NITROGEN ASSIMILATION IN HIGH LIPID (TRIACYLGLYCEROL) TRANSGENIC ARABIDOPSIS

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Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Master of Applied Science

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This study aimed to gain a greater understanding of the reason(s) for growth limitation in high lipid *Arabidopsis thaliana* line 'D1o3-3#47' under nitrate (NO_3^-) nutrition, as reported by the Plant Biotechnology group at Agresearch, Palmerston North. Growth (dry weight, DW) and nitrogen (N) related measurements were carried out for D1o3-3#47 and wild type (WT) Arabidopsis plants, initially under a range and then under a selected NO_3^- concentration. The study also investigated whether the vegetative growth in any way correlates with the leaf fatty acid content of the Arabidopsis lines studied. Finally, the growth of D1o3-3#47 plants was compared to WT plants initially under a range and then under a selected concentration of four N forms: NO_3^-, ammonium (NH_4^+), urea and glutamine.

D1o3-3#47 plants showed only 50% of the growth of WT plants at higher (≥ 2 mM) NO_3^--N concentrations. Under NO_3^- nutrition, D1o3-3#47 plants showed either similar or occasionally greater shoot N% (of g DW), but consistently less shoot total-N than WT plants. Shoot NO_3^--N, leaf soluble protein content and leaf nitrate reductase activity (NRA) were greater in D1o3-3#47 plants than WT plants. A weak negative correlation was found between growth and leaf fatty acid content of the Arabidopsis lines studied. D1o3-3#47 plants showed between 53-59% of the growth of WT plants under selected N concentration of NO_3^-, ammonium (NH_4^+), urea and glutamine, and their growth was not better than WT plants even under Thrive® nutrition. It appears that D1o3-3#47 plants show limited growth regardless of the N availability or form. Based on these results the possible reasons for the limited growth of D1o3-3#47 plants as compared to WT plants were discussed.

Keywords: Growth, nitrogen assimilation, nitrate, Arabidopsis, *Arabidopsis thaliana*, transgenic plants, genetic modification, lipids, oleosin, triacylglycerol, vegetative tissue, ammonium, urea, amino acids, glutamine, nitrate reductase.

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Chapter 1

Introduction

1.1 Introduction

Nitrogen (N) is an essential macronutrient and N availability is a major determinant of plant growth and crop productivity. Nitrogen is a key component of many macromolecules, including nucleic acids, amino acids, proteins, the photosynthetic pigments (chlorophyll a and b), energy carriers (e.g. nicotinamide adenine dinucleotide [NADH] and adenosine triphosphate [ATP]) and the plant hormones auxins and cytokinins (Andrews, Raven & Lea, 2013; Krapp et al., 2014; Raven, Handley & Andrews, 2004).

Plants take up and utilize various inorganic and organic forms of N, mainly nitrate (NO_3^-), ammonium (NH_4^+), urea (CH_4N_2O) and amino acids (Kraiser, Gras, Gutierrez, Gonzalez & Gutierrez, 2011). Nitrate is the most abundantly available inorganic N form taken up and assimilated by plants in cultivated soils, while NH_4^+ can be the dominant inorganic N source in uncultivated and/or acidic soils. Plant NO_3^- assimilation involves its reduction, first into nitrite (NO_2^-) and then into NH_4^+ by nitrate reductase (NR) and nitrite reductase (NiR) enzymes, respectively (Masclaux-Daubresse et al., 2010). Ammonium, either resulting from NO_3^- assimilation or directly taken up from the soil, is first converted into glutamine by glutamine synthetase (GS), then into glutamate by glutamate synthase (GOGAT), and eventually into other amino acids through the transaminase reactions (Xu, Fan & Miller, 2012). Urea is another important N source for plants, entering agricultural soils as N fertiliser and/or animal waste. Plants first hydrolyse urea into ammonia (NH_3) and carbon dioxide (CO_2) by urease, and then this NH_3 follows the NH_4^+ assimilation pathway (Witte, 2011). In both agricultural and natural habitats, plants can also take up and assimilate amino acids as an organic N source. Once taken up, these amino acids can be utilized directly or are converted into other amino acids and amides via the transaminase reactions (Näsholm, Kielland & Ganeteg, 2009). Thus, each N form has a unique assimilation pathway, which may or may not be linked to the assimilation pathway of another N form (Masclaux-Daubresse et al., 2010).

In most plants, triacylglycerol (TAG) is the major form of neutral storage lipid, serving as energy and carbon (C) reserves for seed germination and seedling development. Triacylglycerol consists of three fatty acids esterified to a glycerol molecule. Seed TAGs are synthesized within the membranes of the endoplasmic reticulum (ER), through the sequential incorporation of fatty acids to a glycerol backbone (Kennedy pathway). Diacylglycerol acyltransferase (DGAT) and phospholipid diacylglycerol

acyltransferase (PDAT) are two important enzymes that regulate the terminal rate-limiting steps of TAG biosynthesis (Lung & Weselake, 2006). Once assembled, TAGs are released into the cytosol enclosed in discrete structures called oil bodies (Frandsen, Mundy & Tzen, 2001). The most common proteins associated with the surface of oil bodies are oleosins, which stabilize the oil bodies and prevent them from coalescence during seed dehydration (Siloto et al., 2006).

As compared to seeds, leaves and other vegetative tissues accumulate less TAG, usually as a temporary storage intermediate during the turnover of membrane lipids (Lin & Oliver, 2008). Multiple strategies have been employed to elevate TAG levels in the vegetative tissues of crop species (see chapter 2 section 2.2), as lipids offer more than twice the energy density and economical extraction methods as compared to carbohydrates (Chapman & Ohlrogge, 2012; Durrett, Benning & Ohlrogge, 2008). Hence, plants with elevated lipid content in their vegetative tissues have tremendous applications in the biofuel, forage, food, and nutraceutical industries.

At Agresearch Grasslands Palmerston North, Nick Roberts and the Plant Biotechnology group have been involved in a research program to enhance the metabolizable energy of forage grasses. Initially, they developed transgenic perennial ryegrass (*Lolium perenne*) expressing the *Arabidopsis thaliana DGAT1* (*AtDGAT1*) gene that can accumulate up to 40% more leaf lipid than the wild type (WT) ryegrass (Winichayakul et al., 2008). Next, they engineered polyoleosins (multiple head-to-tail tandem oleosin repeats) and showed that these polyoleosins can be incorporated in the native oil bodies of Arabidopsis transgenic seeds—enhancing the integrity of oil bodies (Scott et al., 2010). More recently, Winichayakul et al. (2013) have shown that constitutive co-expression of DGAT1 and cys-oleosins (oleosin stabilized by strategically placed cysteines) results in long-term storage of oil bodies in Arabidopsis leaves, stem, and roots even after senescence. The study reported 2.1% and 6.5% TAGs of dry weight (DW) in the mature leaves and roots, respectively, of a transgenic line named 'D1o3-3#47'. Interestingly, it was also reported that the mature leaves of D1o3-3#47 plants fixed 24% more CO_2 m^{-2} s^{-1} causing a 50% increase in total leaf biomass per plant as compared to the WT Arabidopsis (when grown in a commercial potting mix) (Winichayakul et al., 2013). This 'cys-oleosin technology' was also developed for perennial ryegrass, alfalfa (*Medicago sativa*), camelina (*Camelina sativa*), tobacco (*Nicotiana tabacum*) and soybean (*Glycine max*). However, a preliminary study by the Plant Biotechnology group found that the 50% increase in total leaf biomass was observed only when the Arabidopsis D1o3-3#47 plants were treated with Thrive®, a commercial plant fertilizer containing urea and traces of NH_4^+, and not with a nutrient solution containing NO_3^- as the sole N source (N. Roberts, Agresearch Palmerston North, personal communication, May 2015). In fact, they found that under NO_3^- nutrition, the D1o3-3#47 plants showed limited growth as compared to the WT plants (N. Roberts, Agresearch Palmerston North, personal communication, May 2015). This was a concern

because although NH_4^+ and urea are usually applied as crop fertilizers, NO_3^- is still the most abundantly available N form taken up and utilized by plants in cultivated agricultural soils (Andrews et al., 2013).

