Dissertation

Nitrogen signalling in *Arabidopsis thaliana*

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Content

Figures

Tables

COMMONLY USED ABBREVIATIONS

AMT	ammonium transporters
bp	base pair
cDNA	complementary DNA
DNA	deoxyribonucleic acid
EMS	Ethyl Methane Sulphonate
FNR	ferredoxin--NADP(+) reductase
gDNA	genomic DNA
HATS	High-affinity transport system
KO	Knock Out
kb	kilo bases
LB	Laura Bretani medium
LN_2	Liquid nitrogen
LUC	Luciferase reporter gene
mRNA	messenger RNA
½ MS	half-strength Murashige and Skoog medium
N	Nitrogen
NIA	nitrate reductase
NII	nitrite reductase
NRT	nitrate transporter
OX	over-expressor
PCR	Polymerase Chain Reaction
qRT-PCR	Quantitative Reverse-Transcription Polymerase Chain Reaction
RNA	ribonucleic acid
RT-PCR	Reverse-Transcription Polymerase Chain Reaction
SE	standard error
T-DNA	Transfer DNA
TF(s)	Transcription Factor(s)
v/v	Volume per volume
w/v	Weight per volume
WT	Wild type
YEB	Yeast extract nutrient broth

1. INTRODUCTION

1.1 Nitrogen in the environment (nitrogen cycle) and plants biology

Nitrogen is the fourth most abundant element in plants, after carbon, oxygen, and hydrogen. It is an essential component of many biological compounds, including: amino acids, purines and pyrimidines which are the building blocks of proteins, nucleotides, and nucleic acids.

Nitrogen occurs in many forms in the biosphere. The atmosphere contains 78% of molecular nitrogen (N_2), which is not directly available to higher plants, although legumes and some non-legumes can access this via symbiotic nitrogen fixation with bacteria. On the other hand the two major forms of inorganic nitrogen present in the soil, nitrate and ammonium, are easily assimilated by higher plants. Plants are also able to assimilate organic N sources, like amino acids, which are abundant in soils that contain high concentrations of organic matter. The conversion of molecular nitrogen into ammonium or nitrate is called nitrogen fixation. Via the consumption of plants by animals, nitrogen moves further on in the nitrogen cycle. Through the death and subsequent decomposition of the organisms, nitrogen is returned to the soil (Taiz and Zeiger, 2002). The nitrogen cycle is depicted below (Figure 1.1).

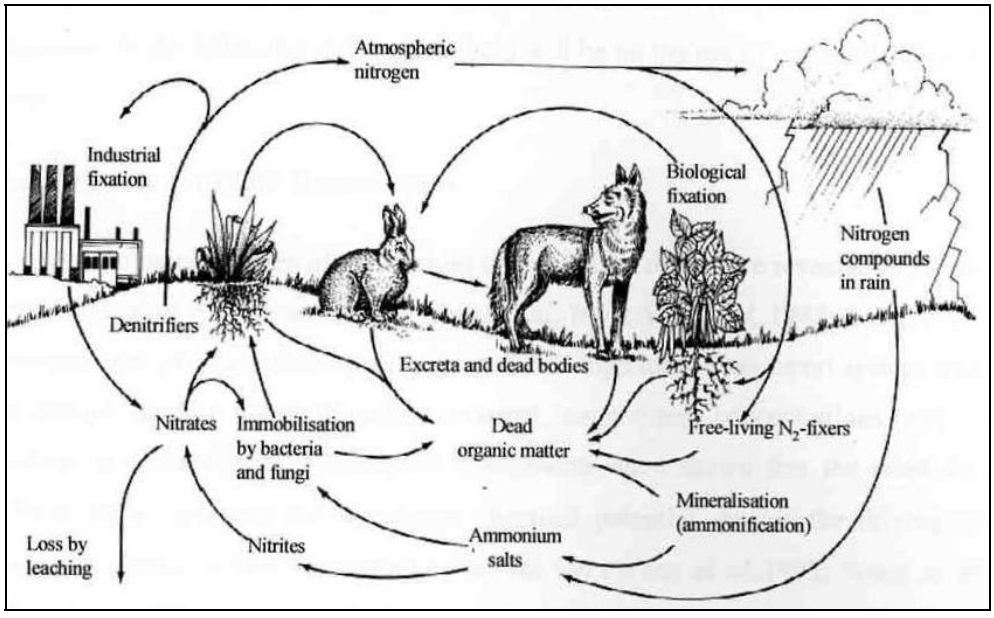

Figure 1. 1 The nitrogen cycle

Nitrogen cycles through the atmosphere as it changes from gaseous form to reduced or oxidised ions, before being incorporated into organic compounds of living organisms (taken from Taiz and Zeiger, 2002, p. 294).

Natural processes leading to the fixation of 190 million tonnes nitrogen/year are: oxidation by lightning (8%) to produce HNO_3, photochemical reaction in the stratosphere producing nitric oxide (2%), and biological nitrogen fixation (90%) performed by bacteria, either free-living or in symbiotic association with plants (e.g. legumes). During symbiotic nitrogen fixation, N_2 is reduced to ammonium. During a process called ammonification, organic forms of nitrogen like amino acids derived from once living organisms are also converted to ammonium by different bacteria and fungi. This ammonium can be used as a nitrogen source by various autotrophs, including plants, or can be oxidised to nitrite and then to nitrate in a process called nitrification in some prokaryotes. The conversion of nitrate to molecular nitrogen is called denitrification (Figure 1.1). The concentration of ammonium and nitrate in soil varies over 3-4 orders of magnitude depending on different factors like the pH of the soil and the amount of fertilisation. Nitrate is more abundant in many soil types, especially in tropical and temperate regions, because of the predominance of nitrifying bacteria in these soils Marschner, 1995. However, ammonium not nitrate, is preferentially taken up by most plants, even when nitrate concentration is 10 times higher than that of ammonium (Crawford and Forde, 2002).

Despite its importance in plants nitrogen availability in the environment often limits plant growth. As the nutrition of a large part of the world's population relies on cereals and other crops like that are not able to fix nitrogen in symbiosis, huge amounts of fertiliser nitrogen are now used in agriculture, as shown in figure below (Figure 1.2).

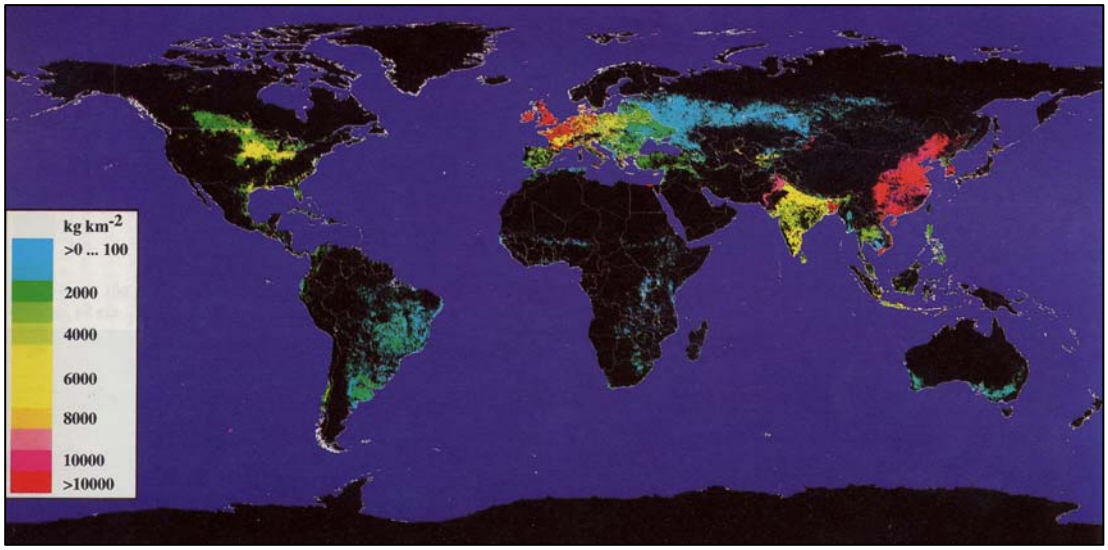

Figure 1. 2 World nitrogen fertilizer use in 1996 (taken from Kawashima H, 2000)

In 1995, a total of 77 million tons of N-fertilisers was applied to the world's cereal croplands Laegreid and Bockman, 1999. By 2050 this number may rise to over 200 million tons. Plants do not assimilate nitrogen fertilisers with 100% efficiency. Loss of fertilizer N, as a result of volatilisation, denitrification and leaching, ranges from between 10% to 80%. Leachate pollutes rivers and lakes and adds to the economic cost of agriculture (Tilman et al., 2001).

1.2 Nitrogen transport and assimilation by plants.

Nitrate is taken up by root cells via specific transporters in the plasmalemma, then either metabolised in the cytoplasm of root cells or transported passively along the gradient of concentration, to the shoots via the xylem. The excess of nitrate could be effectively exerted from the root cells. Aerial organs, like leaves are able to metabolise or store large amounts of nitrate (Taiz and Zeiger). Plants have evolved numerous nitrate uptake systems, to cope with variable nitrate levels in soil. At the physiological level, they are divided into two distinct groups: low affinity transport systems (LATS), which operate at high NO_3^- concentrations (above 1mM) and high affinity transport systems (HATS), which operate in a the micromolar range. The HATS are typically low capacity, saturable systems, while LATS are high capacity systems, with linear, non-saturable uptake kinetics, as reported in number of recent reviews covering all aspects of nitrate uptake in plants (Orsel et al., 2002a; Glass et al., 2002; Crawford and Forde, 2002; Forde, 2000; Daniel-Vedele et al., 1998). Nitrate is co-transported with H^+ (symport). Two families of nitrate transporter genes: the NRT1 and NRT2 families have been cloned from plants, which are believed to encode the LATS and HATS systems, respectively (see the reviews listed above). NRT1 proteins belong to the oligopeptide transporters family (PTR super-family), while NRT2 proteins belong to the nitrate – nitrite porters (NNP super-family). The *Arabidopsis* genome encodes seven NRT2 family and four NRT1 family transporters 2000. Expression of NRT1 and NRT2 gene families in shoots as well in roots implicates the encoded proteins in processes other than uptake into root cells, although the exact nature of these is unclear, in the absence of protein localisation data in most cases.

Ammonium transport systems have also been divided in to LATS and HATS types, based on physiological data, as reported in number of recent reviews covering all aspects of ammonium uptake in plants (Crawford and Forde, 2002; Glass et al., 2002; von Wiren et al., 2000; Howitt and Udvardi, 2000). The HATS in *Arabidopsis*, appears to be encoded by genes

of the AMT1 and AMT2 families (Ninnemann et al., 1994; Gazzarrini et al., 1999; Sohlenkamp et al., 2000). Biochemical and biophysical characterization of AMT1 and AMT2 transporters indicate that they are NH_4^+ uniporters (Ninnemann et al., 1994; Gazzarrini et al., 1999; Sohlenkamp et al., 2000; Shelden et al., 2001). *Arabidopsis* has five *AMT1* and one *AMT2* genes. It has been suggested that the LATS may represent also a simple diffusion of NH_3 through the plasma membrane (White, 1996). It has also been postulated that the LATS for ammonium might result form a is a product of the activity of ammonium-sensitive potassium (K^+) channels, K^+ transporters or even water channels (Howitt and Udvardi, 2000). However, one *Arabidopsis* AMT1 family member (*ATAMT1.2*) shows biphasic kinetics with a non-saturable component (Shelden et al., 2001). AMT genes are expressed in roots and/or in shoots, which indicates that they play roles in ammonium transport throughout the plant. AMT-GFP fusion studies using transformed plant cells, indicate that some of the AMT are located in the plasma membrane, implicating them in ammonia uptake from the apoplast of root and shoot cells (Sohlenkamp et al., 2000).

Other nitrogen sources can be also taken up by plants. Amino acid permeases (*Aap*) have a broad substrate specificity and are expressed differentially between plant tissue types. Other transporters that move small oligopeptides across the membrane are also present in plants. Although the role(s) of many of these transporters remains unclear, some may supply plants with N in soils that contain high concentrations of organic matter (Grossman and Takahashi, 2001).

When nitrate enters the plant cell, it is reduced to ammonium in a two step process catalysed by nitrate reductase (NIA) and nitrite reductase (NII) (Figure 1.3). Reduction consumes eight electrons in total and occurs in both, the cytoplasm and in plastids. Ammonium is incorporated into glutamine then glutamate, via glutamine synthetase (GS) and glutamate synthase (GOGAT) cycle, which operates in cytoplasm and plastids, or via the mitochondrial enzyme glutamate dehydrogenase (GDH), directly into glutamate. Primary nitrogen assimilation in plants is also tightly co-ordinated with primary carbon metabolism. Nitrate assimilation requires synthesis of organic acids, like alpha keto-glutarate (2-OG), which acts as acceptor for ammonium in GS mediated reaction (Figure 1.4) and malate as a counter-anion which prevents alkalisation during nitrate assimilation. Photosynthesis and glycolysis provide energy and redox equivalent for the energy consuming process of nitrate reduction.

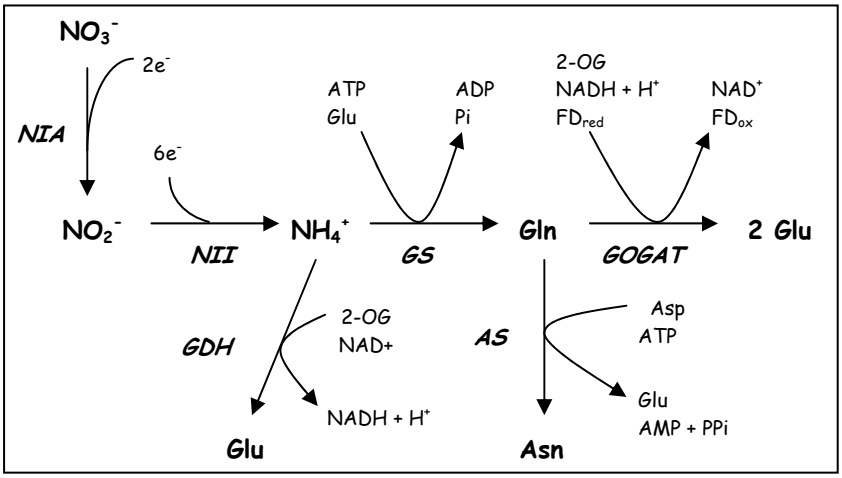

Figure 1. 3 Possible pathways for the assimilation of inorganic nitrogen into organic compounds

Abbreviations: NIA – nitrite reductase; NII- nitrite reductase; GS – glutamine synthetase; GOGAT – glutamate synthase; AS – asparagine synthetase; GDH – glutamate dehydrogenase; (all enzyme abbreviations in italic); e^- - electron; FD_{red} / FD_{ox} - reduced ferredoxin / oxidised ferredoxin; NAD^+ / NADH – reduced / oxidised NAD; -2-OG – alpha keto-glutatrate, Pi – inorganic phosphate

1.3 Nitrogen regulation of transport and metabolism

When plants grow in N rich environment, downstream metabolites of nitrate assimilation, like ammonium and amino acids, repress transcription of many genes involved in nitrate assimilation as shown for *Arabidopis* (Scheible et al., 2004; Wang et al., 2000; Wang et al., 2003; Wang et al., 2004), tobacco (Scheible et al., 1997a; Scheible et al., 1997b) and tomato (Wang et al., 2001.) Expression of the same genes is de-repressed when nitrogen becomes limiting for plant growth as shown for *Arabidopsis* (Scheible et al., 2004; Wang et al., 2000; Wang et al., 2003; Wang et al., 2004)), tobacco (Scheible et al., 1997a; Scheible et al., 1997b) and tomato (Wang et al., 2001). A second level of signalling activates many of these genes, when nitrate becomes available as the sole or major source of nitrogen in the environment. Due to the fact that C and N metabolism are tightly co-ordinated, nitrate also regulates transcriptional activity of many genes from carbon metabolism (Figure 1.4) as shown before for *Arabidopsis* (Scheible et al., 2004; Wang et al., 2003; Wang et al., 2000; Wang et al., 2003; Scheible et al., 2004), and tobacco (Scheible et al., 1997a; Scheible et al., 1997b). On the other hand, expression of most of the nitrate and ammonium transporters as well as genes involved in primary nitrogen assimilation is light / diurnally regulated (as reviewed by Stitt, 1999; Stitt et al., 2002; Foyer et al., 2003). This regulation can be abolished by addition of external sugars. It was proposed that, light control of nitrate uptake reflects

regulation exerted by the downward transport of photosynthates (Delhon et al., 1996). Presumably, reciprocal controls between N and C metabolism ensures their coordination at the whole-plant level (Coruzzi and Bush, 2001; Coruzzi and Zhou, 2001; Stitt et al., 2002; Foyer et al., 2003). This allows a reprogramming of nitrogen and carbon metabolism to facilitate the assimilation of nitrate and its incorporation into amino-acids (references above).

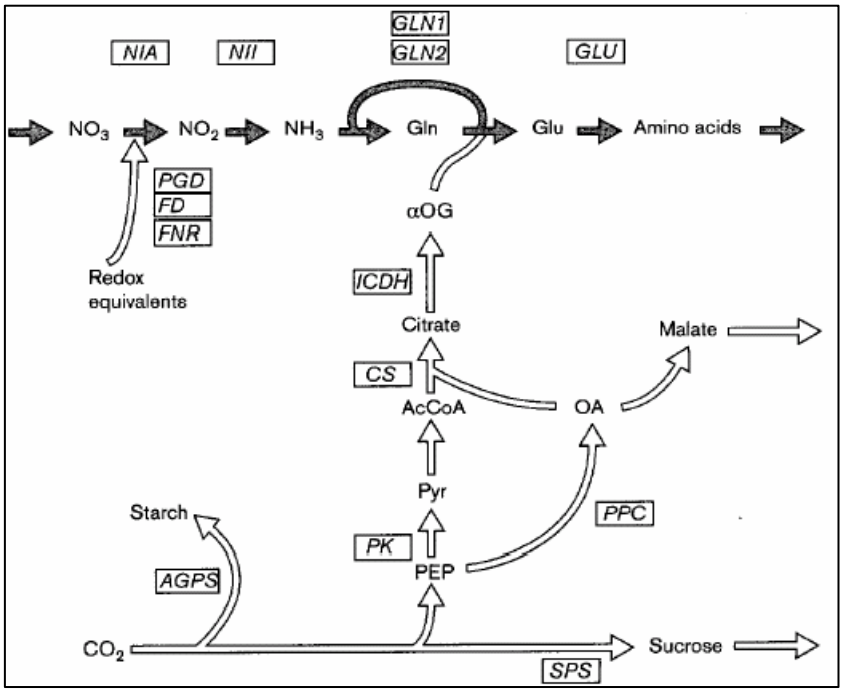

Figure 1. 4 Nitrate-regulated genes from primary metabolism (taken from Stitt, 1999)

Shaded arrows: primary nitrogen assimilation pathway, open arrows: carbon metabolism pathways. Abbreviations: AGPS – ADP-glucose pyrophosphorylase (regulatory subunit), SPS- sucrose phosphate synthase, PK – cytosolic pyruvate kinase, PPC – phosphoenolpyruvate carboxylase, CS – citrate synthase, ICDH – NADP-isocitrate dehydrogenase, FD – ferredoxin, FNR – ferrodoxin:NADP oxidoreductase, PGD – 6-phosphogluconate dehydrogenase, NIA - nitrate reductase, NII– nitrite reductase, GLN1 - plastid glutamine synthetase, GLN2 – cytosolic glutamine synthase, GLU – glutamate synthase

Transcriptional regulation of both nitrate uptake systems is quite precise and both of the systems have constitutive (c) and inducible (i) elements. cHATS and cLATS operates even if plants have not been exposed to nitrate from the soil before, while iHATS and iLATS are strongly and transiently induced by micromolar concentrations of external nitrate ($[NO_3^-]_{ext}$) (see Orsel et al., 2002a; Glass et al., 2002; Crawford and Forde, 2002; Forde, 2000; Daniel-Vedele et al., 1998 for references). It has been postulated that iHATS and iLATS do not derive from constitutive elements expressed at basal level but that they involve *de novo* synthesis of transporters (references above). Expression of NRT2 family members in *Arabidopsis*, barley or tobacco roots is under feedback inhibition by increased internal concentration of ammonium ($[NH_4^+]_{int}$) or other downstream metabolites of nitrate acquisition (e.g. glutamine) (Matt et al., 1998) as shown by the experiments with inhibitors of enzymatic

activity of: GS (like aza-serine or methionie sulfoximine), GOGAT and aspartate aminotransferasae (Zhuo et al., 1999; Vidmar et al., 2000a; Vidmar et al., 2000b). Tobacco *NpNRT2* transcript abundance declines when glutamine is fed to the roots (Matt et al., 1998) while in *Arabidopsis,* arginine is the more effective than asparagine or glutamine, which are reducing *ATNRT2.1* expression levels to 18, 38 and 77% respectively (Zhuo et al., 1999). Experiments with knockout mutant affected in both: *ATNRT2-1* and *ATNRT2-2* expression showed that both transporters appear to be the major components of iHATS in *Arabidopsis* roots (Filleur and Daniel-Vedele, 1999; Filleur et al., 2001). The expression pattern of the other five *NRT2* genes makes them potentially involved in the cHATS (Orsel et al., 2002b; Okamoto et al., 2003). *ATNRT2-5* is the only nitrate-repressible transporter in both shoots and roots, and it is therefore postulated to be involved in nitrate transfer from storage pools (Orsel et al., 2002a; Okamoto et al., 2003; Orsel et al., 2004). *ATNRT 1-1* seems to be a major component of the iLATS (Wang et al., 1998) and cLATS may be encoded by *ATNRT1-2* and *ATNRT1-4* (as reviewed by Crawford and Forde, 2002; Orsel et al., 2002a). There is no sharp border between the HATS and LATS. *ATNRT1-1* (*CHL1*) has been proposed as the first dual-affinity nitrate transporter, with a switch between HATS and LATS occurring via phosphorylation (Wang et al., 1998; Huang et al., 1999; Liu et al., 1999). Expression of *NRT2* genes are also diurnally regulated (maximum *ATNRT2-1* expression in roots during day, minimum in the first hours of darkness) and such regulation depends on sucrose (Lejay et al., 1999; Lejay et al., 2003). It has been proposed, that a reciprocally acting signal might regulate *ATNRT2-1* expression in roots. The evidence of such a signal has not been show, but role of auxins or amino acids concentration in the phloem sap, was discussed (Forde, 2002b; Forde, 2002a).

The expression of ammonium transporters is also regulated by nitrogen, with N-deprivation inducing *AMT* gene expression within hours in shoots and roots, as reviewed by: Howitt and Udvardi, 2000; von Wiren et al., 2000; Crawford and Forde, 2002; Glass et al., 2002. Expression of ammonium transporters are also diurnally regulated (maximum *AMT1-1* expression at the end of the light period, which declines with the onset of darkness) and such also depends on the carbon status of plants (Gansel et al., 2001). *ATAMT1-1*, *ATAMT1-2,* and *ATAMT1-3* are strongly inhibited by high (over 5 mM) external ammonium or by glutamine. Such inhibition occurs not only on transcriptional, but possibly also post-transcriptional level, or affects directly kinetics of ammonium transporters (see references above).

Nitrate induces expression of many of the genes involved in its assimilation, including those encoding: nitrite reductase (*NII*), nitrate reductase (*NIA*), enzymes required for ammonium assimilation via the GOGAT pathway (*GS, GOGAT*) as well as glutamate dehydrogenase (*GDH)* and asparagine synthetase (*AS*) as reviewed by Stitt, 1999; Wang et al., 2000; Stitt et al., 2002. Nitrate reductase transcription and enzymatic activity in leaves is strongly induced by nitrate and repressed by ammonium and amino acids in both *Arabidopsis* and tobacco (Scheible et al., 1997a; Scheible et al., 1997b; Campbell, 1999) Increase in transcript is accompanied by increased NIA protein and activity and increased activity of NII and glutamine synthetase (see Kaiser and Huber, 2001 for references). *NIA* expression is also regulated diurnally, by sucrose and cytokinins. Post-translational down-regulation of NIA activity by high carbohydrate content, involves NIA phosphorylation and binding of a 14-3-3 protein dimmer (see Kaiser and Huber, 2001 for references). Nitrite reductase (*NII)* has a similar pattern of regulation to *NIA* in both – *Arabidopsis* and tobacco (Crete et al., 1997) Nitrite (NO_2^-) is a toxic ion, which must be converted in plants very quickly to ammonium. When nitrate enters the plant cell, *NIA* and *NII* transcript accumulates. However, the cell cannot allow NIA activity to out-pace that of NII, as nitrite would accumulate to toxic levels. Plant cells avoids this scenario by maintaining a basal level of *NII* transcription in the absence of nitrate, which is than rapidly translated to protein when NO_3^-/ NO_2^- appears in the cell as discussed by Crete et al., 1997).

Studies on *NIA*-deficient mutants provide an opportunity to distinguish between sensing of nitrate and its downstream metabolites. Such mutants are not affected in nitrate uptake but they cannot metabolise nitrate. First plant mutant with very low nitrate reductase activity was tobacco Nia30(145) (Scheible et al., 1997a; Scheible et al., 1997b). The analysis of the enzymes involved in primary carbon and nitrogen metabolism (Figure 1.4) and the levels of some key metabolites from N and C pathways, in Nia30(145) mutants, allowed to propose that nitrate acts as a signal o initiate coordinated changes in C and N metabolism. The first *Arabidopsis NR* double mutant described was *G'4-3* (Wilkinson and Crawford, 1993). *G'4-3* is not a true null mutant, as it shows detectable growth on nitrate and retains slight nitrate reductase activity in both shoots and roots (Wilkinson and Crawford, 1993; Lejay et al., 1999). A true *NIA null* mutant, with no detectable NR activity in shoots and in roots, was described recently by Wang et al., 2004. This mutant was not able to grow on KNO_3 as the sole nitrogen source. The mutant grew normally only in the presence of an alternative N-source, like ammonium succinate at neutral pH. Genome-wide transcriptional analysis of *NIA*

null mutant allowed not only to confirm that nitrate acts as signal to activate transcription of the genes involved in C and N metabolism but also to identify some genes activated transcriptionally by downstream metabolites of nitrate acquisition (Wang et al., 2004). Recent work, using the *Arabidopsis NIA null* mutant, showed that nitrate reductase activity is required for nitrate uptake into fungal but not into plant cells (Unkles et al., 2004).

Neither isoform of *Arabidopsis GS* is induced by nitrate, but both are repressed by amino acids (Oliveira and Coruzzi, 1999). Expression of the chloroplastic isoform of *GS* (*GS2*) is regulated diurnally and by sugars (sucrose, fructose, and glucose) while the cytosolic isoform (*GS1*) is also regulated diurnally and by alpha keto-glutarate. Experiments with monochromatic light of a various wavelengths in etiolated *Arabidopsis* seedlings, indicated phytochrome – mediated regulation of *GS2* well as *AS1* and *AS2* (Thum et al., 2003; Thum et al., 2004). *GOGAT* is activated at the transcriptional level by both light and by nitrate (see Stitt et al., 2002 for references) *AS1* and *GDH1* on the other hand are repressed by light. The function of that reciprocal regulation in plants is presumably that high expression of *AS1* in the dark enables plants to convert Gln to Asn, which is the major transport and storage form of N, when availability of carbon skeletons becomes limiting (Lam et al., 1998) Additionally, ammonium activates both isoforms of *AS* (Lam et al., 1998).

Nitrate leads to increases in transcripts of enzymes from glycolysis and the TCA cycle in both *Arabidopsis* and tobacco, including: pyrruvate kinase (*PK*), citrate synthase (*CS*), or *iso*-citrate dehydrogenase (*ICDH*), which provides carbon scaffolds for amino acids synthesis. Nitrate also induces transcription of the genes encoding enzymes involved in synthesis of red-ox equivalents like ferredoxin--NADP(+) reductase (*FNR*) or enzymes from oxidative pentose pathway, like 6-phosphogluconate dehydrogenase (*PGD*) or electron-transferring proteins, like ferredoxin (*FD*). It also inhibits starch synthesis pathway via ASP-glucose phopshorylase (*AGPS*), has no influence on sucrose phosphate synthase (*SPS*), indicating that sucrose production continues when nitrate concentration increases in plant cell, as reviewed by Stitt, 1999; Stitt et al., 2002; Foyer et al., 2003.

It is not known whether regulatory cross-talk between nitrogen and carbon metabolism is mediated via phytohormons, Ca^{2+} signalling or whether it involves protein phosphorylation, 14-3-3 binding, or regulatory proteolysis

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1.4 Nitrogen control of plant development

Nitrate serves as a very important signal for plant development. A wide range of developmental processes are affected by nitrate as depicted in Figure 1.5

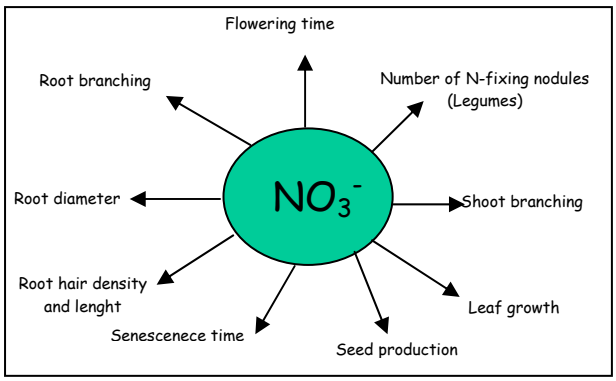


Figure 1. 5 Developmental processes controlled by nitrate

In *Arabidopsis,* local supply of nitrate, but not its reduced forms, like ammonium and glutamine, at low concentrations (0.1-10 mM) increase lateral root elongation rate and length and, without effecting in their spacing along the primary (seminal) root or growth of the primary root itself (Zhang and Forde, 1998; Zhang et al., 1999). High nitrate (50 mM), on the other hand, has no effect on lateral root number but inhibits its elongation just after lateral root emergence. In contrast to the stimulatory effect of low nitrate, inhibitory effect is systemic and stronger in a double nitrate reductase mutant *G'4-3* (Zhang et al., 1999). Therefore it has been postulated, that accumulation of nitrate itself in plants is enough to inhibit lateral root growth. (Zhang et al., 1999; Malamy and Ryan, 2001; TRANBARGER et al., 2003). Nitrate inhibition of lateral root growth can be alleviated by increased concentrations which indicates, that C/N ratio may be a key factor, in regulating lateral root initiation. Additionally, high (above 3 mM) ammonium concentrations, supplied without K^+ ions could also inhibit primary root growth in *Arabidopsis* (Cao et al., 1993).

At least two transcription factors have been implicated in the control of lateral root development by nitrate. Antisense repression of the *Arabidopsis ANR1* gene, (MADS-box TF family member) eliminate the positive response of lateral root growth, to the localised supplied of nitrate (Zhang and Forde, 1998). Nitrate effects on lateral root proliferation are also blocked in the *axr-4* (auxin-resistant mutant) (Zhang and Forde, 1998; Zhang et al., 1999) A regulatory model connecting *ANR1*, *AXR4* and other genes controlling particular stages of lateral root initiation has been proposed (Zhang et al., 1999) and is shown below (Figure 1.6)

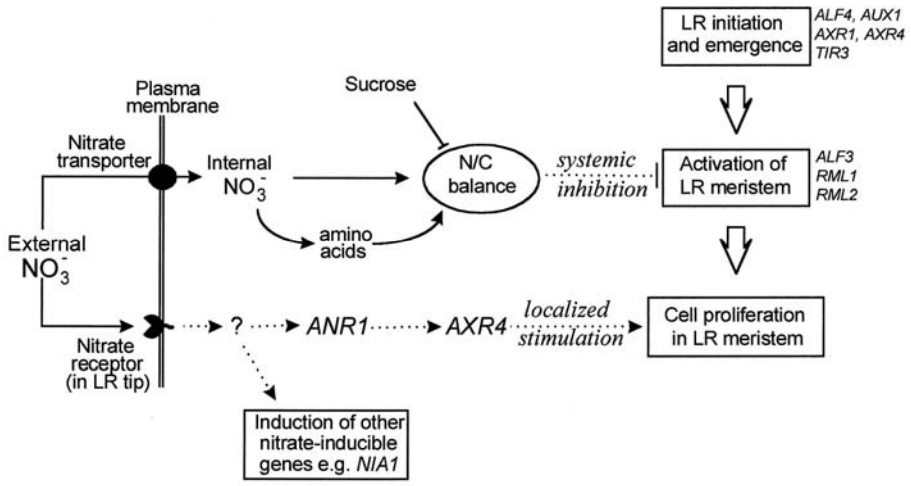


Figure 1. 6 Dual pathway model for regulation of LR growth and development by nitrate (taken from Zhang et al., 1999)

Dual-pathway model for regulation of LR growth and development by NO3–. Because the *ANR1* gene is rapidly induced by NO3– (12), the putative NO3– receptor and the mechanism for transcriptional activation of ANR1 are likely to be shared with other NO3—inducible genes such as the *NIA1* genes encoding NR. *ANR1* was tentatively placed upstream of *AXR4* in the signal transduction pathway. This arrangement makes a number of predictions that can be tested experimentally by using *axr4* mutants and *ANR1* antisense lines. Other genes implicated in controlling particular stages in LR initiation or developments are shown on the right. Broken arrows indicate signalling steps, solid arrows indicate transport or metabolic steps, and large open arrows indicate developmental steps.

In this model, external nitrate supply is monitored by individual lateral root tips and the signal is transduced via A*NR1* and *AXR4* to produce increased meristematic activity. The identity and sub-cellular location of nitrate sensor, as well as the mechanism of *ANR1* induction remain unknown. It was shown that auxins transported from the aerial tissues are essential for LR initiation and auxin transport is blocked of the hypocotyls/root junction when plants are grown under conditions inhibiting LR growth (Malamy and Ryan, 2001). Auxin-responsive mutants (*aux1, axr1, axr2*) are resistant to the inhibition of lateral initiation and emergence by high nitrate, suggesting that auxin and nitrate response pathways may overlap during environmental control of lateral root growth as proposed by Zhang et al., 1999).

It is well known, that high soil nitrate increases shoot to root ratio in many plant species and it has been demonstrated that the internal pool of nitrate mediate that effect (see Stitt, 1999; Crawford and Forde, 2002; Stitt et al., 2002 for references). The following figure shows long – distance signalling in *Arabidopsis* (Figure 1.7), as reviewed by Forde, 2002b; Forde, 2002a)

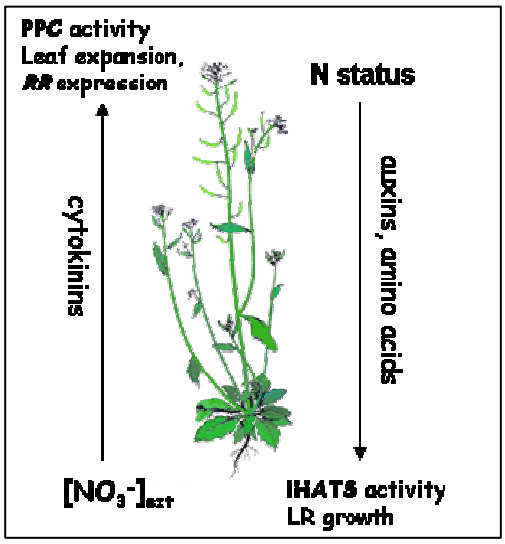


Figure 1. 7 Long distance nitrogen signalling processes in *Arabidopsis*

Abbreviations: $[NO_3^-]_{ext}$ – external nitrate, iHATS – inducible high affinity nitrate transport system, LR- later root, PPC – phosphoenolpyrruvate carboxylase, *RR* – response regulators.

Cell division slows rapidly in leaves of *Arabidopsis* plants following transfer from nitrate to ammonium, as sole nitrogen source. This is correlated with a decrease of the zeatine fraction of xylem zap. Flux of cytokinins from the root to the shoot has been postulated as a part of long-distance nitrogen signalling. Long-distance signalling mechanisms are also proposed to link N uptake by roots with N-demand in the shoots. An unknown signal(s) is produced by roots in response to changes in N-nutrition that is/are transported via the phloem to aerial organs. Cytokinins might act as such a signal, as their levels in xylem sap change with changing N-nutrition and they are able to activate response regulator (*RR*) genes (*ARR* in *Arabidopsis* and *ZRR* in maize) only in presence of nitrate assimilated by roots (Forde, 2002b; Forde, 2002a). Cytokinins, via RR activity can stimulate cell proliferation, change leaf morphology, and activate PEP-carboxylase activity in maize (Sakakibara et al., 1998). Reciprocally operating signal might by mediated by auxins or amino acids but possible regulatory genes involved in the response are unknown (Forde, 2002b; Forde, 2002a).

In many plant species, intensive N-fertilisation is reported to delay senescence and flowering, and to increase seed production, when applied during the reproductive phase. Mechanisms which regulate these processes are mostly unknown Recent comparative studies between *Arabidopsis* and *Sinapsis alba* showed that inequality in organic C and N supply to apical meristem may be important at the floral transition (Corbesier et al., 2002)

1.5 Nitrogen signalling in prokaryotes and lower eukaryotes

Nitrogen sensing and signalling has been an intensively studied topic, especially in lower organisms, like fungi, algae, and bacteria.

1.5.1 Bacteria

The first N-regulatory (NTR) protein, PII (GlnB) was described in *E. coli* more than 25 years ago (Pahel et al., 1978). PII is part of intensively studied NTR system, coordinating nitrogen and carbon metabolism in eubacteria. The model of NTR system is shown on figure below (Figure 1.8).

PII does not have an enzymatic activity. It interprets the intracellular concentration of the key energy, C, and N metabolites (ATP, 2-oxoglutarate, and Gln, respectively) and interacts with two other proteins to regulate their function (Magasanik, 1993; Ninfa and Atkinson, 2000; Arcondeguy et al., 2001). PII, unmodified by UMP, binds to ATase. The PII-ATase complex stimulates the adenylylation of GS and allows GS to be feedback inhibited by other metabolites, i.e. "inactive" GS (Jiang et al., 1998). Unmodified PII also associates with NRII, which is part of a two-component signalling system whereby NRII autophosphorylates itself and other response regulator, NRI. The transcription of N-regulated genes (which includes GS and the proteins necessary for utilization of other extra cellular N sources) requires the activated (phosphorylated) form of the transcription factor NRI. The binding of PII to NRII suppresses the kinase and activates the phosphatase activities of NRII, thereby dephosphorylating NRI and preventing transcription of the N-sensitive regulon.

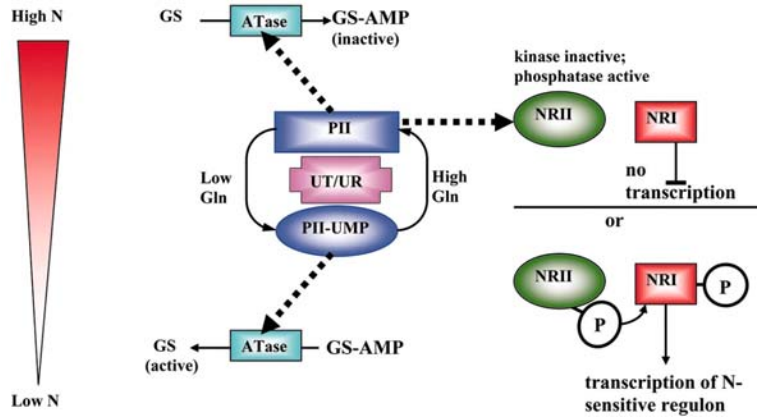


Figure 1. 8 Model of bacterial NTR regulatory system (taken from Moorhead and Smith, 2003)

Abbreviations and detailed description in the text Thick dashed lines, Protein: protein interactions. Substrates for the covalent modification reactions (UTP and ATP) are not shown for simplicity.

A low Gln concentration allows the uridylyltransferase (UT) to uridylate modify PII by uridylylation. PII-UMP stimulates the deadenylylation of GS-AMP (by ATase), thereby promoting GS activity. PII-UMP has no affinity for NRII, which permits high protein kinase activity of NRII, leading to increased phospho-NRI and, therefore, transcription of the N-regulated genes. Conversely, a high level of Gln (N-sufficient conditions) allosterically activates the uridylyl-removing activity to deuridylylate PII-UMP. Unmodified PII activates the ATase to adenylylate and, therefore, inactivate GS. Gln also allosterically affects the ATase, stimulating the adenylylation reaction.

The C signal, 2-oxoglutarate, is sensed allosterically by PII. Each subunit of the PII trimer can bind one molecule of ATP and 2-oxoglutaratewhich is a prerequisite for PII uridylation by UT. This signalling mechanism ensures that C is available for amino acid biosynthesis before GS is activated. Similarly, for PII to interact with NRII, it must have associated 2-oxoglutarate. If Gln concentrations are low, the C-saturated form of PII is readily uridylylated by the UT, and PII-UMP in turn activates GS in response to the high-C skeleton, low-Gln signal, as reviewed by Arcondeguy et al., 2001.

In *E. coli*, very low concentrations of extra cellular nitrate are sensed via two functionally overlapping sensors which are distinct from NTR system. In the presence of nitrate or nitrite, NarX/NarQ - nitrate/nitrite receptors kinases, phosphorylate soluble DNA-binding response regulators NarP/NarL. NarP/NarL regulates operons containing electron transport components and anaerobiosis-specific nitrate reductase (Chiang et al., 1997).

1.5.2 Fungi

Although *S. cerevisae* cannot transport or assimilate nitrate, it senses nitrogen and carbon status of the environment via the well characterized TOR pathway (Beck and Hall, 1999), shown schematically in figure 1.8.

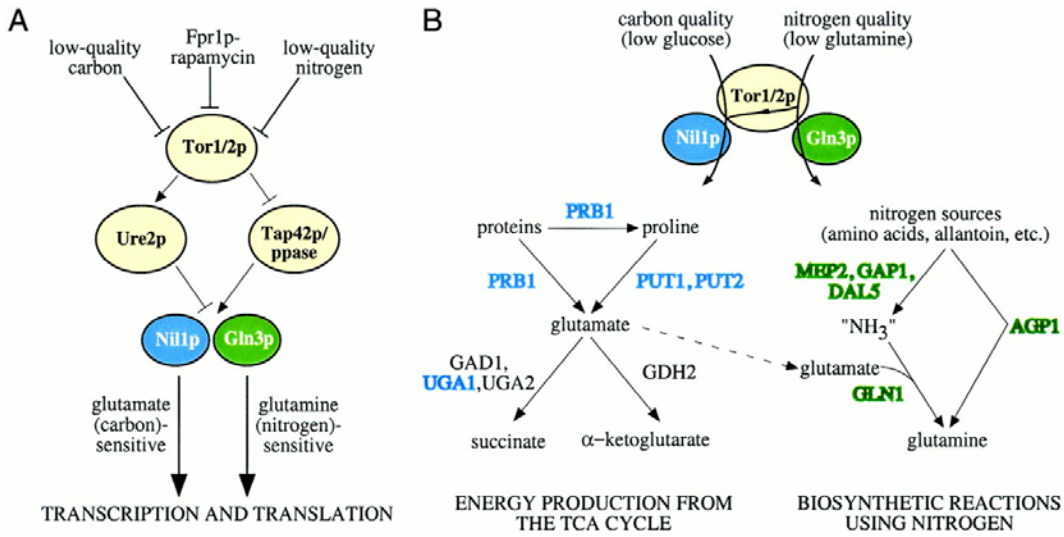


Figure 1. 9 TOR signalling pathways in yeasts (taken from Kuruvilla et al., 2001)

(***A***) A model for how the Tor proteins regulate Nil1p and Gln3p using the cytoplasmic anchor protein Ure2p and Tap42p/phosphatase. This regulation is differential, depending on the quality of available carbon and nitrogen sources. (***B***) When Nil1p is activated, the genes that are up-regulated suggest that the cell is trying to generate energy via production of TCA cycle intermediates. (*PRB1* is a vacuolar, broad-specificity protease that is envisioned to supply amino acids like proline or glutamate.) When Gln3p is activated, the genes that are up-regulated suggest that the cell is trying to collect alternative nitrogen sources to synthesize glutamine. Low-quality nitrogen up-regulates genes regulated by Nil1p, perhaps as a source of glutamate from which to make glutamine. Thus, the sets of genes controlled by Nil1p and Gln3p are overlapping. Shown in blue or green are genes primarily dependent on Nil1lp or Gln3p, respectively.

TOR (target of rapamycin) protein kinase inhibits nuclear translocation of products of two GATA transcription factors genes: *GLN3* and *GAT1 (NIL1*) by promoting the association of TAP42 protein with SIT4 protein phosphatase. When C- or N- deprivation or rapamycin, blocks TOR activity, inactive SIT4 dissociates from TAP42 and de-phopshorylates GLN3 and NIL1 in complex with URE2. URE2 anchors phosphorylated GLN3/NIL1 in the cytoplasm. Once dephosphorylated, free GLN3/NIL1 moves into nucleus where it represses transcription of many genes involved in transcription and translation, including *eIF4, RNA pol I* and *RNA pol III* (Beck and Hall, 1999). TOR preferentially uses GLN3 or NIL1 to down regulate translation in response to low-quality N and C, respectively (Kuruvilla et al. 2001). TOR also regulates two Zn finger TFs *MSN2* and *MSN4*, in response to various stresses, including C limitation, by retaining them in cytoplasm in complex with BMH2 (a 4-3-3 protein). Experiments with GS activity blockers (MSX) showed that intracellular glutamine depletion leads to nuclear translocation of GLN1 and two bHLH TFs, RTG1 and RTG2, both mediating Gln synthesis (Crespo et al., 2002). Other TOR controlled TFs, including: NIL1, MSN2 and MSN4 were unaffected by Gln starvation. Therefore TOR appears to discriminate between different nutrient conditions to elicit a response appropriate for a given condition.

As mentioned above, use of nitrate or nitrite as N source is restricted to a few yeast species. One of them, *Hansenula polymorpha* has a nitrate transporter gene (*YNT1*) clustered with nitrite (*YNI1*) and nitrate (*YNR1*) reductase genes in a 11kb fragment of the genome (Siverio, 2002). This cluster also contains two GATA TFs, *YNA1* and *YNA2*. It was proposed that YNA1 alone or together with YNA2 acts to activate expression of *YNT1*, *YNR1* and *YNI1* in response to external nitrate or nitrite. More recent experiments showed that YNR1 activity is regulated post-translationally by the TOR pathway in *H. polymorpha* (Navarro et al., 2003).

