Dissection of nitrate signalling in

Arabidopsis thaliana

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I declare that this is my own work and that any contribution made by other parties is clearly acknowledged.
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<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AMT</td>
<td>ammonium transporter</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>C</td>
<td>carbon</td>
</tr>
<tr>
<td>CaMV</td>
<td>Cauliflower Mosaic Virus</td>
</tr>
<tr>
<td>CCD</td>
<td>charged-coupled device</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>cHATs</td>
<td>constitutive high affinity transport system</td>
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<td>CHX</td>
<td>cycloheximide</td>
</tr>
<tr>
<td>Col Wt</td>
<td>Columbia 0 ecotype (wild type)</td>
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<tr>
<td>CTR</td>
<td>constitutive triple response, a MAPK kinase</td>
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<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
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<td>EIN</td>
<td>ethylene insensitive</td>
</tr>
<tr>
<td>EREs</td>
<td>ethylene response elements</td>
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<tr>
<td>ERF</td>
<td>ethylene response factor</td>
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<td>ERS</td>
<td>ETR1-related sequence</td>
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<tr>
<td>ETR</td>
<td>ethylene receptor</td>
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<tr>
<td>Fd</td>
<td>ferredoxin</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>GOGAT</td>
<td>glutamate synthase (glutamine-2-oxoglutarate aminotransferase)</td>
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<tr>
<td>GS</td>
<td>glutamine synthetase</td>
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<tr>
<td>GUS</td>
<td>β-glucuronidase</td>
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<tr>
<td>HATS</td>
<td>high affinity transport system</td>
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<tr>
<td>iHATS</td>
<td>inducible high affinity transport system</td>
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<td>KIN</td>
<td>kinetin</td>
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<td>LATs</td>
<td>low affinity transport system</td>
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<tr>
<td>LB</td>
<td>Luria-Bertani medium</td>
</tr>
<tr>
<td>LR</td>
<td>Lateral root</td>
</tr>
<tr>
<td>LUC</td>
<td>luciferase</td>
</tr>
<tr>
<td>N</td>
<td>nitrogen</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MES</td>
<td>2-morpholinoethanesulfonic acid</td>
</tr>
<tr>
<td>MOI/s</td>
<td>mutant/s of interest</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-Morpholino)-propanesulfonic acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MS</td>
<td>Murashige and Skoog medium</td>
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<td>N</td>
<td>nitrogen</td>
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<tr>
<td>NAA</td>
<td>naphthalene-acetic acid</td>
</tr>
<tr>
<td>NR</td>
<td>nitrate reductase</td>
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<td>NRA</td>
<td>nitrate reductase activity</td>
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<td>nitrate transporter</td>
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<td>NIR</td>
<td>nitrite reductase</td>
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<td>Ω</td>
<td>omega sequence</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>3-O-MDG</td>
<td>3-O-Methyl-D-Glucose</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
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<td>P</td>
<td>phosphate</td>
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<td>PCR</td>
<td>polymerase Chain Reaction</td>
</tr>
<tr>
<td>PNK</td>
<td>polynucleotide kinase</td>
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<td>qPCR</td>
<td>quantitative PCR</td>
</tr>
<tr>
<td>RAN1</td>
<td>responsive to antagonist 1, a copper transporter</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcriptase PCR/ cDNA synthesis</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SSC</td>
<td>sodium aline, sodium citrate</td>
</tr>
<tr>
<td>Suc</td>
<td>sucrose</td>
</tr>
<tr>
<td>UPM</td>
<td>uroporphyrinogen III methyltransferase</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>Wt</td>
<td>wild type</td>
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Abstract

Nitrate is an important plant nutrient necessary for growth and development. Agronomically, nitrate is important for producing healthy protein rich crops and is a major constituent of fertilisers. Abundance of nitrate in the soil is variable, requiring plants to evolve adaptive growth responses in order to optimise uptake. The nitrate molecule induces the expression of genes involved in its uptake and assimilation and many other nitrate responsive genes have been identified. However, how plants sense nitrate is unknown. This thesis presents the design of a genetic screen to identify components of early nitrate signalling and perception. *A. thaliana* luciferase reporter lines of nitrite reductase NIR, and the ammonium transporter, AMT1.1, were characterised and found to be not suitable for use in a genetic screen. A new transgenic luciferase reporter line driven by the high affinity nitrate transporter NRT2.1 was generated and characterised and was found to be suitable to use in a genetic screen. Split root experiments with the NRT2.1 reporter lines showed that this gene responds to local signalling.

Signalling mechanisms involved in early nitrate signalling were identified by pharmacological analysis using *A. thaliana* cell suspension cultures. These revealed a novel sucrose-dependant cycloheximide (CHX) effect, where CHX exerts no effect on nitrate gene induction of *NIR* and *NRT2.1* when sucrose levels are high but when sucrose levels are low CHX treatment results in a reduction in the nitrate gene induction response. This may indicate a putative role for new protein synthesis in the nitrate gene induction response when sucrose is limiting. Investigation of the nitrate transport analog, chlorate, showed that this molecule is not a signalling analog as it failed to induce the nitrate responsive genes, *NIR* and *NRT2.1*. 
Chapter 1 - Introduction

1.1 Importance of nitrogen

Nitrogen is a major nutrient for plants that is vital for growth and development. As the key element in the amino group, nitrogen is required to make amino acids, proteins, nucleic acids, coenzymes and membrane constituents. Thus it is clear that nitrogen is essential for plant structure, function, growth and development. Nitrogen makes up approximately 2-5% of a whole plant’s dry weight (Marschner 1995). Nitrate stored in the vacuole is important for maintaining cation-anion balance, and osmoregulation. Marschner (1995) stated that ‘the importance of the reduction and assimilation of nitrate for plant life is similar to that of the reduction and assimilation of CO₂ in photosynthesis’.

1.2 Nitrogen availability in the soil

Plants obtain N in two ways: 1) from the atmosphere, via symbiotic relationships with N₂ fixing bacteria (although only some species of plants such as legumes, have these symbiotic associations) and 2) from the soil in the form of nitrate (NO₃⁻) or ammonium (NH₄⁺). Plants can also assimilate amino acids and urea (Parsons and Sunley, 2001) however, nitrate and ammonium are the most prevalent N sources (Marschner, 1995). The soil environment is highly heterogeneous for these two N nutrients (Glass et al., 2002). Most agricultural soils have a mixture of the two, with nitrate as the predominant N nutrient. The availability of these nutrients in the soil fluctuates by several orders of magnitude among different soils and as a result of seasonal changes. The nutritional demand of plants also varies with diurnal and seasonal growth changes (Glass et al., 2002). As nitrate is highly soluble it is easily lost from the soil by leaching or bacterial nitrification (López-Bucio et al., 2003). Soil type and pH also affect N availability; for example sandy soils drain water effectively but consequently may contain fewer nutrients such as soluble nitrate, whereas loamy soils hold water and soluble nutrients better rendering it an ideal soil for plant growth. In order to survive in an ever-changing environment, plants require perception of the nutrient type and availability and be able to integrate this with internal demand in order to produce adaptive growth responses.
1.3 Nitrogen in agriculture

Nitrogenous nutrients are added to fertilizers facilitating the production of protein rich, high quality crops. However, this amelioration of the soil through fertilizer is not always efficient; Raun and Johnson, (1999) stated that ‘worldwide, nitrogen use efficiency for cereal production is approximately 33%’ and that the unaccounted 67% ‘represents a [£8.6 billion] annual loss of N fertilizer’. Surplus N from fertilisers can have detrimental environmental effects such as contamination of waterways; in lakes and streams, high nitrate can cause eutrophication, where an increase of water plant and algae growth and subsequent decomposition leads to decreased levels of oxygen. High nitrate in human drinking water or food can cause ‘blue baby syndrome’ (methemoglobinemia), where reduction of nitrate to nitrite interferes with the ability of blood to carry oxygen. Thus, there is much ongoing research into plant nitrogen acquisition and assimilation in order to understand the regulation of nitrogen metabolism. Knowledge of how plants perceive and respond to nitrate and the signalling involved, will help the development of sustainable agriculture with better fertilization regimes or perhaps the production of transgenic plants with enhanced N utilisation.

1.4 Nitrogen uptake

Prior to advances in molecular biology, transport systems for NO$_3^-$ and NH$_4^+$ were identified and described by several research groups using physiological techniques such as kinetic measurements of $^{15}$NO$_3^-$ influx, net NO$_3^-$ uptake and membrane polarisation (reviewed in Glass and Siddiqi, 1995). It was found that high affinity and low affinity transport systems (HATS and LATS) exist for both NO$_3^-$ and NH$_4^+$ uptake. In general, the HATS operate when nutrient concentrations in the soil are between 0.01-1 mM whilst LATS activity is most evident above 5mM. High affinity transport shows saturable kinetics and requires energy from H$^+$ symport, and low affinity transport shows linear, non-saturable kinetics that was once thought to be a result of diffusive fluxes (Glass et al., 2002). However, LATS for NO$_3^-$ has been shown to be active and mediated by H$^+$ symport even when external concentrations of NO$_3^-$ are high, this is because NO$_3^-$ is an anion and is thus repelled from the negatively polarised cell membrane. It was further found that there are two HATS for NO$_3^-$.
uptake: a low capacity, constitutive HATS (cHATS) and an inducible HATS (iHATS). The iHATSs is rapidly induced by exposure to NO$_3^-$ followed by equally rapid down-regulation. The HATS for NH$_4^+$ uptake is constitutive. Both the NO$_3^-$ and NH$_4^+$ transporter systems are regulated and are subject to feedback down-regulation by glutamine, an N metabolite.

With the onset of genetic molecular biology it has been possible to identify some of the genes responsible for these transport systems. In *Arabidopsis thaliana*, there are seven high affinity transporters for NO$_3^-$ uptake (the NRT2 family) and five high affinity transporters for NH$_4^+$ (the AMT1 family). Some of the NRT2 genes are induced by NO$_3^-$ and so belong to the iHATS (Orsel *et al.*, 2002); one such nitrate transporter is NRT2.1. Low affinity nitrate transporters have also been identified and comprise the NRT1 family.

**1.5 Nitrogen in the cell**

In the cytosol, nitrate can be 1) transported to the vacuole for storage, 2) translocated via the xylem to the shoot and other tissues or 3) assimilated into amino acids via several enzymatic reductions. Vacuolar reserves not only buffer against short term perturbations but also function to maintain osmotic and ionic equilibrium in the cell. Plants respond to changes of external supply or internal demand long before vacuolar reserves are exhausted (Glass *et al.*, 2002). Ammonium is toxic to plants, affecting membrane proton gradients and vacuolar sequester of metabolites; therefore it is not stored or transported but is assimilated near the site of uptake or generation.

Nitrogen is transported either as inorganic nitrate or nitrogen rich organic molecules such as amides and ureides. Most nitrogenous compounds are transported from the roots to the shoots via the xylem. The phloem carries some nitrogenous compounds especially those from senescing leaves; however nitrate itself is only transported in the xylem (Taiz and Zeiger, 2002). It has been proposed that the cycling of amino acids within the vascular system may be a mode for signalling N status to the roots in order to regulate N uptake (Marschner *et al.*, 1997, Glass *et al.*, 2002).
1.6 Nitrogen metabolism

1.6.1 Nitrate assimilation

The first step in nitrate assimilation is its reduction to nitrite, catalysed by the enzyme nitrate reductase (NR) (Figure 1.1). This reaction uses NAD(P)H, which derives from either photosynthetic electron transport or the oxidative pentose pathway. This first reduction step occurs in the cytosol. The second step is the reduction of nitrite to ammonium, catalysed by nitrite reductase (NIR). This reaction occurs in chloroplasts and plastids and requires reduced ferredoxin (Fd\text{red}) as an electron donor; Fd\text{red} also originates from photosynthetic electron transport. This reduction step is important as it prevents the build up of nitrite, which is highly reactive and toxic. Ammonium from nitrate reduction or from direct uptake from the soil is converted into glutamine by glutamine synthetase (GS) and then to glutamate by glutamate synthase (GOGAT), glutamine and glutamate are then converted into other essential amino acids by transamination.

Nitrogen metabolism and carbon metabolism coupling is evident, with the reductants for nitrogen reduction originating from C metabolic processes such as glycolysis and photosynthesis, and ATP energy required for nitrate assimilation originating from respiration. Carbon skeletons, such as triose, are essential to synthesis amino acids, the end products of N metabolism.

![Figure 1.1 Uptake and assimilation of nitrate and ammonium.](image-url)
1.6.2 Diurnal regulation of nitrate assimilation

NO$_3^-$ and NH$_4^+$ uptake and assimilation are diurnally regulated. Clement et al., (1978), observed that NO$_3^-$ uptake in ryegrass increased during the day, peaking in the late afternoon followed by a decrease in the dark with the minimum uptake occurring towards the end of dark period. In soybean, Delhon et al., (1995) saw similar diurnal regulation of NO$_3^-$ uptake, reduction and transport. NH$_4^+$ uptake display similar diurnal patterning in Arabidopsis (Gazzarrini et al., 1999). The amplitude of the diurnal pattern for NO$_3^-$ and NH$_4^+$ uptake was highest on high irradiance days. It is thought that this diurnal regulation is linked to light and photosynthesis rather than to the circadian clock and it has been shown that application of sugars can counteract the dark repression effect (Stitt and Krapp, 1999, Crawford et al., 1995, Vincentz et al., 1993, Cheng et al., 1992). In tobacco, Matt et al., (2001) measured the diurnal regulation of the activities and gene expression of the nitrate $^-+$ transporter, NRT2, and the assimilatory enzymes, NR and GS, alongside measurements of concentrations of sugars and amino acids. A correlation was observed between NRT2 transcript abundance and sugar level. Therefore, light indirectly sets the diurnal regulation of nitrate assimilation via the production of sugars. With the production of sugars during the day, by photosynthesis, more C skeletons are made available to combine with reduced nitrogen to make amino acids, whereas in the dark, no sugars are produced. Instead sugars are mobilised from starch and used for respiration, thus reducing the amount C skeletons available for nitrate assimilation. Hence, diurnal regulation of N metabolism is adaptive to plants for maintaining C:N balance.

1.6.2.1 Diurnal posttranslational regulation of NR

Light and photosynthesis also result in posttranslational regulation of nitrate assimilation, by affecting the provision of reductant. In the dark, reduced ferredoxin (Fd$_{\text{red}}$) from photosynthetic electron transport is limiting, resulting in a reduction of NIR activity. This could lead the accumulation of nitrite to toxic levels; however, plants have evolved a rapid and reversible mechanism to inhibit NR activity to prevent nitrite build up. Darkness stimulates the phosphorylation of a conserved serine residue (Ser543 in spinach, Ser534 in Arabidopsis) in the NR enzyme. This results in the formation of a phosphopeptide motif to which 14-3-3 proteins bind, rendering NR
inactive. Phosphorylation alone does not inactivate NR but requires 14-3-3 binding (Figure 1.2). Light and high carbohydrate levels stimulate the dephosphorylation of NR and thereby reactivate the enzyme. How dephosphorylation of bound NR is achieved is unknown, although evidence suggests that 14-3-3 proteins are also posttranslationally diurnally regulated in response to hormone and nutrient signals (Mackintosh and Meek, 2001).

![Figure 1.2 Posttranslational regulation of NR (Mackintosh and Meek 2001)](image)

1.7 Plant growth responses to nitrogen

1.7.1 Low nitrogen

When nitrogen supply is suboptimal, shoot growth is reduced and lateral root growth is stimulated (Figure 1.3a). Senescence of mature leaves occurs as nitrogen is mobilised and retranslocated to the growing organs (Marschner, 1995). This increased root growth increases the exploratory capacity and surface area of the root, which optimises nitrogen uptake.

1.7.2 High nitrogen

In general, high nitrate (10 mM) results in morphological changes opposite to those seen in low nitrate conditions. Lateral root growth is suppressed, and shoot growth is stimulated (Figure 1.3b). In agriculture, this can increase yield in foliage crops and cereals; however, stem elongation from high nitrogen can cause detrimental effects such as lodging.
1.7.3 Nitrogen ‘Hotspots’

Under low nitrogen conditions, exposure of part of the primary root to high nitrate results in the stimulation of lateral root growth into the localised ‘hotspot’ of nitrate. This hotspot response is in contrast to the suppression of lateral root growth by uniformly high nitrate conditions. The root inhibitory effect of nitrate is thought to be a response to nitrate sufficiency (López-Bucio et al., 2003) although it may also be an indirect effect of osmotic stress (Doerner pers. comm. 2006, Lai et al., 2006).

![Figure 1.3 Plant root growth responses to a) low and high N provision (adapted from López-Bucio et al., 2003) and b) N hotspots; the plants were grown in low N and locally treated with 1 mM KCl or KNO3 (adapted from Zhang and Forde, 1998).](image)

1.8 Plant gene responses to nitrogen

There are over 1000 genes that are responsive to nitrate treatment in Arabidopsis (Wang et al., 2000). The genes that are most up-regulated by nitrate addition are those involved in nitrate uptake and assimilation such as the nitrate transporter genes, NRT1 and NRT2, the nitrate reductase NIA genes, and the nitrite reductase NIR gene and its cofactor, UPM1 (uroporphyrinogen III methyltransferase). Genes involved in ammonium assimilation, organic acid synthesis, ferredoxin reduction and the oxidative
pentose pathway are also induced. Other genes are repressed by nitrate, such as the ammonium transporter gene, AMT1.1 (Wang et al., 2000).

Physiological studies complement this molecular data, showing that nitrate uptake and NR activity increase with nitrate treatment (reviewed in Redinbaugh and Campbell, 1991). The induction of the nitrate uptake and assimilation genes is rapid and does not require de novo protein synthesis to occur; thus these genes are primary response genes (Gowri et al., 1992, Sueyoshi et al., 1999).

It is known that nitrate itself is the signal for the induction of its uptake and assimilation (reviewed in Crawford, 1995). However, how and where nitrate is sensed is not known. NR-deficient mutants have been useful to distinguish between nitrate-dependant and nitrate-independent processes; for example it was found that starch mobilisation after nitrate treatment is due to ammonium from nitrate assimilation rather than from nitrate itself (Wang, et al., 2004).

Both the nitrate transporter, NRT2.1 and the ammonium transporter, AMT1.1 are repressed by negative feedback from N metabolites such as glutamine and asparagine (Nazoa et al., 2003, Rawat et al., 2003). These transporters are also induced by N starvation, although this induction is probably a result of release from the repression by N metabolites.

### 1.9 Signalling Pathways

The above growth responses (Section 1.7) and gene expression responses (Section 1.8) are all beneficial responses that enable plants to adapt to the changing nutrient conditions in the soil environment. For these responses to occur, plants must be able to ‘perceive’ the environment. Perception is the ability to sense a change in the environment, integrate this with internal demand and produce an appropriate response. This process requires signalling pathways.

Signalling pathways are complex and can be compared to electrical circuits (Tyson et al., 2003). Instead of electronic components such as resistors, transistors and capacitors linked by wires, biological signalling pathways have genes, proteins and metabolites linked by chemical reactions and molecular interactions. Complex electrical circuits such as those found in a radio are constructed by simpler modules that carry out specific functions such as ‘receiver’, ‘transducer’ and ‘amplifier’ that
link an incoming signal with an appropriate output. Likewise we can break down molecular signalling 'circuits' into smaller functional modules, such as 'perceiving components' that directly respond to an external cue and start the signalling cascade, 'intermediate components' that process, amplify and transduce the signal, and downstream 'effector components' that produce the various regulatory responses or 'outputs' that are the adaptive responses shown by the plant to the cue (Figure 1.4).

Passive 'permissive components' also exist that are required for the signalling to occur but are not components of the signalling pathway per se. These are the 'equipment'; for example, a computer needs a fan for the processor to work but the fan is not involved in any of the computing processes. A plastid transporter may be required for the transport of a signalling intermediate, but it is not part of the signalling pathway.