1.2 Objectives of the study

The overall aim of this project is to gain a greater understanding of the reason(s) behind any growth limitation in D1o3-3#47 Arabidopsis under NO_3^- nutrition. Chapter 2 is a literature review of plant nitrogen nutrition and the biotechnological advancements in elevating lipid content in plants. Chapter 3 and 4 are the 'results' chapters, each with its own set of experiments. Chapter 5 is the Final Discussion of the whole study.

The objectives of this study are listed as follows:

- To confirm that D1o3-3#47 plants show limited growth as compared to WT plants under NO_3^- nutrition, as reported by Agresearch (Chapter 3).
- To determine whether the growth of D1o3-3#47 plants is lower than WT plants across all NO_3^- concentrations (Chapter 3).
- To examine NO_3^- assimilation in D1o3-3#47 and WT plants under a selected NO_3^- concentration (Chapter 3).
- To determine whether there is any correlation between vegetative growth and leaf fatty acid content of the Arabidopsis lines studied (Chapter 3).
- To assess whether the growth of high lipid D1o3-3#47 plants is better than WT plants under other N source(s) (Chapter 4).

Chapter 2

Literature Review

2.1 Plant nitrogen nutrition

Nitrogen (N) is an essential nutrient for plant growth and development as it is a constituent of deoxyribonucleic acid (DNA), the genetic material; ribonucleic acid (RNA), the genetic information carrier; amino acids and hence structural proteins and enzymes; the photosynthetic pigments chlorophyll a and b; the high energy compounds adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NADH); and the plant hormone groups auxins and cytokinins, involved in plant growth and development (Andrews et al., 2013). Hence, although only 1-6% of total plant dry weight (DW) consists of N, it plays an essential role in a plant's survival, growth and reproduction. Plants can take up and assimilate a variety of different N forms; mainly nitrate (NO_3^-), ammonium (NH_4^+), urea (CH_4N_2O) and amino acids.

Nitrate is the most abundant N form available to and utilized by plants in well aerated/cultivated soils, with concentrations ranging between 1-20 millimolar (mM) in interstitial soil water (Andrews et al., 2013; Krapp et al., 2014). Two types of NO_3^- uptake systems have been identified in plants; the NO_3^- specific low-affinity transport systems (LATS) which work at > 1 mM soil NO_3^- levels and the high-affinity transport systems (HATS) which work at < 0.5-1 mM soil NO_3^- levels (Andrews et al., 2013; Hawkesford et al., 2012; Miller, Fan, Orsel, Smith & Wells, 2007). Nitrate taken up by roots can either

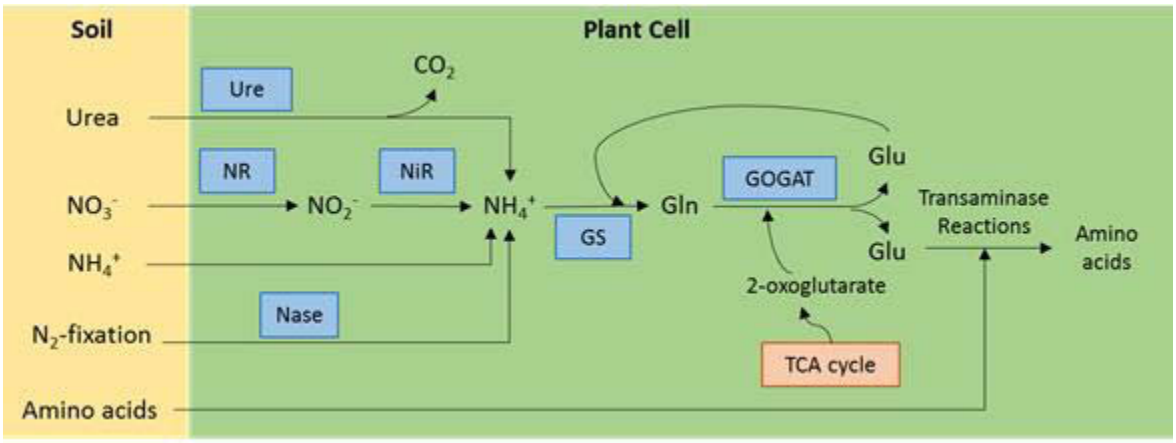

Figure 2.1 A simplified overview of the assimilation of inorganic and organic nitrogen forms in plants. Enzymes involved are shown in blue boxes. Associated biochemical pathway is shown in pink box. NR, nitrate reductase; NiR, nitrite reductase; Nase, nitrogenase; Ure, urease; GS, glutamine synthetase; GOGAT, glutamate synthase; NO_3^-, nitrate; NH_4^+, ammonium; N_2, nitrogen; gln, glutamine; glu, glutamate and TCA, tricarboxylic acid cycle.

be stored in the vacuole or assimilated in the root cells, or transported via the xylem to the shoot, where again it can be either stored or assimilated (Miller, 2010). Generally, only a little transport of NO_3^- has been observed from shoot to root via the phloem (Wang et al., 2012). During assimilation, NO_3^- taken up via roots is first reduced to nitrite (NO_2^-) in the cytosol by the NADH-assisted nitrate reductase (NR) enzyme (Fig. 2.1). Nitrite is then translocated to plastids where it is reduced to NH_4^+ by the ferredoxin (Fd)-assisted nitrite reductase (NiR) enzyme. Ammonium is then assimilated into amino acids through the glutamine synthetase (GS) and glutamate synthase (GOGAT) regulated pathways (Lam et al., 1996; Masclaux-Daubresse et al., 2010; Miller et al., 2007; Xu et al., 2012). Generally, for most plant species, both NR and NiR are light and substrate-induced enzymes (Andrews et al., 2013). Andrews (1986) proposed that the site of NO_3^- assimilation varies with genotype and environmental conditions, in particular with soil NO_3^- levels. For example, temperate cereals and grain legumes assimilate NO_3^- primarily in roots at ≤ 1 mM soil NO_3^- levels, however, shoot NO_3^- assimilation gains importance over the range of 1-20 mM soil NO_3^- levels (Andrews, 1986; Andrews, Morton, Lieffering & Bisset, 1992). In contrast, certain tropical and sub-tropical cereals and grain legumes assimilate NO_3^- mainly in their shoots over the entire range of NO_3^- concentrations (Andrews et al., 2004). Assimilation of one molecule of NO_3^- produces 0.67 molecules of OH^- in plants, which must be excreted or neutralised to maintain the biochemical pH-stat (Raven, 1985). Most of the OH^- generated in root NO_3^- assimilation is effluxed into the soil, while most of OH^- generated in shoot NO_3^- assimilation is neutralised by the synthesis of organic acids, particularly, malate (Andrews et al., 2004; Raven, 1985).

