 4.2.A) and a decrease in LRD (Figure 4.2.E). BRL, LRN and TLRL increased at intermediate particle size (Figure 4.2.B-D) and the same response was seen for *AtPIP2.2* expression (Figure 5.1.D). At medium (1.0 mM) nitrate supply, an increase in PRL corresponded to a decrease in LRN, TLRL and LRD (Figure 4.2.) and also to an increase in the expression of *AtNAR2.1* (Figure 5.1.C) and *AtTIR1* (Figure 5.4.C). BRL again increased at intermediate particle size (Figure 4.2.B), which coincided with increased *AtEIN2*, *AtABI4* and *AtIPT5* expression (Figure 5.4.D-F). At high (10.0 mM) nitrate supply, an increase in PRL and BRL coincided with a decrease in LRN and LRD (Figure 4.2), which corresponded to an increase in the expression of *AtNRT2.1*, *AtPIP2.2* (Figure 5.1.B and D) and *AtTIR1* (Figure 5.4.C).

When water supply was manipulated via altered matric potential at low (0.1 mM) nitrate supply, PRL increased with a decrease in BRL, TLRL and LRD (Figure 4.3), which coincided with a decrease in the expression of *AtNRT1.1* (Figure 5.2.A), *AtTIR1*, *AtEIN2* and *AtABI4* (Figure 5.5.C-E). LRD decreased at the intermediate matric potential (Figure 4.3.E) and this coincided with a similar change in *AtPIP2.2* expression (Figure 5.2.D). Under medium (1.0 mM) nitrate supply, an increase in PRL corresponded with a decrease in BRL and LRD (Figure 4.3) and a decrease in the expression of *AtNRT1.1* and *AtNAR2.1* (Figure 5.2.A and C), an increase in *AtNRT2.1* and *AtPIP2.2* expression (Figure 5.2.B and D) and decrease in *AtEIN2* and *AtIPT5* expression (Figure 5.5.D and F). At high (10.0 mM) nitrate supply, an increase in PRL occurred with a decrease in LRN, TLRL and LRD (Figure 4.3), an increase in *AtNRT1.1* and a decrease in *AtNRT2.1* and *AtPIP2.2* (Figure 5.2), and an increase in the expression of *AtPIN2* (Figure 5.5.B).

Therefore, a change in root proliferation parameters appears to be linked with a change in nutrient and hormone associated gene expression responses to specific nutrient supplies. The interaction of these components of nutrient acquisition was further investigated in selected mutant lines at extremes of water availability under high nitrate supply.

5.5. The root proliferation and gene expression responses of selected mutants to substrate hydraulic characteristics under high nitrate supply

The coordination of *AtNRT2.1* and *AtPIP2.2* expression and the apparent opposite responses under high nitrate supply for each manipulation of water supply, the separate hydraulic regulation of the partner genes *AtNRT2.1* and *AtNAR2.1*, and the role of these three genes in controlling root proliferation responses was investigated using selected loss of function mutant lines (see Chapter 2) at extremes of water availability under high (10.0 mM) nitrate supply. The root proliferation and gene expression responses of *atnrt2.1*, *atnar2.1*, *atnar2.1xNpNRT2.1* and *atpip2.2* mutant lines were characterised at extremes of unsaturated hydraulic conductivity (0.18 and 1.35 $m.d^{-1}$) under high (10.0 mM) nitrate supply. The *atnar2.1xNpNRT2.1* mutant line over-expresses *NpNRT2.1* in the *atnar2.1* loss of function mutant but does not restore HATS activity (Orsel et al. 2006). All seedlings were germinated and grown on sand rhizotrons for 7 days. At 12 dpg, root proliferation parameters were measured and seedlings were harvested. RNA was extracted for RT PCR gene expression analysis (see Chapter 2 for details).

The root growth responses to water deficit and the gene expression responses to a range of abiotic stresses of Ws, Col 0 and Ler 1 have been previously shown to be conserved (Des Marais et al. 2012), and may be a reflection of the fact that all three ecotypes are spring accessions collected along the 52° line of latitude. To confirm this idea, the PRL, LRN, TLRL and LRD responses to extremes of unsaturated hydraulic conductivity were compared between Ws and Col 0 ecotypes and all responses were found to be conserved (Appendix 5). As a result, mutant line responses were compared to Arabidopsis Ws responses (Figures 5.6., 5.7. and 5.8.).

The increased PRL response to low unsaturated hydraulic conductivity (0.18 $m.d^{-1}$) was lost in all mutant lines except the *atnar2.1* mutant ($P < 0.001$, d.f. 406; Figure 5.6.A). For the *atnar2.1xNpNRT2.1* line, PRL was significantly longer than wild-type ($P < 0.001$, d.f. 406). As with wild-type, BRL remained unresponsive in all the mutant lines (Figure 5.6.B). The increased LRN and TLRL observed in the wild-type at high unsaturated hydraulic conductivity (1.35 $m.d^{-1}$) was conserved in the *atnrt2.1* mutant ($P < 0.001$, d.f. 406; Figure 5.6.C and D), but lost in the other mutant lines. Finally, the increased LRD observed in the wild-type at high unsaturated hydraulic conductivity (1.35 $m.d^{-1}$) was lost in all the mutant lines (Figure 5.6.E).

Figure 5.6. Root proliferation responses of selected mutant lines at extremes of unsaturated hydraulic conductivity (K_{unsat}) under high (10.0 mM) nitrate supply. *, P < 0.001. D.f. 406. Treatments: 1.35 $m.d^{-1}$; 0.18 $m.d^{-1}$.

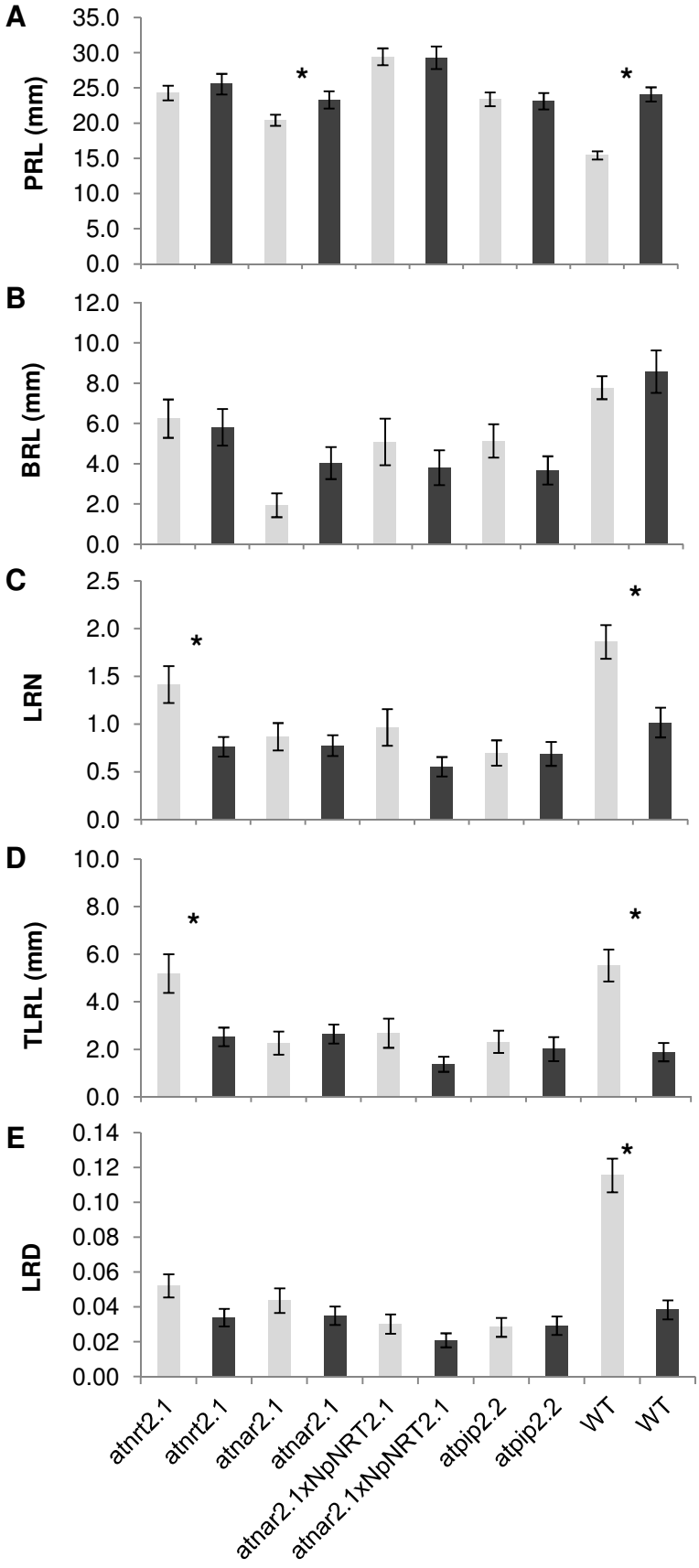


Figure 5.7. Nutrient acquisition gene expression responses of selected mutant lines to the extremes of unsaturated hydraulic conductivity (K_{unsat}) under high (10.0 mM) nitrate supply. Treatments: 1.35 $m.d^{-1}$; 0.18 $m.d^{-1}$. Level of *AtNRT2.1* expression may be underestimated due to saturation.

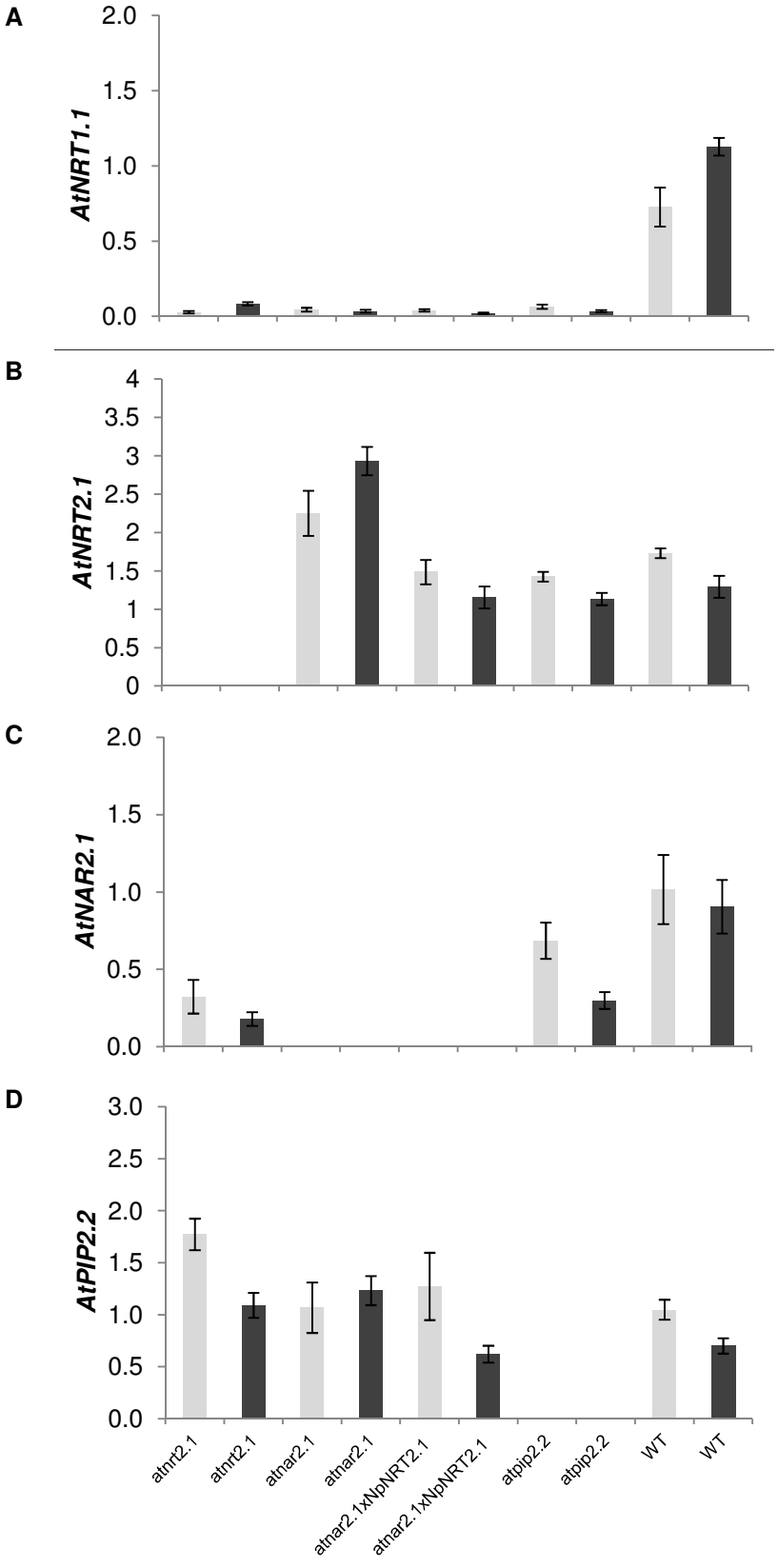
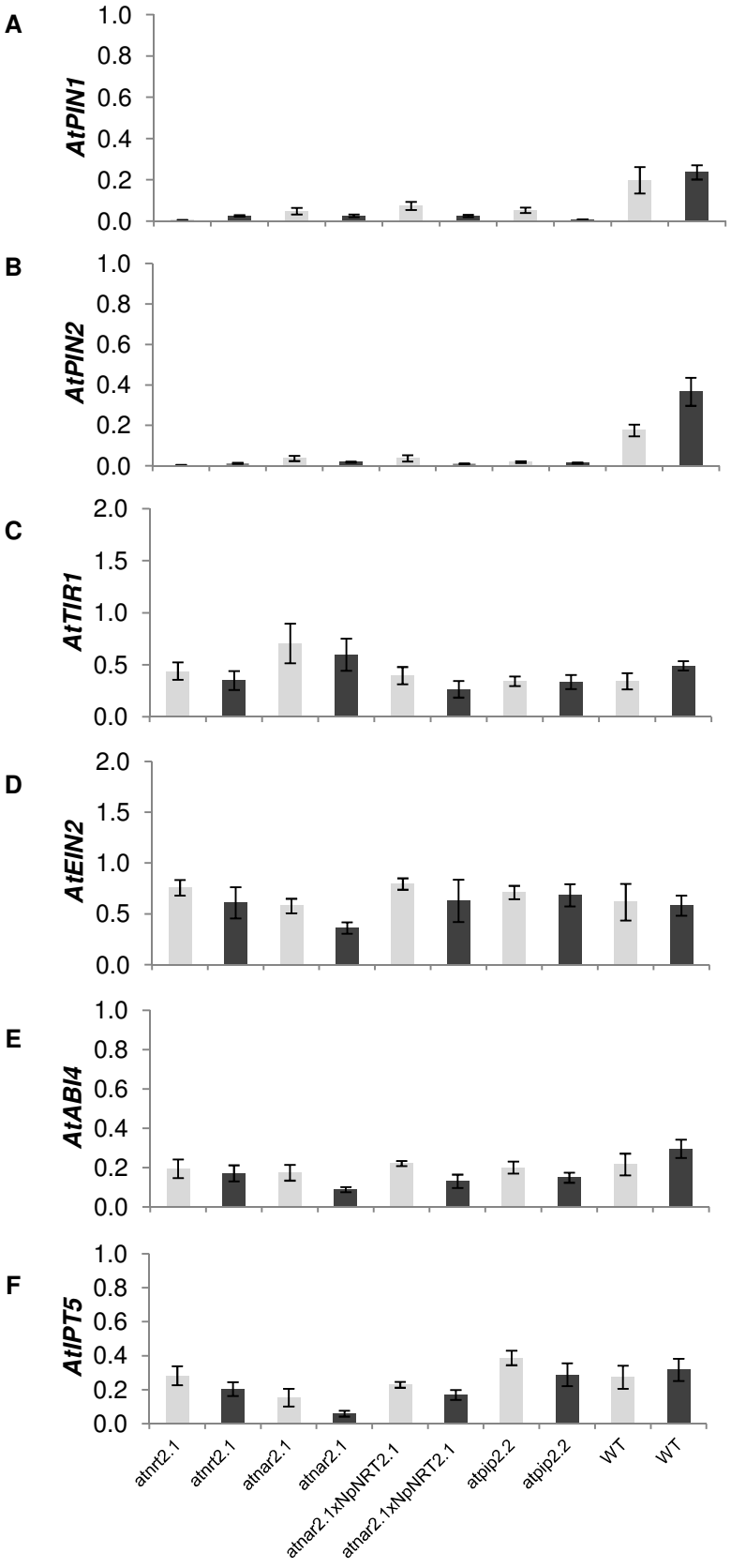


Figure 5.8. Hormone-associated gene expression responses of selected mutant lines to the extremes of unsaturated hydraulic conductivity (K_{unsat}) under high (10.0 mM) nitrate supply. Treatments: 1.35 $m.d^{-1}$; 0.18 $m.d^{-1}$.



The positive response of *AtNRT1.1* expression to decreased water availability was lost in all mutant lines, with expression significantly decreased compared to wild-type in all mutant lines (Figure 5.7.A). The response of *AtNRT2.1* expression was lost in the *atnar2.1* and *atnar2.1xNpNRT2.1* mutant lines and *AtNRT2.1* expression actually increased in the *atnar2.1* mutant compared to wild-type (Figure 5.7.B). In the *atnrt2.1* mutant line, *AtNAR2.1* expression remained unresponsive but expression was decreased relative to wild-type (Figure 5.7.C). Although not responsive to water availability in the wild-type, *AtNAR2.1* expression decreased at decreased water availability in the *atpip2.2* mutant (Figure 5.7.C). Interestingly, the response of *AtPIP2.2* expression was lost in the *atnar2.1* mutant line (Figure 5.7.D).

The loss of PRL, LRN, TLRL and LRD responses to extremes of unsaturated hydraulic conductivity in the *atpip2.2* mutant (Figure 5.6) also coincided with a decrease in *AtNAR2.1* expression at decreased water availability (Figure 5.8.C). Loss of the PRL response to water availability in the *atnrt2.1* mutant coincided with severely decreased *AtNRT1.1* (Figure 5.7.A), *AtPIN1* and *AtPIN2* expression (Figure 5.8.A and B). Altered *AtNRT2.1* expression in the *atnar2.1* and *atnar2.1xNpNRT2.1* mutant lines (Figure 5.7.B) also coincided with the loss of LRD responses within these lines (Figure 5.6.E). The LRN, TLRL and LRD response to water availability under high (10.0 mM) nitrate supply in the *atnrt2.1* mutant was equivalent to wild-type indicating that these responses were independent of *AtNRT2.1* (Figure 5.6.C-E).

The *atnar2.1* mutant was the only line that displayed a change in expression of the hormone signalling genes relative to wild-type expression, whereby expression of *AtEIN2*, *AtABI4* and *AtIPT5* all decreased at decreased water availability (Figure 5.8.D-F). The change in the expression of these genes also coincided with the decreased PRL response, loss of LRN, TLRL and LRD responses (Figure 5.6), a loss of *AtPIP2.2* and *AtNRT2.1* responses (Figure 5.7.B and D) to water availability in the *atnar2.1* mutant line. The mutant line responses are summarised in Table 5.3..

Table 5.3. Summary of mutant root physiology responses to increased unsaturated hydraulic conductivity (K_{unsat}) under high (10.0 mM) nitrate supply.

Parameter	*atnrt2.1*	*atnar2.1*	*atnar2.1xNpNRT2.1*	*atpip2.2*
PRL	Unresponsive	Increased	Unresponsive	Unresponsive
BRL	Unresponsive	Unresponsive	Unresponsive	Unresponsive
LRN	Decreased	Unresponsive	Decreased	Unresponsive
TLRL	Decreased	Unresponsive	Decreased	Unresponsive
LRD	Decreased	Unresponsive	Unresponsive	Unresponsive
AtNRT1.1	Unresponsive	Unresponsive	Unresponsive	Unresponsive
AtNRT2.1	-	Increased	Unresponsive	Decreased
AtNAR2.1	Decreased	-	-	Decreased
AtPIP2.2	Decreased	Decreased	Decreased	-
AtPIN1	Unresponsive	Unresponsive	Unresponsive	Unresponsive
AtPIN2	Unresponsive	Unresponsive	Unresponsive	Unresponsive
AtTIR1	Unresponsive	Unresponsive	Unresponsive	Unresponsive
AtEIN2	Unresponsive	Decreased	Unresponsive	Unresponsive
AtABI4	Unresponsive	Decreased	Unresponsive	Unresponsive
AtIPT5	Unresponsive	Decreased	Unresponsive	Unresponsive

5.6. Comparing the gene expression and root proliferation responses of selected mutant lines

The role of selected genes in controlling root proliferation responses to unsaturated hydraulic conductivity under high (10.0 mM) nitrate supply was investigated using selected homozygous loss of function mutants. The expression of *AtNRT1.1*, *AtPIN1* and *AtPIN2* was decreased in all mutant lines relative to wild-type and coincided with altered PRL and LR responses to water availability under high (10.0 mM) nitrate supply. Therefore, altered expression of these genes may be responsible for altered root proliferation responses observed in mutant lines grown in the sand rhizotron system.

In the *atnar2.1* mutant, wild-type *AtNRT2.1* and *AtPIP2.2* expression responses were disrupted, *AtEIN2*, *AtABI4* and *AtIPT5* expression became responsive and coincided with a decrease in the PRL response and a loss of LRN, TLRL and LRD responses to water availability. *AtIPT5* has an important role in root cytokinin biosynthesis which can induce root growth (Takei et al. 2004; Kushwah et al. 2011) and the expression of *AtEIN2*, *AtABI4* and *AtIPT5* has been linked with the nitrate regulation of LR growth. The pathway that determines cytokinin-induced root growth acts on *AtABI4* and *AtEIN2*. Therefore, the level of *AtNAR2.1* expression could play a role in the hormonal regulation of the root proliferation and nutrient gene expression responses to water availability. All LR responses to water availability were lost in the *atnar2.1* and the LRD response was also lost in the *atnar2.1xNpNRT2.1* lines, supporting previous work (Orsel et al. 2006) demonstrating that it is the *AtNAR2.1* gene product which is important in determining LR growth responses to nitrate supply.

The *atnrt2.1, atnar2.1* and *atnar2.1xNpNRT2.1* mutant lines lack AtNRT2.1 at the plasma-membrane and are impaired in high affinity nitrate uptake (Filleur et al. 2001; Orsel et al. 2006; Wirth et al. 2007) but these are unlikely to be governing the PRL response to water availability because differences in the PRL response occur between the *atnrt2.1*, *atnar2.1* and *atnar2.1xNpNRT2.1* mutant lines. However, it does indicate that AtNRT2.1 must be plasma-membrane located to demonstrate wild-type hydraulic regulation. In addition, when *NpNRT2.1* is overexpressed wild-type uptake capacity is not restored (Orsel et al. 2006), but the hormone responses observed in the *atnar2.1* mutant are no longer observed suggesting that *NRT2.1*

expression in the cell but not at the plasma-membrane can interfere with the *AtNAR2.1*-hormone interactions.

The *atnar2.1* mutant line was the only mutant line that displayed a change in expression of nutritionally regulated hormone genes relative to wild-type (Figure 5.10.). This change in hormone gene expression with unsaturated hydraulic conductivity corresponded to the reduction in the PRL response and a loss of the LRN, TLRL and LRD responses (Figure 5.8.). These results indicate that *AtNAR2.1* is required for the wild type root response to unsaturated hydraulic conductivity at high (10.0 mM) nitrate supply and this is likely to be related to the constraint of hormone gene expression.

Interestingly, the hormone response demonstrated by the *atnar2.1* mutant line is similar to that for wild-type seedlings subjected to the same extremes of unsaturated hydraulic conductivity (0.18 and 1.35 $m.d^{-1}$) at low (0.1 mM) and medium (1.0 mM) nitrate supply. For the wild-type under low (0.1 mM) nitrate supply, the expression of *AtEIN2* and *AtABI4* decreased with decreasing water availability, whilst under medium (1.0 mM) nitrate supply, the expression of *AtEIN2* and *AtIPT5* decreased with decreasing water availability. Under high (10.0 mM) nitrate supply, the expression of these hormone genes was unresponsive to water availability. Therefore, it would seem that *AtNAR2.1* may have a nitrate-dependent role in regulating root physiological responses to nutrient availability.