The ethylene hormone signalling pathway is a good example to illustrate these signalling concepts (Figure 1.4). Ethylene is a hormone that regulates fruit ripening, leaf and flower senescence, abscission, root hair development, seedling growth and hook opening (Taiz and Zeiger, 2002). Ethylene is 'sensed' by binding to ER-membrane bound ethylene receptors such as ETR1, ETR2, ERS1, ERS2 and EIN4. This inactivates these histidine protein kinases, preventing the activation of CTR1, a RAF1-like MAPK serine/threonine protein kinase (Kieber et al. 1993). The MAPK cascade is thus prevented, allowing the EIN2 protein to become active. Activation of EIN2 turns on the EIN3 family of transcription factors, which in turn induces the expression of the ERF1 transcription factor. The ERF1 transcription factor binds to the ethylene response elements (EREs) of ethylene-regulated genes, which include genes that encode cellulase, ripening-related genes and ethylene biosynthesis genes (Taiz and Zeiger, 2002). In this example, the ethylene receptors are the 'perceiving components', as their binding to intracellular ethylene and inactivation enables ethylene signalling to occur. CTR1 and the MAPK cascade are 'intermediate components' that negatively regulate ethylene signalling. EIN2 is also an 'intermediate component' that positively regulates ethylene signalling by activating EIN3. The transcription factors EIN3 and ERF1 are 'effector components' that regulate the gene expression of ethylene response genes. Figure 1.4 also shows the RAN1 protein (Hirayama et al., 1999), a 'passive component' that is required for the
transfer of the copper ion cofactor to the ethylene receptors where copper is necessary for high affinity ethylene binding. Thus RAN1 is required for ethylene signalling but it is not involved in the signalling processes.

In nitrate signalling to date, no receptor or intermediate signalling components have been identified. It is known that nitrate itself is the signal that leads to the induction of genes involved in its uptake and metabolism (reviewed in Crawford, 1995). What is not known is how or where this nitrate is sensed and what signalling processes occur that lead to the induction of the transcription of these nitrate responsive genes.

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**Figure 1.4** Ethylene signalling pathway in *Arabidopsis* (adapted from Gazzarrini and McCourt, 2003). Ethylene binds to and inactivates the ethylene receptors (ETR1, ETR2, ERS1, ERS2, EIN4) resulting in the inactivation of CTR1, this prevents the MAPK cascade and thereby releases EIN2 to activate the EIN3 transcription factor. EIN3 induces transcription of ERF1 transcription factor which in turn induces ethylene-regulated genes. RAN1 is required for the transfer of the copper cofactor to the ethylene receptors. These proteins are signalling components of the ethylene signalling pathway and can be divided into the categories; perceiving components, intermediate components, effector component and passive components, as described in Section 1.9.
1.10 Objectives

The aim of this research is to investigate nitrate signalling in plants. The main focus of this study was to identify core signalling components involved in the nitrate perception, signal amplification or signal movement. This was to be accomplished using a genetic screen for mutants with altered kinetics of gene expression responses to a change in nitrate nutrition. A large part of my research was directed towards the characterisation of suitable reporter lines for this screen.

This research also investigates mechanisms involved in nitrate signalling through the application of pharmacologically active compounds such as inhibitors of protein synthesis, using quantitative RT-PCR to monitor gene expression responses to nitrate provision. This was done using a cell suspension culture system where conditions could be tightly regulated. These experiments led to the identification of the need for de novo protein synthesis for the amplification of the signal when C availability is limiting. Whole plant studies using chlorate provision revealed that transport and reduction activity do not elicit the nitrate signal, and split-root experiments demonstrated that local signalling of nitrate occurs.
Chapter 2 – Materials and Methods

2.1 Plant Growth methods

2.1.1 Seed sterilisation

*Arabidopsis thaliana* (Columbia 0) seeds were ethanol sterilised. Seeds were first washed in 70% ethanol + 0.05% Triton X-100 for 20min, with shaking, followed by washing with 95% ethanol for 5-10min, with shaking. Seeds were air dried under a sterile hood and either directly plated or stored in 0.1% sterile agar (micro agar, Duchefa). Seeds were vernalised at 4°C for 2-5 days.

2.1.2 Growth media

All plants were grown in half-strength Murashige and Skoog (1962) medium (½xMS), prepared from the following components (chemicals supplied by Fluka): 100x micro-elements (10 mM H₃BO₃, 19.2 nM CoCl₂, 9.34 nM CuSO₄, 11.08 mM Na₂EDTA, 9.99 mM FeSO₄, 100 mM MnSO₄, 0.1 mM NaMoO₄, 0.5 mM NaI, 4.8 mM ZnSO₄); 10x macro-elements (29.9 mM CaCl₂, 15 mM MgSO₄, 206 mM NH₄NO₃, 188 mM KNO₃, 12.5 mM KH₂PO₄); 50x MES (0.5 M MES, titrated with NaOH to pH5.5), 1% Sucrose (axenic cultures used 0.6% sucrose). Media was titrated to pH5.6 using 0.1 M KOH. Solid media was made with 1% w/v micro agar (Duchefa). Nitrogen content was adjusted by the addition of nitrogen compounds to -N media (½xMS without NH₄NO₃ and KNO₃). Carbon content was adjusted by varying the sugar content.

*Arabidopsis* cell cultures were grown in full-strength Murashige and Skoog (1xMS) liquid medium with 3% sucrose, 0.5 mg/L 1-naphthaleneacetic acid (NAA), 0.05 mg/L Kinetin.

2.1.3 Cell cultures experiments

*Arabidopsis* cell cultures were obtained from the lab of Professor Carol MacKintosh, University of Dundee, originating from the cultures originally described by May and Leaver, (1993). The cultures were grown at 37 °C in the dark, with gentle shaking. The cultures were maintained by sub-culturing 2.5 ml in 50 ml media every 7 days. Nutrient and pharmacological experiments were carried out as follows: after 6 days of growth the cells were transferred to N starvation media (1xMS, 3% Suc, - N macroelements) by centrifuging the cells at 3000 rpm (1811 G), 5min with a slow
brake, washing the cells with a 10x macroelement wash (29.9 mM CaCl\textsubscript{2}, 15 mM MgSO\textsubscript{4}, 12.5 mM KH\textsubscript{2}PO\textsubscript{4}), then re-suspending cells in the new media. The cells were grown a further 2 days before transfer to fresh N starvation media and addition of nutrients/chemicals or transfer to experimental conditions. 1.5ml samples were taken according to time-course intervals using wide-tipped pipette tips.

2.1.4 Axenic plant cultures
Analysis of gene expression from \textit{Arabidopsis} plant roots was achieved using plants grown hydroponically in conical flasks. Approximately 6 seedlings were germinated and grown in each flask, in 10 ml of 0.5x MS, 0.6% sucrose media for 10 days (the media was replenished after 7 days). The flasks were closed with sterile foam bungs to maintain sterile culture conditions whilst allowing gas exchange. Plants were grown in long day conditions with gentle shaking (40 rpm). After the 10 day pre-growth, the plants were N starved for 4 days by replacing the media with −N 0.5x MS, 0.6% sucrose media. Nutrients and pharmacological compounds were added as required to the flasks and root tissue harvested at set time points. 0.1 g of tissue was taken per time point from individual flasks. The data is representative of pooled individual data and is not continuous. Root tissue was frozen in liquid N\textsubscript{2} and stored at −80 °C until RNA extraction.

2.1.5 Plant growth conditions for CCD imaging
Seedlings from reporter lines were germinated and grown on vertical square plates containing 75 ml of 0.5x MS (Section 2.1.2). The plants were grown under constant light conditions. After 10 days pre-growth, the seedling were transferred to −N 0.5x MS plates for N starvation. For initial studies, transfer was done using tweezers to move individual plants. For later studies, seedlings were grown on nylon mesh placed on top of the agar media, enabling the easy transfer of multiple plants and reducing mechanical stress and damage to the plants. After 4 days of N starvation, reporter gene expression was induced by transfer to induction plates. In early experiments these induction plates contained agar induction media; this was later modified to be plates containing blotting papers soaked with induction media, this change improved luciferin availability. Seedlings were treated with luciferin and imaged for luciferase activity using a photon counting cooled CCD camera (Andor\textsuperscript{TM} and Hamamatsu\textsuperscript{TM}).
2.1.6 CCD imaging
Luciferin was stored as 50 mM stocks in 50 mM NaHPO₄ pH 7 buffer, at -80 °C. Working concentrations of 1 mM luciferin in 0.01% Triton x-100 were made from these stocks, and applied evenly to the plants with a fine mist spray. Plants were prepared for imaging by first pre-spraying with 1mM luciferin, 10 hours before the study, to eliminate accumulated luciferase protein.

After pre-spraying the plants were returned to growth conditions. At each time point the plants were re-sprayed and imaged after 5 min dark treatment to allow auto-fluorescence to decay. The plants were imaged with an exposure of 10 seconds using a photon-counting cooled CCD camera (Andor™ and Hamamatsu™).

Images were analysed using Andor Analysis, Simple PCI and Image J. They were analysed for relative luminescence by selecting regions of interest (ROIs) to include the subject plant and measuring the integral light intensity of that area. The background intensity was also measured and subtracted from the ROI result. The ROI area was also measured, thus the intensity per mm² could be calculated. Average intensities were taken from replicates.

2.1.7 Split root experiments
These experiments were used to study systemic signalling in \textit{PRONRT2.1::LUC} reporter lines. Seedlings were germinated on square vertical 0.5x MS plates in long day conditions. At 6 days, the primary roots were removed below the hypocotyls to encourage adventitious root growth. The plants were grown a further 14 days before being transferred to 3-compartment Petri-dishes, one seedling per plate. One compartment of the Petri-dish contained 18 ml of 1% water agar, the other compartments containing 7 ml of −N 0.5x MS, 0.6% sucrose liquid media. The shoots were placed on the solid agar and the roots system was split equally between the two liquid compartments. The seedlings were N starved and grown horizontally for 4 days, with gentle shaking. The plants were pre-treated with 1 mM luciferin 10 hours before induction; this was done to eliminate any accumulated luciferase protein. Gene expression was induced by adding KNO₃ to a concentration of 10 mM to the left root compartment. The plants were then treated with 1 mM luciferin and imaged with the CCD camera for LUC activity over a 48h time-course.
2.2 RNA methods

2.2.1 Isolation of RNA from cell cultures and plant material
For both tissue types, the same RNA isolation method was used. In general, cell cultures yielded more RNA per mg of tissue than plant material. RNA isolation was conducted under a fume hood and care was taken to keep samples chilled and prevent RNAase contamination.

0.1 g of tissue was homogenised in 1 ml of TRIzol reagent (38% H$_2$O-saturated phenol, 0.8 M guanidine thiocyanate, 0.4 M ammonium thiocyanate, 0.1 M Na Ac pH 5, 5% glycerol). 200 µl of chloroform was added and the samples vortexed, then left at room temperature for 2 min before centrifuging at 14000 rpm (18300 G) for 15 min at 4 °C. The aqueous phase was removed to a new tube. RNA precipitation was achieved by adding 0.8 M sodium citrate/1.2 M NaCl, before adding isopropanol, both at half the volume of the aqueous phase. The samples were incubated at room temperature for 10 min then centrifuged at 14000 rpm for 10 min at 4 °C. The supernatant was removed and the pellet washed with 75% ethanol and briefly air dried, before re-suspending in 40 µl of DEPC water.

RNA samples were further purified by LiCl precipitation. Samples were spun at 14000 rpm for 5 min to remove any insoluble material and the supernatant removed to a new tube. LiCl was added to a final concentration of 2 M and the RNA left to precipitate at 4 °C overnight. The samples were then centrifuged at 14000 rpm for 15 min at 4 °C, and the supernatant discarded leaving a clear pellet, this was re-suspended in 30 µl of DEPC water. 1 µl was run on an agarose gel to check quality of the RNA. RNA samples were stored at -80 °C.

2.2.2 RNA quantification
RNA was quantified using a Beckman DU®520v1.03 spectrophotometer and the equation: $\text{OD}_{260} = 1 \equiv 40 \mu g/ml$ RNA

2.2.3 cDNA synthesis
Complementary DNA (cDNA) was made by reverse transcription (RT) of extracted RNAs. RNA was reverse transcribed using Reverse-iTTM 1st Strand Synthesis kit (ABgene) and oligo-dT primers. Each RT reaction consisted of approximately 1 µg of RNA, 1 µl of oligo dT primer (500 ng) and sterile water to a final volume of 6.5 µl.
This mixture was heated to 70°C for 5 min to eliminate any secondary structures, before addition of 2 μl of 5x First Strand Synthesis buffer, 1 μl dNTP mix (5 mM each) and 0.5 μl Reverse-iT™ RTase blend. The final reaction volume was 10 μl. cDNA synthesis was achieved by incubation at 47 °C for 30 min, followed by incubation at 75 °C for 10 min to inactivate the RT enzyme. The resultant cDNA was diluted to 100 μl with sterile water and stored at -20°C.

2.2.4 Quantitative RT-PCR analysis

Each QPCR reaction comprised of 3 μl of diluted cDNA, 10 μl 2X ABsolute QPCR mix (ABgene, contains 0.025 units/μl ThermoStart® enzyme), 1 μl forward primer (4 pmol), 1 μl reverse primer (4 pmol), 2μl 10X SYBR® green (made fresh), 3 μl sterile H2O. Each reaction was performed in quadruplicate and aliquoted into clear 96-well PCR plates and sealed with optical sealing tape. QPCR reactions were performed using the iCycler iQ™ Real-Time Detection System (BIO-RAD), at 490 nm for SYBR® green fluorescence detection. Before each QPCR run the system was calibrated using a well-factor plate.

The QPCR run consisted of an initial 15 min 95 °C denaturing step (required to activate the ThermoStart® enzyme) followed by 35 cycles of denaturing at 95 °C for 45s, annealing at 58 °C for 20s, extension at 72 °C for 30s.

Each reaction was replicated using primers specific for eIF4α cDNA to assess cDNA content of this constitutively-expressed gene (Metz et al., 1992). Genomic DNA contamination was also measured using eIF4α genomic DNA specific primers. This was important for reactions using NRT2.1 (which had to be designed in the 5’UTR to ensure specificity) and AMT1.1 (which has no intron) primer sets. All other primer pairs were designed across exon-intron borders, and are cDNA-specific. QPCR primer combinations are shown in the table below.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Quantitative PCR primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIR</td>
<td>Forward 5’GCTTATCGACGAACCTTGGTGT3’</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’AGCTTCCGGTTTTCGAGGTIT3’</td>
</tr>
<tr>
<td>eIF 4A</td>
<td>Forward 5’TTCGCTCTTCTCTTTGCTCTC3’</td>
</tr>
<tr>
<td>(cDNA)</td>
<td>Reverse 5’GAACCTCATCTTTCGCCCTCAAGTA3’</td>
</tr>
</tbody>
</table>
A threshold of 1000 RFU (relative fluorescence units) was used, corresponding to the exponential phase of amplification. The number of PCR cycles required for relative fluorescence to reach the threshold was taken as the threshold cycle of amplification (Ct) value. Data was normalised for cDNA loading by subtracting the mean Ct for each gene from the eIF4α mean Ct for that sample, this value was know as the threshold cycle difference. A sample with a large Ct difference has high gene expression. Fold difference of gene expression was calculated by using the first sample from a time course experiment as the base level to which all other samples are compared. Thus the Ct difference of the first sample was subtracted from the Ct difference of all the samples. As each cycle represents a doubling of cDNA amounts the final value is made an exponent of 2. Thus the base level value is always 1 and the values of the rest of the samples represent to fold difference of gene expression in relation to the base value. This calculation is summarised in the equation below.

\[
\text{Fold difference in gene expression} = 2^{(b-a)-c}
\]

\(a\) = mean Ct of tested gene, \(b\) = mean Ct of eIF 4α (cDNA) gene
\(c = (b-a)\) at the starting time-point
2.2.5 Northern Blot Analysis

Separation of 15 µg RNA was performed in a 1% formaldehyde agarose gel as described in Section 4.9 of Current Protocols in Molecular Biology, Volume 3. The gel and running buffer contained 1x MOPS. After electrophoresis, gels were washed with DEPC-H$_2$O for 3 x 15min., then 10x SSC (in DEPC-H$_2$O) for 2x 15min, with slow shaking. RNA was blotted overnight onto a nylon membrane then UV cross-linked and immobilised at 90 °C for 1h. The nylon membrane was hybridised to $^{32}$P-labelled cDNA probes specific to the gene of interest. RNA loading was determined by stripping the membranes and hybridising to $^{32}$P-labelled 303 bp cDNA of the constitutively expressed eIF4a gene. Blots were hybridised at 65 °C and washed once at room temperature with 2x SSC, 1% SDS, and once at 65 °C with 2x SSC, 1% SDS, followed by 2 washes with 0.2x SSC, 1% SDS, at 65 °C.

cDNA was labelled by annealing specific primers to denatured cDNA template and synthesising new DNA strands using Klenow polymerase and ($\alpha$ $^{32}$P) dCTP. Final probes used were as follows; 1670 bp LUC, 515 bp NIR, 231 bp NRT2.1, 240 bp NRT2.4, 484 bp AMT1.1

2.3 DNA methods

2.3.1 PCR amplification

Primers were designed to amplify target DNA sequences and add restriction endonuclease enzyme sites to facilitate further cloning. PCR reactions were performed in a T3 thermocycler (Biometra) using either Taq DNA polymerase (NEBiolabs) or the high fidelity PfuTurbo® DNA polymerase (Stratagene) depending on the size of the PCR product. PCR reactions were purified using QIAquick® spin columns (Qiagen)

2.3.2 Restriction digests and ligations

DNA digestions with restriction endonucleases were performed using enzyme manufacturer’s specific buffers and recommended temperatures. Ligations were performed using T4 DNA Ligase (NEBiolabs) in 10x Ligase buffer (NEBiolabs) and incubated at 4°C overnight. A 3:1, target insert to vector ratio was used.
2.3.3 Agarose gel electrophoresis
DNA separation on agarose gels was performed using gels made with and run in 1X TAE (40 mM Tris-base, 20 mM Acetic acid, 1mM EDTA)

2.3.4 DNA isolation from agarose gels
DNA fragments from restriction digests were isolated from agarose gels using Qiaex® II gel extraction kit (Qiagen).

2.3.5 Transformation of *Escherichia coli* competent cells
Plasmid DNA (or 5 μl of ligation mix) was added to 100 μl freshly thawed DH5α competent *E. coli* cells and incubated on ice for 15-30 min. The cells were heat shocked at 42 °C for 1 min then immediately placed on ice for 2 min. 1 ml of L-broth (LB) was added. Cells were incubated at 37 °C with shaking, for recovery. 100 μl of LB mix were spread on LB antibiotic selective plates. Remaining mix was centrifuged and re-suspended in a small quantity of LB, this concentrated mix was also spread on LB selective plates. The plates were incubated at 37 °C overnight.

2.3.6 Isolation of *Escherichia coli* plasmid DNA
Bacterial colonies were screened for correct plasmid insertions by isolating the plasmid DNA using the boiling miniprep method. 3 ml of selective LB broth was inoculated from single colonies and cultures were grown overnight at 37°C, with shaking. 1.5 ml of culture was centrifuged down at 7000 rpm (4600 G) for 3 min. The supernatant was removed and the pellet resuspended in 350 μl of Boiling Buffer (8% (w/v) sucrose, 0.5% (v/v) Triton X-100, 50 mM EDTA, 10 mM Tris-HCL, pH 8) with lysosyme. Cells were lysed by boiling for 1 min and immediately placed on ice for 2 min. Cells were centrifuged at 14000 rpm for 20 min at 4 °C, and the slimy pellet removed using a toothpick. DNA precipitation was achieved by adding 40 μl of 3 M Na Ac (pH 5.2) and 400 μl isopropanol, mixing by inversion. Plasmid DNA was pelleted by centrifuging at 14000 rpm (18300 G) for 5 min, washed with 70% ethanol and air dried. DNA was resuspended in 50 μl of R40 buffer (TE, 40 mg RNAase A, pH 7). Screening was continued by restriction digest analysis.

Fresh cultures were made from bacterial colonies identified as containing the correct plasmid insert and the plasmid DNA isolated using QIApREP® mini-prep kit (Qiagen)
2.3.7 Sequencing
Plasmid DNA was sequenced using the BigDye ® Terminator v3.1 sequencing kit (Applied Biosystems) and analysed at the SBS sequencing facility.