All plants studied can take up and utilize NH_4^+ and, in uncultivated and/or acidic soils, NH_4^+ can be the main source of N available to plants, with concentrations varying between 20 µM to 2 mM and above (Hawkesford et al., 2012; Jones, Owen & Farrar, 2002). Like NO_3^-, roots take up NH_4^+ through NH_4^+ specific LATS and HATS (Williams and Miller, 2001). Ammonium taken up by the roots is assimilated into amino acids through the glutamine synthetase (GS)/ glutamate synthase (GOGAT) pathway (Fig. 2.1). Glutamine synthetase regulates the ATP-dependent conversion of NH_4^+ and glutamate into glutamine, while GOGAT regulates the NADH or Fd-dependent conversion of glutamine and 2-oxoglutarate into two molecules of glutamate (Andrews et al., 2004; Lam et al., 1996; Masclaux-Daubresse et al., 2010). Most plant species assimilate NH_4^+ mainly in roots, although high soil NH_4^+ levels can shift their NH_4^+ assimilation to the shoot (Schjoerring, Husted, Mäck & Mattsson, 2002). Assimilation of one NH_4^+ generates 1.33 H^+ ions, which must be expelled via roots or neutralized in shoots to maintain cytoplasmic pH (Raven, 1985). However, there is a limitation on the ability of plants to neutralise H^+ in the shoot (Andrews et al., 2009 & 2013; Raven 1985) Generally, significant quantities of NH_4^+ transport to the shoot can cause NH_4^+ toxicity, which is characterized by reduced photosynthesis rate and growth, and necrotic lesions on the leaf surface (Bittsánszky, Pilinszky, Gyulai

& Komives, 2015). Several mechanisms have been proposed to explain NH_4^+ toxicity in plants, and there is robust evidence that pH imbalances and inhibition of K^+ uptake and transport are important factors (Andrews et al., 2004, 2009 & 2013; Bittsánszky et al., 2015; Raven, 1986). Certain genetic modification strategies, such as overexpressing enzymes and transporters involved in NH_4^+ compartmentation, assimilation, and detoxification, and silencing the genes involved in NH_4^+ uptake and translocation have been proposed to enhance NH_4^+ tolerance in crops (Bittsánszky et al., 2015). High irradiance has also been reported to improve NH_4^+ tolerance in wheat (*Triticum aestivum*) due to an increase in NH_4^+ assimilation rates (Setién et al., 2013).

Plants in agricultural soils can also take up and utilize urea (CH_4N_2O) as an N source (Merigout et al., 2008; Witte, 2011). Globally, urea comprises > 50% of N fertilizers applied to the agricultural soils (Heffer & Prud'homme, 2016). Grazing animals also deposit large amounts of urea into agricultural lands (Vitousek et al., 2009). Urea specific LATS and HATS have been characterized in Arabidopsis, rice (*Oryza sativa*) and maize (*Zea mays*) (Kojima, Bohner, Gasset, Yuan & Wirén, 2007; Wang et al., 2012; Zanin et al., 2015). Urea taken up by roots is hydrolyzed into ammonia (NH_3) and CO_2 by the urease enzyme (Witte, 2011). The resulting NH_3 is assimilated into amino acids through the GS/GOGAT pathway (Fig. 2.1) (Merigout et al., 2008). Assimilation of one urea-N generates 0.33 H^+ ions (Andrews et al., 2013). Less information is available in terms of urea uptake and assimilation partitioning between roots and shoots of most plant species, in comparison to NO_3^- and NH_4^+.

As compared to inorganic N forms (NO_3^- and NH_4^+), the importance of organic N forms; such as amino acids, amides and proteins, as potential plant N sources has been realized only recently—mainly through laboratory experiments under controlled conditions (Forsum, Svennerstam, Ganeteg & Näsholm, 2008; Näsholm et al., 2009; Paungfoo-Lonhienne et al., 2008). Evidence is strong that mycorrhizal and some non-mycorrhizal plants can take up and utilize amino acids, and in certain alpine, arctic and boreal forest ecosystems, amino acids can be their main N source (Näsholm et al., 2009; Weigelt, Bol & Bardgett, 2005). Forums et al. (2008) showed that Arabidopsis—a nonmycorrhizal species—prefers L-amino acids, such as L-glutamine and L-asparagine, over D-amino acids as N sources. Paungfoo-Lonhienne et al. (2008) showed that even intact proteins can be used, either directly or indirectly, as an N source in species like Arabidopsis and *Hakea actites*. Amino acids are most abundant near the surface of organic-rich natural soils, while in agricultural soils their concentrations are generally in the range of 1-100 µM (Jones et al., 2002; Miller, 2010). Once taken up, amino acids can be converted into other amino acids and amides, such as asparagine and arginine through the transamination reactions (Bloom, 2015).

In addition to NO_3^-, NH_4^+, urea and amino acid utilization by plants, a range of plant species, in particular, legumes (Fabaceae family) and actinorhizal plants can also take up and utilize atmospheric N_2 via symbiotic N_2-fixing bacteria. These symbiotic N_2-fixing bacteria, rhizobia in the case of legumes and *Frankia* for actinorhizal plants, exist and fix N_2 in root nodules (Franche, Lindström & Elmerich, 2009). The N_2-fixers use the nitrogenase (Nase) enzyme to reduce atmospheric N_2 into NH_3/NH_4^+, which is then assimilated via the GS/GOGAT pathway in plants (Fig. 2.1) (Bothe et al., 2007).

In conclusion, N is a crucial element for plant growth and development. Plants can acquire and utilize N in various inorganic and organic forms. Each N form has a unique uptake and assimilation pathway, which may or may not be linked to other N assimilatory pathway(s), but all result in providing the N required for the synthesis of amino acids, proteins and other essential N containing compounds (Bloom, 2015; Masclaux-Daubresse et al., 2010; Miller, 2010;).

2.2 Plants with elevated lipid content

The rising world population and industrial development have dramatically increased global energy requirements. The possibility of fossil fuels depletion and increasing atmospheric CO_2 concentration due to fossil fuels consumption have motivated researchers to find alternative energy options. Plant-derived oils can be sustainable and potentially 'environment-friendly' energy resources. The present global production of seed-derived oil is 194.3 million metric tonnes (Foreign Agricultural Service, United States Department of Agriculture, 2017). The majority of seed-derived oil is used for nutrition and only a small proportion, mainly coming from oil crops like rapeseed (*Brassica napus*) soybean (*Glycine max*), oil palm (*Elaeis guineensis*), camelina (*Camelina sativa*), switchgrass (*Panicum virgatum*) and Jatropha (*Jatropha curcas*), is used for biofuel production.