5.7. Summary and conclusions

The expression of key nitrate and water acquisition genes was determined by specific water and nitrate availabilities within the sand rhizotron system.

Similar *AtNRT2.1* and *AtPIP2.2* expression was observed for all treatments, including the apparent opposite regulation at high (10.0 mM) nitrate supply seen for the two different manipulations of water supply. Therefore, the expression of these genes appears to be regulated by independent hydraulic parameters and also nitrate dependent.

AtNRT2.1 expression has been shown to be induced by nitrate supply in the short term and decreased in response to high external nitrate supply and downstream nitrogen metabolites (Filleur and Daniel-Vedele 1999; Lejay et al. 1999; Zhuo et al. 1999; Gansel et al. 2001). Therefore, it is likely that *AtNRT2.1* expression is responding to altered delivery of dissolved nitrate ions to the root surface or a direct response of *AtNRT2.1* to water availability. The hydraulic regulation of *AtNRT2.1* expression is a novel observation.

AtPIP2.2 expression is responsive to water availability (Alexandersson et al. 2005) and the expression of *AtNRT2.1* and *AtPIP2.2* was positively coordinated across all treatments. All root proliferation responses to water availability were lost but the *AtNRT2.1* expression response to water availability was conserved in the *atpip2.2* mutant. Furthermore, *AtNAR2.1* expression actually became responsive to water availability (decreasing at low unsaturated hydraulic conductivity) in the *atpip2.2* mutant and *AtNRT1.1* expression was decreased relative to wild-type. This indicates that *AtPIP2.2* is required for wild-type root proliferation and *AtNAR2.1* expression responses to water availability under high (10.0 mM) nitrate supply, which is a novel observation.

AtNRT1.1 is known to interact with auxin in the regulation of PR and LR growth in response to nitrate supply (Remans et al. 2006a; Walch-Liu and Forde 2008). The expression of *AtNRT1.1*, *AtPIN1* and *AtPIN2* was decreased in all mutant lines relative to wild-type and coincided with altered PRL and LR responses to water availability under high (10.0 mM) nitrate supply. Therefore, the decreased expression of these genes may also be responsible for the loss of certain root proliferation responses in the mutant lines.

The LR growth responses to water availability were lost in the *atpip2.2* mutant, although the expression of *AtNAR2.1* was also disrupted in this mutant line. Therefore, the loss of root proliferation responses in the *atpip2.2* mutant could be an indirect result of altered *AtNAR2.1* expression. Wild-type *AtNAR2.1* expression was important for the LR response to water availability and the *atnar2.1* mutant line was the only mutant line that displayed a change in expression of hormone-associated genes known to determine nitrogen-regulation of root proliferation.

In the *atnar2.1* mutant, the expression of *AtEIN2*, *AtABI4* and *AtIPT5* became decreased relative to the wild-type under low water availability and this was associated with a loss of LR

growth responses to water availability. These hormone-associated genes have important roles in regulating LR growth responses to nitrogen supply (Signora et al. 2001; Tian et al. 2009; Shkolnik-Inbar and Bar-Zvi 2010) and their expression altered similarly across several nutrient regimes. In addition, *AtIPT5* expression also became responsive to water availability in the *atnar2.1* mutant and associated with a reduction in the PRL response. *AtIPT5* is important for root cytokinin biosynthesis (Takei et al. 2004) which can induce root growth via a pathway that interacts with *AtABI4* and *AtEIN2* (Kushwah et al. 2011), which were altered in the *atnar2.1* mutant. Therefore, at high (10.0 mM) nitrate supply, *AtNAR2.1* is important for the root proliferation responses to water availability. This function appears to be independent of *AtNRT2.1* and could be dependent on specific hormone-associated gene expression that has been previously described to regulate root growth.

Taken together, these data demonstrate the novel findings that:

- The expression of the high affinity nitrate transporter *AtNRT2.1* and the aquaporin *AtPIP2.2* responsible for root water uptake and osmotic fluid transport were coordinated across treatments;
- Under high (10.0 mM) nitrate supply, *AtNRT2.1* and *AtPIP2.2* expression was oppositely regulated by the different manipulations of water supply;
- Although usually found to closely follow each other, *AtNAR2.1* and *AtNRT2.1* expression demonstrated separate responses to water and nitrate availability;
- Certain wild-type root proliferation responses to water availability under high (10.0 mM) nitrate supply were lost in the *atnar2.1* mutant line and this coincided with a change in hormone gene expression;
- *AtPIP2.2* was required for wild-type root proliferation and *AtNAR2.1* expression responses to water availability under high (10.0 mM) nitrate supply.

The novel findings and conclusions of Chapters 3, 4 and 5 will now be discussed in relation to the wider literature (Chapter 6).

6. General Discussion

The physical properties of the growth substrate (agar culture compared to the sand rhizotron system) were found in this thesis to influence Arabidopsis (*Arabidopsis thaliana*) root proliferation responses to nutrient supply (Chapter 3). Within the sand rhizotron system, manipulation of hydraulic properties had large effects on root proliferation (Chapter 4). The expression of nitrate and water transporter genes was regulated by hydraulic properties of the substrate that determined nutrient availability and specific interactions were observed with hormone signalling genes (Chapter 5). In this Chapter, the interactions between these responses are defined using simplified regression models and are explored in the context of the sand rhizotron system and other published work.

6.1. Why develop the sand rhizotron system?

Previous research has shown that the physical characteristics of the substrate can determine the availability of water and dissolved nutrient ions to the root (Nye and Tinker 1977; Tinker and Nye 2000; Miller and Cramer 2005). Root physiology is significantly affected by substrate physical properties such as mechanical impedance (Eavis 1972; Young et al. 1997; Bingham and Bengough 2003; Whalley et al. 2004a; Bengough et al. 2006; Bingham et al. 2010), soil strength (Masle and Passioura 1987; Bengough 1997; Kirby and Bengough 2002; Clark et al. 2008a; Whalley et al. 2008), aggregate size (Braunack and Dexter 1989; Murungu et al. 2003), pore size (Matthews et al. 2010), water potential (Sharp et al. 1988; Verslues et al. 1998; Whalley et al. 1998; van der Weele et al. 2000; Roycewicz and Malamy 2012) and hydraulic conductivity (Whalley et al. 2004b; Matthews et al. 2010; Chapman et al. 2011).

The relationship between these characteristics is complex and interdependent. In porous substrates, particle size determines the connectivity of pores which affects the ease with which water can move through the substrate (i.e. the substrate hydraulic conductivity). The ease with which water moves through the substrate is also determined by the water potential and specifically the matric potential component which defines the strength with which water is held between particles. Furthermore, these soil hydraulic characteristics are especially important for the highly mobile nitrate ion and can determine root proliferation responses to the delivery of

water and dissolved nitrate ions by mass flow and diffusion (Nye and Tinker 1977; Tinker and Nye 2000; Miller and Cramer 2005).

Simplified laboratory experimental systems, such as agar Petri dishes and hydroponics, have generated a great deal of fundamental understanding regarding the root physiological component of nutrient acquisition, but these systems may be of limited use for our understanding of the importance of soil physical characteristics (Zhu et al. 2011; Chapman et al. 2012). Therefore, it may be unsurprising that disparities occur when experiments are compared across different experimental system.

Attempts have been made to investigate the influence of water potential on root growth within the agar culture system. In these studies, water potential is manipulated by an increase in substrate concentration or the introduction of an osmoticum such as polyethylene glycol (Clark et al. 1998; Verslues et al. 1998; van der Weele et al. 2000). The strength of agar increases as water content decreases but the responses of root growth to the water content of agar were not explained by its mechanical strength (Clark et al. 1998). This emphasizes the difficulty in understanding the complexity of root growth environments, even in simplified laboratory systems such as the agar Petri dish.

In studies on a selection of barley sub-species, root proliferation data were inconsistent when roots were compared between gel and soil culture methods (Hargreaves et al. 2009). Total root lengths and average root diameters of seedlings grown in soil were decreased relative to gel-grown seedlings. A similar disparity between soil and gel methods was described for root characteristics in dwarf wheat cultivars (Wojciechowski et al. 2009). Again, total root length of dwarf lines was decreased in soil, by between 24-33%. These two examples found that results were confirmed in different soil culture methods, but that these differed from the results obtained using gel culture. These disparities highlight the challenge of translating observations from simplified experimental systems to the complex root growth environment within soil.

Disparities do not only arise when comparing soil to gel culture, but also when comparing porous experimental systems to the field. The use of vermiculite to replicate low water potential in a seed-bed demonstrated that the shoot emerged even at water potential of -1.64 MPa provided the seed germinated (Liang et al. 1997), but a strong soil at relatively high water potentials has been shown to prevent shoot elongation (Whalley et al. 1999). In other work,

good root penetration was identified in rice cultivars after screening with a flooded wax layer system or a sand-core system, but these cultivars did not always exhibit good hard-pan penetration in the field (Clark et al. 2002). This was speculated to be caused by an improved root penetration response to a gradual increase in soil strength in the field which was not replicated in the simplified laboratory screening systems (Dennis et al. 2008). This further highlights the complexity of the root growth environment in the field (Mittler 2006), where soil physical properties and nutrient and water availability are often difficult to quantify as more than one aspect changes almost concurrently.

Simplified experimental systems are useful for high-throughput screening and identifying fundamental responses and it is likely that a greater understanding of root nutrient acquisition will require investigation using a combination of systems (De Smet et al. 2012). However, the physical characteristics of experimental systems are important factors that influence root nutrient acquisition responses and the limitations of each system must be considered in the interpretation of results.

It was within this context that the aim was set to develop an experimental system to study both the plant physiological and soil physical components of root nutrient acquisition. Central to this aim was the importance of using a porous growth substrate, so that hydraulic characteristics could be manipulated and thus the delivery of water and dissolved nutrients could be altered in a way that may more closely represent the field situation. To investigate only the effects of manipulation of water and nitrate supply, it was also necessary to remove the external influence on root growth of anything other than nutrient and water availability. Furthermore, by developing the system for use in combination with the model experimental plant Arabidopsis, the potential exploitation of a superior molecular tool-kit that includes well established gene expression analyses and a large number of mutant and reporter lines was facilitated. Moreover, this enabled results to be considered within the context of the advanced root physiological understanding held for this model plant (van Norman and Benfey 2009; Benfey et al. 2010).

6.2. How is nutrient availability manipulated within the sand rhizotron system?

To understand the root physiological responses to water and nitrate supply in the sand rhizotron system, it is important to consider how their availability is manipulated. In the sand rhizotron

system, several steady-state conditions were explored where Arabidopsis seedlings were grown in water-saturated environments under a range of nitrate concentrations. As young Arabidopsis seedlings were investigated in a covered system, little transpiration occurs (Christman et al. 2008) and water potential at the surface of the root was likely to be constant. As a result, the flux of water towards the root was regulated by the difference in water potential between the root and the sand, as well as the hydraulic conductivity of the sand (Lang and Gardner 1970). Relative to decreased unsaturated hydraulic conductivity and more negative water potential, a greater connectivity of solution occurs at increased unsaturated hydraulic conductivity and less negative water potential (Chapter 3, Table 3.2., p71), and thus the availability of water and dissolved ions to the root is increased under these conditions.

Adjusting matric potential within a narrow range had only a small influence on the change in water potential but a large effect on hydraulic conductivity. In addition, water potential was mainly determined by the osmotic potential component which was altered by changing the nitrate concentration in the nutrient solution. Therefore, sand hydraulic conductivity and nitrate concentration were the principal treatments used to adjust the flux of water to the root in the sand rhizotron system. The increase in nitrate supply not only manipulates water flux but is also likely to have a direct nitrate nutritional effect on some root physiological parameters that respond to nitrate itself (e.g. *AtNRT* expression).

The sand used in this work can drain to residual water content at decreased matric potential. In this situation, water potential will no longer be controlled by the tension height (Chapter 3, Figure 3.1.B, *h*) or water table (Whalley et al. 2011), but instead by isolated water structures that form in drying sand (Bird et al. 2005). Under these conditions, the availability of water and nitrate to the root is likely to be less predictable due to the stochastic nature of encounters between the roots and any isolated pockets of water that may form.

Therefore, the hydraulic characteristics that determine the availability of water and nitrate to the root within the sand rhizotron system are likely to be very different to that encountered in the most widely used experimental system, the agar Petri dish. This is largely due to the difference in physical properties between the two growth substrates that determine the hydraulic conductivity and the relative contribution of matric and osmotic components to total water

potential. To investigate the impact of these differences, the root proliferation and gene expression components of root nutrient acquisition were directly compared.

6.3. A direct comparison of the sand rhizotron system to agar culture

Comparing the sand rhizotron system with agar culture identified some important differences and similarities in the response of root physiological characteristics (Chapter 3). For example, the PRL of sand-grown seedlings was significantly longer than that of agar-grown plants and *AtNAR2.1*, *AtABI4* and *AtIPT5* expression was increased for agar-grown seedlings relative to sand-grown seedlings. However, the relative response of individual characteristics to nutrient supply was conserved between systems and no relative change in expression was detected between seedlings grown in agar or sand for *AtPIN1*, *AtPIN2*, *AtTIR1* or *AtEIN2*.

Therefore, several of the overall nutrient responses observed in agar culture were detected using the sand rhizotron system, but there were some important differences. These were speculated to be driven by disparities in hydraulic characteristics and the volume of nutrients supplied. It was concluded that the consideration of the root physiology responses in a porous substrate should identify root responses to water and nitrate that were previously identified in simplified experimental systems such as the agar Petri dish. Additionally, there may be the potential to identify important responses that are not observed in agar culture due to the influence of the physical properties of the porous root growth environment on water and nitrate availability.

6.4. Comparing the sand rhizotron system root data with previous work using other experimental systems

Up to this point, the rationale for developing the sand rhizotron system has been described. Examples of disparities in results between porous and non-porous systems, including a direct comparison between the sand rhizotron system and agar culture, have been presented to illustrate the limitations of some simplified experimental systems in considering the soil physical component of root nutrient acquisition. In this section, the influence of water and nitrate

availability on root proliferation and gene expression in the sand rhizotron system is explored and compared with other published studies that used a range of experimental systems.

6.4.1. The influence of water and nitrate availability on root proliferation

PRL has been shown to increase at small negative water potentials in agar (van der Weele et al. 2000) and soil (Taylor and Ratliff 1969) systems, and under large negative water potentials in vermiculite (Sharp et al. 1988). PRL was also correlated with water potential in the sand rhizotron system. However, sand hydraulic conductivity determined PRL more strongly than water potential in the sand rhizotron system. This may be explained by the fact that unsaturated hydraulic conductivity changes with water potential and the sand rhizotron system serves mainly to manipulate unsaturated hydraulic conductivity. In addition, this hydraulic regulation of PRL was seen under each nitrate supply, which is in agreement with previous agar studies that demonstrated PRL to be unresponsive to nitrate concentration in (Forde and Zhang 1998; Walch-Liu and Forde 2008).

In finding that water availability regulates PRL at each nitrate supply, the sand rhizotron system supports previous studies undertaken in a range of growth substrates that report a lack of PRL regulation by nitrate. However, a stronger influence of sand hydraulic conductivity than water potential on PRL is reported in the sand rhizotron system. This may not have been identified in non-porous growth substrates such as agar or hydroponics because hydraulic conductivity is dependent on pore distribution and connectivity. Although PRL has been described to be determined by hormones in agar (Blilou et al. 2005; Ruzicka et al. 2007), hydroponic (Liu et al. 2010), vermiculite (Spollen et al. 2000; Leach et al. 2011) and soil (Santisree et al. 2011) studies, these were not found to be the main regulators of PRL in the sand rhizotron system.

BRL increased with increasing nitrate concentration, but increased with decreasing water flux at high (10.0 mM) nitrate supply. In addition, a change in BRL was often associated with a similar change in *AtABI4* expression. *AtABI4* expression has been shown in agar studies to regulate LR growth in a nitrate-dependent manner (Signora et al. 2001; Shkolnik-Inbar and Bar-Zvi 2010) and here it seems to similarly associate with BRL changes. *AtABI4* has been shown on agar to act up-stream of important auxin and ethylene genes to determine root growth (Kushwah et al. 2011) and the basal root growth of common bean (*Phaseolus vulgaris*) has been shown to be

determined by auxin and ethylene in a hydroponic-style germination pouch system (Basu et al. 2011).

Due to limited reports of BR growth in agar and hydroponic studies, it is difficult to consider the BRL data in the context of previous work. However, BRL seems to respond in a similar fashion to that reported for LR proliferation. The positive regulation of BRL by *AtABI4* expression in the sand rhizotron system could be similar to that reported for the hormonal regulation of BR growth in common bean or the nitrate-dependent *AtABI4* regulation of LR growth. Similar regulation of BRL and TLRL could enable the plant to coordinate an increase in the total size and exploratory capacity of the root system.

The proliferation responses of BRL and TLRL could be coordinated as an adaptive strategy to increase root system size. Nitrate patches that may be formed under conditions of decreased water availability could be of sufficient concentration to induce the BRL proliferation response to increased nitrate supply, perhaps in a similar fashion to high nitrate regulation of TLRL classically reported in sand (Drew and Saker 1975) and agar (Zhang et al. 1999; Malamy and Ryan 2001). In the same way that LR proliferation has been studied in these systems, basal root proliferation could be investigated in the sand rhizotron following the introduction of patches of increased nitrate concentration in proximity to established basal roots growing under decreased water availability.

Previous work using the agar Petri dish system reported that LRN was responsive to water availability (Deak and Malamy 2005; Roycewicz and Malamy 2012). In support of this, both manipulations of water availability influence LRN in the sand rhizotron system. The importance of auxin transport in specifying LR growth has been identified in agar (Casimiro et al. 2001; Bao et al. 2007; Dubrovsky et al. 2011), but no interaction with *AtPIN1* or *AtPIN2* was observed in the sand rhizotron system.

Agar studies have previously identified the osmotic regulation of TLRL (Deak and Malamy 2005; Roycewicz and Malamy 2012). In the sand rhizotron system the regulation of TLRL by water potential is reported, but water potential in the sand rhizotron system is mostly determined by the osmotic component. Therefore, the regulation of TLRL by water potential in the sand rhizotron system confirms the identification of osmotic regulation of TLRL reported in previous agar studies.

LRD was negatively regulated by water potential independent of nitrate input and LRD has been shown to be negatively regulated by increased osmotic potential independent of nitrate in agar systems (Deak and Malamy 2005; Roycewicz and Malamy 2012). The water potential within the sand rhizotron system is mainly determined by the osmotic potential of the nutrient solution and increases with increasing nitrate concentration. Therefore, the regulation of LRD by water potential may be viewed as a response to the osmotic component. As with previous studies using agar (van der Weele et al. 2000; Deak and Malamy 2005; Roycewicz and Malamy 2012), LRD responded to water potential in a similar manner as LRN and TLRL, but the relationship with water availability was stronger for LRD in the sand rhizotron system.

LRD has been previously shown to be regulated by auxin in agar systems (Casimiro et al. 2001; Bao et al. 2007; Dubrovsky et al. 2008; Ivanchenko et al. 2008), but no interactions with the key auxin associated genes were identified in the sand rhizotron system. However, LRD responded to water potential in a similar manner to *AtABI4*, *AtEIN2* and *AtIPT5* at low (0.1) or medium (1.0 mM) nitrate supply. *AtABI4* acts upstream of key auxin associated genes to determine root proliferation (Kushwah et al. 2011). *AtABI4*, *AtEIN2* and *AtIPT5* have been reported in agar studies to regulate LR growth by disrupting auxin transport (Signora et al. 2001; Shkolnik-Inbar and Bar-Zvi 2010). Thus, an interaction between LR growth and certain hormone associated genes is supported by previous work in agar.

6.4.2. The influence of water and nitrate availability on root gene expression

The repression of *AtNRT2.1* at high nitrate concentrations has been demonstrated to be mediated by *AtNRT1.1* in agar and hydroponic systems (Munos et al. 2004; Krouk et al. 2006). Consistent with this previous work, the decrease in overall expression of *AtNRT2.1* at high (10.0 mM) coincided with an increase in *AtNRT1.1* expression, while decreased *AtNRT1.1* expression at low (0.1 mM) and medium (1.0 mM) nitrate supply corresponded to increased overall *AtNRT2.1* expression.

AtNRT1.1 expression has been shown to be regulated by nitrate and auxin in agar and hydroponic systems (Guo et al. 2002; Okamoto et al. 2003; Wang et al. 2004; Remans et al. 2006a; Bao et al. 2011). Under high (10.0 mM) nitrate supply, the expression of *AtNRT1.1* appeared to show a similar patter to that of *AtPIN1* and *AtPIN2* in the sand rhizotron system.

AtNRT1.1 has been shown to regulate the expression of *AtNRT2.1* which requires *AtNAR2.1* for wild-type uptake function (Okamoto et al. 2003; Munos et al. 2004; Orsel et al. 2004; Krouk et al. 2006; Orsel et al. 2006; Wirth et al. 2007). In the sand rhizotron system, the response of *AtNRT1.1* and *AtNAR2.1* were similar under several treatments, suggesting that the regulation of *AtNRT2.1* previously reported may be mediated by *AtNAR2.1*.

Agar and hydroponic studies have demonstrated that the expression of *AtNRT2.1* is induced by nitrate and repressed by high nitrate supply (Nazoa et al. 2003; Okamoto et al. 2003; Orsel et al. 2004; Wang et al. 2004; Alboresi et al. 2005; Little et al. 2005; Remans et al. 2006b; Krouk et al. 2010a; Bao et al. 2011). Consistent with these studies, *AtNRT2.1* expression was increased at low (0.1 mM) and medium (1.0 mM) nitrate supply in the sand rhizotron system when water supply was manipulated by particle size.

AtNAR2.1 is required for wild-type AtNRT2.1 function and *AtNAR2.1* expression has been shown to closely follow *AtNRT2.1* expression in agar and hydroponic studies (Nazoa et al. 2003; Orsel et al. 2004; Little et al. 2005; Krouk et al. 2006; Orsel et al. 2006; Remans et al. 2006b; Girin et al. 2007; Bao et al. 2011). Although it is widely reported from agar and hydroponic studies that the expression of *AtNRT2.1* and *AtNAR2.1* closely follow each other, the response of the genes to nutrient supply in the sand rhizotron system was often distinct.

In fact, the expression of *AtNRT2.1* and *AtPIP2.2* was coordinated across treatments. A similar response of *AtNRT2.1* and *AtPIP2.2* expression across all treatments may indicate a coordination of nitrate and water uptake and/or sensing systems in response to altered water availability. Previous studies have not identified an interaction between *AtNRT2.1* and *AtPIP2.2* or *AtNRT2.1* and water availability. This could be due to differences in the pore structure between the sand rhizotron system and agar or hydroponics that determine water and nitrate availability; responses to altered connectivity of solution are unlikely to be identified in non-porous growth media such as agar or liquid culture.

Interestingly, no regulation of LR growth by *AtNRT2.1* expression was identified in the sand rhizotron system, despite this being reported in agar culture (Little et al. 2005; Remans et al. 2006b) and LR growth remained unaltered in the atnrt2.1 mutant in the sand rhizotron system. However, the wild-type LR response was perturbed in *atnar2.1* and *atpip2.2* mutants, and this was associated with altered expression of *AtEIN2*, *AtABI4* and *AtIPT5* in the *atnar2.1* mutant.

The ethylene signal transducer *AtEIN2* has been shown to control LR growth in response to nitrogen supply on agar plates (Tian et al. 2009). *AtNAR2.1* was required for the wild-type response of *AtEIN2*, *AtABI4* and *AtIPT5* expression at low water availability under high (10.0 mM) nitrate supply which coincided with a loss of LR growth responses to water availability. Interestingly, the investigation of *atnar2.1* mutants in agar and hydroponic systems previously identified a LR phenotype under high (10.0 mM) nitrate supply and the *AtNAR2.1* gene product was speculated to be more important for LR responses than *AtNRT2.1* (Orsel et al. 2006). Thus, in the sand rhizotron system, *AtNAR2.1* appears to have an *AtNRT2.1*-independent role in facilitating root proliferation responses to water availability under high (10.0 mM) nitrate supply, which may be mediated by hormone associated gene expression.