Various filamentous fungi are able to use nitrate or nitrite as N source. Several N-sensing elements have bee known to operate in the two filamentous fungi *Neurospora crasa* and *Aspergillus nidulans*. Fungal N- regulatory genes can be divided into two major groups, as reviewed by Marzluf, 1997

1. Pathway un-specific (globally acting) regulators, mediating global N-repression and de-repression, which are responsible for tuning many different, unlinked but co-regulated genes. In absence of N-sources like Gln or NH_4^+ , they act positively on the expression of genes required to utilise alternative N-sources like nitrate and to catabolise amino acids. They share several common features:
    - All belongs to the GATA TF family
    - Their expression rises upon N-deprivation (de-repression) and is repressed by downstream metabolites of nitrate assimilation,
    - C – terminus contains a putative binding motif for glutamine or another N-metabolite binding motif which alternatively might function in protein-protein interactions with NMR protein, during N-repression
    - One Zn finger motif is located in a positively charged region, necessarily to promote expression of target genes.
2. Pathway specific regulatory factors, that acts in a positive fashion and provides selective activation of specific sets of the genes, like nitrate transporters and nitrate reductase, and which become active after binding of a specific inducer. They also share a couple common features:
    - Almost all belong to GAL4 TF family, which has a fungal - specific type of zinc finger motif,
    - C-terminus is probably involved in protein-protein interactions,

- They can interact with the basal transcriptional apparatus independently or via protein-protein interactions with globally acting factors (e.g. NIT2 with NIT4 and NIRA with AREA)

Examples of the first group of genes are: *NIT2* (*N. crasa) AREA* (*A. nidulans)* and *GLN3* (*S. cerevisae*) but a plethora of homologues has been found in other fungi so far. Example for the second group of the genes are: *NIT4* (*N. crasa)*, *NIRA* (*A. nidulans)*, *YNA1* (*H. polymorpha)*

1.5.3 Algae

Expression of N assimilatory genes in the unicellular algae *Chlamydomonas reinhardtii* is controlled by the *NIT2* locus. These gene encodes a homologue of *NIT2* TF from *N. crasa* and its expression is repressed by ammonium and induced by nitrate (Quesada et al., 1998). Recent results show that in *C. reinhardtii*: (i) nitrate is sensed intracellularly following uptake via high affinity nitrate transporters, (ii) negative feedback regulates nitrate reductase (NIA1) activity is due to signalling by a minor amounts of nitrate in N-free medium, (iii) nitrite does not exert any direct effect on the expression of the *NIA1* gene, and (iv) ammonium or a product of its assimilation inhibits nitrate induction by preventing nitrate uptake (Llamas et al., 2002). Thus, nitrate transport, not nitrate reduction has been postulated as a key step controlling nitrate assimilation in *C. reinhardtii*. Two regulatory genes: *Nrg1* and *Nrg2* required for ammonium modulation of *NIA* expression have been also identified in *Chlamydomonas* (Prieto et al., 1996).

1.6 Nitrogen signalling in plants

Nitrogen signalling in plants is a virtually unexplored territory. Although there are homologues of prokaryotic and fungal regulatory proteins in plants, there is still lack of evidence that they mediate N-signalling processes.

The *Arabidopsis* PII homologue (*GLB1*) encodes a chloroplastic protein. Transcription of *GLB1* is induced by sucrose and repressed by amino acids like Gln, Asp, and Asn (Hsieh et al., 1998). *GLB1* has been proposed as putative C/N sensor based on fact, that *Arabidopsis* lines, that overexpress *GLB1* under 35S promoter lack glutamine sensing (Hsieh et al., 1998). The crystal structure of GLB1 was resolved recently (Smith et al., 2003). Interestingly, it overlaps perfectly with the x-ray structure of bacterial PII. The same work showed, that GLB1

is able to bind small molecules, like: ATP, ADP and 2-OG, with high affinity. Its also binds oxaloacetate (OAA) with lower affinity, but does not bind Gln, or other amino acids. It has been proposed that homotrimeric PII protein could regulate its targets post-translationally, via very conservative T-loop (Smith et al., 2003).

Homologues of fungal N-regulators: *AREA*, *NIT2* and *GLN3* from *Arabidopsis* are able to complement *gln3nil1* double mutant in yeast, but they are not members of GATA TF family. Additionally, their expression is unchanged in response to nitrate induction or N - starvation (Truong et al., 1997). They are almost identical to the two genes involved in gibberellins signal transduction: *GAI* and *RGA* (Peng et al., 1997; Silverstone et al., 1998). There is no evidence that these genes are involved in N-regulation in plants.

Genetic screening of an EMS mutant population, yielded the *lin1* mutant, which constitutively produces lateral roots under high N conditions (Malamy and Ryan, 2001). The *Lin1* gene has not been cloned.

Microarray analysis comparing *Arabidopsis* seedlings grown on high (10 mM) or low (0.5 mM) nitrate, supplemented with 5 mM Gln, showed strong response to nitrate for two transcription factors: *bZIP-210* (bZIP family member) and *ATL2-237* (LIM family member) (TRANBARGER et al., 2003). The same work showed that their expression was preferentially observed in roots and correlates to the root response to nitrate availability. Other array experiments revealed further TF genes that respond to nitrogen deprivation or to nitrate, but none of these have been so far been characterised functionally (Wang et al., 2000; Wang et al., 2003; Scheible et al., 2004; Wang et al., 2004)

1.7 *Arabidopsis* transcription factors

Expression of many genes/proteins involved in nitrogen acquisition and assimilation is regulated at the transcriptional level. This implicates transcription factors in N-regulation in plants, although as already noted, none of these have been identified. TFs are sequence-specific DNA-binding proteins capable of activating or/and repressing transcription of target genes. Their domain architecture includes at least one DNA-binding domain (DBD) which mediates the binding to specific DNA sequences in the promoter region of their target genes, and a transactivation domain (TAD) that can interact with the basal transcription machinery. In many cases, additional domains mediate other interactions, such as homo- or heterodimerisation, interaction with other TFs, or the binding of co-activators or low-

molecular weight ligands, e.g. steroid hormones (Lewin, 2000). TFs are often expressed in a tissue-specific, developmental-stage-specific, or stimulus-specific manner. Functional redundancy is not unusual within TF families, which can complicate their genetic analysis. *Arabidopsis* MYB proteins WEREWOLF and GLABROUS1 have been shown to be functionally interchangeable, and owe their particular roles in plant development to differences in their expression patterns (Lee and Schiefelbein, 2001).

The availability of *Arabidopsis* genome sequence (*Arabidopsis* Genome Initiative, 2000), allows global, or genomic analysis of transcriptional regulation in plants. Initial estimates put the number of TF genes in *Arabidopsis* at 1572 TFs or approximately 6.1% of the total number of 25.498 genes (2000; Riechmann, 2002). Therefore content of TF genes in *Arabidopsis* and *H. sapiens* (4.6-6.6% of total number of the genes) are similar (Morgan, 2001; 2004). More recent data, available at: http://arabidopsis.med.ohio-state.edu/AtTFDB/ and http://genetics.mgh.harvard.edu/sheenweb/AraTRs.html), enlarge this to number around 2200 genes or nearly 8% of the genome. Grouping *Arabidopsis* TF proteins according to the sequence of TF DNA-binding domains resulted in the classification of 45 families and 15 subfamilies (Figure 1.10) according to Riechmann, 2002

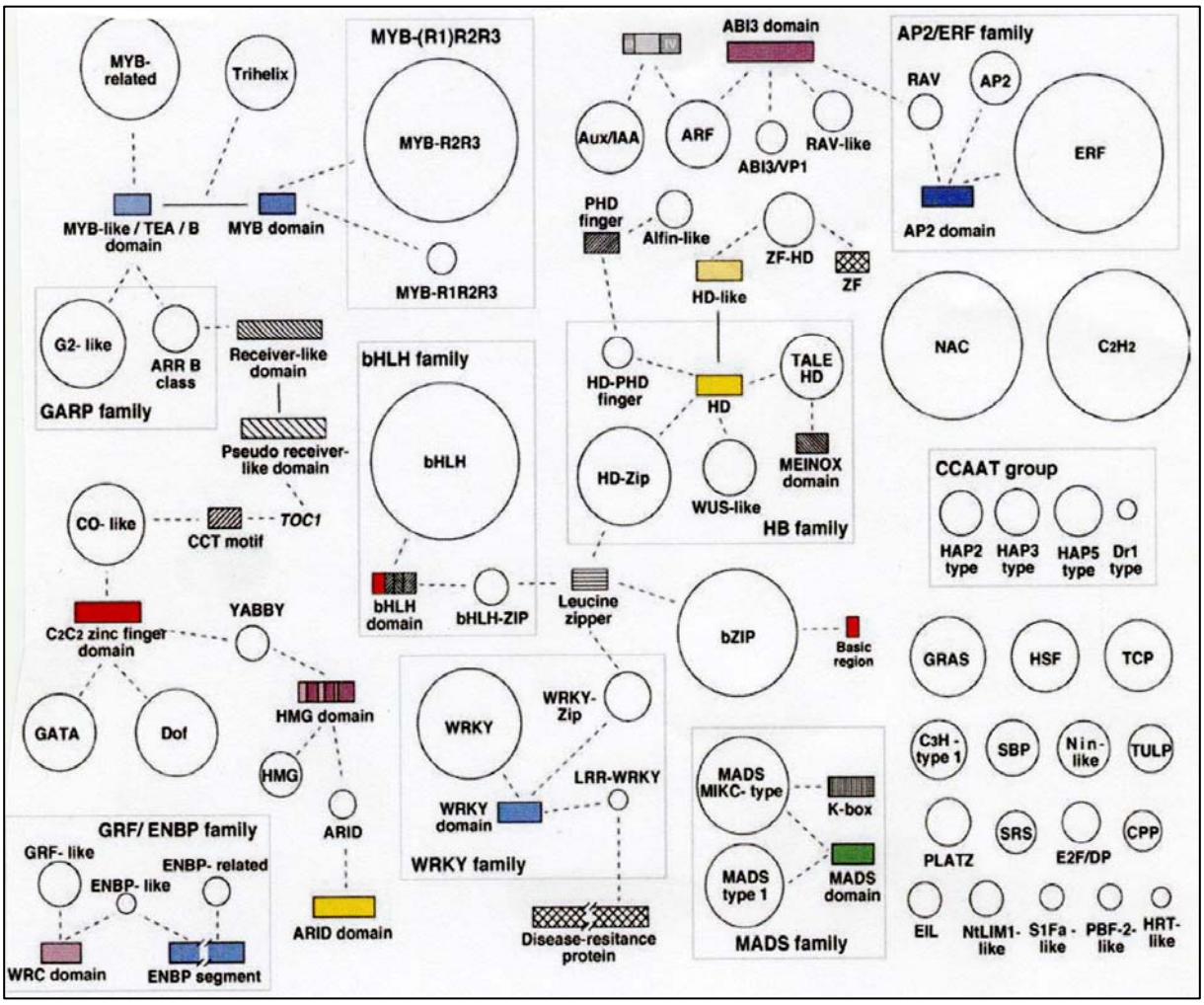


Figure 1. 10 The *Arabidopsis* complement of transcription factors (taken from Riechmann, 2002)

Gene families are represented by circles whose size is proportional to the number of members in the family. Domains that have been shuffled, and therefore "connect" different groups of TFs are indicated in rectangles, whose size is proportional to the length of the domain. DNA binding domains are colored; other domains (usually protein-protein interaction domains) are shown with hatched patterns. Dashed lines indicate that a given domain is a characteristic of the family to which it is connected.

Shuffling of TF DNA-binding domains during evolution has generated novel TFs with plant-specific combinations of modules, within TF families like homeodomain, MADS or ARID. For example, combinations of the: homeobox domain with leucine zipper, PHD finger or plant specific-zinc finger domains are not found in yeast, *Drosophila,* or *C. elegans* (Riechmann et al., 2000; Riechmann, 2002). Members of kingdom-specific families represent 45% of the *Arabidopsis* complement of transcriptional regulators (Table 1.1)

Table 1. 1 Content and distribution of TFs in eukaryotic organisms (from Riechmann 2002)

	A. thaliana	*D. melanogaster*	*C. elegans*	*S. cerevisiae*
Number of genes	25.498 [1] – 29454[2]	~14000[3]	~19000[4]	~6000[5]
Number of TFs (% of the genome)	**2256[6] (5.3 – 8.6%)**	**642 (4.6%)**	**673 (3.5%)**	**210 (3.5%)**
% of TFs from kingdom-specific families	~45	~14	~47	~32
% of TFs from families common to all three kingdoms	~53	~81	~49	~65
% of TFs from families present in two of the three kingdoms	~2	~5	~4	~3

Table legend:

[1] 2000
[2] Alonso et al., 2003
[3] Adams et al., 2000
[4] 1998
[5] Goffeau et al., 1996
[6] number estimated in this work

Although nearly half of all *Arabidopsis* TF genes are represented by ESTs only a small fraction (114 genes, so 5%) have been characterized functionally. Most of the TF genes were characterized through the traditional, forward genetic approach whereby genes are first defined by the mutant phenotype and then isolated. A detailed list of functionally-characterized TFs and the proposed functions for TF families are available elsewhere (Riechmann et al., 2000; Riechmann and Ratcliffe, 2000; Riechmann, 2002). There is still very little known about the modes of TF action that is on the genes that they regulate and on the mechanisms that they use to achieve that regulation. The combinatorial nature of transcriptional regulation also adds to the complexity of this research area.

1.8 Aims of this thesis

The broad aim of this thesis was to identify transcription factors and other regulatory proteins that control nitrogen acquisition and assimilation in *Arabidopsis thaliana*. Parallel reverse and forward genetics approaches were taken to achieve this goal. More specifically, the aims of the reverse genetic approach were:

- To develop a novel resource, based on real-time RT-PCR, for rapid and robust expression profiling of all *Arabidopsis* transcription factor genes,
- To test the qRT-PCR resource for its precision, accuracy and robustness,
- To use that resource to identify a set of nitrogen regulated TF genes,
- To select TF genes regulated specifically by nitrogen and characterize their responses to various N-sources in greater details, and characterize them functionally using reverse genetics

The aims of the forward genetics approach were as follows:

- To establish a suitable screening system for promoter activity of the high affinity nitrate transporter *ATNRT2.1,* using firefly *Luciferase* as a reporter gene,
- To EMS mutagenise transgenic plants carrying the promoter-reporter construct,
- To identify mutant lines impaired in N-regulation of *ATNRT2.1* promoter activity,
- To confirm the lack of N-regulation for the endogenous *ATNRT2.1* gene, and other N-regulated genes,
- To cluster mutant lines for complementation crosses, according to the expression of other genes involved in nitrogen and carbon metabolism.

2. MATERIALS AND METHODS

2.1 Commonly used equipment, kits and consumables

2.1.1 Equipment

Amerscham Pharmacia Biotech, Life Chalfont, UK; DynaQuant™ 200 fluorimeter,

Applied Biosystems, Foster City, USA; 2 X ABI Prism 7900HT and 7300 real-time PCR systems,

Agilent Technologies, Waldbronn, Germany; Agilent 2100 BioAnalyser and RNA 6000 Nano Chips,

Beckman Instruments Inc., Fullerton, USA; Avanti J30I centrifuge,

Biometra, Göttingen, Germany; UNO II PCR and T Gradient machines,

Bio-Rad, Richmond, USA: Gene Pulser II, Smart Spec™3000,

Eppendorf, Hamburg, Germany; Microcentrifuges: 5417, 5417C, 5417R, Megefuge 5810R, BioPhotometer,

Fuji, Dusseldorf, Germany; BAS 1500 BioImaging Analyser, BAS cassettes 2040,

Hammamatsu Photonics Deutschland, Herrsching, Germany **;** Ultra high sensitive CCD camera C2400-30H and HPD-LIS luminescenece imaging software,

ICN, Irvine, USA; α-^{32}P-dCTP,

Leica, Heidelberg, Germany, DC300 microscope,

Retsch, Haan, Germany**;** MM200 homogeniser,

Sorvall, Langenselbold, Germany **;** centrifuge RC5B Plus,

Stratagene, Heidelberg, Germany **;** UV-crosslinker,

NanoDrop, Wilmington, USA**;** NanoDrop ND-1000 spectrophotometer,

2.1.2 Consumables

AB Gene, Hamburg, Germany **;** 96 well PCR plates, adhesive PCR seals,

Ambion, Huntingdon, Cambridgeshire, UK**;** Turbo DNA-*free*™ DNase, mMESSAGE kit with T7 RNA polymerase, RNA ladder 6100 for RNA 6000 Nano Chips,

Applied Biosystems, Foster City, USA; SYBR Green PCR mix, 384 well plates and adhesive covers,

Eurogentec, Seraing, Belgium; 96 well PCR plates optical grade with caps, Smart™ DNA ladder, oligonucleotides,

MWG, Ebersberg, Germany; oligonucleotides,

Merck, Darmstadt, Germany; other chemicals,

New England Biolabs, Beverly, USA; Restriction nucleases and restriction buffers,

Novagene, Houston, USA; *Kod* DNA polymerase,

Schleicher & Schuell, Dassel, Germany; Nytran™ super charge nylon mambrane

Sigma Aldrich, Taufkirchen, Germany; RNase free DNase I, Ethyl Methane Sulphonate, Ethidiumbromide, Diethylpyrocarbonate (DEPC), other chemicals,

Stratagene, Heidelberg, Germany**;** *Pfu* DNA polymerase,

Roth, Karlsruhe, Germany **;** other chemicals,

2.1.3 Kits

Amerscham Pharmacia Biotech, Life Chalfont, UK; RediPrime™ labeling kit,

Invitrogen, Karlsruhe, Germany; TRIzol™ reagent, Superscript™III reverse transcriptase, *Taq* polymerase, 10bp and 50bp DNA ladder , BP clonase ™ Mix, LR clonase ™ Mix, oligonucleotides

Machery-Nagel, Düren, Germany; Porablot™ NY amp nylon membrane, Nucleobond AX plasmid purification kit,

Qiagen, Hilden, Germany; Oligotex mRNA Mini Kit, Qiaquick Plasmid Mini Prep Kit, Gel Extraction Kit, Oligo dT primer,

Roche Applied Science, Hague Road, USA; DIG-labeling system, Restriction nucleases and restriction buffers, antibiotics

2.2 Plant material

Arabidopsis thaliana (L.) ecotype Col-O

Arabidopsis thaliana (L.) ecotype C24

Arabidopsis thaliana (L.) ecotype Nössen

2.3 Plant growth media and conditions

2.3.1 Sterile liquid cultures

Table 2. 1 Sterile full nutrition and low-nitrate medium composition

Full nutrition (FN)		**Low nitrate (-N)**	
Compound	Final [mM]	Compound	Final [mM]
KNO_3	2	KNO_3	0.1
NH_4NO_3	1	NH_4NO_3	0.05
Glutamine	1	Glutamine	0
KCl	0	KCl	3
KH_2PO_4/K_2HPO_4 (pH 5.8)	3	KH_2PO_4/K_2HPO_4 (pH 5.8)	3
$CaCl_2$	4	$CaCl_2$	4
$MgSO_4$	1	$MgSO_4$	1
K_2SO_4	2	K_2SO_4	2
MES (pH5.8)	3	MES (pH5.8)	3
Microelements	1x *	Microelements	1x *
Sucrose	*0,5%*	*Sucrose*	0.5%

***** - Microelements: 40µM $Na_2FeEDTA$, 60µM H_3BO_3,14µM $MnSO_4$, 1µM $ZnSO_4$, 0.6µM $CuSO_4$, 0.4µM $NiCl_2$, 0.3µM $HMoO_4$, 20nM $CoCl_2$

Wild-type Col-0 seedlings (100-120 seeds) were grown in 30 ml of sterile liquid FN medium (250 ml Erlenmeyer glass flasks) on orbital shakers with constant, uniform fluorescent light (~50 µE in the flask) and temperature (22°C). Shaker speed was low (30 rpm) during the first three days, and then increased to 80 rpm. Care was taken to prevent significant clumping of seedlings. After seven days the media was replaced with fresh 30 ml FN medium or low N medium (-N). FN medium was again changed on day 8 in FN cultures to prevent N-limitation, because by this stage N was rapidly depleted by the growing seedlings. The –N medium contained 0.2 mM inorganic N to minimize variation between flasks due to different amounts of the original medium being left in the flask, and was completely exhausted within hours, assuring N-deprivation after two days. For glutamine deprivation (-Gln), the medium contained 2 mM KNO_3 and 1 mM NH_4NO_3 but no glutamine. On day 9 FN cultures and some of the –N cultures were harvested. At the same time all other flasks of N-starved cultures were opened, and re-closed either without addition or after addition of 180 µl 500 mM KNO_3 (3 mM final concentration) or 180 µl 500 mM KCl (3 mM, control). The Gln induced flasks obtained 1mM Gln (final concentration) at this stage. Added liquid was allowed to disperse without changing the shaking speed. Groups of –N flasks that received no addition, KNO3, KCl or Gln were harvested after 30 min and 3 hr. Plant materials from each flask were quickly (<10 sec for the entire procedure) blotted on tissue paper, washed twice in an excess of deionised water, blotted on tissue paper again and frozen in

liquid nitrogen (LN_2). Materials were stored in (LN_2) until pulverization using mortar and pestle. Ground material was stored at –80° C until further use.

2.3.2 Growth on agar plates

To imbibe *Arabidopsis* (Col-0) wild-type seeds, they were kept in 0.15% agar in darkness of 4° C for 3-4 days. Plants were then grown vertically, on half-strength Murashige and Skoog medium (Murashige and Skoog, 1962), supplemented with 0.5% (w/v) sucrose and solidified with 0.7% agar at 22°C under a 16 h day (140 µmol m^{-2} s^{-1}) - 8h night regime. For experiments with plants expressing *LUC* reporter gene, all nitrogen-sources in MS medium were replaced with specified organic and inorganic N-source, as described in the Results section. Plants were grown under the same temperature and light conditions as described above. Plant material was harvested 14 days after germination, ground and frozen as described above.

For the root architecture studies the following medium was used to grow plants:

Table 2. 2 Medium used for root architecture studies on agar plates

	0.2 NO_3^-	**1 NO_3^-**	**1 NO_3^- - Gln**	**6 NO_3^-**	**6 NO_3^- -Gln**	**35 NO_3^-**	**1 NO_3^- + 34 KCl**
Compound	**Final concentration [mM]**						
KNO_3	0,2	1	1	6	6	35	1
$MgSO_4$	3	3	3	3	3	3	3
KCl	5	5	5	5	5	0	34
KH_2PO_4/K_2HPO_4 pH 5.8	3	3	3	3	3	3	3
$CaCl_2$	2	2	2	2	2	2	2
NaFeEDTA	8 mg/L	8 mg/L	8 mg/L	8 mg/L	8 mg/L	8 mg/L	8 mg/L
Microelements*	1x	1x	1x	1x	1x	1x	1x
MES	3	3	3	3	3	3	3
Select agar	16 g	16 g	16 g	16 g	16 g	16 g	16 g
Glutamine	4	4	0	4	0	4	4
Sucrose	0.5%	0.5%	0.5%	0.5%	0.5%	0.5%	0.5%

* - Microelements: 75µM H_3BO_3, 17.5µM $MnSO_4$, 1.25 µM $ZnSO_4$, 0.75 µM $CuSO_4$, 0.5 µM $NiCl_2$, 0.375 µM Na_2MoO_4, 25nM $CoCl_2$

Seeds were surface sterilized and stratified on 0.15% agar for 4 days. About 15-20 seeds were sown on girded square plates (10cmx10cm) containing one of 7 different nitrogen concentrations. At least 3 OX lines and all available homozygous knock out (KO) lines were grown in parallel to the WT, on separated plates under a 12h day / 12h night regime in a

phytotron. Germination ratio (number of seeds with emerged root) was counted for OX, KO and the WT after 48 h and 96 h. After 4 days, plates were opened and the most similar looking 6 plants were selected (one plant per 1cm of the grid) and the rest was discarded. Antibiotic sensitive plants were also discarded. The bottom of each plate was covered with Parafilm™ (Pechiney Plastic Packaging, Chicago, USA), to prevent contamination from the water which condensed at the bottom of the lid. The other sides of the plate were wrapped with paper tape. Plants were grown for 16 days in total (counting from sowing on the plate). Primary root length was checked every second day. Number of the lateral roots was checked on day 16.

2.3.3 Growth on soil

Plate-grown, 14-days-old *Arabidopsis* seedlings were picked to GS90 soil: vermiculite mixture (1:1 v/v) (Fritz Kausek GmbH & Co. KG, Mittenwalde, Germnay) and grown in the glasshouse under the following conditions: 16h day with 21^0C and 8h night with 17^0C, relative humidity 50%. Plants were watered with tap-water every second day for six weeks. Plants were harvested into paper bags and dried for 2 weeks to obtain seeds. Seeds were harvested in 2 mL screw-cup glass vials and stored at 14^0 C and 10% relative humidity.

2.4 RNA isolation procedures

2.4.1 TRIzol maxi-prep protocol

Total ribonucleic acid (RNA) was isolated from shoots or roots using TRIZOL reagent (Invitrogen), as previously described (http://www.*Arabidopsis*.org/info/2010_projects/comp_proj/AFGC/RevisedAFGC/site2Rna; Chomczynski and Sacchi, 1987). RNA concentration was estimated by measuring A_{260} in a spectrophotometer (Eppendorf) and applying the formula: [RNA] = A_{260} x D x 40µg/µL, where D is the RNA dilution factor. RNA quality was judged from $A_{260/280}$ (ratio 1.8-2.0, indicates low protein contamination) and $A_{260/230}$ (ratio ≥ 2.0, indicating low polysaccharide contamination). To remove all traces of DNA contamination, 150 µg of total RNA was digested with Dnase I RNase-free (Sigma), according to the manufacturer's instructions. Absence of genomic DNA contamination was subsequently confirmed by PCR, using primers

designed on an intron sequence of a control gene: At5g65080 (primers sequences in Appendix B). RNA integrity was checked on a 1.5% (w/v) agarose gel both prior to, and after DNaseI digestion. Poly-A^+ RNA was purified with an Oligotex mRNA Mini Kit (Quiagen) using the supplier's batch protocol.

2.4.2 RNA extraction using TRIzol mini-prep protocol

Frozen plant material (100-200 mg) was ground in LN_2 in pre-cooled mortar or homogenised using metal beads (Ø 5mm) in the MM200 homogeniser (Retsch). TRIzol reagent (Invitrogen) was then added and mixed well by vortexing. After 5 min incubation at room temperature the homogenate was centrifuged at 13.000 x g for 5 min at 4°C in pre-cooled centrifuge. The supernatant was removed to a fresh Eppendorf tube and 400µl of chloroform was added and mixed by vortexing before incubation at RT for 5 min. After 10 min centrifugation at 13.000 x g, at 4^0C, the aqueous phase (~1 mL) was transferred to a fresh Eppendorf tube. RNA was precipitated with 0.5 ml of isopropanol and 0.5 mL of HSS buffer (0.8 M Sodium Citrate and 1.2 M NaCl) per 1 mL of aqueous phase, overnight at – 20^0 C. The precipitate was pelleted by centrifugation at 13.000 rpm for 30 minutes at 4 °C. The supernatant was removed and the pellet was washed twice with 1 ml 70% EtOH, air dried, and re-suspended in ca. 50 µl water (approx. 1µg RNA/ 1µL). RNA amount and purity was determined by photometric measurements at 230, 260 and 280nm (see above).

2.5 cDNA synthesis

Reverse transcription reactions were performed using 500 ng of poly-A^+ RNA or 5µg of total RNA, with SuperScript™ III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The efficiency of cDNA synthesis was assessed by real-time PCR amplification of control genes encoding *Ubiquitin*10 (primers sequences in Appendix B) and *GAPDH* (primers sequences in Appendix B). Only cDNA preparations that yielded similar C_T values (e.g. 20±1) for the control genes were used for subsequent comparison.

2.6 Real-time PCR primer design

Putative TF genes were identified in the *Arabidopsis* genome by taking advantage of gene annotations and INTERPRO domain searches (Riechmann and Ratcliffe, 2000) at the MIPS (http://mips.gsf.de/cgi-bin/proj/thal/) TAIR (http://www.arabidopsis.org) and AGRIS (http://arabidopsis.med.ohio-state.edu/AtTFDB/) databases. The resulting set of sequences was supplemented by performing BLASTP and TBLASTN searches (http://www.ncbi.nlm.nih.gov/blast/), to uncover further possible TF genes in the *Arabidopsis* genome. To facilitate RT-PCR measurement of transcripts of all putative TF genes under a standard set of reaction conditions, oligonucleotide primers were required to meet a stringent set of criteria as outlined in the beginning of the Results section. Primers were designed according to these criteria by Dr. Jacqueline Weber-Lehmann at MWG Biotech AG (Ebersberg, Germany) using the Prime™ program of GCG® Wisconsin Package™ Version 10.2 (Madison, WI). Global alignments of tentative primer sequences with genomic and transcript sequences of all *Arabidopsis* genes were performed using the Smith-Waterman nucleotide (swn) search algorithm in BioView Toolkit (BTK) Software Version 5.0, (Paracel, Pasadena CA, USA). Assessment and choice of primer pairs was realized with PERL scripts specifically designed for our purposes at MWG Biotech AG. The sequences of each primer pair are given in Czechowski et al. (2004). All other qPCR primers were designed using Primer Express 2.0 software (Applied Biosystems) with the following parameters: melting temperatures (Tm) of 60±2 C, primer lengths of 20-24 nucleotides, guanine-cytosine (GC) contents of 45-55%, and PCR amplicon lengths of 60-150 base pairs. In addition, when possible at least one primer of a pair was designed to cover an exon-exon junction, according to the gene structure models at TAIR (http://www.arabidopsis.org). Where possible, primers were designed close (no more than 500 bp) from the 3' end of longest gene transcript annotated in TAIR (www.arabidopsis.org) and primer sequences were blasted against the *Arabidopsis* genome sequence using TAIR BLAST (http://www.arabidopsis.org/Blast/) with standard parameters to check their specificity.

2.7 Real–time PCR conditions and analysis

PCR reactions were performed in an optical 384-well plate with an ABI PRISM® 7900 HT Sequence Detection System (Applied Biosystems), using SYBR® Green to monitor dsDNA synthesis. Reactions contained 5 µL 2X SYBR® Green Master Mix reagent (Applied Biosystems), 1.0 ng cDNA and 200 nM of each gene-specific primer in a final volume of 10

µL. A master mix of sufficient cDNA and 2X SYBR® Green reagent was prepared prior to dispensing into individual wells, to reduce pipetting errors and ensure that each reaction contained an equal amount of cDNA. An electronic MultiPro™ Pipette (Eppendorf) was used to pipette the cDNA-containing master mix, while primers were aliquoted with an Eppendorf 12-channel pipette. Reactions were also scaled-down to 6 µL, containing 3 µL of 2X SYBR® Green Master Mix reagent (Applied Biosystems), 1µL of cDNA and 2 µL of each gene-specific primer (200 nM final concentration of each primer). An electronic MultiPro™ (5-100 µL) Pipette (Eppendorf) was used to pipette 5µL of the primer-containing master mix and electronic Eppendorf Multipipette (0.5-10µL) was used to pipette 1µL of cDNA template. The following standard thermal profile was used for all PCR reactions: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 sec and 60°C for 1 min. Data were analysed using the SDS 2.1 software (Applied Biosystems). To generate a baseline-subtracted plot of the logarithmic increase in fluorescence signal (ΔR_n) versus cycle number, baseline data were collected between cycles 3 and 15. All amplification plots were analysed with an R_n threshold of 0.3 to obtain C_T (threshold cycle) values. In order to compare data from different PCR runs or cDNA samples, C_T values for all TF genes were normalised to the C_T value of *ubiquitin10*, which was the most constant of the five house-keeping genes (*actin*2, *Ubiquitin10, β-6-tubulin, elongation factor 1 alpha, adenosyl-phosphoribosyltransferase*) included in each PCR run. The average C_T value for *ubiquitin*10 was 20.04 (+/- 0.89) for all plates/templates measured in this series of experiments. PCR efficiency (E) was estimated in two ways. The first method of calculating efficiency utilised template dilutions and the equation $(1+E) = 10^{(-1/slope)}$, as described previously (Pfaffl et al., 2001). The second method made use of data obtained from the exponential phase of each individual amplification plot and the equation $(1+E) = 10^{slope}$ (Ramakers et al., 2003). TF gene expression was normalised to that of *ubiquitin*10 by subtracting the C_T value of ubiquitin-10 from the C_T value of the TF gene of interest. Expression ratios of sample A to sample B were then obtained from the equation $(1+E)^{-\Delta\Delta CT}$ where $\Delta\Delta C_T$ represents ΔC_{TA} minus ΔC_{TB}, and E is the PCR reaction efficiency. Dissociation curves of the PCR products were analysed using SDS 2.1 software. Additionally, all RT-PCR products were resolved on 4% (w/v) agarose gels (3:1 HR agarose, Amresco, Solon, OH) run at 4 V cm^{-1} in TBE buffer, along with a 50 bp DNA-standard ladder (Invitrogen GmbH). Some of the PCR reactions were also sequenced, using real-time PCR primers.

2.8 Northern blotting

2.8.1 RNA electrophoresis and transfer according to Roche manual

4 µg of total RNA prepared using TRIzol miniprep protocol, was separated by gel electrophoresis under denaturing conditions (Lehrach et al., 1977). Gels contained 1.5% agarose and 2% formaldehyde. RNA was transferred to positively charged nylon membranes (Schleicher&Schuell) as described for Southern blotting and fixed using a UV-Transluminator, the wavelength 302 nm, for 4 min.

2.8.2 Probe labelling with Dioxygenin-11-dUTP

Probes were labelled during PCR-amplification of 200 pg plasmid DNA using gene specific primers. The PCR mixture contained all nucleotides at a concentration of 100 µM plus 17.5 µM Dioxygenin-11-2'-deoxy-Uridine-5'- Triphosphate, alkaline labile (Roche) and 82.5 µM dTTP. The following PCR program was used:

1 cycle of 95^0 C for 1min.,
30 cycles of: 95^0 C for 1min.; T_m of the primers (calculated from the formula: 2 x GC + 4 x AT), for 1min., 72^0 C for 1min.,
1 cycle of 72^0 C for 5 min.

Labelling efficiency was checked following agarose gel electrophoresis by monitoring the shift to larger size of the Dig-labelled DNA band, compared to the control PCR reactions (without Dioxygenin-11-dUTP). Probes were used for hybridisation at a concentration of 2 µl/ml of Dig Easy Hyb solution, as recommended in Roche manual.

2.8.3 Pre hybridisation and hybridisation conditions

Filters were pre-hybridized for 30 min. at 50^0 C in pre-warmed Dig Easy Hyb solution in hybridization tubes. PCR-Dig labelled probe (see above) was diluted in 50 µl of ddH2O and denatured at 95^0C for 5 min. The probe was then immediately chilled on ice and added to fresh pre-warmed (50^0 C) Dig Easy Hyb solution. The pre-hybridization solution was then

replaced with 3.5 ml hybridization solution per 100 cm^2 membrane, containing the probe, and hybridization was performed overnight at 50^0 C. Afterwards, hybridization solution was decanted and stored in -20^0C (stable for one year). The blot was washed twice in low stringency buffer (2X SSC; 0.1% SDS) for 5 min at room temperature, then twice in pre-warmed, high stringency buffer (0.5% SSC; 0.1% SDS) for 15 min at 50^0C.

2.8.4 Detection

Blots were washed in 250 mL maleic acid buffer (0.1M Maleic acid; 0.15 M NaCl; pH 7.5; 0.3 % Tween 20) for 2 min at RT, than blocked 1 time in 250 mL of Blocking solution (Roche) for 30 min at RT. 20 mL of antibody solution (diluted 1:15000 in blocking solution) was then added and the membrane incubated at RT for 30 min. The membrane was washed twice for 15 min in maleic acid buffer and equilibrated with 20 mL of detection buffer (0.1 M Tris-HCl pH 9.5; 0.15 M NaCl) for 3 min then dried briefly. The membrane was placed (DNA/RNA side facing up) in a plastic bag and 500µl / 100 cm^2 drops of CDP-*Star* (Roche) were evenly applied on to the surface of the blot. The plastic bag was laid for 5 min., excess of the liquid was squeezed out, bag was sealed and the membrane was incubated in RT for 1h. Chemiluminescent signal was detected using an Ultra Sensitive CCD Camera (Hammamatsu Photonics) with 10 min to 2 h acquisition time in the "dynamic" mode for photon acquisition. The camera sensitivity was 255 and threshold for background subtraction was 30. Images were analysed using HPD-LIS luminescence imaging software (Hammamatsu Photonics).

2.9 DNA isolation

For Southern blots, DNA was extracted from a single rosette leaf, inflorescence or young seedlings, using the CTAB method as described previously (http://carnegiedpb.stanford.edu/methods/ppsuppl.html). For PCR-based screening of homozygous SALK knockout lines, the alkaline lysis method was used (Lukowitz et al., 2000; Klimyuk et al., 1993)

2.10 DNA cloning and sequence analysis

Cloning of PCR- amplified DNA fragments was performed using GATEWAY™ technology, according to the manufacturer's instructions (GATEWAY cloning manual,

Invitrogen). Restriction maps and descriptions of the vectors used for cloning are presented in Appendix A. Gateway® Technology is based on the bacteriophage lambda site-specific recombination system which facilitates the integration of lambda DNA into the *E. coli* chromosome and the switch between the lytic and lysogenic pathways (Ptashne, 1986). Lambda-based recombination involves two major components: the DNA recombination sequences (*att* sites) and the proteins that mediate the recombination reaction (*i.e.* Clonase™ enzyme mix). Lambda integration into the *E. coli* chromosome occurs via intermolecular DNA recombination that is mediated by a mixture of lambda and *E. coli*-encoded recombination proteins (*i.e.* Clonase™ enzyme mix). Recombination occurs between specific attachment (*att*) sites on the interacting DNA molecules. Recombination is conservative (*i.e.* there is no net gain or loss of nucleotides) and requires no DNA synthesis. The DNA segments flanking the recombination sites are switched, such that after recombination, the *att* sites are hybrid sequences comprised of sequences donated by each parental vector. For example, *att*L sites are comprised of sequences from *att*B and *att*P sites. Two recombination reactions constitute the basis of the Gateway® Technology:

- **BP Reaction:** Facilitates recombination of an *att*B substrate (*att*B-PCR product or a linearised *att*B expression clone) with an *att*P substrate (donor vector) to create an *att*L-containing entry clone,
- **LR Reaction:** Facilitates recombination of an *att*L substrate (entry clone) with an *att*R substrate (destination vector) to create an *att*B-containing expression clone,

The template for PCR reaction was either cDNA obtained from RT reactions, cDNA clones (if available) or genomic DNA. Sequences of the primers used for GATEWAY™ cloning are shown in Appendix B.

A commonly used touch-down PCR program (Don et al., 1991) to generate amplicon for GATEAWAY™ cloning system using *Puff* high fidelity DNA polymerase and cDNA as a template was as follows:

1 cycle of 95^0 C for 3 min.

2 cycles of 95^0 C for 45 sec.; (T_m primers + 4) ^{0}C for 45 sec., 72^0 C for 3min.,

2 cycles of 95^0 C for 45 sec.; (T_m primers + 2)^{0}C for 45 sec., 72^0 C for 3-4min.,

2 cycles of 95^0 C for 45 sec.; (T_m primers)^{0}C for 45 sec., 72^0 C for 3-4min.,

36 cycles of 95^0 C for 45 sec.; (T_m primers – 2)^{0}C for 45 sec., 72^0 C for 3-4min.,

1 cycle of 72^0 C for 10min.

A commonly used touch-down PCR program (Don et al., 1991) to generate amplicons for GATEAWAY™ cloning system using *Kid* high fidelity DNA polymerase and genomic DNA as a template was as follows:

1 cycle of 98^0 C for 15 sec.,

2 cycles of 95^0 C for 15 sec.; (T_m primers + 4)^{0}C for 2 sec., 72^0 C for 20 sec.,

2 cycles of 95^0 C for 15 sec.; (T_m primers + 2)^{0}C for 2 sec., 72^0 C for 20 sec.,

2 cycles of 95^0 C for 15 sec.; (T_m primers)^{0}C for 2 sec., 72^0 C for 20 sec.,

34 cycles of 95^0 C for 15 sec.; (T_m primers – 2)^0 C for 2 sec., 72^0 C for 20 sec.,

1 cycle of 72^0C for 10 min.

Gel-purification of PCR product was performed after a successful amplification of the product of predicted size. Following PCR with gene-specific primers containing part of attB sequence, universal attB primers containing all of the attB sequence were used in a second round of PCR to generate amplicons suitable for GATEWAY™ BP reactions (primer sequences in the Appendix B). Gel-purified PCR product was used for BP reaction with pDONR207 (vector description in the Appendix A). The scheme of the cloning with BP clonase is represented below (Figure 2.1).

The second approach to clone PCR product into a GATEWAY™ entry vector used the TOPO cloning system (Invitrogen). One round of PCR amplification was performed using gene-specific primers (sequences in Appendix B). Forward primer always contained an additional 4 bp sequence: CACC, that is recognised by topoisomerase, and leads to insertion of PCR product into pENTR™/D-TOPO vector (vector description in the Appendix A), as described TOPO cloning manual (Invitrogen).

The scheme below (Figure 2.1 B) presents the last step of GATEWAY cloning, the LR reaction, during which the DNA sequence of interest is transferred to the destination vector. We used two vectors to overexpress transcription factor genes in *Arabidopsis*. One contained the 35S CaMV promoter for constitutive gene expression in vector pMDC32 created and kindly provided by Dr Mark Curtis from University of Zurich, Switzerland (Curtis and Grossniklaus, 2003) and the second incorporated the AlcA promoter system from *A. nidulans* (Caddick et al., 1998) for ethanol induced overexpression (pSRN-GW vector created and kindly provided by Dr Ben Trevaskis from MPI-MP, Golm). Features of both vectors are shown in the Appendix A

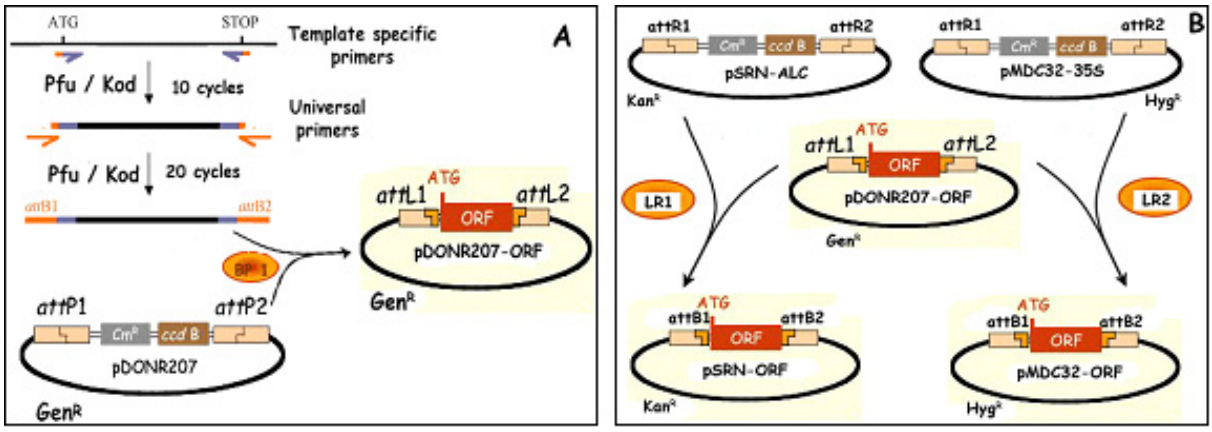


Figure 2. 1 Scheme of cloning TF genes, using GATEWAY™ technology

Abbreviations: attB1, attB2, attP1, attP2, attL1, attL2, attR1, attR2 – recombination sites, BP –reaction catalyzed by BP Clonase™, LR1 and LR2 – reactions catalyzed by LR Clonase™, Gen^R – gentamycin resistance, Hyg^R – hygromycin resistance, Kan^R – kanamycin resistance, *Pfu, KOD* – high fidelity DNA polymerases, all vectors described in Appendix A,

We routinely checked transformed bacterial colonies for the presence of the desired insert in the vector, using gene specific primers (sequence in Appendix B). Standard DNA manipulations like plasmid mini preparation, restriction digests, and gel electrophoresis were performed as described in Sambrook et al., 1989). For plasmid mini preparations and DNA gel purifications, commercially available kits were also used, according to the manufacturer's instructions (Qiagen). In all cases, positive ENTRY clones were sequenced using: M13 universal primers (pENTR™/D-TOPO) or ENTR207 primers (pDONR207). Additionally, for inserts longer than 1.5 kb, usually internal sequencing primers were designed or primers for KO screening were used (sequences in Appendix B). DNA sequencing was performed by AGOWA GmbH (Berlin, Germany) using Big Dye™ chemistry on a Perkin Elmer ABI 377 HT sequencer. Sequencing chromatograms were analysed using Chromas 1.45 software. Only the clones giving no sequence differences to the deposited in the TAIR database were chosen for the further cloning steps. Positive clones after LR reaction were routinely digested with *EcoRV* enzyme, to confirming that recombination events did not destroy the vector structure (vector structures are given in Appendix A). Only those clones showing a correct restriction pattern were used for transformation of *Agrobacterium tumefaciense* strain GVpmp90. Plasmid minipreps were performed from *A. tumefaciense* and the restriction pattern checked again using *EcoRV*. Only *A. tumefaciense* clones giving the same pattern as the corresponding *E.coli* clones were used for plant transformation.

2.11 PCR – based screening for homozygous knock-out (KO) lines

Two pairs of primers were used to identify homozygous KO lines: two gene specific primers unable to amplify product of expected size from homozygous KO and a gene specific primer plus a T-DNA specific primers (sequences in the Appendix B), that amplify DNA only from a KO lines but not from the WT. T-DNA specific primers were designed on the sequence of the vectors used to create the mutant lines. Genomic DNA from WT grown in parallel was always used as control in both PCR reactions. All gene-specific primers were designed using web-based software (http://signal.salk.edu/tdnaprimers.html) with the following parameters: optimal primers size - 21bp; optimal T_m - 65^0C; GC content between 20 and 80%; maximum distance from the insertion site – 10 bp. PCR was performed on DNA prepared by alkaline lysis as described above, using the combination of two gene specific priers or one gene specific primers and primer LbB1/LbB2 (sequences in Appendix B). PCR products were visualised by EtBr staining following electrophoresis on agarose gels.

The most commonly used touch down PCR program for PCR-based screening was:
1 cycle of 95^0 C for 45 sec.,
2 cycles of 94^0 C for 45 sec.; (T_m primers +4)^{0}C for 45 sec., 72^0 C for 1min.,
2 cycles of 94^0 C for 45 sec.; (T_m primers + 3)^{0}C for 45 sec., 72^0 C for 1min.,
24 cycles of 94^0 C for 45 sec.; (T_m primers -2)^{0}C for 45 sec., 72^0 C for 1min.,
1 cycle of 72^0 C for 10min..