2.3.8 Transformation of Agrobacterium tumefaciens
1 μg of Plasmid DNA was added to 100 μl of freshly thawed competent Agrobacterium cells. The mix was incubated on ice for 30 min then snap-frozen in liquid N₂ for 1 min and immediately heat shocked at 37 °C, until thawed. 1 ml of YEP was added and the cells incubated at 28 °C with shaking for 3 hours. Cells were centrifuged at 7000 rpm (4600 G) for 1 min, and then pellet resuspended in 100 μl and spread on YEP Kanamycin (vector resistance)/ Gentamycin 80 (A. tumefaciens selection) plates. Plates were incubated at 28 °C for 2-3 days.

2.3.9 Isolation of total Agrobacterium tumefaciens DNA
Plasmid rescue was done to check that DNA rearrangements had not occurred in the transformed A. tumefaciens lines. 3 ml cultures were grown overnight in selective YEP broth. 1.5 ml of this was centrifuged at 14000 rpm (18300 G) for 3 min, the supernatant was discarded and the pellet resuspended in 100 μl of ice-cold buffer P1 (50 mM Tris.HCl, 10 mM EDTA) and incubated at room temperature for 30 min. 200 μl of buffer P2 (0.2 M NaOH, 1% SDS) was added and the mix incubated on ice for 5 min before adding ice-cold buffer P3 (KAc, pH 4.8). The mix was vortexed upside down and incubated on ice for 5 min, before centrifuging at 14000 rpm for 5 min at 4 °C. The supernatant was removed to a clean tube and the DNA precipitated by adding 315 μl isopropanol and mixed by inversion. The DNA was pelleted by centrifuging at 14000 rpm for 10 min at 4 °C, and washed with 70% ethanol then air dried before re-suspending in 10 μl of R40.

2.3.10 Transformation of Arabidopsis thaliana
Arabidopsis thaliana (Columbia 0) plants were transformed using the floral dipping technique (Clough and Bent, 1998). Plants are grown in short day conditions for approximately 6 weeks then moved to long day conditions to promote flowering. To encourage multiple inflorescence growth, primary bolts are removed when 2-5 cm high. The A. tumefaciens was prepared by inoculating 3 ml of YEP (plus selective antibiotics) from a single colony containing the desired plasmid construct, this was
incubated for 2 days at 28 °C, with shaking. 2.5 ml of this was used to inoculate 50 ml of culture media, the remaining culture was used to make glycerol stocks for storage at -80 °C. The 50 ml culture was incubated overnight at 28 °C and was used to make a further 500 ml culture which was also incubated overnight. This culture was pelleted at 4200 rpm (3306 G) for 20 min at 20 °C. The large pellet was re-suspended in 500 ml ¼ x MS containing 200 μL/L Silwet. The plant inflorescences were dipped into this mix for 5 min. The plants were then covered with plastic bags for a day to encourage \textit{A. tumefaciens} infiltration. A second transformation was performed a week after the first procedure. The siliques were allowed to mature and the seeds collected.

2.4 Plasmid Constructs

2.4.1 Generation of the pSP Ω-LUC+ 301 vector

The 80 bp omega enhancer sequence originates from the tobacco mosaic virus 5’UTR and was used by Millar et al., (1992). Six oligonucleotides were annealed to create an omega fragment with NheI and NcoI overhangs (Table 2.2). The oligos; Omega P2,P3,Q2 and Q3 were phosphorylated by incubating 1 ng of oligo with 1 μl of polynucleotide kinase (PNK) in T4 ligase buffer (containing 1 mM ATP), for one hour at 37 °C, mock reactions without PNK were set up for the oligos, Omega P1 and Q1. 1 μl of these reactions were subsequently dissolved 1:10 in low TE buffer. Annealing was performed by adding 10 ml of each oligo solution together and denaturing at 80 °C for 5 mins, after which the mixture was allowed to cool slowly to room temperature.

A 1:50 dilution of the annealed omega sequence was cloned into the pSPLUC+ 301 (generated in the lab by T.Keller from pSPLUC+® (Promega) vector to contain terminal KpnI and SacI sites for cloning) vector (digested with NheI and NcoI). The resultant construct was sequenced to confirm the incorporation of the omega fragment and named pSP Ω-LUC+ 301.
2.4.2 Generation of pSP NaN LUC+ 301 and pSP NN Ω LUC + 301 vectors

These vectors were generated to include additional endonuclease restriction enzyme sites to facilitate cloning. Linker fragments were constructed by the annealing of designed oligonucleotides using the method described above in 2.4.1. Two linkers were made: NaN and NN. NaN was made by annealing 2 specific oligos (5'-TAGCCAAGCTTGACGATTGTCGACTATCCTAGG 3' and 5'-CATGGCCTAGGATAGTCGACAATCGTCAAGCTTGGC 3'), this produced a DNA fragment containing internal HindIII, Sall and AvrII sites and NdeI, NcoI overhangs. NN, was also made by annealing 2 oligos (5'-TAGCCAAGCTTGACGATTGTCGACTATCCTACGAGCTTGGC-3') and (5'-CTAGCCAAGCTTGACGATTGTCGACTATCCTACGAGCTTGGC-3') to produce a DNA fragment containing internal HindIII and Sall sites with NdeI and NcoI overhangs. These NaN and NN linker fragments were then cloned into pSP LUC+301 and pSP Ω LUC+301 (digested with NdeI and NcoI) to produce pSPNaNLUC+301 and pSPNNΩLUC+301 respectively (Figure 2.1).
2.4.3 Generation of $\text{PRO}_{\text{NRT2.1}}::\text{LUC}$ and $\text{PRO}_{\text{NRT2.1}}::\Omega::\text{LUC}$ constructs

A 1222 bp upstream sequence of the $\text{NRT2.1}$ gene (At1g08090) representing the promoter region was PCR amplified from Arabidopsis genomic DNA using a specific 5' primer (5'-GCAAGCTTACATATCAATCG-3') introducing a HindIII restriction site, and a specific 3' primer (5'-GCCCTAGGCAAGTTTG-3') introducing an AvrII restriction site. PCR amplification was done using the high fidelity DNA polymerase, PfTurbo® (Stratagene) and was further incubated with Taq DNA polymerase to add dA overhangs to facilitate cloning into the pGEMT vector (Promega). The promoter fragment was released from the pGEMT vector by digestion with HindIII and AvrII and transcriptionally fused with the luciferase reporter gene in pSP NaNLUC+301 (cut with HindIII and AvrII) or pSP NNΩLUC+301 (cut with HindIII and NheI (AvrII compatible)) to generate $\text{PRO}_{\text{NRT2.1}}::\text{LUC}$ and $\text{PRO}_{\text{NRT2.1}}::\Omega::\text{LUC}$ constructs respectively (Figure 2.2). Both constructs were cloned into the pTV50 binary vector via HindIII and SacI restriction sites and transformed using A. tumefaciens into Arabidopsis thaliana (Col 0) plants.
Part 1 – The Genetic Screen

The aim was to identify core signalling components of the nitrate signalling pathways with the use of a genetic screen. Screen design and characterisation of reporter lines are described.
Chapter 3 - Design of a genetic screen for kinetic mutants in nitrate signalling

3.1 Summary

This chapter describes the rationale for a kinetic genetic screen and the use of luciferase as a reporter gene. Experiments done with \textit{PROCaMV35S::LUC} lines to determine optimal imaging conditions such as luciferin application and concentration are discussed. This information contributed to the subsequent assessment of reporter lines and the design of a future genetic screen.

3.2 Introduction

Nitrogen is an essential nutrient for plants. The availability of N is highly variable among soil types and pH. N availability also varies with climate and seasonal changes. As well as being a required nutrient, N is also a cue for adaptive growth responses (Section 1.7). Signalling pathways are present in plants that enable plants to sense N availability in the environment, transduce and process this signal, and make these adaptive growth responses. As changes of growth due to metabolism can mask changes of growth due to signalling, focusing on gene responses is preferable as these are early responses that would precede growth responses caused by metabolism. At present, it is known which genes are induced and repressed by N (Section 1.8), but it is not known how N is sensed or what signalling components are involved that lead to these gene responses. The use of a genetic screen to identify these early N signalling components would be very beneficial in the elucidation of components of the N signalling pathway.

3.2.1 Rationale for a genetic screen

Genetic screening is a useful technique to direct identification of novel genes in pathways of interest. This is done by screening a large mutagenised population for specific mutants with abnormal phenotypes. These mutants are then characterised, and the genes responsible for the phenotype are mapped. For example, a genetic screen to find components involved in ethylene signalling was done by screening for mutants with abnormal ethylene triple response. This resulted in the identification of
mutants and genes involved in ethylene biosynthesis (eg eto1) and signalling (eg ein2) (Guzman and Ecker., 1990). A genetic screen for flower development mutants led to the identification of the floral ABC genes (Bowman et al., 1989).

With the use of a well designed genetic screen, it was aimed to identify core components of the nitrate signalling pathway. In particular, it was aimed to identify ‘perceiving’ components or early ‘intermediate’ components (Section 1.9) that are involved in the precipitation, flux and amplification of the nitrate signal. It was aimed to identify these components by screening for mutants with altered gene responses to nitrate. Gene responses to a signal often precede responses to changes in metabolism such as growth responses. As gene responses are fairly imperceptible, a reporter-gene system was used.

Reporter genes code for proteins with activity that can be visualised and measured. Such proteins are: green fluorescent protein (GFP), β-glucoronidase (GUS) and luciferase (LUC). In a reporter construct, these genes are driven by the promoter of the gene of interest so that the reporter gene ‘reports’ the expression of the endogenous gene and ‘reports’ when a cognate pathway is active. They are important in showing where and when a gene is expressed. Using reporter genes can produce a ‘highly specific background to dissect virtually any process of interest’ (Page and Grossniklaus, 2002). This is very beneficial for targeting mutants specific to a desired pathway. For this screen the reporter gene will be driven by a promoter from a nitrate responsive gene, thereby targeting the nitrate signalling pathway. The resultant reporter lines will then be mutagenised and screened for mutants with abnormal reporter gene responses to nitrate.

Many screens are designed to look for mutants with constitutive responses such as loss of response when stimulus is present or constitutive expressing in the absence of a cue. For example, the ctrl1 ethylene mutant was found as a mutant that constitutively displayed the ethylene triple response (Kieber et al., 1993). Although these types of screens can be informative in identifying core signalling components, they can often yield false positives, ie the mutants may appear to be affected in a core component but are in fact affected in a passive component (Section 1.9). A passive component is required for signalling to occur but is not a signalling component. For example, a computer needs a fan for it to work but the fan is not involved in any of its processes.
However, if the fan was to breakdown the processor would melt and the computer would fail to work. Likewise, a mutation of a passive component may abolish a particular signalling pathway and therefore the mutant phenotype would be 'constitutively off'. The identification of RAN1, a passive component of the ethylene signalling pathway required for the transfer of a copper ion to the ethylene receptors, was identified in such a genetic screen where it displayed an ethylene response in the presence of an antagonist of ethylene binding (Hirayama et al., 1999)

Therefore, instead of screening for mutants that are either on or off, the aim is to screen for mutants with different kinetics of response. Examples of a kinetic mutant would be where the response occurs faster or slower, a timing mutant, or where the response is greater or smaller, a magnitude mutant (Figure 3.1). By screening for kinetic mutants we increase the chances of identifying mutants involved in the core early 'intermediate' components (Section 1.9) that are responsible for the precipitation, flux and amplification of the nitrate signal. Millar et al., (1995) used a kinetic approach in their screen for light mutants in the \textit{cab2::\Omega::Luc} background. These transgenic lines were screened for timing mutants and from this screen the \textit{toc1} mutant was isolated as having a short-period phenotype. TOC1 is a regulatory protein involved in regulating the plant circadian clock (Taiz and Zeiger., 2002).

Also, instead of screening for mutants with inappropriate responses to a constitutive cue, a key element of this screen will be to 'ask' the plants to signal by changing the nitrate provision. This targets the screen to signalling components rather than passive components. Also, an early response to a change is more likely to be due to a signal rather than to downstream effects of metabolism.
3.2.2 Criteria of the screen

Page and Grossniklaus (2002) state that the success of a genetic screen depends on two factors: first, the definition of a suitable genetic background, and second, an easy and tight procedure to identify the mutants of interest.

In this screen, the genetic background is a reporter line expressing Luciferase, a fluorescent reporter gene (Section 3.2.3), under the control of the promoter of a nitrate responsive gene. The choice of gene promoter is critical for a successful kinetic screen and should be from a gene that has defined and robust gene responses. The criteria for this choice and the candidate gene promoters chosen are discussed in the next chapter (Section 4.2.1).

Characterisation of the background reporter line is also important, especially the characterisation of the nitrate gene response so that ‘normal’ kinetics can be established. From this, mutants that deviate significantly from these ‘normal’ kinetics can be identified.

In order to have an efficient high throughput screen, growth conditions and induction techniques need to be simple, quick and consistent. Therefore, optimising growth conditions for the screen is imperative. A system is required where N supply can be adjusted whilst keeping other factors constant. This is crucial, as many metabolic pathways are coupled and changes in a variable other than N could lead to confounding results. It is also important to avoid mechanical or nutritional stress to the plants as this can cause signalling cascades that may also inadvertently affect N signalling.
3.2.3 Arabidopsis thaliana as an experimental system

Arabidopsis thaliana (thale cress) is a model plant for genetic studies. Several features make it a good model: its small size, its short generation time (5-6 weeks) and its high fecundity. It grows well in media as well as soil, and it is easily transformed by Agrobacterium tumefaciens. A. thaliana has the smallest known plant genome (Page and Grossniklaus, 2002) which has been sequenced, and can be easily searched using The Arabidopsis Information Resource (TAIR) website. It is the plant of choice for this screen due to its small size which enables it to respond rapidly to environmental changes, and because it is easy to transform, allowing the generation of transgenic reporter lines.

3.2.4 Luciferase as a reporter gene

The reporter gene luciferase (LUC) was chosen as it can be used non-invasively to track gene expression in vivo as well as in vitro. The luciferase gene was cloned by DeWet et al., (1985) from the North American firefly, Photinus pyralis, and has been expressed in plants, mammalian cells, fish and insects. Its substrates are oxygen, ATP and luciferin, all but the latter being present in plants. The luciferase protein is very stable in the absence of its substrates; with its substrates it undergoes a reaction that leads to the emission of one photon at 562 nm at pH 7 (Figure 3.2)

\[
\begin{align*}
LUC + \text{luciferin} + MgATP & \rightleftharpoons (LUC\text{-lucifein-AMP}) + MgPP_i \\
(LUC\text{-lucifein-AMP}) + O_2 & \rightarrow (LUC\text{-oxyluciferin}^*\text{-AMP}) + CO_3 \\
(LUC\text{-oxyluciferin}^*\text{-AMP}) & \rightarrow (LUC\text{-oxyluciferin-AMP}) + hv
\end{align*}
\]

**Figure 3.2** Luciferase reaction. The formation of the \((LUC\text{-luciferin-AMP})\) complex is a fast equilibrium reaction. After oxidative decarboxylation, the oxyluciferin is excited \((^*)\) which leads to the emission of a photon at 562 nm (adapted from Van Leeuwen et al., 2000)

By spraying LUC expressing transgenic plants with luciferin we can set off this reaction and thus see luminescence in plants. Luciferase activity reports real-time expression. Due to its short half-life, the luciferase molecule only reacts once and emits one photon. Therefore, in the presence of luciferin, the LUC protein will not accumulate but will report gene expression as a flux of the LUC protein molecules.
being made in the cell. It is thus a good reporter gene that accurately reflects the expression of the endogenous gene (Schneider et al., 1990, Xiong et al., 1999). Unlike more stable reporters such as GUS, it shows gene expression as the proteins are being made and not the accumulation of the protein over time. Hence, luciferase reporters are ideal for time course studies of kinetics.

Millar et al., (1992) used a CAB2::LUC fusion to follow the kinetics of the endogenous chlorophyll binding protein gene CAB2. Later, they mutagenised this reporter line and screened the M2 plants for altered circadian rhythms. From this screen they identified the toci mutant that has short circadian rhythms (Millar et al., 1995a). Similarly, we want to first study the response kinetics of our reporter lines so that when we mutagenise the reporter line we can select those with variant kinetics in a genetic screen.

3.3 Results

3.3.1 Autofluorescence decay
Plant leaves display auto-fluorescence upon movement into the dark due to the re-emission of light from chlorophyll molecules. This auto-fluorescence is not detectable to the human eye but is detectable by a photon counting CCD camera. This phenomenon is short lived; a time course experiment using wild type Colombia Arabidopsis plants showed that auto-fluorescence ceases to be detectable with our CCD camera after 3 minutes of dark treatment. Assuming that auto-fluorescence decay will be the same in reporter line plants, it was determined that plants should be subjected to 3 minutes of dark treatment before imaging for luciferase activity.

3.3.2 Luciferase degradation in PROCaMV35S::LUC – aiming for the plateau
In the absence of luciferin, luciferase will accumulate in the cell. Therefore the initial light emission upon spraying with luciferin will be due to accumulated luciferase and not the real-time expression of the gene. For real-time expression to be seen this accumulated luciferase needs to be degraded before experimental conditions are applied. Figure 3.3 shows a hypothetical model of luciferase degradation. The addition of nitrate would be during the 'plateau' phase where LUC production equals LUC degradation, and therefore LUC activity reflects steady-state real-time gene expression.
Figure 3.3 Hypothetical response kinetics graph incorporating luciferase degradation. This model is based on expected kinetics for \textit{NIR::LUC}. The blue dashed line represents a change to nitrate containing media. This point is during the plateau phase where luciferase made = luciferin used. The green line represents an inductive response to the nitrate treatment. The continuing black line would be a control plant with no added luciferin or a plant where no response is made. The pink line represents limiting luciferin, what we would expect if the amount of luciferin were not sufficient to follow the response but instead is used up quicker due to an increase in luciferase activity.

To ascertain when this plateau occurs, LUC degradation kinetics were analysed. This was done using \textit{PROCaMV35s::LUC} lines. \textit{PROCaMV35s::LUC} plants were grown on vertical plates containing 0.5xMS for 10 days. These were sprayed with luciferin and imaged over a time-course. Three different concentrations of luciferin were used to determine the lowest concentration required to degrade the stored luciferin and reach the ‘plateau’. It was found that luminescence was high at the 0 hour time point due to accumulated luciferase (Figure 3.4); luminescence quickly decreases within the first 2 hours indicating the degradation of the accumulated luciferase after reacting with luciferin. A plateau is achieved between 3 and 10 hours at all luciferin concentrations, although this plateau continues to decline and may be due to limiting substrate. From this it was determined that 1 mM luciferin should be used as a working concentration. A further experiment where luciferin was applied at either 2h or 4h intervals showed that the substrate was limiting (Figure 3.5); after each application the luminescence increased. However, it is clear that with frequent application (1-2 hourly) a plateau would be achieved.

However, such frequent application of luciferin throughout a time course would be costly and could be harmful to the plant due to the detergent Triton x-100 which is used to aid uptake of the luciferin solution by the plant (Van Leeuwen \textit{et al.}, 2000).
However, a contributing factor to the limiting luciferin problem is that the agar media that the plants are induced and imaged on also absorbs the luciferin solution; sequestering the luciferin away from the plant.

This problem was overcome using a technique designed by Fan Lai (2005, pers. comm.) where the seedlings are transferred from agar media to a media-soaked blotting paper in a square Petri dish lid. He showed that, using this method, he was able to effectively degrade accumulated luciferin by pre-spraying with 1mM luciferin for 10h and he was able to track reporter gene regulation by subsequent luciferin applications at each time-point.

It is important to recognise that $\textit{PRO}\text{CaMV35s:} \textit{LUC}$ is constitutively expressed, thus luciferase is constantly being made. This increases the problem of trying to achieve the steady-state plateau. However, the reporter line for the screen will be nitrate responsive and will be only expressed when induced. Prior to induction the plants will be N starved to ensure that these genes are ‘off’ or at steady-state at the start of the experiments. Therefore the pre-spray of the plants is done 10 hours before induction and the plants are sprayed and imaged just before induction to ensure that no accumulated luciferase is present.