AtPIP2.2 expression has been shown to be responsive to water availability in hydroponically grown seedlings (Javot et al. 2003; Alexandersson et al. 2005; Da Ines et al. 2010) and *AtPIP2.2* was potentially required upstream of *AtNAR2.1* for wild-type root proliferation and hormone signalling responses to water availability under high (10.0 mM) nitrate supply in the sand rhizotron system. A specialized role for *AtPIP2.2* in osmotic root water uptake was previously reported in hydroponic culture (Javot et al. 2003) and it is possible that the same is true for the sand rhizotron system.

Although there is no evidence in the literature for auxin-regulation of *AtPIP2s*, this has been reported in rubber trees (Tungngoen et al. 2011) and a loss of root proliferation responses in the *atpip2.2* mutant coincided with decreased *AtPIN1* and *AtPIN2* expression. AtPIN1 plays an important role in auxin transport to the root tip that determines auxin-dependent directional root growth (Blilou et al. 2005). Therefore, this aquaporin could have a role in the hormonal regulation of root proliferation responses to altered water availability.

Nitrate-dependent regulation of *AtPIP2.2* has not been previously reported, but the expression of AtPIP2.2 in the sand rhizotron system was regulated by water availability differently at different nitrate concentrations. Water channels have been previously shown to be regulated by nitrate (Wang et al. 2001). However, this aquaporin is responsive to water availability (Alexandersson et al. 2005; Alexandersson et al. 2010) and has been demonstrated to facilitate osmotic root water uptake (Javot et al. 2003). Water potential in our sand rhizotron system is mainly determined by the osmotic potential of the nutrient solution. Therefore, it is more likely

that the expression of *AtPIP2.2* is responding to an altered osmotic potential as determined by nitrate concentration rather than a direct response to nitrate.

6.4.3. Summary

The investigation of root physiological responses to altered water and nitrate availability as manipulated in the sand rhizotron system has generated several results that are consistent with simplified experimental systems such as agar and hydroponics. For example, the results support previous agar studies that found PRL and LRD to be mainly regulated by water supply rather than nitrate concentration. Responses that are conserved between systems are important for the validation of the sand rhizotron system as another useful experimental tool for the investigation of root nutrient acquisition.

However, the use of the sand rhizotron system also identified some novel findings. For example, *AtNRT2.1* and *AtPIP2.2* expression was responsive to soil hydraulic properties and their expression was coordinated across treatments. A new role was identified for *AtNAR2.1*, independent of *AtNRT2.1*, in the regulation of LR responses to altered water availability under high (10.0 mM) nitrate supply. This was regulated at the transcript level and probably mediated by the hormone-associated genes *AtEIN2* and/or *AtABI4*. The sand rhizotron data also suggest that *AtPIP2.2* may act upstream to coordinate acquisition responses to altered water and nitrate availability.

The majority of these novel results were responses to altered water availability as manipulated by altered sand hydraulic conductivity or matric potential. These findings may not have been identified in experimental systems (such as agar or hydroponic culture) that do not utilise a porous growth substrate, as the substrate physical properties that determine water and nitrate availability are very different in these systems compared to the sand rhizotron system (Chapman et al. 2012). The use of the sand rhizotron system also enabled root physiological responses to be considered within an added layer of information regarding the physical properties that determine water and nitrate availability.

6.5. A proposed coordination of root water and nitrate responses to altered hydraulic properties

The consideration of the water and nitrate nutrient acquisition responses to altered availability within the context of an extra layer of understanding relating to the sand physical properties that govern their availability enabled a conceptual model to be proposed for the coordination of the nitrate and water root acquisition. Taken together, the data indicate that there could be a coordination of root proliferation and gene expression in response to altered water or nitrate availability.

PRL decreased, and LRD and LRN increased, under relatively high water availability conditions presumably to exploit good water availability (Mommer et al. 2012). Conversely, LRD and LRN is decreased, and PRL increased, under relatively low water availability probably to forage for better water availability down the soil profile (Hodge 2009). As water availability decreases, nitrate distribution may become more heterogeneous, with TLRL increasing to capture localized patches of high nitrate (Robinson et al. 1999). This competitive advantage may be enhanced by increased BRL under increased nitrate supply.

A decrease in water availability (i.e. under low unsaturated hydraulic conductivity or more negative water potential) increased the heterogeneity of nitrate supply within the root growth environment and regulated expression of *AtNRT2.1* and *AtPIP2.2*. This may be either to maintain acquisition of these growth-limiting nutrients or as a sensory response to determine downstream root proliferation responses. This is not seen at low water availability under high (10.0 mM) nitrate supply because when patches are encountered the nitrate concentration is sufficiently high to enable the repression response. Consequently, *AtNRT2.1* and *AtPIP2.2* expression becomes tightly regulated by water flux over a much narrower expression range.

Under high (10.0 mM) nitrate supply, the root proliferation response to low water availability requires *AtNAR2.1* and may be mediated by the expression of *AtEIN2* and/or *AtABI4*. An increase in ethylene signalling (*AtEIN2*) and the reduced expression of auxin-related transcriptional repressors (*AtTIR1*) positively determines cytokinin biosynthesis (*AtIPT5*) which inhibits LR growth in response to high nitrate. The lack of regulation of *AtNAR2.1* expression by increased water availability under high (10.0 mM) nitrate supply may require *AtPIP2.2* upstream. This, in addition to the fact that *AtPIP2.2* expression is responsive to altered water

availability and may overlap with auxin transport, suggests that this aquaporin could play a significant role in sensing, or facilitating downstream signalling responses to, altered water availability and thus determine downstream root physiological responses to altered water and/or nitrate availability.

Whilst this conceptual model is simplified, it provides a framework to understand how the root proliferation and gene expression components of water and nitrate acquisition may be coordinated in response to altered soil hydraulic properties. It also helps to identify testable elements for future lines of enquiry.

6.6. Why might water and nitrate transporter expression be coordinated?

The regulation of aquaporin expression provides a degree of control in the uptake of water into the root and could help to maintain the mass flow of water (and dissolved solutes such as nitrate) to the root when transpiration is low (Nye and Tinker 1977; Jungk 1996; Tinker and Nye 2000; Javot and Maurel 2002; Maurel et al. 2008). *AtPIP2.2* has been shown to predominantly facilitate root water uptake under conditions of reduced transpiration to enable osmotic transport within the root (Javot et al. 2003) and its expression is sensitive to water supply (Jang et al. 2004; Alexandersson et al. 2005; Alexandersson et al. 2010).

At the root surface, transporter-dependent ion uptake may create localised depletion and a gradient along which nitrate ions can diffuse towards the root (Nye and Tinker 1977; Tinker and Nye 2000). AtNRT2.1 achieves high-affinity root nitrate uptake and *AtNRT2.1* expression is repressed by high external nitrate concentrations (Lejay et al. 1999; Zhuo et al. 1999; Cerezo et al. 2001; Gansel et al. 2001; Orsel et al. 2002b; Nazoa et al. 2003; Orsel et al. 2004; Bao et al. 2011). This function requires AtNAR2.1 and *AtNAR2.1* expression normally closely follows that of *AtNRT2.1* in response to nitrate supply (Okamoto et al. 2006; Orsel et al. 2006; Wirth et al. 2007; Yong et al. 2010). The work in this thesis describes a link between *AtPIP2.2* and *AtNRT2.1* expression across all treatments and that their expression decreased and became regulated by water availability under high (10.0 mM) nitrate supply. Furthermore, under high (10.0 mM) nitrate supply, root proliferation was linked to *AtNAR2.1* expression responses to water availability, which in turn required *AtPIP2.2* expression upstream.

Considering these results within the context of the wider literature, it is possible to speculate that a feedback loop may be established between the water and nitrate uptake systems whereby activity is modified in response to altered availability. Nitrate delivery to the root is decreased under conditions of decreased water availability (Nye and Tinker 1977; Tinker and Nye 2000). An increase in the expression of *AtPIP2.2* serves to counter the decreased water availability and maintain water uptake when transpiration is low (Javot et al. 2003). Expression of *AtNRT2.1* is induced by the subsequent delivery of dissolved nitrate ions to the root surface to facilitate high-affinity nitrate acquisition (Cerezo et al. 2001; Nazoa et al. 2003; Orsel et al. 2004). An increase in nitrate uptake by AtNRT2.1 alters the osmotic potential of the cell and drives an increase in *AtPIP2.2* expression to increase water uptake and maintain radial osmotic transport within the root (Javot et al. 2003). As increased AtPIP2.2-mediated water uptake increases the delivery of water and dissolved nitrate ions to the root surface, *AtNRT2.1* expression becomes repressed as external nitrate concentration increases (Lejay et al. 1999; Zhuo et al. 1999; Gansel et al. 2001). This decreases net nitrate uptake and a decrease in *AtPIP2.2* expression follows because less water is required to be taken up in order to maintain radial osmotic transport within the root (Javot et al. 2003).

Under high (10.0 mM) nitrate supply, a relative decrease in water availability facilitates a relative increase in the concentration of nitrate at the root surface and AtNRT2.1-mediated nitrate uptake is subsequently decreased. The expression of *AtNRT2.1* is then further decreased in response to a relatively increased nitrate concentration. Consequently, *AtPIP2.2* expression decreases as less water uptake is needed to maintain radial osmotic transport within the root. Under relatively increased water availability, the nitrate concentration at the root surface is decreased and the result is that expression of both genes becomes positively regulated by water availability. This is probably a result of an overall decreased level of expression over a much narrower range than that observed under low (0.1 mM) or medium (1.0 mM) nitrate supply and enhanced *AtNRT2.1* repression in response to high (10.0 mM) nitrate supply at decreased water availability.

6.7. Coordinated water and nitrate transporter expression could be regulated by nitrate

Manipulating matric potential within our sand rhizotron system alters the availability of water and the subsequent delivery of dissolved nitrate ions to the root surface. A major component of root water uptake and osmotic fluid transport under conditions of low transpiration (Javot et al. 2003), *AtPIP2.2* has been shown to be responsive to altered water availability (Alexandersson et al. 2005) and important for systemic water flux (Da Ines et al. 2010). Root responses to nitrate availability have been suggested to be mediated by the perception of nitrate itself as the signal (Wang et al. 2004; Alboresi et al. 2005; Krouk et al. 2010a).

As water flux to the root increases, dissolved nitrate ions begin to be delivered to the root surface and the expression of *AtNRT2.1* is induced. *AtNRT2.1* expression is induced by nitrate supply and strongly repressed by sustained high supply (Filleur and Daniel-Vedele 1999; Lejay et al. 1999; Zhuo et al. 1999). *AtNRT2.1* expression is also repressed by reduced nitrogen metabolites (Crawford and Glass 1998; Gansel et al. 2001; Miller et al. 2007; Gojon et al. 2009), but additionally requires a specific *AtNRT1.1*-dependent inhibition of local *AtNRT2.1* transcript accumulation under high nitrate supply (Krouk et al. 2006). Therefore, the decrease in *AtNRT2.1* expression observed under high (10.0 mM) nitrate availability requires the activation of two different signalling pathways that integrate plant nitrogen demand and locally high nitrate availability.

Nitrate regulates *AtNRT2.1* expression in a concentration dependent manner, but how is *AtPIP2.2* expression repressed under high nitrate supply? It is possible that the expression of *AtPIP2.2* could also be sensitive to nitrate itself as water channel genes have been shown to be induced by nitrate (Wang et al. 2001). Aquaporin-mediated changes in root hydraulic conductance have also been reported in response to high nitrate supply (Carvajal et al. 1996; Clarkson et al. 2000), but change in conductance has been shown not to be directly mediated by aquaporins (Gorska et al. 2008b). Aquaporin homologues have been shown to transport ammonia via proton exclusion (de Groot et al. 2003; Jahn et al. 2004; Hove and Bhave 2011) and two members of the Arabidopsis *TONOPLAST INTRINSIC PROTEIN 2* (*AtTIP2*) family have been shown to mediate extracytosolic transport of ammonia (Loque et al. 2005). In fact, changing just a single amino acid in a mammalian aquaporin was shown to switch function to an

anion channel (Liu et al. 2005). Although no previous work has reported that AtPIP2.2 can transport nitrate as AtNRT2.1 does, this could be tested *in vitro* in the frog (*Xenopus laevis*) oocyte expression system which has been used to characterise nitrate transporter activity (Miller and Zhou 2000).

In addition to high external nitrate supply, *AtNRT2.1* repression is also regulated by nitrogen demand and metabolite feedback repression (Crawford and Glass 1998; Gansel et al. 2001; Miller et al. 2007; Gojon et al. 2009). Under high nitrate supply, nitrate availability is increased for use by the plant until such a point that supply exceeds demand. At this point nitrate (De Angeli et al. 2006; Wege et al. 2010) or downstream metabolites (Loque et al. 2005) may be transported into the vacuole and stored preferentially as a readily available energy store (Miller et al. 2009). As this happens, the effective cytoplasmic solute concentration, and the osmotic potential of the cytoplasm, becomes decreased. The decreased expression of *AtPIP2.2* could therefore be the result of less water being required to be taken up into the cell in order to maintain the radial osmotic transport of water from the soil towards the vascular tissue of the root (Steudle and Frensch 1989; Javot et al. 2003; Maurel et al. 2008).

Nitrate is known to play an important role in the regulation of osmotically-driven cellular expansion that determines root growth (Steudle and Frensch 1989; Miller et al. 2009; Bloom et al. 2012). A change in cytoplasmic osmotic potential as a result of altered vacuolar nitrate allocation could explain the nitrate-mediated changes in root hydraulic conductance (Clarkson et al. 2000; Gorska et al. 2008a; Gorska et al. 2008b). Root hydraulic conductance has been shown to correlate with the external supply and uptake rates of water and nitrate (Nobel and Alm 1993; Gallardo et al. 1996; Nardini and Pitt 1999; Clarkson et al. 2000; Li et al. 2005; Gorska et al. 2008b; Gorska et al. 2010). Although the modification of root hydraulic conductance in response to nitrate supply has been previously reported not to be regulated by changes in aquaporin expression (Gorska et al. 2008b), the vacuolar-nitrate transport-dependent change in cytoplasmic osmotic potential could act as the upstream signal to modify *AtPIP2.2* expression in response to nitrate availability (Gorska et al. 2010). This may be further elucidated by investigating *AtPIP2.2* expression and root hydraulic conductance in mutant lines with defective vacuolar-nitrate transport in the sand rhizotron system.

Root hydraulic conductance and aquaporin abundance have been shown to respond rapidly to decreased water availability (McLean et al. 2011) and the *atpip2.2* mutant exhibits decreased root water uptake and root hydraulic conductivity (Javot et al. 2003; Da Ines et al. 2010). In the *atpip2.2* mutant under high (10.0 mM) nitrate supply, *AtNAR2.1* expression is decreased at decreased water availability, while the expression of *AtNRT2.1* is no longer responsive to water availability and *AtNRT1.1* expression is overall greatly decreased. Therefore, it seems that *AtPIP2.2* could play a role in the coordination of the expression of important nitrate acquisition genes. Furthermore, the disturbance of root hydraulic conductance, as a function of a change in the osmotic potential of the cell, could be the driver of disrupted transporter expression and root proliferation in the *atpip2.2* mutant. The question of whether the *AtPIP2.2* gene product or *AtPIP2.2*-mediated root hydraulic conductance is more important for wild-type root proliferation and gene expression responses could be addressed in the sand rhizotron system by analysing these acquisition responses in seedlings with decreased root hydraulic conductance, either in mutant lines that retain wild-type *AtPIP2.2* expression or perhaps where aquaporin activity is inhibited by $HgCl_2$ (Javot and Maurel 2002; Tyerman et al. 2002; Knipfer et al. 2011).

6.8. A novel role for *AtNAR2.1* in coordinating root proliferation and transporter expression

AtNRT2.1 has been shown to be crucial in the perception or transduction of the high nitrate repression signal (Little et al. 2005; Remans et al. 2006b) and to activate LR initiation under low nitrate conditions (Remans et al. 2006b). However, AtNRT2.1 is dependent upon AtNAR2.1 for its translocation to the plasma membrane (Wirth et al. 2007) where it functions in nitrate uptake activity (Okamoto et al. 2006; Orsel et al. 2006). Moreover, mutants for both genes have a similar LR phenotype (Orsel et al. 2006). Although the exact function of AtNAR2.1 itself remains elusive (Gojon et al. 2011), it has been suggested that the nitrate transporter is actually formed of an AtNRT2.1/AtNAR2.1 hetero-oligomer in the plasma membrane (Yong et al. 2010) and AtNAR2.1 can strongly interact with all bar one (AtNRT2.7) of the other AtNRT2 family members to facilitate nitrate transport (Kotur et al. 2012). However, unlike the well-characterised AtNRT1.1 or AtNRT2.1, no role has yet been identified in root proliferation or nitrate signalling responses to altered availability.

Due to the requirement of AtNAR2.1 for AtNRT2.1 uptake function, the expression of *AtNAR2.1* usually closely follows that of *AtNRT2.1* (Okamoto et al. 2006; Orsel et al. 2006; Wirth et al. 2007; Yong et al. 2010). However, it should be noted that there was no nitrate-dependent response to water availability for *AtNAR2.1* expression, like that seen for *AtNRT2.1*. Although expression of these genes correlates with transport activity, this dissimilarity in expression responses to sand hydraulic characteristics could be due to the suggested importance of post-transcriptional regulation of the high-affinity uptake system (Laugier et al. 2012). Nevertheless, this represents the novel finding that independent expression responses to sand hydraulic characteristics exist for *AtNRT2.1* and *AtNAR2.1*.

AtNRT2.1 has been identified as an important coordinator of LR responses (Little et al. 2005; Remans et al. 2006b), although the partner *AtNAR2.1* gene product itself has been suggested to be important for this (Orsel et al. 2006). In the sand rhizotron system, a potential role was identified for *AtNAR2.1* in mediating hormonal regulation of root physiology in response to water and nitrate availability. The PRL response to water availability under high (10.0 mM) nitrate supply was greatly reduced and all other root proliferation responses were lost in the *atnar2.1* mutant. In addition, *AtPIP2.2* expression remained responsive to water availability in the *atnar2.1* mutant, but the expression of *AtNRT1.1* was greatly decreased while *AtNRT2.1* expression became unresponsive to water availability. Intriguingly, *AtNAR2.1* expression became responsive to water availability in the *atpip2.2* mutant. The changes in root proliferation and transporter expression in the *atnar2.1* mutant coincided with a change in *AtABI4*, *AtEIN2* and *AtIPT5* expression with altered water availability. Taken together, these results could indicate a role for *AtNAR2.1* in mediating hormone-regulated root proliferation responses to water availability within the sand rhizotron system.

The expression of the ABA-signalling gene *AtABI4* is enhanced by ABA and repressed by auxin (Shkolnik-Inbar and Bar-Zvi 2010). In addition, the polar transport of auxin to the root tip by AtPIN1 is decreased in response increased *AtABI4* expression (Shkolnik-Inbar and Bar-Zvi 2010). Auxin transport in the root is essential for PR growth, establishment of LR primordia and the elongation of emerged laterals (Friml et al. 2003; Blilou et al. 2005; Scheres and Xu 2006; De Smet et al. 2007; Nibau et al. 2008; Fukaki and Tasaka 2009). Therefore, the sand rhizotron results linking nitrate-dependent changes in hormone-associated gene expression are consistent with the literature.

Although no direct link between *AtNAR2.1* and hormone-dependent root proliferation responses have been described in response to nutrient availability, evidence exists that links AtNAR2.1/AtNRT2.1 to hormone signalling in response to biotic stress. *AtNAR2.1* was first identified as *Wound Responsive 3* (*WR3*) and was shown to be transcriptionally induced in response to wounding (Léon et al. 1998). Although the induction of *WR3* was via a pathway that was independent of the plant hormone jasmonic acid, cross-talk between defence and other stress responses share some common elements between jasmonic acid, salicylic acid, ABA and ethylene pathways (Knight and Knight 2001; Boursiac et al. 2008; Anjum et al. 2011; Aroca et al. 2012; Moffat et al. 2012; Saeed et al. 2012; Wathugala et al. 2012). The capacity of plants to mount a successful defence response against the bacterial plant pathogen *Pseudomonas syringae* has also been linked to nitrogen status (Modolo et al. 2006) and interestingly *atnrt2.1* mutants demonstrate a reduced susceptibility to a *Pseudomonas syringae* strain associated with the hormone salicylic acid (Camanes et al. 2012). In addition, the high affinity transport system in wheat is regulated by ABA and glutamine (Cai et al. 2007). Therefore, there is some evidence that links *AtNAR2.1/AtNRT2.1* to hormone-dependent changes in root growth in response to abiotic or biotic stress, but it remains unclear whether this is mediated by hormones or nitrate itself (Wang et al. 2012). A greater insight could perhaps be gained by investigating the interaction between *AtNAR2.1* and biotic stress across a range of mutants with defective hormone signalling and nitrate metabolism in the sand rhizotron system.

Root growth is dependent upon the osmotically driven influx of water that determines cellular expansion (Sharp et al. 1990; Spollen and Sharp 1991; Ingram and Malamy 2010). Although nitrate has been implicated as an important osmoticum for this process (Steudle and Frensch 1989; Miller et al. 2009; Bloom et al. 2012), PRL increased at low water availability independent of nitrate supply. This indicates that PRL can be sustained independent of nitrate availability at the root surface and its subsequent use as an osmoticum. Maize PRL has been previously demonstrated to be maintained by osmotic adjustment at low water potentials. This was the result of segment specific accumulation of other solutes, including downstream nitrate assimilates such as the amino acid proline (Sharp et al. 1990; Voetberg and Sharp 1991), and by more than could be attributable to the anticipated accumulation from inhibited growth (Munns 1988; Yamaguchi and Sharp 2010). This accumulation of proline was the result of increased proline transport to the root tip (Verslues and Sharp 1999) and was shown to require an

accumulation of ABA (Ober and Sharp 1994). Therefore, at decreased water availability the ABA-mediated accumulation of nitrate assimilates in the Arabidopsis root tip could be the signal to maintain PRL independent of nitrate supply. Despite the difference in root size between maize and Arabidopsis, this could be tested by quantifying Arabidopsis root tip proline (Verslues and Juenger 2011) and/or ABA content at a series of water availabilities in the sand rhizotron system.

In summary, the expression of *AtNAR2.1* and *AtNRT2.1* has been described to closely parallel one another in agar or hydroponic systems, assumed to be due to the requirement of *AtNAR2.1* for AtNRT2.1 uptake function (Orsel et al. 2006; Wirth et al. 2007; Yong et al. 2010). The investigation of root physiological responses to altered water and nitrate availability in the sand rhizotron system has identified a potential novel role for *AtNAR2.1* in the determination of certain root proliferation responses to nutrient supply. The data indicate that this is independent of *AtNRT2.1* and may be mediated by interaction with hormone-associated genes. Therefore, the manipulation of sand hydraulic characteristics to alter water and nitrate supply has identified the first example of an 'un-coupling' of the expression responses of these two genes.

6.9. Conclusions

The main findings of this research are summarised below:

- A novel sand rhizotron system was developed to investigate *Arabidopsis thaliana* root nutrient acquisition responses to altered water and nitrate availability as a result of manipulated soil physical characteristics (Chapter 3); published in Chapman et al. 2011 (Appendix 2);
- Methods to analyse root proliferation and gene expression components of root nutrient acquisition were developed, including the novel *in situ* imaging of GFP-reporter lines (Chapters 3, 4 and 5); the novel use of GFP lines was published in Chapman et al. 2012 (Appendix 3);
- Disparities in several root proliferation and gene expression parameters between seedlings grown on agar and sand culture may be explained by differences in hydraulic properties of the root growth environment and the volume of nutrients available in each system (Chapter 3); published in Chapman et al. 2011 (Appendix 2);
- These results highlighted the importance of considering soil physical properties in root nutrient acquisition studies; reviewed by Chapman et al. 2012 (Appendix 3);
- In the sand rhizotron system, PRL and LRD were regulated by water availability at each nitrate (0.1, 1.0 and 10.0 mM) supply (Chapter 4);
- This may have been the result of no significant difference in the nitrate concentration at the root surface as determined by nitrate-selective microelectrode measurements (Chapter 4); a novel result published in Chapman et al. 2011 (Appendix 2);
- The expression of the high affinity nitrate transporter *AtNRT2.1* and the aquaporin *AtPIP2.2* responsible for root water uptake and osmotic fluid transport were coordinated across all treatments (Chapter 5);
- *AtNRT2.1* and *AtPIP2.2* expression was increased and more varied under low (0.1 mM) and medium (1.0 mM) nitrate supply, before decreasing overall and becoming regulated by water availability under high (10.0 mM) nitrate supply, and this was particularly evident at low water availability (Chapter 5);
- Although usually found to closely follow each other, *AtNAR2.1* and *AtNRT2.1* expression demonstrated independent responses to water and nitrate availability (Chapter 5);

- Certain wild-type root proliferation responses to water availability under high (10.0 mM) nitrate supply were lost in the *atnar2.1* mutant line and this coincided with a change in hormone gene expression (Chapter 5);
- Wild-type root proliferation and *AtNAR2.1* expression required *AtPIP2.2* upstream for wild-type responses to water availability under high (10.0 mM) nitrate supply (Chapter 5);
- Some gene expression results (e.g. the regulation of *AtNRT2.1* by unsaturated hydraulic conductivity or the 'un-coupling' of *AtNRT2.1* and *AtNAR2.1*) may not have been detected in agar or hydroponic culture due to the difficulty of manipulating soil hydraulic characteristics in these systems;
- The coordination of root proliferation and transporter and hormone gene expression may be regulated by nitrate as a response to external availability, downstream metabolites and/or its function as an osmoticum (Chapter 6).