2.12 Southern blotting

Three weeks old rosette leafs from about 20 antibiotic resistant plants, from each selected T2 line containing *LUC* reporter construct insertion, were collected for Southern blot analysis. Genomic DNA was extracted by the CTAB method, as described above, and 20 µg of DNA, digested with *BamHI*. Resulting DNA fragments were separated on a 0.7% agarose gel. The gel was then incubated for 10 min in 0.25 N HCl, twice for 15 min in denaturing solution (0.5 M NaOH, 1.5 M NaCl) and twice in neutralising buffer (1.5 M Tris-HCl pH 7.5, 1.5 M NaCl). DNA was transferred to nylon membranes (Machery-Nagel; Düren,) by means of capillary transfer using 20 x SSC as the buffer (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate). DNA was covalently linked to the membrane using an UV-crosslinker (Stratagene). Nylon membranes were prehybridised for at least 1 h at 65^0 C and hybridised overnight in 250 mM sodium phosphate buffer (pH 7.2) containing 7% (w/v) SDS, 1% (w/v) BSA and 1mM

EDTA. A 1.7 kb DNA fragment containing *LUC* gene, cut out from pZPXOmegaL+ vector by *BamHI* / *StuI* digestion was used for probe. Fragment was gel purified and labelled using the RediPrime random priming labelling kit (Amersham Pharmacia Biotech). Following hybridisation, membranes were washed twice in 2 x SSC, 1% (w/v) SDS for 30 min, twice in 0.2 x SSC, 1% (w/v) SDS for 30 min, and then subjected to autoradiography in BAS 2040 cassettes (Fuji) between intensifying screens for 24 h. Radioactive images were obtained using a BAS 1500 Bio Imaging Analyser (Fuji).

2.13 Transformations

2.13.1 Transformation of bacteria

Transformation of *Escherichia coli* strain DH5α was performed using a heat shock method, as described previously (Hanahan, 1983). *Agrobacterium tumefaciens* strain GV3101.pMP90 was transformed by electroporation with a Gene Pulser II, according to the manufacturer's instruction. *E .coli* strains were grown in LB media (Sambrook et al., 1989) while *Agrobacterium tumefaciense* strains were grown in YEB medium (Vervliet et al., 1975). For growth on solid media, 1.5% agar was added. Filter-sterilised antibiotics were added at the following concentrations: Kanamycin, 50µg/mL; Gentamycin, 125µg/mL; Rifampicin, 100µg/mL.

2.13.2 Plant transformations

Transformation of *Arabidopis thaliana* Col-0 with *Agrobacterium tumefaciens* was performed using the floral dip method (Clough and Bent, 1998).

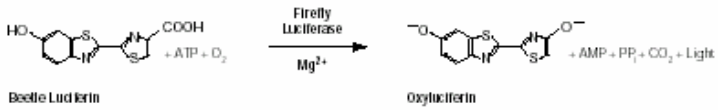
2.14 Selection of TF over-expressing plants and EtOH induction experiments

Antibiotic selection was used to select transgenic plants harbouring T-DNA containing TF gene constructs. Hygromycin (50µg/mL) was used to select plants carrying pMDC32 constructs and Kanamycin (50µg/ml) for the pSRN-GW constructs. About 300-500 T0 seeds were grown horizontally on ½ MS with 0.5% sucrose (round Petri plates; ø 15 cm) in a phytotron under 12h day / 12h night conditions at 22^0C. After one week, resistant plants were

transferred to fresh plates to avoid bacterial and fungal contamination and grown for another week. About 10 antibiotic resistant plants carrying pMDC32 constructs and about 15 plants carrying pSRN-GW construct were transferred to soil and grown in the greenhouse for seed production. Col-O of the same age was always grown in parallel and used as a control. When plants were about 4-6 weeks old, the material was harvested for Northern blot screening. About 3-4 young inflorescences and 3-4 cauline leaves from constitutive over-expressors were harvested and frozen in LN_2. To screen the EtOH inducible lines harbouring constructs in pSRN-GW vector, we performed induction experiments in following way. About 3-4 inflorescences and the same amount of cauline leaves were harvested into 6 well plates (Machery Nagel). From each transgenic, one-month-old plant, material was harvested twice; one it was put in 5 mL tap water as a negative control, and the second time in 5 mL of 3% (v/v) ethanol solution. Both – negative control and induced samples were incubated at room temperature for 6 h, with gentle shaking (20-30 rpm). All plates were covered with Parafilm™ (Pechiney Plastic Packaging, Chicago, USA) to prevent evaporation. After 6 h, plant material was transferred to 2 mL Eppendorf tubes and frozen immediately in liquid N_2.

2.15 Luciferase assay

When transgenic plant-expressing firefly *Luciferase (LUC)* are sprayed with D-luciferin, LUC catalyses the adenylation of D-luciferin to produce D-luciferin adenylate (using ATP produced within the cell) which reacts with molecular oxygen to form oxyluciferin. This photon-emitting reaction is summarised below:



The resulting bioluminescence can be monitored by a photon imaging camera (Xiong et al., 2001)

A 1.7 kb fragment 5' of the start codon of the *ATNRT2-1* gene was amplified by PCR, sequenced and cloned at the 5' end of the *LUC* gene of binary vector pZPXOmegaL+ (*HindIII* restriction site), to serve as a promoter to drive expression of the *LUC* gene. Vector restriction map is presented in Appendix A. The cloning steps and plant transformation was done by Dr. Georg Leggewie and Katrin Piepenburg. Seeds from transformed plants (T1 generation) of the transformed plants was screened for resistance to gentamycin, on plates with ½ MS, 1% sucrose and 125 μg/ml gentamycin. Only resistant plants showing LUC

activity (as described above) were grown for seeds. LUC activity was detected, using 1mM D-luciferin (sodium salt, Promega) as a substrate, according to Chinnusamy et al., 2002. Bioluminescence imaging was performed on Ultra Sensitive CCD Camera (Hammamatsu Photonics) with 5 min. acquisition time in the "static" mode for photon acquisition. The camera sensitivity was 255 and threshold for background subtraction was 30. Images were analyzed using HPD-LIS luminescence imaging software (Hammamatsu Photonics).

To test the influence of various N-sources on *LUC* activity, seeds from the selected lines were grown vertically on ½ MS medium, containing 0.5% sucrose and solidified with agar. All nitrogen sources from the ½ MS medium (Murashige and Skoog, 1962) were replaced by amino acid, potassium nitrate or ammonium chlorate. Plants were grown for 10 days under long day conditions in a growth chamber and *LUC* activity assay was performed as described in above.

2.16 EMS mutagenesis

Dry *Arabidopsis* seeds were placed in Erlenmeyer flasks containing following concentrations of EMS (Sigma): 0.1% (v/v), 0.2% (v/v), 0.3% (v/v), 0.4% (v/v), and 0.5% (v/v), in 100 mL of bi-distilled water. The flasks were gently shaken (50 rpm) under fume hood over 12 hours at RT. Seeds were washed 15 times over the course of 3 hours by decanting the solution, adding fresh water, mixing, allowing the seeds to settle and decanting again. After about 8 washes the seeds were transferred to a new container and the original one was decontaminated. After washing, the seeds were suspended in 0.15% agar solution and immediately pipetted on to soil at about 1 seed per square cm. The number of germinated seeds was counted after 14 days.

2.17 Bioinformatics tools and computer analysis

Arabidopsis sequence comparisons were performed using the BLAST (http://www.arabidopsis.org/Blast/) or WU-BLAST (http://www.arabidopsis.org/wublast/index2.jsp) programs with the standard parameters. For alignment of two sequences the BLAST2 program (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html) was used with standard parameters. SALK and RIKEN *Arabidopsis* knock-out lines were identified using the T-DNA Express

tool (http://signal.salk.edu/cgi-bin/tdnaexpress). Putative TF Binding Sites / Cis-Elements were identified using the following databases: the PLANT Cis-acting regulatory DNA elements (PLACE, Higo et al., 1999); the object-oriented transcription factors database (ooTF, Ghosh, 2000), and the TRANSFAC database, using the TRES package (http://bioportal.bic.nus.edu.sg/tres/). DNA in *silico* restriction analysis were performed using NEB cutter 2 software (http://tools.neb.com/NEBcutter2/index.php). All analyses of sequence chromatograms were performed using Chromas 1.45 software. Data calculations and visualization was performed using Excel, Word (Microsoft Office 2003), and Sigma Plot 2000 programs. All photos were prepared using Adobe™ Photoshop 7.0

3. RESULTS

3.A. Identification of TF's involved in N-regulation – A reverse genetic approach

Nitrogen acquisition and assimilation in plants is regulated at many different levels, including at the level of transcription. Regulation of gene transcription involves transcription factors (TFs). To identify TF genes that may be involved in N-regulation, we begun with the assumption, that such TF genes may be regulated by N-supply and/or demand in plants. Two different technologies were used to identify such TFs: real time RT-PCR and Affymetrix arrays. All ATH1 array data are coming from experiments done by Molecular Genomics group led by Dr Wolf-Ruediger Scheible and were used for this work with his permiton. The first approach required the development of gene specific primers for real-time PCR of all *Arabidopsis* TFs, which is described in the next section, 3.A.1 are coming from cooperation with Dr Wolf-Ruediger Scheible and Rajendra Bari. Section 3.A.2 presents process of the selection of N-regulated TFs (work done in collaboration with Dr Wolf Ruediger Scheible and Dr Rosa Morcuende), while section 3.A.3 describes the physiological characterization of N-regulated TFs. Section 3.A.4 presents preliminary results of functional characterization of N-regulated TFs, using reverse genetics, all the results were achieved together with Dr Jens-Holger Dieterich and other collaborators from Molecular Genomics Group (MPI-MP, Golm, Germany) led by Dr. Wolf-Ruediger Scheible.

3.A.1 Development and testing of a resource for qRT-PCR profiling of *Arabidopsis* TF genes (in collaboration with Dr Wolf-Ruediger Scheible and Rajendra Bari from Molecular Genomics Group, MPI-MP, Golm, Germany)

3.A.1.1 PCR primer design and reaction specificity

At the start of this project approx. 1500 putative TF genes had been identified in *Arabidopsis* (). To enable real time RT-PCR analysis of all TF genes with maximum specificity and efficiency under a standard set of reaction conditions, a stringent set of criteria was used for primer design. This included predicted melting temperatures (Tm) of 60±2 C, primer lengths of 20-24 nucleotides, guanine-cytosine (GC) contents of 45-55%, and PCR amplicon lengths of 60-150 base pairs. In addition, when possible at least one primer of a pair was designed to cover an exon-exon junction, according to the gene structure models at MIPS

(http://mips.gsf.de) and/or TAIR (http://www.arabidopsis.org). This was the case for ~74% of all primer pairs. The specificity of PCR primers was tested using first strand cDNA derived from either plate-grown *Arabidopsis* seedling shoots or roots, or whole seedlings grown in axenic cultures. Total RNA was always treated with DNase I prior to purification of poly (A)+ RNA. Before proceeding with first-strand cDNA synthesis, complete degradation of genomic DNA in RNA preparations was confirmed by PCR analysis. All 1465 TF primer pairs produced initially were tested for their efficacy in amplifying the specific target cDNA from roots and shoots. For each tissue, a single pool of cDNA was used to seed all qRT-PCR reactions, each of which contained a unique pair of TF primers. Approximately 83% of all primer pairs produced a single DNA product of the expected size, as exemplified in Figure 3.1 A.

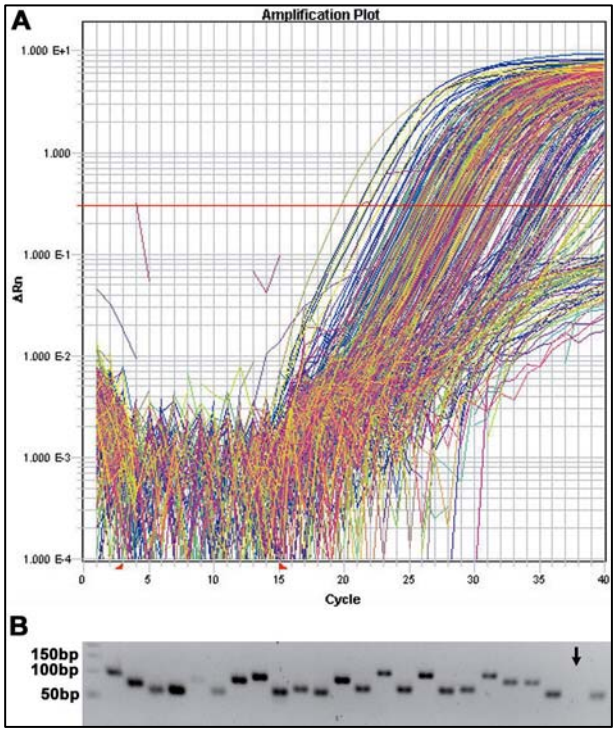


Figure 3. 1 Specificity of qRT-PCR

(A) Typical qRT-PCR amplification plots of 384 TF genes showing increase in SYBR® Green fluorescence (ΔRn, log scale) with PCR cycle number. Note the similar slope of most curves as they cross the fluorescence threshold of 0.3, which reflects similar amplification efficiencies. Note also the low proportion of amplification curves that do not cross the fluorescence threshold. Such reactions yield no detectable product when visualised on agarose gels.
(B) Separation of RT-PCR products on 4% (w/v) agarose gels revealed single products of the expected size for most reactions, with few reactions yielding no product (arrow). Size standards in base pairs (bp) are indicated at the left.

Only 4% of reactions yielded more than one PCR product. Thirteen percent (193) of reactions yielded no PCR product from root or shoot cDNA after 40 PCR cycles, indicating that the target genes were probably not expressed in these organs and growth conditions. Primer pairs for fifty-six of these genes were complementary to exon sequences only, which enabled us to check the primers on genomic DNA. Forty-four of these primer pairs were tested and all produced a unique PCR product of the expected size from genomic DNA. This result confirmed not only that the primers were effective, but also that the target genes were not expressed in plants under the conditions studied. The remaining 137 primer pairs

contained at least one primer spanning an intron, which prohibited a similar check of primer efficacy using genomic DNA. Nonetheless, the percentage (~71%) of intron-spanning primer pairs amongst those that failed to yield PCR amplicons in our experiments was not higher than the percentage of such primer pairs (~74%) that did yield specific amplicons. Therefore, failure to predict intron-splicing sites correctly probably does not account for failure to detect these transcripts/cDNA in our experiments.

Data from gel-electrophoresis analysis of the amplified PCR products (Figure 3.1B) were confirmed by melting curve analysis, which was performed by the PCR machine after cycle 40. A more stringent test of the specificity of PCR reactions was performed by sequencing the products of nine Myb/Myb-like genes (*AT3G01140*; *AT3G02940*; *AT3G61250*; *AT4G05100*; *AT5G02320*; *AT5G15310*; *AT5G16770*; *AT5G54230*; *AT5G65230*) and eight basic helix-loop-helix (bHLH)-type genes (*AT3G19860*; *AT3G56970*; *AT3G56980*; *AT5G08130*; *AT5G09750*; *AT5G10570*; *AT5G37800*; *AT5G46830*). Genes were chosen from these two families because each family contains many members (>100) with a high degree of sequence similarity in the DNA-binding domains. The chosen genes also exhibited a wide range (>10^3) of expression levels. In each case, the sequence of the PCR product matched that of the intended target cDNA, although primers were sometimes placed in conserved regions, confirming the exquisite specificity of the primer pairs.

3.A.1.2 Dynamic range, sensitivity and robustness of real-time PCR

The threshold cycle, C_T, is the cycle number at which SYBR® Green fluorescence (ΔRn) in a real-time PCR, reaches an arbitrary value during the exponential phase of DNA amplification (set at 0.3 in all of our experiments: see Figure 3.1A). For an ideal reaction, the number of dsDNA molecules doubles after each PCR cycle. In this case, a difference in C_T (C_T) of 1.0 indicates a 2-fold difference in the amount of DNA at the start of a reaction, a ΔC_T of 2.0 is equivalent to a four-fold difference, etc. Therefore, C_T is inversely proportional to the logarithm of the amount of target DNA present at the start of a PCR (Figure 3.2 A), or 2^{Ct} is inversely proportional to the amount of target DNA. To make data from qRT-PCR easier to understand, we often plot it as 2^{40-Ct}, which is directly proportional to target DNA amount. The number 40 above is somewhat arbitrary, but was chosen because PCR reactions are typically stopped at cycle 40.

The sensitivity and robustness of quantification by qRT-PCR were investigated in two ways. In the first approach, C_T was measured for a cloned *Luciferase* gene from the plasmid pZPXomegaL+ (see Appendix A) and an amplified 75bp intergenic DNA fragment (genetic marker ATC4H; www.*arabidopsis*.org) of *Arabidopsis*, which were diluted serially from 1 million copies to a single copy and added to a complex matrix of *Arabidopsis* root cDNA (1 ng or approximately 10^9 cDNA molecules). Amplification of the 60 base pair (bp) *Luciferase* gene fragment, using *Luciferase* specific primers (*LUC* -F 5'-ATTGTTCCAGGAACCAGGGC-3'; *LUC* -R 5'-GAACCGCTGGAGAGCAACTG-3') and the 75bp intergenic region resulted in C_T values of ~16 when 1 million copies of template DNA were introduced into reactions (Figure 3.2 A). An inverse linear relationship between the logarithm of copy number and C_T was observed down to 10 or 2 copies of the *LUC* gene and the intergenic fragment, respectively, reflecting a PCR efficiency of greater than 98% in both cases (Pfaffl, 2001). With fewer than ten copies of the *LUC* gene at the start of PCR, a non-specific product was amplified (not shown), which resulted in an effective detection limit of ten molecules in this case. The effective detection limit for the intergenic region was two copies; the template was undetectable in further dilutions, which can most easily be explained by a complete absence of the template in these reactions (Figure 3.2 A).

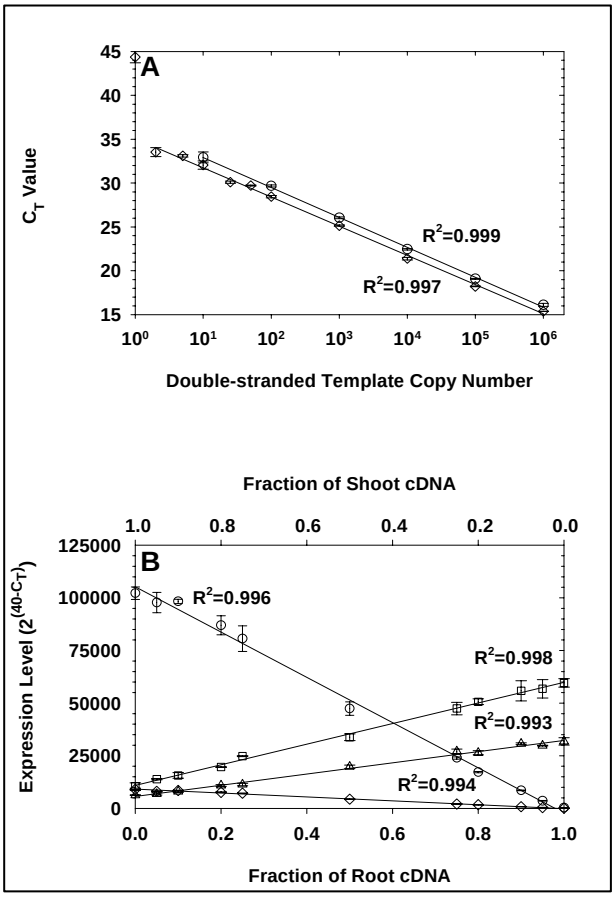


Figure 3. 2 Sensitivity and robustness of qRT-PCR

(A) Relationship between amplification kinetics (Ct) and copy number of a *Luciferase* gene (o) and an intergenic DNA fragment (◊) in reactions containing a complex pool of 1ng *Arabidopsis* cDNA.
(B) Relationship between the expression level, $2^{(40\text{-}Ct)}$, and the fraction of root or shoot cDNA in a mixture of the two totalling 1 ng, for the four genes At1g13300 (circle); At1g34670 (diamond); At4g32980 (triangle) and At5g44190 (square). Symbols in both panels represent the mean and standard deviation of three replicate measurements

Thus, we were able to detect as few as two double-stranded copies of a target gene within a complex mixture of 1ng cDNA. Assuming that the average length of an mRNA (cDNA molecule) is 1.3 kb (2000; Haas et al., 2002) and that the average number of transcripts per plant cell is $2x10^5$ (Kiper, 1979; Kamalay and Goldberg, 1980; Ruan et al., 1998), we estimate the detection limit of our system to be close to one transcript per 1000 cells, or 0.001 transcripts per cell.

The second approach to assess the sensitivity, robustness, and linearity of quantification by qRT-PCR involved mixing different amounts of root and shoot cDNA prior to determining C_T values for four root or shoot-specific genes in each mixture. Mixtures of root and shoot cDNA were made to give the following amounts (ng) of root cDNA in a total of 1 ng cDNA: 1.0; 0.95; 0.90; 0.80; 0.75; 0.50; 0.25; 0.20; 0.10; 0.05; and 0. Real-time PCR using 1ng cDNA was performed as described above, with primers for two shoot-specific (*AT1G13300*; *AT1G34670*) and two root-specific genes (*AT4G32980; AT5G44190*).

A linear relationship between $2^{(40-Ct)}$ and root/shoot cDNA amount was obtained for each gene over the whole range of mixtures (Figure 3.2 B), which showed that the precision of real-time PCR measurements is not influenced by the complex milieu of molecules present in typical PCR reactions.

3.A.2.3 Precision of real-time RT-PCR

The technical precision or reproducibility of qRT-PCR measurements was assessed by performing replicate measurements in separate PCR runs, using the same pool of cDNA (intra-assay variation; Figure 3.3 A) or two different pools of cDNA obtained independently from the same batch of total RNA (inter-assay variation; Figure 3.3 B)

Precision, as reflected by the correlation coefficient, was high in both cases, with the intra-assay variation (R^2= 0.9953) exceeding the inter-assay variation (R^2= 0.9571), as expected. As Affymetrix chips have become a 'gold-standard' for *Arabidopsis* transcriptome analysis, we were interested to compare the results of qRT-PCR measurements of TF transcript levels with corresponding data from 'whole-genome' chips. Using the same preparations of RNA that had been used for RT-PCR analysis, Affymetrix chips detected (called 'present' twice in at least one organ by Affymetrix software) less than 55% of the putative transcription factors listed in Czechowski et al., 2004, supplementary material. Inter-assay variation between replicate Affymetrix chips was greater than that of real-time RT-

PCR, which indicated a lower precision of the Affymetrix technology, especially for low-abundance transcripts (see Figure 3.3 C, D).

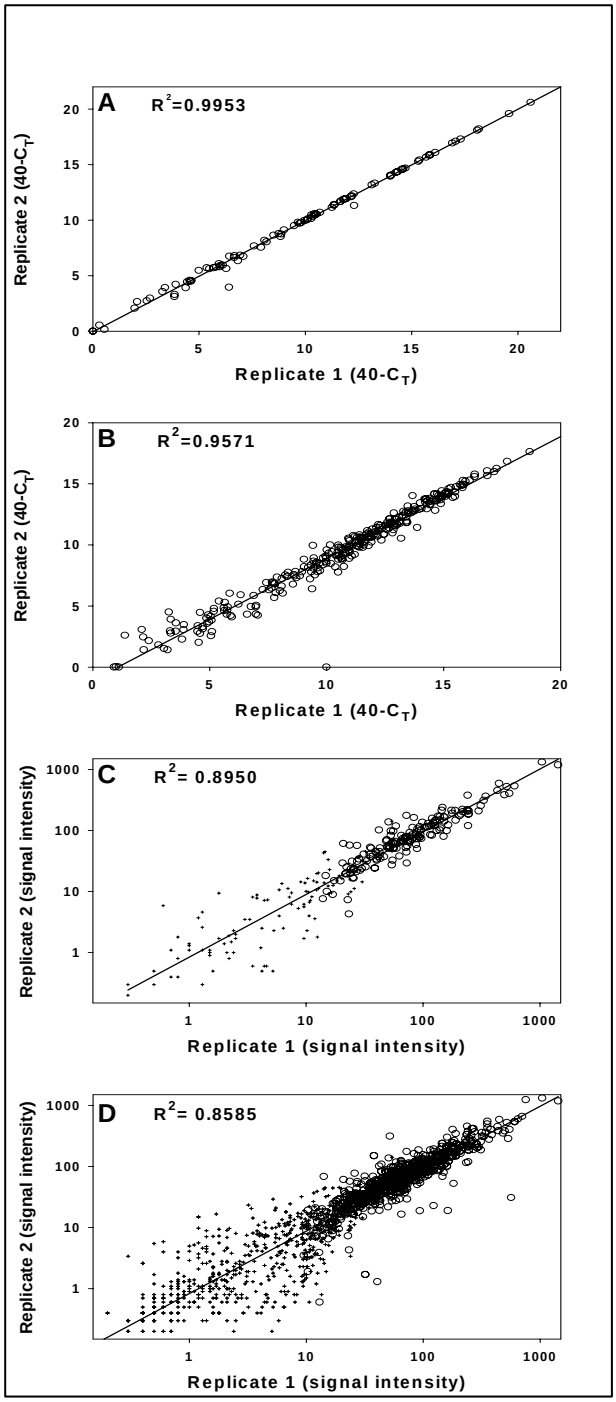


Figure 3. 3 Technical precision of qRT-PCR and Affymetrix full genome arrays

(A) Real-time RT-PCR was used to obtain duplicate measurements of TF cDNA levels from the same reverse transcription (RT) reaction, or **(B)** from two separate RT reactions. The same sample of total RNA from shoots was used throughout to preclude biological variation. Thus, (A) and (B) illustrate intra- and inter-assay technical variability, respectively. Two separate measurements of 101 genes (A) and 298 genes (B) are compared. **(C)** and **(B)** The Affymetrix full genome array (ATH1) was used to measure TF transcript levels via cRNA derived from two separate RT reaction products starting with the same RNA sample as for (A) and (B). **(C)** Inter-assay technical variability is illustrated for the 277 TF genes on the Affymetrix array that correspond to the 298 genes shown in panel (B). The 169 genes that were categorized 'present' in both replicates by the Affymetrix software are depicted as circles and those called 'absent' as crosshairs. A regression line and the corresponding correlation coefficient (R^2) is shown for the entire set of 277 genes. Inter-assay technical variability of all 1275 TF genes represented on the Affymetrix array is depicted in **(D)**.

3.A.1.4 Efficiency of PCR reactions

The number of cycles needed to reach a given fluorescence intensity depends not only on the amount of cDNA in the extract, but also on the amplification efficiency (E). In the ideal case, when the amount of cDNA is doubled in each reaction cycle, E=1. As mentioned above, PCR primers were designed to produce short amplicons, typically between 60-150 bp, to maximise E. While preliminary measurements (see Figure 3.1 A, for example) showed that efficiencies of virtually 100% were achieved in some reactions, we expected that a significant fraction of the 1465 TF-specific PCR reactions would have lower efficiency.

Different methods are available for estimating PCR efficiency (for a compilation see http://www.weihenstephan.de/gene-quantification/). The classical method uses C_T values obtained from a series of template dilutions, (e.g. Pfaffl et al., 2001). An alternative method utilises absolute fluorescence data captured during the exponential phase of amplification of each real-time PCR reaction (Ramakers et al., 2003). Comparison of the two methods yielded very similar amplification efficiencies for a sub-set of 46 TF primer pairs (data not shown). Hence, we used the latter method to establish amplification efficiencies for all 1465 primer pairs, since it does not require standard curves for every primer pair, and because it allows estimation of the efficiency for each individual PCR reaction.

The E value is derived from the log slope of the fluorescence vs. cycle number curve for a particular primer pair, using the equation $(1+E) = 10^{slope}$ (Ramakers et al., 2003). Inspection of Figure 3.1 A reveals that each PCR reaction shows a lag and then enters an exponential phase, which appears in the logarithmic plot as a linear increase. The positions of the lines are offset, reflecting the different amount of cDNA for each transcription factor. The slopes of the lines are, in most cases, very similar showing that E is similar for most of the primer pairs. However, a small subgroup with a lower slope can be distinguished. Of the 1465 primer pairs, 71 had E values >0.90, 402 between 0.90-0.81, 495 between 0.80-0.71, 244 between 0.70-0.61, 86 between 0.60-0.51 and 51 between 0.50-0.41. 116 primer pairs had E values ≤0.40, but *nota bene* they were barely or not at all detected in shoots or roots. Efficiency values were taken into account in all subsequent calculations, including calculations of the ratios of transcript levels in the shoot and root.

3.A.1.5 Comparison of technologies: qRT-PCR versus Affymetrix chips

We did not necessarily expect a good correlation between signals obtained for the levels of the individual transcripts by qRT-PCR and Affymetrix chips. Unlike quantitative RT-PCR, hybridisation-based technologies like Affymetrix chips are qualitative and there is not a strict linear relationship between signal strength and transcript amount for different genes (Holland, 2002). Nonetheless, genes determined to be highly expressed by qRT-PCR typically yielded high signals on Affymetrix chips. A large majority (90%) of the 503 genes that were categorised as ‘absent’ by Affymetrix software were detected by real-time PCR (see above) albeit at lower levels, as other TF genes, as expected. Overall, there was little

quantitative agreement between the two data sets for 1083 TF genes that were analysed from shoots (Figure 3.4) or roots (data not presented).

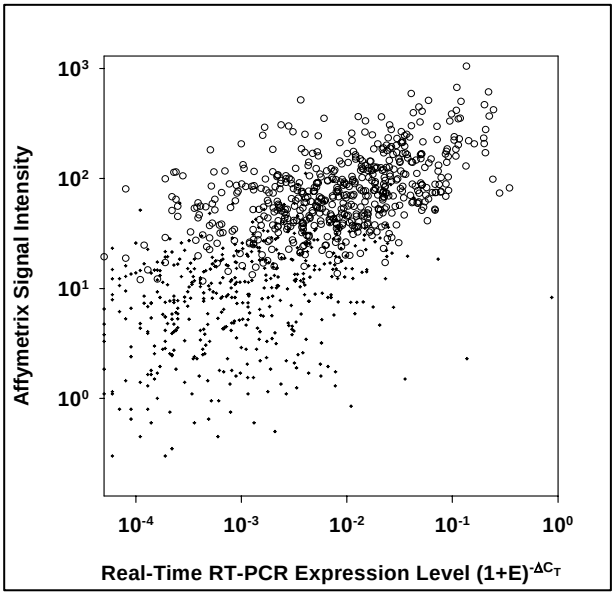


Figure 3.4 Comparison of shoot TF transcript levels measured by qRT-PCR and Affymetrix whole genome arrays

Normalised raw data from RT-PCR ($(1+E)^{-\Delta Ct}$) were compared to normalized raw data from Affymetrix chips (log_{10} of fluorescence signal) for the 1083 TF genes which were detectable in shoots on the qRT-PCR platform and also present on the Affymetrix ATH1 gene array. Genes categorised as 'present' or 'absent' by Affymetrix software are depicted as circles or crosshairs, respectively

3.A.1.6 Identification of root and shoot-specific TF genes by real-time RT-PCR

The qRT-PCR resource for TF transcript profiling was used to identify root and shoot-specific TF genes, to test its efficacy in identifying known organ-specific TFs, and to identify novel root- or shoot-specific TFs for future study. From amongst the 1214 TF gene transcripts that were detected by qRT-PCR in roots and shoots, 438 (36%) were differentially expressed (shoot/root ratio > or < 4; Figure 3.5).

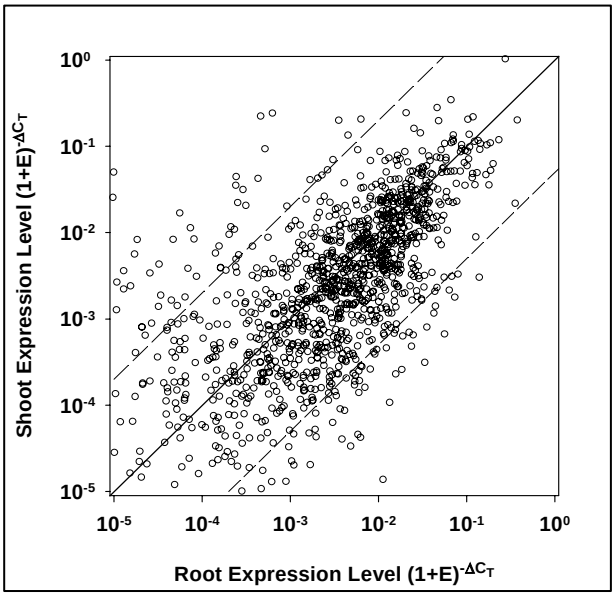


Figure 3. 5 Comparison of TF transcript levels in shoots and roots

Normalised expression values ($(1+E)^{-\Delta CT}$) from qRT-PCR amplification of cDNA from shoots and roots are compared for 1214 genes resulting in specific amplicons. Dashed lines indicate 20-fold differences in the shoot to root transcript levels.

Approximately 10.5% (127/1214) of the TF genes exhibited a greater than twenty-fold difference in expression level in shoots compared to roots (indicated by the dashed lines in Figure 3.5). We considered these as putative shoot- or root-specific genes. Many of these genes were not previously reported to be organ-specific, and several of the genes are not represented on the Affymetrix ATH1 array (Table 3.1).

Table 3. 1 Shoot-specific and root-specific TF genes identified by real-time RT-PCR

AGI	Gene Name	RT-PCR	ATH1	MPSS[c]		AGI	Gene Name	RT-PCR	ATH1	MPSS[c]	
		Shoot / Root	Shoot / Root	Shoot[d]	Root[d]			Root / Shoot	Root / Shoot	Shoot[d]	Root[d]
AT1G73870	COL7	8678[a]	101[b]	12	0	*AT4G37940*	AGL21	136	174[b]	0	19
AT4G00180	YAB3	4284[a]	6.8[b]	67	0	*AT5G06840*		106	5.1[b]	0	29
AT2G30420		3855[a]	6.9[b]	11	0	*AT4G13620*		89	N	0	47
AT2G45190	YAB1	1578[a]	147[b]	56	11	*AT5G15130*		86	45[b]	5	118
AT2G41940	ZFP8	1351	9.7[b]	89	2	*AT1G13300*		81	12	12	171
AT2G43010	PIF4	458	5.9	172	0	*AT2G22630*	AGL17	68	8.0[b]	N	N
AT1G68520	COL6	458	29	993	0	*AT4G10350*		65	2.6[b]	N	N
AT5G25390		451[a]	2.4[b]	0	0	*AT5G56960*		53[a]	N	N	N
AT5G54630		423	10[b]	91	0	*AT4G33880*		52	N	0	42
AT3G15270	SPL5	408	8.3[b]	0	0	*AT5G18560*		43	0.9	0	4
AT1G73830	BEE3	373[a]	3.6	31	0	*AT3G09290*		41	16[b]	0	6
AT5G46690		333	10.0[b]	6	0	*AT5G19790*		40	18[b]	18	115
AT3G59060	PIL6	272	130[b]	138	0	*AT5G02350*		38	23[b]	N	N
AT3G06120		264[a]	0.81[b]	N	N	*AT5G14340*		38	4.5[b]	0	44
AT1G18710		223	5.1[b]	35	0	*AT2G42660*		31	5.6[b]	1	45
AT5G49330		218	5.3[b]	0	0	*AT2G33720*		31[a]	0.7[b]	N	N
AT5G15310		189	12[b]	90	8	*AT4G01350*		30	1.5	0	4
AT5G46880		184[a]	2.4[b]	2	0	*AT1G68150*		29	9.7[b]	0	43
AT3G58070		177[a]	8.7[b]	5	0	*AT5G52170*		29	2.0[b]	2	13
AT1G25440	COL1	158	N	250	0	*AT5G19520*		28	13[b]	4	463
AT1G62360		157	11	21	0	*AT1G29280*		26	7.4	18	346
AT3G18010		152[a]	N	19	0	*AT1G68880*		26	47[b]	3	72
AT3G57600		136	2.4	9	0	*AT3G24310*		26	11[b]	0	4
AT5G10570		131	N	2	7	*AT3G20840*		26[a]	2.7[b]	0	0
AT4G01460		128	11[b]	29	0	*AT3G25790*		25	14[b]	0	20
AT1G71030		126	9.3	223	2	*AT5G65790*		25	27[b]	1	76
AT2G36610		126	2.0[b]	18	29	*AT1G64000*		25	4.8[b]	0	4
AT1G68190		122	11[b]	71	6	*AT1G74500*		24	38[b]	0	176
AT5G56860		110	53[b]	28	0	*AT3G45170*		24[a]	N	N	N
AT5G11190		97	N	N	N	*AT1G28160*		22[a]	1.3[b]	0	13
AT3G24140		73	5.4	27	0	*AT3G12720*		22	19[b]	0	13
AT5G57150		73	0.32	32	269	*AT1G66470*		21	7.3[b]	0	44
AT4G14540		70	138[b]	100	10	*AT1G17950*		21	31[b]	0	18
AT1G76110		64	33[b]	25	0	*AT1G69810*		20	26[b]	0	94
AT2G39250		63	5.0[b]	11	0	*AT1G79580*		20	2.1	0	4
AT5G15800	AGL2	61	0.25[b]	0	0						
AT2G02450		51	29[b]	44	0						
AT3G61950		48[a]	7.5	9	0						
AT4G25490	CBF1	45	3.3[b]	3	0						
AT1G53160	SPL4	43	2.5	48	0						
AT2G33810	SPL3	43	43[b]	34	3						
AT5G15850	COL1	37	15[b]	297	0						
AT5G44190	GLK2	35	8.6	193	7						
AT4G32980	ATH1	33	13	47	0						
AT1G75490		30	2.0[b]	1	0						
AT1G08810		27	6.8[b]	21	0						
AT4G25470	CBF2	27	1.7[b]	33	0						
AT1G33760		27	6.1[b]	N	N						
AT2G17950	WUS	26[a]	0.78[b]	3	0						
AT3G02380	COL2	25	28[b]	77	0						
AT5G47220	ERF2	25	3.8[b]	112	11						
AT2G46870		20	9.6[b]	19	2						

Table legend:

[a] nominal value (transcripts undetectable in one kind of organ: C_T value = 40, in both biological replicas)
[b] transcripts called absent by Affymetrix software in at least one organ and in both biological replicas).
[c] unspecific MPSS signatures were not considered.
N: gene not represented on Affymetrix chip or MPSS database.
[d] p.p.m.

Organ-specific expression was confirmed for the 87 TF genes shown in Table 3.1 by repeating the qRT-PCR with a biological replicate. Biological replication was also performed using Affymetrix analysis. The mean S/R ratio obtained for the confirmed organ-specific genes was compared to publicly available data from Massively Parallel Signature Sequencing (MPSS; http://mpss.udel.edu/at/java.html) of *Arabidopsis* (Table 3.1). The MPSS database contained signatures for 73 of the 87 genes that were found by qRT-PCR to show strong (>20-fold) differences in expression levels between the shoot and root. For this subset of 73, there was remarkably good qualitative agreement between the two technologies. In all but four cases, genes with a high S/R transcript ratio measured by RT-PCR also had a high ratio as determined by MPSS. In most of these cases, signature sequences were completely absent for roots. For genes with a very low S/R ratio there was even better qualitative agreement between qRT-PCR and MPSS data. In general, data from Affymetrix arrays were also in qualitative agreement with qRT-PCR and MPSS data. In very few cases where data from the three different technologies at odds with one another.

To investigate further the reasons for discrepancies between qRT-PCR and Affymetrix chip data, the S/R ratios were calculated for both complete data sets, and plotted against each other (Figure 3.6 A). At first glance, there was only weak agreement between the ratios obtained with the two technologies (R^2 =0.472 for the entire set of 975 considered genes). A different picture emerged when the data set was split into groups of genes according to their Affymetrix shoot expression level (Figure 3.6 B). For example, when the 50 TF genes with the highest Affymetrix shoot expression levels were analysed, there was quite good agreement with the S/R ratios estimated from real time RT-PCR data (R^2=0.727). When genes with lower expression level were introduced (see Figure 3.6 B), the correlation coefficient dropped continuously. In general, there was a clear correlation between the ‘discrepancy’ in the S/R ratios determined by the two technologies and the frequency of genes that were flagged ‘absent’ by Affymetrix software (Figure 3.6 C). For example, about 7% of the genes showed a >10-fold discrepancy in the S/R ratio obtained from qRT-PCR and Affymetrix chips, and of these about 80% were called ‘absent’ by the Affymetrix software. In contrast, 75% of the genes had similar S/R expression ratios (<3 fold discrepancy) in both data sets, of which only 46% were called ‘absent’ by the Affymetrix software (Figure 3.6 C).

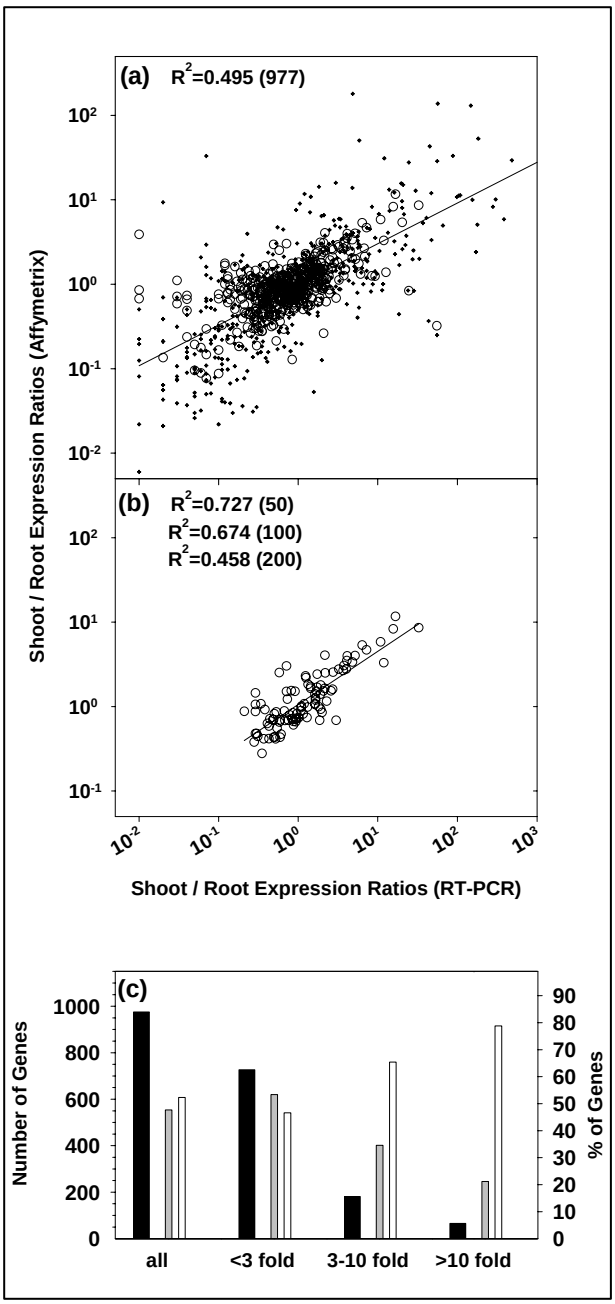


Figure 3. 6 Comparison of shoot to root expression ratios obtained from qRT-PCR and Affymetrix data

(A) Expression ratios are shown for the subset of 975 genes, which were clearly detectable by qRT-PCR (CT value <40) and were also present on the Affymetrix ATH1 gene chip. Circles represent genes that were called 'present' in the shoot as well as the root by Affymetrix software. Crosshairs stand for genes that received an 'absent' call in either shoot or root or both organs. **(B)** A subset of 100 genes (called 'present', see (A)) with highest shoot expression levels, according to Affymetrix technology, is depicted. Correlation coefficients (R^2) of shoot-root expression ratios for this subset of 100 genes, a subset of 50 and one of 200 genes, obtained by the two technologies are given. Panel **(C)** displays a distribution of the 975 genes, according to the ratio of $(S/R)_{RT\text{-}PCR}$ to $(S/R)_{Affymetrix}$. Genes are categorized in four subsets (black bars): all genes, the 727 genes for which the expression ratios obtained with the two technologies varies less than 3-fold, the 182 genes for which the expression ratios differ 3-10-fold, and the 66 genes with more than 10-fold difference. The percentage of genes called 'present' (grey bar)

3.A.1.7 Further development of the TF RT-PCR platform (in collaboration with Dr Wolf-Ruediger Scheible)

During this project, the number of putative TF genes in *Arabidopsis* continued to increase. The AGRIS database for TFs (http://arabidopsis.med.ohio-state.edu/AtTFDB/), contains about 1700 AGI codes for TF grouped in 45 different TF families. In contrast, the recently published list of *Arabidopsis* TFs from Jen Sheen's lab (http://genetics.mgh.harvard.edu/sheenweb/AraTRs.html) contains, in contrast, almost 3000 genes which includes genes for core-transcription factor machinery. To extract set of

regulatory TFs, data from these two sources was compared and all new putative transcription factor AGI codes were selected. Gene families like: chromatin remodelling proteins, histone acetylases and proteins from the general transcription machinery were excluded, because they presumably do not possess the regulatory specificity of bona fide TFs, as discussed before (Riechmann and Ratcliffe, 2000; Riechmann, 2002). As a result, we selected 789 additional TF genes, some from previously described families (such as MADS-box or AP2/EREBEP) and others, from newly identified families, like: *AS2* (asymmetric leaves 2), *LBD* (lateral organ boundaries), *BZR*, *GeBP* , *B3* (Iwakawa et al., 2002; Shuai et al., 2002; Wang et al., 2002; Curaba et al., 2003; Yamasaki et al., 2004, respectively). All sequences were extracted from the TAIR database (http://www.arabidopsis.org) and primers were designed as described in materials and methods. Additionally, all primers showing efficiency lower than 60% (about 70 primer pairs) and those resulting in multiple amplicons (about 60 primer pairs), were replaced with newly re-designed primer pairs. In all cases, where we had two primer pairs for the same gene, the pair working with the lower efficiency was removed (about 100 primer pairs). Finally, primers for the genes not anymore annotated as TFs in TAIR database (TIGR *Arabidopsis* database release, version 3.0) were also removed from that platform. Enlarged and improved version of the platform contains now primer pairs for 2256 TF genes, representing 53 gene families and sub-families arrayed on six 384-well plates. As a result of collaboration wit Dr Yves Gibbon (System Regulation Group), set-up of real-time PCR reactions is now fully robotized (Evolution P3 liquid handling system, Perkin Elmer). One researcher is able to measure expression of all 2256 TF genes in a single biological sample in a just one working day, when using both available ABI Prism 7900HT machines. Platform is being currently tested, on broad range of the biological samples (i.e. salt and osmotic stress, phosphate starvation and replenishment, seed dormancy, biotic stress), so the results of the performance of the newly and re-designed primer pairs will be available soon.

3.A.2 Nitrogen regulated transcription factors: needles in a haystack

The qPCR platform described above was used to identify nitrogen-regulated TFs. RNA was extracted from axenically grown plants were grown axenically and analyzed on Affymetrix ATH1 arrays and by qPCR, to enable direct comparison between both datasets, in collaboration with Dr Wolf-Ruediger Scheible and Dr Rosa Morcuende.

3.A.2.1 Physiological responses to N-deprivation and nitrate re addition in *Arabidopsis* seedlings grown in liquid cultures.

Figure 3. 7 Phenology of nine-day old N-limited and N-replete *Arabidopsis* seedlings grown in sterile liquid culture (taken from Scheible et al. 2004)

Seedlings were grown for seven days in full nutrients and then transferred to low N (-N) or maintained in full nutrients media (+N) for another two days.