![Figure 3.4 Luciferase degradation curve in $\textit{PRO}\text{CaMV35s:} \textit{LUC}$ plants treated with 0.2 mM, 1 mM and 5 mM luciferin concentrations. This curve is based on luminescence data from CCD images of LUC activity.](image-url)
3.3.3 Optimising luciferin application

Luciferin is applied exogenously to the plants as a liquid solution. After trying different solutions it was determined that the solution used by Millar et al., (1998), was the best for our experiments as it is quickly absorbed by the plants roots and shoots and a uniform luminescence was seen in plants treated (data not shown). 50 mM luciferin stock solutions were made in 50 mM NaHPO₄ pH7 buffer and stored at -80°C; working concentrations of 1 mM luciferin in 0.01% Triton x-100 were made from these. Triton x-100 is a detergent used to improve the uptake of luciferin by the plant cells.

The application method was also tested. It was determined that spraying the plants with a fine mist spray was preferable to application by pipetting since spraying gives a more even application resulting in uniform luminescence from the PROCaMV35S::LUC plants (data not shown). It is necessary to spray the whole plant rather than application via the roots. For my experiments, a simple spray nozzle compatible with a 50ml Falcon tube was used. Each square plate of plants was sprayed five times: once at each corner and once in the centre. Testing the spray on paper showed that this provides a fairly even application. However, for a full scale high-throughput screen an air-brush spray will be used: this method was shown to be quick and effective in a screen by Fan Lai (2005, pers. comm.).
3.4 Discussion

Genetic screens are useful for identifying novel components of biological processes. Care should be taken during screen design in order to direct the screen and optimise finding mutants that will be affected in the desired biological process. For a screen to elucidate a signalling pathway a kinetic approach using reporter lines was chosen. Examining gene responses as opposed to growth responses and 'asking' the plants to signal will ensure targeting of signalling processes and not downstream metabolic processes.

Luciferase is the chosen reporter gene for this kinetic screen. These preliminary experiments using \textit{PRO\textsubscript{CaMV35s}:LUC} reporter lines have been important in determining the concentration and application method of luciferin. This work was also informative for the training for use of the CCD imaging equipment and image data analysis. It is shown in the next chapters that this information was required for the characterisation of reporter lines and for the future screen.
Chapter 4 - Characterisation of \( \text{PRO}_{\text{NIR}}::\text{LUC} \) and \( \text{PRO}_{\text{AMT1.1}}::\text{LUC} \) reporter lines

4.1 Summary

Arabidopsis plants expressing luciferase reporter genes driven by the promoters of nitrate responsive genes, NIR and AMT1.1, proved to be unsuitable for use in a genetic screen for nitrate signalling mutants. Characterisation for LUC activity using CCD imaging revealed that LUC activity was not present in the \( \text{PRO}_{\text{NIR}}::\text{LUC} \) lines and that LUC activity is not correctly regulated in the \( \text{PRO}_{\text{AMT1.1}}::\text{LUC} \) lines. Northern blot analysis confirmed the aberrant expression of the \( \text{PRO}_{\text{AMT1.1}}::\text{LUC} \) lines but showed that \( \text{PRO}_{\text{NIR}}::\text{LUC} \) is transcribed and regulated analogous to the endogenous gene. The cause for the lack of LUC activity in \( \text{PRO}_{\text{NIR}}::\text{LUC} \) lines was not found. It is concluded that a new reporter line driven by the promoter of the nitrate transporter gene, NRT2.1 should be generated.

4.2 Introduction

Nitrogen is an important nutrient for the growth and development of plants. Plants have developed complex signalling pathways to regulate its uptake and assimilation which ensure optimal nutritional status amidst the inevitable nutritional stresses of a heterogeneous environment. Expression of nitrate transporters and assimilatory enzymes are all induced by nitrate provision (Wang et al., 2000).

A genetic screen for mutants with altered gene responses could identify early components of the nitrate signalling pathway. Using a kinetic screen it would be possible to identify mutants whose response may be altered in timing or magnitude, implying a mutation in components responsible for the movement or amplitude of the signal. The reporter gene luciferase, is ideal for a kinetic screen as it is non-invasive and reports real-time expression. Choice of gene promoter to drive luciferase is critical to the effectiveness of the screen and is outlined here.
4.2.1 Candidate gene promoters for the genetic screen

In order to produce reliable and informative reporter lines, good gene promoters should be carefully selected. The criteria for our selection of gene promoters were: 1) nitrate responsive, 2) early response genes, 3) robust, predictable response, 4) quick response and 5) high expression. Nitrate responsiveness is critical as these genes are involved and affected by nitrate signalling. Early and primary response genes are part of the first response of a plant to a change in N nutrition and are upstream in the signalling pathway, also these genes respond quickly which would speed up the screening process and allow more plants to be screened, increasing the chance of identifying mutants of interest. A robust, predictable response is important so that deviants from the norm can be identified and high expression will aid reporter gene detection. The nitrate responsive genes we chose are those for NIR, an enzyme involved in nitrate assimilation, AMT1.1, an ammonium transporter and NRT2.1, a nitrate transporter.

Nitrite reductase (NIR) is the second enzyme in the nitrate assimilation pathway. It catalyses the reduction of nitrite to ammonium, using reduced ferredoxin as the reductant. This process takes place in plastids and is important in preventing the accumulation of nitrite, which is toxic. This enzyme is highly regulated in coordination with nitrate reductase (NR). It is rapidly induced by NO₃⁻ and suppressed by NH₄⁺ (Privalle et al. 1989). Induction of NiR can lead to 100 fold increases in transcript levels. In a microarray study by Wang et al., (2000), this gene was found to be the most strongly upregulated in response to NO₃⁻. The predictable regulation, high expression and rapid response make the promoter of this gene an excellent candidate for a reporter gene for N-signalling.

NIR was chosen rather than NR (nitrate reductase), the first enzyme in the nitrate assimilation pathway. NR gene expression and regulation also fits our criteria for a candidate promoter. NiR was chosen in preference to NR for 3 reasons: 1) NiR has higher expression and a greater magnitude of induction than NR (Wang et al., 2000). 2) In Arabidopsis, NR is coded by two genes, NIA1 and NIA2, whereas NiR is coded by only one gene, NiR (Wang et al., 2000). 3) Other factors may influence NR expression; circadian rhythms, light via the phytochrome system and cytokinin (reviewed in Redinbaugh and Campbell, 1991).
The ammonium transporter AMT1.1 responds negatively to nitrate. It is suppressed by high nitrate or when adequate nitrogen is available (Cerezo et al., 2001). Thus ammonium uptake is inhibited in preference to nitrate uptake. In the microarray experiment reported by Wang et al., (2000), AMT1.1 was identified as the second most suppressed gene by high (10 mM) nitrate. AMT1.1 is induced by N-starvation; this induction is gradual and peaks after 2 days (Gazzarrini et al., 1999). Repression of AMT1.1 expression by nitrate is quicker than its induction by N-starvation and would be the preferential response to study in the screen. This response is more likely to be due to early signalling events than the long induction response, which is likely to be due to metabolism and depletion of internal N supplies.

The nitrate transporter NRT2.1 is a high affinity transporter that is expressed in roots. It is strongly and rapidly induced by nitrate after a period of N-starvation. This induction shows that the NRT2.1 gene is downstream of perception. NRT2.1 is also slowly induced by N starvation; this induction is due to release from repression by N-metabolites such as glutamine (Nazoa et al., 2003).

The PRONIR::LUC and PROAMT1.1::LUC reporter lines were generated by Dr. T Keller, prior to the commencement of this project.

4.3 Results

4.3.1 Verification of expression profiles of candidate genes

Verification of the reported gene expression of the chosen candidates (Section 4.2.1), was done on the RNA level by Northern blot (Figure 4.1). After 4 days of N starvation, hydroponically grown plants were treated with either 250 μM nitrate or 10 mM nitrate for 20 minutes and 2 hours respectively, according to the procedure used by Wang et al. 2000 where NIR and NRT2.1 were identified as primary response genes to nitrate and AMT1.1 was identified as responding negatively to high amounts of nitrate after 2 hours.

We found that NIR was induced by low (250 μM) and high (10 mM) KNO₃ treatment (Figure 4.1), this induction was rapid and was evident after 20 minutes. No induction was seen by KCl treatment, which was used as a control for K⁺.
NRT2.1 was also induced by nitrate treatment. Although transiently induced by N starvation the high expression seen in the 2h non-treated sample (Figure 4.1) is unusual as this transient induction should not be present after 4 days as seen in the 20 min non-treated sample. However, strong expression seen for all probes from the 2h non-treated sample (Figure 4), including the EIF4a-1 loading control, which suggests high loading of this sample has occurred, this high loading may have resulted in the apparent high expression of NRT2.1 in this sample.

AMT1.1 repression by nitrate was evident after 2h with 10 mM KNO₃ treatment (Figure 4.1). NIR and AMT1.1 expression was comparable to that identified by Wang et al., (2000). Although our results for NRT2.1 are not quite as expected, it is evident that NRT2.1 is nitrate inducible. NIR remained the preferred candidate due to its predictable regulation, high expression and high magnitude of induction.

EiF4a-1, a constitutively expressed eukaryotic translation initiation factor (Metz et al., 1992), was used as a loading control.

![Northern blot of RNA from Colombia Wt Arabidopsis plants induced by 250 mM or 10 mM KNO₃ (or KCl control) following the protocol of Wang et al., (2000). The blot was probed for NIR, NRT2.1 and AMT1.1 transcripts to analyse expression and regulation of these genes. EiF4a was used as a loading control](image)

*Figure 4.1* Northern blot of RNA from Colombia Wt *Arabidopsis* plants induced by 250 mM or 10 mM KNO₃ (or KCl control) following the protocol of Wang et al., (2000). The blot was probed for NIR, NRT2.1 and AMT1.1 transcripts to analyse expression and regulation of these genes. EiF4α was used as a loading control.
4.3.2 Analysis of $\text{PRO}_{\text{NIR}}::\text{LUC}$ reporter lines

4.3.2.1 Imaging for LUC activity

$\text{PRO}_{\text{NIR}}::\text{LUC}$ Arabidopsis reporter lines were generated by Dr. T Keller (P Doerner lab) using 2.6 kb of the upstream promoter region of $\text{NIR}$ to drive the $\text{LUC}$ reporter gene. These reporter lines were imaged for LUC activity using a photon counting CCD camera. Seedlings were grown from 15 homozygous lines on vertical plates containing 0.5 x MS agar media (2.1.2 Materials and Methods). After 10 days pre-growth, the plants were transferred to N starvation plates for 2 days before induction by transfer to 10 mM KNO$_3^-$ plates. The plants were pre-sprayed with the substrate luciferin 10 hours prior to induction and at the time of imaging, 2 hours after induction. This 2 hour time-point was chosen based on our Northern blot result (Figure 4.1). Surprisingly, no luminescence was detected from the reporter lines (Figure 4.2). Luminescence was seen in the control $\text{PROCaMV35s}::\text{LUC}$ plants, indicating that plant growth conditions, manipulations, luciferin application and camera settings were conducive for luciferase activity and detection.

An extended time-course of 72 hours was performed. The original time-point of 2 hours had been chosen based on our Northern blot result (Figure 4.1) and previous studies that showed NIR gene expression peaks at this time (Privalle et al., 1989, Sakakibara et al., 1997), yet the peak for protein expression may be later than that for RNA expression. However, no luminescence was detected from the $\text{PRO}_{\text{NIR}}::\text{LUC}$ reporter lines over this time course. Increasing the sucrose content from 1% to 3% to overcome any ‘dark’ inhibitory effects (plants are imaged in the dark) also did not enable luminescence to occur. The N starvation step was also modified to be just nitrate starvation, using NH$_4$ Citrate as the N source in case total nitrogen starvation is too detrimental to the plants (although conditions facilitated LUC activity from the $\text{PROCaMV35s}::\text{LUC}$ plants). Still no luminescence could be detected.

4.3.2.2 RNA analysis of $\text{PRO}_{\text{NIR}}::\text{LUC}$ lines

Northern blots of RNA from $\text{PRO}_{\text{NIR}}::\text{LUC}$ plants were probed for $\text{NIR}$ and $\text{LUC}$ mRNA transcripts. These plants were grown in hydroponic cultures and after a 4 day nitrate starvation, were induced with 10 mM KNO$_3^-$ or 10 mM KCl (control for K$^+$. LUC transcripts were found in all the reporter lines (Figure 4.3). Hybridisation with
LUC transcripts was detected in roots and shoots in KNO₃ treated plants but were absent in KCl treated plants. LUC expression was identical to NIR expression in terms of localisation and nitrate induction. Thus, on the RNA level the reporter gene is expressed and is nitrate inducible, analogous to the endogenous gene. Peculiarly, as revealed by CCD imaging for LUC activity, this gene expression does not seem to carry through to the protein level. It appears that either the LUC transcripts are not translated or that a non-functional protein is being made.

4.3.2.3 Sequence analysis of the PRO₈₊:LUC construct

Prior to making the reporter construct, Dr Thomas Keller had sequenced 2.6 kb NiR promoter PCR fragment and had found it to be 100% identical to the genome sequence.

To further check the integrity of the construct, the junction of the 2.6 kb NIR promoter and the luciferase gene was sequenced as this is a common site where sequence error can occur during cloning. The terminal end of the luciferase gene was also sequenced. It was found that the predicted base sequence was present on both strands for the junction and terminus and that the added terminal Kpn I and Sac I sites were intact (data not shown). The whole LUC gene was sequenced and again no errors were found. This sequencing was done on the construct contained in the high copy plasmid, pLit28. This construction was named A1, and from this plasmid the construct was transferred into the plant transformation vector pTV20, flanked by TDNA borders. This construction was called NF5, and was used to transform A. tumefaciens, which was then used to transform Columbia WT Arabidopsis thaliana plants.

4.3.2.4 PCR and Restriction analysis for recombination events

To test for a potential recombination event in A. tumefaciens; new A. tumefaciens transgenic lines were made with NF5 and labelled NF5-β and the original transgenic A. tumefaciens (NF5-α) was re-grown from a glycerol stock. From these cultures the plasmids were rescued in E. coli and the DNA extracted. These DNA samples, plus the high copy A1 construct and the plant transformation NF5 construct, were used for PCR and restriction analysis.
In the PCR analysis a 2.6 kb fragment spanning parts of the NIR promoter and the LUC gene were amplified out (Figure 4.4b). Comparison of the band sizes show no differences implying that a large recombination event has not occurred. In the restriction analysis the samples were cut with the 4 base pair cutters NlaIII and HaeIII (Figure 4.4a). Other digests were also performed including one that excised the whole construct (data not shown). These digests all showed identical restriction patterns between the four samples, implying that small recombinations have not occurred. However, the results of these tests do not rule out the possibility of point mutations occurring in A. tumefaciens.

4.3.2.5 Generation of new PRO\textsubscript{NIR}::LUC reporter lines

Finally new reporter lines were generated using the NF5-β re-transformed A. tumefaciens and imaged for LUC activity. These plants also failed to show LUC activity (data not shown). The lack of LUC activity in the new PRO\textsubscript{NIR}::LUC reporter lines indicates that a recombination event in A. tumefaciens during Arabidopsis transformation probably did not happen in the original lines. This was assumed as the probability of the recombination happening again is small. However, this indicates that a factor in the construct is the cause of either the inhibition of reporter gene translation or the inhibition of protein function.

Thus, why we see gene expression on the transcript level but not on the protein level remains unknown. Further exploration would prove too time costly and was therefore not continued. It is apparent that the PRO\textsubscript{NIR}::LUC lines could not be used for a mutagenesis screen.
Figure 4.2 CCD image of four nitrate-induced $PRO_{NIR}::LUC$ plants next to a control $PRO_{CaMP35s}::LUC$ plant, demonstrating that no luminescence is detectable in the $PRO_{NIR}::LUC$ plants.

<table>
<thead>
<tr>
<th>Line name</th>
<th>Treatment</th>
<th>49F</th>
<th>54A</th>
<th>68I</th>
<th>70E</th>
<th>80B</th>
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</thead>
<tbody>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
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<td>Cl$^-$</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eIF4α</td>
<td></td>
<td></td>
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</table>

Figure 4.3 Northern blot of RNA from five lines of $PRO_{NIR}::LUC$ plants induced by 10 mM KNO$_3^-$ or KCl (control). Blot was probed for NIR and LUC transcripts, to compare transgene expression with the endogenous gene expression. NIR and LUC transcripts are present in RNAs from KNO$_3^-$ induced plants.

Figure 4.4 a) Restriction analysis of plasmid rescue minipreps from the original transformed A. tumefaciens (αA-C) and from the retransformed A. tumefaciens (βA-C), and from the low copy plasmid containing the $PRO_{NIR}::LUC$ construct (NF5) before transformation. NcoI and HaeIII are 4 bp cutters. b) PCR analysis of the rescued plasmids αA and βA, the low copy plasmid NF5 and the high copy plasmid A1. A 2.6 kb fragment across the $NIR::LUC$ junction was amplified. The control reaction contained no template. The band sizes are identical from each plasmid indicating that no large recombinations have occurred during the cloning.
4.3.3 Analysis of \textit{PROAMT1.1::LUC} reporter lines

4.3.3.1 Imaging for LUC activity and regulation

These reporter lines were generated by Dr. T. Keller (P Doerner Lab) using 2 kb of the \textit{AMT1.1} gene promoter to drive the \textit{LUC} gene. Seedlings from all 14 homozygous lines were imaged for LUC activity. \textit{PROAMT1.1::LUC} plants were germinated and grown on 0.5 x MS agar plates. At 12 days old, the plants were pre-treated with 1 mM luciferin 10 hours before and during imaging. Luminescence was detected in 11 of these 14 lines (Figure 4.5).

\textit{LUC} expression was only detected in plant shoots and not in roots. \textit{AMT1.1} was shown by Northern blot to be expressed throughout the plant and highly expressed in roots (Gazzarrini \textit{et al.}, 1999). In the root it is preferentially expressed in root hairs (Lauter \textit{et al.}, 1996) which make up >70 % of the root surface (Marschner, 1995), it could be this root hair expression that makes root LUC activity hard to detect as the roots hairs are small single cells: it is also possible that another substrate such as ATP or oxygen is limiting here. However, luminescence is detected in the roots of the \textit{PROCaMV3S::LUC} plants and also in \textit{PROAT4::LUC} transgenic plants (Fan Lai 2003, \textit{pers. com.}). \textit{AT4} is a phosphate starvation gene.

Imaging for LUC activity in \textit{PROAMT1.1::LUC} plants treated with 10mM KNO$_3$ and under N starvation was performed to analyse the regulation of the reporter gene. CCD images were taken over 3-day time-courses and the integral light intensity of the luminescence was measured (data not shown). The predicted repression by nitrate or induction from N starvation was not detected. Thus, the \textit{PROAMT1.1::LUC} reporter gene did not reflect endogenous \textit{AMT1.1} expression, in fact, it appeared to be constitutively expressed.

4.3.3.2 RNA analysis of \textit{PROAMT1.1::LUC} reporter lines

Northern blots demonstrate that the reporter gene is mis-expressed (Figure 4.6). Root and shoot tissue was harvested separately from 14 day old plants that had be N starved for 4 days prior to repression by 10mM KNO$_3$. From these, RNA was extracted and blots were probed for \textit{AMT1.1} and \textit{LUC} mRNA. The results showed \textit{LUC} transcripts were only present in the shoot and are present under repression as well as induction (Figure 4.6), indicating that the reporter gene is being constitutively
expressed in the shoots. This confirms the earlier observations from imaging. Exploration into the cause of this mis-expression was time costly. It was concluded that none of the $PRO_{AMTI.1}::LUC$ lines could be used for a screen.

![Image](https://example.com/image)

**Figure 4.5** CCD images of 7 $PRO_{AMTI.1}::LUC$ lines with $PRO_{CALM335}::LUC$ as a control (top right panel). LUC activity is visible in the leaves. Variability of light intensities between lines is attributed to variable TDNA insertion sites for the transgene among these lines.