6.10. Future lines of investigation

A novel sand rhizotron system was developed that facilitated the investigation of root physiological and molecular responses to manipulations of hydraulic properties that determine water and nutrient supply to the root. By exploiting the superior molecular tool-kit available for the model laboratory plant Arabidopsis, it has been possible to determine the influence of altered water and nitrate availability on some important root proliferation and gene expression components of nutrient acquisition. This approach has enabled a greater understanding of the coordination of water and nitrate acquisition responses to be achieved. However, it has also raised several potential future lines of investigation and here some possible ways forward are discussed.

Within the sand rhizotron system *in situ* imaging of promoter-tagged GFP-reporter lines under specific nutrient regimes was developed. However, only steady-state conditions under certain nutrient supply have been investigated and at one time point. It would be exciting to utilise readily available GFP-reporter lines to investigate rapid expression changes that underpin root acquisition responses to nutrient availability. For example, if a locally high nitrate patch was introduced into a sand rhizotron under low nitrate supply, it may be possible to image the expression of key auxin genes that regulate the LR proliferation response to colonise the patch. The exploitation of FP-reporter lines in the current system could enable several fundamental acquisition mechanisms to be addressed. If there more time, it would be extremely useful to create promoter-tagged FP-reporter lines for *AtPIP2.2* and *AtNAR2.1* before crossing them with the *proAtNRT2.1:eGFP* line. By using different FPs with different excitation ranges it would be possible to image simultaneous expression changes and thus address the regulation of proliferation responses to altered water and/or nitrate availability. This would be particularly useful to address the point at which *AtNRT2.1* and *AtPIP2.2* expression becomes decreased by high nitrate concentration and to elucidate the nature of their interaction in response to specific changes in water or nitrate supply.

The nature of the coordination of *AtNRT2.1* and *AtPIP2.2* could be further elucidated by the use of ^{15}N uptake experiments to determine whether the coordination was a sensory and/or uptake response. Although coordination of *AtNRT2.1* and *AtPIP2.2* was identified at the transcript level in this project, it could be informative to investigate changes in protein abundance and post-translational modification because regulation at this level may be important for aquaporin and

nitrate transporter function (Ho et al. 2009; McLean et al. 2011; Laugier et al. 2012). Split-root experiments could be used to investigate whether the coordination of *AtNRT2.1* and *AtPIP2.2* expression is a systemic or localized response to altered water or nitrate availability (Boukcim et al. 2006; Girin et al. 2010; Ruffel et al. 2011).

It would be informative to investigate the speculated regulation of *AtNRT2.1* and *AtPIP2.2* coordination by an osmotic nitrate signal. Investigation of the expression of *AtCLCs* or *AtTIPs* and the use of FP-reporter or mutant lines (Loque et al. 2005; De Angeli et al. 2009), could address the hypothesis that vacuolar transport of nitrate and/or assimilates may be the drivers for changes in cell osmotic potential and determine *AtNRT2.1* and *AtPIP2.2* expression. Although difficult to undertake in Arabidopsis, microelectrode measurements of the vacuolar solute concentration or pH (Miller and Smith 1992; Walker et al. 1995; Miller and Smith 1996; Miller and Smith 2008), and the quantification of root hydraulic conductance changes (Sutka et al. 2011), could enable the speculated nitrate-mediated osmotic signal to be better understood. The latter would also add more information about difference in water conductivity between the root and the sand (which was quantified) that drives water uptake in the sand rhizotron system.

The 'un-coupling' of *AtNAR2.1* and *AtNRT2.1* expression was detected in the sand rhizotron system but not previously reported in agar or hydroponics so may be a response to the sand physical properties (e.g. hydraulic conductivity, matric potential or osmotic potential). This 'un-coupling' could be further tested in response to other soil physical properties, e.g. mechanical impedance or soil strength, while the possibility of osmotic regulation of the AtNAR2.1/AtNRT2.1 transporter could be tested *in vitro* using Xenopus oocytes across a range of osmotic potentials (Miller and Zhou 2000). The interaction of *AtNAR2.1* and hormone-associated gene expression could be further investigated by analysing root physiological responses of the *atnar2.1* mutant and mutants with defective hormone signalling across a range of biotic and abiotic stresses in the sand rhizotron system. These approaches have the potential to identify a new role for *AtNAR2.1* or the nitrate signal in a general stress response.

The sand rhizotron system is based on robust physical methods that could be scaled up to generate a larger system for the investigation of nutrient acquisition responses in larger plant species. This research has identified some interesting relationships for nutrient acquisition genes that could be explored in related *Brassicacae* species (e.g. Tilsner et al. 2005), or in

crops where some information is known about the relevant acquisition genes, such as wheat (e.g. Yin et al. 2007), maize (e.g. Hachez et al. 2006) or barley (e.g. Knipfer et al. 2011). Although the reduced availability of advanced molecular tools in most of these species still represents an experimental challenge, there are some advantages in using a larger experimental system. The split-root approach could be used to gain a greater insight into the root physiological responses to spatial variation in soil hydraulic properties (Bingham and Bengough 2003), e.g. the impact on the nitrate-dependent hydraulic regulation of *NRT2.1* of exposure of a split root system to different hydraulic conductivities. In addition, as the competition of roots is driven by nutrient concentration (Hodge et al. 1999; Nord et al. 2011), the influence of altered soil hydraulic properties on the competition between roots for nitrate could be investigated in a larger system.

6.11. The potential of a weed species

It is clear that Arabidopsis root physiology is developmentally plastic in response to altered water and nitrate supply. This is presumably a function of the roots of this weed species being well adapted to obtaining sufficient nutriment from the patchy soil environment associated with its natural habitat. As a result, Arabidopsis roots may be well adapted to nutrient acquisition under more patchy resource supply (Fitter et al. 2002). The characterisation of root nutrient acquisition responses to altered water and nitrate availability in the 'weed' species *Arabidopsis* could help identify useful root traits to enhance the acquisition of nutrients in crop species (Rensink and Buell 2004; Smith and De Smet 2012). Furthermore, comparing the vast amount of information on Arabidopsis root growth in agar or hydroponic systems with data obtained using porous growth systems has identified the importance of the soil physical component of nutrient acquisition (Chapman et al. 2012).

Beyond the obvious advantages of the superior molecular tools and understanding possessed for this model laboratory plant, it is the natural adaptation to patchy resource provision that holds the potential for enhancing nutrient acquisition in our crop species. Furthermore, identifying root traits for improved nutrient acquisition in experimental systems that consider the important soil physical properties determining water and nitrate availability may help to accelerate the transition from experimental understanding to field results that can help to

address the challenge of food security. The identification of these beneficial root traits can inform crop breeding programmes that are likely to continue to be the selection platform for nutrient use efficiency (Bingham et al. 2012; Wasson et al. 2012).

By investigating beneficial root traits for acquisition under patchy (or low) nutrient supply, there is potential to improve yield in low input agricultural systems that dominate the developing world where the security of food supply is most fragile (Lynch and Brown 2012). This approach will also be important where heterogeneous environments are adopted for agriculture, e.g. the newly adopted Cerrado region in Brazil (Merten et al. 2010). This phenomenon may happen more frequently as land-use pressure associated with an increasing global population intensifies agricultural practice (Gaiser et al. 2011) and as the changing global climate impacts upon the availability of water and the mobile nitrate ion in current agricultural regions (Adeloye 2010). Furthermore, enhancing water and nitrate acquisition under low supply can also help to improve the sustainability of agricultural practice (Sutton et al. 2011).

The potential of a weed species may be exploited to realise crop improvements in the field. Understanding the strategies used by a weed species to acquire sufficient water and nutrients from a more heterogeneous environment could provide useful information for the improvement of nutrient acquisition in crops, particularly those crops that may have become accustomed to the high water and nutrient availability that is presently changing with the global climate or that is no longer sustainable. Furthermore, considering the influence of the soil physical properties determining water and nitrate availability may continue to identify novel interactions that are important for root nutrient acquisition strategies. Therefore, by adopting both fine-scale molecular Arabidopsis and scaled-up crop system approaches, while maintaining the consideration of soil physical properties determining water and nitrate availability, the challenge of enhancing nitrate and water acquisition to help achieve food security may be addressed more rapidly.

Appendix 1. Publications and prizes associated with this project

Journal articles

Chapman, N., W. R. Whalley, K. Lindsey and A. J. Miller (2011). Water supply and not nitrate concentration determines primary root growth in Arabidopsis. *Plant, Cell & Environment*, **34**(10): 1630-1638. (Appendix 2).

Chapman, N., A. J. Miller, K. Lindsey and W. R. Whalley (2012). Roots, water and nutrient acquisition: let's get physical. *Trends in Plant Science*, **in press**. (Appendix 3).

Chapman, N., A. J. Miller, K. Lindsey and W. R. Whalley ().Coordination of Root Water and Nitrate Acquisition Responses to Altered Soil Hydraulic Properties. *Plant Physiology*, **in preparation**.

Book chapters

Chapman N. and A.J. Miller (2011) Nitrate Transporters and Root Architecture. In: *Transporters and Pumps in Plant Signaling* (Eds. M. Geisler & K. Venema), pp. 165-190. Springer, Berlin, Germany.

Miller, A. J. and N. Chapman (2011). Transporters involved in nitrogen uptake and movement. In: *The molecular and physiological basis of nutrient use efficiency in crops* (Eds. M. J. Hawkesford and P. Barraclough), pp 193-210. Wiley-Blackwell, New Jersey, USA.

Conference presentations

Chapman, N. (2009). **Poster**: Investigating root development within a multi-stress system. *Workshop presentation: Root system architecture. Conference proceedings: 20th International Conference on Arabidopsis Research, Edinburgh, UK.*

Chapman, N. (2009). **Oral**: Investigating root development within a multi-stress system. *Workshop presentation: Root system architecture. Conference proceedings: 20th International Conference on Arabidopsis Research, Edinburgh, UK.*

Chapman, N., W. R. Whalley, K. Lindsey and A. J. Miller (2010). **Poster**: Separating the effect of nitrate and water stress on root system architecture. *Conference proceedings: 15th International Workshop on Plant Membrane Biology, Adelaide, Australia.*

Chapman, N. (2010). **Oral**: Separating the effect of nitrate and water stress on root system architecture. *Conference proceedings: 15th International Workshop on Plant Membrane Biology, Adelaide, Australia.*

Chapman, N., W. R. Whalley, K. Lindsey and A. J. Miller (2010). **Poster**: Separating the effect of nitrate and water stress on root system architecture. *Conference proceedings: Nitrogen 2010, Inuyama, Aichi, Japan.*

Chapman, N., W. R. Whalley, K. Lindsey and A. J. Miller (2011). **Poster**: Parallel expression changes of *AtNRT2.1* and *AtPIP2.2* in the Arabidopsis root system. *SEB Plant Transporter Group Meeting, Lancaster, UK.*

Chapman, N. (2011). **Oral**: Root responses to nitrate and water: can they be separated? *Rank Prize Funds Mini-symposium: Roots for Improving Resource Acquisition in Crops, Grassmere, UK.*

Chapman, N. (2012). **Oral**: Coordinated *AtNRT2.1* and *AtPIP2.2* Expression in Response to Changing Water and Nitrate Supply. *VIPCA 2012: Plant Growth, Nutrition and Environmental Interactions, Vienna, Austria.*

Chapman, N. (2012). **Oral**: Arabidopsis root nutrient acquisition responses to altered water and nitrate supply. *8th Symposium, International Society of Roots Research, Dundee, UK.*

Prizes

2nd Place, Postgraduate Poster for a Non-specialist Audience, University of Durham, 2009.

Best conference talk, VIPCA 2012: Plant Growth, Nutrition and Environmental Interactions, Vienna, Austria.

Appendix 2. Chapman et al. 2011, *PCE*, 34(10): 1630-1638.

Chapman, N., W. R. Whalley, K. Lindsey and A. J. Miller (2011). Water supply and not nitrate concentration determines primary root growth in Arabidopsis. *Plant, Cell & Environment*, 34(10): 1630-1638.

Plant, Cell & Environment

Plant, Cell and Environment (2011) **34**, 1630–1638 doi: 10.1111/j.1365-3040.2011.02358.x

Water supply and not nitrate concentration determines primary root growth in *Arabidopsis*

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ABSTRACT

Understanding how root system architecture (RSA) adapts to changing nitrogen and water availability is important for improving acquisition. A sand rhizotron system was developed to study RSA in a porous substrate under tightly regulated nutrient supply. The RSA of *Arabidopsis* seedlings under differing nitrate (NO_3^-) and water supplies in agar and sand was described. The hydraulic conductivity of the root environment was manipulated by using altered sand particle size and matric potentials. Ion-selective microelectrodes were used to quantify NO_3^- at the surface of growing primary roots in sands of different particle sizes. Differences in RSA were observed between seedlings grown on agar and sand, and the influence of NO_3^- (0.1–10.0 mM) and water on RSA was determined. Primary root length (PRL) was a function of water flux and independent of NO_3^-. The percentage of roots with laterals correlated with water flux, whereas NO_3^- supply was important for basal root (BR) growth. In agar and sand, the NO_3^- activities at the root surface were higher than those supplied in the nutrient solution. The sand rhizotron system is a useful tool for the study of RSA, providing a porous growth environment that can be used to simulate the effects of hydraulic conductivity on growth.

Key-words: *Arabidopsis* root; hydraulic conductivity; matric potential; nitrate; sand; water potential.

INTRODUCTION

The supply of nitrogen (N) and water to plant roots is affected by spatial and temporal heterogeneity in soil, and this is complicated by microbially mediated cycling between the different chemical forms of N (Ellis, Dendooven & Goulding 1996; Miller *et al*. 2007). For many plants, nitrate (NO_3^-) is the primary source of N because it is more mobile in solution than ammonium and often found at higher concentrations. The root is the main organ for nutrient acquisition, and understanding how root growth adapts to changing NO_3^- and water availability is important for improving nutrient acquisition (reviewed by Miller & Cramer 2005; Kant, Bi & Rothstein 2011). Root growth has a high degree of plasticity that is partly regulated by water and N supply, and this is sometimes described as a foraging response to seek nutrients within the growth environment (reviewed by De Kroon *et al*. 2009). Such adaptations can be quantified by measuring the root system architecture (RSA), which is defined by changes in the growth rate and the spatial distribution of the root system (Malamy 2005).

In soil, the delivery of water to the root is largely driven by water potential differences within the root–soil system. These differences in water potential for a given hydraulic conductivity of the soil and transpirational demand from the shoots determine the flux of water that can be supported through the plant–soil (Sperry *et al*. 1998; Cramer, Hawkins & Verboom 2009). Water potential (Ψ_t) is the sum of the osmotic potential of water (Ψ_o) caused by dissolved solutes and the matric potential (Ψ_m) caused by the capillary pressure of water held between substrate particles. The relationships between soil hydraulic conductivity (its conductance to water) and saturation are established, and robust empirical relationships exist between soil water content, matric potential and hydraulic conductivity (van Genuchten 1980).

The effect of soil hydraulic conductivity on root development has long been recognized (Passioura 1991), but it has received little attention. In particular, the direct influence of hydraulic conductivity of the substrate on RSA has been neglected. The effects of water stress on plant growth have been studied using model systems such as an osmoticum (PEG) or equilibrated vermiculite (Verslues, Ober & Sharp 1998), where plant growth is related to the water potentials of the growth environment and typically the effects of water potential. It has been shown that primary root length (PRL) is stimulated at small negative water potentials, although the rate of PR growth decreases with increasing water stress (Sharp, Silk & Hsiao 1988; Wiegers, Cheer & Silk 2009). In addition, the formation of lateral roots (LRs) increased with water supply on agar plates (Deak & Malamy 2005), and LRs are known to proliferate in wetter soil zones (Greacen & Oh 1972).

NO_3^- supply influences individual RSA characteristics differently. PRL has been shown not to be sensitive to NO_3^- concentration (Zhang & Forde 1998; Linkohr *et al*. 2002), although PR growth of at least one *Arabidopsis* accession (No-0) has been shown to be sensitive to low NO_3^-

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concentrations (Walch-Liu & Forde 2008). In contrast, LR development and elongation are stimulated by local NO_3^- application in numerous plant species (Zhang & Forde 1998; Zhang *et al.* 1999; Casimiro *et al.* 2003; Visser *et al.* 2008), and this result was first demonstrated in sand culture (Drew & Saker 1975). *Arabidopsis* vertical agar plate experiments showed an increased LR density in response to local high-NO_3^- patches (Zhang & Forde 1998; Remans *et al.* 2006a,b). Conversely, seedlings growing on uniformly high-NO_3^- supply exhibit suppressed LR development (Zhang *et al.* 1999). Much less is known about other root parameters, such as basal root (BR) growth, in response to both NO_3^- and water supply.

The RSA responses of the model plant *Arabidopsis* to water and NO_3^- availability tend to be studied using agar and hydroponic culture methods (Zhang & Forde 1998; Casson & Lindsey 2003; Little *et al.* 2005; Orsel *et al.* 2006; Remans *et al.* 2006a; Chopin *et al.* 2007). However, the root growth environment of these laboratory techniques is very different from that encountered by a growing root in the field. Physical parameters, such as Ψ_t and hydraulic conductivity, facilitate the delivery of N and water to the root and may be limiting in the field; therefore, becoming important regulators of RSA. When different root growth systems are compared, they have often given very different results (Hargreaves, Gregory & Bengough 2009; Wojciechowski *et al.* 2009).

In this paper, we describe the RSA of *Arabidopsis thaliana* (Ws) wild-type seedlings subjected to different NO_3^- and water supplies. Significant differences in RSA were measured under different water and NO_3^- treatments, and between seedlings grown on agar and sand. In combination with RSA measurements in these different growth substrates, we quantified the NO_3^- activity at the surface of a growing root in order to determine whether PRL was more sensitive to water or NO_3^- supply.

MATERIALS AND METHODS

Experimental approach

Experiments were conducted to study root architectural responses to altered N and water supply in a sand rhizotron system. The RSA was compared for *Arabidopsis* seedlings growing in sand and agar culture systems (see below for details) under different N and water supplies. The concentration of NO_3^- supplied was changed and seedlings were grown in sands with different particle sizes and at different water matric potentials. The NO_3^- available at the surface of primary roots grown in sands of different particle sizes was quantified using ion-selective microelectrodes. For all experiments, the same nutrient solution was used (as described in Orsel *et al.* 2006) with final concentrations of each component: 0.5 mM $CaSO_4$, 0.5 mM $MgCl_2$, 1 mM KH_2PO_4, 10 μM $MnSO_4{\cdot}7H_2O$, 24 μM H_3BO_3, 3 μM $ZnSO_4{\cdot}7H_2O$, 0.9 μM $CuSO_4{\cdot}5H_2O$, 0.04 μM $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 72 μM Fe sequestrene. For the different NO_3^- supply experiments, KNO_3 was added at final concentrations of 0.1, 1 and 10 mM. The K^+ concentration was kept constant by the addition of K_2SO_4 to the 0.1 and 1 mM KNO_3 solutions. For all experiments, final volume was made up using distilled H_2O, adjusted to pH 5.7 and buffered with 1 mM MES.

Agar culture

Arabidopsis seeds were grown on agar Petri dishes as described previously (Zhang & Forde 1998). The method was modified as agar was added to the nutrient solution at a final strength of 2%. This concentration was higher than that used previously by some authors, because lower percentages failed to give full agar setting with this nutrient solution. Square plates (120 × 120 mm) were set at an angle of 60°, and four seedlings per plate were grown under the same environmental conditions as described for all the experiments. The plants were orientated such that only the roots were in contact with the agar.

Sand rhizotron system

Acid-washed Redhill T sand (Sibelco UK Ltd, Sandbach, UK) was used for each sand experiment. To set up the experimental system (Fig. 1), a 5 L filter funnel (20 cm diameter) was vacuum-saturated with nutrient solution in order to support a defined water tension. Rhizotrons were constructed using modified clear jewel CD cases (142 × 124 × 10 mm) and positioned on a layer of sand to enable a continuous connection of solution from the aspirator to the seedling. Orientation was at an angle of 60° to encourage the roots to grow near to the surface for easy access. This orientation was found from trial experiments, at steeper angles roots grew along the sand/plastic interface (data not shown). Each experimental unit was filled with sand and saturated with nutrient solution before four seedlings were transferred to the growth media on the exposed surface. The system was covered with opaque sheeting, apart from the exposed seedlings that were covered with a transparent polythene sheet, to minimize evaporative losses and algal growth. Different particle sizes were obtained from Redhill T sand by sieving into the following fractions: <250, 250–425 and >425 μm. Matric potential was adjusted by altering the difference in height between the sand culture and the level of the nutrient solution reservoir in the aspirator bottle (see Fig. 1). We used three matric potentials of −1.5, −3.0 and −4.5 kPa (indicated as arrows in Supporting Information Fig. S1a).

Physical characterization of growth substrates

The water potential (Ψ_t) of sand samples was determined using a WP4 Dewpoint PoteniaMeter (Decagon Devices, Inc, Pullman, WA, USA). The water release characteristics for the sands, which is the relationship between matric potential and water content, were determined using the burette method (Marshall, Holmes & Rose 1996). Saturated hydraulic conductivity K_{sat} was measured using the constant head permeability test (Marshall *et al.* 1996). Water release

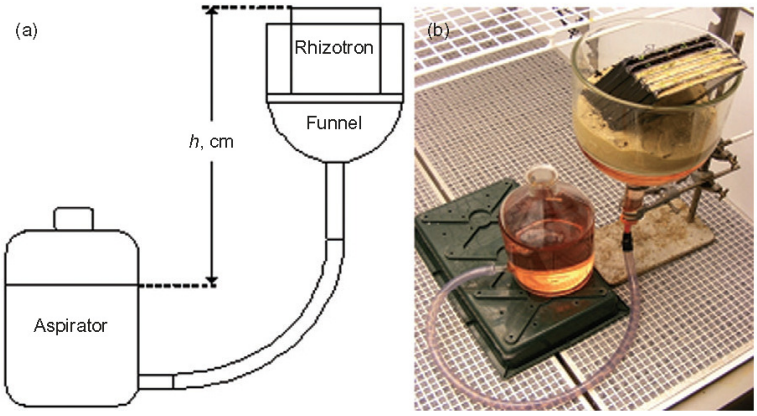

Figure 1. A schematic (a) and photograph (b) of the sand rhizotron system for growth of *Arabidopsis* seedlings in sand where tension height (h, cm) can be altered.

data were fitted to the Mualem van Genuchten models (Supporting Information Table S1; van Genuchten 1980), and unsaturated K_{unsat} was predicted (Supporting Information Fig. S1). The value of Ψ_m in the different grades of sand was adjusted by altering the water tension height, to give hydraulic conductivities of the sand to water between 0.2 and 1.4 m D^{-1} (Table 1).

Plant growth conditions

For both sand and agar experiments, *Arabidopsis thaliana* (Ws) seeds were sterilized, stratified and germinated on Whatman no. 2 filter paper (Whatman, Maidstone, UK) soaked in distilled water. At 5 d post-germination (dpg), seedlings were selected for similar size and PRL, and transferred to the respective experimental systems (four seedlings per experimental unit). At 12 dpg, the following RSA characteristics (see Zobel & Waisel 2010) were measured manually for each seedling: PRL, BR length (BRL), LR number (LRN) of length ≥1 mm and total LR length (TLRL). For all germination and physiology experiments, growth conditions were cycled: 16 h light (290 μmol m^{-2} s^{-3}), 8 h darkness, at 22 °C and 75% relative humidity. A minimum of two independent replicates were used for each experiment comparing different nitrate supplies or the effect of particle sizes, with three or four in all other treatments.