Arabidopsis seedlings (plant material kindly provided by Dr Rosa Morcuende) were grown in liquid culture with low levels of sucrose in the medium and continuous light to minimize diurnal changes in carbohydrate and N metabolism (Matt et al., 1998; Scheible et al., 2000), which would otherwise complicate interpretation of experimental data. N-deprived seedlings exhibited the typical phenology of N-limited plants including reduced chlorophyll, accumulation of anthocyanins in the leaves, and pronounced root and especially lateral root growth (Fig. 3.7 and data not shown). Two independent experiments were carried out at an interval of two months.

3.A.2.2 Transcriptional regulators

qPCR expression profiling was performed on the RNA extracted from plants grown in full nutrition, plants deprived of N for two days and N-deprived plants exposed to nitrate for 30 min. The latter time point was chosen to identify TF genes involved in early-responses to nitrate re-supply. The N deprived control plants obtained 3 mM KCl also for 30 min. Prior to screening all TF genes by RT-PCR, RNA extraction and qRT-PCR analysis was performed for several "marker" genes involved in primary assimilation of nitrogen. As controls for N-starvation status, two ammonium transporter genes were analysed: *ATAMT1-5* and *ATAMT1-1* (primer sequences in the appendix B), which are strongly induced during N-starvation (Sohlenkamp, unpublished data). Other genes were used as indicators for nitrate induction, including those encoding the high affinity nitrate transporter *ATNRT 2.1*, two nitrate reductases (*NIA1* and *NIA2*), nitrite reductase (*NII*), and ferredoxin--NADP(+) reductase

(*FNR*). All of these genes are well known to react quickly to nitrate and carbohydrates (se e.g. Stitt et al. 2002 for references). Typical results from such controls are shown in Figure 3.8

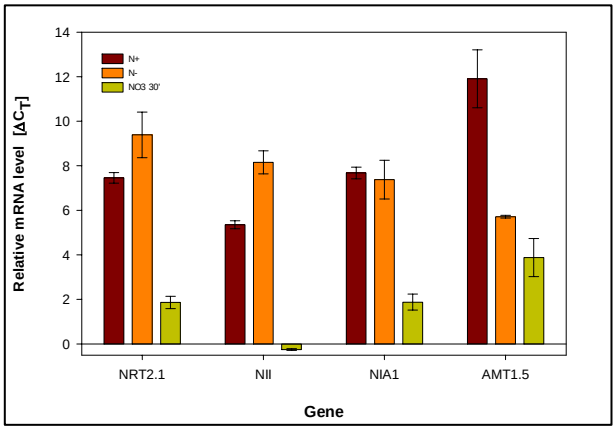


Figure 3. 8 Transcriptional response to N – deprivation and to nitrate replenishment for selected marker genes.

Transcript abundances expressed relatively to *UBQ10* in the $\log_2$ scale (ΔC_T), are inversely proportional to the height of the bars. Error bars – SE from three technical replicates of qPCR reaction (n=3). N+ - full nutrition, N- nitrogen deprivation, NO3 30' – 30 minutes after nitrate re –addition

Following confirmation that the various N – treatments resulted in expected changes in expression of control marker genes, qPCR was performed for all 1465 TF genes. Transcripts of 1243 TF genes were detectable in at least one condition analysed. Comparison of transcriptional changes for this subset of TF genes under N-derivation and nitrate replenishment is depicted in figure 3.9 A and B, respectively.

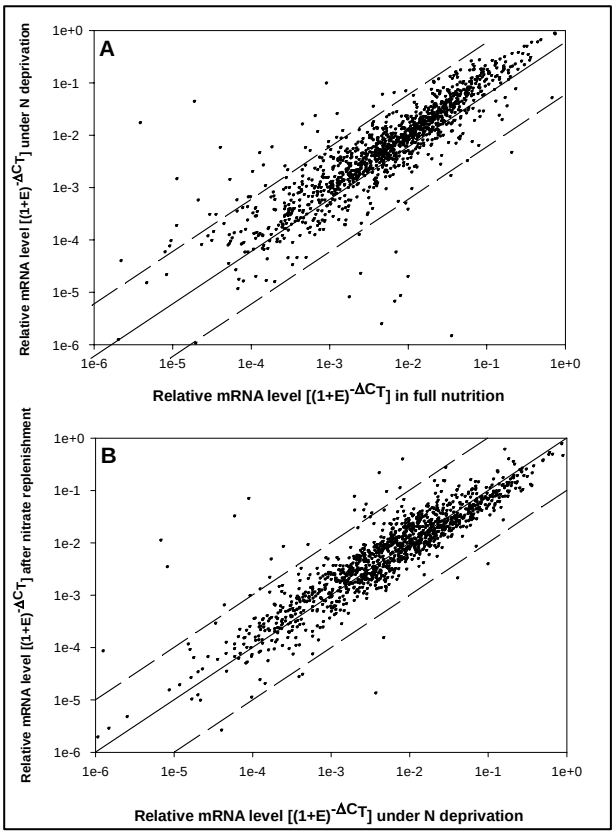


Figure 3. 9 Comparison of TF transcript levels under N deprivation and 30 minutes after nitrate replenishment

(A) Expression values ($(1+E)^{-\Delta CT}$) normalised to *UBQ-10*, from qRT-PCR amplification of cDNA from plants grown on full nutrition medium (FN) and 48 hours of nitrogen deprivation (N-) depicted for 1243 TF genes resulting in specific amplicons. Dashed lines indicate 10-fold differences in the N- to FN transcript levels.
(B) Expression values ($(1+E)^{-\Delta CT}$) normalised to *UBQ-10*, from qRT-PCR amplification of cDNA from plants under 48 hours of nitrogen deprivation (N-) and 30 minutes after nitrate re-addition (30'N) depicted for 1243 TF genes resulting in specific amplicons. Dashed lines indicate 10-fold differences in the 30' N to N- transcript levels.

From the figure 3.9 it can be seen that transcript levels for *Arabidopsis* TF genes, represented by $(1+E)^{-\Delta CT}$, varied over 6 orders of magnitude as observed also for shoot and root (compare with Figure 3.5).

The highest TF expression level was close to that of the house keeping genes (*UBQ-10* and *ACT-2*) and the lowest just on the limit of detection, of 1 transcript per 1000 cells (as described above). To limit the number of genes for further studies, we choose 10-fold cut off to identify N-regulated TF genes. Expression analysis for 71 TF genes selected in this way are shown in Appendix C. Twenty four TF genes were found to be repressed more than 10-fold after N deprivation, including TF gene from the following families: bHLH (7), CO-like (4), MYB and MYB-like (2), C2H2 (2), MADS (2), NAC (1), ARR (1), ARID HMG (1), NIN-like (1), ABI3VP (1), GATA (1) and SBP (1). A similar number (22) of genes were found to be induced more than 10 fold after 48 h N- deprivation, from following families: MYB and MYB-like (7), WRKY (5), AP2/EREBP (3), MADS (3), bHLH (1), bZIP (1), ARP (1) and HSF (1).

Relatively few genes were found to respond negatively to the transition from N – deprivation to nitrate re-addition: just 7 were repressed more than 10 fold 30 min. after re addition of 3 mM KNO_3, namely MADS (2), bZIP (1), AP2/EREBP (1), NAC (1), C2H2 (1), DOF (1). On the other hand, 25 genes were up-regulated more than 10-fold, by nitrate in those experiments, from following gene families: AP2/EREBP (3), MYB-like (3), NAC (3), MADS (3), NIN-like (3), SBP (3), ARR (1), bZIP (1), C2H2 (1), GATA (1), HB (1), TCP (1), GARP (1).

To confirm strong N-regulation of the candidate genes mentioned above, we performed an analysis of a biological replicate experiment (Appendix C). Plants were grown in exactly the same way using the same stock of Col-O seeds. Only ten of the 45 genes, that responded to N-deprivation in the same way and at similar magnitude (>10 fold change in the transcript level). Twenty three other genes responded in the same way but lower than cut-off used (3-10 fold change in the expression level). Twenty genes did not respond in the transcript level (1-2 fold change). Seven genes responded in the opposite way to that observed in the first experiment. Twenty of the 25 nitrate induced genes responded in the same way and at similar magnitude (>10 fold) in the replicate experiment. Three of the remaining 5 genes responded I the same way but with less than 10 fold change in the transcript level. Just 2 genes showed no response in the transcript level (1-2 fold changes) and no gene responded in the opposite way. Nitrate repression of expression was not confirmed for any of the seven genes identified in the first experiment. In fact, five showed no response in the transcript level (1-2 fold changes) and no two responded in the opposite way.

Thirty seven TF genes exhibiting at least 10 fold changes in expression in both independent experiments, as measured by qPCR, are listed in Table 3.2. Expression data for only fraction of the genes was obtained also from Affymetrix arrays (P calls). Other genes yielded absent (A) calls or were not represented on ATH1 array (NR). Homologues of some selected TF genes, aroused by segmental gene duplications are also listed (http://www.tigr.org/tdb/e2k1/ath1/Arabidopsis_genome_duplication.shtml). Data were also compared to the previous microarray experiments, identifying transcriptional responses to nitrate (Wang et al., 2003; Wang et al., 2004) and to the qPCR data identifying shoot and roots specific TF (Czechowski et al., 2004).

Table 3. 2 N-regulated TF genes of *Arabidopsis*

AGI	Gene	TF family	Duplication	-N vs. +N		N 30' vs. –N		MAS 5 call		
				RT-PCR	ATH1	RT-PCR	ATH1	+N	-N	N 30'
AT2G22200[2,4]		AP2 EREBP	*AT4G39780*	1.00	1.18	44.94	18.43	A	A	P
AT2G39250[2,4,S]		AP2 EREBP	*AT3G54990*	0.17	0.67	241.11	3.05	A	A	P
AT4G25490[1,S]	CBF1	AP2-EREBP		0.85	1.09	54.44	1.51	A	A	A
AT2G33720[1,R]		ARP		18.62	0.97	1.12	1.05	A	A	A
AT1G59940[2,4,R]		ARR		0.25	1.34	28.30	3.65	P	P	P
AT4G01460[1,S]	AtbHLH057	bHLH	*AT2G46810*	0.09	0.51	1.79	0.86	A	A	A
AT1G68880[2,R]	AtbZIP8	bZIP		0.12	0.38	31.03	4.73	P	A	P
AT5G38800[1]	AtbZIP43	bZIP		9.98	1.61	4.96	1.12	A	A	A
AT1G25440[3,R]	COL16	CO-like	*AT1G68520*	0.12		1.60		NR		
AT1G68190[2,S]		CO-like		0.09	0.29	0.95	1.23	A	A	A
AT1G68520[2,R]	COL6	CO-like	*AT1G25440*	0.03	0.15	8.53	2.08	P	P	P
AT1G73870[1,R]	COL7	CO-like		0.08	0.50	1.42	1.12	A	A	A
AT4G26150[1]	GATA-22	GATA	*AT5G56860*	0.30	1.02	41.99	2.45	P	P	P
AT1G69780[1,4]	ATHB-13	HB		0.61	1.44	406.01	0.85	P	P	P
AT3G51910[1]	HSFA7A	HSF	*AT1G32330*	21.49	0.61	1.24	1.60	P	P	P
AT1G01530[1]	AGL28	MADS		1.03	1.04	31.84	1.50	A	A	A
AT3G30260[1]	AGL8	MADS		0.73	1.15	36.64	1.05	A	A	A
AT5G65060[3]	MAF3	MADS		13150.50		1.20		NR		
AT1G13300[2,4,R]		MYB	*AT3G25790*	0.32	0.29	44.84	18.51	P	A	P
AT1G56650[2,4]	PAP1	MYB		27.89	8.10	1.18	0.61	A	P	P
AT1G66380[1,]	AtMYB114	MYB		15.67	1.30	0.75	0.90	A	A	A
AT1G66390[2,4]	PAP2	MYB		155.92	51.36	0.68	0.50	A	P	P
AT3G13890[1]	AtMYB26	MYB		0.13	2.31	15.69	1.12	A	A	A
AT1G68670[2,4]		MYB-like	*AT1G25550*	0.08	0.28	160.44	9.49	P	P	P
AT2G33550[3]		MYB-like		1.10		27.87		NR		
AT3G25790[2,4,R]		MYB-like		0.04	0.84	457.35	5.34	A	A	P
AT1G02230[1]		NAC	*AT4G01540*	0.47	0.95	10.17	1.12	A	A	A
AT4G17980[1]		NAC	*AT5G46590*	1.30	0.82	42.48	1.50	A	A	A
AT2G43500[2,4]		NIN-like	*AT3G59580*	7.83	8.00	0.81	0.48	A	P	P
AT4G24020[2,4]		NIN-like	*AT1G64350*	0.35	0.97	187.24	3.38	P	P	P
AT4G38340[2,4]		NIN-like	*AT1G76350*	2.06	2.78	942.62	18.65	A	A	P
AT1G76350[2]		NIN-like	*AT4G38340*	0.06	0.19	72.19	0.69	P	P	P
AT1G02040[3]	SPL8	SBP		1.19		24.46		NR		
AT3G57920[1]	SPL15	SBP	*AT2G42200*	0.49	0.82	14.46	2.01	A	A	P
AT1G35560[1,4]		TCP		0.73	0.89	30.76	1.01	P	P	P
AT2G40750[2,4]	AtWRKY54	WRKY		10.64	10.76	3.88	2.54	A	P	P
AT5G22570[2,4]	AtWRKY38	WRKY		17.90	3.90	3.08	2.25	P	P	P

Table legend:
(A) absence as determined by Affymetrix MAS5 software
(P) presence as determined by Affymetrix MAS5 software
(NR) not represented on ATH1
[1] induced or repressed transcripts detected by RT-PCR only
[2] transcripts categorized as induced or repressed by RT-PCR and Affymetrix gene chips (<= or >= 3-fold)
[3] induced or repressed transcripts detected by RT-PCR; not represented (NR) on the Affymetrix array.
[4] similar patter of nitrate induction in both: NR null mutant and the wild type according to Scheible et al., 2004
[S] expression is preferentially in the shoot (shoot/root expression ratio >20) according to Czechowski et al., 2004 or Wang et al., 2003; Wang et al., 2004
[R] expression is preferentially in the root (shoot/root expression ratio <0.05) according to Czechowski et al., 2004 or Wang et al., 2003; Wang et al., 2004

Data from an ATH1 array hybridisations provided by Dr Wolf-Ruediger Scheible, were also used to identify N-regulated TF genes. Of the ~1800 potential TFs on the ATH1 chip, 93 showed marked (>3-fold) changes in transcript abundance. Figure 3.10 summarizes the response (-N vs. +N) for 1169 genes that are included in both technology platforms. Real time RT-PCR confirmed that most TF genes did not respond strongly to N availability. Some of the genes depicted on the plots, identified as interesting only by qPCR, were not identified by hybridisation Affymetrix ATH1 ('absent' calls in all samples analysed).

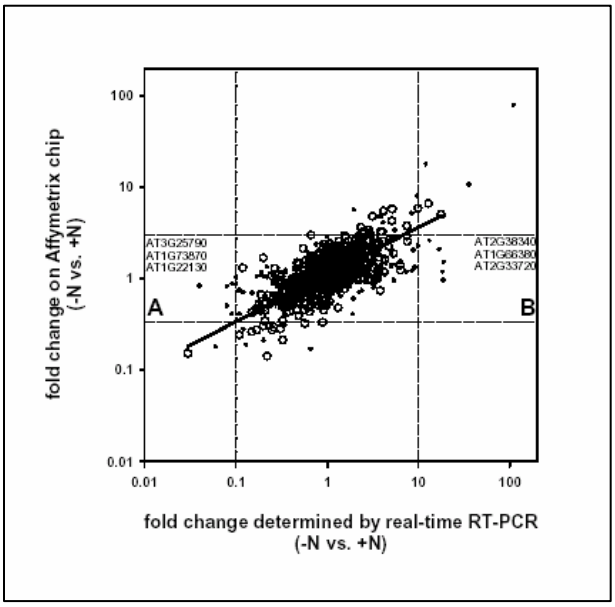


Figure 3. 10 Comparison of TF gene expression ratios, as determined by qRT-PCR and Affymetrix technology (taken from Scheible et al., 2004)

1169 TF genes included in both platforms are shown for a comparison of N-starved versus N sufficient *Arabidopsis* seedlings. Circles and crosshairs denote genes that were called 'present' or 'absent', respectively, in replicate ATH1 arrays. Dashed lines indicate ten-fold (RT-PCR axis) or three-fold (Affymetrix axis) changes in expression ratios. A regression line (R2=0.59) is shown for the 693 'present' genes. Quadrants A and B contain genes (see Table I) that were inconspicuous according to Affymetrix analysis, but were identified as interesting by RT-PCR.

In total, 17 TF genes were revealed as NO_3^--responsive by RT-PCR only, 15 genes were revealed by both technologies, and five genes were not represented on the ATH1 array. Three genes (*At1g35560, AT3G519101, AT1G697801*, encoding a TCP-domain, heat shock factor and homeobox TF, respectively) gave conflicting results: strong induction was found by qRT-PCR after 30 min NO_3^- re-supply or under N deprivation, but not on ATH1 arrays even though they were called 'present' (and the corresponding probe set appears gene-specific). The result from the real time RT-PCR platform for *At1g35560* was confirmed by analysis of more biological replicates using a different primer pair (R. Bari & W.-R. Scheible, unpublished), and by inspection of the Stanford Microarray Database (spot history for clone143C3XP in experiments 3787, 3789, 10849 and 10851). This gene was also found to be one induced by nitrate on ATH1 array performed by Wang et al., 2004) in both – wild type and *NIA null* mutant. It is apparent that data obtained with both technologies are generally consistent. The additional NO_3^-/N-responsive TF genes identified by qRT-PCR analysis include additional segmental-duplicated gene pairs.

3.A.2.3 Candidate genes selection

A total of 37 N-regulated TF genes were considered for further study. Because functional characterisation was to involve plant transformation and significant subsequent analyses, the number of candidate genes chosen for further study was reduced by applying the following filters. First, we checked whether regulation of each TF was specific to changes in nitrogen nutrition, by comparing TF expression various abiotic-stress experiments, including phosphate, sulphate, carbohydrates, osmotic and salt stress. Data were kindly provided by collaborators from Molecular Genomic Group, MPI-MP Golm, Germany (led by Dr Wolf-Ruediger Scheible): Dr Daniel Osuna, Dr Rosa Morcuende, Rajendra Bari, and Tomasz Kobylko, by Monika Bielecka (from Amino Acid and Sulphur Metabolism group led by Dr Rainer Hoefgen) and by Dr Wenming Zheng (from Molecular Plant Nutrition Group, MPI-MP Golm, Germany led by Dr Michael Udvardi). All the nutrient-stress experiments were done with the same axenic culture system, including the same light conditions in the same phytotron chamber, and the same basic media, except for differences in a single nutrient as shown on Figure 3.11.

Six of the 37 N-regulated TFs also responded to changes in either P and S nutrition or salt and osmotic stresses (Table 3.3) These were eliminated from further consideration with two exceptions: *PAP1* and *PAP2* genes. *PAP1* and *PAP2* transcript levels responded positively to S and P deprivation in addition to N deprivation as well as to long term (3 hours) osmotic and salt stress. The general responses of *PAP1* (*AT1G56650*) and *PAP2* (*AT1G66390*) to nutrient and other abiotic stresses was interesting in light of the knowledge that both are involved in the regulation of anthocyans and flavonoid biosynthesis, which are activated under a variety of stresses conditions. It is apparent that many (11) N-regulated TF genes, besides *PAP1* and *PAP2* are also carbon regulated. Those are particularly interesting, potentially mediating crosstalk between C and N metabolism discussed in introduction. *MAF3* expression for example was highly up-regulated by nitrogen and carbohydrates deprivation. *MAF3*, is one of the genes that may control vernalisation pathway to floral induction (Ratcliff et al. 2004). Regulation of *MAF3* by C and N supply could provide a piece of the puzzle that links plant nutrition and the flowering time.

To reduce number of the candidate genes to manageable number for functional characterisation we selected 16 genes from remaining 31. That set represents all observed types of responses to nitrogen and most of the TF families possibly involved in N signalling (Table 3.3).

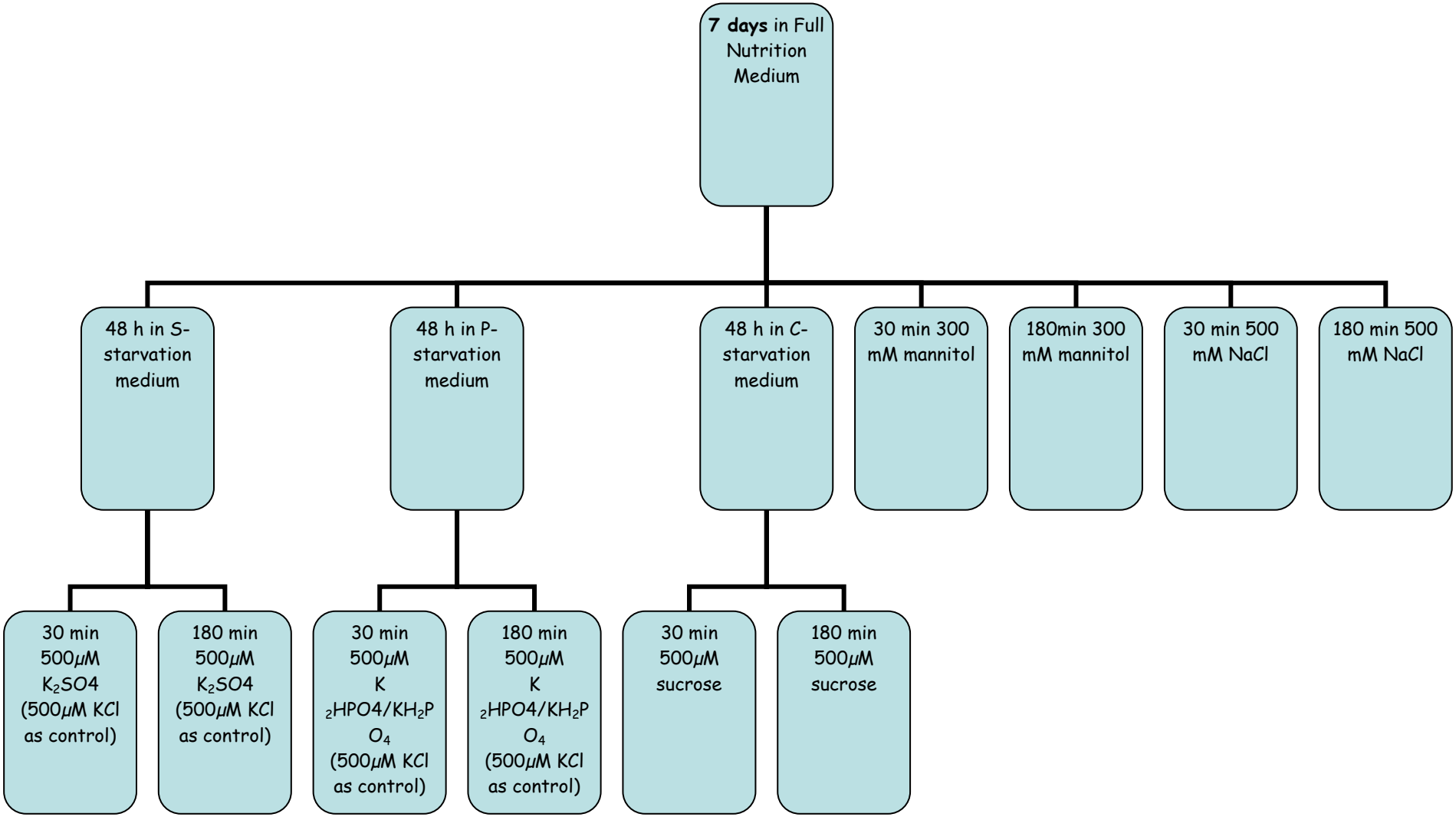


Figure 3. 11 The experimental set-up for testing various abiotic stresses in *Arabidopsis* liquid cultures, used for selection of nitrogen-regulated TF genes.

Table 3. 3 qPCR results of various abiotic stresses for all nitrogen regulated TF genes

AGI	Gene Name	TF family	-S/FN[1]	S 30'/-S[1]	S 180'/-S[1]	-C/FN[2]	C 30'/-C[2]	C 180'/-C[2]	-P/ FN[3]	P 30'/-P[3]	P 180'/-P[3]	Man 30'/FN[4]	Man 180'/FN[4]	NaCl 30'/FN[4]	NaCl 180'/FN[4]
AT2G22200		AP2-EREBP	1,22	1,14	1,40	0,54	1,45	1,14	0,40	1,65	2,15	1,55	1,22	3,07	2,07
AT2G39250		AP2 EREBP	0,54	1,05	1,10	0,14	1,88	2,60	0,64	2,70	2,49	0,65	0,72	1,08	0,52
AT4G25490	CBF1	AP2-EREBP	3,45	0,58	0,40	0,56	1,42	1,50	0,66	1,75	1,74	8,94	2,09	22,90	10,48
AT2G33720		ARP	8,53	0,99	1,61	0,10	7,50	1,81	0,40	3,12	3,47	0,61	2,81	0,94	1,96
AT1G59940		ARR	1,50	1,03	1,02	0,06	1,35	1,82	0,84	0,73	0,76	1,17	0,86	1,94	1,01
AT4G01460	AtbHLH057	bHLH	0,27	0,87	0,98	0,56	0,43	0,35	1,00	0,89	1,96	0,70	0,31	0,88	0,25
AT1G68880	AtbZIP8	bZIP	1,71	1,80	1,65	0,10	4,15	2,67	0,63	2,07	0,96	2,88	3,22	2,26	8,04
AT5G38800	AtbZIP43	bZIP	15,35	1,08	1,06	0,02	4,12	5,60	1,00	1,72	0,54	0,53	3,79	2,35	2,69
AT1G25440	COL16	CO-like	0,29	1,33	1,13	0,43	0,74	1,08	0,20	3,98	4,60	3,26	3,81	3,51	3,14
AT1G68190		CO-like	0,25	0,92	0,98	0,34	0,41	0,04	0,66	1,32	1,64	0,17	0,13	0,26	0,10
AT1G68520	COL6	CO-like	0,24	0,91	1,57	0,23	0,24	0,56	0,38	1,58	2,45	1,97	1,26	3,37	1,01
AT1G73870	COL7	CO-like	0,19	1,28	1,28	0,25	1,53	0,69	0,44	0,83	2,23	0,12	0,40	0,12	0,14
AT4G26150	GATA-22	GATA	2,44	1,85	1,65	2,75	1,20	1,22	0,57	1,65	3,02	0,66	0,68	0,91	0,68
AT1G69780	ATHB-13	HB	1,15	1,52	1,48	332,08	1,20	2,15	0,74	2,13	1,03	0,61	0,63	0,89	0,95
AT3G51910	HSFA7A	HSF	0,62	0,51	2,64	0,82	3,59	4,29	0,54	0,12	0,14	3,49	3,79	8,57	4,07
AT1G01530	AGL28	MADS	0,52	1,13	0,98	0,50	0,13	0,80	0,25	1,46	4,38	1,27	1,20	0,89	0,54
AT3G30260	AGL8	MADS	2,79	0,91	0,55	0,03	1,06	1,93	1,07	0,24	0,09	0,41	0,38	0,64	0,27
AT5G65060	MAF3	MADS	1,29	0,55	0,74	2024,16	1,10	0,81	0,34	1,04	1,04	ND	ND	ND	ND
AT1G13300		MYB	0,27	1,86	1,93	0,05	0,76	3,73	1,41	0,27	0,60	1,34	0,93	2,57	1,35
AT1G56650	PAP1	MYB	164,24	0,00	0,49	0,77	2,38	22,12	6,89	0,56	1,08	1,48	15,35	1,45	12,81
AT1G66380	AtMYB114	MYB	109,03	0,11	0,32	0,39	3,74	13,36	5,16	0,40	0,71	0,29	4,22	0,70	2,42
AT1G66390	PAP2	MYB	443,51	0,27	0,36	0,18	1,48	20,05	2802,07	0,53	0,30	0,22	4,20	0,49	1,99
AT3G13890	AtMYB26	MYB	1,42	0,42	0,56	0,03	9,31	10,36	0,32	0,62	1,68	4,42	1,79	11,83	1,62
AT1G68670		MYB-like	1,19	1,52	1,26	0,41	0,71	0,89	2,09	0,24	0,43	2,13	1,61	4,36	2,31
AT2G33550		MYB-like	1,37	0,66	0,77	1,05	1,03	1,93	0,61	0,45	1,19	0,88	0,74	1,60	0,81
AT3G25790		MYB-like	0,23	0,93	1,07	0,05	0,41	2,84	13,12	0,32	0,39	5,42	0,61	8,49	1,49
AT1G02230		NAC	0,99	1,45	0,48	0,26	6,15	5,42	2,89	0,51	0,34	2,69	0,96	1,25	1,43
AT4G17980		NAC	1,34	2,87	0,65	4,69	0,96	1,19	0,83	0,90	1,75	2,27	1,56	4,15	2,45
AT2G43500		NIN-like	4,43	1,49	0,67	2,42	1,67	1,52	2,28	0,67	1,20	0,81	2,17	9,11	4,75
AT4G24020		NIN-like	1,32	1,30	0,81	1,44	1,28	0,74	1,19	1,05	1,14	0,84	1,08	0,73	0,89

AT4G38340		NIN-like	2,27	1,91	1,66	0,12	1,27	8,26	3,82	0,20	0,65	0,61	0,84	1,33	1,13
AGI	**Gene Name**	**TF family**	**-S/FN**	**S 30'/-S**	**S 180'/-S**	**-C/FN**	**C 30'/-C**	**C 180'/-C**	**-P/ FN**	**P 30'/-P**	**P 180'/-P**	**Man 30'/FN**	**Man 180'/FN**	**NaCl 30'/FN**	**NaCl 180'/FN**
AT1G02040	SPL8	SBP	1,07	1,50	1,43	0,14	1,27	1,55	0,73	0,95	0,95	0,76	0,83	0,95	0,82
AT3G57920	SPL15	SBP	0,67	1,11	0,83	0,56	1,09	1,89	1,19	0,88	1,34	0,88	0,57	1,50	0,65
AT1G35560		TCP	1,28	1,23	0,99	0,53	1,90	4,06	0,34	0,74	1,89	1,34	1,52	1,22	0,63
AT2G40750	AtWRKY54	WRKY	3,45	1,14	0,55	0,26	0,41	1,04	2,41	0,45	0,32	0,31	0,61	0,40	0,33
AT5G22570	AtWRKY38	WRKY	6,38	1,12	0,55	0,27	0,56	1,04	0,42	0,81	1,61	3,42	9,78	24,09	10,28

Table legend:

FN – full nutrition medium
-S/FN – expression after sulphate starvation versus full nutrition ,
-C/FN – expression after sucrose starvation versus full nutrition ,
-P/FN – expression after phosphate starvation versus full nutrition
S 30'/-S – expression 30 min. after sulphate re-addition versus sulphate starvation
S 180'/-S – expression 180 min. after sulphate re-addition versus sulphate starvation
C 30'/-C – expression 30 min. after sucrose re-addition versus sucrose starvation
C 180'/-C – expression 180 min. after sucrose re-addition versus sucrose starvation
P 30'/-P – expression 30 min. after phosphate re-addition versus phosphate starvation
P 180'/-P – expression 180 min after phosphate re-addition versus phosphate starvation
Man 30'/FN – expression 30min after mannitol addition to FN medium,
Man 180'/FN – expression 180min after mannitol addition to FN medium,
NaCl 30'/FN – expression 30min after NaCl addition to FN medium,
NaCl 180'/FN – expression 180min after NaCl addition to FN medium,

Data kindly provided by:

[1] Monika Bielecka (Amino Acid and Sulphur Metabolism Group, MPI-MP Golm, Germany),

[2] Dr Daniel Osuna Jimenez (Molecular Genomics Group, MPI-MP Golm, Germany),

[3] Dr Wenming Zheng (Molecular Plant Nutrition) and Rajendra Bari (Molecular Genomics Group, MPI-MP Golm, Germany),

[4] Dr Rosa Morcuende and Tomasz Kobylko (Molecular Genomics Group, MPI-MP Golm, Germany),

3.A.3 Further characterisation of N-regulated TF genes

Selected TF genes were subjected to further analysis, including a more detailed time course of regulation by nitrate and an experiment in which glutamine, but not NH_4^+ / NO_3^- was left out of the growth medium and then later re-supplied. These experiments were performed with wild type plants and a nitrate reductase mutant impaired in both *nia1* and *nia2*.

3.A.3.1 TF transcript changes in response to changes in nitrate or glutamine in the growth medium.

A time course of nitrate re-addition was performed on axenically cultured plants as described in Materials and Methods. Plant material, kindly provided by Dr Rosa Morcuende, was harvested: 12, 30, 75 and 180 min after nitrate addition to N-deprived plants. Two independent biological replicates were subjected to qRT-PCR analysis as described in Materials and Methods. In glutamine starvation experiments, plants were grown in medium containing no glutamine, but ammonium and nitrate. Following 48 h N-deprivation, plants were exposed to medium containing 4 mM glutamine for 30 minutes. The following genes were used as controls to monitor the effects of the various N-regimes: *ATNRT2-1, NIA1, NIA2, NII, FNR, ATAMT1.5* and *ATAMT1.1*. Transcript levels of both ammonium transporters, *ATAMT1.5* and *ATAMT1.1* increased following N-deprivation, while transcripts of *ATNRT2-1, NIA1, NIA2, NII* and *FNR* were unaffected by this treatment. (Figure 3.12, next page). On the other hand transcript levels for *ATNRT 2-1, NIA1, NIA2, NII* and *FNR* increased following nitrate re-addition to N-deprived plants. Response in expression was rapid (occurring within 12 min) and strong for these “marker” genes and remained stay induced relatively high during next 3 hours after nitrate re-addition.

Glutamine deprivation and re addition for 30min, had no effect on the expression of *ATNRT2-1, NIA1, NIA2, NII* and *FNR*. However, Gln- deprivation induced the expression of both ammonium transporters *ATAMT1-5* and *AMT1-1*. (Figure 3.12, next page).

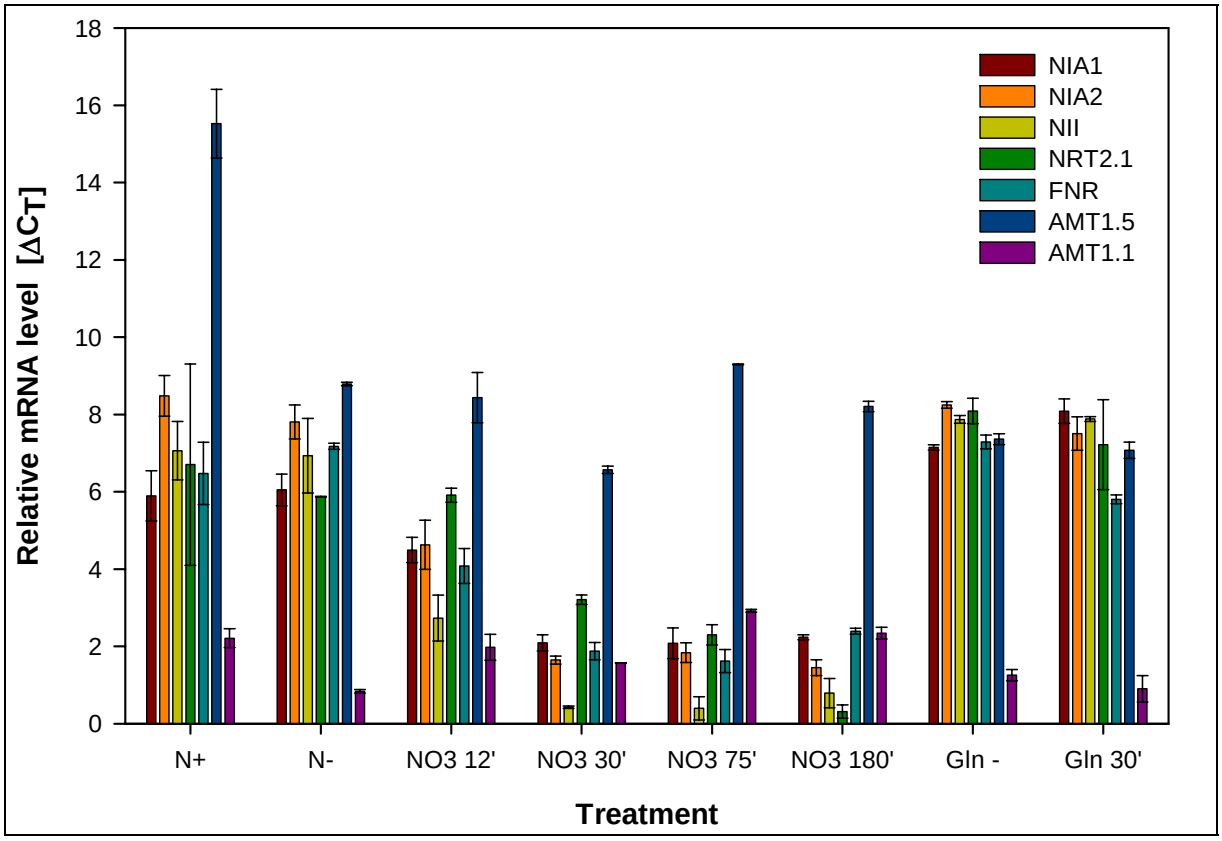


Figure 3. 12 Changes in gene expression of 6 marker genes after nitrate / glutamine starvation and re-addition.

Transcript abundances expressed relatively to *UBQ10* in the $\log_2$ scale (ΔC_T), are inversely proportional to the height of the bars. Error bars – SE from two biological and two technical replicates of qPCR reaction (n=4). N+ - full nutrition, N- nitrogen deprivation, NO3 12' – NO3 180' – 12 - 180 minutes after nitrate re –addition, Gln- glutamine deprivation, Gln 30' – glutamine re- addition.

Transcript levels for 16 N-regulated TF genes were also measured in the same experiment (Table 3.4). Three distinct responses were observed for these genes. The majority (12 out of 16 genes) were rapidly (within 12 or 30 min) but transiently induced by nitrate re-addition, and some like *AT4G38340* responded extremely strongly (1600 fold induction). Transcripts levels for most of them declines within 75 to 180 min after induction by nitrate. None of these genes responded to removal or re-addition of glutamine.

The second type of transcript response was more complex. Two genes: *AT1G13300* and *AT3G25790*, are also responded quickly to NO_3 replenishment within 12 min. However, their expression declined afterwards during the next 30 or 75 min before increasing again after 3 hours (Table 3.4). None of these genes responded to removal or re-addition of glutamine.

A third pattern of induction was observed for one gene *AT1G68880*. Strong but sustained induction of expression following nitrate replenishment was observed for this gene (Table 3.4)

The other two genes *PAP1* and *AT1G51910* were induced strongly by nitrogen deprivation. However, expression of those genes declined again within 30 min (*AT1G51910*) and 3 hours (*PAP1*) of nitrate re-addition. Moreover, *PAP1* was the only gene of the 16 TF genes investigated that was induced by glutamine starvation

Table 3. 4 N –regulation of selected TF genes

AGI	FN *		N- *	NO3 12' *		NO3 30' *		NO3 75' *		NO3 180' *		Gln-[1]	Gln [2]30'
	mean	SE		mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
AT1G01530	3,95	2,97	1,00	13,83	6,51	48,55	17,46	85,39	7,95	8,03	0,37	2,72	2,30
AT1G02230	0,56	0,29	1,00	0,17	0,05	32,51	7,44	0,17	0,04	5,49	4,60	0,16	0,99
AT1G13300	2,73	0,64	1,00	10,95	0,27	31,86	4,78	9,33	0,59	19,58	6,59	0,33	1,27
AT1G35560	1,19	0,55	1,00	6,98	2,14	24,14	1,51	7,61	0,19	3,09	1,32	0,97	0,74
AT1G56650	0,04	0,03	1,00	1,65	0,02	1,68	0,24	0,26	0,03	0,07	0,02	109,54	1,19
AT1G68880	35,70	5,35	1,00	123,28	31,67	233,42	64,80	202,21	60,75	171,04	23,50	1,80	1,09
AT1G76350	16,93	4,78	1,00	97,99	47,43	72,19	14,47	16,69	1,74	13,82	13,45	2,02	1,46
AT2G22200	0,97	0,01	1,00	10,30	0,80	41,26	0,10	23,02	0,08	10,57	8,20	0,74	1,32
AT2G23740	0,63	0,35	1,00	0,59	0,02	29,29	27,75	176,21	13,72	14,87	12,61	0,67	21,96
AT2G33550	2,89	1,44	1,00	4,71	0,24	48,68	3,47	19,58	3,36	33,51	21,41	0,55	0,49
AT2G33720	2,36	0,22	1,00	6,92	2,43	27,37	0,37	4,23	0,08	4,20	3,67	1,87	1,65
AT2G43500	3,79	1,37	1,00	65,85	18,56	53,01	6,61	10,21	0,52	3,69	3,14	8,10	1,49
AT3G25790	5,18	0,68	1,00	41,66	21,57	358,96	74,15	151,87	38,76	400,37	153,78	0,12	0,88
AT3G51910	0,03	0,01	1,00	0,03	0,00	1,24	0,47	2,32	0,63	2,77	1,68	4,55	0,15
AT4G26150	10,14	2,37	1,00	21,50	2,25	58,37	6,78	60,23	5,02	18,04	8,64	0,55	1,13
AT4G38340	13,92	4,34	1,00	922,85	9,67	1649,9	212,50	1018,4	90,32	584,54	250,55	5,60	0,80

Table legend:

Transcript levels of TF genes under various N regimes expressed as relative to the level at N-deprivation.

FN – full nutrition,
-N –48h low nitrate medium plus 3hours 3mM KCl ,
NO3 12' – 12min after 3mM KNO_3 re-addition to N-deprived plants,
NO3 30' – 30min after 3mM KNO_3 re-addition to N-deprived plants,
NO3 75' – 75min after 3mM KNO_3 re-addition to N-deprived plants,
NO3 180' – 180min after 3mM KNO_3 re-addition to N-deprived plants,
-Gln – 48h full nutrition medium minus glutamine,
Gln 30' – 30min after 4mM glutamine re-addition to Gln starved plants
[1] – compared to full nutrition medium
[2] –compared to Gln- medium
Mean – average expression determined from two independent biological replicates and two technical replicates, except glutamine experiments where two technical but no biological replicates were performed
SE – standard error determined from two independent biological replicates and two technical replicates (n=4), except glutamine experiments where two technical but no biological replicates were performed (n=2).

* -plant material kindly provided by Dr Rosa Morcuende

3.A.3.2 Nitrate regulation of TF genes in a n*ia1nia2* double mutant

An *Arabidopsis nia1nia2* double mutant G'4-3 (Willkinson and Crawford, 1993), bulked and donated by Dr Wolf-Ruediger Scheible, was used to determine whether nitrate rather than a product of its assimilation was responsible for the changes in TF expression observed in earlier experiments. That mutant was created in the following way: line with T-DNA insertion into *NIA2* gene was used as subject of EMS mutagenesis. Mutant affected in second NR gene, *NIA1*, was selected by screening for growth on nitrate as sole nitrogen source. G' 3-4 is not a true null mutant, as it shows detectable growth on nitrate and still retains some NR activity (1% of shoot wild type and 5-10% of root wild type; Willkinson 1992, Lejay et al. 1999).

Plants were exposed to the same set of conditions mentioned in the last section and the transcript levels of reference genes was measured (Fig. 3.13). The mutant shows typical phenology of N-deprived plants after 48 hours as shown in Figure 3.7

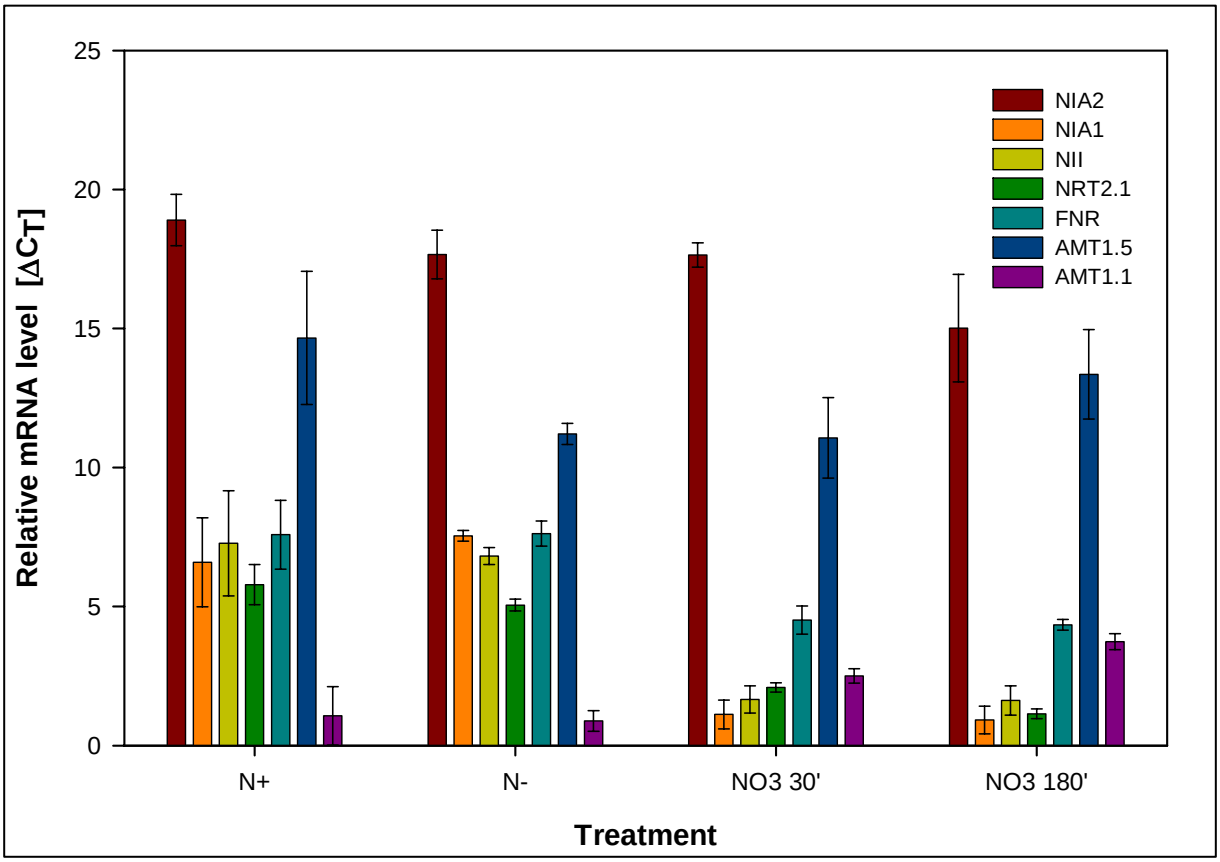


Figure 3. 13 Changes in expression of marker genes in *nia1nia2* mutant after N deprivation and nitrate re-addition.

Transcript abundances expressed relatively to *UBQ10* in the $\log_2$ scale (ΔC_T), are inversely proportional to the height of the bars. Error bars – SE from two biological and two technical replicates of qPCR reaction (n=4). N+ - full nutrition, N- nitrogen deprivation, NO3 30' – 30 minutes after nitrate re –addition.