<table>
<thead>
<tr>
<th>Shoots</th>
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<td>weak</td>
</tr>
<tr>
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</tbody>
</table>

<table>
<thead>
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<th>1hr</th>
<th>2hr</th>
<th>4hr</th>
<th>6hr</th>
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<td>Cl$^-$</td>
<td>NO$_3^-$</td>
<td>Cl$^-$</td>
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<tr>
<td><strong>AMTI.1</strong></td>
<td>strong</td>
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<td>weak</td>
</tr>
<tr>
<td><strong>LUC</strong></td>
<td>weak</td>
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</tbody>
</table>

**Figure 4.6** Northern blot of RNA from $PRO_{AMTI.1}::LUC$ plant roots and shoots, harvested from plants repressed by 10 mM KNO$_3$. 10mM KCl was used as a control for K$^+$. The blots were probed for $AMTI.1$ and $LUC$ transcripts to compare transgene expression with endogenous gene expression. $LUC$ transcripts are not present in the roots. In the shoot, $LUC$ expression is not regulated as the endogenous $AMTI.1$. This blot is representative of those from four $PRO_{AMTI.1}::LUC$ lines.
4.4 Discussion

It is has been shown that \(\text{PRO}_{\text{NIR}}::\text{LUC}\) and \(\text{PRO}_{\text{AMT1.1}}::\text{LUC}\) reporter lines cannot be used for a screen or any physiological study of the activity of these two genes. In the case of \(\text{PRO}_{\text{NIR}}::\text{LUC}\), the reporter gene does not 'report' and in the case of \(\text{PRO}_{\text{AMT1.1}}::\text{LUC}\) the 'report' is inaccurate and does not reflect the endogenous gene expression. In both cases the evidence points to integral defects in the reporter construct rather than the TDNA insertion position in the plant genome. This is evident by the fact that the same mis-expression pattern was observed in all the homozygous lines for both \(\text{PRO}_{\text{NIR}}::\text{LUC}\) and \(\text{PRO}_{\text{AMT1.1}}::\text{LUC}\).

It is unknown whether translation is occurring in the \(\text{PRO}_{\text{NIR}}::\text{LUC}\) lines. Antibody studies for LUC protein would have been able to determine this. However, this further investigation would have been costly in terms of funds and time, and not relevant to the screen or dissection of nitrate signalling. If translation is occurring then modifications have occurred that render the LUC protein inactive. Large promoter sequences were used to ensure the presence of all regulatory sequences. It is possible that the sequence also contains cis-acting elements that caused mRNA or protein modifications that are required for normal NIR protein activity but in turn inhibit LUC protein activity. For example; immunochemical investigations in wheat, pea, rice, spinach and maize have shown that a precursor NIR is made. Upon transport into chloroplasts, this precursor NiR is cleaved resulting in formation of the active NiR enzyme (reviewed in Redinbaugh and Campbell, 1991). It may be that elements in the NiR promoter region code for cis-acting regulatory factors to be attached to the LUC protein that results in it also being transported and cleaved, rendering the protein inactive.

It is evident that promoter activity and regulation is occurring as expected; Northern blots showed that the \(\text{PRO}_{\text{NIR}}::\text{LUC}\) gene expression profile follows that of endogenous \(\text{NIR}\) gene expression. It is interesting to note that the only other Arabidopsis reporter lines for nitrite reductase used the tobacco \(\text{NIR}\) promoter sequence rather than the Arabidopsis sequence (Dorbe et al., 1998).

Analysis of the \(\text{LUC}\) sequence showed it to be intact. The same LUC vector (pSP \(\text{LUC+ 301}\)) was used in the \(\text{PRO}_{\text{AMT1.1}}::\text{LUC}\) construct. These lines showed LUC
activity demonstrating the integrity of the $LUC + 301$ sequence and its potential to code for an active protein. Unfortunately in the $PRO_{AMT1.1}::LUC$ lines, LUC imaging and RNA analysis show constitutive shoot expression which is not reflective of endogenous $AMT1.1$ expression.

Thus, it was apparent that new reporter lines had to be made to use in the screen. As discussed in 4.2.1 and 4.3.1, the remaining candidate gene promoter is that for the nitrate inducible high affinity nitrate transporter, NRT2.1. The construction and successful characterisation of these reporter lines is the subject of chapter 5.
Chapter 5 - Construction and characterisation of the PRO\textsubscript{NRT2.1::LUC} reporter line as suitable to the screen

5.1 Summary

Two LUC reporter lines using the \textit{NRT2.1} gene promoter were generated in \textit{Arabidopsis thaliana}. These reporter lines were characterised and found to express the transgene. LUC activity was detected predominately in the roots of the plants and activity increased with nitrate provision, indicating that the reporter gene is regulated and nitrate inducible. RNA analysis using quantitative real-time PCR confirmed that the reporter gene reflects endogenous gene expression. Suitable candidate lines are selected for use in a genetic screen.

5.2 Introduction

It is imperative for the success of a genetic screen to have a good reporter line. The criterion of a good reporter line is that the reporter gene activity accurately reflects the activity and regulation of the endogenous gene. The activity of the reporter gene is determined by the promoter region that drives it. In the last chapter, it was shown that the \textit{PRO\textsubscript{NIR::LUC}} and \textit{PRO\textsubscript{AMT1.1::LUC}} reporter lines did not fit this criterion. Thus, it was necessary to generate new reporter lines using the upstream promoter region of the nitrate transporter, NRT2.1.

The selection of \textit{NRT2.1} as a candidate gene promoter was briefly outlined in Section 4.2.1. Here a more detailed explanation will be given. NRT2.1 is a nitrate transporter thought to be the main contributor to the inducible high affinity transport system (iHATs). A TDNA knock-out mutant for NRT2.1 and the lower expressed NRT2.2, retains only 27% iHATs activity.

iHAT activity for nitrate uptake was identified and studied before the discovery of the nitrate transporters (Section 1.4). \textit{NRT2.1} expression conforms with described iHAT regulation as it is: 1) induced by nitrate, 2) repressed by high internal N status, 3) repressed by N metabolites and 4) stimulated by photosynthesis (Cerezo \textit{et al.}, 2001). \textit{NRT2.1} is strongly induced by nitrate, especially after a period of starvation. It is repressed by high levels of nitrate and high internal nitrogen status. It is also
repressed by NH$_4^+$ and N metabolites such as glutamine and arginine. $NRT2.1$ expression is also de-repressed by N starvation; this is thought to be due to the release from repression by N metabolites. The magnitude of this de-repression is lower than the magnitude of induction by nitrate. $NRT2.1$ expression is also diurnally regulated; its transcript levels decrease at night, and the addition of sucrose inhibits this dark effect. This well-established, robust regulation of $NRT2.1$ and its strong induction by nitrate makes it an attractive gene promoter candidate for this screen.

5.2.1 $NRT2$ Family

$NRT2.1$ is a member of the $NRT2$ multigene family, which in Arabidopsis consists of seven members. $NRT2.1$ was the first member to be isolated and is the most characterised.

The $NRT2$ family is a member of the nitrate/nitrite porter (NNP) subfamily which is a part of the major facilitator superfamily (MFS). The first member of the $NRT2$ family was identified in the fungus Aspergillus nidulans based on a chlorate resistant mutant (crna) which was subsequently found to be affected in nitrate uptake (Unkles et al., 1991). Further members have been found in C.reinardtii, barley, N.plumbaginifolia, soybean, wheat, rice and tomato (Orsel et al., 2002).

The seven Arabidopsis $NRT2$ genes are distributed throughout the genome. $NRT2.1$ and $NRT2.2$ are situated in a tail-to-tail formation on chromosome I, with $NRT2.5$ at the bottom of chromosome. $NRT2.3$ and $NRT2.4$ are in head-to-tail formation on chromosome V with $NRT2.7$ at the top of the chromosome. $NRT2.6$ is located alone on chromosome III. All these genes show sequence homology and amino acid and domain homology on the protein level. Recent studies to further characterise these individual genes have shown that $NRT2.1$ is the most highly expressed member of the family. It is clear that redundancy is present within the Arabidopsis $NRT2$ family; when $NRT2.1$ is absent, high affinity nitrate uptake still occurs albeit at a reduced level. However, individual genes may play specific roles for example, $NRT2.7$ exhibits strong leaf expression (Orsel et al., 2002, Okamoto et al., 2003), indicating a specific role for nitrate transport in this organ.

$NRT2.2$ and $NRT2.4$ have the closest homology to $NRT2.1$ on the protein level, sharing 87% and 82% identity respectively. It is not surprising that $NRT2.1$ and
NRT2.2 display such close sequence similarity as they are situated in tandem on chromosome I, suggesting that a duplication event has occurred. The expression profile of NRT2.2 is a matter of debate; some report it as 'barely detectable' (Orsel et al., 2002), others report it as 'highly expressed' (Okamoto et al., 2003). These discrepancies may be due to variation in experimental conditions such as the developmental stage of the plants used and the RT-PCR primer set specificities. NRT2.4 was reported to have significant expression in shoots as well as roots; this expression was inducible by nitrate and N starvation. In fact, it was reported to be 'the most inducible gene by limiting nitrate conditions' (Orsel et al., 2002). When initial candidate promoters were chosen (Section 4.2.1), this expression profile for NRT2.4 had not been published. As shoot expression as well as root expression would be useful for screening conditions, further characterisation of mutants and investigation of systemic signalling effects, it was decided to examine NRT2.4 expression alongside NRT2.1 to determine which would be the best candidate for the new reporter line.

5.3 Results

5.3.1 Comparison of NRT2.1 and NRT2.4 gene expression

Northern blots of RNA extracts of shoots and roots from nitrate induced plants were probed for NRT2.1 and NRT2.4 transcripts (Figure 5.1). It was found that NRT2.1 is more highly expressed than NRT2.4. This is evident by the shorter exposure time required for NRT2.1 expression to be seen, even though the NRT2.4 probe had a high specific activity than the NRT2.1 probe (data not shown). The Northerns show preferential root expression for both genes. Shoot expression for NRT2.4 was not seen. NRT2.4 de-repression by N starvation (KCl control) was greater than its induction by nitrate. The overall low expression of this gene renders it an unsuitable candidate gene promoter for the screen. Therefore, we decided to remain with the original decision to use the NRT2.1 promoter for the construction of new reporter lines.
5.3.2 Construction of PRO\textsubscript{NRT2.1}::\text{LUC} and PRO\textsubscript{NRT2.1}::\omega::\text{LUC} reporter lines

Two PRO\textsubscript{NRT2.1} reporter gene constructs were made; the PRO\textsubscript{NRT2.1}::\text{LUC} construct is a standard promoter::reporter gene fusion whilst PRO\textsubscript{NRT2.1}::\omega::\text{LUC}, was made to contain a translational enhancer sequence know as the ‘omega’ sequence. The omega sequence is an 80bp sequence derived from the Tobacco Mosaic Virus 5’UTR and was first used by Millar \textit{et al.}, (1992) to enhance the translation of their PRO\textsubscript{CAB2}::\text{LUC} reporter genes for their genetic screen for circadian clock mutants. The omega sequence was made by annealing six oligonucleotides (Materials and Methods 2.4.1) and placed upstream of the ATG start codon of the luciferase gene.

We originally tried to clone a large promoter region of 1.9 kb for \textit{NRT2.1}, to ensure all regulatory sequences were incorporated. Despite many different cloning strategies we were not able to ligate this promoter to the \text{LUC} gene. A successful PRO\textsubscript{NRT2.1}::\text{GUS} reporter line had been generated by Nazoa \textit{et al.}, (2003) using 1.2 kb of \textit{NRT2.1} promoter. We found that we were able to clone a 1.222bp \textit{NRT2.1} promoter, first time, into the LUC vectors (Materials and Methods 2.4.3).
5.3.3 Pre-screen of heterozygous lines for Luciferase activity

Second generation plant transformants (T2) were tested for reporter gene activity prior to generating homozygous T3 lines. T2 lines showing 3:1 segregation on selective media were grown on vertical 0.5x MS agar plates for 10 days before transfer to N starvation conditions for 2 days. This was followed by nitrate induction by transfer to 10 mM KNO₃ containing plates. The plants were sprayed with 1 mM luciferin as described in Materials and Methods, 2.1.6.

Luciferase activity was detected in plants from every T2 heterozygous line. The plants were imaged prior to induction and 2h and 4h after induction. A sample of eight plants per line was taken. Figure 5.2 shows plants from two T2 lines. Luminescence can be seen mostly in roots with a small amount in the shoots. This expression pattern fits the expected expression profile as determined from our Northern data and other published studies (Orsel et al., 2002, Nazoa et al., 2003, Okamoto et al., 2003). Individual plants that did not show luciferase activity were presumed to be homozygous non-transgenic plants.

Figure 5.3 shows the quantitative data from the T2 pre-screen, which was obtained from the CCD images as described in Materials and Methods, 2.2.4. There is variation in the luminescence intensity between the lines; this occurs because TDNA can be incorporated throughout the genome and thus come under the influence of other promoters and enhancers. Although these results come from a mixture of homozygous and heterozygous plants, they gave a preliminary result for which lines might be best for the screen. For example, PRONRT2.1::LUC T2 #10 and #28 and PROnrt2.1::Ω::LUC T2 # 7, showed the brightest luminescence after induction, a quality which could aid mutant detection in the future screen.

It was also found that relative luminescence intensity increased after provision of 10 mM KNO₃ (Figure 5.3), demonstrating that the reporter construct is nitrate inducible. This preliminary screen of the T2 lines enabled us to know that the reporter gene is active and that its expression appears to be regulated like the endogenous gene in terms of localisation and induction.
Figure 5.2 CCD images of T2 $\text{PRO}_{\text{NRT2.1}}::\text{LUC}$ plants. The first picture shows a light image and the second shows plants imaged for LUC activity. The plants were induced by 10 mM KNO$_3$ for 2h.

Figure 5.3 Quantitative data from T2 pre-screen of $\text{NRT2.1}$ promoter driven reporter lines. Plants were induced by 10 mM KNO$_3$ and imaged for LUC activity before induction and at 2h and 4h after induction.
5.3.4 Imaging homozygous lines for luciferase activity in response to nitrate induction

Ten homozygous lines were identified for \( PRO_{NRT2.1::LUC} \) and twelve homozygous lines were identified for \( PRO_{NRT2.1::\Omega::LUC} \). Based on the results from the preliminary screen (Section 5.3.3), 5 lines were taken for further analysis. The three putative strong expressers; \( PRO_{NRT2.1::LUC} \) #10, #28 and \( PRO_{NRT2.1::\Omega::LUC} \) #7 were taken as well as \( PRO_{NRT2.1::\Omega::LUC} \) #20, which had low expression and \( PRO_{NRT2.1::\Omega::LUC} \) #1, which was down regulated after induction (Figure 5.3).

Plants from these lines were tested for LUC activity. Luminescence was seen in all of the plants, confirming the homozygocity of the lines. These plants were grown on nylon mesh overlaid on 0.5x MS agar media on vertical plates. The plants were N starved for 5 days prior to induction. The plants were induced using liquid 10 mM KNO\(_3\) media. This was done by lifting the nylon mesh off the agar media and placing it into a square Petri dish on top of two layers of moist blot paper. A CCD image representing the 0h time-point was taken at this time, prior to the addition an induction media. Plants were treated with luciferin as previously described (Materials and Methods, 2.1.6). Images were taken at 2h, 4h and 8h time-points.

Surprisingly, it was found that light intensity was greatest in the 0h time-points before induction (Figure 5.4). After investigation it was determined that this result is due to the change in experimental method; the 0h time-point was taken just after transfer of the plants whereas in previous experiments the 0h time-point was taken prior to transfer as the transfer coincided with induction. Nass and Scheel (2000) showed that rapid wound-induced light emission occurs due to enhanced luciferin entry upon wounding. The transfer of the plants causes some wounding to the plants, allowing the enhanced influx of luciferin, by imaging the plants directly after transfer we were imaging this phenomenon. In subsequent experiments the 0h time-point is taken after a recovery time of 30min and this wound effect is abolished (evident in Figure 5.7a-c.).

Despite these technical drawbacks, it is apparent from Figure 5.4 that light intensity increases between 2h and 8h of induction by nitrate. This suggests induction of the reporter gene. It is also apparent that luminescence is greatest in the two \( PRO_{NRT2.1::LUC} \) lines #10 (NLuc10) and #28 (NLuc28), which was also seen in
the T2 pre-screen. It is interesting to note that the lines containing the omega sequence display lower levels of expression than those without (Figure 5.4). To determine if these results for reporter gene expression are truly representative of endogenous gene expression it was necessary to look at gene induction on the RNA level in the plants.

![Graph showing quantitative data from T3 homozygous NRT2.1 promoter driven reporter lines, imaged for LUC activity after 10 mM KNO₃ induction. Plants show rapid wound-induced light emission at 0hr; this was due to an altered transfer method and was accounted for in subsequent experiments. LUC activity increases between 2h and 8h, indicating induction of transgene by nitrate. This induction is not seen in 10 mM KCl treated plants (not shown).](image)

**Figure 5.4** Quantitative data from T3 homozygous NRT2.1 promoter driven reporter lines, imaged for LUC activity after 10 mM KNO₃ induction. Plants show rapid wound-induced light emission at 0hr; this was due to an altered transfer method and was accounted for in subsequent experiments. LUC activity increases between 2h and 8h, indicating induction of transgene by nitrate. This induction is not seen in 10 mM KCl treated plants (not shown).

### 5.3.5 Examining reporter gene regulation by quantitative PCR

The five candidate lines were also characterised at the transcript level. The previous unsuccessful NIR and AMT1.1 reporter lines were examined on the RNA level by Northern blot to analyse the erroneous expression we had seen by luciferase imaging. In the NRT2.1 reporter lines, LUC activity was detected and correctly regulated; therefore RNA analysis comprised part of the characterisation for a suitable line for a screen. Quantitative RT-PCR (qPCR) was used instead of Northern blot. This was done as qPCR is a highly sensitive and quantitative approach, allowing a detailed analysis of the reporter gene and endogenous gene expression.
RNA was extracted from T3 homozygous reporter lines grown hydroponically in conical flasks (Materials and Methods 2.1.4). The plants were induced by either 10 mM KNO$_3$ or 10 mM KCl (as a control for $K^+$) after a period of N starvation. After induction, plant roots were harvested at 1h, 2h and 4h. Plants were also harvested from non-treated flasks, representing a 0h time-point.

It was found that the LUC reporter gene expression is induced by nitrate (Figure 5.5). LUC transcript levels remain at the basal level after 2h of 10 mM KCl treatment, whereas with 2h of 10 mM KNO$_3$ treatment transcript levels rise dramatically to a maximum of 300x the basal level. In Figure 5.5, the $PRO_{NRT2.1::LUC}$ lines #10 and #28 show the greatest gene expression, which correlates with the findings from imaging data for LUC activity (Section 5.3.4). This data also supports the lower LUC activity findings for the $PRO_{NRT2.1::Q::LUC}$ lines which all show nitrate induction of reporter gene expression with a maximum of only 92 fold difference, which is only a third of the maximum expression level of nitrate induced $PRO_{NRT2.1::LUC}$ lines (Figure 5.5).

Nitrate induction of LUC peaks after 2 hours of KNO$_3$ treatment followed by a decrease in transcripts seen in the 4 hour time-point (Figure 5.5b). As LUC activity continues to increase after 2 hours induction (Figure 5.3, 5.4) these results indicate a delay between gene transcription and mRNA translation (See also Figure 5.7).

$PRO_{NRT2.1::LUC}$ lines #10 and #28 were taken for further analysis to compare LUC gene expression (Figure 5.6a) with endogenous $NRT2.1$ gene expression (Figure 5.6b). It was found that the pattern of LUC gene expression in response to nitrate correlates with the pattern of $NRT2.1$ gene expression (Figure 5.6a-b). Although, relative LUC transcript levels are 14x greater than $NRT2.1$ levels for the 2h time-point in $PRO_{NRT2.1::LUC}$ #10, demonstrating that the reporter gene is more induced. This exaggerated response of the reporter gene may be fortuitous if LUC activity is also highly induced, as this would facilitate detection by imaging. It is important to note that these figures show fold difference relative to the basal level of each gene; this basal level is the amount of transcript present at the T0 time-point. NIR (Nitrite reductase) gene expression was also found to be induced in the nitrate treated plants (Figure 5.6c); this indicates that a global nitrate induction has occurred in the plants.
Figure 5.5 a) Quantitative RT-PCR for LUC transcripts from RNA samples, show induction of reporter gene expression in response to NO$_3^-$. b) Time course of nitrate induction of the reporter genes.