NO_3^--selective microelectrodes

Double-barrelled NO_3^--selective microelectrodes were prepared using filamented double-barrelled borosilicate glass (Miller & Zhen 1991). Microelectrodes were mounted on a micromanipulator (model NMN-21; Narashige, Tokyo, Japan). Both microelectrode reference barrels and reference electrodes were backfilled with 200 mM KCl. The experimental unit was secured to the stage of an Olympus microscope (model SZX9, Olympus, Southend-on-Sea, UK). Calibration curves were obtained using solutions of known NO_3^- activities (Miller & Zhen 1991). The NO_3^- activities at the surface of intact primary roots were measured at the root tip (RT) and at 2 mm back from the tip (RT – 2 mm), and only data with unaltered calibration and recalibration curves before and after each measurement were considered acceptable. Care was taken to minimize the area of sand exposed for the measurement to avoid drying, but steady recordings were obtained suggesting that evaporative losses were minimal (Supporting Information Fig. S2). For the analysis of agar, a scalpel was used to cut 0.2 g sections from the plate, and each was melted in 1.8 g of distilled water (1 in 10 dilution) by heating in a microwave oven. A minimum of at least three replicates were used for each measurement.

Statistical analyses

All statistical analyses were carried out using GenStat (12th edition; VSN International Ltd, Harpenden, UK). For root physiology experiments, means were plotted and general analysis of variance (ANOVA) was carried out followed by comparison of means using the LSD (5%). LSD and SED values are given in Supporting Information Table S2. Some data were transformed to a log scale to stabilize variance

Sand	Water potential (MPa)	Matric potential (kPa)	Calculated K_{unsat} (m d^{-1})
Redhill T	–0.11	–1.5	1.35
Redhill T	–0.33	–3.0	0.79
Redhill T	–0.40	–4.5	0.18
<250 μm	–0.06	–3.0	0.99
250–425 μm	–0.26	–3.0	0.36
>425 μm	–0.33	–3.0	0.17

Table 1. Measured values for total water and matric potentials, and calculated values of the hydraulic permeability (K_{unsat}), showing the ranges for the sands used in this study

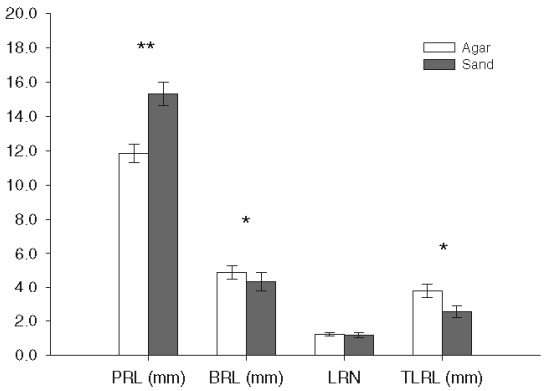

Figure 2. Comparison of the root architectural characteristics of *Arabidopsis* seedlings grown on agar and sand under the same nutrient supply. The average primary root length (PRL), basal root length (BRL), lateral root number (LRN) and total lateral root length (TLRL) were plotted for each treatment ($n = 120$; d.f. 57), and data were compared on the $\log_{10}$ scale to stabilize variance across treatments. Significance: $*P < 0.05$; $**P < 0.01$.

across treatments (figure legend will indicate). Graphs were made using SigmaPlot (version 11; Systat Software Inc, San Jose, CA, USA).

RESULTS

RSA was different between sand and agar

The RSA of seedlings grown on agar and sand under the same growth conditions was compared. The PRL of sand-grown seedlings were significantly longer ($P < 0.01$; d.f. 57) than that of agar-grown plants (Fig. 2). Whereas BRL and TLRL were significantly longer ($P < 0.05$; d.f. 57) in agar-grown seedlings when compared with those grown in sand. The LRN and LR density were not significantly different between treatments.

PRL was stimulated by changes in water flux to the root

The data in Fig. 3 clearly show that the PRL significantly increased with particle size (Fig. 3a, $P < 0.001$; d.f. 32) and tension (Fig. 3b, $P < 0.001$; d.f. 50), but the situation for other RSA characteristics was more complicated. Like PRL, BRL was significantly stimulated by sand particle size ($P < 0.01$; d.f. 32). In contrast to the primary root, the BRL showed no response to changes in matric potential (comparing Fig. 3a,b). The LRN and TLRL were not significantly different for roots growing in sand of different particle sizes (Fig. 3a), but when matric potential was increased both LRN and TLRL significantly decreased (Fig. 3b). The same response to matric potential was observed for LR density, but only for sand with a particle size <250 μm (Fig. 4).

The percentage of roots with laterals increased linearly with K_{unsat} ($r^2 = 0.93$; Fig. 5). Although PRL (negative, $r^2 = 0.66$) and LRN (positive, $r^2 = 0.94$) were correlated with K_{unsat}, the analysis of grouped data revealed that there was not a common curve that applied to both approaches to manipulate K_{unsat} (i.e. using different sand or adjusting matric potential). No clear relationship could be identified for BRL and TLRL plotted against K_{unsat}.

Root architectural responses to changes in nitrate supply are different for sand and agar

The RSA of seedlings grown on agar and sand was compared, but with the KNO_3 supply adjusted to give concentrations of 0.1, 1 and 10 mM (Fig. 6). There were some significant differences between the root growth patterns on agar and sand (Fig. 6a,b). The PRL was significantly longer

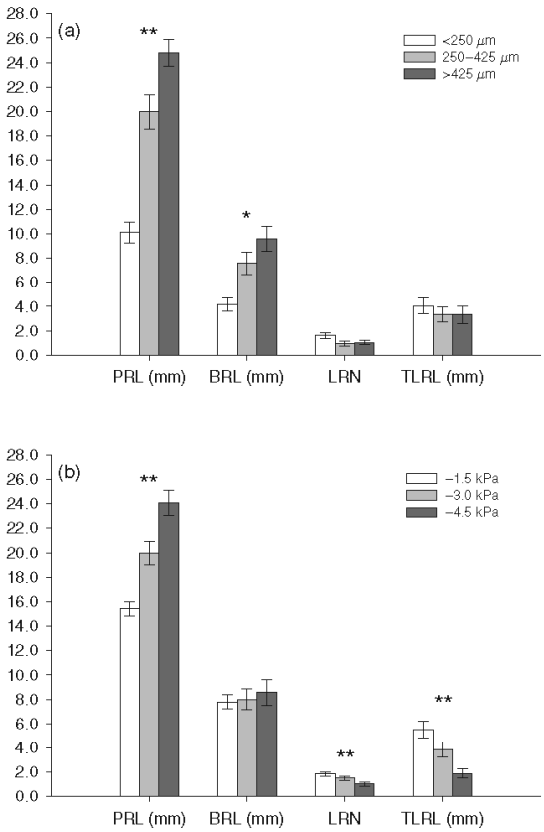

Figure 3. *Arabidopsis* root architectural characteristics of seedlings grown in sand with a constant N availability, but varying water delivery rates. Sands of three (a) particle sizes (>250, 250–425 and >425 μm) and (b) tension heights 15, 30 and 45 cm (1.5, 3.0 and 4.5 kPa) were used while keeping N availability constant across treatments. The average primary root length (PRL), basal root length (BRL), lateral root number (LRN) and total lateral root length (TLRL) were plotted for each treatment; (a) $n = 48$, d.f. 32; (b) $n = 72$, d.f. 50. Significance: $*P < 0.01$; $**P < 0.001$.

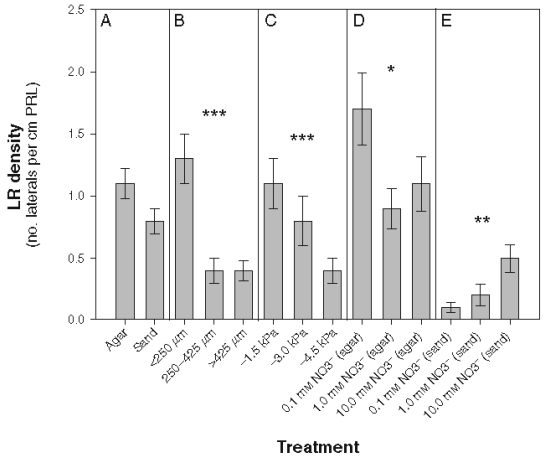

Figure 4. Lateral root (LR) density [number of LRs per cm primary root length (PRL)] was plotted for each treatment. Experiment A demonstrated no significant difference. For experiment B, d.f. 32, SED 0.021, LSD 0.042. For experiment D, d.f. 46, SED 0.033, LSD 0.066. Significance: $*P < 0.04$; $**P < 0.01$; $***P < 0.001$.

on sand when compared with agar at all three KNO_3 concentrations (consistent with Fig. 2), and there was no difference between treatments in sand, but on agar the PRL was greater ($P < 0.001$; d.f. 42) at 1 mM when compared with 10 mM KNO_3 supply (Fig. 6b). At the highest KNO_3 supply, LRN, TLRL and LR density are increased in sand, but this response was not observed in agar where LR density was significantly greater at 0.1 mM KNO_3 (Fig. 4). In the sand system, LRN, TLRL (Fig. 6) and LR density (Fig. 4) were significantly increased at 10 mM compared to 0.1 and 1.0 mM KNO_3 supplies.

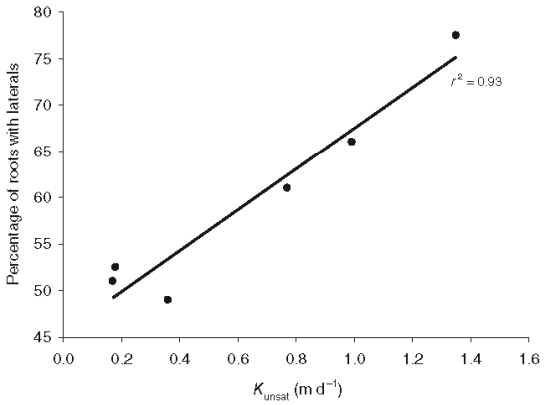

Figure 5. Plot of percentage of roots with laterals against K_{unsat} showing evidence of negative linear correlation. Linear regression line plotted ($r^2 = 0.93$).

PRL was stimulated by changes in water flux, but not root surface nitrate

In order to quantify the NO_3^- availability encountered by the sensing and uptake regions of the root, the activity was measured at the RT and 2 mm above the RT (RT – 2 mm) using NO_3^--selective microelectrodes. The NO_3^- activity reported by the microelectrodes on the surface of the root was greater than that supplied in the nutrient solution. However, the NO_3^- activity was not significantly different across particle size treatments or between locations on the root for seedlings supplied with 10.0 mM (Fig. 7a) and 0.1 mM (not shown). Therefore, PRL responses to water flux (Fig. 3) are independent of NO_3^- availability at the root surface.

In agar without plants, the NO_3^- activity was similar to the original 10 mM NO_3^- supply (Fig. 7b). However, when plants were introduced into the agar system, the NO_3^- activity

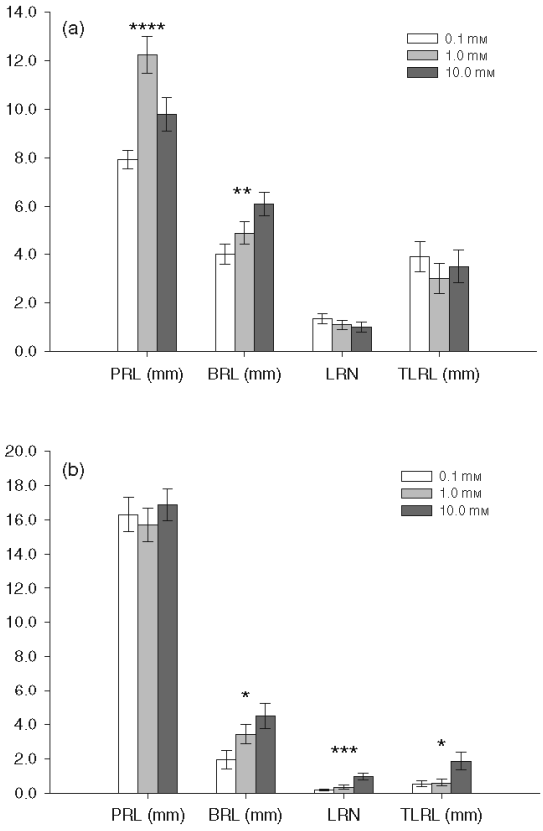

Figure 6. Root architectural characteristics of *Arabidopsis* seedlings grown under varying NO_3^- supply on either agar (a) or sand (b). Root system architecture (RSA) response to nitrate concentration was compared between agar (a) ($n = 24$; d.f. 42) and sand (b) ($n = 72$; d.f. 49). The average primary root length (PRL), basal root length (BRL), lateral root number (LRN) and total lateral root length (TLRL) were plotted for each treatment. Significance: $*P < 0.1$; $**P < 0.05$; $***P < 0.01$; $****P < 0.001$.

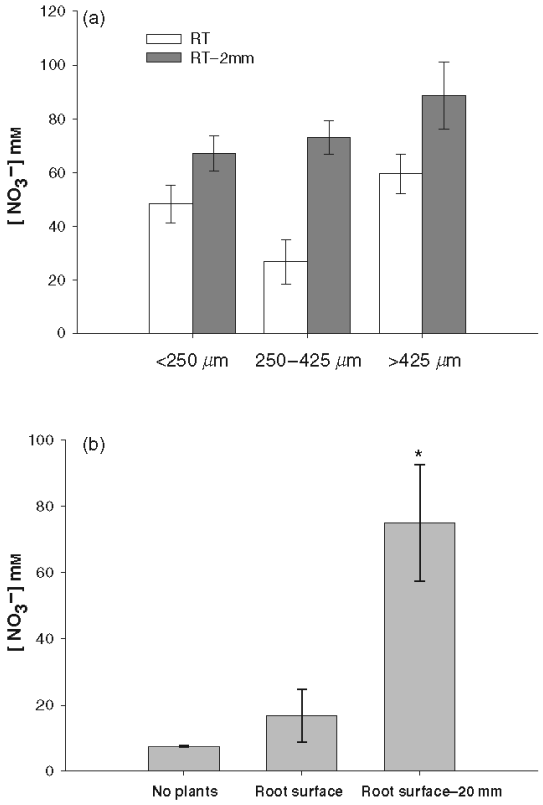

Figure 7. Ion-selective microelectrode measurements of NO_3^- activity: (a) at the root surface of *Arabidopsis* seedlings grown on sands of three particle sizes supplied with 10 mM KNO_3, and (b) in the agar around Petri dish-grown *Arabidopsis* seedlings. For the sand measurements, NO_3^- was measured at the root tip (RT) and 2 mm up from the tip (RT – 2 mm). For the agar measurements: NO_3^- was measured at the PR surface and 20 mm away from the PR; agar with no plants was used as a control; for each treatment $n = 3$, d.f. 6; significance: $^*P = 0.01$.

found at the root surface was significantly lower ($P < 0.01$; d.f. 6) than that observed 20 mm away from the root surface (Fig. 7b). In addition, the activities observed for the agar when plants were present were also greater than the initial 10 mM NO_3 added to the agar.

DISCUSSION

The use of sand to study root architecture

Sand culture is more similar to soil than agar or hydroponics (Table 2) in the sense that both sand and soil can be defined by comparable hydraulic parameters. Sand culture has minimal microbial impact on root nutrient acquisition relative to soil, but provides an easier environment than soil to study RSA in response to nutritional and physical (especially hydraulic) treatments.

In our sand rhizotron system, the hydraulic conductivity values obtained for the sands are similar to the saturated hydraulic conductivity ranges typically found for sandy and loamy soils (Campbell 1985; Marshall *et al.* 1996). When roots extract water, the value of the Ψ_m adjacent to the root becomes more negative (Carminati *et al.* 2010), so in our system tension height should be taken as an approximate estimate of the Ψ_m at the soil–root interface. However, because the hydraulic conductivities we use are at relatively high bulk matric potential (determined by the tension height), they are likely to be a reasonable estimate of matric potentials at the sand–root interface (Whalley *et al.* 2000). *Arabidopsis* has a relatively low transpiration demand (Christman *et al.* 2008), and the seedlings were grown in a covered system; therefore, it is unlikely that significant moisture gradients will develop near the root in this experimental system. The range in matric potential we used was very small (–1.5 to –4.5 kPa; Table 1), and water potential was dominated by the osmotic component. Thus, the effect of manipulating matric potential or the size of sand particles was primarily to change the hydraulic conductivity of the sand to water, and hence the flux of water to the root surface.

Comparing our sand system with agar culture, we found significant reproducible differences in several aspects of RSA for *Arabidopsis* seedlings growing under complete nutrient supply (Fig. 3). This suggests that differences in the physical properties of the two culture systems (agar and sand) are influencing the response of RSA to water and NO_3^- treatments.

Why is RSA different between sand and agar?

Certain sands offer physical impedance to growth that is not found when roots grow on the surface of agar. Changes in RSA (e.g. a reduced PRL) have been described for mechanically impeded roots (reviewed by Clark, Whalley & Barraclough 2003). Pore size is related to particle size (Marshall *et al.* 1996), and the pore size of the sand used in this work is sufficient for the fine *Arabidopsis* roots (typical diameter 150 μm; Bowman 1994) to grow between particles and thus not be impeded. The physical structure of agar and the fact that the roots grow mostly over the surface mean that mechanical impedance effects can be excluded from these experiments. Therefore, physical impedance effects cannot explain the RSA differences observed in these experiments.

Root gas exchange may differ between sand and agar. The gaseous hormone ethylene is known to influence RSA (Ivanchenko, Muday & Dubrovsky 2008), and localized gradients may develop differently in sand and agar. Roots growing along an agar surface are in contact with the air, while those in wet sand may have a more restricted opportunity for gas exchange. However, the sand environments were unsaturated, except in one case (Table 1, row 1), so it is reasonable to assume that the roots were adequately aerated. Other work has concluded that in similar sand systems, oxygen availability does not limit growth (Whalley *et al.* 1999). Taken together, this information suggests that

Feature	Agar	Hydroponics	Sand	Soil
Matrix structure	Homogeneous	Homogeneous	Heterogeneous	Heterogeneous
Pores (air)	Moderate	Limited	Variable	Variable
Pores (water)	Moderate	Saturated	Variable	Variable
Impedance	Minimal	Minimal	Variable	Variable
Bacteria	Minimal	Minimal	Minimal	High
Light exposure	High	Minimal	Minimal	Minimal

Table 2. Relative differences between culture techniques used to study root architecture

Sand is more similar to the natural growth environment encountered in the soil than agar or hydroponics.

gas exchange properties are unlikely to explain the differences in RSA between sand and agar systems.

Light supply can be an important factor influencing root growth. On agar, the roots are not maintained in the dark, and light inhibition of root growth is ethylene and jasmonate mediated (Adams & Turner 2010). In sand, roots are likely to be exposed to less light, but PRL for agar and the smallest particle sand are similar (comparing Figs 2 & 3a). This result suggests that a difference in light interception by roots does not explain the RSA differences between sand- and agar-grown plants. When developing the method, we compared conventional agar-grown seedlings with those grown on agar with root light exposure limited, and found no significant difference in RSA (data not shown).

Unlike agar culture, the sand rhizotron system was not completely sterile. Although the sand was acid washed, contained no carbon source for microbial growth and the system was covered in opaque sheeting, when comparing agar and the sand culture data, we cannot exclude the possibility that there was some microbial growth in the sand system. However, during method development, we found that autoclaving the acid-washed sand had no effect on RSA when compared with unsterilized sand (data not shown). Therefore, we believe that microbial activity is not a significant contributor to the RSA of seedlings grown in our sand rhizotron system.

One of the biggest differences between the two systems is in the volume of nutrients supplied in each system. In the sand system, the volume of water and NO_3^- available to the plant is much greater than the finite volume supplied in agar plates. The impact on RSA of the volume of nutrients supplied between the two systems is further compounded by differences in the hydraulic properties which determine delivery of water and NO_3^- to the root. It is known that Ψ_t influences RSA (Sharp *et al.* 1988; Verslues *et al.* 1998), and our results demonstrate the influence of water flux on RSA. Therefore, differences in the volume of nutrients supplied and hydraulic parameters between sand and agar systems may explain the changes in RSA we have observed by affecting the delivery of water and NO_3^- to the root.

The effects of water and nitrate supply on root architecture

Several papers have reported the influence of water stress as Ψ_t on RSA (Sharp *et al.* 1988; Verslues *et al.* 1998; Wiegers *et al.* 2009). This is especially true for PRL which has been shown to decrease as water stress increases (Sharp *et al.* 1988). In our sand system, the opposite effects were obtained in response to the manipulation of substrate hydraulic conductivity: PRL increased with increasing particle size and water tension (Fig. 3). Nitrate is delivered to the root surface dissolved in water, but we have shown that the tight regulation of PRL by changes in water flux to the root was independent of NO_3^- activity at the root surface (Fig. 7).

PR elongation was shown to be strongly dependent on inorganic N (both NO_3^- and NH_4^+) supply in tomato and maize, and this response was optimal at lower concentrations (Bloom, Jackson & Smart 1993; Bloom, Frensch & Taylor 2006). Conversely, *Arabidopsis* PR growth is arrested by NH_4^+ supply (Li *et al.* 2010) and is insensitive to NO_3^- supply across a large range from 0.01 to 100.0 mM (Zhang & Forde 1998; Linkohr *et al.* 2002; Orsel *et al.* 2006), although the latter response is known to be genotype dependent (Walch-Liu & Forde 2008). Here, we demonstrate an insensitivity of *Arabidopsis* Ws PRL to high (0.1–10.0 mM) NO_3^- supply (Fig. 6b), and while lower NO_3^- concentrations were not investigated, an insensitivity to low (0.05–1.0 mM) NO_3^- supply has been previously reported for Ws (Remans *et al.* 2006b).

The insensitivity of *A. thaliana* PRL to NO_3^- has previously been shown in agar culture experiments (Zhang & Forde 1998; Linkohr *et al.* 2002). However, our agar PRL results differed from those previously reported (Fig. 6a). This difference could be genotype dependent, and this has been demonstrated for No-0 plants that were sensitive to low NO_3^- concentrations compared to other genotypes (Walch-Liu & Forde 2008). Another possible explanation is that we used 2% agar, whereas 0.8% (Linkohr *et al.* 2002) and 1% (Zhang & Forde 1998) were used previously. It has been shown that a change of just 0.1% in gel concentration equates to a 1 to 2×10^{-4} MPa change in Ψ_m (Spomer & Smith 1996). We may assume that the Ψ_t experienced by roots in our agar system is approximately 20 times that of the lower concentration agar used in previous work. Ψ_t is known to influence PR growth (Sharp *et al.* 1988), and in gel substrates Ψ_m is a large component of Ψ_t. Therefore, the difference in Ψ_t is likely to account for the contrast in PRL we have observed compared to previous work.

A decrease in water availability has been shown to significantly repress the formation of LRs (Deak & Malamy 2005).

This result is confirmed for *Arabidopsis* roots growing in sand by the LRN (Fig. 3b), and the percentage of roots with no laterals increased as K_{unsat} decreased (Fig. 5). TLRL has been shown to respond to localized patches of high NO_3^- concentration (Zhang *et al.* 1999), and indeed the TLRL was greater for agar-grown seedlings compared to sand (Fig. 2). This suggests patchiness in NO_3^- concentration caused by a finite supply of water in agar culture which moves towards the plant, creating local patches of high NO_3^- concentration at the periphery of the agar plate, as confirmed by the NO_3^- microelectrode measurements (Fig. 7b).

While the water supply, determined by substrate hydraulic conductivity and gradient in water potential, is important for PRL, LRN and TLRL, changes in NO_3^- supply produce the same response in BRL (Fig. 6). BRL of sand-grown ($P < 0.1$; d.f. 49) and agar-grown seedlings ($P < 0.05$; d.f. 42) increased with increasing NO_3^- concentration. LRN and TLRL have been shown to respond to patches of high NO_3^- supply in agar (Zhang *et al.* 1999), and BRL could be responding in the same way here. Particularly as BRL increased at larger particle sizes, but was unaffected by matric potential, sands of a larger particle size have a larger pore size and poorer connectivity of solution, resulting in the occurrence of NO_3^- patches.