Lipids offer more than twice the energy density and more economical extraction methods than equivalent amounts of carbohydrates (Durrett et al., 2008; Chapman & Ohlrogge, 2012). The major form of lipid in seed-derived oils is triacylglycerol (TAG)—a neutral storage lipid synthesized mainly through the Kennedy pathway (Fig. 2.2). It consists of three fatty acids esterified to a glycerol backbone (Lung & Weselake, 2006). Triacylglycerol is mainly stored in seeds, enclosed in discrete subcellular organelles called lipid droplets or oil bodies, as long-term energy deposits for germination and seedling development. Moreover, the role of TAG in maintaining lipid homeostasis by acting as a buffer for cytotoxic fatty acids has also been elucidated (Fan et al., 2013). The surface of an oil body is covered with a monolayer of phospholipids embedded with hydrophobic proteins. Oleosins are the major class of oil body associated proteins in seeds, which stabilizes and prevents the oil bodies from coalescence during seed dehydration (Siloto et al., 2006).

Biotechnological advancements have increased the oil content of seeds by up to 40-60% (of DW) (Napier, Haslam, Beaudoin & Cahoon, 2014; Vigeolas, Waldeck, Zank & Geigenberger, 2007). However, as compared to seeds, vegetative tissues contain only small amounts of oil, which is generally used as a temporary storage intermediate during the turnover of membrane lipids. For example, in Arabidopsis, TAG accounts for < 0.1% (of DW) in leaves, stems and roots (Lin & Oliver, 2008; Yang & Ohlrogge, 2009; Xu & Shanklin, 2016). Vegetative tissues, specifically from energy crops like perennial grasses, have a greater proportion of harvestable biomass per hectare as compared to their seeds and fruits, and can potentially accumulate higher amounts of TAG (Chapman, Dyer & Mullen, 2013). Thus, it is proposed that a substantial improvement in global oil yield can be achieved if terrestrial crops are engineered to produce oil in their vegetative tissues rather than only in seeds (Durrett et al., 2008). Such crops with elevated TAG in their vegetative organs could be of immense economic value in the biofuel and forage production, human nutrition and the nutraceutical industry (Durrett et al., 2008).

Transgenic approaches to elevate TAG content in the vegetative tissues of plants have proven to be more promising than the conventional breeding methods. Several research groups have employed such approaches, which could be broadly categorized as either 'single-', 'paired-' or 'multiple-genes' strategies. Some recent advancements in these transgenic strategies to achieve improved TAG content in vegetative organs of plants have been briefly reviewed here.

A 'single-gene' transgenic strategy, to elevate TAG content in vegetative tissues of a plant, is to either overexpress, ectopically-express or knock-down an enzyme or transcription factor, which is involved in either fatty acid or TAG biosynthesis or degradation. Slocombe et al. (2009) reported 1-2% (of DW) TAG content in senescent leaves of Arabidopsis *PEROXISOMAL ABC TRANSPORTER 1* (*pxa1*) and *COMATOSE 2* (*cts-2*) mutants (with blocked fatty acid breakdown) as compared to 0.1% (of DW) TAG content in wild type (WT). The same study also highlighted the importance of the *DIACYLGLYCEROL ACYLTRANSFERASE 1* (*DGAT1*) gene in the partitioning of fatty acids to TAG biosynthesis in leaves (Slocombe et al., 2009). Ectopic expression of the *DIACYLGLYCEROL TYPE TWO* (*DGTT2*) gene, from the microalga *Chlamydomonas reinhardtii*, in Arabidopsis resulted in 1% (of DW) TAG as compared to 0.04% (of DW) TAG in WT (Sanjaya et al., 2013). Andrianov et al. (2010) demonstrated two different lipid enhancing strategies in the leaves of tobacco (*Nicotiana tabacum*).

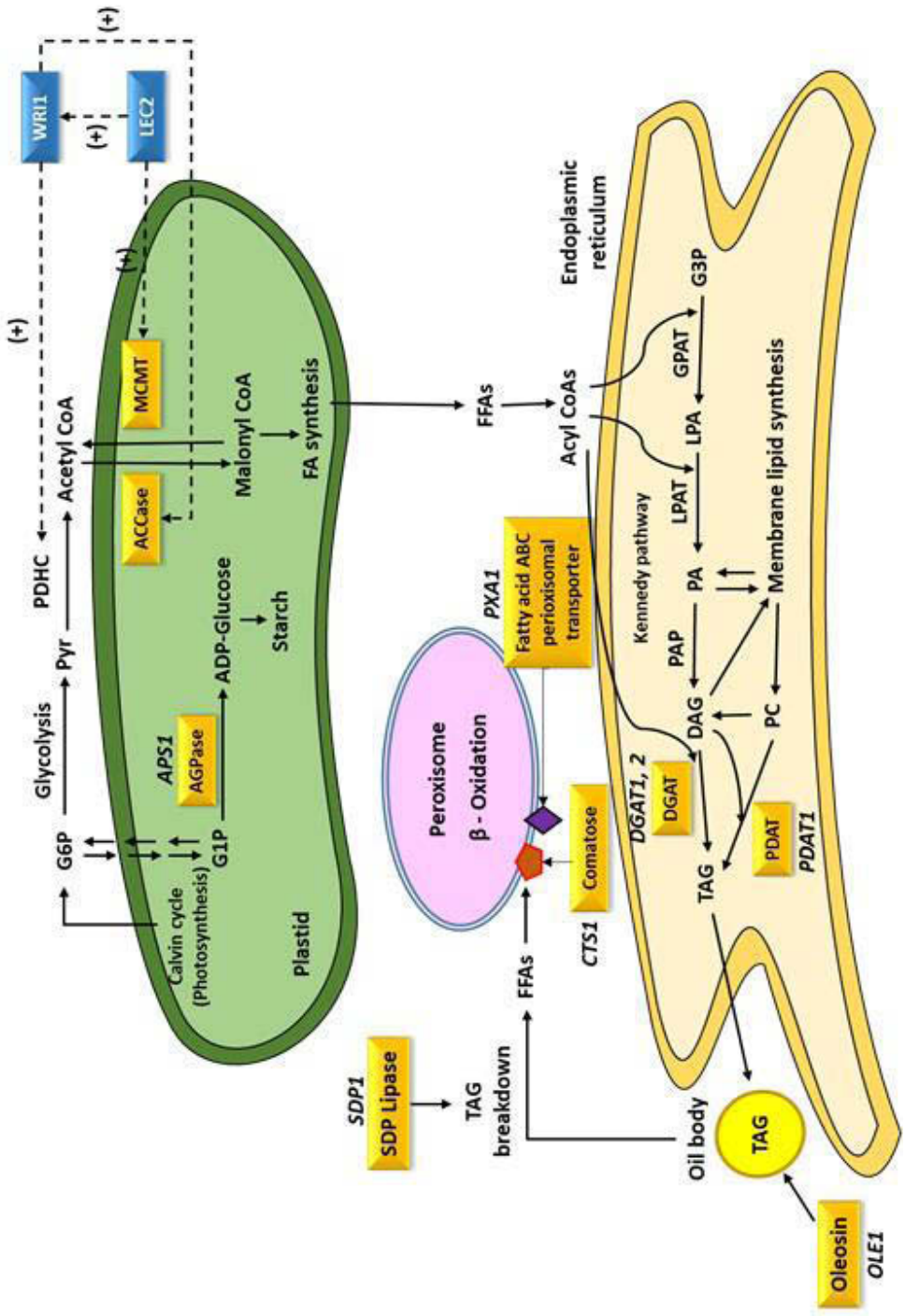

Figure 2.2 An overview of the targeted metabolic pathways to elevate TAG content in the vegetative tissues of plants. Genes for the targeted enzymes or transporters are italicized. Targeted enzymes and transporters are shown in yellow boxes and transcription factors are shown in blue boxes. Dotted line with a positive sign represents the up-regulation by transcription factors. Full forms of the targeted genes, transcription factors, enzymes and transporters names are given in Table 2.1. G6P, glucose-6-phosphate; G1P, glucose-1-phosphate; Pyr, pyruvate; ADP-Glucose, adenosine diphosphate-glucose; FFAs, free fatty acids; G3P, glycerol-3-phosphate; GPAT, glycerol-phosphate acyltransferase; LPA, lysophosphatidic acid; PA, phosphatidic acid; LPAT, lysophospatidic acid acyltransferase; PAP, phosphatidic acid phosphatase; DAG, diacylglycerol; PC, phosphatidylcholine; TAG, triacylglycerol.