Transcripts for *NIA2* were essentially un-detectable as expected, given the T-DNA insertion into this gene (Figure 3.13). On the other hand, transcript levels for the mutant *NIA1* gene, which contains only a point mutation, were normal and responded to changes in N in the same way as the wild type (Figure 3.13) Other reference genes, including *ATNRT2.1, NII,* and *FNR* also responded in the same way in both the mutant and the wild type. Interestingly, the ammonium transporter genes *ATAMT1.1* and *ATAMT1.5* did not respond in the same way in the mutant as in the wild type: transcript levels did not increase in the mutant following N-deprivation (Fig 3.13).

Most (13) of 16 N-regulated TF genes showed no difference in responses to nitrogen in the mutant background as compared to the wild type (Table 3.5). For example, *AT3G25790* was repressed slightly during N-deprivation and induced by more than 100-fold by nitrate re-addition in both the mutant and the wild type. One gene, *AT1G01530* did not respond to nitrate re-addition in the mutant although, it was strongly induced by this treatment in the wild type. This gene also responded differently in the mutant compared to the wild type, following N-deprivation, i.e. *AT1G01530* was induced in the mutant but not in the wild type. In contrast another gene, *PAP1* (*AT1G56650*) which was strongly induced by nitrate deprivation in the wild type was not induced in the mutant (Table 3.5). Finally *AT3G51910* was induced by nitrate re-addition in the mutant but not the wild type.

Table 3. 5 N-regulation of TF genes in WT and *nia1nia2* mutant plants

AGI	*nia1nia2*							Col-0						
	FN		N-	NO3 30'		NO3 180'		FN		N-	NO3 30'		NO3 180'	
	mean	SE	mean	mean	SE	mean	SE	mean	SE	mean	mean	SE	mean	SE
AT1G01530	0,08	0,04	1	0,89	0,20	0,38	0,09	3,95	2,97	1	48,55	17,46	8,03	0,37
AT1G02230	1,22	0,90	1	3,36	2,76	2,19	0,90	0,56	0,29	1	32,51	7,44	5,49	4,60
AT1G13300	3,52	2,13	1	42,38	6,69	90,75	3,03	2,73	0,64	1	31,86	4,78	19,58	6,59
AT1G35560	2,74	0,47	1	5,24	0,55	16,04	0,77	1,19	0,55	1	24,14	1,51	3,09	1,32
AT1G56650	1,55	0,57	1	19,11	7,75	16,11	4,78	0,04	0,03	1	1,68	0,24	0,07	0,02
AT1G68880	2,33	0,74	1	6,67	3,41	28,03	2,48	35,70	5,35	1	233,42	64,80	171,04	23,50
AT1G76350	1,01	0,37	1	16,49	3,13	17,57	6,69	16,93	4,78	1	72,19	14,47	13,82	13,45
AT2G22200	1,22	0,26	1	4,55	0,85	6,24	3,47	0,97	0,01	1	41,26	0,10	10,57	8,20
AT2G23740	2,07	1,09	1	1,37	0,41	79,30	79,23	0,63	0,35	1	29,29	27,75	14,87	12,61
AT2G33550	0,63	0,22	1	4,01	1,03	21,90	13,75	2,89	1,44	1	48,68	3,47	33,51	21,41
AT2G33720	3,95	3,37	1	7,29	4,43	3,18	2,97	2,36	0,22	1	27,37	0,37	4,20	3,67
AT2G43500	3,23	0,07	1	20,87	2,74	21,28	8,85	3,79	1,37	1	53,01	6,61	3,69	3,14
AT3G25790	5,05	2,53	1	244,04	68,60	257,29	101,31	5,18	0,68	1	358,96	74,15	400,37	153,78
AT3G51910	18,09	3,80	1	34,16	2,69	61,09	27,18	0,03	0,01	1	1,24	0,47	2,77	1,68
AT4G26150	12,47	6,65	1	30,63	2,53	174,67	100,71	10,14	2,37	1	58,37	6,78	18,04	8,64
AT4G38340	19,35	15,77	1	82,86	42,72	90,36	56,27	13,92	4,34	1	1649,9	212,50	584,54	250,55

Table legend:

Transcript levels of TF genes under various N regimes expressed as relative to the level at N-deprivation.
FN – full nutrition,
-N – 48h low nitrate medium plus 3hours 3mM KCl ,
NO3 30' – 30min after 3mM KNO_3 re-addition to N-deprived plants,
NO3 180' – 180min after 3mM KNO_3 re-addition to N-deprived plants,
Mean – average expression determined from two independent biological replicas and two technical replicates,
SE – standard error determined from two independent biological replicas and two technical replicates (n=4)

3.A.4 Functional characterisation of the N-regulated TF genes

For the functional characterisation of TF genes a two-pronged approach consisting of both gain and loss of function was taken (Figure 3. 14). Homozygous T-DNA or transposon knock-out lines were used for loss-of-function studies. The gain of function approach involved cloning of selected TF gene into binary vectors for constitutive or inducible over expression, plant transformation, selection of the transgenic lines showing increased expression of the gene of interest, and functional characterisation of the transgenic lines. GATEWAY™ technology was used for all cloning steps. This part of the work was done together with Dr Jens-Holger Dieterich (Molecular Genomics Group, MPI-MP Golm, Germany).

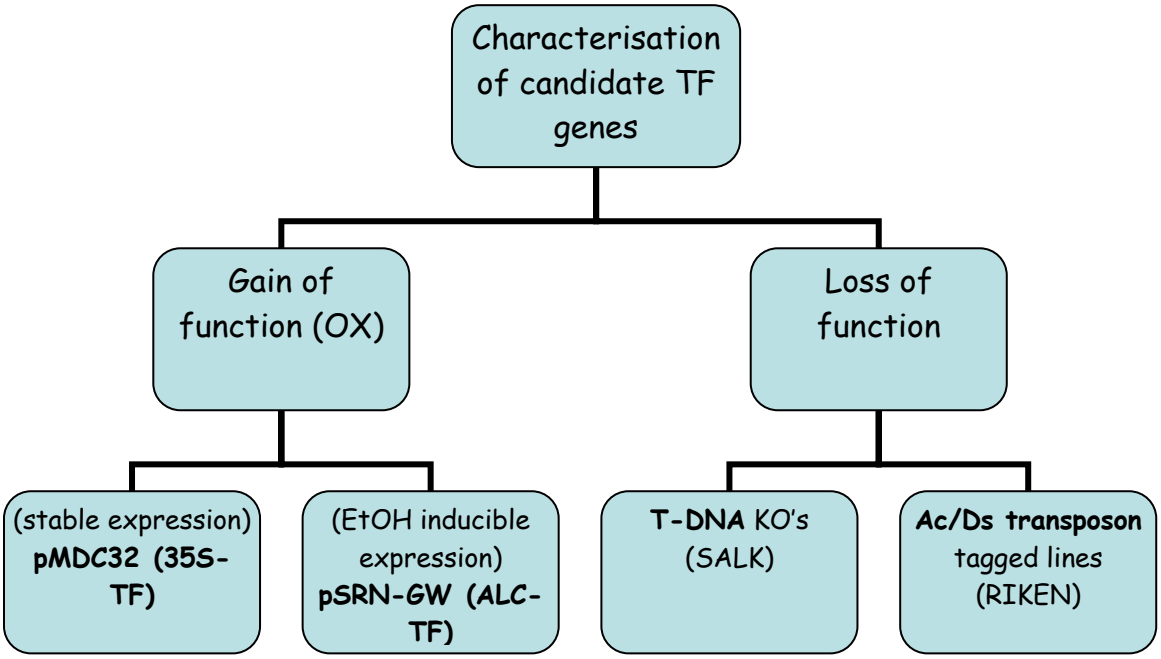


Figure 3. 14 Overview of the approaches used for functional characterisation of N-regulated TF genes

3.A.4.1 Genes cloning with the GATEWAY™ system

Two approaches were used to clone TF ORFs into GATEWAY donor vectors:

1. TF ORFs (from the first ATG to the stop codon) were PCR-amplified using primers containing *attB* sequences (listed in Appendix B), which facilitates cloning into the entry vector pDONR207 (description in Appendix A) via GATEWAY™ BP reaction,

2. TF ORFs were cloned directly into the vector pENTR™/D-TOPO (description in Appendix A), using the TOPO cloning system (Invitrogen) following PCR amplification using gene-specific primers without *attB* sequences (listed in Appendix B). The forward primer always contained the sequence CACC at 5' end which is recognised by topoisomerase, and facilitate entry of the PCR product into the vector.

Initially we were able to amplify only 8 out of 15 TF genes when cDNA as a template for PCR reactions (Figure 3.15 A, Table 3.6). Using genomic DNA as template gave 100% successful amplifications, even for the longest ORFs including intron sequences, like 3,7 kb (Figure 3.15 A, Table 3.6). Seven out of seven genes we tried were successfully amplified from genomic DNA. The easiest approach was to amplify ORFs from full length clones obtained from publicly available stocks, like ABRC or RIKEN. This gave also 100% successful amplifications (Figure 3.15 C, Table 3.6). Two TF genes were already cloned into pENTR vectors and ready for further steps of GATEWAY cloning. pENTR-bZIP8 construct was kindly provided by Dr. Wolfgang Dröge-Laser (Albrecht-von-Haller-Institut; Univeristy of Göttingen, Germany) and pENTR-MAF3 construct was provided by Dr. Oliver Ratcliff (Mendel Biotech Inc., USA).

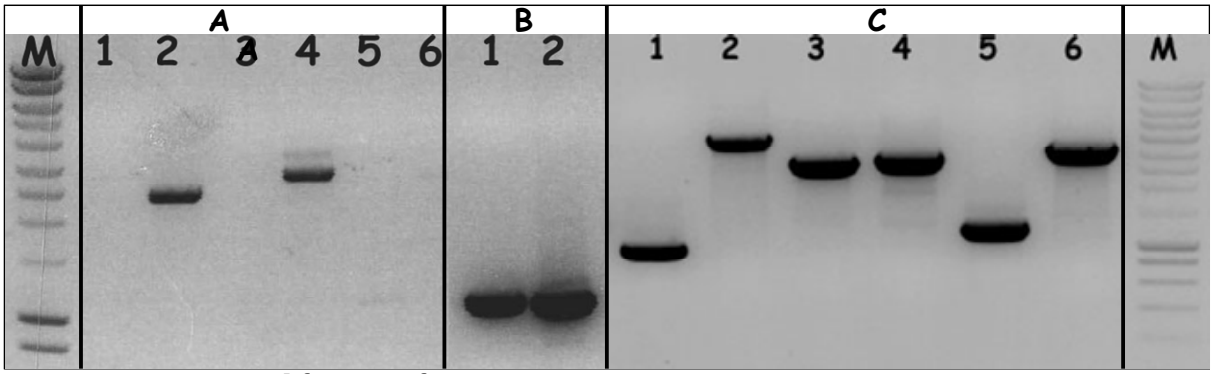


Figure 3. 15 PCR amplification of some TF genes

50 ng of genomic cDNA (A), 1pg of plasmid DNA (B) or 50 ng of cDNA (C) was used as template for PCR reaction using gene specific primers and high fidelity *Pfu* DNA polymerase. Amplified ORFs: (A) 2 – AT1G76350, 4- AT2G43500 (B) 1-AT1G13300, 2- AT1G35560, (C) 1-AT1G01530, 2-AT2G43500 , 3-AT1G76350, 4-AT2G23740, 5-AT2G33720, 6-AT4G38340, M- DNA leader, band sizes (from top): 12, 10, 8, 6, 4, 3, 2.5, 2, 1.5, 1, 0.8, 0.6, 0.4, 0.2. kb.

Restriction analysis of plasmid DNA was used to check for successful cloning of ORFs (e.g. Figure 3.16) as described in Materials and Methods. High efficiency of LR reactions was obtained most of the times, three to five of five *E.coli* colonies showed the presence of insert into both destination vectors, when analysed by restriction with *EcoRV* (Figure 3.16 A) *A* .

tumefaciens transformed with TF ORFs cloned into destination vector showed 100% correct *EcoRV* restriction patterns (Figure 3.16 B).

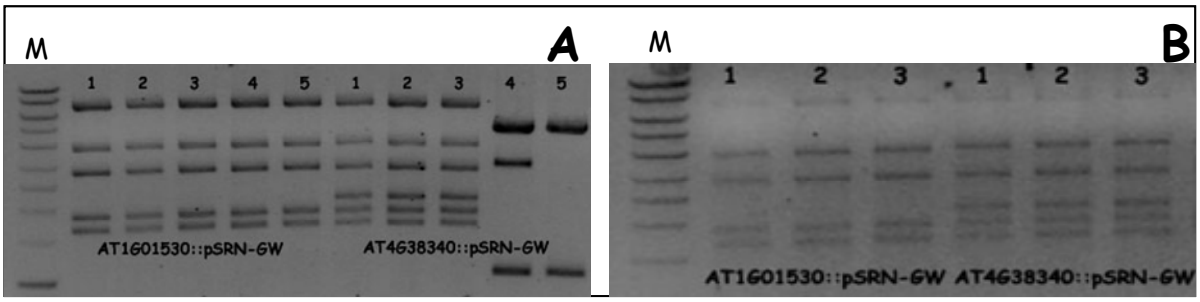


Figure 3. 16 Restriction analysis of the recombinant destination vectors cloned into *E.coli* (A) or *A .tumefaciens*. (B)

1µg of plasmid DNA from TF ORFs genes cloned into pSRN-GW was digested with *EcoRV*. Restriction patterns were correct, except clones for 4 and 5 on panel A. M- DNA leader, band sizes (from top): 12, 10, 8, 6, 4, 3, 2.5, 2, 1.5, and 1 kb.

Table below shows current status of cloning selected TF genes and screening for transgenic lines, done in collaboration with Dr Jens-Holger Dieterich (Molecular Genomics Group, MPI-MP Golm, Germany)

Table 3. 6 Current status of cloning and plant transformation for 17 N-regulated TF genes.

AGI	cDNA[1]	gDNA[2]	cDNA pool	cDNA clone	gDNA	ENTR clone	S	ALC clone	35S clone	ALC in plants	35S in plants	T1 ALC	T1 35S
AT1G76350	2427	2675			+								
AT1G01530		1023			+	+	+	+	+	+	+		
AT4G38340		3119	+		+	+	+	+	+	+	+		
AT3G25790	1055		+			+	+	+	+	+	+	+	+
AT1G13300	1035			+		+	+	+	+	+	+	+	+
AT1G02230		3745			+	+	+	+	+	+	+	+	+
AT2G22200	786		+	+		+	+	+	+	+	+	+	+
AT1G56650	747		+			+	+	+	+	+	+	+	+
AT1G35560	1026			+		+	+	+	+	+	+	+	+
AT2G23740		2770			+	+	+	+	+	+	+		
AT3G51910	819		+			+	+	+	+	+	+	+	+
AT4G26150	1059		+			+	+	+	+	+	+	+	+
AT2G33720		1471			+	+	+	+	+	+	+		
AT2G43500		3736	+		+	+	+	+	+	+	+		
*AT2G33550**	945			+		+	+	+	+	+	+	+	+
AT2G33550	945	945	+			+	+	+	+	+	+	+	+
AT1G68880			1	1	1	+	+	+	+				
AT5G65060			2	2	2	+	+	+	2		2		

Table legend:

[1] length of the cDNA sequence in bp
[2] length of the genomic sequence in bp
cDNA pool, cDNA clone, gDNA – PCR amplification using cDNA pools, clones or genomic DNA as template; ENTR clone – ORF introduced into GATEWAY™ entry clone and correct ORF sequence obtained from the

clone (S). ALC and 35S clone – ORF introduced into pSRN-GW and pMDC32 destination vectors respectively. ALC and 35S in plants – Col-0 *Arabidopsis* transformed with pSRN-GW and pMDC32 constructs and T1 seeds obtained (T1 ALC and T1 35S)
* sequence of that cDNA clone (RIKEN) differs by 3 amino acids from the genomic DNA sequence from TAIR.
1- cDNA clone in pENTR1A vector kindly provided by Dr. Wolfgang Dröge-Laser (Albrecht – von – Haller Institut , Univeristy of Göttingen, Germany)
2- cDNA clone in pENTR vector kindly provided by Dr. Oliver Ratcliff (Mendel Biotech Inc., CA, USA) as well as T1 seeds of constitutive over-expressor (under 35S promoter), published in: Ratcliff et al. 2004.

3.A.4.2 Selection of over expressing lines

RNA was isolated from transformed T1 plants containing of constitutive or ethanol inducible TF constructs and subjected to Northern blot analysis using the DIG-labelling system of Roche. (Figure 3.17 left and right, respectively). RNA prepared from soil grown Col-0 was always run in parallel as a control in case of constitutive over-expressors and transgenic plants treated with water were controls for ethanol inducible lines.

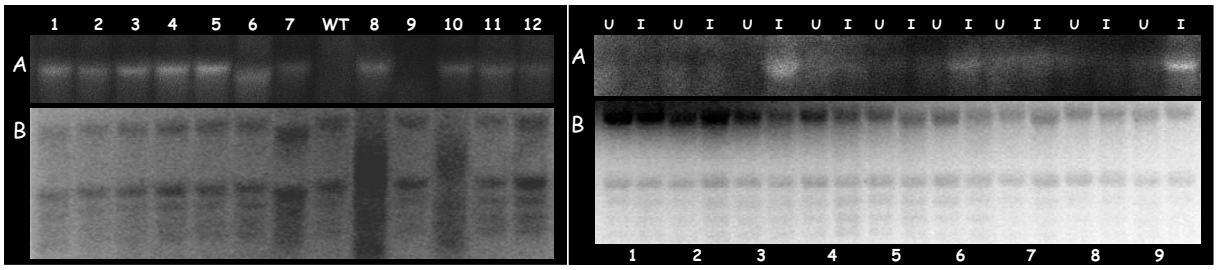


Figure 3. 17 Northern blot analysis of plants constitutively overexpressing TF gene *AT4G38340* (left) and inducible overexpressing TF gene *AT2G45300* (right)

(B) 4 µg of total RNA was extracted from: 12 transgenic plants from T1 generation (lines 1-12) and Col-O control (WT) **(left panel)** or from 9 transgenic plants from T1 generation (lines 1-9), after ethanol (I) or water (U) treatment **(right panel)** than separated by electrophoresis **(B)** and hybridised to PCR generated probe, specific to ORF **(A)** as described in materials and methods

Typically, endogenous expression of target TF genes was below the detection limit of Northern blots (Fig 3.17 A, line WT). On the other hand, transgene expression driven by the 35S promoter was clearly detected on such blots. Efficiency of over-expression driven by 35S promoter was good (70% of OX lines in average from 15 created constructs) in T1 generation of transgenic plants. In contrast, efficiency of over-expression driven by AlcA promoter was poor, reaching just 24% in T1 generation (in average from 13 created constructs). Moreover, for the ethanol inducible TF lines, high basal expression of TF genes in uninduced samples (Figure 3,17 left, lines 4U and 7U) was sometimes observed. Only ethanol inducible transgenic lines with minimal basal expression were chosen for further analysis (e.g. Figure 3.17 right, lines 3, 6 and 9). Seeds were harvested from selected T1 over-expressor plants. Visible phenotypes were recorded while T1 plants were grown in the greenhouse. T2 seeds

were screened for antibiotic resistance and T2 plants were grown in the greenhouse and selected for TF over-expression as described above (about 7 plants from each T1 line). T2 lines that no longer segregate for antibiotic resistance will be taken for detailed molecular and physiological phenotyping of T3 generation.

3.A.4.3 Growth phenotypes of selected transgenic lines

The majority of transgenic lines showed no obvious phenotype in the T1 generation, when grown under standard greenhouse conditions. We also observed no visible phenotypes in all EtOH inducible lines grown under greenhouse conditions. However, constitutive over-expression of three TF genes yielded interesting phenotypes.

About 50% of the T1 plants containing *35S-AT1G01530* (*AGL28,* MADS) plants showed curled rosette leafs (Fig. 3.18). However, this phenotype was not observed in stable T2 generation (pictures not shown).

Figure 3. 18 Phenotypic variation in the *35S-AT1G01530*

Seven transgenic plants from T1 generation and Col-O wild type, were grown for one month in standard greenhouse conditions and photographed.

The other phenotype was observed for the family member in the transgenic lines. Expression of *AT2G33720* was strongly (18-fold) induced upon the nitrogen starvation and unaffected 30 min after nitrate re-addition. About 50% of T1 plants expressing the ARP TF *35S-AT2G33720* shows abnormal inflorescence shape, producing "cauliflower-like" structures (Fig. 3.19 A) Flowers were reduced size and male-sterile, because no pollen sacs were produced (Fig. 3.19 B).This phenotype was observed only for the first inflorescence. Subsequent inflorescences emerging from the side stems were wild-type like and produced normal amounts of seeds.

Figure 3. 19 Flower phenotype of *35S-AT2G33720*

(A) 4 weeks old transgenic plants inflorescence **(B)** Single flower from transgenic inflorescence compared to the same age WT-flower.

The last TF over-expressor which gave visible phenotype so far was the line. That MYB-like TF gene expression is strongly (28 fold) induced 30 min after nitrate re-addition and its unaffected under N-starvation period. About 50% of T1 plants containing the MYB-like over-expression construct *35S-AT2G33550* generation showed a strongly stunted growth (Figure 3.20) The stunted plants produced small amount of the seeds when grown under standard greenhouse conditions. The phenotype is also stable in the T2 generation (selected dwarfed plants from T1 produced also dwarfed T2 progeny). The dwarf phenotype segregated also in the T2 generation, where about 50% plants looked like wild type.

Figure 3. 20 Phenotypic variation in the *35S -AT1G33550a*

Nine transgenic plants (1-13) from T1 generation and wild type (Col-0), were grown for six weeks in standard greenhouse conditions and photographed.

We cloned *AT2G33550* from cDNA and also obtained cDNA clone from RIKEN. However, the two sequences were not the same. The RIKEN clone contained additional 9 bp annotated as intron sequence in TAIR database. The difference results in a 3 amino acid insertion in low conserved region of *AT2G33550* sequence in the RIKEN clone (Figure 3.21).

```
ATGGCTCTGG AACAGTTAGG ATTAGGAGTG AGCGCCGTTG ACGGTGGAGA GAACAGTAGC GCGCCGTCAA ATGACGGTGG AGATGACGGC GTTAAGACGG      100
MetAlaLeuG luGlnLeuGl yLeuGlyVal SerAlaValA spGlyGlyGl uAsnSerSer AlaProSerA snAspGlyGl yAspAspGly ValLysThr

CGAGACTTCC TCGTTGGACG AGACAAGAGA TTCTGGTTCT GATTCAAGGG AAGAGAGTGG CGGAGAACAG AGTCCGGCGA GGGAGAGCGG CGGGTATGGC      200
laArgLeuPr oArgTrpThr ArgGlnGluI leLeuValLe uIleGlnGly LysArgValA laGluAsnAr gValArgArg GlyArgAlaA laGlyMetA

TCTCGGGTCG GGTCAAATGG AGCCTAAATG GGCTTCTGTT TCGTCTTACT GTAAACGTCA CGGTGTAAAT CGTGGGCCGG TTCAGTGCCG GAAAAGATGG      300
aLeuGlySer GlyGlnMetG luProLysTr pAlaSerVal SerSerTyrC ysLysArgHi sGlyValAsn ArgGlyProV alGlnCysAr gLysArgTr

AGCAATCTCG CCGGAGATTA TAAGAAGATT AAAGAATGGG AGTCTCAGAT TAAGGAAGAG ACTGAGTCCT ATTGGGTTAT GAGGAATGAT GTTCGTAGAG      400
SerAsnLeuA laGlyAspTy rLysLysIle LysGluTrpG luSerGlnIl eLysGluGlu ThrGluSerT yrTrpValMe tArgAsnAsp ValArgArg

AGAAGAAGCT TCCTGGTTTT TTCGATAAGG AGGTTTATGA TATTGTTGAC GGTGGTGTGA TTCCTCCGGC GGTTCCGGTT CTTTCGCTTG GATTGGCTCC      500
luLysLysLe uProGlyPhe PheAspLysG luValTyrAs pIleValAsp GlyGlyValI leProProAl aValProVal LeuSerLeuG lyLeuAlaP

GGCGTCAGAC GAGGGATTGT TGTCTGATTT AGATCGGAGA GAAAGTCCTG AGAAGTTGAA TTCTACTCCG GTGGCTAAAT CAGTTACTga tgttataGAC      600
oAlaSerAsp GluGlyLeuL euSerAspLe uAspArgArg GluSerProG luLysLeuAs nSerThrPro ValAlaLysS erValThrAs pValIleAs

AAAGAGAAGC AAGAAGCTTG TGTAGCAGAT CAAGGTGAAG GTAGAGTGAA AGAGAAACAG CCAGAAGCAG CAAACGTGGA AGGTGGATCG ACATCACAAG      700
LysGluLysG lnGluAlaCy sValAlaAsp GlnGlyGluG lyArgValLy sGluLysGln ProGluAlaA laAsnValGl uGlyGlySer ThrSerGln

AAGAGAGGAA GCGTAAACGG ACATCTTTTG GTGAAAAGGA AGAGGAAGAA GAAGAAGGAG AAACAAAGAA GATGCAGAAT CAGTTGATAG AGATACTAGA      800
luGluArgLy sArgLysArg ThrSerPheG lyGluLysGl uGluGluGlu GluGluGlyG luThrLysLy sMetGlnAsn GlnLeuIleG luIleLeuG

AAGAAACGGG CAGTTGTTGG CGGCACAGCT TGAGGTTCAG AATTTAAACT TAAAACTAGA CAGAGAGCAA AGAAAAGATC ACGGTGATAG CTTAGTCGCT      900
uArgAsnGly GlnLeuLeuA laAlaGlnLe uGluValGln AsnLeuAsnL euLysLeuAs pArgGluGln ArgLysAspH isGlyAspSe rLeuValAl

GTTCTCAATA AGCTCGCTGA TGCTGTGGCA AAAATCGCGG ATAAGATGTA G                                                          951
ValLeuAsnL ysLeuAlaAs pAlaValAla LysIleAlaA spLysMetTe r
```

Figure 3. 21 Sequence of *AT2G33550*

The circled sequence is present only in the RIKEN cDNA clone but not in TAIR database or in the cDNA obtained form RT in our lab.

Because both sequences seem to be present *Arabidopsis* transcriptome, we decided to transform independently plants with both sequences cloned in the destination vector. The transformed lines were named: *35S-AT2G33550a* (TAIR sequence) and *35S-AT2G33550b* (RIKEN clone). The dwarfed phenotype was also visible in about 50 % of the T1 *35S-AT2G33550b* plants (picture not shown) and propagated into T2 generation.

Both of the constructs introduced to plants independently resulted in the aberrant flower phenotype, depicted in Figure 3.22 shows. All flowers from dwarf plant were strongly reduced in size but fertile and able to produce pollen and small numbers of seeds.

Figure 3. 22 Flower phenotype of 35S-AT2G33550

(A) Single flower from transgenic inflorescence *35S-AT2G33550a* compared to WT-flower of the same age (Col-0). **(B)** Single flower from transgenic inflorescence *35S-AT2G33550b* compared to WT-flower of the same age (Col-0).

The strength of the dwarf phenotype in *35S-AT2G33550a* was also correlates positively with the expression of the transgene, determined by northern blot analysis. Plants showing no over-expression were looked like the wild type (Figure 3.23 line 14), whereas the plants with strong over-expression exhibit also strong phenotypes (Figure 3.23 lines 12 and 13).

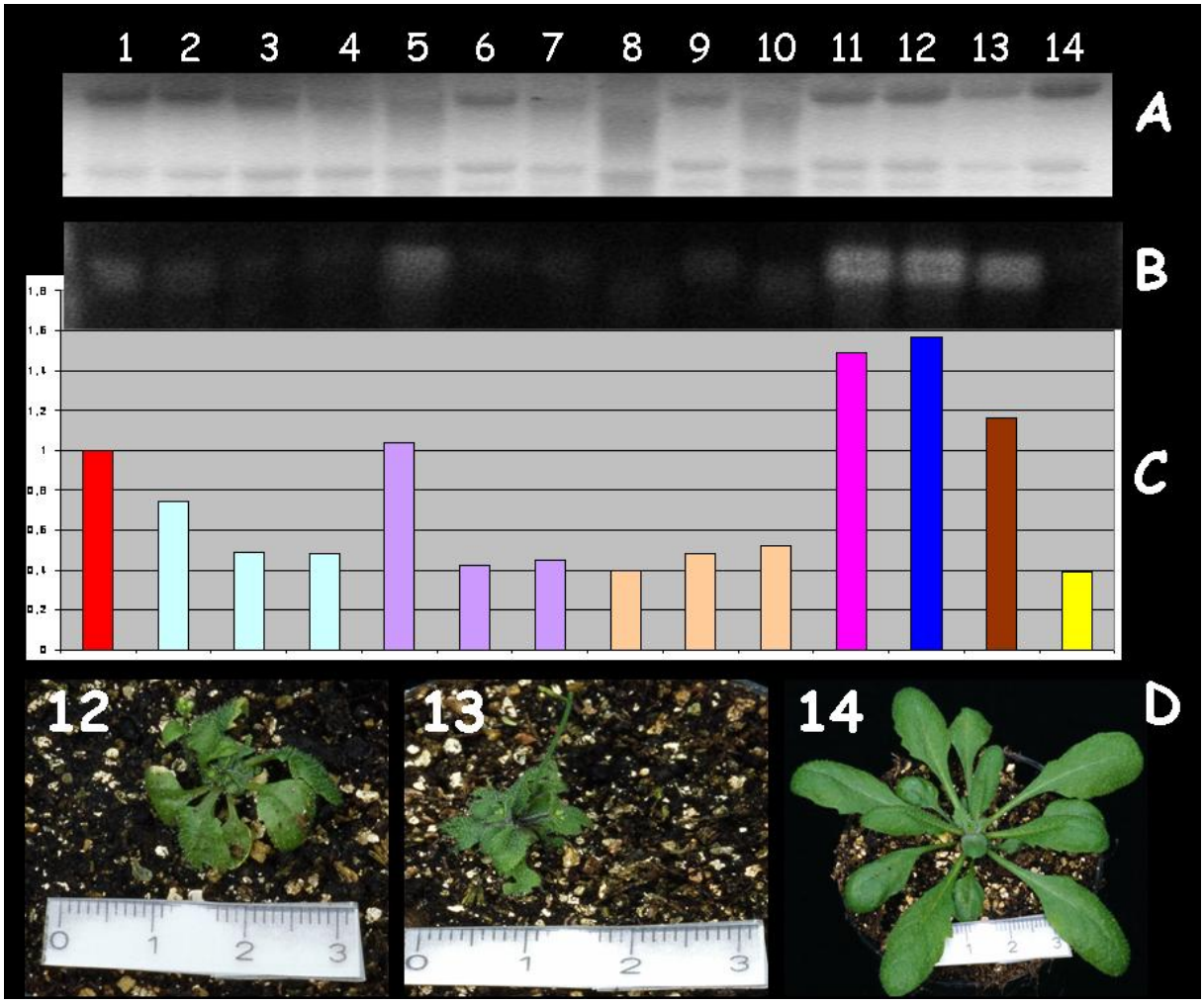


Figure 3. 23 Over-expression of *AT2G33550* leads to severely dwarfed phenotype

(A) 4 µg of total RNA was extracted from 14 transgenic plants from T2 generation (lines 1-14) and than separated by electrophoresis **(B)** Hybridisation was performed using PCR generated probe specific to ORF **(C)** Intensities of luminescent signal was measured on CCD photon counting camera as described in Materials and Methods **(D)** Phenotypes of some of 14 transgenic lines (number corresponds to that on panel A).

To check for additional visible phenotypes we screened for root architecture changes, as described in Materials and Methods. The length of primary roots was checked every other day, and the number of lateral roots was counted on day 16. Germination ration was checked also on all of the plates 48 h and 96 h after sowing. Analysis of the WT phenotype showed differences in growth depending on nitrogen source applied (Figure 3.24)

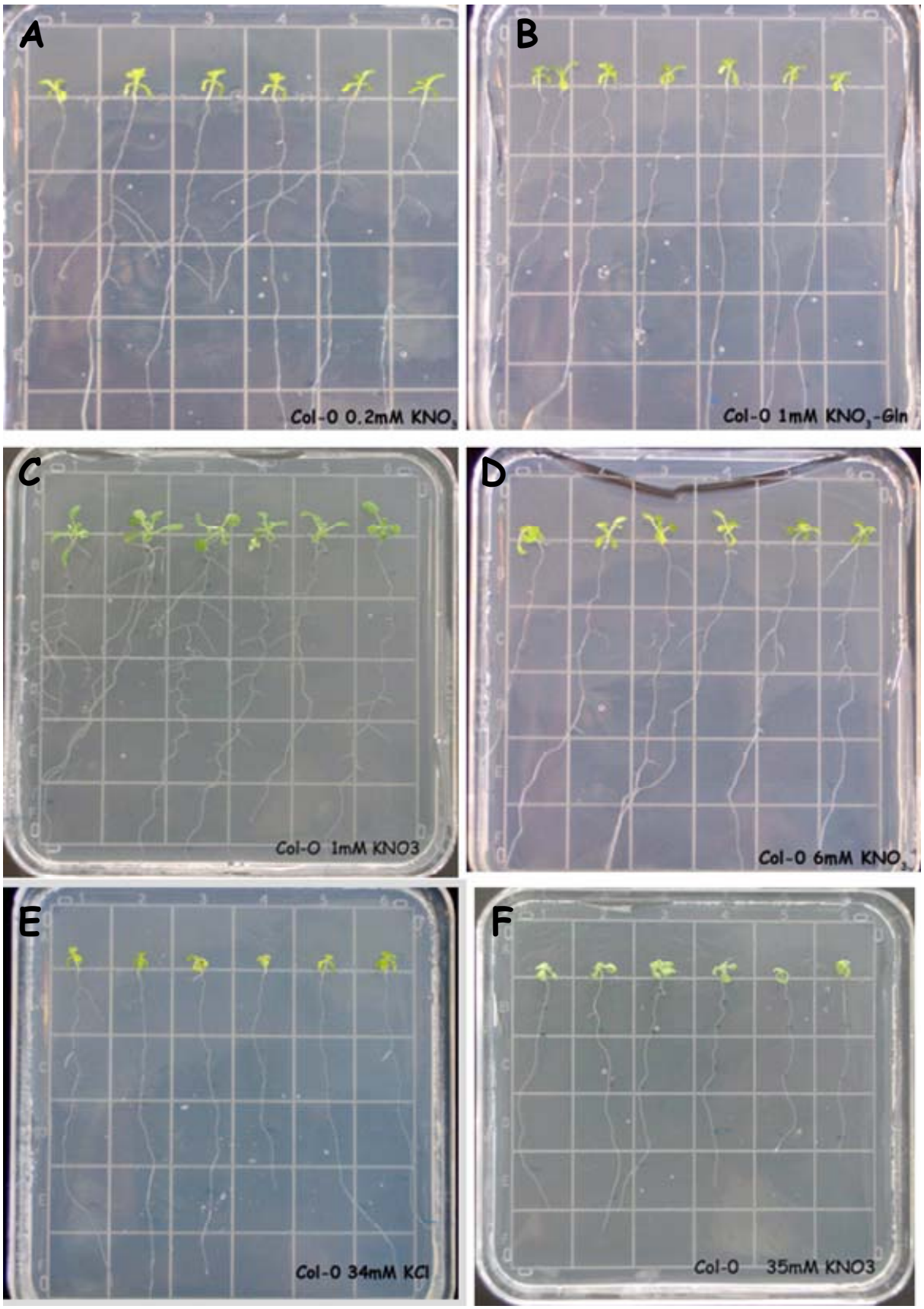


Figure 3. 24 Root architecture of Col-0 seedlings grown under various nitrate regimes

Col-0 seedlings were grown vertically on agar plates containing various nitrogen sources (indicated on the right bottom corner of each plate) for 14 days and photographed.

Optimal plant growth was observed on plates supplemented with 1 mM KNO_3 and 4 mM Gln (Figure 3.24 panel C). Growth on 1 mM without Gln reduced slightly shoot and root growth (Figure 3.24 panel B). When nitrate concentration was reduced to 0.2 mM and 4 mM Gln was added plants formed reduced numbers but longer lateral roots (Figure 3.24 panel A). Higher nitrate (6 mM) plus 4mM only slightly reduced number of lateral roots (Figure 3.24 panel D), but highest nitrate concentration (35 mM) reduced LR number to zero and resulted in very good shoot growth (Figure 3.24 panel F). However, the same effect was observed when plants were supplemented with 1 mM KNO_3, 4 mM Gln and 34 mM KCl. (Figure 3.24 panel E).

Three of independent transgenic lines, for each of: *35S-AT2G22200, 35S-AT1G3300* and *35S-AT3G25790* were tested for root architecture changes on the nitrogen regimes described above but showed no changes in root architecture compared to WT controls.

Number of lateral roots for three independent lines for *35S-AT2G22200*, measured 16 days after sowing is shown on Figure 3.25.

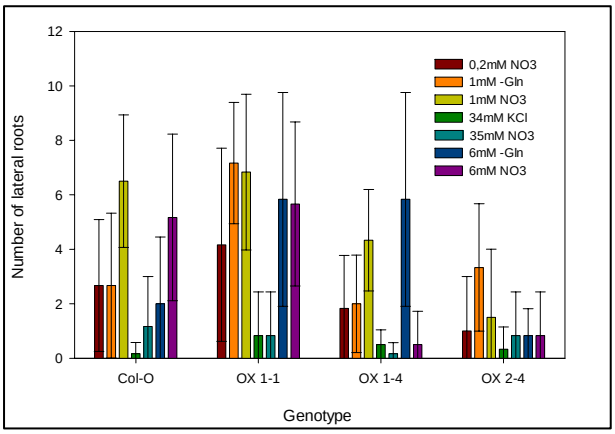


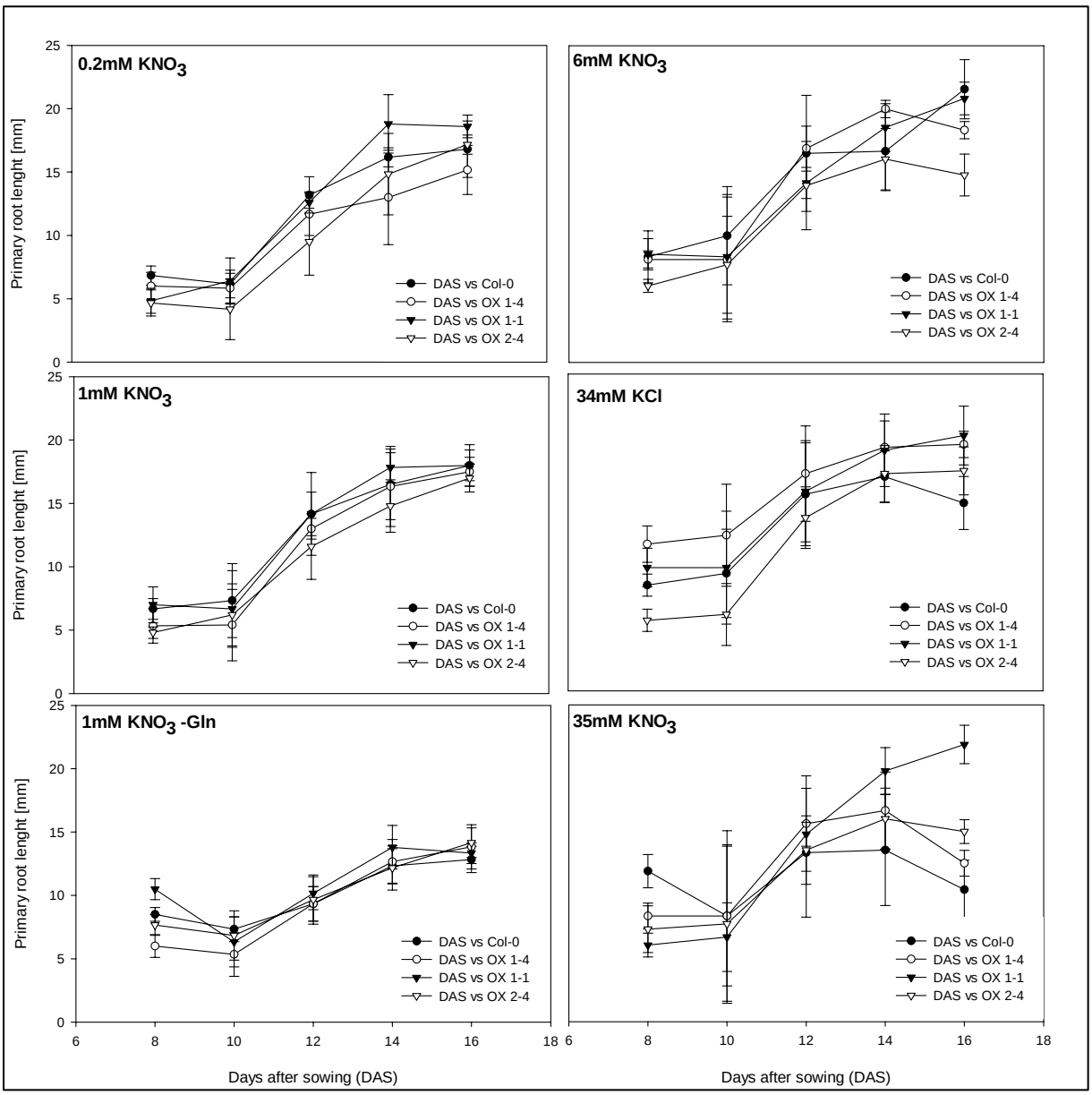
Figure 3. 25 Development of lateral roots of 35S-AT2G22200

Number of lateral roots was measured for six plants of: Col-0 wild type (closed circles), and three transgenic lines for *35S-AT2G22200*, 16 days after sowing. Bars represents average from six measurements and error bars represents SE (n=6).

Figure 3.26 on the next page shows kinetics of primary root growth for *35S-AT2G22200* and Col-0 wild type grown on various nitrogen regimes.

Both kind of analysis failed to far to show any significant differences between transgenic lines and corresponding wild type. Additionally, high biological variation within six plants grown on the same plate is apparent from figures 3.27 and 3.26. Results for two other TF constructs: *35S-AT1G3300* and *35S-AT3G25790* are very similar (data not shown) and show no differences to the wild type. We could not obtain any data *35S-AT2G33550a* because all three independent lines grew extremely poorly on all nitrogen regimes tested (data not shown).

Figure 3. 26 Kinetics of primary root growth of *35S-AT2G22200*



Length of primary root was measured for six plants of: Col-0 wild type (closed circles), *35S-AT2G22200* line 1-4 (open circles), *35S-AT2G22200* line 1-1 (closed triangles) and *35S-AT2G22200* line 2-4 (open triangles), grown on each indicated nitrogen regime. Data points represents average from six measurements and error bars represents SE (n=6).

3.A.4.4 Characterisation of TF knock-out mutants

Arabidopsis mutants impaired in selected N-regulated TF genes were obtained from two sources. First was SALK T-DNA insertion collection (88 000 lines covering 21700 genes). SALK lines were obtained as described before (Eckerd et al. 2003). *Arabidopsis* Col-0 plants were transformed with construct containing T-DNA, genomic DNA was be prepared, T-DNA flanking plant DNA was recovered and sequenced. Insertion site sequences were aligned with the *Arabidopsis* genome sequence data will are available via a web accessible graphical interface: T-DNA Express (http://signal.salk.edu/cgi-bin/tdnaexpress). Preference was given to mutants with insertions in exons to maximise the chance of complete loss of the gene function. Second resource used was RIKEN *Arabidopsis* Transposon mutants collection (11800 lines single copy *Ds* transposon potentially affecting 5031) Lines were created by crossing plants carrying *Ds* element, GUS as a reporter genes and hygromycin resistance gene, to plants carrying *Ac* element (transposase) in *Arabidopsis* ecotype Nössen as described in Kuromori et al. (2004). Transposon insertion sites of mutants were estimated by a BLASTN homology against the genome sequence database of *Arabidopsis* Columbia ecotype. The closest genes (predicted by AGI) to the transposon insertion sites were picked up. Data are available via the web accessible graphical interface: T-DNA Express (http://signal.salk.edu/cgi-bin/tdnaexpress). There are two major advantages of using transposon- as compared to T-DNA- tagged lines. First, there is no stochastic additional insertions (causing often an artificial phenotypes) in the selected line (Kuromori et al. 2004). Secondly, the insertion of the transposon in intron or exon sequence in the correct orientation lead to fusions of the GUS reporter gene to the promoter of the studied gene. In many cases the resulting GUS activity can be detected by sensitive histochemical staining, revealing the tissue-specific expression of genes (Kuromori et al. 2004).

3.A.4.5 Selection of homozygous T-DNA KO lines

Homozygous mutants were identified by PCR with primers that distinguished between wild type and mutant alleles (See Materials and Methods). A typical result for PCR-screening of a T-DNA mutant line is shown in Figure 3.27, which clearly shows the difference between wild type (lanes WT), heterozygous (lanes Hz) and homozygous (lanes Hm) mutant individuals.

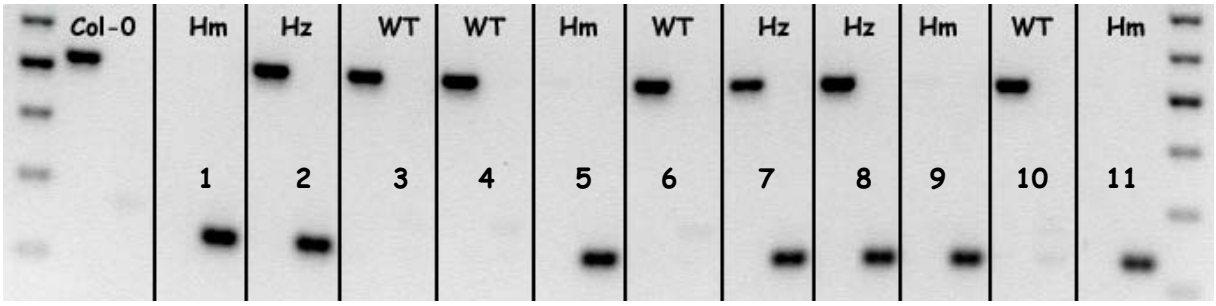


Figure 3. 27 PCR screening for the homozygous T-DNA insertion line for AT3G51910

Genomic DNA was prepared from 11 individual plants from lines SALK_080138 (lines 1-11) and from Col-0 wild type (line Col-0) used as a template for PCR reaction with T-DNA specific (right) or gene-specific (left) primer combinations as described in Materials and Methods. WT – wild type loci, Hz – loci heterozygous for T-DNA insertion, Hm – loci homozygous for T-DNA insertion. M – DNA marker, the sizes of the bands from top: 0.6, 0.5, 0.4, 0.3, 0.2 kb

SALK KO lines often contained multiple T-DNA insertions, which can make interpretation of observed phenotypes difficult (Ecker et al. 2003). To circumvent this problem, we routinely backcrossed all selected homozygous lines to the wild type. Col-O was always the mother plant and the KO-line the father for backcrosses. Two rounds of backcrossing, followed by "selfing" and selection of plants with homozygous alleles should be sufficient to remove additional insertions. However, the backcross process is very time consuming (ca. 4 months), so selection of lines homozygous for the mutant allele of interest, without regard for secondary mutations proceeded in parallel with backcrossing. Phenotypic analysis of such plants should at least show if loss of TF function has an effect on phenotype. If an interesting phenotype is observed, then complementation of the mutant phenotype with the functional version of TF gene or phenotypic analysis of independent mutations in the same gene would confirm the role of the TF gene. In contrast to SALK T-DNA lines, RIKEN Ac/Ds lines contain only a single insertion, therefore backcrossing was not performed on these mutants.