CONCLUSIONS

The sand rhizotron system is a step towards bridging the gap between the lab and the field, combining a porous growth environment with controlled water and nutrient delivery to give an additional tool to explore *Arabidopsis* RSA. The contrasting RSA responses in sand and agar may be explained by differences in the volume of nutrient supplied and the conductivity of each substrate to water. In the sand system, we have shown that changes in hydraulic conductivity over a narrow range of matric potentials can have large effects on RSA; the best example of this point was in the percentage of roots with laterals. In the case of PRL, water flux regulates growth independent of NO_3^- supplied across a range of 0.1–10 mM. In contrast, BRL is more closely regulated by NO_3^- supply and exhibits a similar response to that previously reported for LRs and high NO_3^- patches.

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SUPPORTING INFORMATION

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Figure S1. Water release characteristics of the sands used in these experiments. The water release characteristic was measured using the burette method. (a) Arrows indicate the tensions used during the water tension experiment, and K_{sat} was measured using the constant head permeability test (b). This information was computed using the van Genuchten model to calculate K_{unsat} (c).
Figure S2. Typical NO_3^- microelectrode recording obtained from the surface of an *Arabidopsis* root growing in sand and supplied with a full nutrient solution containing 0.1 mM NO_3^-. Stable measurements were recorded at the root tip (RT) and 2 mm up (RT – 2 mm) from the RT of an intact primary root. The recording shows the calibration of the microelectrode before (t = 0–10 min) and after (t = 25–32 min) the measurement with solutions of known NO_3^- activity (100, 10, 1, 0.1 and 0.01 mM).
Table S1. Sand physical properties including parameters used in the van Genuchten equation.
Table S2. Standard error differences (SEDs) and least square difference (LSD, 5%) values of root system architecture (RSA) characteristics for each physiology experiment (Exp).

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Appendix 3. Chapman et al. 2012, *TiPS*, **17**(12): 701-710.

Chapman, N., A. J. Miller, K. Lindsey and W. R. Whalley (2012). Roots, water and nutrient acquisition: let's get physical. *Trends in Plant Science*, **17**(12): 701–710.

Review

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Roots, water, and nutrient acquisition: let's get physical

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Improved root water and nutrient acquisition can increase fertiliser use efficiency and is important for securing food production. Root nutrient acquisition includes proliferation, transporter function, exudation, symbioses, and the delivery of dissolved nutrients from the bulk soil to the root surface via mass flow and diffusion. The widespread adoption of simplified experimental systems has restricted consideration of the influence of soil symbiotic organisms and physical properties on root acquisition. The soil physical properties can directly influence root growth and explain some of the disparities obtained from different experimental systems. Turning this to an advantage, comparing results obtained with the same model plant *Arabidopsis* (*Arabidopsis thaliana*) in different systems, we can tease apart the specific effects of soil physical properties.

The need to enhance root nutrient acquisition

The challenge of securing sufficient food for an estimated global population of 9 billion people by 2050 requires a significant increase in global agricultural productivity [1]. This will not be easy given the adverse effects of climate change on crop production, and the pressure to devote land to bio-fuel production, together contributing to an intensified burden on land-use. Severe flooding and drought episodes currently occur more frequently, devastating crop yields throughout the world [2]. At current productivity there are over 1 billion inadequately fed people in the world [3] and agriculture is under political pressure to improve its sustainability [4]. A significant improvement in agricultural productivity is therefore needed to establish food security.

Enhancements in future agriculture range from better access to water and soil management to improved plant breeding and efficient nutrient acquisition [1]. One promising target is the improvement of crop nutrient acquisition under changing environmental conditions [5–7]. The efficiency by which plants acquire nutrients from the soil is a major determinant of crop yield. Root nutrient acquisition includes root proliferation and transporter function, exudates to mobilise sparingly available nutrients, symbiotic associations with other organisms, mass flow of water to the root and diffusion of nutrient ions to the root surface (see Glossary) [8]; all of which depend on both plant traits and soil physical characteristics (Figure 1). Through a better understanding of root nutrient acquisition under variable water and nutrient availability, it may be possible to modify root traits to improve nutrient acquisition in specific environmental conditions and increase agricultural productivity.

Root growth demonstrates a high degree of plasticity in response to changes in the supply of vital nutrients [9], which has not been lost in crop plants and offers some future possibilities for the improvement of nutrient acquisition. Given the potential productivity gains, it is not surprising that roots are the subject of much research and the physiological processes that underpin nutrient acquisition are frequently reviewed [5,9–21]. Although the movement of nutrients and water in soil is well understood [22,23], the investigation of root acquisition using model systems may not accurately reflect the interaction between the root and soil physical components [9,24].

In this review, we aim to dissect the soil physical properties that influence root proliferation, transporter function, and the delivery of nutrients to the root by mass flow and diffusion. The quantitative contribution of soil physical properties to each of these different root functions is identified. The limitations and strengths of different

Glossary

Diffusion: the movement of dissolved ions along a concentration gradient towards the root, driven by net influx of ions at the root surface.
Hydraulic conductivity: this characterises the ability of a soil to transmit water and depends on pore size and the water content. Empirical relationships exist which allow it to be expressed as a function of water content for a given soil.
Mass flow: the convective transport of dissolved ions from bulk soil towards the root, driven by water potential gradients from leaf to bulk soil that is initiated by the 'transpirational influx' of water.
Matric potential: the capillary pressure of water held between substrate (e.g., soil) particles [137].
Mechanical impedance: the physical resistance to root growth of a 'strong' soil.
Osmotic potential: largely determined by dissolved solutes within the soil solution. They are negative, with low potentials being more negative and high potentials less negative.
Root proliferation: dependent on the continued growth and branching of the root in response to internal and external cues that determines root system architecture (RSA).
Soil strength: mechanical resistance of a soil to root growth, and usually determined by water content and soil structure.
'Strong' soil: soil with increased strength, usually as a result of decreased water content and increasing bulk density.
Water potential: the difference between the chemical potential of water in a material and the chemical potential of pure water at defined reference conditions of pressure and temperature.
'Weak' soil: soil with decreased strength, usually as a result of increased water content and decreasing bulk density.

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Keywords: mass flow; diffusion; architecture; soil physics; hydraulic; matric potential; nitrogen; nitrate; particle size.

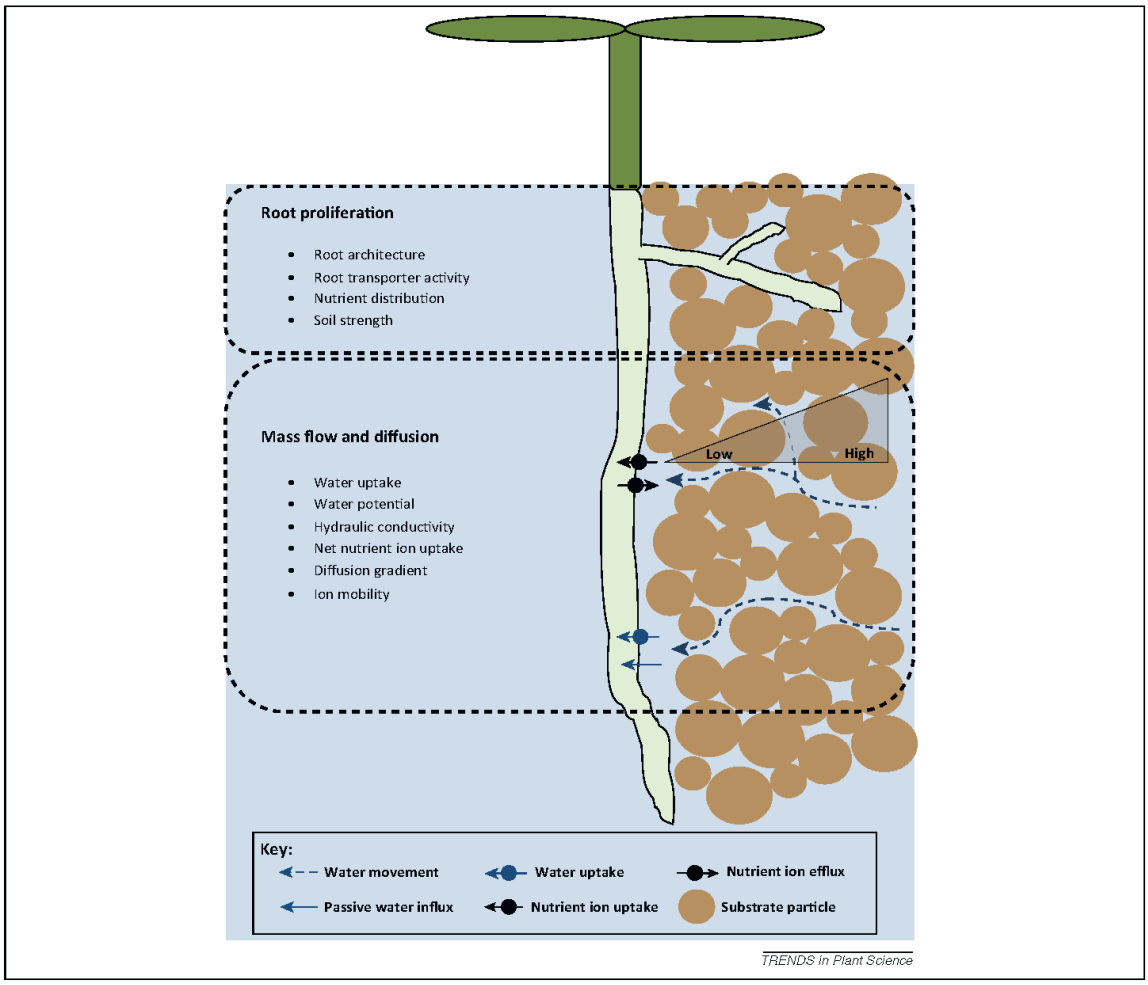

Figure 1. Nutrient acquisition depends on root proliferation and transporter function, exudation, symbioses, and diffusion of nutrient ions and mass flow of water to the root surface. These processes can be facilitated by root exudates to mobilise sparingly soluble nutrients and symbiotic associations with other organisms such as bacteria. Note that for root proliferation, soil strength is a biophysical factor while the other three listed are all biotic. These components are regulated by plant physiology and the physical properties of the substrate.

experimental systems in facilitating the study of these processes are reviewed, pointing out potential problems associated with translating laboratory understanding to the field. The major nutrient nitrogen (N) has been identified as one of the most important targets for improving root acquisition efficiency [5,6,12,25–27] and will be used here to illustrate root nutrient acquisition processes. The contribution of soil physical characteristics to N availability is varied due to the presence of both mobile and immobile forms in the soil. This is particularly true for the delivery of nutrients to the root and the consequent root responses.

Delivery of nutrients to the root surface

Mass flow and diffusion are responsible for the fluxes of water and dissolved nutrients to the root surface from bulk soil and are inextricably linked to its physical properties (or those of the growth substrate). The water potential gradient from the atmosphere to the bulk soil initiates the 'transpirational influx' that is responsible for mass transport of dissolved nutrient ions [8] and can have large effects on root growth and development [28]. A diffusion gradient of dissolved ions within the soil solution towards the root surface is generated by the influx of ions causing localised depletion at the root surface [8]. Therefore, the flux of water and dissolved ions from the soil towards the root is determined by differences in water potential, soil hydraulic conductivity and the shoot-transpiration demand.

These processes have been modelled [29], but the transport processes occurring in laboratory experimental systems are often different from those which occur in soil. Hydraulic conductivity and diffusivity are non-linear functions of matric potential and the shape of these functions, although similar in form, differs greatly between different soil types. Hydraulic conductivity in sandy soils can drop

by several orders of magnitude as soil dries by just a small amount from saturation to matric potentials as high as –100 kPa (i.e., moist soil). Water supply also influences the concentration of nutrients at the root surface. When most N uptake occurs as nitrate, the process depends on delivery of dissolved nitrate ions in the soil water to the root surface, whereas a low hydraulic conductivity in dry soils necessitates effective root architecture for nitrate acquisition.

For diffusive fluxes, the structure of the growth substrate has some influence due to the tortuous nature of diffusion via networks of water-filled pores in porous substrates [30]. This can be accounted for by the introduction of an impedance factor to estimate the diffusion of an ion in soil compared to water [22]. For nitrate, the effective diffusion coefficient in soil is several orders of magnitude lower than that in free water due to soil physical factors impeding diffusion [8]. The contribution of the diffusion flux to root N acquisition is dependent on the ionic form of the nutrient [31] as there is a large difference in effective soil diffusion coefficients between nitrate (3.26×10^{-10} m^2 s^{-1}) and ammonium (2.70×10^{-12} m^2 s^{-1}) [32]. Thus, the contribution of diffusion flux to acquisition of nitrate by the root is likely to be greater than that for ammonium, one reason for this is the binding of ammonium to clay particles [22]. In the field, the rate of diffusion is further influenced by fertiliser application, which determines concentration in the soil; and by temperature [32,33], although the latter is likely to have a greater effect on cell membrane transporter activity than diffusion rate itself.

Root responses to nutrient delivery

Root system architecture (RSA) is dependent on the continued growth and branching of the root in response to internal and external cues [34]. Primary root growth is modified in response to physical challenges [35] and is essential for access to deep water that determines aboveground yield [36]. Lateral branching from the primary axis is extremely plastic [37] and together their growth determines the size of the root system in response to environment stimuli [38]. The characterisation of RSA responses to the supply of nutrients is the focus of significant research effort and has identified some fundamental nutrient-specific responses [16]. For example, primary root growth in many higher plants is unreactive to changes in nitrate concentration [39,40], but lateral roots proliferate in response to high nitrate patches when plants are grown on low nitrate supply. This proliferation is not seen when seedlings are supplied with uniformly high nitrate [41,42].

Root proliferation is particularly important for the acquisition of nutrient ions that are only able to move small distances within the soil, whether due to physical isolation or decreased mobility. The restricted movement of ions due to their physical isolation is often the result of soil structure, where the formation of connected networks of solution is restricted by the distribution of pores (Figure 2) and can affect nutrient acquisition regardless of the ion's mobility. The previously described lateral root proliferation response to nitrate, itself a very soluble and mobile ion, may indicate a foraging strategy evolved to compete for isolated nutrient pockets in the heterogeneous field environment. While also important for more mobile ions at low water availability, root proliferation remains an important adaptive trait for roots to access less mobile nutrient ions, such as ammonium and phosphate [43–46]. There is even a distinct RSA response to small differences in mobility between different N forms [47], whereby the less mobile ammonium induces lateral root initiation while nitrate regulates proliferation [48].

Root growth responds to water supply, termed hydrotropism [49]. Primary root growth relative to shoot growth has been shown to increase as soil water potential decreases [50,51], in a process independent of nitrate supply [40]. Lateral roots proliferate in response to wet soil [52] and particularly to altered osmotic potential [53]. Furthermore, water content determines the strength of a soil which increases as soil drying occurs, potentially leading to the

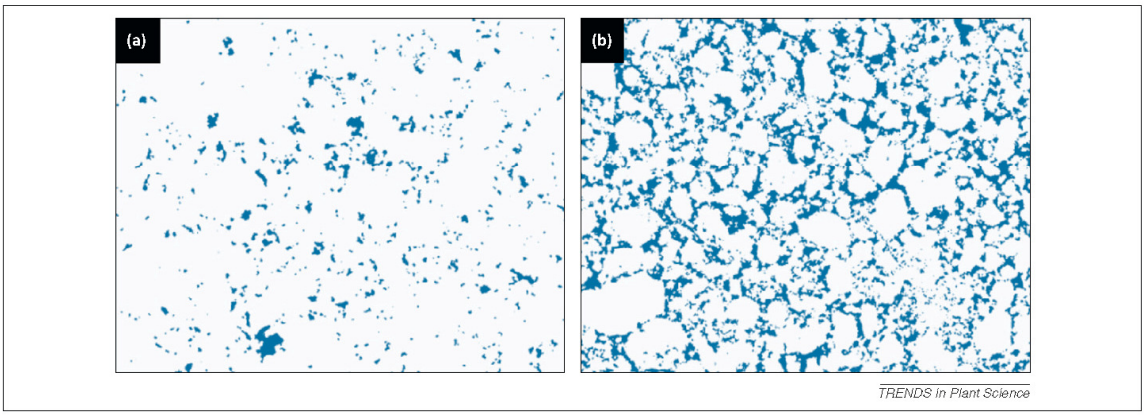

Figure 2. Diffusion and mass flow of nutrients to the root surface are dependent upon the particle size and pore structure of a substrate. The soil pore system of two samples has been characterised using X-ray computed tomography (CT) scanning where particles (white) and pore space (blue) can be distinguished, redrawn from [138]. Both of these images are of the same soil, but **(a)** is compact and **(b)** is loose. Compaction decreases the ability of the soil matrix to transport water and nutrients (a lower saturated hydraulic conductivity) because the pores in compact soil **(a)** are smaller. Changes in pore size also alter the relationship between matric potential and soil water content. In summary: even for a single soil type there can a wide range of soil physical characteristics depending on how the soil is managed or damaged by agricultural practice.

mechanical impedance of root growth [54]. An extreme deduction from a soil physics analysis is that the root proliferates in wet soil because the soil is 'weak' rather than because its matric potential is high [52]. Although it is now understood that the root response to water is more sophisticated than this [40,50], the ability of 'strong' soil to restrict root growth may have been overlooked [55]. In the field environment, the soil can be sufficiently strong to seriously impede root elongation at matric potentials as high as −80 kPa, where a 75% reduction in root growth has been reported [55].

Significant research effort has been paid to the investigation of root transporter function in facilitating net influx of water and nutrient ions into the root. The adoption of *Arabidopsis* for genetic studies has led to a vastly improved understanding of the physiological and molecular mechanisms underlying root nutrient acquisition, including the identification of high and low affinity transporter families [56–59]. Plants have complex systems for the uptake of nitrate across a wide range of concentrations which is mediated by nitrate transporters (NRTs) [60–67]. For example, the expression and activity of the two component high-affinity nitrate transporter AtNRT2.1/NAR2.1 is directly related to nitrate uptake and content [68,69]. The consequent characterisation of root transporters has provided significant understanding of root water and nutrient uptake [15,70] and led to the identification of important nutrient sensing mechanisms [12]. Transporter expression directly impacts on nutrient acquisition; for example, mutant plants lacking specific NRTs are much smaller than wild type plants [68,69].

As the trans-membrane transport of nitrate is electrically sensitive, the flux of ions can be determined using ion-selective microelectrodes [71,72]. This provides a non-destructive method for assaying internal ion concentration [73] and thus estimating the influx rate when external supply is known [72]. A great deal of information on N transport, storage, and remobilisation has been gained from measurements on maize (*Zea mays*) [74,75], rice (*Oryza sativa*) [76], canola (*Brassica napus*) [77], and soybean (*Glycine max*) [78]. Although there is evidence that soil strength can differently affect the expression of phosphate and sulphate transporters [79], the effect of multiple stress environments on NRTs has not been investigated.

There is also scope to improve our understanding of water uptake responses to physical changes in the root growth environment. The movement of water across cell membranes is partly facilitated by water channels known as aquaporins [13,80,81] and their ability to respond rapidly at the transcriptional level to environmental stimuli means they are useful markers of the potential conductance of water across plasma membranes. For example, the *Arabidopsis* plasma membrane intrinsic protein (PIP) gene *AtPIP2.2* is restricted to the root and facilitates root water uptake under low transpiration conditions, with its expression is significantly downregulated in response to drought and recovers upon rehydration [82–86]. However, few studies have investigated the direct influence of simultaneous physical changes associated with changing water availability in soils. Importantly for both water and nitrate uptake, plant biologists have calibrated the uptake kinetics (K_m and V_{max}) of plant roots and membrane transporters with concentration units usually derived from hydroponic experiments which may not reflect the field situation [87].

A great deal of understanding regarding root responses to nutrient supply has come from simplified experimental systems that were developed to cope with the experimental challenges presented by soil. Soils provide a complex root-growth environment for the study of nutrient acquisition, with each type having an intrinsic nutrient composition and microflora that impact on root nutrient acquisition [88]. A major difficulty with the experimental use of soil is that as the roots dry the soil, several abiotic factors change simultaneously [24,89]. For example in a drying soil the water potential decreases, mechanical impedance increases, oxygen availability increases and the water flux (and hence dissolved nutrients) to the root decreases. This raises the question; which factor is the plant actually responding to? Therefore, the characterisation of plant traits in simplified experimental systems may limit our understanding of root nutrient acquisition in the field, unless the physical properties of the soil and their role in nutrient mass flow and diffusion are considered.

The physical limitations of experimental systems

To overcome the interpretational difficulties of 'multi-stress' environments (e.g., soil), 'single-stress' experimental systems became popular and have facilitated considerable advancement in the understanding of root nutrient acquisition. Experimental systems with simplified root growth environments have been developed in order to non-destructively analyse the independent influence of experimental factors on root growth under regulated laboratory conditions [37,56]. Complementing field studies [45,90], nutrient regulation of root development and physiology has been investigated using a wide variety of experimental systems, including experiments with porous media such as soil [91,92] or sand [40,93], non-porous agar or gel cultures [94,95], and hydroponics [96,97].

The term 'hydroponics' was coined in the early 20th century during the promotion of aquaculture for use in commercial crop growth [98]. Initial problems of poor aeration and insufficient recycling of the nutrient solution were overcome [99] and hydroponics has been widely adopted as a laboratory tool. It provides advantages including sustained nutrient supply, relative sterility, enabling the use of plants with shorter life-cycles for rapid small-scale studies. Hydroponics has been used extensively for the investigation of aquaporins [97,100–103], NRTs [96,104], and to a lesser extent for the measurement of RSA [105,106] and ion fluxes [75]. However, the lack of physical structure within the hydroponic root growth environment means that the soil physical aspects of root nutrient acquisition encountered in the field cannot be reproduced or approximated.

The use of gel substrates to investigate root nutrient acquisition has proved to be an effective research tool. The widespread adoption of translucent, sterile and small-scale agar/gel systems has enabled remarkable progress to be made in studying the relationship between root morphology and nutrient supply. This is particularly true for the

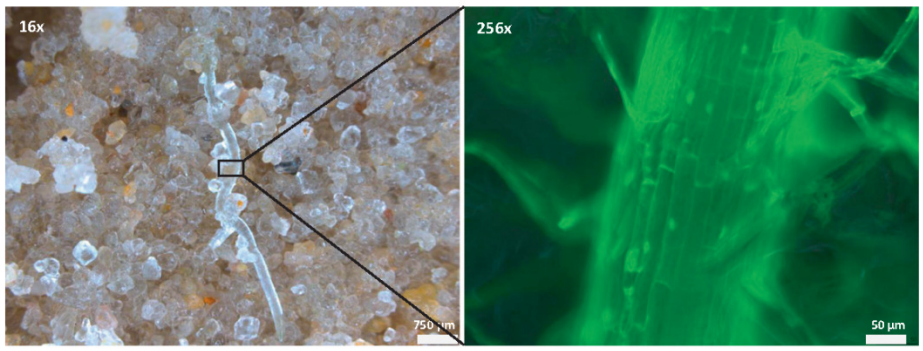

Figure 3. *In situ* imaging of an AtNRT2.1:GFP seedling within a sand rhizotron system. It is possible to investigate root nutrient acquisition in a porous growth substrate and still retain the superior molecular tool-kit offered by a model species such as *Arabidopsis*. Here, nitrate transporter (NRT) expression under good water supply (–1.5 kPa) is visualised non-destructively in a sand rhizotron system. Images were taken by NC with a stereo light-microscope (M205 FA, Leica, UK) at 16× and 256× magnification using a GFP2 filter (excitation 480 nm and emission 510 nm) and LAS-AF software (Leica, UK). The AtproNRT2.1:eGFP line [67] was a gift to AJM from Takatoshi Kiba (Riken Institute, Japan).

activity of nutrient ion transporters and aquaporin water channels, the genetic regulation of RSA [42,107], and even the development of roots grown in space [108]. It is also possible to characterise the physical properties of agar/gel substrates for the investigation of nutrient acquisition. When agar/gel substrates are used to apply water deficit treatments, the water potential of the substrate is usually manipulated by the introduction of an osmoticum, such as polyethylene glycol (PEG) [109] or via an increase in substrate concentration [110,111], and water potential can be measured [112]. However, an attempt to use agar of different concentrations to investigate the effects of mechanical impedance in fact showed that decreased root elongation was due to an unknown non-mechanical effect [111], illustrating the limitation of agar plates for manipulating the physical aspects of nutrient acquisition.