Table 2.1 List of genes manipulated to elevate TAG content in the vegetative tissues of plants. Authors cited here have targeted either a single or a combination of genes. E, enzyme; T, transporter; TF, transcription factor.

Gene	Product	Function	References
ACC	Acetyl CoA carboxylase (ACCase) (E)	Catalyses the conversion of acetyl-CoA into malonyl-CoA for fatty acid synthesis	Klaus et al., 2004 Liu et al., 2016
APS1	Adenosine diphospho (ADP)-glucose pyrophosphorylase (AGPase) (E)	Catalyses the first step of starch biosynthesis	Sanjaya et al., 2011 Zale et al., 2015
CTS1	Comatose 1 (T)	Transports fatty acids into peroxisome for β-oxidation	Slocombe et al., 2009
DGAT1	Diacylglycerol acyltransferase 1 (E)	Catalyses the only committed step of TAG biosynthesis	Andrianov et al., 2010; Kelly et al., 2013 Liu et al., 2016 Winichayakul et al., 2013 Vanhercke et al., 2013 Zale et al., 2015
LEC2	Leafy cotyledon 2 (TF)	Regulates seed development, involved in TAG synthesis and oil body production	Andrianov et al., 2010 Kim et al., 2015 Slocombe et al., 2009
MCMT	Malonyl-CoA:acyl carrier protein malonyltransferase (E)	Catalyses the conversion of malonyl-CoA into acetyl-CoA for fatty acid synthesis	Kim et al., 2015
OLE1	Oleosin 1 protein	Stabilizes the structure of oil bodies and prevents them from coalescence	Fan et al., 2013 Liu et al., 2016 Scott et al., 2010 Vanhercke et al., 2013 Winichayakul et al., 2013 Zale et al., 2015
PDAT1	Phospholipid:diacylglycerol acyltransferase 1 (E)	Involved in TAG biosynthesis from membrane lipids	Fan et al., 2013 Kelly et al., 2013
PXA1	Peroxisomal ABC-transporter 1 (T)	Transports fatty acids into peroxisome for β-oxidation	Slocombe et al., 2009 Zale et al., 2015
SDP1	Sugar-dependent 1 lipase (E)	Initiates lipid breakdown in seeds after germination	Kelly et al., 2013
WRI1	Wrinkled 1 (TF)	Activates glycolytic and fatty acid biosynthesis genes	Li et al., 2015 Liu et al., 2016 Kelly et al., 2013 Sanjaya et al., 2011 Sanjaya et al., 2013 Vanhercke et al., 2013 Yang et al., 2015 Zale et al., 2015

In the first strategy, the Arabidopsis *DGAT* gene was overexpressed under a strong promoter, resulting in 5.6% (of DW) fatty acid content (correlated to their TAG content), in transgenic tobacco as compared to 2.8% (of DW) fatty acid content in WT. In the second strategy, an Arabidopsis transcription factor called *LEAFY COTYLEDON 2* (*LEC2*), which is a seed development regulator, was overexpressed under an inducible promoter resulting in 6.8% (of DW) fatty acid content in the senescent leaves of transgenic tobacco (Andrianov et al., 2010).

Further improvements of TAG accumulation in different vegetative tissues of plants have been achieved by targeting a combination or pair of genes belonging either to the same or different metabolic pathways. Sanjaya, Durrett, Weise & Benning (2011) showed that concurrent down-regulation of transient starch accumulation by suppressing adenosine diphosphate (ADP)-glucose pyrophosphorylase (AGPase), and up-regulation of fatty acid biosynthesis, by ectopically-expressing *WRINKLED 1* (*WRI1*), leads to a 5.8-fold increase in TAG accumulation in transgenic Arabidopsis seedlings compared to WT seedlings (Sanjaya et al., 2011). Vanhercke et al. (2013) combined the transient over-expression of Arabidopsis *DGAT1* and *WRI1* genes in *Nicotiana benthamiana*, resulting in 2.48% (of DW) TAG content in transgenic leaves in comparison with 0.6% (of DW) in WT leaves. Some research groups have not only focused on manipulating fatty acid and TAG biosynthesis genes but also targeted the oleosin protein to further stabilize the oil body's structure and prevent lipolysis. Fan, Yan, Zhang & Xu (2013) demonstrated that the co-expression of *PHOSPHOLIPID: DIACYLGLYCEROL ACYLTRANSFERASE1* (*PDAT*) and *OLEOSIN1* (*OLE1*) genes lead to 6.4% (of DW) leaf TAG in transgenic Arabidopsis leaves compared to 0.05% (of DW) in WT leaves, and highlighted the significance of *PDAT1* for future oil enhancing techniques. The 'cys-oleosin technology', as described in the 'Introduction' chapter of this study, has also employed the gene-pair strategy by co-expressing *DGAT1* and oleosin (Winichayakul et al., 2013). However, a unique feature of this strategy is that the oleosin structure has been stabilized, by strategically inserting cysteine units, which improved oil body integrity and prolonged the storage of 2.1% and 6.5% (of DW) TAGs in mature leaves and roots, respectively, in the transgenic Arabidopsis line 'D1o3-3#47' (Winichayakul et al., 2013).

Some authors have suggested that the maximum increase in TAG accumulation can only be achieved through integrated transgenic approaches that concurrently target multiple genes in carbon (C) metabolism and fatty acid biosynthesis ('push'), TAG biosynthesis ('pull') and lipolysis prevention ('protect') (Vanhercke et al., 2014; Napier et al., 2014; Xu & Shanklin, 2016). However, so far only a few groups have been successful in applying this 'push, pull and protect' strategy. Kelly et al. (2013) reported that the disruption of fatty acid turnover by silencing a TAG lipase called SUGAR-DEPENDENT1 (SDP1) and co-expressing *DGAT1* and *WRI1* genes resulted in 5-8% (of DW) TAG levels in Arabidopsis leaves, which after supplementing with sucrose further increased up to 17% (of DW) in

roots, although this TAG increment was accompanied by biomass reduction. Vanhercke et al. (2014) reported that co-expressing *WRI1*, *DGAT1* and *OLE1* genes result in 15.8% (of DW) TAG levels in tobacco leaves at the expense of starch level but without severely impacting growth. Recently, elevated TAG accumulation has been achieved in sugarcane (*Saccharum officinarum*), a C_4 biomass crop, by co-expressing *WRI1*, *DGAT1-2*, *OLE1* and co-suppressing (via RNAi technology) *AGPase* and a subunit of *PXA1* (Zale et al., 2016). This has resulted in up to 1.9% (of DW) TAG accumulation in leaves of transgenic sugarcane, which is 95-fold higher than the leaves of WT sugarcane (Zale et al., 2016). An extension of Vanhercke et al. (2014) 'push, pull and protect' strategy applied in potato (*Solanum tuberosum*), by overexpressing *WRI1*, *DGAT1* and *OLE1* genes, showed a 3.3% (of DW) TAG accumulation in potato tuber which was about 100-fold higher than the WT potato tuber (Liu et al., 2016). Hence, such transgenic strategies and proof of concepts have opened new areas of exploration for the non-seed tissues of crops as the alternative platforms to produce high energy storage lipids.