Figure 5.6 Quantitative RT-PCR for LUC and NRT2.1 transcripts show that reporter gene expression follows that of the endogenous gene. Quantitative RT-PCR for NIR shows that a global NO$_3^-$ induction response is occurring in the plants.
5.3.6 Selection of candidate lines for the screen

The final selection for the candidate line was to induce and image the PRO_{NRT2.1;}::LUC T4 homozygotes over a time-course (Figure 5.7a-b). LUC activity increased after 10 mM KNO_3 induction, peaking at the 6h time-point (Figure 5.7c). This peak is later than the 2h peak in RNA transcripts seen in qPCR experiments (Figure 5.7d). Again, this shows the delay between RNA transcript production and active protein production. It is clear that LUC activity and regulation is detectable in both these lines and that both these lines are suitable for use in a genetic screen for nitrate signalling mutants.

Figure 5.7 a) and b) show CCD images of PRO_{NRT2.1;}::LUC plants from lines #10 and #28. These plants have been induced by 10 mM KNO_3 after 2 days N starvation. Luminescence (LUC activity) increases up to 6h post induction. This image data is quantified in c). Graph d) shows quantitative RT-PCR data from these lines. It shows that LUC and endogenous NRT2.1 mRNA transcript levels peak at 2h post induction. The pattern of LUC expression follows that of NRT2.1 although it level of induction is greater. As fold difference is calculated relative to transcript levels at the 0h time-point, it is not possible to compare amounts of gene expression between the two genes analysed.
5.4 Discussion

Using a 1.222 kb promoter sequence upstream of the \textit{NRT2.1} gene it was possible to generate LUC expressing reporter lines in \textit{Arabidopsis thaliana}. Cloning of a larger promoter region of 1.9 kb was not possible, however, it was shown from the \textit{PRO_{NRT2.1::LUC}} reporter lines that 1.2 kb of promoter region is sufficient to drive and regulate reporter gene expression so that it accurately reports endogenous \textit{NRT2.1} gene expression. This was shown on the transcript level as well as on the protein level. It was interesting that the reporter lines containing the omega sequence had a lower expression than those without when this sequence is a translation enhancer. Apparently, this effect has been seen in other omega containing reporter lines (Millar A, \textit{pers. comm.} 2005).

During the characterisation of these reporter lines improvements in growth and induction conditions were made which will be useful for the future screen. It was found that seedling growth on nylon mesh enables quick transfer of multiple plants to new media without major disturbance to the root system. Previously, plants were transferred individually using tweezers; this system was slow and difficult and caused the root system to gather. However, it was found that the transfer even with nylon mesh causes some minor wounding which led to increased luciferin uptake and a burst of luminescence known as the 'rapid wound-induced light emission effect' (Nass and Scheel., 2000); however, it was found that a recovery time of 30 minutes after transfer was sufficient to eliminate this effect.

Unfortunately characterisation of these lines was not taken further, due to time constraints. The next step of the characterisation would have been to check the two \textit{PRO_{NRT2.1::LUC}} lines and one omega line for transgene content; this analysis would have been done by Southern blot. It is important to do this as it is preferable to use a reporter line that contains only one transgene as multiple transgenes can lead to potential problems such as gene silencing. In a screen, this would lead to the identification of false positives rather than actual mutants. If multiple transgenes were found in these lines, backcrossing into wild-type \textit{A.thaliana} (Col 0) would be done until lines with single transgene insertions were generated.
After this characterisation, the chosen reporter line would have been further tested. Repeated induction experiments would have been done using CCD imaging for analysis to generate a normal response curve. This is important so that mutants with altered responses in timing or magnitude can be detected as deviating from the norm. These plants would also be used to optimise growth and screening conditions. Once this was done, bulked seed would have been mutagenised by ethyl methanesulphonate (EMS) and taken through the screen. Unfortunately, time did not permit this to be done and it can only be hoped that these functional reporter lines will be used in a future screen.

These lines can also be used to investigate nitrate signalling as measured by NRT2.1 expression as reported by the reporter gene, and they were used in split root experiments to examine systemic signalling (Chapter 9).
Chapter 6 - Discussion and Conclusions for Chapters 3 – 5

These chapters have focused on the development of a genetic screen to identify early intermediate signalling components for nitrate signalling (Section 1.9). The original objective was to perform this screen using a $PRON_{NIR}::LUC$ reporter line in *Arabidopsis thaliana*. Unfortunately, after rigorous testing and analysis it was found that these reporter lines were not suitable for the screen (Section 4.3.2). The reporter gene transcripts were present after induction by nitrate but LUC activity was not detected. Analysis of another set of reporter lines, $PRO_{AMT1.1}::LUC$, showed that these were also unsuitable for use in the screen as the reporter gene, $LUC$, showed mis-expression in localisation and regulation (Section 4.3.3). It was realised that due to the time taken to characterise these two reporter lines, it would not be possible to map any mutations identified in a screen. However, it was hoped that, with the generation of a new reporter line, the screen could still be performed and the mutants described.

New reporter lines using 1.2 kb of the $NRT2.1$ promoter region were generated and showed regulated $LUC$ expression and activity. These reporter lines fulfil the original criteria for the envisaged screen, and with a little further characterisation (Section 5.4) could be used to screen for mutants affected in nitrate signalling. Unfortunately, the cloning of the $PRO_{NRT2.1}::LUC$ was difficult; many cloning strategies were tried before the successful construct was made and the transgenic *Arabidopsis thaliana* lines produced. This meant that there was no longer sufficient time to perform the genetic screen.

In the future, these lines may be used for a genetic screen and here I shall outline how such a screen could be conducted with these lines.

6.1 The future screen

There are several different types of mutants that we could identify in a genetic screen using the $PRO_{NRT2.1}::LUC$ reporter lines; these are summarised by the model in Figure 6.1 and described in Table 6.1. Figure 6.1 shows the predicted ‘normal’ nitrate induction response which we would expect from wild-type $PRO_{NRT2.1}::LUC$
reporter lines. This curve is based on the nitrate induction result for \textit{PRONRT2::LUC} #28 seen in Section 5.3.6, where the induction peaks at 6 hours.

![Graph showing time vs. light intensity with early, high, and late/Low/Loss categories.](image)

\textbf{Figure 6.1} Hypothetical model for identifying kinetic mutants in a genetic screen for nitrate signalling mutants.

<table>
<thead>
<tr>
<th>Class</th>
<th>Type/Characteristics</th>
<th>Implications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Timing</td>
<td>Early Peaks early, quicker induction</td>
<td>Implies release from repression or gain of function.</td>
</tr>
<tr>
<td></td>
<td>Late Peaks late, slower induction. Requires long time-course to identify.</td>
<td>Implies loss of factors or defective/impaired components involved in the speed or movement of the signal. May imply reduced perception.</td>
</tr>
<tr>
<td>Magnitude</td>
<td>High Peaks normal, more LUC activity</td>
<td>Implies release from repression or gain of function resulting in an increase amplification of the signal.</td>
</tr>
<tr>
<td></td>
<td>Low Peaks normal, less LUC activity</td>
<td>Implies loss of factors involved in amplification of the signal. May also be affected in perception or affected in passive factors such as C metabolism.</td>
</tr>
</tbody>
</table>

\textbf{Table 6.1} Classes and types of MOIs, their characteristics and implications.
Although constitutively on or off mutants could also be identified, we want to screen for kinetic mutants affected in the timing and/or magnitude of the response (Section 3.2.1), as these would imply mutations in intermediate core signalling components.

There are four types of mutants of interest (MOIs); Early, High, Late and Low, these are summarised in Table 6.1. Combinations of these types could also occur, such as Early/High, Early/Low, Late/High and Late/Low, these would all be MOIs. Although, it is predicted that mutants that are Early and/or High will be the easiest to identify, these mutants are not expected as these mutants would indicate release from repression and experiments with protein synthesis inhibitors have indicated that there is no repression acting on early nitrate signalling (reviewed in Redinbaugh and Campbell, 1991). Late and/or Low mutants will be harder to distinguish from each other and require further characterisation by an extended time-course. However, these mutants would be informative in revealing core components involved in amplification, perception and movement of the signal.

It is proposed that the screen should be done in at least two rounds. The first round should be simple and have a high-throughput. This screen would not be based on the response kinetics but would be designed so that potential mutants of interest (MOIs) could be identified, thus reducing the number of plants taken through to the second round kinetic screen.

The plants should be grown on nylon mesh on vertical plates. As already described (Section 5.4), nylon mesh is the most effective way for transferring plants from one media plate to another, which is necessary for transfer to nitrate starvation and induction conditions. The frequency of visible mutants is low (hence the need to screen a large population), therefore the majority of plants on a plate will be non-mutated or non-phenotypic mutants that resemble the wild-type plants and can thus be used as a control to which mutants are compared to by eye and MOIs are identified as shown in Figure 6.2.

For the first round screen, the mutant M2 plants should be imaged at just one time-point after induction. The suggested time-point for the first round screen, is the three hours after induction time-point. This time-point is chosen as at this time-point luminescence is bright enough so that mutants with low or late expression can be identified but has not yet peaked so that mutants with an early response can be
identified as having brighter luminescence (Figure 6.3). Therefore, it is suggested to screen for mutants that are either significantly brighter or dimmer than the non mutant control plants. As shown in Figure 6.1, these plants could be affected in the timing and/or magnitude of the response. Only further characterisation by means of a time-course can truly determine the type of signalling mechanism that has been affected. Therefore, the second round screen using M3 plants would incorporate a time-course of three or more time-points. Three time-points is manageable and sufficient to identify kinetic mutants. From this screen, plants that show constitutively high or low luminescence would be discarded.

True kinetic mutants identified in the second round screen would then be characterised and select mutants chosen, and the mutated gene mapped. Hopefully, revealing a core nitrate signalling component gene.

Figure 6.2 Hypothetical plate showing how MOls are identified against non mutants (ie wild-type). Darkness of roots represents strength of light intensity in the CCD image. In this example, #2 has a weak signal, possibly due to late/low/loss expression, #9 has a strong signal, possibly due to early/high expression. These two plants would be mutants of interest (MOIs). Plant #6 shows loss of expression; this is not an MOI and would be discarded. The other plants resemble wild-type and so would not be further screened.

Figure 6.3 – Hypothetical model illustrating potential mutant response kinetics against the normal nitrate induction of NRT2.1 as shown by LUC activity in PRO\textsubscript{NRT2.1::LUC} # 28 lines. The highlighted areas at the 3 hour time-point show that at this time-point each type of mutant is distinguishable from the ‘norm’ as having either more or less LUC activity.
6.2 Conclusions

Preparing for a genetic screen is not a simple task. Careful deliberation is needed when choosing suitable gene promoters for reporter lines tailored so that the most information can be gained from future mutants in order to answer specific biological questions. Likewise, the choice of reporter gene is also important. As shown here, the cloning and characterisation of reporter lines is not always straightforward. My work was frustrated by the fact that two of my reporter lines proved to be unsuitable and the production of a third was difficult and time costly. However, a suitable reporter line was generated that can be used to further examine nitrate signalling in Arabidopsis, either in a genetic screen or by other methods.

Although the screen was not carried out, it is clear that using the $PRONRT2.1::LUC$ reporter lines in a kinetic based genetic screen could yield interesting and informative mutants that would contribute to the elucidation of the plant nitrate signalling pathway.
Part 2 – Pharmacological Experiments

The aim was to identify signalling mechanisms involved in nitrate signalling by studying the effect of the application of pharmacological chemicals on the nitrate induction response. Main findings are reported here.
Chapter 7 – The requirement of de novo protein synthesis for normal nitrate signalling when sucrose is limiting

7.1 Summary
Cycloheximide (CHX) is a protein synthesis inhibitor used here to determine whether de novo protein synthesis is involved in the nitrate induction response of NIR and NRT2.1. Using Arabidopsis cell suspension cultures and hydroponic plant cultures we identified a sucrose-dependent CHX effect. With low sucrose, CHX treatment reduced the amplitude of the nitrate induction of both NIR and NRT2.1. This suggests a role for protein synthesis in amplification of the nitrate signal when sucrose is limiting. At high sucrose provision, CHX had no effect on the induction of these genes.

7.2 Introduction
Nitrate is an important nitrogen nutrient for plants and its distribution in the soil is heterogeneous. Signalling pathways enable the plant to sense changes in the environment and then produce adaptive responses that optimise nutrient uptake, assimilation and growth. Prior to visible growth responses, there is an array of gene expression responses. As discussed in Section 1.8, there are many nitrate responsive genes that make up the nitrate transcriptome. Most of these genes are involved in nitrate uptake (NRT2 genes) and assimilation (NR, NIR, UPM, Fd, GOGAT, GS). Using nitrate metabolism mutants, nitrate itself was identified as the signal for the induction of these genes (reviewed in Crawford, 1995). However, it is not known how nitrate is sensed and the signal produced, transduced and propagated to result in the induction of these genes. It was our aim to investigate this primary signal and the signalling mechanisms involved. Here we examined the effect of the protein synthesis inhibitor, cycloheximide (CHX) on the nitrate induction response of the nitrite reductase gene NIR and the high affinity transporter NRT2.1 gene, using Arabidopsis cell suspension cultures and quantitative PCR.

It is thought that protein synthesis is not required for the nitrate induction response to occur, as measured by the induction of NR, NIR and NRT2.1. This has
been shown in a few studies using protein synthesis inhibitors such as cycloheximide and also chloramphenicol (Privalle et al., 1989, Gowri et al., 1992, Ritchie et al., 1994, Sakakibara et al., 1997, Sueyoshi et al., 1999). Apart from Sueyoshi et al., (1999), who studied detached barley leaves, these studies were in maize. However, none of these studies were in Arabidopsis or used the highly sensitive approach of quantitative RT-PCR to measure gene expression. The use of cell suspension cultures provides a general system where environmental conditions can be uniformly manipulated. As the cells are undifferentiated and identical they are expected to respond alike. Privalle et al., (1989) also used cell suspension cultures using maize (Zea mays) cells and from these were able to produce continuous data showing the kinetics of the nitrate response. In whole plants, cells are differentiated, therefore expressing different proteins, and are subject to cellular and systemic signals which may interfere with and confound the nitrate inducing signal. Thus we primarily used cell suspension cultures for pharmacological analysis of the nitrate induction response, and complemented these experiments with whole plant analysis.

7.3 Results

7.3.1 Optimisation of qPCR primer sets
Gene specific primer sets were designed for the nitrate responsive genes NIR and NRT2.1 (Section 2.2.3). Quantitative PCR with these primer sets was performed on cDNAs derived from RNA samples previously analysed by Northern blot (Section 4.3.1, Figure 4.1). The RNA samples were taken from hydroponically grown plants, induced by nitrate as described in Section 4.3.1. It was found that the qPCR expression profiles matched the Northern blot profiles for both of these genes (Figure 7.1). These data verified the qPCR conditions and the specificity of the primer sets. Gel electrophoresis of qPCR products and melt curves also verified the integrity of the primers and that no primer dimerisation had occurred (data not shown).
7.3.2 NIR gene expression in cell cultures

*Arabidopsis* cell suspension cultures were N starved for 2 days prior to induction with 250 μM KNO₃ (Materials and Methods, 2.1.3). A 0h sample was taken prior to nitrate addition and subsequent samples were taken every 30 minutes. RNA was extracted and purified from these cells as described in Materials and Methods, 2.2.1. Quantitative PCR was performed using primer sets for *NIR* and *NRT2.1*. Nitrate induction of *NIR* gene expression was seen (Figure 7.2a) with induction peaking at 60 minutes. This was comparable to *NIR* gene induction detected in plant roots (Figure 7.2b).

The induction of *NIR* in cell suspension cultures was greater than that seen in hydroponic grown plant roots. In cell cultures there was a range of 250-500 fold difference whereas in plants only a 30-60 fold difference was seen (Figure 7.2). Sugar is known to enhance the expression of *NIR* and *NR* and overcome dark inhibition (Stitt and Krapp, 1999, Crawford *et al.*, 1995, Cheng *et al.*, 1992, Vincentz *et al.*, 1993), therefore this difference was attributed to the high sucrose media in which cell cultures are grown. The pattern of induction for plants roots and cell cultures is the same.
7.3.3 *NRT2.1* expression is not observed in cell cultures

Quantitative PCR Ct levels (Section 2.2.4) for *NRT2.1* gene expression were low, and were similar to background levels (data not shown). Gel electrophoresis of the qPCR reactions for *NRT2.1* revealed that no PCR products were made in these reactions, whereas control reactions for *EIF4a* gene expression did yield PCR products.

*NRT2.1* gene expression was detected from hydroponically grown plant roots during the optimisation experiments (Section 7.3.1 and Figure 7.1). The difference between the samples is the origin; the optimisation samples derived from intact roots whilst the test samples were from cell suspension cultures. Therefore, it appears that *NRT2.1* is not expressed in cell cultures. This was confirmed by subsequent RT-PCR on RNA from nitrate induced root and cell culture samples, which revealed that
NRT2.1 transcripts were not amplified out from the cell culture samples (Figure 7.3a).

NRT2.1 is a member of the seven gene NRT2 family of nitrate transporters. It is possible that in cell cultures another member of the NRT2 is more prevalent and active than NRT2.1. Due to the high sequence homology within the NRT2 family, degenerate qPCR primers specific to the entire family were made. However, PCR amplification showed that the NRT2 family also is barely expressed in cell cultures, whereas expression of NRT2 genes is present in the whole plant sample (Figure 7.3b). As NRT2.1 is not expressed in cell cultures, its use for pharmacological analysis of the nitrate induction response was limited to in planta studies.

Figure 7.3 PCR amplification from cDNAs made by reverse transcription using oligo dT primers to target mRNA sequences. a) PCR amplification of NRT2.1 from plant and cell culture derived RNAs. Eif4E primers were used as a control for cDNA integrity and loading. b) PCR amplification of whole NRT2 family from plant and cell culture derived RNAs. All plant and cell culture samples were taken after 1h nitrate induction. Note the absence of bands for NRT2.1 in cell culture samples. gDNA = plant genomic DNA sample.
7.3.4 *de novo* protein synthesis is not required for nitrate induction of the *NIR* gene from *Arabidopsis* cell cultures.

Previous studies with maize suspension cultures (Privalle *et al.*, 1989) and excised barley leaves (Sueyoshi *et al.*, 1999) have shown that *de novo* protein synthesis is not required for the nitrate induction response of *NIR*. To test whether this is the case for *Arabidopsis* we treated cell cultures with 20 µM of CHX, 30 minutes prior to induction with nitrate. We found that CHX did not prevent the nitrate induction response of *NIR* to either 250 µM or 10 mM nitrate (Figure 7.4a-b). The decrease in *NIR* mRNA is still seen after the induction peak, although this decrease appears to be slower. Therefore new protein synthesis is not required for induction or turnover of *NIR* mRNA but newly synthesised proteins may be needed to facilitate complete turnover. CHX alone had no effect on *NIR* expression, implying that there is no labile repressor acting on *NIR* (Figure 7.4b).

![Figure 7.4 Quantitative PCR results showing *NIR* gene expression in cell cultures in response to a) 250 µM nitrate and b) 10 mM nitrate addition, with or without the addition of CHX. These graphs are representative of 4 replicates.]
7.3.5 Inhibition of new protein synthesis reduced the nitrate induction of the *NIR* and *NRT2.1* gene in whole plants.

To test that the cell culture result reflected *in planta* gene expression, *Arabidopsis* seedlings were germinated and grown hydoponically in conical flasks, then N starved and induced by nitrate re-supply, with or without CHX treatment (Materials and Methods, 2.1.4). Surprisingly, CHX treatment did have an effect on the nitrate induction response of *NIR*. It resulted in a decrease in *NIR* induction. Induction of *NIR* by nitrate still occurred as was evident by an increase in *NIR* transcripts peaking at 60 minutes after induction; however, this induction was 5 times lower than that from nitrate-only treated plants (Figure 7.5a-b).