Translucent agar/gel substrates are used for root 'phenomics', overcoming the problem of opacity associated with many porous substrates and have been used with impressive high-throughput automated imaging systems to characterise fine RSA changes in response to different treatments across several species [56]. Indeed, most of our understanding of RSA changes in response to nutrient provision has come from agar/gel systems [94,107]. However, attempts to readdress the problem of opaque porous substrates are being made [37,113]. X-ray imaging technology is being used to visualise RSA and soil physical characteristics, and the *in situ* fluorescent-imaging of nutrient transporter reporter lines can visualise expression changes in response to manipulations of water and nutrient supply (Figure 3).

Various porous substrates have been used for root studies, including rubber or glass beads [114], vermiculite [115,116], perlite [117,118], and sand [40,119]. Of these, sand culture has historically been favoured to study the nutritional response of RSA [41]. While simplifed experimental systems have explored the effects of low water potential, sand culture has also been developed to explore the effect of high mechanical impedance on root and whole-plant growth [120,121]. Here, the sand is confined by an applied pressure, but is well supplied with nutrient solution which is important because a strong root growth environment can reduce root proliferation, tiller number, and leaf elongation even if water and nutrient supply is adequate [120]. These substrates have become useful for nutrient acquisition studies as they have a pore structure which has similarities to soil, but lack the biotic and abiotic complexities of soil.

Despite the limitations of simplified 'single-stress' experimental systems, they have been reasonably successful in translating results from the laboratory to the field [122]. Studying root nutrient acquisition in the field is the ideal scenario to understand the real-world influence of physical components and reduce the time taken to transfer understanding to yield gains. However, there are experimental limitations to field studies which include difficulties in accessing roots and regulating the environment [89]. The use of pot/rhizotron systems to study root nutrient acquisition in soil has removed some of the limitations associated with field studies and increased our understanding of RSA nutrient responses [123–125]. One investigation, which considered the physical properties of the soil, used pot experiments to determine the correlation of abscisic acid concentration in the root with water content, matric potential, and penetrometer resistance of the soil [91]. Soil was also used in a proteomics study investigating responses to drought caused by cessation of watering [92], but the individual soil physical properties were not characterised. In addition to the previously discussed general problems with soil, there are limitations in the volume of substrate that influences oxygen availability to the root and water content [126].

The development of simplified experimental systems was driven by interpretational difficulties associated with soils and has been extremely useful in advancing our understanding of plant responses to nutrient supply. However, the advantages of each experimental system have been a trade-off for restricting (to varying degrees) the consideration of the soil physical characteristics which remain central to nutrient acquisition in the field

environment. This begs the question: to what extent are the physical restrictions of experimental systems limiting our understanding of root nutrient acquisition?

How are the physical properties of experimental systems relevant?

The structure of non-porous substrates (agar/gel) means that the physical properties are different from porous substrates (sand or soil) (Table 1). The contribution of diffusion and mass flow in non-porous substrates is unlikely to be representative of porous substrates, let alone field conditions, particularly as pore size and distribution regulate the hydraulic conductivity and thus the availability and mobility of dissolved nutrient ions. Given the significant influence of the physical properties of the substrate on root nutrient acquisition processes and the simplified physical root growth environment provided by experimental systems, it is perhaps not surprising that disparities between experimental systems have been reported.

For *Arabidopsis*, several RSA parameters have been found to differ between seedlings grown in sand rhizotrons and those grown in agar Petri dishes: roots were shorter in sand than in agar [40]. However, the relative change in each root trait was similar, for example, primary root length was longer than lateral root length in both sand and agar culture. The differences in root traits between agar and sand culture methods was attributed to a difference in substrate structure and the volume of available nutrients between each system. RSA of barley (*Hordeum vulgare*) subspecies has also been found to be inconsistent for plants grown in gel chambers versus soil culture [127]. The total root length and average root diameter of seedlings grown in soil in sacks or pots were significantly smaller than those grown in gel chambers. The same disparity between gel chambers and soil media (columns and field) has been described during the screening of root characteristics in dwarf wheat (*Triticum aestivum*) cultivars [128]. The total root length of dwarf lines increased by ~40% in gel chambers and decreased by ~24–33% in soil. These two studies found that for soil the results of each method used were consistent with each other, but they differed from the results of the gel studies, further highlighting the impact of physical properties of the experimental system. The direct impact of the different substrates used in many model experimental systems on root nutrient acquisition has not been studied.

The physical limitations of experimental systems are particularly important for laboratory-based trait-screening methods because interactions between physical and nutritional factors are complex. For example, root responses to the water content of agar were not explained by its mechanical strength, but agar strength increases as water content decreases [111]. Therefore screening plants for improved traits under osmotic stress using agar systems may lead to the identification of traits for mechanical impedance that would not be translatable to the field. Another example can be found from the use of vermiculite to replicate low water potential in a seed-bed: the data suggest that, provided a seed germinates, the shoot will emerge even at water potential of −1.64 MPa [116]. By contrast, a 'strong' soil at relatively high water potentials prevents shoot elongation [54].

However, laboratory root screening systems may be difficult to interpret even when comparing porous experimental systems to the field. For example, rice cultivars identified as having good root penetration via a flooded wax layer system or a sand-core system did not always translate to good hard-pan penetration in the field [129]. This was speculated to be due to an improved root penetration response to a gradual, rather than abrupt, increase in soil strength which was not replicated using the laboratory system [130]. This may reflect the differences between the complex field and more simple laboratory test environments. The complex nature of concurrent changes in physical properties within soil perhaps explains why even comparisons between porous experimental systems produce disparities in the field.

Therefore, the physical characteristics of the experimental system are important factors that influence root nutrient acquisition and the limitations of each system must be considered in the interpretation of results. The sensitivity of the component factors affecting nutrient acquisition (e.g., root proliferation, transporter function, mass flow, and diffusion) are difficult to quantify and may

Table 1. Physical parameters that determine nutrient acquisition by roots[a]

Physical parameter	Agar/Gel	Sand	Soil
Pore size	Small	Narrow range	Wide range
Pore distribution	Regular	Approximately normal	Log normal
Pore structure	Limited	Narrow	Complex
Matric potential	Increases with substrate concentration	Function of saturation	Function of saturation
Saturated hydraulic conductivity	Low	Decreases with pore size	Decreases with pore size
Unsaturated hydraulic conductivity	Determined by diffusion gradients	Function of saturation	Function of saturation
Strength	Increases with substrate concentration[b]	Decreases with increasing saturation by a limited amount, but can be manipulated independently with applied mechanical stress	Decreases greatly with increasing saturation
Nutrient ion distribution	Homogeneous	Heterogeneous over a narrow range of scales	Heterogeneous over a wide range of scales

[a]The physical parameters that determine nutrient acquisition by roots vary between substrates and these differences could account for disparity of results between experimental systems.

[b]However, this cannot be used as an experimental variable [111].

be different in each screening system. As a result, there is no single experimental system that can address all questions [37] and a greater understanding of the interaction of root and soil components of nutrient acquisition is likely to be gained by the combined use of several systems. Comparing the responses of a genetically modified reporter plant (like *Arabidopsis*) in these experimental systems can provide a way of identifying the differences between them.

Opportunities uniting plant and soil science to achieve productivity gains

Because field data is difficult to interpret, our understanding of root biology has been derived from using simplified experimental systems. This has led to a plant-centric view of root nutrient acquisition that lacks consideration of the contribution of symbionts, root exudates, and soil physical components. Extensive research effort has been directed to the characterisation of RSA despite the fact that the majority of applied nutrients (e.g., N) in the field are delivered to the root via mass flow and diffusion fluxes rather than root proliferation [131]. Characterising plant transporters involved in the influx of water and nutrient ions and assessing cellular ion concentrations has gone some way in improving understanding of the processes driving mass flow and diffusion, but neglects the influence of the soil physical properties on the delivery of nutrients to the root surface. Experimental systems have simplified root growth environments and restricted the necessary consideration of soil physical properties – a restriction that is emphasised by root growth disparities between substrates. Different experimental systems will continue to be used for the investigation of root nutrient acquisition, so it is necessary for researchers to remain aware of the physical limitations of each if we are to translate experimental understanding to potential gains in crop yields.

Recent advances in microbial metagenomics and structural tomography of roots in soil are generating a better understanding of soil that will undoubtedly reveal more limitations in our current experimental systems. It is vitally important that the emergent X-ray tomographic technologies [132] are used in conjunction with an understanding of how the imaged soil structures affect the ability of the soil to supply water and nutrients, as well as what the implications of the soil structures are in terms of physical stress (e.g., matric potential and soil strength). Tomographic approaches need to provide more than images to fully realise their potential and produce 3D maps of the abiotic stress environment. This is a challenge to soil physicists, who must also explore alternative imaging technologies based on elastic waves or NMR which offer the possibility for detection of soil strength and matric potential respectively [133].

The potential for improving plant yield through more effective root nutrient acquisition is likely to be realised through better understanding of the interaction between soil physical properties and root traits (summarised in Box 1). In a changing environment, soil water content will become even more important for both mass flow and diffusion components of root nutrient acquisition. Increased water content increases hydraulic conductivity, which facilitates the movement of water and dissolved ions over relatively large distances via mass flow, whereas decreased water content serves to concentrate nutrient ions and thus decrease the diffusional flux [22]. Furthermore, hydraulic conductivity and water content of soil influence root proliferation [40] and the activity of some ion transporters [79]. It is important to understand how changes in soil water status affect both the plastic response of the root system as well as the ability of the soil to supply water and nutrients. Such understanding will allow results obtained with model systems to be applied to simulation models [134] and the field environment [135].

If we are to capitalise on the potential productivity gains from understanding the importance of physical properties for root nutrient acquisition, it is essential that the plant scientist's wealth of knowledge regarding the nutritional regulation of plant physiology and the soil scientist's understanding of complex changes in the physical properties of the soil become even more closely integrated with the common aim of working towards improved food security [136]. To better interpret root function, soil scientists must develop new technologies customised to describe soil physical characteristics, such as water flow, stress, and soil strength, in more detail and in ways that allow images to be constructed. Improved methods for imaging of nutrient availability in the soil and rhizosphere are required to better describe how soil physical processes influence nutrient delivery to the root. An integration of these approaches with the use of gene-specific mutants and reporter plants offers an exciting way forward for determining the role of soil physical parameters in nutrient acquisition.

Box 1. Outstanding questions

- Improved root nutrient acquisition is a promising component of a sustainable solution to securing our food supply in the face of a changing climate.
- The adoption of experimental systems with simplified root growth environments has advanced our understanding but there is still much scope to improve understanding of the contribution of soil physical processes to root nutrient acquisition.
- The physical limitations of experimental systems influence our understanding of root proliferation and transporter activity and the delivery of dissolved nutrients to the root surface via mass flow and diffusion fluxes.
- Owing to the complex nature of concurrent changes in soil factors, a lack of consideration of the physical limitations of experimental systems could lead to the failure to identify desirable root traits in laboratory-based root phenotype screening methods.
- The greatest potential productivity gains are likely to be achieved by a better understanding of the interaction between plant traits and soil physical properties that determine the delivery of dissolved ions to the root.
- If potential gains in securing our food supply are to be realised in the field, an even closer integration of the expertise of plant and soil scientists is needed to develop new methods and understand the limitations of current root experimental systems.

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Appendix 4. cDNA populations

The concentration of each cDNA population determined using a Nanodrop Spectrophotometer.

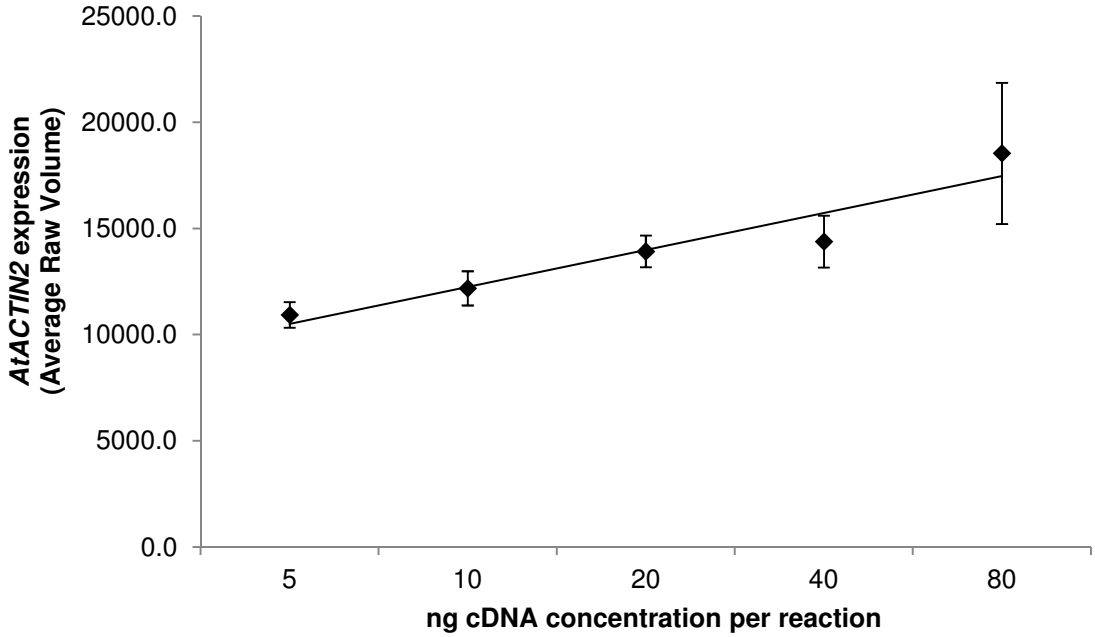
ID	Treatment	[cDNA] ng/µl
1	2% agar, 10 mM KNO_3	1143.91
2	2% agar, 10 mM KNO_3	1163.40
3	Redhill T sand, -3.0 kPa, 10 mM KNO_3	1403.78
4	Redhill T sand, -3.0 kPa, 10 mM KNO_3	1321.64
5	<250 µm sand, -3.0 kPa, 10 mM KNO_3	1477.97
6	<250 µm sand, -3.0 kPa, 10 mM KNO_3	1533.46
7	250-425 µm sand, -3.0 kPa, 10 mM KNO_3	1317.82
8	250-425 µm sand, -3.0 kPa, 10 mM KNO_3	1699.56
9	>425 µm sand, -3.0 kPa, 10 mM KNO_3	1428.31
10	>425 µm sand, -3.0 kPa, 10 mM KNO_3	1493.10
11	Redhill T sand, -1.5 kPa, 10.0 mM KNO_3	1457.26
12	Redhill T sand, -1.5 kPa, 10.0 mM KNO_3	1514.73
13	Redhill T sand, -1.5 kPa, 10.0 mM KNO_3	1404.05
14	Redhill T sand, -3.0 kPa, 10.0 mM KNO_3	1358.38
15	Redhill T sand, -3.0 kPa, 10.0 mM KNO_3	1316.41
16	Redhill T sand, -3.0 kPa, 10.0 mM KNO_3	1351.15
17	Redhill T sand, -4.5 kPa, 10.0 mM KNO_3	1433.78
18	Redhill T sand, -4.5 kPa, 10.0 mM KNO_3	1337.05
19	Redhill T sand, -4.5 kPa, 10.0 mM KNO_3	1330.35
20	Redhill T sand, -3.0 kPa, 0.1 mM KNO_3	1357.45
21	Redhill T sand, -3.0 kPa, 0.1 mM KNO_3	1343.31
22	Redhill T sand, -3.0 kPa, 0.1 mM KNO_3	1375.55
23	Redhill T sand, -3.0 kPa, 1.0 mM KNO_3	1283.79
24	Redhill T sand, -3.0 kPa, 1.0 mM KNO_3	1178.11
25	Redhill T sand, -3.0 kPa, 1.0 mM KNO_3	1449.57
26	Redhill T sand, -3.0 kPa, 10.0 mM KNO_3	1282.79
27	Redhill T sand, -3.0 kPa, 10.0 mM KNO_3	341.51
28	Redhill T sand, -3.0 kPa, 10.0 mM KNO_3	336.84
29	Redhill T sand, -1.5 kPa, 0.1 mM KNO_3	2458.83
30	Redhill T sand, -1.5 kPa, 0.1 mM KNO_3	2838.75
31	Redhill T sand, -1.5 kPa, 0.1 mM KNO_3	2645.73
32	Redhill T sand, -4.5 kPa, 0.1 mM KNO_3	955.22
33	Redhill T sand, -4.5 kPa, 0.1 mM KNO_3	2137.99
34	Redhill T sand, -4.5 kPa, 0.1 mM KNO_3	3728.29
35	Redhill T sand, -1.5 kPa, 1.0 mM KNO_3	1643.36
36	Redhill T sand, -1.5 kPa, 1.0 mM KNO_3	1798.79
37	Redhill T sand, -1.5 kPa, 1.0 mM KNO_3	1351.93
38	Redhill T sand, -4.5 kPa, 1.0 mM KNO_3	1384.66
39	Redhill T sand, -4.5 kPa, 1.0 mM KNO_3	2056.24
40	Redhill T sand, -4.5 kPa, 1.0 mM KNO_3	2005.66
41	<250 µm sand, -3.0 kPa, 0.1 mM KNO_3	1697.02
42	<250 µm sand, -3.0 kPa, 0.1 mM KNO_3	1421.10
43	<250 µm sand, -3.0 kPa, 0.1 mM KNO_3	1547.05
44	<250 µm sand, -3.0 kPa, 1.0 mM KNO_3	1683.89
45	<250 µm sand, -3.0 kPa, 1.0 mM KNO_3	1489.78
46	<250 µm sand, -3.0 kPa, 1.0 mM KNO_3	1385.81
47	250-425 µm sand, -3.0 kPa, 0.1 mM KNO_3	1510.01
48	250-425 µm sand, -3.0 kPa, 0.1 mM KNO_3	1682.33
49	250-425 µm sand, -3.0 kPa, 0.1 mM KNO_3	1612.86
50	250-425 µm sand, -3.0 kPa, 1.0 mM KNO_3	1407.67
51	250-425 µm sand, -3.0 kPa, 1.0 mM KNO_3	2243.92
52	250-425 µm sand, -3.0 kPa, 1.0 mM KNO_3	1863.62

The concentration of each cDNA population determined using a Nanodrop Spectrophotometer.

ID	Treatment	[cDNA] ng/µl
53	>425 µm sand, -3.0 kPa, 0.1 mM KNO_3	1543.05
54	>425 µm sand, -3.0 kPa, 0.1 mM KNO_3	2015.65
55	>425 µm sand, -3.0 kPa, 0.1 mM KNO_3	1723.61
56	>425 µm sand, -3.0 kPa, 1.0 mM KNO_3	1475.01
57	>425 µm sand, -3.0 kPa, 1.0 mM KNO_3	1642.40
58	>425 µm sand, -3.0 kPa, 1.0 mM KNO_3	1930.71
59	atnrt2.1, Redhill T sand, -1.5 kPa, 10 mM KNO_3	1641.05
60	atnrt2.1, Redhill T sand, -1.5 kPa, 10 mM KNO_3	1717.11
61	atnrt2.1, Redhill T sand, -1.5 kPa, 10 mM KNO_3	1368.66
62	atnrt2.1, Redhill T sand, -4.5 kPa, 10 mM KNO_3	1310.33
63	atnrt2.1, Redhill T sand, -4.5 kPa, 10 mM KNO_3	1337.32
64	atnrt2.1, Redhill T sand, -4.5 kPa, 10 mM KNO_3	1297.01
65	atnar2.1, Redhill T sand, -1.5 kPa, 10 mM KNO_3	2720.37
66	atnar2.1, Redhill T sand, -1.5 kPa, 10 mM KNO_3	3001.26
67	atnar2.1, Redhill T sand, -1.5 kPa, 10 mM KNO_3	2314.06
68	atnar2.1, Redhill T sand, -4.5 kPa, 10 mM KNO_3	2303.13
69	atnar2.1, Redhill T sand, -4.5 kPa, 10 mM KNO_3	2338.71
70	atnar2.1, Redhill T sand, -4.5 kPa, 10 mM KNO_3	2149.01
71	atnar2.1xNpNRT2.1, Redhill T sand, -1.5 kPa, 10 mM KNO_3	1249.59
72	atnar2.1xNpNRT2.1, Redhill T sand, -1.5 kPa, 10 mM KNO_3	1265.00
73	atnar2.1xNpNRT2.1, Redhill T sand, -1.5 kPa, 10 mM KNO_3	1266.75
74	atnar2.1xNpNRT2.1, Redhill T sand, -4.5 kPa, 10 mM KNO_3	1342.60
75	atnar2.1xNpNRT2.1, Redhill T sand, -4.5 kPa, 10 mM KNO_3	1277.64
76	atnar2.1xNpNRT2.1, Redhill T sand, -4.5 kPa, 10 mM KNO_3	1260.09
77	atpip2.2, Redhill T sand, -1.5 kPa, 10 mM KNO_3	1307.75
78	atpip2.2, Redhill T sand, -1.5 kPa, 10 mM KNO_3	1253.81
79	atpip2.2, Redhill T sand, -1.5 kPa, 10 mM KNO_3	1362.35
80	atpip2.2, Redhill T sand, -4.5 kPa, 10 mM KNO_3	1554.21
81	atpip2.2, Redhill T sand, -4.5 kPa, 10 mM KNO_3	1708.06
82	atpip2.2, Redhill T sand, -4.5 kPa, 10 mM KNO_3	1394.08

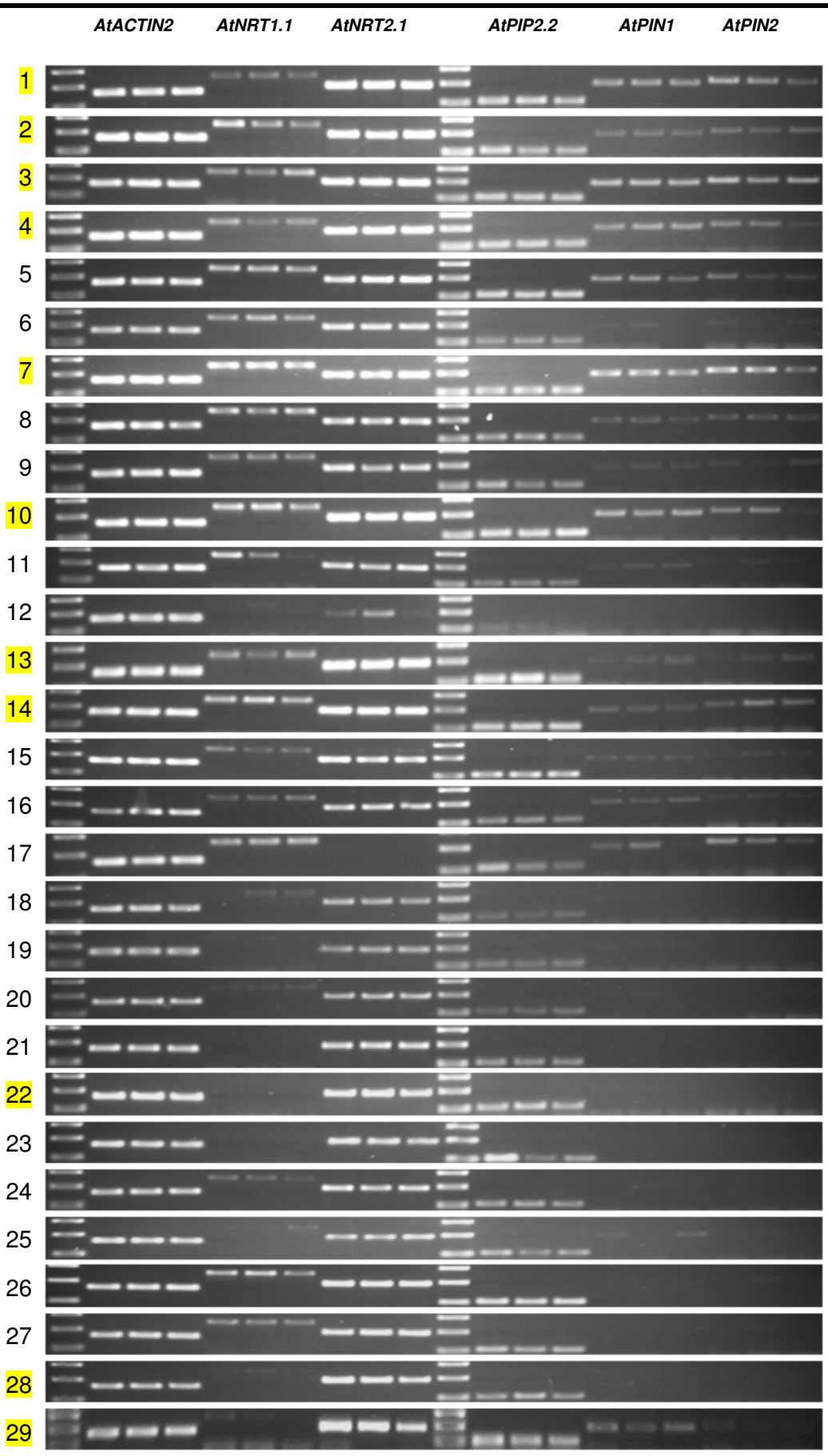
Absence of gDNA in cDNA populations was confirmed by RT PCR for *AtAPT1*. 1-82 refers to the cDNA populations in Table 2.1.; 83, no cDNA control; 84-85, gDNA control.