Conclusively, plant-derived oils can be a significant energy resource, and many biotechnological approaches have been employed to enhance their content. However, achieving long-lasting high lipid content in the vegetative biomass of plants is a challenging task. The major constraints are the tightly regulated fatty acid and TAG biosynthesis pathways and their reliance on C metabolism, the involvement of different cellular compartments in lipid biosynthesis and the regulation of each intermediate biochemical reaction by specialized enzyme(s). Optimization of these factors to increase oil yield in vegetative tissues without disrupting other physiological functions of the plant is a complex problem. However, results of some recent studies are encouraging, and have illustrated that informed integrated metabolic engineering approaches have the potential to overcome these barriers in the future (Kelly et al., 2013; Vanhercke et al., 2014; Zale et al., 2016).

Chapter 3

Growth and nitrate (NO_3^-) assimilation in high lipid Arabidopsis and the correlation between growth and leaf lipid content under NO_3^- nutrition

3.1 Introduction

Nitrate (NO_3^-) is the main source of nitrogen (N) available to and utilized by plants in disturbed/cultivated soils (Andrews et al., 2013). Plant NO_3^- assimilation involves a series of steps, in which NO_3^- is first reduced to nitrite (NO_2^-) by the action of nitrate reductase (NR) and then to ammonium (NH_4^+) by nitrite reductase (NiR) (Masclaux-Daubresse et al., 2010). The resulting NH_4^+ is then converted into glutamine and glutamate through glutamine synthetase (GS) and glutamate synthase (GOGAT) enzymes, respectively, and eventually into other amino acids via the transaminase reactions (Miller et al., 2007).

As described in chapter 1, a transgenic *Arabidopsis thaliana* line, named 'D1o3-3#47', was genetically engineered to accumulate up to 2.1% and 6.5% triacylglycerol (TAG) of dry weight (DW) in mature leaves and roots, respectively (Winichayakul et al., 2013). This transgenic line was reported to fix 24% more carbon dioxide (CO_2) m^{-2} s^{-1} causing a 50% increase in leaf biomass per plant as compared to wild type (WT) Arabidopsis, when grown in a commercial potting mix (Winichayakul et al., 2013). However, a preliminary study by the Plant Biotechnology group at Agresearch Grasslands found that D1o3-3#47 plants show reduced vegetative growth as compared to WT plants when supplied with a nutrient solution having NO_3^- as the sole N source but not with Thrive®, a commercial plant fertilizer containing mainly urea-N with traces of NH_4^+-N (N. Roberts, Agresearch Palmerston North, personal communication, May 2015). The reason(s) behind this limited growth of D1o3-3#47 plants under NO_3^- nutrition is (are) unknown.

Physiological changes, such as reduced growth and decreased starch levels and photosynthetic capacity, have been reported previously for plants with genetically enhanced TAG content in their non-seed tissues. Specifically, Sanjaya et al. (2011) reported delayed development and leaf expansion in high lipid transgenic Arabidopsis with suppressed adenosine diphosphate (ADP)-glucose pyrophosphorylase (AGPase) and overexpressed *WRINKLED 1* (*WRI1*) genes, grown on Murashige and Skoog (MS) medium. Kelly et al. (2013) reported smaller rosettes and a 20-30% reduction in leaf and root biomass in Arabidopsis *SUGAR-DEPENDENT1* (*sdp1*) mutants overexpressed with

DIACYLGLYCEROL ACYLTRANSFERASE 1 (*DGAT1*) and *WRI1* genes. Yang et al. (2015) studied the ectopic expression of *WRI1* in transgenic *Brachypodium distachyon* and reported cell death due to free fatty acids from rapid leaf TAG turnover. A substantial reduction in transitory starch levels was reported in transgenic tobacco leaves and potato tubers with combined overexpression of *WRI1*, *DGAT1* and *OLEOSIN* genes (Vanhercke et al., 2014; Liu et al., 2016). However, none of these studies has explicitly investigated whether the growth reduction in the high lipid plants was dependent on N availability or form. Moreover, no attempts have been made to investigate whether the growth of transgenic plants was in any way correlated to their leaf lipid content.

In this chapter, four experiments were carried out. Experiment 1 was aimed to confirm that D1o3-3#47 plants show limited growth as compared to WT plants under NO_3^- nutrition, as reported earlier by the Plant Biotechnology Group at Agresearch Grasslands. This experiment also aimed to determine whether the D1o3-3#47 plants show limited growth in comparison to WT plants across all NO_3^- concentrations. Experiments 2 and 3 examined how much NO_3^--N was taken up and how much NO_3^--N was assimilated in D1o3-3#47 in comparison to WT plants under a selected NO_3^- treatment. Experiment 4 assessed if there is a correlation between growth and leaf fatty acid content of different Arabidopsis WT and transgenic lines under NO_3^- nutrition. A Thrive® treatment was included in this experiment to determine the growth response of D1o3-3#47 and WT plants with this N source.

3.2 Materials and methods

3.2.1 Plant material

Arabidopsis thaliana (Arabidopsis) ecotype Columbia (Col-0) wild type (WT) and transgenic (T_4 generation) seeds from plant lines expressing the void vector (control) 'VC-BB1#2', only DGAT1 'D1-SA#43' and DGAT1 with cys oleosins, 'D1o3-3#47, D1o3-3#18 and D1o3-3#41' were provided by Dr Somrutai Winichayakul, Agresearch Grasslands, Palmerston North. For brevity, VC-BB1#2 and D1-SA#43 were referred to as BB1 and DGAT1, respectively. Experiment 1, 2 and 3 used seeds from only WT and D1o3-3#47 lines. Experiment 4 used the seeds from WT and transgenic lines (BB1, DGAT1, D1o3-3#18, D1o3-3#41 and D1o3-3#47).