A summary of mutant lines that have been collected and studied to date in collaboration with Dr Jens Holger Dieterich (Molecular Genomics Group, MPI-MP Golm, Germany), is given in the table 3.7.

Table 3. 7 Overview of the selected KO lines, used for "loss of function" approach

AGI	SALK (S) / RIKEN (R)	Line inGH	HZ	BC1 seeds	BC1S1 seeds	Hz BC1S1	BC2 seeds
AT1G02230	S_055886	+	WT only				
AT1G02230	S_035023	+	8	+			
AT1G02230	S_145420	+					
AT1G02230	S_054446	+	4	+	+		
AT1G13300	S_067074	+	2	+	+	6	+
AT1G13300	R_11-3528-1	+	2				
AT1G68880	R_13-5286-1						
AT1G76350	S_027488	+					
AT1G76350	R_11-4080-1	+					
AT2G22200	S_064696	+					
AT2G22200	S_108879	+	8	+			
AT2G23740	S_050304	+					
AT2G23740	S_026224	+					
AT2G33550	S_047951	+	1	+	+	3	+
AT2G43500	S_028397	+	3	+	+	10	+
AT2G43500	S_026238	+	1	+	+	3	+
AT3G51910	S_080138	+	4	+	+	13	
AT4G26150	S_003995	+	4	+	+	7	+
AT4G38340	S_003418	+	4	+	+	8	+
AT5G65060	S_043198	+					
AT5G65060	S_044822	+					
AT5G65060	S_070820	+	WT only				

Table legend:

BC1 - first backcross to Col-0,
BCS1 - first backcrossed plant selfed,
BC2 - second backcross to Col-0,
Hz - number of homozygous plants
GH - greenhouse

3.A.4.6 Visible phenotypes in some of the selected lines

None of the selected homozygous KO lines from table 3.13, gave an aberrant phenotype when grown under standard conditions in the greenhouse. Mutants were also screened screening for the root architecture changes as described in the last section. None of the four lines screened showed changes in root architecture compared to WT controls. High biological variation within six plants grown on the same plate was also apparent, similar to that on figures 3.25 and 3.26 (data not shown). Germination ratio on all of plates was also determined 48 h and 96 h after sowing. Two individual homozygous KO lines for the gene *AT1G13300* exhibited reduced germination ratio as compared to Col-0 seeds (of the same age) after 48 h but not after 96 h (Figure 3.28 A). Similar results were obtained for two

individual homozygous KO lines for the gene *AT2G33550*, except that lower germination rates were observed at both 48 and 96 h after sowing (Figure 3.28 B)

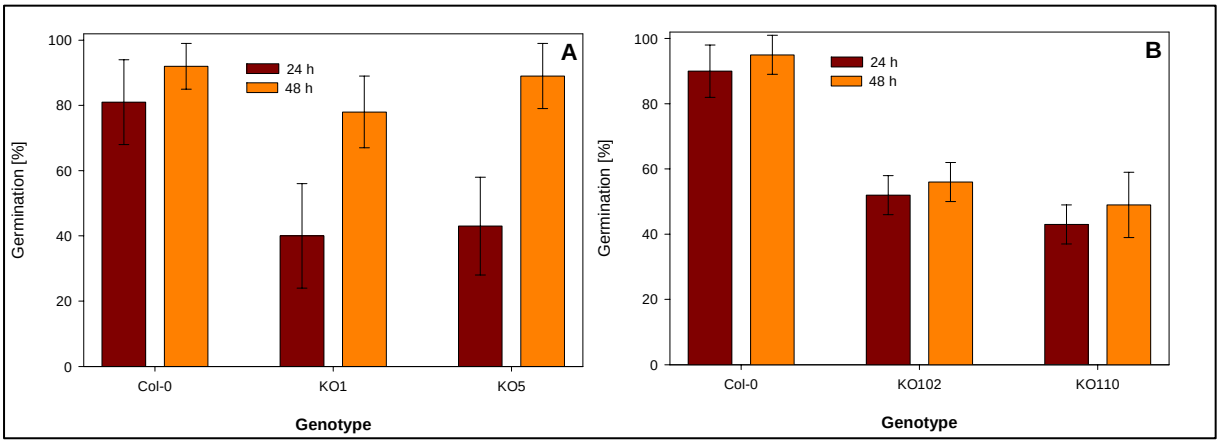


Figure 3. 28 Germination ratio for homozygous T-DNA KO lines for two TF genes

Germination ratio shown for two KO lines (progeny of homozygous individuals selected by PCR) for TF gene *AT1G13300* **(A)** and *AT2G33550* **(B)** Percentage of the plants with emerged roots was measured across about 70 seeds on 6 agar plates. Bars represents average percentage from six plates and error bars represent SE (n=6).

A second phenotypic screen, measured flowering time for plants grown under standard greenhouse conditions. Plants were stratified 4 days in 4°C then sow on ½ MS supplemented with 0.5% sucrose, and grown under constant light at 22°C. After one week, 36 plants of equal size from each line were transferred to fresh plates, to give them more growing space. After a further two weeks, plants were transferred to long-day chamber. Three plants of each line, randomised, were planted in 12 cm pots and put in the greenhouse for an additional two weeks. The number of opened floral buds was measured every day between day 21 and 38 after sowing. Three individual homozygous knockout lines for the following genes were tested: *AT2G43500, AT4G26150, AT4G38340* and *AT3G51910*. Mutant lines of *AT3G51910*, showed marked delay in the flowering time compared the WT (Figure 3.29). On average, there was a 2 day delay in the time taken for 50% of the mutant plants to show the first open floral buds, compared to the wild type

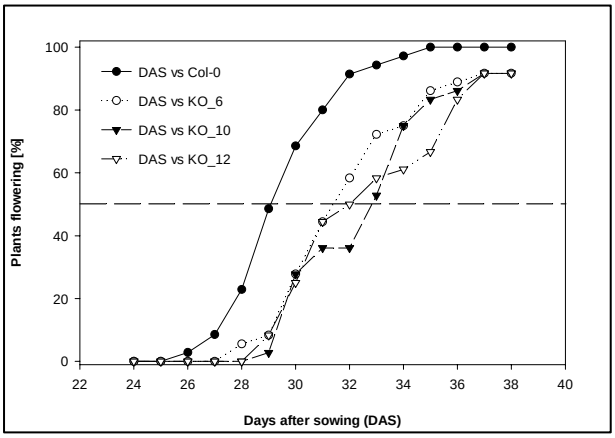


Figure 3. 29 Flowering time point for three T-DNA knock-out mutant lines for *AT3G51910* and WT

Percentage of the flowering plants was measured for 36 plants of Col-0 (closed circles), and three KO lines (progeny of homozygous individuals selected by PCR) for TF gene *AT3G51910*: line 6 (open circles), line 10 (closed triangles) and line 12 (closed triangles), over 14 day period.

AT3G51910 (*HSFA7A*) is a heat-shock family member that was repressed upon the nitrogen deprivation in Col-0. Interestingly *HSFA7A* is preferentially expressed in flowers, according to AtGenExpress (http://web.uni-frankfurt.de/fb15/botanik/mcb/AFGN/atgenex.htm).

3.B Identification of N-regulators of *AtNRT2.1* expression – A forward genetic approach

To complement the reverse genetics approach to identify N-regulation in *Arabidopsis*, a complementary forward genetic approach was devised. The basic strategy was to generate EMS mutants of a $P_{NRT2\text{-}1}$-*LUC* reporter line and screen the mutant population for de regulation of the N-regulated reporter. It was hoped that such a screen would lead to genes encoding not only TFs but also proteins involved in up-stream signalling events.

3.B.1 Preparation of $P_{NRT2\text{-}1}$-*LUC* lines for EMS mutagenesis

A 1.7 kb fragment 5' of the start codon of the *ATNRT2-1* gene was amplified by PCR, sequenced and cloned at the 5' end of the *LUC* gene of binary vector pZPXOmegaL+ (description in the Appendix A). A schematic representation of the resulting $P_{NRT2\text{-}1}$-*LUC* reporter construct, flanked by the left and right borders of T-DNA is shown below (Figure 3.30)

Figure 3. 30 Scheme of the reporter construct used for *LUC* activity screening

Abbreviations: LB – left border T-DNA sequence, RB – right border T-DNA sequence, $Gent^R$ – gentamycin resistance

Arabidopsis was transformed with the reporter construct, and gentamycin resistant plants exhibiting N-regulated *LUC* activity were isolated. Segregation analysis based on antibiotic resistance was used to estimate the number of T-DNA inserts in each line. Only the lines showing a resistant/sensitive ratio in the T_2 generation of 15:1, indicating a double-insertion, were selected. It was expected that a double insertion of $P_{NRT2.1}$-*LUC* into reporter lines would reduce false positives resulting from mutations in the introduced construct.

Seed from T_2 lines containing two copies of the $P_{NRT2.1}$-*LUC* construct were bulked and 300 seeds germinated and tested for *LUC* activity. Line number 9, for which all seeds were *LUC* + indicating homozygosity of *LUC* T-DNA insertions, was selected for EMS mutagenesis. Presence of double insertion in line 9 was confirmed by Southern blotting, as described in Materials and Methods. All individual 15 plants from T3 generations showed two bands on the blot, when probed with *LUC* gene (Figure 3.31).

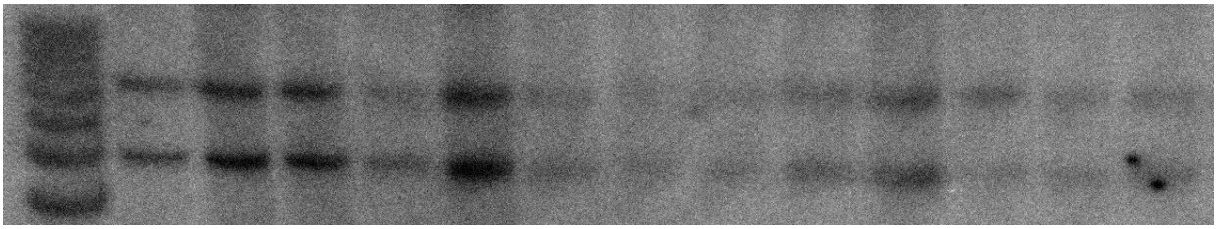


Figure 3. 31 Southern blot analysis of PNRT2.1-*LUC* line number 9 hybridised with *LUC* gene probe

20µg of *BamHI* digested genomic DNA isolated from each of 13 T3 lines was loaded and probed with a PCR amplified and labelled *LUC* gene as described in Materials and Methods. M –1kb DNA ladder, band sizes from top: 12.216, 11.198, 10.180, 9.162, 8.144, 7.126, 6.108 kb.

To investigate if the two T-DNA insertions in the line 9 are linked, individual plants were backcrossed to Col-O wild type. Progeny of the backcross was than selfed and the resulting were analysed for segregation of gentamycin resistance and *LUC* activity. In all cases the antibiotic and *LUC* activity segregated in 3:1 ratio, which indicates that, the insertions were not linked (data not shown).

3.B.2 N-regulation of $P_{NRT2.1}$-*LUC* expression in line 9

The *Arabidopsis ATNRT2.1* gene is repressed by growth of plant on media containing high concentrations of reduced-N, de-repressed by N-deprivation and induced by nitrate in the absence of other sources of N (Nazoa et al. 2003, Lejay et al. 2003, Okamoto et al. 2003, Orsel et al. 2002, Gansel et al. 2001, Zhuo et al. 1999). Thus, we envisaged two types of screening for mutants with altered $P_{NRT2.1}$-*LUC* expression:

1. Expression of *LUC* under normally repressing conditions,
2. Absence of *LUC* expression under NO_3^- inducing conditions,

To establish robust screens, expression of *LUC* activity in $P_{NRT2.1}$-*LUC* line 9 was monitored under different growth conditions. Expression of $P_{NRT2.1}$-*LUC* was repressed by growth of plants on ½ MS which contained high concentrations of NH_4^+, Gln, Glu, and Asp (Figure 3.32, panels B-F). The highest level of *LUC* activity were found in plants grown on 1mM KNO_3 as a sole source of N (Fig 3.32, panel A). Plants grown on pure nitrate showed best germination and fitness overall. Thus, $P_{NRT2.1}$-*LUC* was regulated by N in the same way as the endogenous *ATNRT2.1*. However, growth on ½ MS did not fully suppress expression of P_{NRT2-1}::*LUC* . Therefore, other N-sources were tested to identify conditions for complete repression of *LUC* and more robust mutant screening. Lowest *LUC* activity was observed on 10 mM Arg as the sole N-source (data not shown) but plant growth was extremely retarded

making this condition unsuitable for mutant screening. Slightly better germination and growth was obtained on 5 mM Asn, (Figure 3.32, panel B) although *LUC* activity in roots was never completely absent. Likewise, ammonium or glutamine did not completely repress combination of ammonium and glutamine led to formation of many lateral roots but did not inhibit $P_{NRT2\text{-}1}$:: *LUC* activity completely (Figure 3.32, panel E and F, respectively).

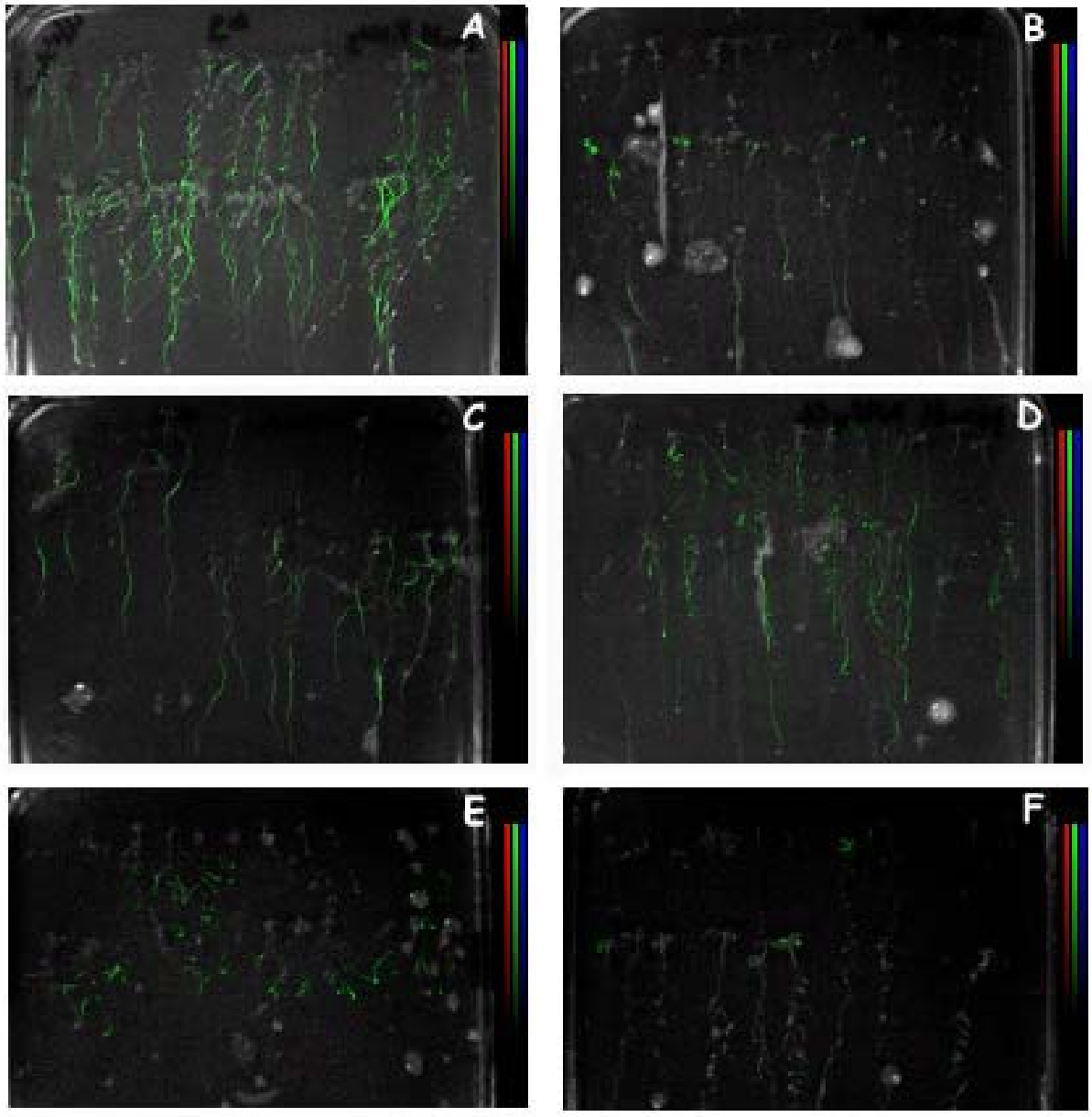


Figure 3. 32 Nitrogen influence of *LUC* reporter gene activity under control of *ATNRT2.1* promoter

Seedlings of the $P_{NRT2.1}$-*LUC* line 9 were grown vertically on agar plates containing :1mM KNO_3 (**A**), 5mM Asn (**B**), 10mM Gln (**C**), 10mM NH_4Cl (**D**), 10mM Glu (**E**), 5mM NH_4Cl and 10mM Gln (**F**) as a sole N-source for 10 days. LUC activity assay was performed as described in Materials and Methods

Plants grown on ½ MS – N plus 1 mM KNO_3 consistently exhibited high *LUC* activity and good growth. Therefore, this growth medium was chosen for the first screen to identify mutants unable to induce P_{NRT2-1}:: *LUC* expression.

3.B.3 Pilot EMS mutagenesis

A pilot EMS-mutagenesis experiment was performed to determine optimal EMS dose for full scale mutagenesis. Because commercially available EMS-stocks vary in mutagenesis efficiency it is crucial to determine the efficiency of each stock individually (Glasebrook and Kroonzuker, 2001). Five different EMS concentrations were used: 0.1%; 0.2%; 0.3%; 0.4% and 0.5%. About 300 seeds were treated in each EMS solution for 15 hours as described in Material and Methods section. Strength of mutagenesis was measured by calculating the number of embryo-lethal mutations as a fraction of plants that survived EMS treatment in the M0 generation. M0 plants were selfed and mature siliques were opened to count the fraction of brown and wrinkled seeds, indicating EMS induced embryo-lethal mutation, under the binocular microscope (Figure 3.33)

Figure 3. 33 Typical view of the mature silique from M1 plants treated with 0.3% EMS

One mature *Arabidopsis* silique was opened and photographed under binocular to count fraction of brown and wrinkled seeds

A concentration of 0.3% EMS, which caused around 20% embryo lethal mutations and gave 60% plants which survives mutagenesis was chosen as optimal. For comparison, 0.1% EMS gave just 5% embryo lethally and 0.5% EMS gave over 90% embryo lethal mutations.

3.B.4 Full-scale EMS mutagenesis experiment

Full scale EMS mutagenesis was performed on about 10000 $P_{NRT2.1}$-*LUC* T3 seeds as described in Materials and Methods. Approximately 60% of seed survived the EMS treatment and produced viable M1 plants (i.e. 6000 individuals). Pools of 15-20 plants were bagged together, resulting in 216 pools of M2 seeds. Seeds were cleaned and stored in 2 mL glass vials in the seed storage room (12^0C and 10% relative humidity).

3.B.5 Screening of the M2 generation on the plates under nitrate induction conditions

M2 plants were screened on plates containing 1 mM KNO_3 as sole N-source to identify mutants defective in nitrate signalling. To ensure full representation of the M2 population, a minimum of 20-30 seeds were sown from each of the 216 M2 polls described. Control plants (not mutagenised from line 9) were always grown in parallel under the same conditions. After stratification, plants were grown vertically for 10 days and *LUC* activity assays were then performed. Plates containing plants that did not show LUC activity, were selected and re-sprayed with D-Luciferin after 3 hours. Plants showing no *LUC* activity in both assays were selected (e.g. Figure 3.34)

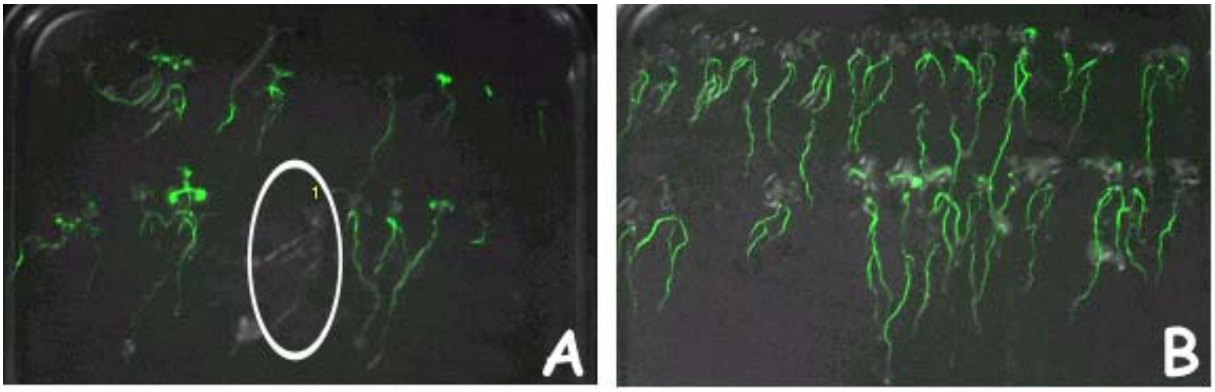


Figure 3. 34 Screening for the mutant phenotype under the inducible conditions

EMS mutated (**A**) M2 plants from poll 120 and control, not EMS mutated (**B**) plants were grown vertically on agar plates with 1mM KNO_3 as the sole nitrogen source for 10 days. LUC activity assay was performed as described in Materials and Methods The plant marked with white circle showed no detectable *LUC* activity in both assays and was indicated as putative mutant (putant)

Putative mutants (putants) were immediately transferred to soil and taken to seed. Many M2 seeds germinated poorly and produced plants with growth defects and high anthocyanin accumulation when grown on plates. Such plants were not selected, even when they lacked *LUC* activity, because they did not survived transfer to the soil. This screen yielded in 69 putants. Eleven of them died in the greenhouse prior the generative phase, and 58 others were able to flower and produce seeds.

3.B.6 Confirmation of mutant phenotypes in the M3 generation

To confirm the mutant phenotypes a minimum of 30 seeds from each plant were grown and screened again for lack of *LUC* activity after growth on 1mM KNO_3. The *LUC* activity test was always performed twice, to minimise experimental artefacts and exclude

false negative results. Twenty seven out of 58 lines showed a wild-type level of *LUC* activity in all individual seedlings tested, while three lines exhibited segregation of the signal: 1, 2 and 9 out of 30 seedlings gave *LUC* signals in lines 42/1, 184/1 and 212/1, respectively. Twenty eight putant lines showed no *LUC* activity in any of the seedlings tested under these conditions, validating their selection as potentially interesting mutants. Figure 3.35 shows the example of results of *LUC* activity screening.

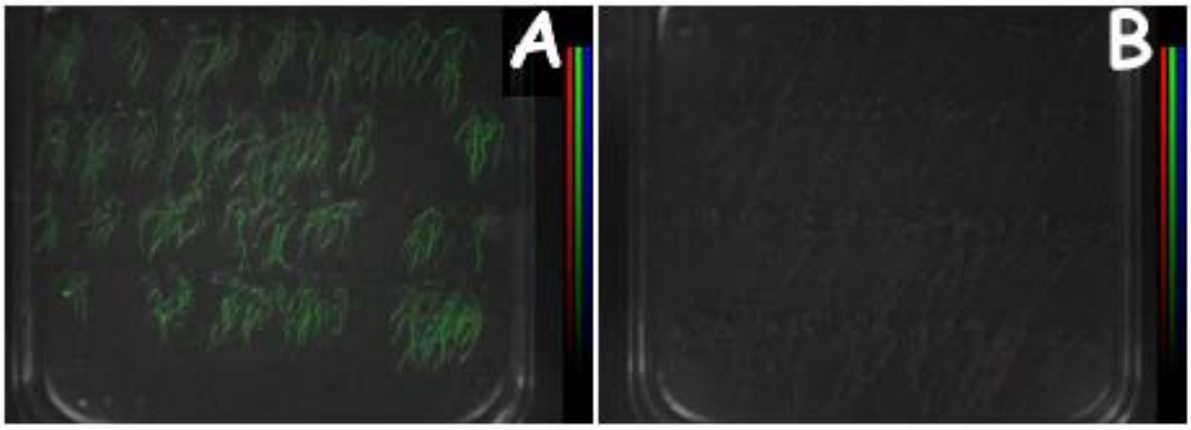


Figure 3. 35 *LUC* activity under inducible conditions in confirmed putant line 54/3

About 60 M3 plants from putant line 54/3 (**B**) and 60 control, not EMS mutated (**A**) plants were grown vertically on agar plates with 1mM KNO_3 as the sole nitrogen source for 10 days. LUC activity assay was performed as described in Materials and Methods All individual plants from EMS line 54/3 showed no detectable *LUC* activity in both assays.

To confirm lack of expression of the endogenous *ATNRT2.1* gene in these lines, RNA was isolated from whole seedlings and subjected to qPCR using primers for *ATNRT2.1* (sequence in Appendix B).

So far, 16 out of 28 interesting putant lines have been analysed (Figure 3.36). Five of the lines tested showed no significant change in *ATNRT2.1* expression compared to control line (less than 3-fold change). Three other putants showed modest repression of the *ATNRT2.1* gene expression (3-5 fold) and the other 8 lines showed dramatic (between 10 and 100 fold) repression of the *ATNRT2.1* gene expression, when compared to the control 9.

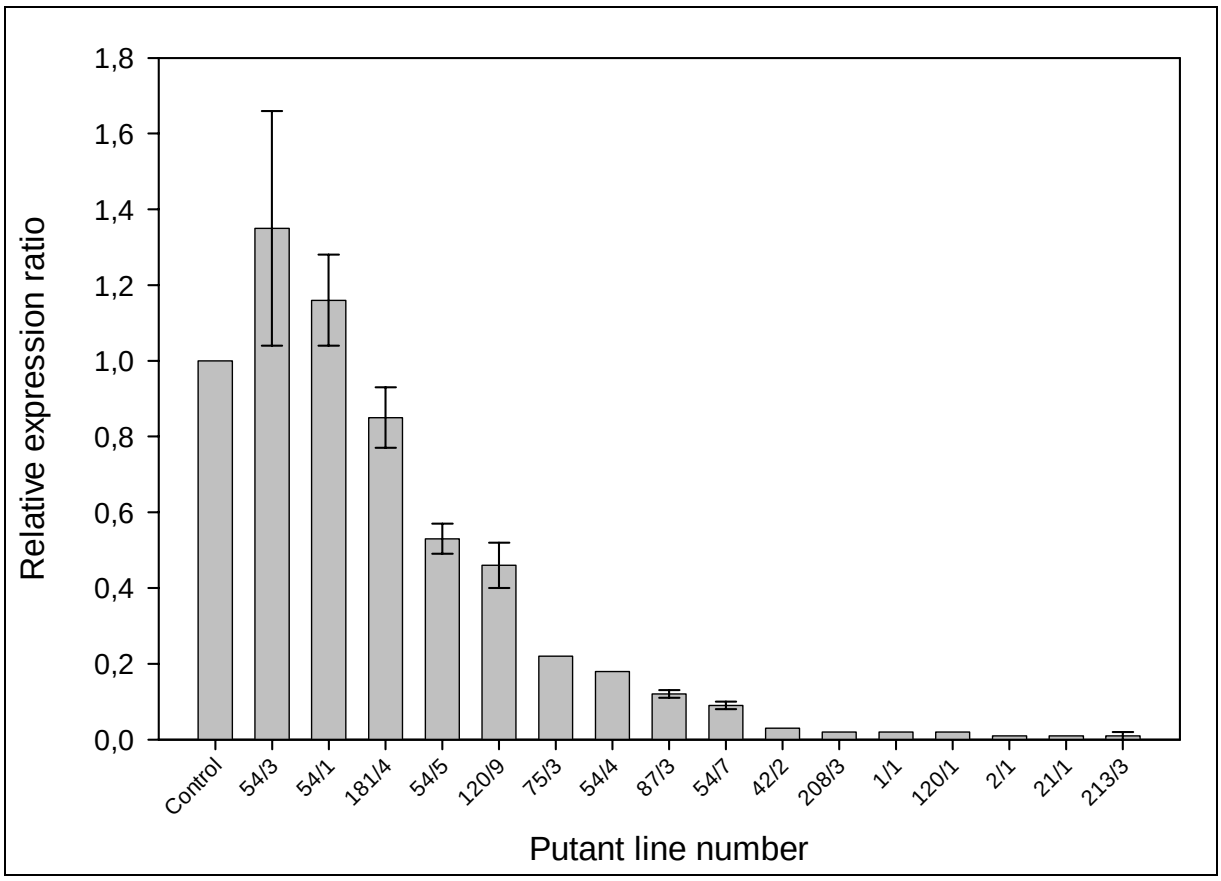


Figure 3. 36 Expression level of *ATNRT2-1* in the putant lines

Error bars indicate SE from three technical repeats of qPCR reaction. Control – not EMS mutagenised line number 9.

Interestingly, none of the putant lines showed a visible mutant phenotype under conditions described above, when compared to the control (Figure 3.37).

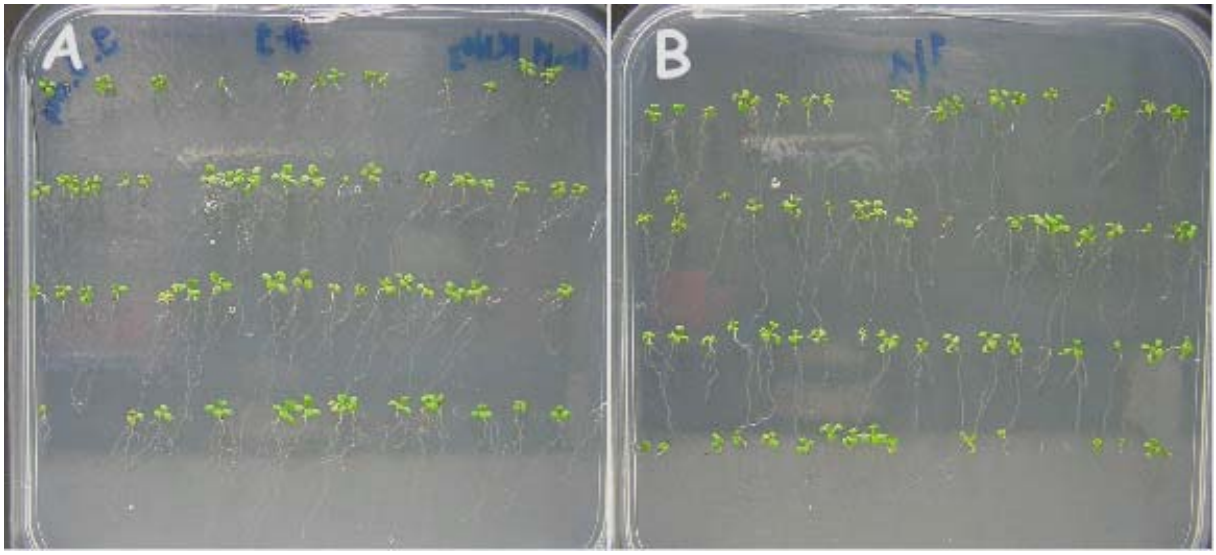


Figure 3. 37 Example growth of putant seedlings.

About sixty seedlings from the selected putant line 1/1 (**B**) and control line 9 (**A**) were grown vertically on agar plates supplemented with 1mM KNO_3 as the sole nitrogen source for 10 days and photographed.

3.B.7 qPCR analysis of the expression of the other genes in selected mutant lines

Possibly, the other members of HATS system expressed also in roots (like: *ATNRT2.2, ATNRT2.3, ATNRT2.4*) or even dual affinity transporters, like *ATNRT1.1* could take over the function of ATNRT2.1 to provide enough nitrogen to the plants. To investigate those possibilities the real time PCR analysis of expression of all NRT2 family members as well as the *ATNRT1.1* and Arabidopsis homologue of *NAR2* transporter gene from *C. reinhardtii* were performed. Real-time RT-PCR was used to test for alteration in gene expression in putants of other genes involved in nitrate primary metabolism, including the: nitrate reductase 1 *NIA1*, nitrate reductase 2 *NIA2*, nitrite reductase *NII*, cytosolic *GS1* and chloroplastic *GS2* glutamine synthetase, NAD^+-dependent glutamate synthase *GOGAT*, as well as the known N-regulated genes of oxidative pentose pathway and glycolysis including the: glucose-6-phosphate isomerase *PGI*, 3-phosphoglycerate dehydrogenase *PGDH,* and two glucose-6-phosphate dehydrogenases: *GPDH1* and *GPDH2*. All primer sequences given in Appendix B. Results of the analysis are summarized in tables 3.8 and 3.9.

Table 3. 8 Expression of the nitrate transporter genes in the selected mutant lines

EMS line	NRT2-1	NRT2-2	NRT2-3	NRT2-4	NRT2-5	NRT2-6	NRT2-7	NRT1-1	NAR2
87/3	- -	-	+	0	0	0	0	0	0
75/3	-	--	+	0	0	0	0	-	-
54/7	--	-	++	--	0	+	0	0	--
54/4	-	-	0	0	-	0	0	0	-
42/2	--	--	0	--	0	ND	--	0	-
213/3	--	--	+	ND	0	0	-	0	-
21/1	--	--	0	--	0	0	0	-	--
208/3	--	--	0	--	0	0	0	0	-
2/1	--	--	ND	--	0	ND	0	--	--
120/1	--	--	0	--	0	ND	0	-	--
1/1	--	--	0	--	0	ND	0	-	--

Table 3. 9 Expression of the genes from primary nitrogen and carbon acquisition in the selected mutant lines

EMS line	FNR	NIA1	NIA2	NII	GS1	GS2	GOGAT	GPDH1	GPDH2	PGDH	PGI
87/3	0	0	0	0	0	0	-	0	0	--	0
75/3	0	0	0	0	0	0	0	0	0	0	-
54/7	0	0	0	0	0	0	0	0	0	0	-
54/4	-	-	--	-	0	0	-	-	-	--	--
42/2	0	0	-	-	0	0	-	-	0	--	--
213/3	0	0	0	-	0	0	0	0	0	--	-
21/1	0	0	-	0	0	0	0	0	0	0	--
208/3	0	0	0	0	0	0	0	0	0	--	0
2/1	--	--	--	--	-	0	--	--	-	--	--
120/1	0	0	0	0	0	0	0	-	0	-	-
1/1	-	-	-	-	0	0	-	--	0	--	--

Tables legend:

-	5-10 fold down-regulated
--	more than 10 fold down-regulated
+	5-10 fold up-regulated
++	more than 10 fold up-regulated
0	below 5 fold cut-off
ND	not detectable (not amplified after 40 cycles)

Its apparent from Table 3.8 that all putants affected in expression of *ATNRT2.1* are also affected in expression of its closest homologue *ATNRT2.2*. Its noteworthy that an *Arabidopsis* homologue of *NAR2* (component of high affinity transport system from *C. reinhardtii*) is affected in all putant lines, except line 87/3. Almost all of the putants, except lines 87/3 and 208/3 are also affected in expression of glucose-6-phosphate isomerase.

Real-time PCR analysis allows to cluster putant lines in couple clusters. First cluster, contain lines affected in nitrate transport, and mostly unaffected in primary nitrate metabolism and oxidative pentose pathway (lines 87/3, 75/3, 54/7, 213/3, 21/1, 208/3). Another group contains genes affected nitrogen transport and assimilation plus in OPP, like 54/4, 42/2, 2/1 and 1/1. Two of those lines (2/1 and 1/1) are strongly affected the genes encoding nitrate transporters, primary nitrate metabolism, genes providing redox equivalents, (*FNR*) and almost all nitrate regulated genes from OPP. Putant line 120/1 represents third class of response. This line is affected in nitrate transporters and to lower extent in OPP genes, but not in genes from primary nitrogen assimilation genes, like *NIA1* and *2, NII* or *GOGAT*.

4. DISCUSSION

4.1 Development of a qPCR platform for profiling all *Arabidopsis* transcription factors

A unique public resource for studying the expression of TF genes in *Arabidopsis* was developed in the course of this project in collaboration with Dr Wolf-Ruediger Scheible. This resource, which is based upon highly multiplexed qRT-PCR (qRT-PCR) with gene-specific primers, enabled us to measure transcript levels in seedling roots or shoots for 1247 TF genes with high specificity and precision. Single PCR products of the expected size were obtained following RT-PCR for all of these genes, and sequencing of a subset of them confirmed the specificity of each PCR reaction. Four percent of the 1465 different TF RT-PCR reactions yielded more than the single expected product. New primer pairs have been made for these, which should enable specific measurements to be made on the transcripts of these genes in the future.

Approximately 13% of TF gene transcripts were not detected in samples of roots or shoots of vegetative plants grown under the conditions used in shoot and root and nitrogen deprivation and replenishment experiments. About a quarter of these genes have PCR primers that do not span exon-exon junctions. All primer pairs tested from this subset yielded unique PCR products of the expected size from genomic DNA as template, showing that the primers have been correctly designed and do indeed function as desired. This indicates that these genes are expressed at extremely low levels or not at all in plats under the conditions tested. Transcripts of another third of these genes have meanwhile been detected in *Arabidopsis* siliques or in seedlings exposed to various nutrient stresses (A. Blacha, T. Czechowski, W.-R. Scheible and M. Udvardi; unpublished results).

The sensitivity and robustness of TF transcript quantification by qRT-PCR were outstanding. As few as two copies of a target DNA could be detected in a complex mixture of 10^9 cDNA molecules (Figure 3.2 A). This corresponds to a detection limit of about one transcript per 1000 cells, or 0.001 transcripts per cell, which is similar to values obtained for yeast (Holland, 2002). In contrast, detection limits of DNA arrays are three orders of magnitude higher, at one transcript per cell (Holland, 2002; Horak and Snyder, 2002). Robustness of cDNA quantification was demonstrated in a second way: A linear relationship between output signal ($2^{(40-Ct)}$) and target cDNA amount was maintained over a wide range of mixtures of root and shoot cDNA (Figure 3.2 B). Such robustness has never been shown for DNA arrays. Precise quantification of transcripts by qRT-PCR depends upon having

uniformly high amplification efficiency, or having a method to determine the amplification efficiency for each individual PCR reaction. The latter was achieved using the method of Ramakers et al., 2003. This allows the amplification efficiency to be determined for each technical and biological replicate, and the relative transcript abundance to be calculated accordingly. The technical precision of qRT-PCR measurements of TF transcript levels was high. Very low intra-assay variation was observed in duplicate measurements of the same pool of cDNA, made in separate runs on the PCR machine (Figure 3.3 A). Inter-assay variation was estimated by measuring cDNA produced from two separate reverse transcription reactions that began from the same sample of RNA. As expected, inter-assay technical variation was slightly higher than intra-assay variation (Figure 3.3 B). Inter-assay variability of Affymetrix chips was greater than that of qRT-PCR (Figure 3.3 C and D), especially for genes expressed at low levels. The signal to noise ratio for hybridisation-based methods of transcript detection is known to decrease exponentially with decreasing amounts of transcript (Holland, 2002; Figure 3.3 C and D). This was not the case for qRT-PCR measurements, although variability in duplicate measurements increased slightly as TF transcript levels decreased in our experiments (Figure 3.3 A and B).

Real-time RT-PCR indicated that TF transcript levels in *Arabidopsis* range over six orders of magnitude (see for example Figure 3.11 A and B). Such a range in TF gene expression levels has never before been reported for plants. Presumably, this great range reflects not only differences in the expression level of different TF genes within any one cell-type, but also differences between cells of different tissues and organs. Given their role(s) as regulators of gene expression, it is to be expected that many TF genes will be expressed in a precise spatial and temporal manner in response to developmental and/or environmental cues. TF genes that orchestrate developmental transitions are known to be amongst the lowest-expressed of all genes, and transcripts of these genes are often only detectable by RT-PCR or RNA *in situ* hybridisation (Putterill et al., 1995; Long et al., 1996; Mayer et al., 1998; Siegfried et al., 1999). The most-highly expressed TF genes are presumably transcribed constitutively throughout the plant. Some of these may bind non-specifically to DNA. Not all of the genes that were targeted are necessarily TF genes. These genes were selected because they encode DNA-binding and other domains that are shared by TF proteins, which does not necessarily mean that they are transcription factor genes. Nonetheless, it is interesting to compare the range of transcript levels that we measured for TFs in *Arabidopsis* with that measured using the same technique in yeast. Levels of TF transcripts in the single-celled yeast

Saccharomyces cerevisiae varied over four orders of magnitude (Holland, 2002), which is two orders of magnitude less than observed by us in the more complex, multicellular plant.

It is also interesting to compare the data on TF transcript abundance obtained by qRT-PCR with those obtained for the same RNA samples using Affymetrix chips (Figure 3.3 and 3.4) The range of values obtained with qRT-PCR was two orders of magnitude greater than that obtained with Affymetrix chips (10^5 vs. 10^3). As shown above, qRT-PCR yields a constant ΔC_T for each X-fold change in initial DNA concentration over the whole range of detectable DNA concentrations (Figure 3.3 A). This is not true for DNA array-based methods, which suffer from an exponential decrease in signal intensity as transcript levels fall, due to second order kinetics of hybridisation (Holland, 2002). This could account for the narrower range of values obtained with Affymetrix chips compared to qRT-PCR (Figure 3.4).

Although qRT-PCR exhibited greater precision in replicate measurements than Affymetrix chips, this does not necessarily imply greater accuracy. To address the issue of accuracy directly, both methods were used to identify TF genes with extreme shoot to root expression ratios and these data were compared with that available in an *Arabidopsis* MPSS database. Massively Parallel Signature Sequencing (MPSS) represents an alternative means by which to estimate the relative abundance of gene transcripts in a particular organ. Like SAGE (Serial Analysis of Gene Expression; Kopecek et al., 2001), MPSS (Brenner et al., 2000a; Brenner et al., 2000b) generates short sequence tags produced from a defined position within an mRNA, and the relative abundance of these tags in a given library represents a quantitative estimate of expression of that gene. The *Arabidopsis* MPSS data set contained 3,645,414 tags from a root cDNA library and 2,885,229 tags from shoots. As described above, there was good qualitative agreement between qRT-PCR and the MPSS data (Table 3.1).

Quantitative accuracy of qRT-PCR and Affymetrix chips was also compared. A plot of the absolute signals given by the two methods revealed a rather weak correlation in the range corresponding to highly expressed genes and no correlation for genes expressed at lower levels (Figure 3.4). Unlike quantitative RT-PCR, hybridisation-based technologies like Affymetrix chips are qualitative and there is not a strict linear relationship between signal strength and transcript amount for different genes. Thus, it is not possible to conclude with confidence that transcripts of one gene are more abundant than transcripts for another gene simply based on greater signal strength for the former on an Affymetrix chip. It is generally assumed that this will not affect the reliability of conclusions drawn from the changes in the Affymetrix signal for a given gene across different chips, i.e., that Affymetrix chips do provide reliable information about the relative levels of a specific gene transcript in different

tissues or conditions. To check this, the shoot to root ratios for all of the transcription factors that we measured, calculated from qRT-PCR data and from Affymetrix arrays (Figure 3.6 A) was compared. Indeed, the agreement was good, provided abundantly expressed transcripts were compared (Figure 3.6 B and C). This confirms the accuracy and reliability of both methods. For about half of the transcription factors, however, the signal obtained by the Affymetrix technology was in a range where accurate results could not be obtained (Figure 3.6 B and C). As already indicated, these discrepancies were most widespread for genes that show a low signal on the arrays.

Transcription factors control many aspects of plant growth and development by regulating the expression of sets of target genes. Many TF genes are also regulated, in time and space, by internal and/or external cues. Thus, it should be possible to identify TF genes involved in important plant processes through 'Guilt by Association'. To identify TF genes that may play roles in root or shoot-specific processes, transcript levels of 1214 TF genes in these organs was compared (Figure 3.5). Approximately 7% (87) of the TF genes repeatedly exhibited greater than twenty-fold differences in expression in shoots compared to roots (Table 3.1). Seventy-three of these were represented in the *Arabidopsis* MPSS data, and as mentioned above almost all of these were confirmed as essentially root- or shoot-specific.

There is no published information on the majority of the 87 shoot- or root-specific genes that we identified by qRT-PCR (Table 3.1). Only 14 of the 52 shoot-specific genes have been characterised to some extent in the past. Eight of these were found to be expressed predominantly or exclusively in shoots. These include: *AGL2* / *SEP1* (*AT5G15800*), *YAB3* (*AT4G00180*), *YAB1* / *FIL* (*AT2G45190*), *ATH1* (*AT4G32980*), *WUS* (*AT2G17950*), *SPL3* (*AT2G33810*), *SPL4* (*AT1G53160*) and *SPL5* (*AT3G15270*). Most of these genes have been implicated in plant development. *AGL2* / *SEP1* is expressed in floral meristem, floral primordia, and ovules and plays a central role in controlling organ identity, such as the development of petals, stamens and carpels (Pelaz et al., 2000). *YAB3* is expressed in all aboveground organs but not in roots and specifies abaxial tissue development in lateral organs (Siegfried et al., 1999). *YAB1* / *FIL* is expressed in above-ground vegetative and reproductive meristems and is required for the growth and maintenance of inflorescence and floral meristems (Sawa et al., 1999). *SPL3*, *SPL4* and *SPL5* are expressed in aerial organs, especially in the inflorescence, and control flowering and other aspects of plant development (Cardon et al., 1997; Cardon et al., 1999). Other shoot-specific genes from Table 3.1 that have been described in the literature are: *ATH1*, which is involved in photomorphogenesis (Quaedvlieg et al., 1995); and two genes involved in phytochrome B signalling, *PIF4* (Huq

and Quail, 2002) and *PIL6* (Yamashino et al., 2003). Shoot-specific expression of the latter two genes has not been reported previously.

Three genes that we identified as shoot-specific encode well-known stress-response regulators: *CBF1/DREB1B* (*AT4G25490*), *CBF2/DREB1C* (*AT4G25470*) and *ERF2* (*AT5G47220*). Expression of the two *CBF* genes, which regulate adaptive responses to cold-stress, is induced dramatically by chilling (Shinwari et al., 1998; Medina et al., 1999). However, under non-stress conditions, *CBF1* and *CBF2* transcripts were barely detectable in shoots or roots (Shinwari et al., 1998; Medina et al., 1999). Results presented here, indicate that the basal, or non-induced level of expression of these genes is significantly greater in shoots than in roots, which makes biological sense because the shoot is exposed to more rapid changes in temperature than is the root. ERF2 is involved in signal reception of ethylene-mediated signalling pathways and also shows modest induction by cold stress (Fujimoto et al., 2000). The *WUS* homeodomain TF gene is expressed in very few cells of the shoot apical meristem during embryogenesis, vegetative growth and flower development, and determines the fate of meristem stem cells (Mayer et al., 1998).