***AtACTIN2* expression (average raw volume) demonstrates a linear relationship (r^2 = 0.91) with increasing cDNA concentration present in the RT PCR reaction.** Bars = SEM.

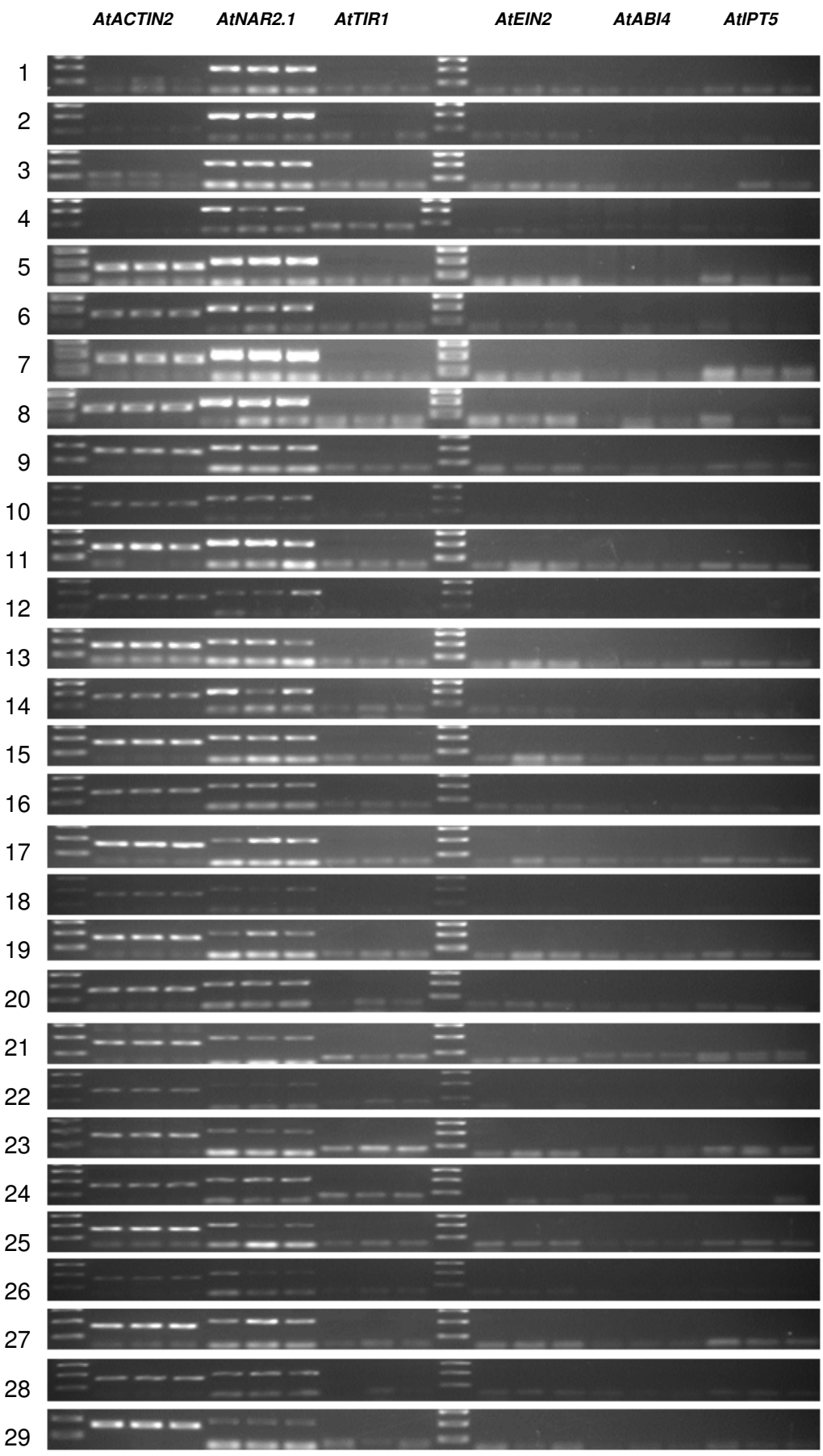


Appendix 5. Gel electrophoresis images.

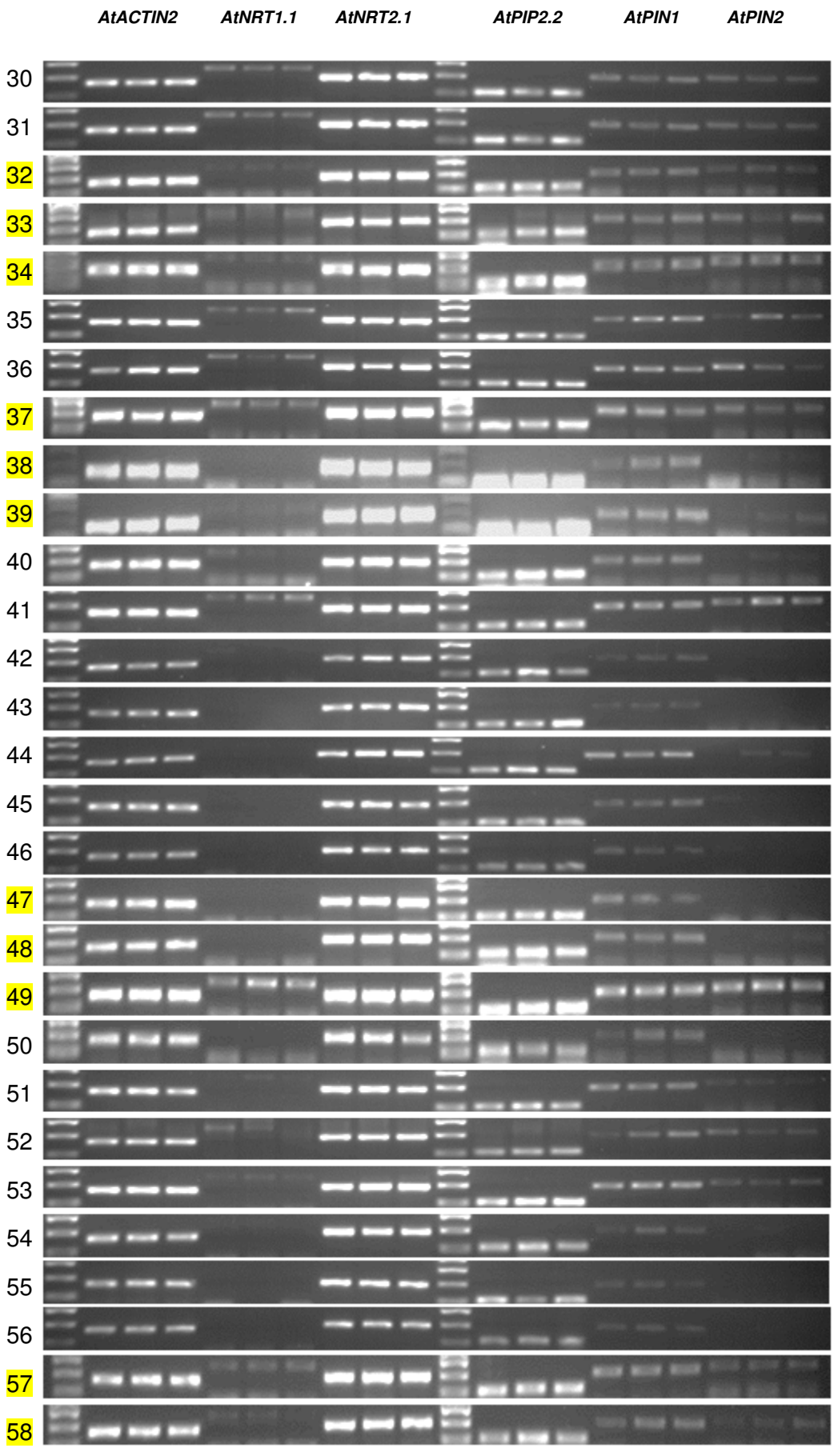
RT PCR gene expression analysis. 1-29 refers to the cDNA populations. Ladder bands: 100, 200 and 300 bp. Highlighted gels are those that may have an *AtNRT2.1* saturation problem.



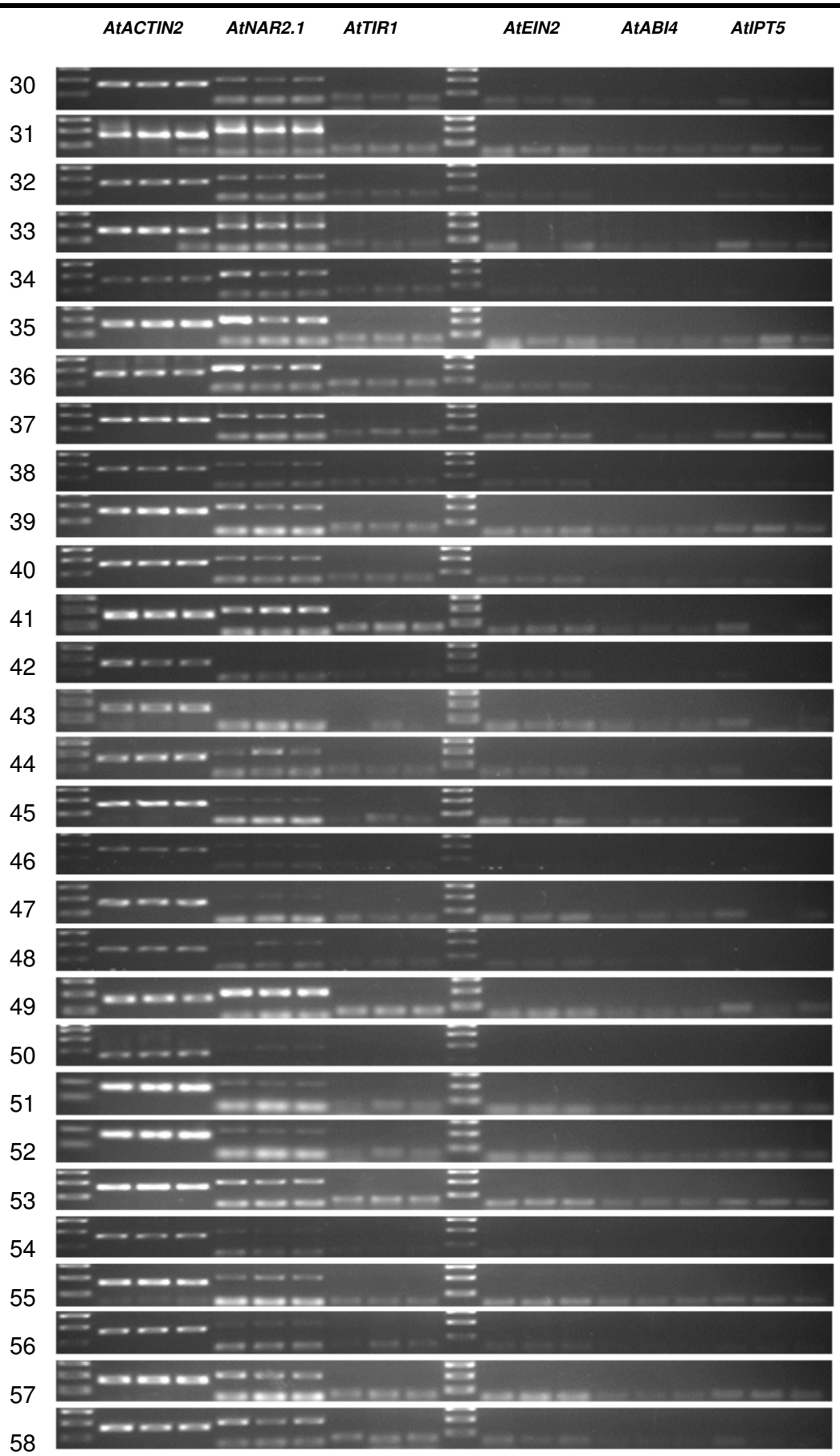
RT PCR gene expression analysis. 1-29 refers to the cDNA populations. Ladder bands: 100, 200 and 300 bp.



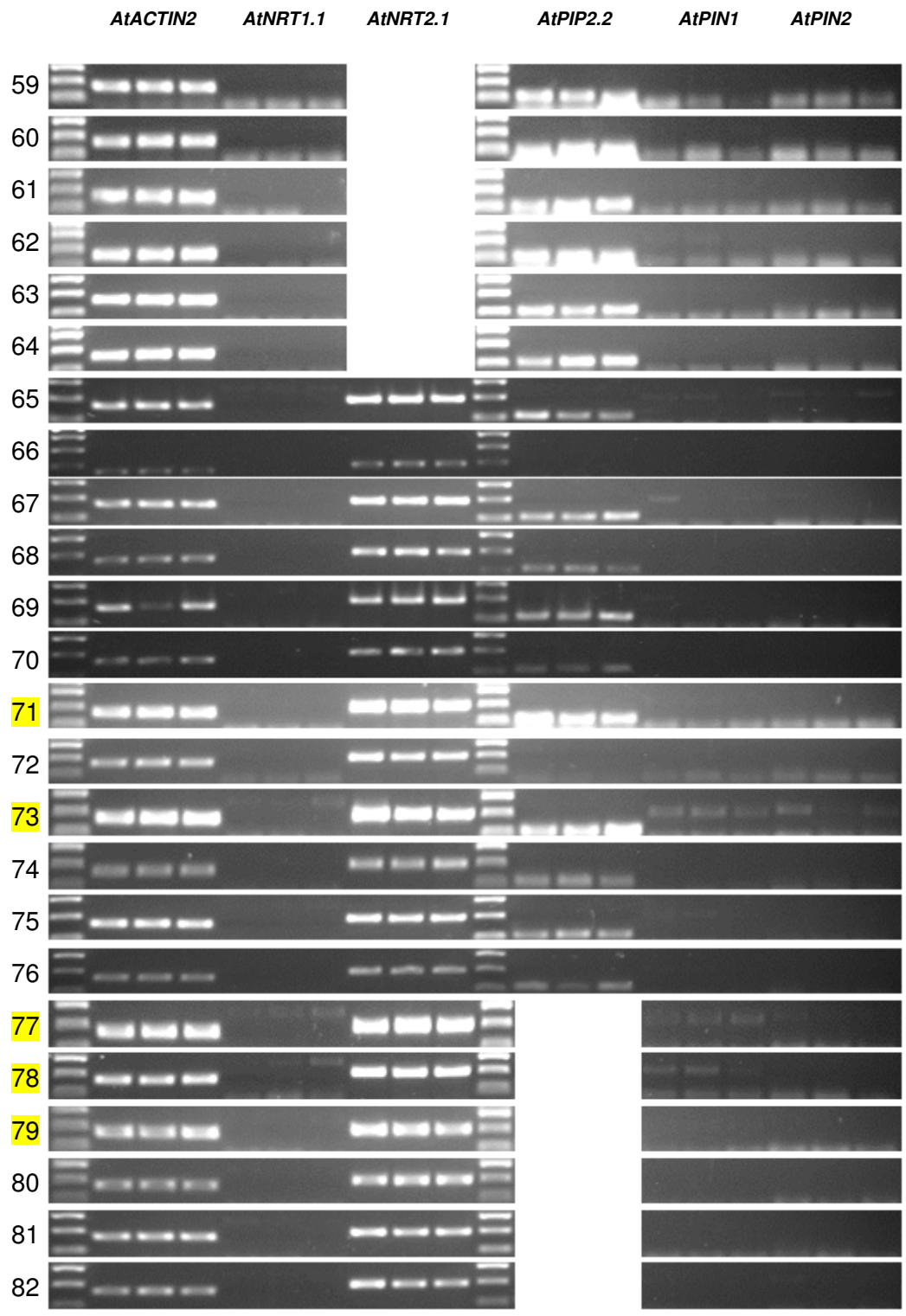
RT PCR gene expression analysis. 30-58 refers to the cDNA populations. Ladder bands: 100, 200 and 300 bp. Highlighted gels are those that may have an *AtNRT2.1* saturation problem.



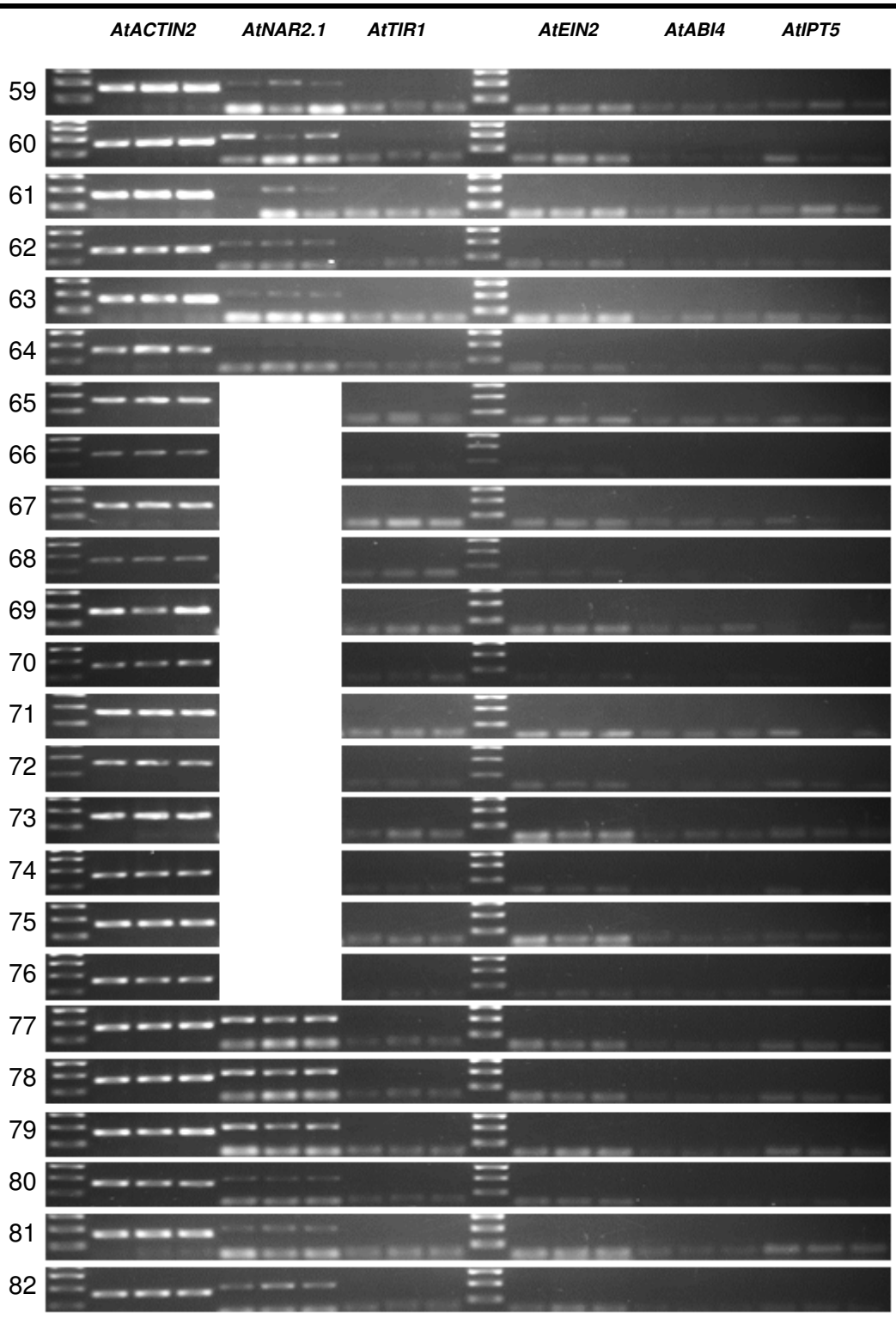
RT PCR gene expression analysis. 30-58 refers to the cDNA populations. Ladder bands: 100, 200 and 300 bp..



RT PCR gene expression analysis. 59-82 refers to the cDNA populations. Ladder bands: 100, 200 and 300 bp. Highlighted gels are those that may have an *AtNRT2.1* saturation problem.

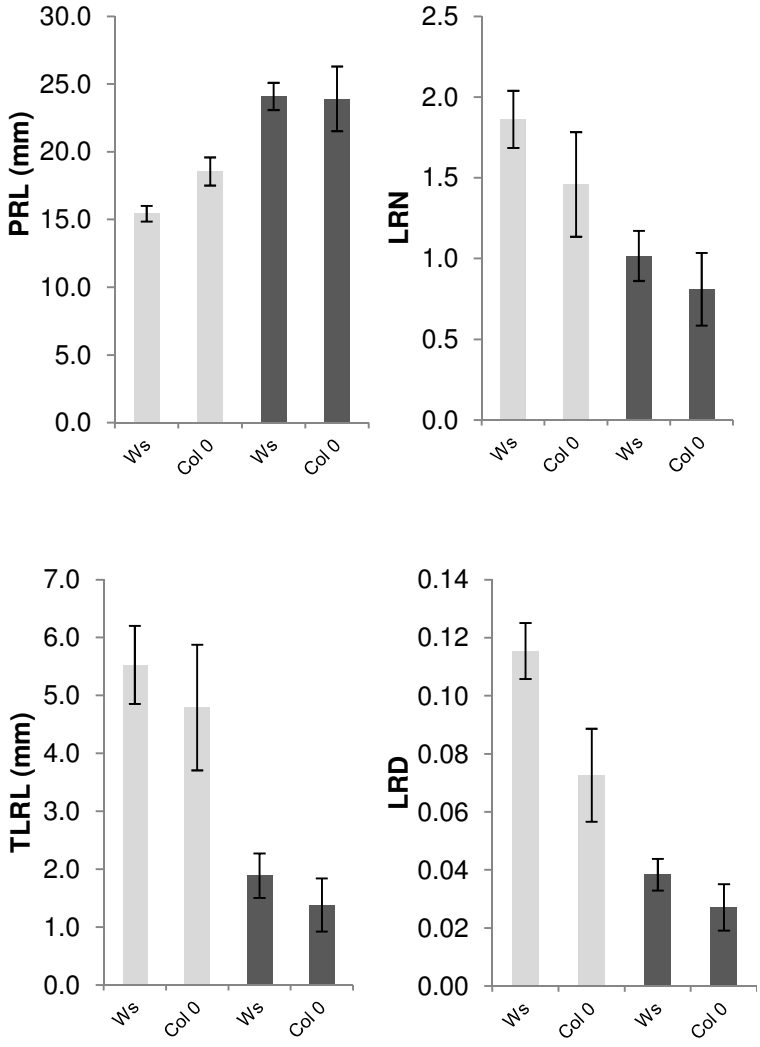


RT PCR gene expression analysis. 59-82 refers to the cDNA populations. Ladder bands: 100, 200 and 300 bp.



Appendix 6. Conservation of root proliferation responses between ecotypes.

Root proliferation responses to extremes of unsaturated hydraulic conductivity (K_{unsat}, $m.d^{-1}$) were conserved between Arabidopsis ecotypes Ws and Col 0. Treatments: ■ 1.35 $m.d^{-1}$; ■ 0.18 $m.d^{-1}$.



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