3.2.2 General methods

All experiments were carried out at the physical containment level 2 (PC2) facility at Lincoln University, New Zealand; under 16 hrs day length, 18-22 °C temperature and 60-70% humidity level. Seeds were stratified in the dark at 4 °C for 48 hrs, while immersed in a 0.01% (w/v) agar solution prepared in double distilled H_2O (ddH_2O). Once stratified, 8-10 seeds per pot were dispensed over the potting mix using the 1 ml pipette. Plants were grown in 0.3 litres of black pots (Egmont, Christchurch) (Fig. 3.1). The 'N-free' potting mix used consisted of 80% composted bark and 20% pumice, to which was added 1 g/L calcium carbonate ($CaCO_3$), 0.3 g/L super-phosphate (9 P 11 S 20 Ca, Ravensdown, NZ) and 0.3 g/L Osmocote (0 N 0 P 37 K), 0.3 g/L Micromax trace elements and 1 g/L Hydraflo (Everris International, Geldermalsen, the Netherlands). To maintain adequate soil moisture, all pots were covered with transparent plastic sheets after sowing until germination. Seeds were germinated over the soil bed pre-moistened with the appropriate NO_3^- treatment. A stock solution of 1 M NO_3^-, as potassium nitrate (KNO_3), was used to prepare working solutions of different concentrations. All solutions were prepared using ddH_2O. Seedlings were thinned down to two per pot once they were one week old.

3.2.3 Experiments

In experiment 1, WT and D1o3-3#47 plants were kept under 50-70 µmol photons m^{-2} sec^{-1} of light and supplied with 0.5, 1, 2, 3, 4, 6, 8 or 10 mM NO_3^--N flushed through with 50 ml in each pot twice per week. There were six replicate pots (2 plants/pot) for each genotype per NO_3^- treatment. Potassium (K^+) concentration was balanced at 10 mM in all NO_3^- treatment solutions by adding calculated volumes of 1 M potassium chloride (KCl) stock, but chloride (Cl^-) concentration was not balanced. Six weeks old plants were harvested, and their shoot and root fresh weight (FW) and DW, total plant DW, shoot to root DW ratio (S:R) and shoot H_2O% were measured.

In experiment 2 (initial and repeat), WT and D1o3-3#47 plants were grown under the same light intensity as in experiment 1, and supplied with 8 mM NO_3^--N flushed through with 50-60 ml in each pot every two days. There were 50 replicate pots (2 plants/pot) for each genotype. At harvest, FW and DW of shoots and roots, S:R, shoot H_2O%, N%, total-N, NO_3^--N content, leaf *in vivo* nitrate reductase activity (NRA) and leaf soluble protein content were measured.

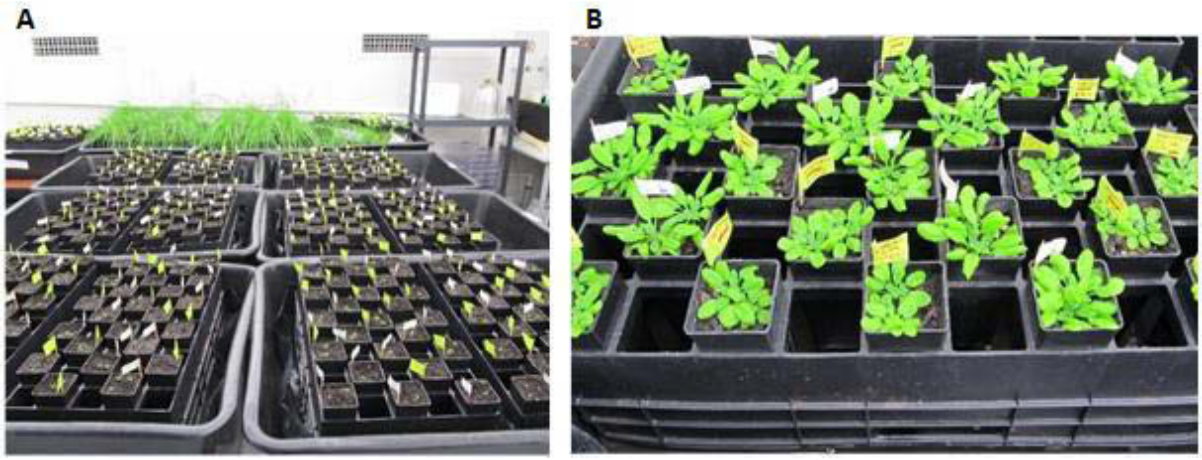

Figure 3.1 Plant growth room experiment set-up. (A) All trays (B) Single tray with randomized N treatments

Experiment 3 (initial and repeat) was carried out as for experiment 2, except that the irradiance level was 250-350 µmol photons m^{-2} sec^{-1}. Measurements in experiment 3 were as for experiment 2.

In experiment 4 (initial and repeat), Arabidopsis WT and transgenic lines BB1, DGAT1, D1o3-3#18, D1o3-3#41 and D1o3-3#47 were kept under 200-300 µmol photons m^{-2} sec^{-1} of light and supplied with 10 mM NO_3^--N or 2 g/L of Thrive®. This Thrive® concentration was the same as used in the preliminary study at Agresearch (S. Winichayakul, Agresearch Grasslands Palmerston North, personal communication, May 2015). Thrive® (2 g/L) solution contained 7.25 mM of urea-N and 0.48 mM of NH_4^+-N as the N source, along with 5% (w/w) phosphorus (P), 8.8% potassium sulphate (K_2SO_4), 4.6% sulphur (S), 0.5% magnesium sulphate ($MgSO_4$) and < 0.2% of trace elements. In the initial experiment 4, the concentration of applied Thrive® was doubled when the plants were two weeks old and tripled in the third week, in an attempt to make their growth comparable to the NO_3^--treated plants. In repeat experiment 4, the concentration of Thrive® was kept constant at 2 g/L. In both experiments, NO_3^- and Thrive® treatments were flushed through with 50 ml in each pot every two days. There were six replicate pots (2 plants/pot) of each genotype per treatment. All plants were harvested at 36 days after sowing (DAS) and their shoot and root FW and DW determined. The fatty acid content in leaves was measured in NO_3^- treated plants.

3.2.4 Fresh and dry weights measurement

Harvested shoots were weighed for FW (g), dried in an oven at 65 °C for three days and re-weighed for DW (g). Roots from selected treatments were washed to remove soil particles and proceeded with the same method as shoots for their FW and DW measurements. Shoot H_2O% was calculated from the difference of shoot FW and DW. Total plant DW was calculated by adding up the shoot and root DW. S:R was calculated by dividing the shoot DW by the root DW.

3.2.5 Nitrate reductase activity

In vivo NRA was measured in fresh leaf tissues as described in Andrews et al. (1984). Briefly, 0.5 g (± 0.001) leaf strips (2-3 mm width) were vacuum infiltrated for 3 mins, while immersed in 7.5 ml of extraction buffer in sealed 50 ml conical flasks. The extraction buffer consisted of 0.1 M potassium phosphate buffer (pH 7.4-7.6), 50 mM KNO_3 and 3% of (v/v) propanol. A time zero aliquot of 1 ml of extract was quickly drawn from each sample into labelled test tubes with a syringe. All conical flasks (still under vacuum) were then incubated in a slow shaking water bath at 30 °C for 30 mins in the dark. Subsequently, 1 ml of each sample extract was again drawn in test tubes. Each sample extract in the test tube was added with 1 ml of 1% (w/v) sulphanilamide in 10% (v/v) HCl and 2 ml of ddH_2O, vortexed and then added with 1 ml of 0.05% (w/v) N-1-naphthylethylenediamine dihydrochloride (NED) for colour development. Nitrite concentration in a sample was measured by comparing the absorption at 543 nm with standards of known NO_2^- concentrations.