Of the 35 root-specific genes that we identified (Table 3.1), only two have been characterised in the past, namely *AGL21* (*AT4G37940*) and *AGL17* (*AT2G22630*). Based on their root-specific expression patterns, roles in root development have been proposed for those two *AGL* genes (Rounsley et al., 1995; Burgeff et al., 2002). Other *AGL* genes have also been characterised as root-specific (Rounsley et al., 1995; Alvarez-Buylla et al., 2000a; Burgeff et al., 2002), including *AGL14* (*AT4G11880*) and *AGL19* (*AT4G22950*). We found transcript levels of both to be ~10x higher in roots than in shoots (data not shown).

Many of the reported genes that were identified as shoot-specific appear to be involved in developmental processes. This may simply reflect the way in which most TF genes have been isolated to date, namely via genetic screens for aberrant growth and development. Defects in TF genes involved other plant processes, such as metabolism may produce more subtle phenotypes, which are difficult to identify. Thus, many of identified, novel root- and shoot-specific genes may eventually be implicated in processes other than development. Obviously, reverse-genetics will play a central role in identifying functions for these genes.

Recently, an expression profile matrix for 400 *Arabidopsis* TF genes, derived from a series of Affymetrix chip experiments, was used to identify TF genes that may play roles in responses to different environmental stresses (Chen et al., 2002). Transcripts of about 10% of the genes were not detected under any of the conditions used in that study. Importantly, expression of several of these genes in roots and/or shoots was detected using qRT-PCR

(*AT4G13480*; *AT1G73410*; *AT4G01500* and *AT3G12820*), which highlights the greater sensitivity of this technique. An interesting anomaly discussed in the paper of Chen et al., 2002) was the expression pattern of the *TINY* gene (*AT5G25810*), which was found by Affymetrix chip analysis to be expressed at high levels in roots but not at all in other organs. TINY is required for both vegetative and floral organogenesis (Wilson et al., 1996), which indicates that it is expressed in aerial parts of the plant. We were able to detect transcripts of this gene in both roots and shoots (seven-fold higher in roots than shoots) using RT-PCR.

A qPCR platform for expression profiling of almost all *Arabidopsis* TF genes was created, that is more sensitive, robust and precise than oligonucleotide microarrays. This resource is also very flexible: we can add, remove, or replace primer pairs at any time. As already mentioned, the existing platform was already extended for 789 primer pairs amplifying newly identified TF genes. The enlarged version of the platform now contains primer pairs for 2256 TF genes, representing 53 gene families and sub-families which are arrayed on six 384 well plates. As a result of collaborative work with Dr Yves Gibbon (System Regulation Group, MPI-MP Golm, Germany), set-up qPCR reactions is fully robotized (Evolution P3 liquid handling system, Perkin Elmer). One researcher is able to measure expression of all 2256 TF genes in as single working day, when using two of ABI Prism 7900HT machines. The resource is currently being used to profile TFs from a broad range biological samples, including salt and osmotic stresses, phosphate starvation and replenishment, seed development and dormancy, and biotic stress.

Robust and reliable protocols for cDNA synthesis, including control: for RT reaction efficiency, for reverse transcriptase processivity and for the lack of contaminating genomic DNA were also established (see Materials and Methods) as part of this project.

4.2 Identification of N-regulated TF genes

The qPCR platform described above was used to identify TF genes potentially involved in nitrogen signalling. Sterile liquid cultures and constant light were used to minimize diurnal changes in carbohydrate and N metabolism (Matt et al., 1998; Scheible et al., 2000).

Molecular and physiological responses to nitrogen deprivation and replenishment

Axenicly grown 10-days old *Arabidopsis* seedlings showed typical, phenotypic response to hours nitrogen deprivation (Scheible et al., 2004), including: reduced chlorophyll,

accumulation of anthocyanins in the leaves, and pronounced root and especially lateral root growth (Figure 3.7). At the molecular level, deprivation of nitrogen leads to increases in the expression of nitrogen-scavenging genes in both prokaryotes (Magasanik, 1993), yeast (Siverio, 2002) or filamentous fungi (Marzluf, 1997). This is also true for higher plants, as reviewed by Howitt and Udvardi, 2000). Among the plant genes that are induced by N-deprivation are genes encoding high affinity ammonium transporters, such as *AMT1.5* and *AMT1.1,* which were used in this project as molecular markers of N-deprivation (see figures 3.8, 3.12 and 3.14). Re-supply of nitrate to N-deprived plants had no observable effect on plant phenotype. Nonetheless, at the molecular level many genes were induced by this treatment, including: *ATNRT2.1, FNR, NIA1, NIA2, NII,* see Figures 3.18, 3.12 and 3.13), which have been found previously to respond to nitrate induction in maize (Matsumura et al., 1997), tobacco (Scheible et al., 1997a; Scheible et al., 1997b), tomato (Wang et al., 2001) and *Arabidopis* (Cheng et al., 1988; Crawford et al., 1988; Wang et al., 2000; Wang et al., 2003; Scheible et al., 2004). Two levels of N-regulation were explored in the experiments described above. First TF genes were sought that might be involved in regulating general plant response to N-deprivation, including: coordinate repression of genes involved in photosynthesis, chlorophyll synthesis, photorespiration, Calvin cycle, plastid protein synthesis, secondary metabolism and mitochondrial electron transport as shown by Scheible et al., 2004).

Identification of N-regulated TF genes

Approximately 3.6% (45) of the *Arabidopsis* TFs were strongly (more than 10-fold) affected by N-deprivation in the first biological replicate (Figure 3.9 A, Appendix C). There was more less the same number of positively (25) and negatively (23) regulated genes, and no TF family was significantly overrepresented among N-regulated TFs. Responses to N-deprivation are orchestrated by the "PII system" in *E. coli* (Arcondeguy et al., 2001), the "TOR pathway" in yeasts (Beck and Hall, 1999), and by "global N responsive elements" in filamentous fungi (Marzluf, 1997).

Fewer TF genes responded to nitrate re-addition at least in the time frame (30 min to 3 hours) studied here (Figure 3.9 B and Appendix C). Short term exposure of plants to nitrate, induces relatively few genes, including those involved in nitrate uptake and assimilation (see above), those providing the reducing equivalents and organic acid skeletons (Scheible et al. 2004). Short term nitrate replenishment induces signalling system in filamentous fungi – involving pathway specific compounds, like *NIT4* and *NIRA* (Marzluf, 1997; Unkles et al.,

2001). Only 2.5% (31) of *Arabidopsis* TF genes responded to nitrate. Seven of them responded in a negative manner (more than 10 fold repression) and 24 in a positive (more than 10 fold induction) way. Again there was no overrepresentation of any particular TF family among the nitrate responding genes. Interestingly, most of the TF genes that were e induced or repressed by N-deprivation did not respond to subsequent NO_3^- re-supply. Only 7 of the TF genes repressed by nitrate derivation were re-induced almost to the same level as in full nutrition, within 30 min of nitrate replenishment. The remaining 19 nitrate-inducible genes were unaffected by nitrogen deprivation. This is in contrast to filamentous fungi, in which globally N-responding elements are induced by nitrogen deprivation and by nitrate re-addition. When nitrogen appear again in the environment they co-operate with pathway specific elements, to mediate transcriptional responses to the target genes (Marzluf, 1997; Unkles et al., 2001). Our findings therefore, might indicate existence of different N-signalling pathways in *Arabidopsis* than in filamentous fungi.

There was a good reproducibility for the nitrate induced TFs in a biological replica - almost all (20 out of 24) genes showed consistent 10 fold or more induction (Appendix C). Much higher variability was associated with the N-deprivation experiments. Only 13 of the 45 selected TF genes re-capitulated the 10 fold or more induction in a independently replicated experiment, although 10 other genes showed the same tendency of response but at lower level (3-10 fold). One possible explanation for such differences in biological variability is that the physiological and biochemical responses to long term N-deprivation are more diverse/complex than the short term responses to nitrate replenishment, which may cause broader biological variation in a former case. Another possible explanation for high variation in TF gene expression after N-deprivation is that many of not confirmed genes were undetected in one or the other condition (expression ratio based on C_T value of 40) in a first biological replica. qRT-PCR can fails not only due to lack of transcript under given condition but also because of the technical problems (e.g. mispipetting of the reagents, problems with reading fluorescence from a given well, inhibitors of *Taq* polymerase, lowering given PCR reaction efficiency, etc.). In fact, all seven nitrate-repressed genes, which failed to be reproduced in second experiment, have expression ratio based on C_T value 40 (Appendix C).

It should be noticed, that all TFs responding specifically to nitrate only in a positive way, as in case of fungal pathway specific regulators, like *NIT4, NIRA* and *YNA1* (Marzluf, 1997; Unkles et al., 2001; Siverio, 2002).

Overview of confirmed N-regulated TF genes (summarised in Table 3.2)

A broader look at N-regulated TF using also lower cut-off ratio (3-10fold) showed a slight overrepresentation of WRKY genes among these responsive to N-deprivation (13 out of 69 genes) and a clear over-representation of NIN-like genes (5 out of 9) among nitrate-inducible TFs. WRKY transcription factors have so far been associated only with plant defence responses (Eulgem et al., 2000; Desveaux et al., 2005). The result presented here suggests that WRKY TFs fulfil biological functions beyond responding to biotic stress. The first plant NIN gene to be described was *L. japonicus* NIN which is involved in root nodule organogenesis, a process which is inhibited by soil nitrate (Schauser et al., 1999). NIN and NIN-like proteins contain the RWP-RK domain, which is conserved in higher plants and algae. The closest homologues of these plant proteins are Mid – proteins from *Chlamydomonas*, which are involved in nitrogen control of gametogeneis induced by nitrogen deprivation (Haring and Beck, 1997; Ferris et al., 2001). Little is known about other N-regulated TF genes. Members of the families: AP2/EREBP (Riechmann and Meyerowitz, 1998), bHLH (Toledo-Ortiz et al., 2003) and MYB (Lee and Schiefelbein, 2001) have been associated with responses to abiotic stress, including phosphorous deprivation (Rubio et al., 2001) but not to N-regulation in the past. Results presented here open for diverse biological roles amongst these large families of TFs. Interestingly, only one member of GATA TF family, members of which are involved in nitrate sensing in fungi (see Introduction), was induced by nitrate in *Arabidopsis* (*AT4G26150*). This suggests that different types of TFs are involved in transmitting nitrate signals in higher plants. A large number of N-regulated TFs identified in this study belong to TF families that have been implicated in developmental regulation, including: TF families associated mainly with flower, leaf and roots development. These include: NAC (Olsen et al., 2005), homeobox (Lee et al., 2001), MADS-box (Alvarez-Buylla et al., 2000a; Alvarez-Buylla et al., 2000b), SBP (Cardon et al., 1999) CONSTANS-like (Griffiths et al., 2003) and TCP TFs (Cubas et al., 1999). It is interesting to speculate that some of these TFs may integrate N-signals into plant development programmes. The *MADS AFFECTING FLOWERING3* (*MAF3*), which is strongly induced upon nitrogen deprivation (Table 3.6, Appendix C) has been associated to regulation of vernalisation in *Arabidopsis.* Overexpression of *MAF3* in the *Landsberg*, but not in Col-0 accession, delayed flowering time, so *MAF3* could also act as floral repressor and contribute to the maintenance of a vernalisation requirement (Ratcliffe et al., 2003). Interestingly, all *MAF3* homologues, like *MAF2, MAF4* and *MAF5*, shown to act as floral repressors that prevent vernalisation by short periods of cold (Ratcliffe et al., 2003) were unaffected by nitrogen deprivation in our experiments (data not shown). Also expression other genes controlling autonomous pathway

of flowering control, like: *FT, AGL28, FLC* (Simpson, 2004) was also not regulated by changes in N-nutrition (data not shown). It will be interesting to determine whether *MAF3* integrates N-signals into the control of flowering time. If it does it would be first example of this kind.

The list of N-regulated TF genes also contained members of TF families that have been implicated in hormone response pathways, including: AP2/EREBP (ABA and ethylene responsive) MYB (ABA responsive) bZIP (ABA and gibberellins), NAC (auxin responsive) and ARR (cytokinin responsive) (see Riechmann, 2002 for references). This results may indicate possible cross talk between hormone and nitrogen-signalling pathways.

Two of the MYB genes, that were strongly up regulated upon nitrogen limitation: *PRODUCTION OF ANTHOCYANIN PIGMENT1* and *2* (*PAP1* and *PAP2*), were previously found to control phenylpropanoid biosynthesis (Borevitz et al., 2000). Activation tagged *Arabidopsis* mutants, overexpressing either of these genes exhibited purple pigmentation in all organs (Borevitz et al., 2000). Transgenic tobacco over-expressing *Arabidopsis PAP* genes, showed the same phenotypic changes, suggesting that *PAP* homologues could activate production of anthocyanin pigment in other plants species (Borevitz et al., 2000). Expression of *TRANSPARENT TESTA8 (TT8)*, shown to regulate synthesis of proanthocyanidine (Abrahams et al., 2002), was also markedly (6-fold) up regulated by N deprivation. Axenically grown N-deprived seedlings accumulate high amounts of anthocyanin pigment in leafs and this is associated with induction of many of the genes involved in anthocyanin biosynthesis (Scheible et al., 2004). Therefore it was interesting to discover that the TFs: *PAP1, PAP2* and *TT8* which regulate anthocyanin production are under nitrogen control themselves.

Genetic evidence shows that the MADS-box gene *ANR1* (*At2g14210*) is required to mediate changes in root architecture in response to NO_3^- availability. *ANR1* expression was induced in roots within 30 min of adding NO_3^- to *Arabidopsis* seedlings (Zhang and Forde, 1998). However, *ANR1* was not induced after adding NO_3^- in our experiments or those of Wang et al., 2003) and there was less than ~2-fold change in *ANR1* expression under full nutrient conditions compared to N deprivation. Our studies also failed to confirm N-regulation of two TF genes (*AT2G18160, AT3G55770*) reported to be repressed in nitrate grown plants (TRANBARGER et al., 2003). The reasons for these conflicting results are unclear. It can be speculated that *ANR1* might be subject to varying degrees of transcriptional and posttranslational regulation depending on yet unknown external or internal factors.

Overlap with data from ATH1 arrays

It is apparent from Table 3.2, which show all 37 confirmed N-regulated TF genes, that data obtained from Affymetrix arrays, provided by Dr Wolf Ruediger Scheible, are generally consistent with that obtained by qPCR. Seventeen out of 37 candidate genes identified by qRT-PCR were confirmed on ATH1 array ("absent" calls by MAS5 software in all experiments). This emphasise again the superiority of qPCR as a technique to detect rare transcripts, that cannot be measured by hybridisation based techniques (Figure 3.10). Advantage of pre existing experiments testing full genome response to nitrate was taken to check the behaviour of candidate TF genes.

Overview of responses to other macronutrients, abiotic stresses and to diurnal rhythm for selected TF genes.

To ensure that changes in expression of candidate TF genes was specific to changes in N-nutrition, TF transcript data were compared between a series of other experiments in which plants exposed to changes in other macronutrients, and to abiotic stresses, including salt and osmotic stress (Table 3.3). This set of data was kindly provided by collaborators from Molecular Plant Nutrition and Amino Acids and Sulphur Metabolism (as mentioned in Results section). This comparisons resulted in identification of a few the genes that responded to general abiotic stresses, rather than specifically to nitrogen. (*CBF1, AT1G66380, AT3G30260, AT5G38880, AT5G22570, PAP1* and *PAP2*). However responses of the majority of the candidate TFs were specific to nitrogen. *PAP1* and responded to deprivation of each of macronutrients tested, and also to salt stress. Therefore these regulators of anthocyanins biosynthesis respond generally to stress conditions that compromise growth of plants. Four of the N-regulated TF genes responded to C starvation in the same way as to N deprivation (*AT1G13300, AT3G25790, AT1G68880* and *MAF3*) while one (*AT2G33720*) responded in an opposite manner. Some of these TF genes may play roles in integrating C and N metabolism in plants and therefore warrant further attention in the future.

Plants were grown under constant light to minimise diurnal changes in gene expression. Comparison of the gene expression data obtained from various nitrogen experiments with that obtained from diurnal experiments (Oliver Bläsing, personal communication), revealed that none of the N-regulated genes apparent from *PAP1* were

subjected to diurnal regulation. Thus the growth regime used here effectively avoided the complications of diurnal changes in transcription

Analysis of promoter regions from N-regulated TF genes

Bioinformatics analysis of the 1kb region, directly upstream of the start codon of each candidate gene was performed using the TRES package (see Materials and Methods), and revealed presence of many well known N-regulated cis-elements such as those found to be targets for N-regulatory TF genes from fungi and algae, like: *GCN4*, *NIT2-niaD-niiA* or *NIT2-nit3*. This fact open the possibility of existing a hierarchy of regulones operating under nitrogen-signalling in Arabidopsis.

Final selection of TF genes for functional characterization using reverse genetics approaches

After filtering out N-regulated TFs that are also regulated by other nutrient and abiotic stresses, a sub-set of 16 genes was chosen for functional analysis in plants (Table 3.4). *PAP* genes were also included, despite their general response to abiotic stress. Although some of the TF genes excluded by this processes may be important for N-regulation in *Arabidopsis*, it was necessarily to scale-down the number of TFs for further analysis for obvious practical reasons.

4.3 Characterisation of selected N-regulated transcription factors

Time course of nitrate induction

Expression of the genes involved in nitrate acquisition is strongly induced by external nitrate already 30 min of nitrate addition to N-deprived plants (see e.g. Figure 3.8). The possibility exist that TFs, which regulate these genes may also be induced by nitrate, which could imply that they are even more rapidly regulated by nitrate. To test this possibility, the kinetics of TF induction by nitrate was studied. A time-course study of TF transcript levels following of nitrate re-addition revealed three types of responses to nitrate. The most common response, was a transient one in which strong up-regulation within 12 min was followed by transcript decline within 75 – 180 min after nitrate replenishment (Table 3.4). This type of the response is mostly expected for the regulatory proteins like TF are – thy are “switched on” by

external stimuli, triggers the expression of the set of effectors genes, and afterward they are "switched off". The rapid induction of this class of genes suggested that they are not regulated by *de novo* synthesised proteins, but rather pre-existing potential N-signalling components including other TFs.

The possibility also exist that some of those TF genes activate other TFs, which are activating themselves via positive feedback loop. This type of regulation by multi-component loop, shown to be common for yeast TF genes (Yu et al., 2003), may explain second type of response to nitrate among N-regulated TFs (Table 3.4). In this two-phase type of response expression of TF genes is induced by nitrate within first 12-30 min and than decline within next 45-60 minutes, to reach a second, smaller peak 3 hours after nitrate re-addition to N-derived plants. Alternative explanation for the observed 2-phase response could simply by that the TF under study was induced with different kinetics in different organs. Longer time needed to induce TF gene expression by nitrate in shoot that in root might be caused by simple fact that nitrate acquired in roots needs to be allocated to shoot. TF profiling was performed on whole seedlings so it was not possible to tell from this experiments whether TFs were expressed in shoots, in roots or both or whether the kinetics of its induction differed in different organs. However, previously published data show that some of those candidates are expressed and respond to nitrate both in shoots and roots (Wang et al., 2003; Wang et al., 2004).

Transcriptional responses to organic N source

Noteworthy, none of the candidates, besides potential general macronutrient regulated *PAP1* gene, responded strongly to glutamine starvation or re-addition (Table 3.4). It may suggest their specific regulation by inorganic nitrogen sources, rather than general N-including components. This is consistent with the fact, that Gln starvation and replenishment did not trigger expression of any potential "target genes", like *NIA1, NIA2, NII, ATNRT2.1* – but only the general N- scavenging transporters like *ATAMT1.1* and *ATAMT1.5* (Figure 3.12).

Transcriptional responses in double mutant of nitrate reductase background

To test whether selected TF genes respond specifically to nitrate rather to downstream products of nitrate assimilation, experiments on the *G'4-3* double mutant of nitrate reductase

mutants (Wilkinson and Crawford, 1993) were performed. This mutant has low, but detectable nitrate reductase activity (1% of the shoot wild type and 5-10% of root wild type), presumably due to residual activity of the EMS-mutated NIA1 gene (Wilkinson and Crawford, 1993; Lejay et al., 1999). The observed lack of induction after N-deprivation for both marker genes: *ATAMT1.1* and *ATAMT1.5* (Figure 3.13) makes the data for genes like *AT3G51910* and *PAP1*, which responded to N-deprivation in *nia1nia2* opposite to WT, hard to interpret. Almost all of the selected TF genes responded in the same way to nitrate replenishment in the double mutant and in WT plants. The only exception was *AT1G01530*, which responded positively to nitrate in WT but not in the mutant. Therefore this TF gene may be regulated by signalling pathways that responds to downstream metabolites of nitrate acquisition, like nitrite for example. However, this kind of sensing cannot be excluded for the other TF genes, since *G'4-3* is still able to metabolise some nitrate. The expression pattern of most of the candidate TF genes in a double nitrate reductase null mutant created by Wang et al., 2004), confirms their nitrate specificity (Table 3.2). Differences in regulation of other TF genes between the *NR* null mutant and *G'4-3* are most probably due to residual nitrate reductase activity in the latter. For example: *AT1G68880* and *AT4G26150*, which responds to nitrate in the WT but not in the null-mutant, probably respond to changes in the level of downstream product of nitrate reduction (Wang et al., 2004).

Functional characterization of TFs using reverse genetic approaches

A gain and loss of function approach was taken for functional characterisation of selected N-regulated TF genes. The GATEWAY™ cloning system (Invitrogen) proved to be an efficient way to create constructs for constitutive and ethanol inducible over-expression of all candidate TF genes. However it was difficult to PCR amplify full length open reading frames for the long cDNAs presumably because of limited processivity of reverse transcriptase (Yamada et al., 2003) and the resulting low abundance of full length cDNA. On the other hand, it was relatively easy to PCR amplify long TFs sequences from genomic DNA. The presence of introns in cloned TF sequences should not interfere with TF expression into functional proteins provided that PCR errors do not interfere with proper intron-splicing in transgenic plants. Selection and screening for the TF over-expressors is currently in progress, but some lines overexpressing TFs under the constitutive 35S promoter have been found to have abnormal phenotypes when grown in soil. Aberrant phenotype include the "dwarfed" phenotype of *35S-AT2G33550* (Figure 3.20), the "cauliflower like" of *35S-*

AT2G33720 (Figure 3.19) or the "curled leave" *35S-AT1g01530* (Fifure 3.18) of which represents neomorphic phenotypes. In neomorphs (new form), the introduced protein confers a new function that is not present in wild type, presumably because of its abundance in inappropriate tissues and/or developmental-stage (see Zhang, 2003 for references). Neomorphic phenotypes can be created by squelching, which is defined as repression/activation of transcription of otherwise unrelated genes by sequestering limiting components, such as co-activators/repressors, that are required for transcription elsewhere. Squelching is commonly observed upon constitutive over-expression of genes that otherwise exhibit organ- specific expression in the WT plants (see Zhang, 2003 for references). The MPSS data and previous qRT-PCR analysis (Czechowski et al., 2004), showed root specific expression for *AT2G33550*.

On the other hand it was already demonstrated that that constitutive over-expression of TF, is an efficient way to improve some agriculturally important traits, like better adaptation to N-limitations. Overexpression of any of the selected TF genes in this work, might potentially lead to similar results. Constitutive over-expression of Dof1 transcription factor from maize in *Arabidopsis* resulted in plants with (i) improved overall growth (ii) improved nitrogen ad ammonium content (iii) elevated amino acids content, especially glutamine and glutamate and (iv) elevated activity of enzymes producing carbon skeletons for nitrogen assimilation, like pyrruvate kinase, phosphoenolpyrruvate kinase, citrate synthase, when grow under low nitrogen on agar plates (Yanagisawa et al., 2004). Over-expression op Dof1 in transgenic potato also led to elevated amino acid levels (Yanagisawa et al., 2004). *Dof1* was associated with coordinated expression of enzymes from organic acids metabolism in maize (Yanagisawa, 2000). However, we did not observe any response to nitrogen for any of Dof TF-family members in our experiments.

To avoid squelching and other problems associated with constitutive over-expression of TF genes, we also used an ethanol-inducible system to express TFs transiently in *Arabidopsis*. Some but not all of inducible TF over-expressor lines showed quite high background expression in the absence of ethanol (Figure 3.17 B). This may also reflect insertion of 35S promoter from pSRN-GW vector (Appendix A) near endogenous promoter that activates TF expression.

Homozygous KO-lines, for most of the candidate TF genes were obtained which enabled the loss-of-function approach to be taken for these. So far, homozygous knock out lines for only a few of TF genes exhibit an abnormal phenotype, like retarded germination (*AT1G13300* and *AT2G33550*) and delay in flowering (*AT3G51910*) (Figures 3.28 and Figure

3.29, respectively). It is too early to conclude that mutations in specific TFs are responsible for these phenotypes, because the level of the transcription of the target TF gene was not yet been measured and mutants have not been backcrossed to the wild type to remove any additional T-DNA insertions in other than investigated loci. Multiple insertions are common in the T-DNA populations used here (Alonso et al., 2003). To overcome this problem, homozygous KO lines are currently backcrossed twice to the Col-0 wild type, which is a time consuming procedure but enable us to clean up any background mutations. Transposon-tagged lines, known to harbour single insertions only (Kuromori et al., 2004) are also being used alternatively used to investigate loss of function effects for some of selected TF genes, although number of such mutants s far lower than those for T-DNA populations.

However, most of the homozygous KO lines showed no visible phenotypes when grown on soil, or when screened for germination ratio or flowering time. This can be explained in couple ways. First, lack of the transcript is not proven so far for any of the selected lines. Second, even if they lack of functional TF transcript, many may exhibit no phenotype because of high functional redundancy among *Arabidopsis* TF genes. A cross-comparison of TFs, in *Arabidopsis* revealed that, closely related genes can be found in nearly half of the major TF families (Riechmann et al., 2000). Pairs or groups of closely related genes correspond to duplication on different chromosomes (~65% of cases), duplications on the same chromosome but at very large distances (~22%) and less frequently to tandem repeats (~13%) (Riechmann et al., 2000; Riechmann, 2002). Clusters of three or more homologous TFs are very rare in the genome (Riechmann et al., 2000; Riechmann, 2002). Thus, insertional mutagenesis into one TF gene will generally not affect the expression of its most closely related homologue, which may substitute for its loss. The extent of functional redundancy among TFs is illustrated by several studies on MADS-box genes. Sequence analysis of *Arabidopsis* MADS-box genes suggests that more than 40% may have (partially) redundant functions (Davies et al., 1999; Liljegren et al., 2000; Pelaz et al., 2000). In this context, it is interesting to note that where segmental duplications is evident for N-regulated TF genes, both may exhibit a similar pattern of regulation (Table 3.2). For example, the two *CONSTANS*-like TF genes *At1g25440* and *At1g68520*, which both display root-specific expression (Table 3.2) and are strongly repressed in N-starvation. Another example is the two root-specific GARP-like (MYB-like) TF genes *At1g13300* and *At3g25790*, which were induced more than 50- and 400-fold respectively in the RT-PCR screen after 30 min NO_3^- addition (Table 3.2). Conserved expression of gene pairs might represent an evolutionally favoured backup system that avoids loss of vital functions due to spontaneous mutations.

However, NO_3^- regulation of duplicated genes is not always preserved, e.g. AP2-EREBP type TF gene *AT2G39250* (Table 3.2) that was ~240-fold induced after 30 min NO_3-addition (RT-PCR) and ~3-fold after 3h (ATH1), whereas its duplicated twin *AT3G54990* was ~3-fold and merely ~2-fold induced, respectively. Divergence of formerly identical regulatory elements by mutation and subsequent natural selection presumably leads to new biological roles for genes/proteins derived from a single progenitor by duplication, and may be a route to increase fitness and adaptation to a given environment.

Some of the selected N-regulated TF genes show root-specific expression, which makes them candidates for control a lot of processes, such as root development. Unfortunately, the screening system that was set up to detect alterations in primary and lateral root development has not revealed any changes in either constitutively overexpressing or knock-out mutants of the selected TF genes. Once again, functional redundancy might be one possible explanation for the lack of interesting results for the KO lines. In the case of 35S-OX we have so far used only segregating lines, which can introduce high variation observed in the analysis (Figure 3.25 and 3.26). High variation was in fact observed from plate to plate also for the wild type plants. This type of screen will be repeated, with non-segregating OX lines and genetically "cleaned" KO lines, once they are obtained, and these will be grown in parallel to the wild type on the same plate, with at least 3-4 replicates, which could minimise variation. Only the high nitrate, shown previously to arrest lateral root growth (Malamy and Ryan, 2001), could be used on side, but usefulness of such a screen are under question mark, since similar inhibitory effect was observed in a KCl salt controls for the wild-type plants (Figure 3.24). Low nitrate concentration promoting lateral root growth could be used on the other side. A more sophisticated screen for developmental phenotypes that we wish to try in the future involves the use of segmented plate with localised nitrate supply, which promotes lateral root formation and elongation (Zhang and Forde, 1998; Zhang et al., 1999). A screen for alterations in nitrate – induced lateral root growth could help to assign functions to specific TF gene, as was the case for *ANR1* (Zhang and Forde, 1998)

4.4 Isolation of novel mutants affected in nitrate-induction of gene expression

A forward genetics approach was taken to identify genes involved in N-regulation of the high affinity nitrate transporter *ATNRT2.1*. It appears that ATNRT2.1 plays a major role in the nitrate inducible, ammonium / amino acids repressible, light / sugar inducible component of high affinity nitrate transport in *Arabidopsis* roots (Lejay et al., 1999; Zhuo et al., 1999;

Filleur et al., 2001; Lejay et al., 2003). Therefore we considered the *ATNRT2.1* promoter an ideal tool to aid in the search for factors involved in nitrogen signalling and regulation as described in introduction. This screening resulted in identification of eleven EMS mutant reporter lines affected in induction of *ATNRT2.1* expression by nitrate. These lines could by divided in the following classes according to expression of other genes involved in primary nitrogen and carbon metabolism: (i) lines affected exclusively in nitrate transport, (ii) those affected in nitrate transport, acquisition, but also in glycolysis and oxidative pentose pathway, (iii) mutants affected moderately in nitrate transport, oxidative pentose pathway and glycolysis but not in primary nitrate assimilation (Tables 3.8 and 3.9).

Selection of $P_{ATNRT2.1\text{-}LUC}$ lines and EMS mutagenesis

A reporter line, harbouring two insertions of the promoter – reporter construct was used for EMS mutagenesis to minimise the possibility of artefactual mutants lacking reporter gene expression due to point mutations in the $P_{ATNRT2.1}$ or *LUC* reporter gene. The first screen done under growth conditions that induce *ATNRT2.1* promoter activity, resulted in the identification of 58 putative mutants (putants) that lacked Luciferase (LUC) activity. It should be noted that the amount of the seed from each of over 216 M2 pools (20-30) was insufficient to represent all 15-20 individual progenitor M1 plants used to create a each pool. Therefore future screening of larger numbers of the M2 seeds, from each pool may result in identification of even more putants. Re-screening for the mutant phenotype in the sub-sequent M3 generation confirmed lack of LUC activity for half of the putants selected in M2 generation. Interestingly, most of the putants showed no segregation of LUC minus with a LUC plus plants phenotype, which may indicate a dominant mutation is responsible for phenotype of these mutants. The false negative "LUC –" plants most probably resulted from experimental artefacts, like uneven distribution of substrate D-luciferin for the LUC mediated reaction, the small size of plants resulting in a underestimation of LUC signal by CCD camera, etc.

Confirmation of the lack of nitrate induction of endogenous ATNRT2.1 gene

Real time RT-PCR analysis performed so far on 16 confirmed putants showed lack of induction for endogenous *ATNRT2.1* expression in eleven of the mutants compared to non-mutagenised control plants grown in parallel (Figure 3.36). The reason for the lack of nitrate

induction of $P_{ATNRT2.1}$ in the remaining five lines remains unclear. It is rather unlikely that they all contain mutations in the introduced $P_{ATNRT2.1}$ - *LUC* genes, due to the fact that they each contains two copies of the construct in the genome. On the other hand it has not been confirmed that both $P_{ATNRT2.1}$ - *LUC* insertions are functional in the original reporter gene used for mutagenesis. It is noteworthy that none of the mutants impaired in nitrate induction of *ATNRT2.1* expression showed a visible sign of N-limitation, when grown on 1 mM KNO_3 as the sole nitrogen source (Figure 3.37).

Responses to nitrate for the other known N-regulated genes in isolated mutants

Real-time RT-PCR was used to determine the extent to which other nitrate – inducible genes were de-regulated in the various mutants. The closest homologue of *ATNRT2.1* gene: *ATNRT2.2* also lacked a normal induction response to nitrate in all mutant lines (Table 3.8). This suggest that common factor regulates the activity of both genes. Interestingly, *AT5G50200*, an *Arabidopsis* homologue of the *NAR2* gene from *Chalmydomonas reinhartdtii* (Maathuis et al., 2003) also lacked wild-type induction by nitrate in all mutant lines (Table 3.8). *NAR2* is required for high-affinity nitrate transport in *C. reinhardtii* (Zhou et al., 2000) and the same role for its homologue, based on the expressional data could be postulated for *Arabidopsis* (Scheible et al., 2004). Results presented here might additionally suggest common nitrate regulatory mechanism of iHATS elements between higher plants and algae.

We also studied in the mutants other nitrate-regulated genes from the primary nitrogen assimilation pathway. Reduction of NO_3^- and nitrite consumes NADH in the cytosol, and ferredoxin (FD) in the plastid. In leaves in the light, photosynthesis provides the reducing equivalents. In respiratory tissues, NADH from the mitochondria is used to reduce FD via NADPH from the oxidative pentose phosphate (OPP) pathway. Nitrate rapidly induces genes that are required to generate NADPH and use it to reduce FD, like two members of small gene family member for Glc-6-P dehydrogenase and (*GPDH1* and *GPDH2*) as well as 6-phosphogluconate dehydrogenase (*PGDH*) (Scheible et al., 2004). Also one member of the small family of phospho-Glc isomerase (*PGI*) was shown to be nitrate inducible (Wang et al., 2000; Wang et al., 2003; Scheible et al., 2004; Wang et al., 2004). PGI is required in the OPP pathway when it operates at a high rate relative to the flux through glycolysis, and fructose-6-phosphate recycled to glucose-6-phosphate and re-enters the OPP pathway. Interestingly, expression of *PGDH* and *PG*I but not the two *GPDH* genes were strongly repressed in most of the mutant lines (Table 3.9) Also well know nitrate-inducible genes like *NAI1, NIA2, NII* as

well as NADH – dependent GOGAT were affected only in a relatively small number of the mutant lines (Table 3.9). On the other hand, expression of genes unaffected by nitrate like glutamine synthetases (*GS1* and *GS2*) as shown before (Scheible et al., 2004), were also unchanged in the mutant lines. In summary – forward genetic screen performed here resulted in the isolation of the mutant lines affected specifically in expression of nitrate-regulated genes involved in its acquisition and assimilation, as well as those from OPP pathway. However, different types of responses were observed among mutant lines which indicates that different regulatory genes may be impaired in the different class of mutants. This idea is currently being tested via mutant crosses, which will help to define different complementation groups. Mapping population derived from crosses of one representative mutant of each complementation group and the wild type *C24* and *Landsberg* then be created to enable map-based cloning of mutant alleles in the future (Lukowitz et al., 2000).

5. SUMMARY AND CONCLUSIONS

Nitrogen is an essential macronutrient for plants and nitrogen fertilizers are indispensable for modern agriculture. Unfortunately, we know too little about how plants regulate their use of soil nitrogen, to maximize fertilizers-N use by crops and pastures. This project took a dual approach, involving forward and reverse genetics, to identify N-regulators in plants., which may prove useful in the future to improve nitrogen-use efficiency in agriculture.

To identify nitrogen-regulated transcription factor genes in *Arabidopsis* that may control N-use efficiency we developed a unique resource for qRT-PCR measurements on all *Arabidopsis* transcription factor genes. Using closely spaced, gene-specific primer pairs and SYBR® Green to monitor amplification of double-stranded DNA, transcript levels of 83% of all target genes could be measured in roots or shoots of young *Arabidopsis* wild-type plants. Only 4% of reactions produced non-specific PCR products, and 13% of TF transcripts were undetectable in these organs. Measurements of transcript abundance were quantitative over six orders of magnitude, with a detection limit equivalent to one transcript molecule in 1000 cells. Transcript levels for different TF genes ranged between 0.001-100 copies per cell. Real-time RT-PCR revealed 26 root-specific and 39 shoot-specific TF genes, most of which have not been identified as organ-specific previously.

An enlarged and improved version of the TF qRT-PCR platform contains now primer pairs for 2256 *Arabidopsis* TF genes, representing 53 gene families and sub-families arrayed on six 384-well plates. Set-up of real-time PCR reactions is now fully robotized. One researcher is able to measure expression of all 2256 TF genes in a single biological sample in a just one working day.

The *Arabidopsis* qRT-PCT platform was successfully used to identify 37 TF genes which transcriptionally responded at the transcriptional level to N-deprivation or to nitrate. Most of these genes have not been characterized previously. Further selection of TF genes based on the responses of selected candidates to other macronutrients and abiotic stresses allowed to distinguish between TFs regulated (i) specifically by nitrogen (29 genes) (ii) regulated by general macronutrient or by salt and osmotic stress (6 genes), and (iii) responding to all major macronutrients and to abiotic stresses. Most of the N-regulated TF genes were also regulated by carbon. Further characterization of sixteen selected TF genes, revealed: (i) lack of transcriptional response to organic nitrogen, (ii) two major types of kinetics of induction by nitrate, (iii) specific responses for the majority of the genes to nitrate

but not downstream products of nitrate assimilation. All sixteen TF genes were cloned into binary vectors for constitutive and ethanol inducible over expression, and the first generation of transgenic plants were obtained for almost all of them. Some of the plants constitutively over expressing TF genes under control of the 35S promoter revealed visible phenotypes in T1 generation. Homozygous T-DNA knock out lines were also obtained for many of the candidate TF genes. So far, one knock out line revealed a visible phenotype: retardation of flowering time.

A forward genetic approach using an *Arabidopsis ATNRT2.1* promoter : Luciferase reporter line, resulted in identification of eleven EMS mutant reporter lines affected in induction of *ATNRT2.1* expression by nitrate. These lines could by divided in the following classes according to expression of other genes involved in primary nitrogen and carbon metabolism: (i) lines affected exclusively in nitrate transport, (ii) those affected in nitrate transport, acquisition, but also in glycolysis and oxidative pentose pathway, (iii) mutants affected moderately in nitrate transport, oxidative pentose pathway and glycolysis but not in primary nitrate assimilation. Thus, several different N-regulatory genes may have been mutated in this set of mutants. Map-based cloning has begun to identify the genes affected in these mutants.

6. FUTURE OUTLOOK

All selected constitutive and inducible OX lines are currently being tested in the axenic culture system. Over-expressors are grown in parallel to the WT plants under conditions that cause minimal endogenous gene expression. Expression of all (around 300) of the potential target genes, strongly nitrogen regulated by nitrogen (Scheible et al., 2004), carbon, phosphate and sulphate (M. Stitt, W.R. Scheible and M. Udvardi – unpublished data) will be investigated by qRT-PCR. So called "molecular phenotypes", will be investigated by comparing WT to 35S OX and by comparing ethanol-treated inducible OX line to the water-treated controls.

All publicly available KO lines for duplicated N-responsive homologues have been defined and we plan to create combinations of mutants to reveal phenotypes associated with specific TFs. As an alternative approach, RNA interference (Hamilton and Baulcombe, 1999, *RNAi*) to silence sets of highly-related TF genes to uncover their function could be used. Chemically inducible *RNAi,* reported recently to work well in *Arabidopsis* and tobacco (Guo et al., 2003).

Finally, all selected homozygous knockout lines, which lack transcript for a given TF gene and that have been backcrossed to the wild type, as well as non-segregating constitutive overexpressors of TFs will undergo a screen for abnormalities in nitrate regulation of flowering time. Such a screen will be done on agar-plate grown plants using various nitrogen sources in the system. The results of such a screen could be particularly interesting for the genes that shown before to regulate flowering in *Arabidopsis,* such as *MAF3*, or newly identified TF gene *AT3G51910*, for which primary results indicate a possible role in control of flowering (see above).

Screening to isolate EMS mutants affected in N-regulation, will be repeated using the same M2 pools, to look for the putants affected in N-repression of *ATNRT2.1* promoter. This approach will need to establish first proper growth conditions, repressing *ATNRT2.1* promoter activity, minimising background LUC activity, and not repressing plant growth. All the nitrogen regimes tested so far failed to reach those requirements (Figure 3.32).

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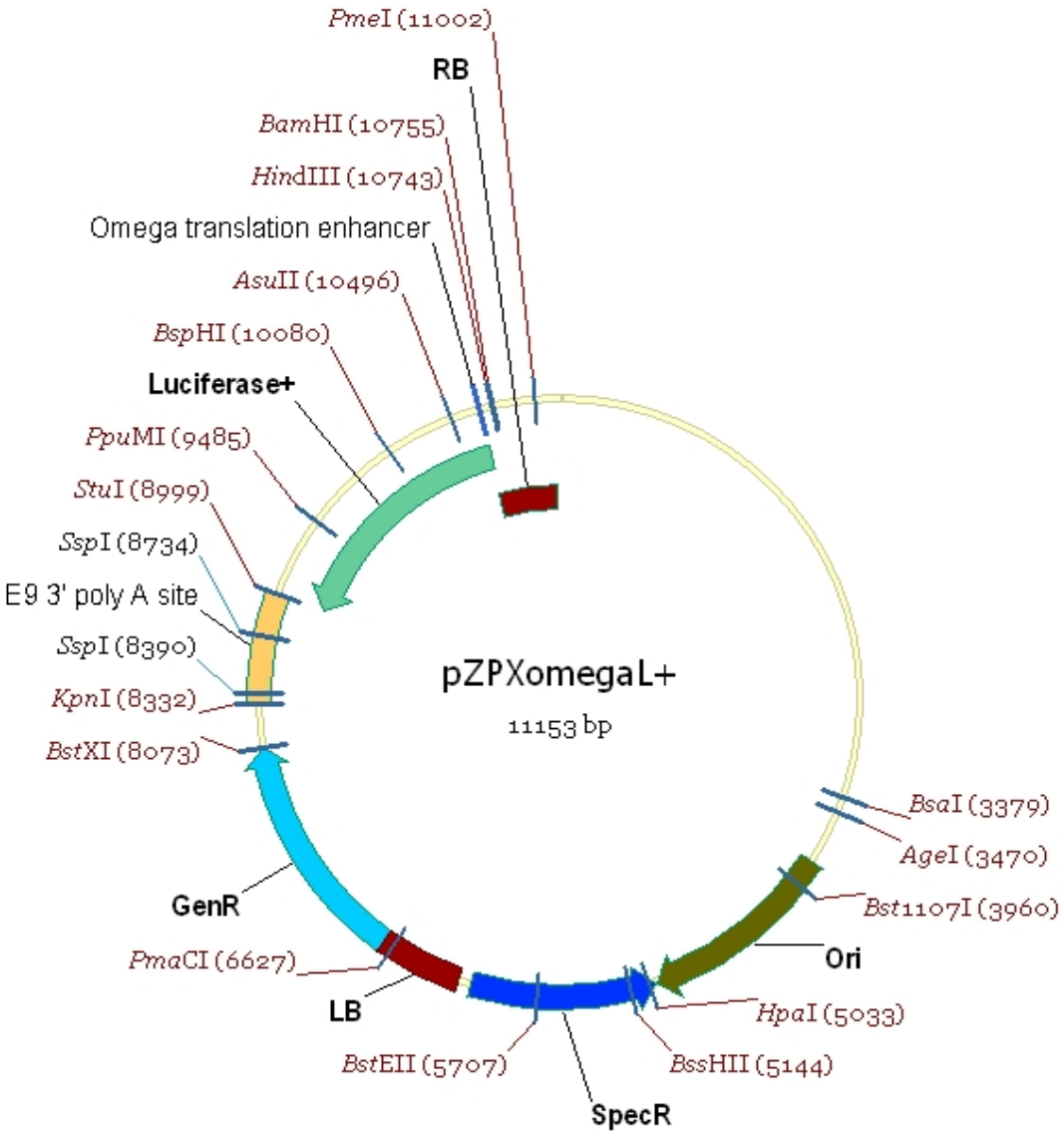
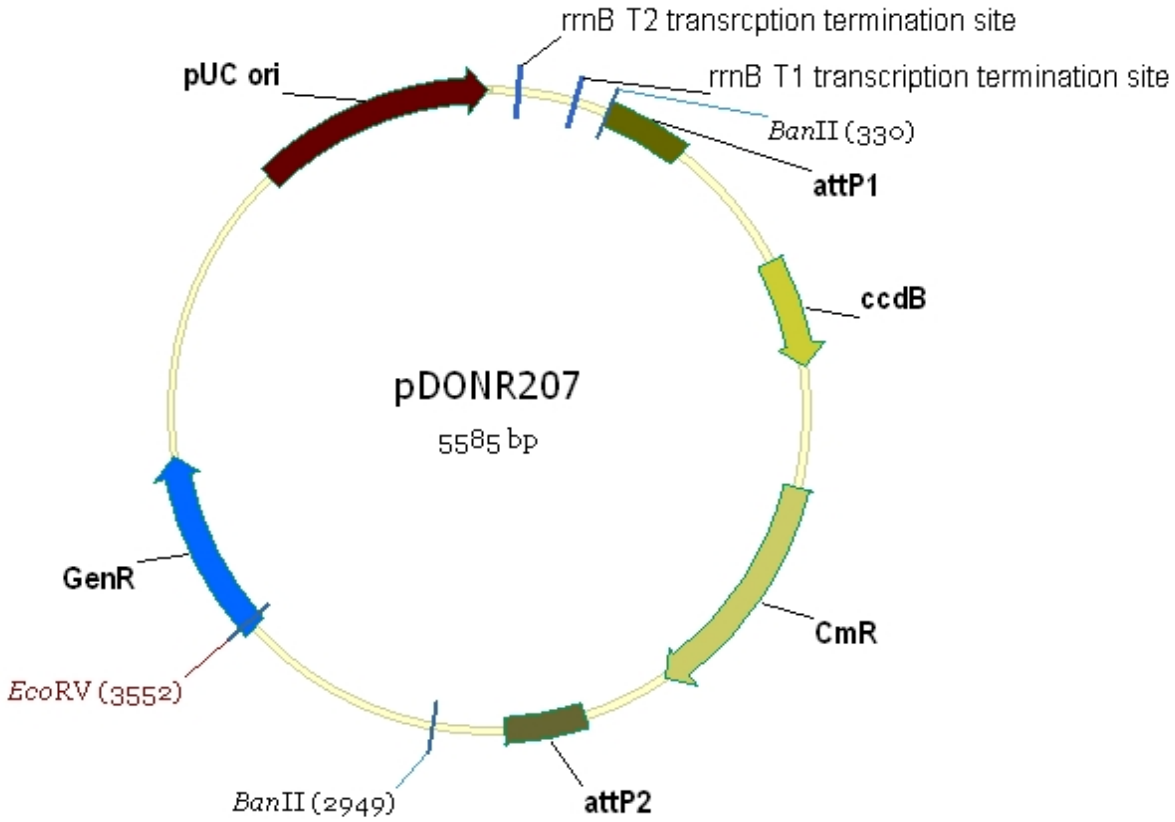
APPENDIX A

Resctriction maps and description of vectors used for this work.