These plant samples were also analysed for *NRT2.1* gene expression to test whether the observed CHX effect was specific to *NIR* gene expression or whether other nitrate responsive genes were also affected. The induction of *NRT2.1* by 250 μM nitrate was also greatly reduced by CHX treatment (Figure 7.5c). *NRT2.1* induction peaked at the same time in CHX treated and non-treated plants; however, its expression levels were 8 times lower with CHX treatment. This was similar to levels for the KCl control and so may not reflect a true induction. Nevertheless, it can be surmised that CHX does inhibit the normal induction of *NRT2.1* by nitrate. The fact that *NRT2.1* was affected similarly to *NIR* suggests that they share signalling components and that this CHX effect affects nitrate signalling. These results imply a novel role for new protein synthesis for nitrate signalling in *Arabidopsis* plants.
Figure 7.5 Quantitative PCR results from plant roots, showing a) gene expression of $NIR$ in response to 250 $\mu$M KNO$_3^-$, with and without CHX addition, b) gene expression of $NIR$ in response to 10mM KNO$_3^-$, with and without CHX addition and c) gene expression of $NRT2.1$ in response to 250 $\mu$M KNO$_3^-$, with and without CHX addition. CHX was added at -30 min and KNO$_3^-$ or KCl (K$^+$ control) was added at 0 min. Note that nitrate induction of $NIR$ and $NRT2.1$ is markedly reduced in CHX treated plants.
7.3.6 Sucrose concentration affects the magnitude of NIR gene induction

The main difference between the growth conditions of cell cultures and whole plants was the sucrose provision: cultured cells were maintained in 3% sucrose (88 mM) whereas whole plants were grown with only 0.6% sucrose (17.6 mM), which was added to aid germination. The cell suspension cultures are non-photosynthetic so require the application of exogenous sugars.

To test the hypothesis that low sucrose provision facilitates this CHX effect in plants, we manipulated the sucrose provision of the cell cultures with the aim of lowering the nitrate induction response to levels analogous to those seen in whole plant cells. If the CHX effect was then detected in these cells it would indicate that this is a sucrose-dependent effect.

Initially we tested three different sucrose concentrations; 0%, 1%, 2%. Mannitol was used as an osmolarity control (Figure 7.6a). Cells were washed and transferred to new sucrose conditions alongside transfer to N starvation media (Materials and Methods 2.1.3), one day before induction with 10mM KNO₃. It was found that reducing the sucrose concentration from 3% to 2% and 1% failed to lower the magnitude of the NIR gene response to nitrate (Figure 7.6a, 3% data not shown). No induction was detected when 0% sucrose was supplied or mannitol was used. The presence of EIF4a transcripts in the 0% sucrose cells throughout the time-course (data not shown) implied that this treatment was not lethal to the cells. Therefore, sucrose is required for the nitrate signal to be perceived and the response to occur.

Sucrose concentrations between the 0% and 1% interval were also tested. Lowering the sucrose provision to 0.3% caused an 80 fold decrease in the amplitude of the response (Figure 7.6b). This level is similar to that seen in whole plants, which are grown in 0.6% sucrose (Figure 7.5).

The nitrate induction of NIR occurred when the cells were supplied with glucose instead of sucrose (Figure 7.6c), showing that other sugars facilitate induction of NIR by nitrate. No nitrate induction of NIR was observed with 3-O-Methyl-D-Glucose (3-O-MDG) provision (Figure 7.6c). 3-O-MDG is a non-metabolised sugar analog that stimulates the hexokinase-dependant sugar signalling pathway (Rolland et al., 2001); thus 3-O-MDG is perceived as sugar but it does not contribute to sugar
metabolism. Its use demonstrated that sugar metabolism rather than sugar perception is required to enable the nitrate induction response of NIR to occur.

**Figure 7.6** Quantitative PCR results from cell cultures showing sugar effects on NIR gene expression in response to 10 mM KNO₃.

a) Magnitude of NIR gene response remains the same when cells are provided 1% and 2% sucrose (Suc). Induction of NIR does not occur when cells are provided with 0% sucrose of 1.06% mannitol (Mann) used as an osmotic control for 2% sucrose.

b) Magnitude of NIR gene response decreases when sucrose provision is reduced to 0.6% and 0.3%. c) NIR gene response occurs when cells are provided 0.6% glucose but does not occur when cells are provided with 0.6% of the sugar perception analog 3-O-Methyl-D-Glucose (3-O-MDG).
7.3.7 Cycloheximide reduces the nitrate induction response in cell cultures under low sucrose provision

0.3% sucrose treated cell cultures were treated with CHX and induced with KNO$_3^-$, after a two day N starvation. It was found that CHX did affect the nitrate induction response of $NIR$ (Figure 7.7a,b); resulting in a reduction and delay in the response. This expression pattern resembled that seen for $NIR$ and $NRT2.1$ from the plant root experiments (Figure 7.5 a-c). Therefore, the reduction of the nitrate induction response by CHX treatment is a sucrose-dependent effect.

Figure 7.7 Quantitative PCR results from cell cultures provided with 0.3% sucrose, showing $NiR$ gene expression in response to 10 mM KNO$_3^-$ addition with and without CHX addition. CHX addition results in a reduction in the induction peak.
7.4 Discussion and Conclusions

7.4.1 The sucrose-dependent cycloheximide effect

In this study, we have observed a sucrose-dependent effect on the nitrate induction of the \textit{NIR} gene. It was found that sucrose is needed to enable the nitrate induction response to occur and that the magnitude of this response is proportional to the concentration of sucrose. This was observed at low concentrations of sucrose, and there was a saturation of the signal at sucrose levels of over 1% (29.2 mM).

When sucrose provision is limited, the addition of cycloheximide exerts an effect on the nitrate induction response of \textit{NIR}. It was found that cycloheximide addition resulted in a reduction of the amplitude of the response. The response also appeared to be slower; this was especially apparent in the down regulation of the response after induction (Figure 7.7).

These results contradict previous reports for the effects of cycloheximide on \textit{NIR}. However, this could be because these previous studies did not use limiting sugar conditions. For example, Privalle \textit{et al.}, (1989) used maize cell suspension cultures grown in media containing 3% sucrose (87.6 mM); we found in \textit{Arabidopsis} cell suspension cultures that this concentration of sucrose prevented the detection of the CHX effect. Other studies have used detached leaves (Sakakibara \textit{et al.}, 1997, Sueyoshi \textit{et al.}, 1999), which are photosynthetically active and can contain high amounts of sugars (Caspar \textit{et al.}, 1985). Also, only monocotyledonous plants have been used in these past studies, therefore there is also the possibility that the sucrose-dependant CHX effect on the nitrate induction response is unique to dicotyledonous plants such as \textit{Arabidopsis thaliana}.

The use of the non-metabolised sugar analogue 3-O-MDG revealed that sugar metabolism and not sugar perception is required for the nitrate induction response of \textit{NIR} to occur (Figure 7.6c). It is well established that nitrogen assimilation is dependent on sugar metabolism. For example, nitrate metabolism is energy consuming, requiring at least 14 ATPs, of which 7 are required for nitrite reduction by NIR. These ATPs are derived during respiration, and over 20% of total root respiration relates to nitrate uptake and assimilation (Bloom \textit{et al.}, 1992). The reductants required for nitrate reduction by NR and NIR come from the
photosynthetic electron transport chain and the oxidative pentose phosphate pathway (Taiz and Zeiger, 2005). Organic acids use up excess hydroxide ions from reduction preventing acidification and synthesis of amino acids requires carbon skeletons formed from photosynthesis and carbon catabolism. Sugar metabolism also requires nitrate metabolism as the utilisation of sugars in growing organs requires simultaneous provision of amino acids (Stitt and Krapp, 1999).

Thus, nitrate metabolism needs to be regulated in response to changes in carbon metabolism; if carbon levels were low and nitrate metabolism continues this would lead to a metabolic imbalance (Stitt and Krapp, 1999), with an accumulation of ammonium and acidification due to the lack of organic acids and a reduction in respiration. Conversely, when carbon levels are high it is important for the rate of nitrogen assimilation to increase (Stitt and Krapp, 1999). This was observed in Figure 7.6b, where sucrose provision affects the amplitude of gene induction of the nitrate assimilation enzyme, NIR.

It was found that under low sucrose provision that normal induction of NIR and NRT2.1 by nitrate was reduced. Cycloheximide addition further reduced this induction. This result implies a requirement for new protein synthesis for amplifying the nitrate signal under low sucrose provision. Figure 7.8 shows a model summarising these results.

Therefore, carbon is a passive signalling component as although it is required for the nitrate induction signal to occur and its metabolism has an amplifying effect on the signal, its effect is indirect. Therefore carbon is not a nitrate signalling component per se, but sugar metabolism is required for nitrate signalling to occur. It may be speculated therefore, that primary nitrate signalling is an energy consuming reaction.

As shown in Figure 7.8, it is predicted that amplifying factors act on the nitrate signalling pathway. These amplifying factors require new protein synthesis to exert an effect. Therefore when CHX is added the induction by nitrate is reduced. This is only observed when sugar availability is limited: at high sugar concentrations this effect is masked by the amplifying effect of sugar metabolism.

The presence of an amplifying system when sucrose is low raises the question of whether there is a basal level of nitrate induction that needs to be maintained when
external nitrate is present. This basal level may exist so that nitrate that is taken up by the constitutive transport systems is assimilated, preventing the build up of nitrate in the cytosol or vacuole and subsequent ionic imbalance. Although it is not adaptive to assimilate nitrate when there are insufficient C skeletons available, this basal level is not maintained when there is no C provided. For example, no nitrate induction was seen when plant cells were under 0% sucrose or glucose provision (Figure 7.6). Thus there must be sufficient C skeletons available at 0.3% sucrose to support the basal level of nitrate induction and subsequent nitrate metabolism.

There is also a possibility that CHX affects the amplitude of the nitrate signal by affecting the sugar signal, in which case new protein synthesis may be required to generate the sugar amplifying effect at low sugar concentrations.

**Figure 7.8** – Hypothetical model for the nitrate induction of primary response genes; *NIR* and *NRT2.1* (not shown) based on our observations. a)-c) The trans-membrane protein labelled (?) represents the yet undiscovered nitrate receptor that propagates the initial nitrate signal. It is shown as a membrane bound protein as the membrane is the site of first contact with NO$_3^-$, however it is possible that it is a cytosolic protein or protein complex. The arrows between this receptor and the gene represents the signalling that occurs between nitrate sensing and gene expression, this signalling may involve several components and steps. a) high sugar provision acts to amplify the nitrate signal which increases the gene expression. Other amplifying factors also act on the system but these are masked by the sugar amplification effect, thus CHX addition has no effect. It is important to note that the sugar effect acts passively on the signal. When sugar provision is low (b) then the effect of the amplifying factors can be seen. We predict that these amplifying factors require protein synthesis, thus when CHX is added, the induction response is further reduced (c). These amplifying factors may be unspecific effects or feedback effects from mRNA turnover.
7.4.2.1 The mRNA turnover hypothesis

One explanation for these amplifying factors is that they represent the effect of feedback from mRNA turnover. It was observed in most of the CHX experiments that subsequent down-regulation after induction of *NIR* and *NRT2.1* was slower in CHX treated cells than non-treated cells. This suggests an effect on the turnover of these mRNA transcripts. There is some evidence that mRNA turnover can regulate gene expression. Lidder *et al.*, (2005) proposed a role for mRNA turnover in circadian clock control and Coller and Parker, (2004) page 862, stated that mRNA turnover plays a 'key role in the control of gene expression, both by setting the basal level of gene expression and as a site of regulatory responses'. CHX may reduce the speed of turnover indirectly by reducing the amount of turnover proteins available, assuming that these proteins themselves are rapidly turned over and so require new protein synthesis to retain their abundance. With reduced turnover, the levels are *NIR* and *NRT2.1* transcripts would rise, thus the gene expression response would appear to be amplified.

An advantage of mRNA turnover having an amplifying effect on the nitrate induction response would be to ensure maintenance of a sufficient population of nitrate uptake and assimilation proteins in order to maximise nitrate acquisition and avoid the build up of toxic nitrite. This is especially essential for *NIR* regulation. The *NIR* gene induction by 250 μM and 10 mM nitrate is similar, suggesting that the nitrate signal is not initially graded by nitrate concentration. Instead the signal strength is modulated by amplifying effects of carbon metabolism and possibly mRNA turnover.

7.4.2 Use of cell suspension cultures

*Arabidopsis* cell suspension cultures were used during this analysis because they provide a system where all experimental variables can be more readily manipulated than in whole plant systems. Cells in suspension cultures derive from a common cell line and are more similar to each other than the different cell types found in whole a plant; thus cells in suspension cultures all produce alike responses. Unlike whole plants, these cells are not subject to systemic signalling which can have confounding effects. Cell suspension cultures are also suited to time-course studies as several samples can be taken at intervals from one culture, yielding comparable continuous
data. For whole plants, one plant or group of plants has to be harvested per time-point. Thus time-course studies with whole plants yields non-continuous independent data.

In this project we have identified a limitation in the use of cell cultures. It was found that gene expression in cell cultures is not always the same as that seen in whole plant cells. We found that the nitrate transporter, NRT2.1, is not expressed in our *Arabidopsis* cell cultures. Further investigation showed that the entire NRT2 multi-gene family is hardly expressed in these cells. As cell cultures are made from undifferentiated cells they may not express genes that are specific for a cell type. For example, NRT2.1 is mainly expressed in the mature root epidermal cells as well as root hairs, cortical cells and root endodermal cells (Orsel *et al.*, 2002, Nazoa *et al.*, 2003), it may be that the cell cultures lack this root tissue identity. These cells originated from cultures propagated by May and Lever, (1993) who used stem explants as parental cells for the cultures so it is possible that they retain some differentiated features of cells of the stem. Another explanation for the lack of NRT2.1 activity is that the activity of the dual-affinity nitrate transporter, NRT1.1, is sufficient for the nitrate transport needs in cell cultures, rendering the NRT2 transporters redundant.

Furthermore, our findings demonstrate the need for awareness of possible limitations of the cell culture system and the requirement to validate important conclusions independently in whole plant systems.

### 7.4.3 Conclusion

The results of these experiments highlight the complexity of nutrient signalling pathways in plants. Integration of metabolic processes and cross-talk with other signalling pathways enable plants to adapt to environmental conditions and stresses. We have shown that carbon metabolism affects the nitrate signalling pathway and we have identified a novel role for *de novo* protein synthesis in amplifying the primary nitrate signal particularly when sucrose provision is limiting.
Chapter 8 – Determining the role of chlorate as a potential signalling analog of nitrate

8.1 Summary
Chlorate is a nitrate transport analog. We investigated whether chlorate can also be used as a nitrate signalling analog. It was found that chlorate added to plants after a period of N starvation does not result in the induction of \(NIR\), \(NR\) or \(NRT2.1\) gene expression. This result infers that chlorate is not a nitrate signalling analog and that the action of transport via nitrate transporters does not propagate the nitrate signal.

8.2 Introduction
Chlorate (\(\text{ClO}_3^-\)) was once used extensively as a non-specific herbicide and defoliator. Borje Åberg (1947) noted that chlorate’s toxic effect on plants was slow and hypothesised that chlorate itself was not the toxin but a by-product of chlorate assimilation. \textit{In vivo} studies revealed that the reduced forms, chlorite and hypochlorite, were much more toxic to plants than chlorate itself. As chlorate structure is similar to nitrate, Åberg further hypothesised that both molecules undergo reduction via a common system. This was further substantiated by the observation that nitrate depresses the toxic affect of chlorate. More recently, it has been shown that chlorate is indeed transported by the nitrate transporters and is also reduced by nitrate reductase (LaBrie \textit{et al.}, 1991). It was also shown that nitrate competitively inhibited chlorate uptake, thus accounting for its depression of the toxicity of chlorate. The toxicity of chlorate was found to be due to the accumulation of chlorite, the product of reduction by nitrate reductase (NR). Whereas nitrite is further reduced by nitrite reductase (NIR), chlorite is not a substrate of this enzyme, this leads to its accumulation causing necrosis of leaves and the eventual death of the plant. Isolation of mutants resistant to the deadly effects of chlorate reduction have lead to the identification of many genes involved in nitrate uptake and metabolism, such as the chlorate transport \textit{chl1} mutant (Tsay \textit{et al.}, 1993). \textit{chl1} was later identified as a mutant of the \textit{NRT1.1} gene, which codes for a dual affinity nitrate transporter (Lui \textit{et al.}, 1999)
As a substrate of nitrate transporters, chlorate has been utilised as a transport analog of nitrate (Deane-Drummond and Glass 1982). Advantages of using ClO$_3^-$ as a transport analog in uptake studies is that liquid scintillation counting of $^{36}$ClO$_3^-$ is a lot cheaper than mass spectrometry of $^{15}$NO$_3^-$. It is also preferable over use of $^{13}$NO$_3^-$ because $^{13}$N has a short half life of 10 minutes, which presents technical difficulties, whereas the half life of $^{36}$ClO$_3^-$ is $4.4 \times 10^5$ years (Kosola and Bloom., 1996).

**8.2.1 Does chlorate also act as a signalling analog**

It is not adaptive for a plant to perceive chlorate as nitrate, because of the toxic effects of chlorate reduction. However, as chlorate is not a naturally occurring molecule, there is no pressure to drive evolution of a mechanism to prevent chlorate being perceived as nitrate. Possible ways how chlorate could stimulate a nitrate signal are via its transport by nitrate transporters or by binding to a yet unidentified nitrate regulatory protein that normally binds nitrate. Its reduction by NR may also be a source for nitrate signalling, however this is not likely as NR mutants can still perceive and respond to nitrate (Crawford, 1995).

If chlorate were a signalling analog of nitrate it would be useful in further studies to dissect the nitrate signalling pathway. For example, it could be used to distinguish between nitrate as a signal and nitrate as a nutrient without the use NR mutants. It could therefore, aid identification of the nitrate sensor or sensory mechanism. For example, it could demonstrate whether nitrate transporters are the source of the primary signal. Having a signalling analog that is not metabolised would also be useful for investigating systemic signalling.

This question has been explored in the past, but has yielded inconclusive results. Many studies have been done on the protein level to investigate whether chlorate is an inducer of nitrate uptake and reduction (McClure *et al*., 1986, Privalle *et al*., 1989, Siddiqi *et al*., 1992, Kosola and Bloom 1996). With the exception of the findings of McClure *et al*., 1986, these studies found that chlorate did not induce nitrate uptake or nitrate reductase activity (NRA). These studies however, did not measure gene expression on the mRNA transcript level. As transcription precedes protein manufacture it is thus an indicator of early signalling.

LaBrie *et al*., 1991, used Northern Blot to examine the effect of chlorate on gene expression of NR and NIR. They found that chlorate did induce NR gene expression
but no NR protein or activity was detected. This lack of NR activity supports what has been previously reported. This result may indicate that chlorate mimics early nitrate signals. However, in the same study, it was found that NIR gene expression was not induced by chlorate treatment. This is interesting in that NR and NIR are part of the nitrate transcriptome and are thus thought to be co-regulated (Redinbaugh and Campbell, 1991).

These studies focused only on leaf and shoot samples. We chose to focus on root gene expression as it is the roots that first encounter and take-up nutrients and hence are more likely to be the site of primary signals. We proposed to use the highly sensitive approach of quantitative RT-PCR to analyse gene expression of NIR, NR and NRT2.1. NIR and NR are both nitrate assimilatory enzymes, NRT2.1 is a nitrate transporter that is part of the iHATs uptake system and is induced by nitrate. NRT2.1 has not been previously tested for induction by chlorate.

8.3 Results

8.3.1 Chlorate does not induce NIR or NR

Arabidopsis thaliana (Columbia 0) plants were germinated and grown hydroponically in sterile liquid half-strength Murashige and Skoog (1962) media, with 0.6% sucrose, as described in 2.1.4 (Material and Methods.). After 4 days of N starvation the plants were induced by addition of either KNO₃ or KClO₃, to a final concentration of either 250μM or 10mM. Roots were harvested from plants at set time-points. Roots from six plants were pooled for each time-point; these six plants were grown together in a single axenic culture. Complementary DNA (cDNA) was made by reverse transcription PCR of RNA extracts from these root samples. Oligo dT was used to prime the reaction so that mRNA transcripts were the templates for cDNA synthesis. The cDNAs were then used in quantitative PCR reactions primed for NIR and NR.

Figure 8.1 illustrates the results of these experiments, showing the levels of gene expression plotted against the time in minutes, after the addition of either KNO₃ or KClO₃. Nitrate induction of NIR gene expression is seen for both 250μM and 10mM concentrations (Fig 8.1a-b). NIR transcript levels peak 60 minutes after induction with nitrate. Conversely, no induction is seen from plants treated with
chlorate. A similar pattern for NR gene expression is seen (Fig. 8.1c); with a peak after 60 minutes of induction with nitrate and no induction by chlorate addition. These graphs demonstrate that chlorate does not induce either NR or NIR.

8.3.2 Chlorate does not induce NRT2.1

Samples from the experiment described above (Section 8.3.1) were also analysed by quantitative PCR for NRT2.1 gene expression. As aforementioned, NRT2.1 is a high affinity nitrate transporter. Figure 8.1d shows the NRT2.1 gene expression from plants induced by 250μM concentrations of nitrate or chlorate, this concentration lies within the high affinity range. Nitrate induction of NRT2.1 peaks between 60 and 90 minutes (not shown). As small peak of NRT2.1 expression was observed 60 minutes after chlorate treatment, which may indicate a slight degree of induction by chlorate (Figure 8.1d). However, this chlorate induction of 2 fold difference is significantly smaller than the 18 fold induction observed in response to nitrate. Therefore, it can be concluded that chlorate does not mimic nitrate induction of NRT2.1.

Figure 8.1 – Quantitative PCR results for NIR, NR and NRT2.1 gene expression in response to KNO₃ and KClO₃ addition to N-starved plants. NIR gene expression response to treatment concentrations of a) 250μM or b) 10mM. c) NR and d) NRT2.1 gene expression to 250μM treatment addition.
8.4 Discussion

My results show that chlorate is not a signalling analog of nitrate as it does not induce \textit{NIR}, \textit{NR} or \textit{NRT2.1} gene expression, whereas these genes are all nitrate inducible. As chlorate is taken up by nitrate transporters it is likely that the origin of the primary nitrate signal is not associated with the act of transport by these transporters. It is also probably that these transporters are not the nitrate sensors. Putative roles for transporters in signalling pathways have been observed in bacteria and yeast; Fukushima \textit{et al.} (2006) showed that an ABC transporter is involved in signalling for sporulation in \textit{Bacillus subtilis}. In yeast, Özcan, Dover and Johnston (1998), identified that two glucose transporter homologs, SNF3 and RGT2, function as glucose sensors.

LaBrie \textit{et al.}, (1991) showed using purified NIR that nitrite reductase does not reduce chlorite. We have shown that \textit{NIR} transcription is also not induced by chlorate. LaBrie \textit{et al.}, (1991), also showed by Northern blot, that chlorate did induce NR gene expression. This was interesting as \textit{NR} and \textit{NIR} are thought to be co-regulated, probably responding to a universal nitrate signal, perhaps by being regulated by the same transcription factor (Redinbaugh and Campbell, 1991). Chlorate is a substrate of NR, so it is reasonable to expect that it may induce its transcription. However the result from our experiment using qPCR to measure gene response is that chlorate does not induce NR transcription (Figure 8.1c). This is contrary to the findings of LaBrie \textit{et al.}, (1991), but supports the hypothesis that \textit{NR} and \textit{NIR} are co-regulated. Also, it is adaptive to plants that chlorate does not induce \textit{NR}, because nitrate reductase catalyzes the reduction of chlorate to the toxic chlorite. There is evidence that nitrate is taken up preferentially to chlorate (Siddiqi \textit{et al.}, 1992, Kosola and Bloom, 1996) suggesting that signalling mechanisms are in place to differentiate between the two molecules.

Hence, we conclude that chlorate is not a nitrate signalling analog and that transport by nitrate transporters and reduction by NR does not initiate a nitrate signal. This further supports the hypothesis that nitrate itself is the signal for nitrate induction and is essential in propagating the primary inducing signal. The nitrate sensor itself remains elusive.
Chapter 9 – NRT2.1 responds to local signalling of Nitrate

9.1 Summary

Split-root experiments using the PRO\textsubscript{NRT2.1::LUC} reporter line and LUC imaging, show the induction of NRT2.1 transcription in NO\textsubscript{3}\textsuperscript{-}-fed roots but not in N-starved roots. It is apparent that local signalling occurs that enables plants to sense nitrate in the environment.

9.2 Introduction

NRT2.1 is an inducible high affinity transporter that accounts for the majority of iHATS activity for nitrate transport in roots (Cerezo \textit{et al.}, 2001). NRT2.1 transcription is induced by nitrate (Wang \textit{et al.}, 2000) and transiently induced by N starvation, this transiently induction is assigned to be due to release of repression from N metabolites (Nazoa \textit{et al.}, 2003).

Several studies have focused on the changes in nitrate uptake rates in response to increased N demand from the plant. Increasing the demand for N is achieved by reducing the N supply to the plant, this is done in a split root system where one part of the root system is given low N or N starvation, whilst maintaining the N supply to the rest of the root. Drew and Saker, (1975) found in low N fed barley, that increased lateral root growth occurs in the portion of roots locally supplied with nitrate (the ‘hotspot effect’ (Section 1.7.3)) as well as an increase in absorption and transport of \textsuperscript{15}N-labelled nitrate. They found that this increase in uptake ensured that N levels in the plant remained comparable to the levels in control plants. This compensatory increase in nitrate uptake was also observed in the NO\textsubscript{3}\textsuperscript{-}-fed roots of \textit{Brassica napus} (Lainé \textit{et al.}, 1994) whilst reduced nitrate uptake ability was observed in the N-starved portion of roots. Lainé \textit{et al.}, (1994) found that protein synthesis inhibitors prevented this compensatory increase in nitrate uptake and they suggested the involvement of inducible nitrate transport proteins in this process. Cerezo \textit{et al.}, (2001) demonstrated with the use of the \textit{Arabidopsis thaliana} nrt2.1 knock-out mutant and a similar split root system, that the transporter, NRT2.1, is required for this compensatory increase in NO\textsubscript{3}\textsuperscript{-} uptake in the NO\textsubscript{3}\textsuperscript{-}-fed portion of roots.
Gansel et al., (2001) used the split root system in *A. thaliana*, to examine the starvation induction response of NRT2.1 and the ammonium transporter, AMT1.1. They also observed that when one part of the root system is N starved that NO$_3^-$ uptake increased and that also NRT2.1 transcript levels increased. This confirms the findings of Lainé et al., (1994) and Cerezo et al., (2001), that the compensatory increase in NO$_3^-$ uptake is facilitated by increased transcription and probable activity of NRT2.1. These studies all conclude that this compensatory response is evidence of shoot-to-root signaling of plant status; as when plant demand for N increases as a result of decreased N supply or N starvation of a portion of root, this is followed by increased NO$_3^-$ uptake following gene induction and presumable activity of NRT2.1. These studies have all focused on the N starvation response to study putative NO$_3^-$ systemic signaling. Also these studies have used long time-courses of up to 6 days (Gansel et al., 2001). This could result in the observation of downstream effects of metabolism rather than actual signaling.

9.3 Results

A split root experiment using *PRO$_{NRT2.1}$::LUC* # 28 line was done following the protocol in (Materials and Methods 2.1.7). The plants were N starved in split root conditions, for 4 days prior to the experiment. This treatment ensured that transient induction by starvation as a response to release from N metabolites is no longer present. This was evident by the fact that no LUC activity is seen in control non-induced plants after luciferin pre-treatment to abolish accumulated luciferin (data not shown).

0.5M KNO$_3^-$ was added to the final concentration of 10mM to the left-hand compartment of the split-root plate. The N-starved right-hand compartment was treated with 10mM final concentration of KCl. Luciferin was applied by solution addition into the media to a final concentration of 1mM, the shoot was sprayed with 1mM luciferin. This treatment was done 10 hours prior to the experiment, at the start and every 2 hours thereafter. LUC activity was analysed by CCD imaging (Section 2.1.6)

LUC activity was observed only in the roots treated with NO$_3^-$ (Figure 9.1a). LUC activity increased throughout the 12 hour time-course. This indicates that
induction of NRT2.1 transcription is occurring in the NO$_3^-$ treated roots. No LUC activity is detected in the right-hand compartment containing no N. Quantification of the image data confirms this observation (Figure 9.1b). No LUC activity is seen in the shoot organs, this corresponds to the known root-only expression pattern of NRT2.1 (Orsel et al., 2002b).

Figure 9.1 - a) Split root experiment using $PRO_{NRT2.1}::LUC$ reporter lines over a 12 h time-course. The top left picture is a light image showing the three compartments of the split root plate and the distribution of the root system. 10 mM NO$_3^-$ was added to the left-hand compartment, the right-hand compartment contains –N media. b) Quantitative data from above split root experiment and 2 replicates.
9.4 Discussion and Conclusions

It was found that NRT2.1 transcription is induced only in the root portion supplied with nitrate and not in the N starved portion of the roots. This shows that the nitrate signal is a local signal that is not transported systemically to the entire root system. It is economical to the plant for this to be a local signal so that production of nitrate transporters does not occur in roots not in contact with nitrate. Whether there is a 'no nitrate' signal that counteracts a systemic 'nitrate' signal in non N-fed roots cannot be ruled out.

From this experiment, a compensatory response analogous to those seen in previous studies cannot be shown. However, it is interesting to note that LUC activity continues to increase throughout the experiment, whereas in previous nitrate induction experiments with these reporter lines (where nitrate was supplied to the entire root system) LUC activity peaked after 6 hours. This may indicate a compensatory response is occurring to having only a partial supply of nitrate.

In the previous studies (described in Section 9.2), the response to a change to limiting N was studied. However, in our experiment the plants were already in conditions of limitation and high N demand. Any transient induction by N starvation is finished by the start of the induction treatment; this is evident by the lack of LUC activity in non-treated plants (data not shown) and in the N-starved portion of roots. Whereas in the previous experiments plants experienced the change from sufficient N to partial N provision and therefore a local N-starvation response was expected and not seen, in our experiment an N-starvation induction response was not expected and was also not seen. It is interesting that a local N-starvation response was not seen in the N-starved roots portions in these previous experiments. The transient N starvation response is attributed to the release from repression of metabolites. These results may indicate that, where partial N supply is present, N metabolites produced from the nitrate taken up by the non N-starved roots are transported to roots where there is no nitrate uptake and that these transported N metabolites ensure the repression of the NRT2.1 gene. This hypothesis was examined by Tillard et al., (1998), who measured the amino acid content in phloem sap from Ricinus communis (castorbean) plants that had been subjected to partial nitrate starvation. They found that the phloem import of amino acids was directed to the nitrate-fed roots and not
the nitrate-starved roots. Thus, they concluded that the cycling of amino acids was not a signal for repression, but that the increase of amino acid import was required to satisfy demands for increased growth in the nitrate-fed roots. However, Forde (2002) suggests that there may still be ‘subtle short-term fluctuations in phloem amino acid composition or fluxes to which NRT2 genes are sensitive.’

We have seen that the local addition of nitrate results in the local induction of NRT2.1 as reported by LUC activity. It is not known whether the induction in these roots is greater than in a plant where the whole root system is nitrate treated. If it is, this would support the ‘compensatory’ hypothesis and that perception of N demand is occurring. It would be interesting to address this question in a follow-up experiment.

It is evident that local signaling of nitrate is occurring, enabling the plant to sense and ‘perceive’ nitrate in the environment. Integration of this information with plant N demand must occur so that up-regulation of NRT2.1 and nitrate uptake can occur in the roots in contact with the nutrient. This may require root-to-shoot signaling or that the unknown shoot-to-root signal of plant N-demand is incorporated into the nitrate signaling pathway, downstream of nitrate perception to amplify the signal, resulting in increased NRT2.1 transcription and nitrate uptake. It would be interesting to see if the other nitrate responsive genes are also induced in the locally N-fed roots, such as those for the nitrate assimilatory enzymes. This would be expected as increased nitrate uptake would need increased assimilation (assuming that the excess nitrate is not used for vacuolar storage or transported to the shoot). If these genes were also induced, it might be hypothesized that the ‘shoot-to-root’ signal is actually due to excess C. It has been shown previously (Crawford and Forde, 2002, Stitt and Krapp, 1999, Cheng et al., 1992, Vincentz et al., 1993) and in Chapter 7, that C levels can amplify the nitrate signal. During N limitation, less N is available to combine with C skeletons to make essential amino acids and proteins; thus there is excess C as well as increased N demand. Therefore, the N demand ‘signal’ may arise as a passive response to decreased N metabolism, which results in excess C, which in turn amplifies the nitrate signal. This amplification only occurs in the nitrate-fed roots as this is where nitrate is present and sensed, and the signal precipitated.
This experiment has shown that local signalling of nitrate occurs, and that this signal is either not transported or it is inhibited, so that nitrate induction does not occur in roots not supplied with nitrate. Further investigation is required to elucidate how systemic signalling of N demand may affect this local signal.
10.1 The genetic screen and recent developments

My initial objective was to conduct a genetic screen to screen for mutants with aberrant kinetics of nitrate response in order to target and identify nitrate signalling components. However, due to unforeseen complications, the screen could not be implemented. These complications included the two aberrant reporter lines \textit{PRONJR..LUC} and \textit{PROAMTJ.I..LUC} and their characterisations (Chapter 4), as well as the generation of the new reporter line, \textit{PRONRT2.1::LUC}, where many cloning strategies were implemented before the successful construct was made (Chapter 5). The cost of these complications was the loss of sufficient time required to conduct the screen. However, the \textit{PRONRT2.1::LUC} reporter line was found to reflect endogenous \textit{NRT2.1} gene expression in localisation and regulation, and a design for a future screen with the \textit{PRONRT2.1::LUC} reporter lines has been outlined in Chapter 6.

The rationale for the genetic screen is still valid, and a screen with \textit{PRONRT2.1::LUC} may prove very effective in identifying signalling components. Two recent papers have identified a possible signalling role for NRT2.1:

Little \textit{et al.}, (2005) showed that mutations in NRT2.1 result in a loss of repression of lateral root initiation by the combination of high sucrose and low nitrate provision. This phenotype was also seen in mutants grown on nitrate-free media; thus Little \textit{et al.}, (2005) concluded that NRT2.1 is a repressor of lateral root (LR) initiation and that this role is independent of nitrate uptake. They also postulate that NRT2.1 may be a sensor of low nitrate or a signal transducer from a sensor, and they conclude that low nitrate repression of lateral root initiation is mediated by NRT2.1.

Conversely, Remans \textit{et al.}, (2006) found that the \textit{atnrt2.1-1} mutant inhibited LR primordia initiation in response to N limitation, therefore suggesting a stimulatory role for NRT2.1. This difference to the findings of Little \textit{et al.}, (2005), was attributed to either the lack of high sucrose in this study or to other differences in the experimental conditions. However, it is clear that NRT2.1 as well as being a major contributor to HATs activity for nitrate uptake, has a complex signalling role that
integrates nutritional cues with root architecture responses. This signalling role increases the suitability of the \textit{PRO}_{\textit{NRT2.1}}::\textit{LUC} reporter lines for a genetic screen to identify signalling components involved in nitrate signalling.

Another recent study has revealed a putative expression partner for NRT2.1 in \textit{Arabidopsis} (Okamoto \textit{et al.}, 2006). The NAR2 protein of \textit{Chlamydomonas reinhardtii} is not a transporter, yet it is required for high-affinity nitrate uptake. Co-expression of a barley \textit{NAR2}-like gene is required for the barley NRT2.1 to transport in Xenopus oocytes (Tong \textit{et al.}, 2005 as cited by Okamoto \textit{et al.}, 2006). Two \textit{NAR2}-like proteins were identified in \textit{Arabidopsis thaliana} by Okamoto \textit{et al.}, (2006) and named NRT3.1 and NRT3.2. NRT3.1 is more expressed than NRT3.2, and its induction by nitrate resembles that of NRT2.1. In the \textit{nrt3.1} mutants, NRT2.1 expression and induction was reduced and high-affinity influx of nitrate was reduced by 98%. This result indicates that these \textit{NAR2}-like genes are required for high-affinity uptake, perhaps via an interaction with NRT2.1 as inferred by the barley result. The decrease in induction of NRT2.1 in the mutants probably correlates to the reduction in internal nitrate due to decreased nitrate uptake. This may indicate that the nitrate sensor that leads to the induction of the nitrate responsive genes is downstream of uptake. It should be noted that a mutation in the NRT3 \textit{NAR2}-like genes in the \textit{PRO}_{\textit{NRT2.1}}::\textit{LUC} reporter lines could be identified in a genetic screen as being a mutant with a reduced magnitude of induction.

\textbf{10.2 \textit{de novo} protein synthesis is required for nitrate responses when sucrose is limiting}

A sucrose-dependent cycloheximide effect was observed for the nitrate induction of the nitrite reductase gene, \textit{NIR}, and also the nitrate transporter, \textit{NRT2.1}. It was found that when sucrose provision was limiting (0.3%), that cycloheximide treatment resulted in the reduction of the magnitude of induction of these genes. This result indicates a role for new protein synthesis in nitrate induction when sucrose is limiting. This was seen from samples taken from \textit{Arabidopsis} cell suspension cultures and hydroponically grown plants.

When sucrose provision is high (> 1%), this cycloheximide effect was not seen and the induction occurred normally. It was this result that has been seen in all
previous studies and which has lead to the conclusion that *de novo* protein synthesis is not required for the induction of the nitrate responsive genes, and that these are therefore primary response genes (Redinbaugh and Campbell, 1991). In none of these studies were plants subjected to low sucrose conditions (discussed in Section 7.4.2).

It is hypothesised from my findings that at low sucrose conditions, amplifying factors are required to produce a normal nitrate induction of *NIR* and *NRT2.1*. These amplifying factors require *de novo* protein synthesis. It is unknown what proteins and mechanisms regulate this amplifying effect.

This result further highlights the presence and complexity of carbon and nitrogen coupling. It was differences between C::N ratios in the conditions used by Little *et al.*, (2005) and Remans *et al.*, (2006) that account for their conflicting results for the role of NRT2.1 in LR initiation (discussed above in Section 10.2). Here the low C conditions used have led to this discovery that there is a novel role for new protein synthesis in nitrate signalling.

### 10.3 Chlorate is not a signalling analog

It has been shown in a root-based assay, that chlorate is not a signaling analog of nitrate. Chlorate treatment does not result in the induction of *NR*, *NIR* and *NRT2.1*. This result contradicts LaBrie *et al.*, (1991) who found that *NR* was induced by chlorate but that *NIR* was not. The fact that we see similar induction patterns for all three nitrate responsive genes suggests that these genes are part of a nitrate transcriptome. Also, as chlorate is a non-metabolized transport analog, it is likely therefore that the action of transport by the nitrate transporters is not the source of the nitrate signal.

### 10.4 NRT2.1 responds to local nitrate signaling

Split-root experiments using the *PROPRT2.1::LUC* reporter lines have shown that *NRT2.1* is induced in roots locally supplied by nitrate. The nitrate signal is either not transported or it is inhibited, so that nitrate induction does not occur in roots not supplied with nitrate. Further investigation is required to elucidate how the systemic signalling of N demand may affect this local signal.
10.5 Final Conclusion

It is evident from the work presented here, that nitrate signalling in *Arabidopsis thaliana* is complex and is intrinsically linked with carbon metabolism. We have found a novel role for new protein synthesis in amplifying the nitrate signal when carbon levels are low. There is still much to be discovered in this field such as the ‘nitrate sensor’ and other components of the signalling pathway. The use of the *PRO\textsubscript{NRT2.1}::LUC* reporter in a future genetic screen could lead to the identification of some of these signalling components.
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