Plasmid	Relevant characteristic	Source
pDONR™207	Entry vector for attB – compatible PCR products in GATEWAY™ system; Gen^R	Invitrogen
pENTR™/D-TOPO	Entry vector for PCR products in GATEWAY™ system; Kan^R	Invitrogen
pMDC32	Binary vector for plant transformation, for constitutive gene over expression; contains 35S promoter from CaMV, Kan^R	Created and kindly provided by Dr. Mark Curtis (Curtis and Grossniklaus, 2003
pSRN-GW	Binary vector for plant transformation, for ethanol-inducible gene over expression, contains *AlcA* promoter system from *A. nidulans*; Kan^R	Created and kindly provided by Dr. Ben Trevaskis, MPI-MP, Golm, Germany
pZPXOmegaL+	Binary vector for construction of promoter LUC fusions; contains omega translation enhancer fused to LUC gene; Gen^R	Kindly provided by Dr. Steve Kay, TSRI, La Jolla, CA

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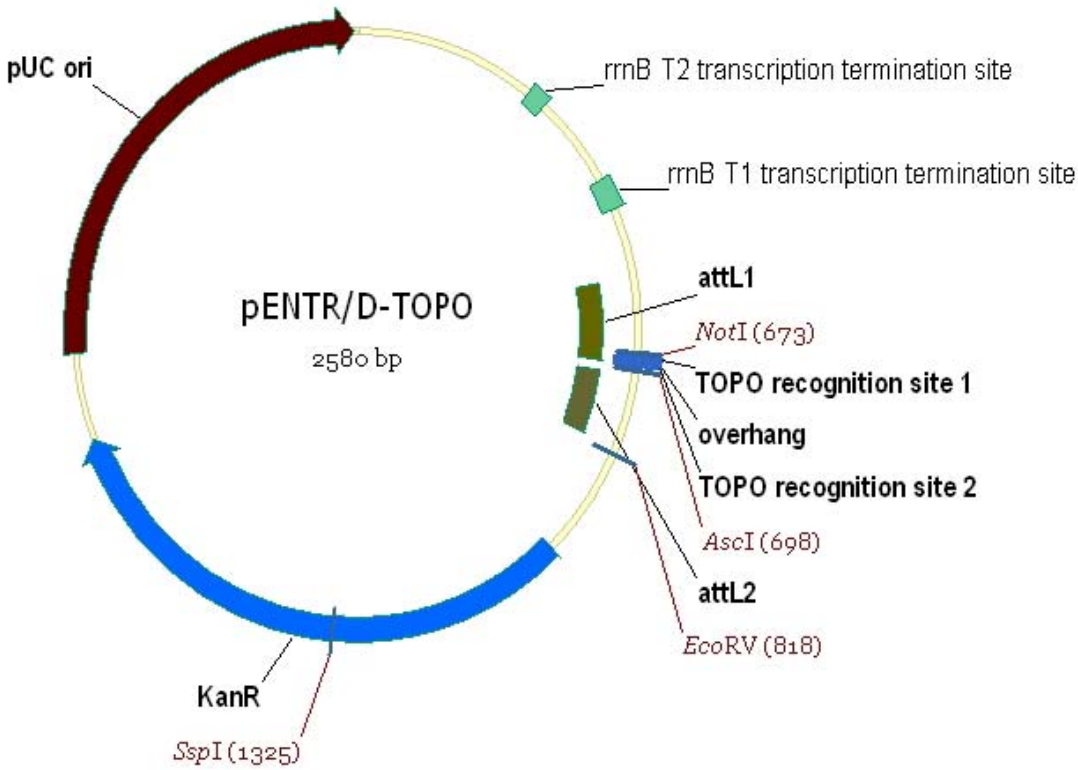
BanII (2949)
attP2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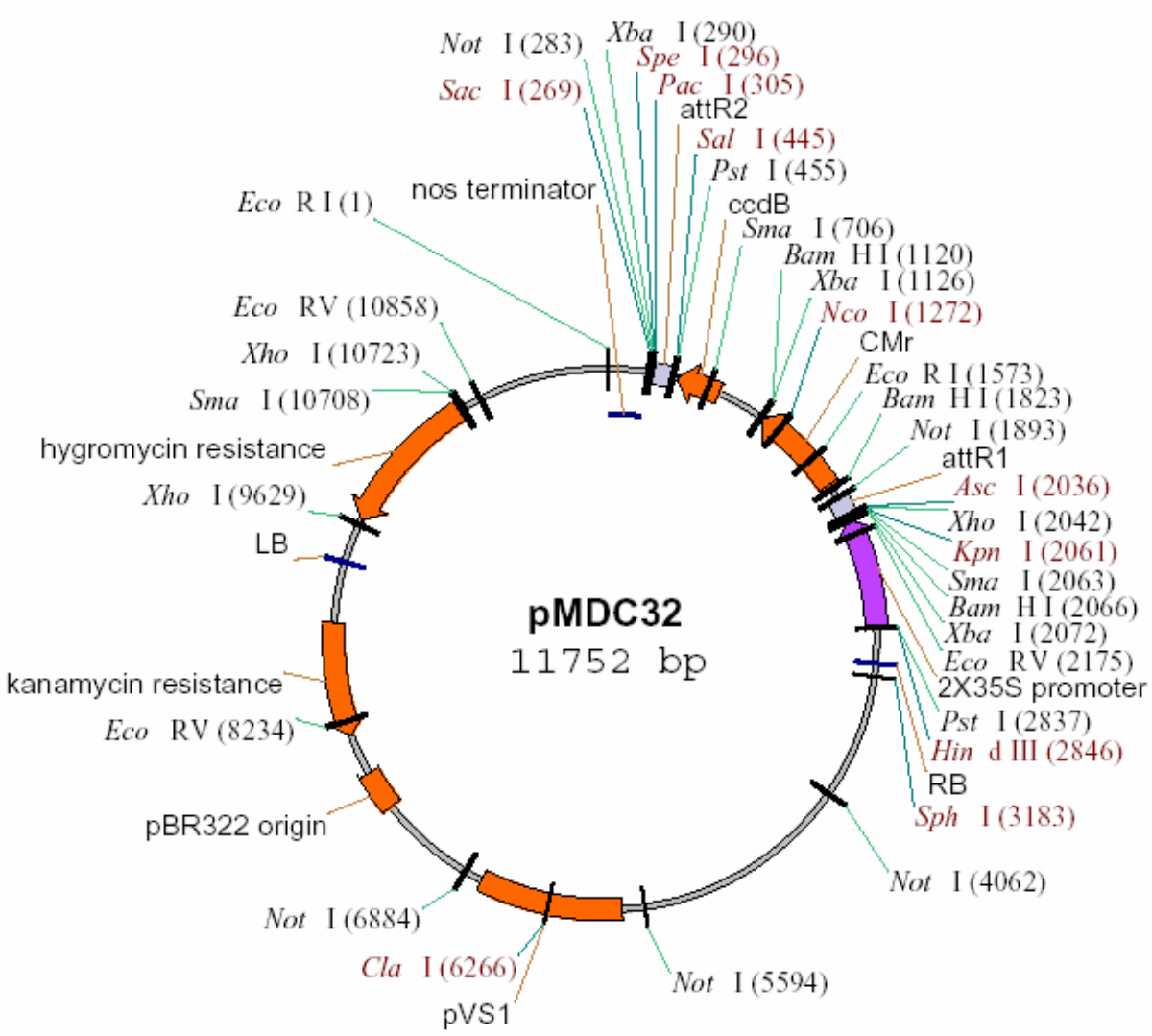
KanR
SspI (1325)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ccdB
Sma I (706)
Bam H I (1120)
Xba I (1126)
Nco I (1272)
Eco RV (10858)
CMr
Xho I (10723)
Eco R I (1573)
Sma I (10708)
Bam H I (1823)
Not I (1893)
hygromycin resistance
attR1
Xho I (9629)
Asc I (2036)
Xho I (2042)
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Kpn I (2061)
Sma I (2063)
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Bam H I (2066)
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Eco RV (2175)
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2X35S promoter
Eco RV (8234)
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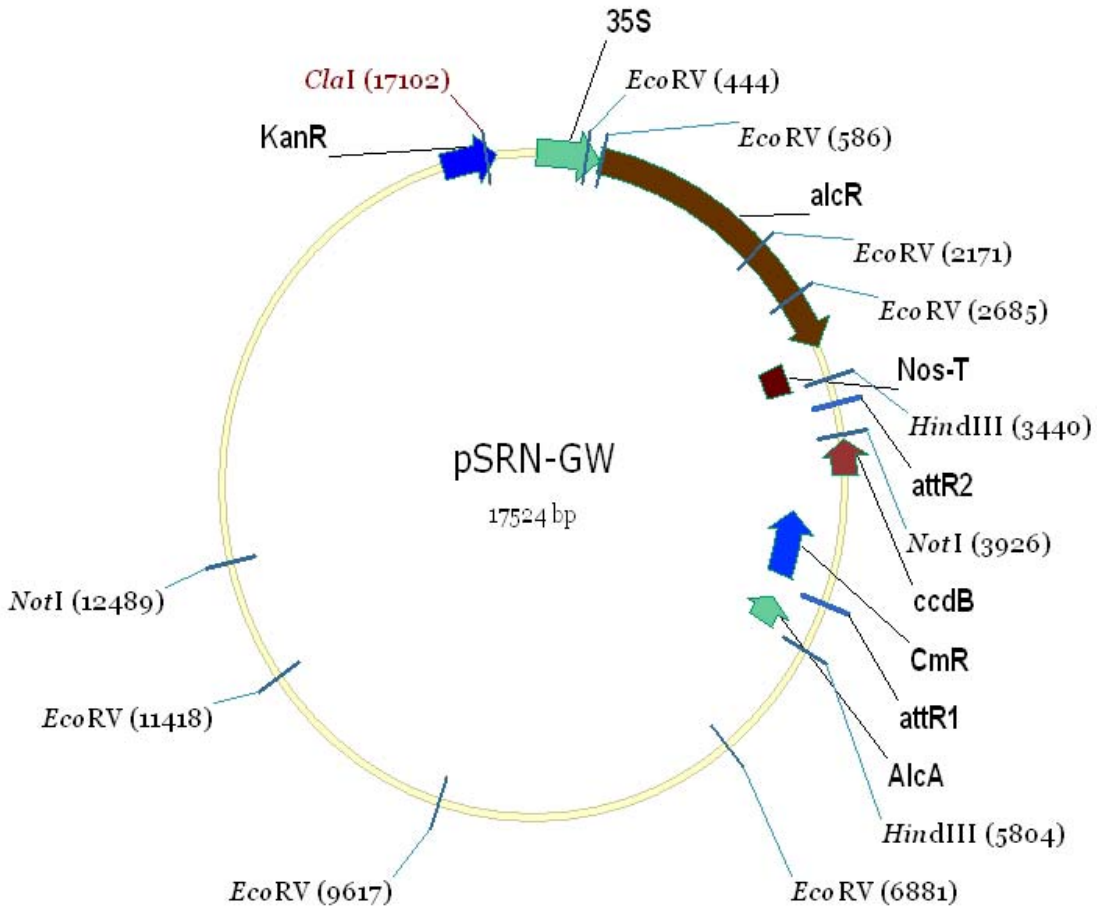
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HindIII (3440)
pSRN-GW
attR2
17524 bp
NotI (3926)
ccdB
NotI (12489)
CmR
attR1
EcoRV (11418)
AlcA
HindIII (5804)
EcoRV (9617)
EcoRV (6881)

APPENDIX B

Commonly used oligonucleotides, not mentioned in the text.

Primers used for cloning with the GATEWAY™ system			
Name	**Forwards Primer Sequence (5' - 3')**	**Name**	**Reverses Primer Sequence (5' - 3')**
AT3G48360F	GGAGATAGAACCATGGAAGCTGTTCTTGTCG	AT3G48360R	CAAGAAAGCTGGGTCTTAAACCCCTTGTGCTTGTT
AT4G26150F	GGAGATAGAACCATGGGTTCCAA TTTTCATTACAC	AT4G26150R	CAAGAAAGCTGGGTCTCACCCGTGAACCATTCCGA
AT2G33720F	GGAGATAGAACCATGAAGATGCCTCCTCCCTTTTC	AT2G33720R	CAAGAAAGCTGGGTCCTATGGACTTGGAGGAAGAGGC
AT1G02230F	GGAGATAGAACCATGATGAATCCGGTGGGT	AT1G02230R	CAAGAAAGCTGGGTCTCAAAAGAGCCAATCCCGA
AT1G01530F	GGAGATAGAACCATGGCGAGAAAGAATCTTGT	AT1G01530R	CAAGAAAGCTGGGTCCTAATAGTAACGAGCCCAAT
AT2G33550F	GGAGATAGAACCATGGCTCTGGAACAGTTAG	AT2G33550R	CAAGAAAGCTGGGTCCTACATCTTATCCGCGATTT
AT3G51910F	GGAGATAGAACCATGATGAACCCGTTTCTCCC	AT3G51910R	CAAGAAAGCTGGGTCTTATGAGGTGGAAGCCAAAC
PAP1 F	GGAGATAGAACCATGGAGGGTTCGTCCAAAGG	PAP1 R	CAAGAAAGCTGGGTCCTAATCAAATTTCACAGTCTCTC
AT1G01530F	CACCATGGCGAGAAAGAATCTTGGT	AT1G01530R	CTAATAGTAACGAGCCCAATAC
AT2G43500F	CACCATGGAAAACCCTTTTGCTTCTA	AT2G43500R	TCATAAGCCTGTTCCAAGATAA
AT4G38340F	CACCATGGTGGGACCTTTTAAAAAG	AT4G38340R	TTAAGAACCAAAGGAAGGACG
AT2G23740F	CACCATGGCTTCACCAGAGGCTGA	AT2G23740R	CTAGAACAAGTAAGATACCTCC
AT1G02230F	CACCATGATGAATCCGGTGGGTTT	AT1G02230R	TCAAAAGAGCCAATCCCGAGGGAT
AT2G33720F	CACCATGAAGATGCCTCCTCCCTT	AT2G33720R	CATTGGACTTGGAGGAAGAGGAGC
AT1G76350F	CACCATGGAAAACAATTCTCTTC	AT1G76350R	TCATGAAAGACATCCACTTCCACTGACA
AT3G25790F	GGAGATAGAACCATGATCAAGAACTTAAGTAATATG	AT3G25790R	CAAGAAAGCTGGGTCTTATGATATTATTTTTGCATCTTTC
AT1G13300F	GGAGATAGAACCATGATTAAAAAGTTCAGCAATATG	AT1G13300R	CAAGAAAGCTGGGTCTTAATTATTCTTGACGTAATGATTA
AT2G22200F	GGAGATAGAACCATGGAAACTGCTTCTCTTTCTTT	AT2G22200R	CAAGAAAGCTGGGTCTTAAGAATTGGCCAGTTTACTAAT
AT1G35560F	GGAGATAGAACCATGGAGTCCCACAACAACAAC	AT1G35560R	CAAGAAAGCTGGGTCTCAAGGAGAACCATCTAT
attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTT CGAAGGAGATAGAACCATG	attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTC

Primers used for sequencing the pENTR GATEWAY™ clones			
Name	**Sequence (5' - 3')**	**Name**	**Sequence (5' - 3')**
Entry207 F	TCGCGTTAACGCTAGCATGGATCTC	Entry207 R	GTAACATCAGAGATTTTGAGACAC
		sq4g38340R2	GAAGGTGGTGACTTTGGAGGCA
sq2g43500F1	TGTTCTTTTCCGGGTCTTCC	Sq2g43500R1	CGCTCTTTATTCCTTCACTGC
		Sq2g43500R1	GTTGTTGGAGAGCGCTTAAG
sq1g02230F1	GGGTTTTTTGTTGTTGATTGACC		
sq1g02230F2	GGTCACTTTCTTTGACAAAAG		

Sq2g23740F1	CCCGCAAGACCAACATATAAARG	sq2g23740R1	GATATCGAGGTTATTGGGGCG
		Sq1g21250R1	CCTAGAGTGCCTTGCACCATAG
		sq1g21250R2	CCAAGCAGAARGACCAAGAAG
Primers used for PCR screening of T-DNA/ transposon knock-out lines			
Name	**Sequence (5' - 3')**	**Name**	**Sequence (5' - 3')**
LbB1	GCGTGGACCGCTTGCTGCAACT		
Ds-5-2a	TCCGTTCCGTTTTCGTTTTTTAC	Ds3-2a	CCGGATCGTATCGGTTTTCG
S_044822F	CAGAACGATTGACGACAGCCA	S_044822R	GCACGAAACGAACACCAAGAA
S_043198F	TCGGAATTATCTTCCACACAAGGA	S_043198R	TGTGAGTTTCACTCAAAACTGTG
R11-4080-1F	CCTTCATGTGGTCTCCAACGC	R11-4080-1R	TGCAGATGCTTTCAAGGTCAGG
R11-3528-1F	TGCAGTAGCTACGCCAAAGC	R11-3528-1R	AATCCCGGCATCAAATTCTAA
S_100686F	CGCATCCCAATCAATCTCCAC	S_100686R	TCCACCAAATCCAAAACCAGC
S_114812F	AACGTGGTGCATTGTTGGTGA	S_114812R	CGGTTGCCTCTGATGAAATCCT
S_035023F	GATGTCAAATCTGAAAACAGAGGA	S_035023R	TGTTGTTGATTGACCTAATTCCA
S_041286F	TTTTCTTTTTGTTTTAAGGAGCAA	S_041286R	ATCGACCAGCAATCAAGGAC
S_058359F	CGGTGTTTTTAAGCCTCGAC	S_058359R	TCCACCATGTCTACTTGCCA
S_070096F	TGTGGTGGTCCTGTCTTTGA	S_070096R	AAAACAGTTCTTTATCAACCCGA
S_044835F	GCAGTCGCATACGCATAGAA	S_044835R	CCAACGAACGTTTTGGAGAT
S_027490F	ATGGAGGTAGTGCGTTCTGG	S_027490R	CGTAGAACCCAAAAGCCAAG
S_108879F	TTTCTCCGGTTTGTTGATGA	S_108879R	GCGAAGCTTAACTTCCCAAA
S_008978F	GGGTTTCCTATGAGTCTTCAGC	S_008978R	AGTAAGCCTCCGTTTCCTCC
S_118487F	TTTCATCCTCATCACCATCG	S_118487R	TAGGAGGGAAGAGGAGAGGC
S_138492F	ATACTCTGTTTTTGGCGGGA	S_138492R	GCTCATGAAAGCACTTCAACC
S_070820F	GGCTAGCCAGGTGACAAAAA	S_070820R	GAAGACAAGCACAAGAACTCTGA
S_025839F	CAAAAAGAAGCTTTGGCTGAA	S_025839R	CAGTAGCCTGTGGGAGATTTG
S_003418F	GAGGCCAAGCTTTCCTTCTTC	S_003418R	AGCTGCTCCGACCCAACTT
S_003995F	CAACGATTGCGTGATTAGGA	S_003995R	GCCTTCCTTTGCCTTATTCC
S_026238F	TCTGACCCTCGACTTGGATT	S_026238R	CGCTCCATCTATGGTTTATGG
S_028397R	TCTGACCCTCGACTTGGATT	S_028397R	CGCTCCATCTATGGTTTATGG
S_047951L	ACCAAACAACATAAAGTGGCA	S_047951R	GAAAGAGAAACAGCCAGAAGC
S_054446F	TCCTGACTCAACAGTCCAGCTA	S_054446R	CGTATCACAAATCCACCCTTTT
S_055886F	TCCTGACTCAACAGTCCAGCTA	S_055886R	TCCTGACTCAACAGTCCAGCTA
S_064696F	TCCCAAACCTCATACACGAAGA	S_064696R	CGACCAACAAAGAAAACTAGGACA
S_067074L	CAAAGCAAATCAGGGAGTTTA	S_067074R	TCAAGAATATGACAAGTCAACAATG
S_080138F	GGAATTTGCAAACGAAGAGTTTTT	S_080138R	GAGGTGGAAGCCAAACTCTCA

Q-RT-PCR primers for non-TF genes			
Name	**Sequence (5' - 3')**	**Name**	**Sequence (5' - 3')**
AMT1.1F	GCTTAGGAGAGTTGAGCCACG	AMT1.1R	CAAATCAAACCGGAGTAGGTG
AMT1.5F	TGATGATTCCATTGGAGTGCCT	AMT1.5R	CTTGAAAATCAAACGGCTGG
GS1 (AT5G37600) F	TGAGAAGCAAAGCCAGGACTC	GS1R	TCCACTTTGGTAGCTGCGAAG
GS2 (AT5G35630) F	TGAAGTTATGCCTGGACAGTG	GS2R	CACCTGCATCAATTCCTACGC
NIA1F	GGCTACGCTTATTCTGGAGGAGGT	NIA1R	TGGTGGTCAAGCTCACAAACACTC
NIA2F	GCCGACGAAGAAGGTTGGTGGTAT	NIA2R	GAAGAATCTCCTCGTGACATGGCG
NIIF	GAAACCCCGATTTCACCAACTTGC	NIIR	CATGAGTCCCCACCACACACACAT
NRT1.1F	CTGCCACACACTGAACAATTCC	NRT1.1R	CCCGCTTCCTGATCCCTTAT
NRT2.1F	GCCTGAGAACACACCCAACAA	NRT2.1R	TCCTTCACCATTCCACCGTG
NRT2.2F	CCAAAGACAAATTCGGAAAGATTCTA	NRT2.2R	AGAAGTACTCGGCGATAACATTGTCT
NRT2.3F	CTCTAAAGACAAATTCTCCAAGGTTTC	NRT2.3R	AAATATCCGGAGATAACGTTGTTGATA
NRT2.4F	CAGTTACTAAAGACAAGTTCTCAAAGGTTTTA	NRT2.4R	AAGTACTCAGCGATGACGTTATCG
NRT2.5F NRT2.6 !!!!!	TTCTAAAGACAAATTCTCCAAGGTCTTT	NRT2.5R	GTAGAAGTATCCAGATATAACGTTGTTGATC
NRT2.6F NRT2.5 !!!!!!	CTTGTTCGGTCAGGATCTTCCT	NRT2.6R	TGTTATCCATCCCCTATAGTTTTTGAT
NRT2.7F	TCGTCTCCACGCGATCG	NRT2.7R	CGAACATCATCACCGGAAAAC
GPDH1(At1g24280)F	GCTGCTGCACTCTTCATCGACAAT	GPDH1(At1g24280)R	TCTTATCTCCGCACTTCGGGTGTT
GPDH2(At5g13110)F	CACTACACACCAGGAGTGCAGAGA	GPDH2(At5g13110)R	ACAAATTCCCCGGAACATGCCTGA
GOGAT(AT5G53640)F	GCCAACACTTGCTGAGAAGCTAGG	GOGAT(At5g41670)R	TGCGAAAACACCTTCCACGGTT
PGDH(At5g41670)F	GATCGTCCTGGTGCATACCACA	PGDH(At5g41670)R	AAGCCCTCAAATCAAGGGACTGT
PGI(At4g24620)F	TTGGGAGCTTGAGCCAGGT	PGI(At4g24620)R	AGTTCCCTGTAGCATCCCAAAG
FNR(AT4G05390)F	TGGAGTCATTCCTCCTGGTGAGAA	FNR(AT4G05390)R	AGCTCGACGGACACATAGACTAGC
At5g50200(NAR2)	AGCCGAGAAGGACCAGGTGTTGTT	At5g50200(NAR2)	ACCCAATCGAGCTTAGCGTCCAT
AT5G65080 F	TTTTTTGCCCCCTTCGAATC	AT5G65080 R	ATCTTCCGCCACATTGTAC
Ubq-10 (AT4G05320) F	CACACTCCACTTGGTCTTGCGT	Ubq-10 (AT4G05320) R	TGGTCTTTCCGGTGAGAGTCTTCA
Actin-2 (AT3G18780) F	TCCCTCAGCACATTCCAGCAGAT	Actin-2 (AT3G18780) R	AACGATTCCTGGACCTGCCTCATC
β-6-tubulin (AT5G12250) F	ACCACTCCTAGCTTTGGTGATCTG	β-6-tubulin (AT5G12250) R	AGGTTCACTGCGAGCTTCCTCA
EF 1α (AT5G60390) F	TGAGCACGCTCTTCTTGCTTTCA	EF 1α (AT5G60390) R	GGTGGTGGCATCCATCTTGTTACA
adenosyl-phosphoribosyltransferase (AT1G27450) F	GTTGCAGGTGTTGAAGCTAGAGGT	adenosyl-phosphoribosyltransferase (AT1G27450) R	TGGCACCAATAGCCAACGCAATAG
Other primers			
Oligo dT	TTT TTT TTT TTT TTT TTT		
LUC R	GATCTTTCCGCCCTTCTTGG	LUC R	CCTGCGTGAGATTCTCGCAT

APPENDIX C

Real-time RT-PCR results for all TF genes, exhibiting more than 10-fold transcript changes, under nitrogen deprivation or 30 min after nitate replenishement.

AGI	Gene Name[3]	TF family	Amplicon[1]	Rep[2]	E	CT FN	ΔCT FN	CT N-	ΔCT N-	ΔΔCT	N-/N+	CT N 30'	ΔCT N 30'	ΔΔCT	N 30'/N-
AT5G65060	MAF3	MADS	79	1	0.54	40.00	20.65	31.22	12.37	-8.28	36.32	40.00	20.21	7.84	0.03
				2	0.85	40.00	21.74	23.27	5.15	-16.59	26264.6	24.57	3.74	-1.41	2.37
AT5G60890	ATR1	MYB	61	1	0.83	40.00	20.65	25.57	6.72	-13.93	4489.89	26.83	7.04	0.32	0.83
				2	0.73	26.43	8.80	25.88	8.12	-0.68	1.45	NM	NM	NM	NM
AT5G54680	AtbHLH105	bHLH	74	1	0.66	34.59	15.24	26.99	8.14	-7.10	36.39	28.93	9.14	1.01	0.60
				2	0.85	26.56	6.92	25.77	6.08	-0.84	1.68	25.48	4.77	-1.31	2.24
AT5G38800	AtbZIP43	bZIP	63	1	0.68	35.77	13.93	30.56	8.52	-5.41	16.79	30.54	9.41	0.89	0.63
				2	0.66	35.96	18.28	35.79	16.00	-2.28	3.18	32.13	11.61	-4.39	9.30
AT5G24470	APRR5	ARR	60	1	0.91	25.18	5.15	40.00	20.71	15.56	0.00	40.00	19.68	-1.03	1.95
				2	0.83	31.24	11.60	29.45	9.76	-1.84	3.05	30.56	9.85	0.09	0.95
AT5G23000	AtMYB37	MYB	109	1	0.82	38.45	19.10	29.79	10.94	-8.16	129.80	31.72	11.93	0.99	0.55
				2	0.81	28.25	10.63	28.41	10.65	0.02	0.99	NM	NM	NM	NM
AT5G22570	AtWRKY38	WRKY	72	1	0.79	34.13	14.78	27.64	8.79	-5.98	32.54	26.65	6.86	-1.93	3.08
				2	0.63	31.33	13.71	29.02	11.27	-2.44	3.27	NM	NM	NM	NM
AT5G22290		NAC	60	1	0.74	31.22	11.87	28.94	10.09	-1.78	2.68	40.00	20.21	10.12	0.00
				2	0.68	NM	NM	28.21	10.45	NM	NM	30.69	10.29	-0.16	1.09
AT5G10570	AtbHLH061	bHLH	101	1	0.85	29.75	9.72	33.06	13.77	4.05	0.08	34.22	13.90	0.14	0.92
				2	0.87	32.30	12.66	34.52	14.83	2.18	0.26	36.78	16.07	1.24	0.46
AT5G07310		AP2-EREBP	63	1	0.81	40.00	18.16	34.61	12.57	-5.59	27.55	35.83	14.70	2.13	0.28
				2	0.89	32.69	15.55	32.32	14.63	-0.92	1.80	35.40	15.43	0.79	0.60
AT5G01900	AtWRKY62	WRKY	83	1	0.73	36.04	16.69	30.13	11.28	-5.41	19.61	30.71	10.92	-0.35	1.22
				2	0.75	29.59	11.97	30.94	13.18	1.21	0.51	NM	NM	NM	NM
AT4G38340		NIN-like	125	1	0.81	30.19	10.84	27.31	8.46	-2.37	4.07	22.96	3.17	-5.29	22.80
				2	0.79	32.87	13.23	37.92	18.24	5.01	0.05	25.97	5.26	-12.98	1862.43
AT4G26150	GATA-22	GATA	79	1	0.87	27.38	7.35	27.70	8.41	1.06	0.51	24.06	3.74	-4.67	18.84
				2	0.90	28.75	9.11	32.73	13.04	3.93	0.08	27.25	6.54	-6.50	65.14
AT4G25490	CBF1	AP2-EREBP	64	1	0.78	35.89	14.05	36.45	14.41	0.37	0.81	29.39	8.26	-6.16	34.83
				2	0.64	38.97	19.33	39.24	19.56	0.23	0.89	31.61	10.90	-8.65	74.04
AT4G24020		NIN-like	141	1	0.78	28.62	9.27	28.80	9.95	0.69	0.67	25.52	5.73	-4.22	11.29

				2	0.76	30.68	11.04	37.92	18.24	7.20	0.02	28.57	7.86	-10.38	363.20
AT4G21030		DOF	100	1	0.72	38.88	17.18	35.95	14.29	-2.90	4.81	40.00	19.14	4.85	0.07
				2	0.88	34.74	15.10	38.47	18.78	3.68	0.10	33.80	13.09	-5.70	36.83
AT4G17980		NAC	148	1	0.77	36.58	17.23	34.56	15.71	-1.52	2.38	31.38	11.59	-4.12	10.55
				2	0.84	37.42	17.78	40.00	20.31	2.54	0.21	33.96	13.25	-7.06	74.42
AT4G17490		AP2-EREBP	88	1	0.88	27.44	5.60	27.13	5.09	-0.51	1.38	30.90	9.77	4.69	0.05
				2	0.86	33.40	13.75	32.43	12.74	-1.02	1.88	29.70	8.99	-3.75	10.24
AT4G11070	AtWRKY41	WRKY	64	1	0.70	39.10	19.75	34.17	15.32	-4.43	10.36	33.70	13.91	-1.41	2.10
				2	0.67	35.79	18.17	34.34	16.58	-1.58	2.24	NM	NM	NM	NM
AT4G01520		NAC	137	1	0.74	30.79	11.44	40.00	21.15	9.71	0.00	30.01	10.22	-10.93	425.63
				2	0.67	29.46	12.33	28.50	10.81	-1.52	2.18	32.14	12.16	1.35	0.50
AT4G01460	AtbHLH057	bHLH	60	1	0.90	27.02	6.99	30.09	10.80	3.81	0.09	31.18	10.86	0.06	0.96
				2	0.96	29.99	10.35	33.53	13.85	3.49	0.10	33.12	12.41	-1.43	2.62
AT3G66656		MADS	77	1	0.92	36.02	16.67	40.00	21.15	4.48	0.05	40.00	20.21	-0.94	1.84
				2	0.80	38.19	21.05	38.08	20.39	-0.66	1.47	38.52	18.55	-1.85	2.96
AT3G61950	AtbHLH067	bHLH	69	1	0.66	32.02	11.99	37.09	17.80	5.81	0.05	37.03	16.71	-1.09	1.73
				2	0.63	31.10	13.85	33.38	15.86	2.01	0.37	NM	NM	NM	NM
AT3G57920	SPL15	SBP	60	1	0.72	33.23	13.88	34.87	16.02	2.14	0.31	31.05	11.26	-4.76	13.31
				2	0.71	36.50	16.86	37.31	17.62	0.76	0.66	33.21	12.50	-5.12	15.60
AT3G56400	AtWRKY70	WRKY	91	1	0.85	28.10	8.75	23.43	4.58	-4.17	12.89	25.24	5.45	0.87	0.59
				2	0.81	26.26	8.64	24.37	6.62	-2.02	3.32	NM	NM	NM	NM
AT3G54620	AtbZIP25	bZIP	100	1	0.71	25.36	3.52	26.33	4.29	0.77	0.66	31.39	10.26	5.97	0.04
				2	0.80	27.44	7.80	26.96	7.28	-0.53	1.36	26.58	5.86	-1.41	2.29
AT3G51910	HSFA7A	HSF	116	1	0.78	40.00	20.36	34.79	15.10	-5.26	20.95	36.28	15.57	0.47	0.76
				2	0.78	33.88	14.09	26.43	8.75	-5.35	22.04	33.68	13.17	-0.93	1.71
AT3G49930		C2H2	77	1	0.85	29.33	7.63	34.04	12.38	4.75	0.05	28.50	7.64	-4.74	18.27
				2	0.75	29.35	11.66	33.84	14.05	2.38	0.26	32.24	11.73	-2.32	3.65
AT3G45170		GATA	150	1	0.70	36.01	15.98	40.00	20.71	4.73	0.08	37.73	17.41	-3.30	5.81
				2	0.67	37.60	19.98	38.78	21.02	1.04	0.59	NM	NM	NM	NM
AT3G30260	AGL8	MADS	140	1	0.81	33.66	14.31	34.57	15.72	1.41	0.43	31.51	11.72	-4.00	10.65
				2	0.84	40.00	20.36	40.00	20.31	-0.05	1.03	34.22	13.51	-6.81	62.62
AT3G25790		MYB-like	81	1	0.70	31.05	9.35	40.00	18.34	8.99	0.01	27.31	6.45	-11.89	555.30
				2	0.59	34.19	17.06	40.42	22.74	5.68	0.07	30.07	10.10	-12.64	359.41
AT3G13890	AtMYB26	MYB	119	1	0.64	36.13	16.10	40.00	20.71	4.61	0.10	36.68	16.36	-4.35	8.67
				2	0.55	33.71	16.03	40.00	20.21	4.18	0.16	33.60	13.08	-7.13	22.70
At2g47080		SBP	100	1	0.76	28.25	8.90	40.00	21.15	12.25	0.00	27.76	7.97	-13.18	1663.11

				2	0.67	27.49	10.35	27.09	9.41	-0.95	1.62	29.44	9.46	0.06	0.97
AT2G46870		ABI3VP1	84	1	0.66	21.07	0.77	27.88	5.84	5.07	0.08	27.28	6.15	0.31	0.86
				2	0.87	25.50	8.37	26.19	8.50	0.13	0.92	27.07	7.09	-1.41	2.42
AT2G43500		NIN-like	97	1	0.63	36.23	16.88	31.08	12.23	-4.65	9.61	32.46	12.67	0.44	0.81
				2	0.68	31.59	13.97	28.28	10.52	-3.45	6.05	NM	NM	NM	NM
AT2G41940	ZFP8	C2H2	98	1	0.74	30.14	8.30	36.17	14.13	5.82	0.04	40.00	18.87	4.74	0.07
				2	0.78	28.37	11.24	29.62	11.93	0.70	0.67	30.03	10.05	-1.88	2.95
AT2G40750	AtWRKY54	WRKY	77	1	0.75	33.03	13.68	28.09	9.24	-4.44	11.90	26.59	6.80	-2.43	3.88
				2	0.73	32.03	14.41	28.10	10.34	-4.06	9.38	NM	NM	NM	NM
AT2G39250		AP2 EREBP	63	1	0.77	29.73	7.89	32.94	10.90	3.02	0.18	26.68	5.55	-5.35	21.26
				2	0.85	34.01	14.37	36.98	17.29	2.93	0.17	28.01	7.30	-9.99	460.95
AT2G38340		AP2-EREBP	60	1	0.76	33.69	11.85	28.51	6.47	-5.38	20.87	29.65	8.52	2.05	0.31
				2	0.87	29.43	12.30	27.23	9.55	-2.75	5.60	28.44	8.46	-1.09	1.98
AT2G33720		ARP	100	1	0.72	40.00	18.16	34.13	12.09	-6.07	26.73	34.62	13.49	1.40	0.47
				2	0.79	35.80	18.66	32.32	14.64	-4.03	10.52	33.64	13.67	-0.97	1.76
AT2G33550		MYB-like	78	1	0.81	29.32	9.97	29.34	10.49	0.52	0.74	24.09	4.30	-6.19	39.43
				2	0.80	NM	NM	27.07	9.31	-0.66	1.47	24.96	4.56	-4.76	16.31
AT2G22200		AP2-EREBP	74	1	0.57	40.00	17.96	40.00	17.96	0.00	1.00	34.77	12.73	-5.23	14.16
				2	0.66	40.00	22.87	40.00	22.87	0.00	1.00	31.46	14.33	-8.54	75.73
AT2G03060	AGL30	MADS	63	1	0.93	40.00	19.97	40.00	20.71	0.74	0.62	34.58	14.26	-6.45	69.00
				2	0.74	NM	NM	35.57	17.82	NM	NM	40.00	19.60	1.78	0.37
AT2G02060		MYB-like	74	1	0.81	40.00	18.30	26.90	5.24	-13.06	2351.04	27.44	6.58	1.34	0.45
				2	0.73	28.93	11.80	31.50	13.82	2.02	0.33	28.04	8.07	-5.75	23.58
AT1G77950		MADS	75	1	0.92	40.00	19.97	34.81	15.52	-4.45	18.27	40.00	19.68	4.16	0.07
				2	0.87	31.90	14.76	32.42	14.74	-0.02	1.01	33.88	13.91	-0.83	1.69
AT1G76350		NIN-like	87	1	0.80	31.80	12.16	37.10	17.41	5.26	0.05	31.20	10.49	-6.93	57.72
				2	0.80	31.19	13.50	37.56	17.77	4.27	0.08	30.66	10.15	-7.62	86.66
AT1G76110		ARID HMG	100	1	0.84	27.36	8.01	31.13	12.28	4.27	0.07	30.53	10.74	-1.54	2.56
				2	0.79	27.24	9.62	30.34	12.58	2.97	0.18	NM	NM	NM	NM
AT1G73870	COL7	CO-like	61	1	0.86	27.10	5.40	31.92	10.26	4.86	0.05	30.41	9.55	-0.71	1.55
				2	0.83	27.44	10.30	31.64	13.96	3.66	0.11	33.53	13.55	-0.41	1.28
AT1G69780	ATHB-13	HB	128	1	0.68	40.00	18.16	40.00	17.96	-0.20	1.11	26.24	5.11	-12.85	791.36
				2	0.84	28.84	9.20	32.65	12.96	3.76	0.10	28.71	7.99	-4.97	20.67
AT1G68880	AtbZIP8	bZIP	80	1	0.87	26.21	4.37	29.85	7.81	3.45	0.12	24.62	3.49	-4.32	14.96
				2	0.89	33.60	13.95	36.99	17.30	3.35	0.12	31.98	11.26	-6.04	47.09
AT1G68670		MYB-like	98	1	0.75	28.24	6.54	32.17	10.51	3.97	0.11	26.04	5.18	-5.33	19.73

				2	0.74	31.49	11.85	36.82	17.14	5.28	0.05	27.59	6.88	-10.25	301.15
AT1G68520	COL6	CO-like	72	1	0.85	24.26	2.56	30.38	8.72	6.16	0.02	27.00	6.14	-2.58	4.88
				2	0.82	24.71	7.58	30.78	13.10	5.52	0.04	28.91	8.94	-4.16	12.19
AT1G68190		CO-like	128	1	0.70	27.12	5.42	32.46	10.80	5.38	0.06	32.20	11.34	0.54	0.75
				2	0.64	30.77	13.64	35.63	17.95	4.31	0.12	37.65	17.67	-0.28	1.15
AT1G66390	AtMYB90	MYB	150	1	0.82	31.69	11.66	23.14	3.85	-7.81	108.94	24.82	4.50	0.64	0.68
				2	0.83	30.21	12.59	21.58	3.83	-8.76	202.91	NM	NM	NM	NM
AT1G66380	AtMYB114	MYB	150	1	0.79	35.29	15.26	29.49	10.20	-5.06	18.84	31.03	10.71	0.51	0.75
				2	0.77	34.42	16.80	30.12	12.36	-4.44	12.50	NM	NM	NM	NM
AT1G65360	AGL23	MADS	80	1	0.78	40.00	19.97	35.25	15.96	-4.01	10.18	40.00	19.68	3.72	0.12
				2	0.82	37.30	20.17	40.00	22.32	2.15	0.27	35.02	15.04	-7.28	79.32
AT1G59940		ARR	78	1	0.78	27.59	8.24	29.18	10.33	2.09	0.30	25.28	5.49	-4.84	16.28
				2	0.77	32.59	12.94	35.44	15.75	2.81	0.20	30.02	9.31	-6.44	40.32
AT1G56650	AtMYB75	MYB	103	1	0.82	32.27	12.24	25.55	6.26	-5.98	35.86	26.30	5.98	-0.28	1.18
				2	0.87	27.15	9.90	22.65	5.12	-4.77	19.93	NM	NM	NM	NM
AT1G35560		TCP	90	1	0.92	25.77	6.42	26.26	7.41	0.99	0.53	21.21	1.42	-5.99	49.26
				2	0.92	NM	NM	24.28	6.53	0.11	0.93	23.10	2.70	-3.83	12.25
AT1G32640	RAP-1	bHLH	87	1	0.68	31.67	11.64	40.00	20.71	9.07	0.01	40.00	19.68	-1.03	1.70
				2	0.67	28.74	11.12	28.00	10.24	-0.87	1.57	NM	NM	NM	NM
AT1G27740	AtbHLH054	bHLH	77	1	0.69	28.87	8.84	40.00	20.71	11.87	0.00	40.00	19.68	-1.03	1.71
				2	0.72	26.92	9.30	27.21	9.45	0.15	0.92	NM	NM	NM	NM
AT1G26260	AtbHLH076	bHLH	116	1	0.86	28.68	8.65	40.00	20.71	12.06	0.00	40.00	19.68	-1.03	1.90
				2	0.79	28.29	10.66	27.06	9.30	-1.36	2.21	NM	NM	NM	NM
AT1G25440	COL16	CO-like	74	1	0.74	26.28	4.58	30.78	9.12	4.54	0.08	29.13	8.27	-0.85	1.60
				2	0.74	26.71	9.57	30.64	12.96	3.39	0.15	32.08	12.11	-0.85	1.61
AT1G22490	AtbHLH094	bHLH	61	1	0.76	28.62	8.59	40.00	20.71	12.12	0.00	40.00	19.68	-1.03	1.79
				2	0.96	26.96	9.71	27.34	9.82	0.11	0.93	NM	NM	NM	NM
AT1G22130		MADS	89	1	0.73	33.97	13.94	37.58	18.29	4.35	0.09	39.11	18.79	0.50	0.76
				2	0.50	40.00	20.36	40.00	20.31	-0.05	1.02	40.00	19.29	-1.02	1.52
AT1G18710	AtMYB47	MYB	60	1	0.79	39.63	19.60	34.04	14.75	-4.85	16.79	36.26	15.94	1.19	0.50
				2	0.78	29.01	11.75	31.74	14.21	2.46	0.24	NM	NM	NM	NM
AT1G13300		MYB	62	1	0.87	27.49	5.79	30.43	8.77	2.99	0.15	23.30	2.44	-6.34	53.05
				2	0.88	30.18	10.53	31.39	11.70	1.17	0.48	26.70	5.99	-5.71	36.64
AT1G02230		NAC	91	1	0.61	37.76	18.41	40.00	21.15	2.74	0.27	35.24	15.45	-5.70	14.96
				2	0.64	NM	NM	37.00	19.24	0.83	0.66	36.23	15.83	-3.41	5.38
AT1G02040	SPL8	SBP	73	1	0.42	40.00	18.16	39.07	17.03	-1.13	1.48	31.09	9.96	-7.08	11.69

				2	0.68	39.76	20.12	40.00	20.31	0.19	0.91	34.04	13.33	-6.98	37.22
AT1G01530	AGL28	MADS	62	1	0.64	37.53	17.50	36.74	17.45	-0.06	1.03	31.02	10.70	-6.75	28.59
				2	0.89	NM	NM	34.49	16.73	NM	NM	31.53	11.13	-5.60	35.09
AT1G01250		AP2-EREBP	89	1	0.75	40.00	18.16	31.27	9.23	-8.93	144.83	31.44	10.31	1.08	0.55
				2	0.80	30.98	13.85	31.72	14.04	0.19	0.89	33.83	13.85	-0.19	1.12

Table legend:
[1] in bp
[2] biological replica
[3] according to TAIR annotations (www.arabidopiss.org)
NM – not measured under conditions showing not changes in first biological replica
E – reaction efficiency

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1984-1992	Primary school in Jablonka Stara (Poland)
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28.05.1996	GCSE
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1999-2001	Master Student at the University of Wroclaw (Poland), Faculty of Biotechnology; Research project: “Novel type of RNA editing in plant mitochondria?”; Project leader: Prof. Dr. Hanna Janska
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Kuhn, C., Hajirezaei, M.R., Fernie, A.R., Roessner-Tunali, U., Czechowski, T., Hirner, B., and Frommer, W.B. (2003). The sucrose transporter StSUT1 localizes to sieve elements in potato tuber phloem and influences tuber physiology and development. Plant Physiol **131,** 102-113.

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Tomasz Czechowski, Thomas Altmann, Mark Stitt, Michael K. Udvardi and Wolf-Rüdiger Scheible (2005). Identification and Validation of Novel and Superior Normalizer Genes in *Arabidopsis* thaliana (*manuscript in preparation*)

List of presentations:

Czechowski, T., Bari, R.P., Stitt, M., Scheible, W.R., and Udvardi, M.K. (2004). **"Real-time RT-PCR profiling of over 1400 *Arabidopsis* transcription factors: unprecedented sensitivity reveals novel root- and shoot-specific genes"**

- 1st International qPCR Symposium & Application Workshop © Transcriptomics, Clinical Diagnostics & Gene Quantification; 3rd - 6th March, 2004 in Freising-Weihenstephan, Germany (Poster)
- 15th International Conference on *Arabidopsis* Research; 11th-14 th July, 2004, Berlin, Germany (Poster)
- 2nd Agronanotech Course; 26 th -29 th October, 2004, Foggia, Italy (Lecture);

Jens-Holger Dieterich and Tomasz Czechowski, Rosa Morcuende, Mark Stitt, Wolf-Rüdiger Scheible & Michael K. Udvardi (2004) „**Systematic in-depth analysis of nitrogen signalling in *Arabidopsis thaliana* (L.)"**

- 15th International Conference on *Arabidopsis* Research; 11 th -14 th July, 2004, Berlin, Germany (Poster)

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