3.2.6 Shoot N measurements

Dried and powdered shoot material from 1 to 3 replicates was pooled together to measure shoot N% and shoot NO_3^--N content. Shoot N% was measured in 0.2 g (± 0.001) dried sample using a Vario Max CN elemental analyser (Elementar GmbH, Hanau, Germany). Briefly, each sample was combusted at 900°C and the resultant gases were detected by a thermo-conductivity sensor. Shoot NO_3^- concentration was determined in 0.5 g (± 0.001) dried samples using an Alpkem Flow solution 3000 twin channel analyser (Alpkem, Texas, USA). Each sample was first shaken in 25 ml of ddH_2O at ambient temperature for 30 mins. The sample extract was then filtered twice through Whatman 42 paper. The NO_3^--N concentration in the filtrate was determined by first reducing NO_3^- to NO_2^-, via an open tubular cadmium reactor (OCTR), and then measuring NO_2^- concentration colourimetrically (see section 3.2.5). Shoot total-N (mg) was calculated using shoot N% and shoot DW data. Shoot NO_3^--N content was calculated as mg/g of DW. Shoot reduced N content (mg/g) was calculated as the difference between shoot total-N and NO_3^--N concentrations (mg/g).

3.2.7 Leaf soluble protein analysis

For leaf soluble protein determination, 1 g (± 0.001) of fresh leaf material was ground with liquid N_2 and suspended in 5 ml of extraction buffer at 4 °C. The extraction buffer was composed of 0.2 M tris-hydrochloride (tris-HCl), 5 mM ethylenediamine-tetra-acetic acid (EDTA), 1 mM cysteine, 50 mM potassium phosphate monobasic (KH_2PO_4), 10 mM flavin adenine dinucleotide (FAD) and 2.5% of polyvinylpyrrolidone (PVP). The homogenate was vortexed and centrifuged twice at 4 °C and 3000 g for 15 mins. The supernatant was used for soluble protein content measurement by Coomassie Blue method as described in Bradford (1976). Bovine Serum Albumin (BSA) standards of 0.0625, 0,125, 0.25, 0.5 and 1 mg/ml concentrations were prepared. Each sample was diluted 10-fold with ddH_2O to fall within the range of the standards. Afterwards, 10 µl of sample/standard with 200 µl of the Bio-Rad protein assay dye reagent (diluted 1 part dye in 4 parts ddH_2O) were pipetted into a 96-well microplate and incubated at room temperature for 5 mins. The absorbance was read at 595 nm via the FLUOstar® Omega microplate spectrophotometer.

3.2.8 Fatty acid extraction and analysis

Leaf fatty acid extraction and analysis were only carried out for the NO_3^- treated WT, BB1, DGAT1, D1o3-3#18, #41 and #47 lines in the initial experiment 4 and for WT, BB1, DGAT1, D1o3-3#41 and #47 lines in the repeat experiment 4 (D1o3-3#18 line failed to germinate in the repeat experiment), at Agresearch Grasslands Palmerston North facility. Briefly, a 0.1 mg (± 0.0001) sample of freeze-dried ground leaf material was extracted in hot methanolic HCl for fatty acid methyl esters (FAME) analysis according to the method described in Browse et al. (1986).

3.2.9 Experimental design and statistical analysis

All experiments were of completely randomized design. In experiment 1, there were six replicate pots of D1o3-3#47 or WT plants at each applied NO_3^--N level. A two-way analysis of variance (ANOVA) was carried out to determine whether shoot DW, total plant DW, S:R and shoot H_2O% of plants were affected by the genotype (WT, D1o3-3#47), applied NO_3^--N level (0.5-10 mM) or their interaction. Afterwards, the means of shoot DW, total plant DW and shoot H_2O% of D1o3-3#47 and WT plants at each applied NO_3^--N level were plotted in graphs and fitted with regression lines.

In experiment 2 and 3 (initial and repeat), there were initially fifty replicates of D1o3-3#47 or WT plants supplied with 8 mM NO_3^--N level. Fifteen of these replicates from each genotype were used to measure shoot DW and shoot H_2O%, and five to six replicates were used to measure total plant DW, shoot N%, shoot NO_3^--N content, total-N, leaf NRA and leaf soluble protein content. Independent

sample *t*-tests were carried out to determine whether the means of the above-mentioned variables differ significantly between D1o3-3#47 and WT plants.

In experiment 4 (initial and repeat), six replicates of each line/genotype: WT, BB1, DGAT1, D1o3-3#18, #41 and #47, were treated with either NO_3^- or Thrive®. Linear regression analyses were carried out to determine whether significant correlations exist between the leaf fatty acid content and shoot DW, and leaf fatty acid content and total plant DW of these lines under NO_3^- treatment. Individual shoot DW and total plant DW values of the replicates, instead of their mean values, were used in this regression analysis. Additionally, in repeat experiment 4, a two-way analysis of variance (ANOVA) was carried out to determine if shoot DW or total plant DW is affected by the genotypes (WT, BB1, DGAT1, D1o3-3#41 and D1o3-3#47), the applied N treatment (NO_3^-, Thrive®) or their interaction.

All significant effects described had p-values < 0.05. The regression analysis, two-way ANOVAs and independent sample *t*-tests were carried out using IBM SPSS 22. Microsoft Excel 2013 was used for plotting graphs and fitting regression lines.

3.3 Results

3.3.1 Growth, shoot H_2O% and S:R of high lipid D1o3-3#47 Arabidopsis under a range of nitrate concentrations

In experiment (exp.) 1, D1o3-3#47 and WT plants were supplied with NO_3^--N levels ranging from 0.5 to 10 mM under 50-70 µmol photons m^{-2} sec^{-1} of light and harvested when around six weeks old.

Shoot DW increased with increasing applied NO_3^--N in D1o3-3#47 and WT plants ($p < 0.01$), however, the increment was significantly greater in WT plants than in D1o3-3#47 plants ($p < 0.01$) (Fig 3.2 A). D1o3-3#47 and WT plants had similar shoot DW at low applied NO_3^--N levels (0.5 mM and 1 mM), but as applied NO_3^--N was increased (from 2 to 10 mM) the shoot DW of the two genotypes diverged significantly. At 8 mM applied NO_3^--N, shoot DW was twice as great with WT than with D1o3-3#47 plants. However, although D1o3-3#47 plants had smaller rosettes than WT plants, they did not show any damage or inter veinal-chlorosis (Fig. 3.2 D).

Total plant DW, measured only at 2, 4 and 10 mM NO_3^--N, increased with increasing NO_3^--N supply for D1o3-3#47 and WT plants ($p < 0.01$) (Fig 3.2 B). However, this increment was significantly greater in WT plants than D1o3-3#47 plants ($p < 0.01$). Total plant DWs of the two genotypes were similar at 2 mM applied NO_3^--N, but diverged at 4 and 10 mM applied NO_3^--N ($p < 0.01$) (Fig 3.2 B). At 10 mM applied NO_3^--N, total plant DW was approximately three times greater with WT than with D1o3-3#47 plants.

There was a significant increase in the shoot to root DW ratio (S:R) of both D1o3-3#47 and WT plants with increasing NO_3^--N supply ($p = 0.04$). At 2, 4 and 10 mM NO_3^--N, the S:R of D1o3-3#47 plants were 2.41 ± 0.82, 2.60 ± 0.64 and 5.68 ± 1.67 respectively ($n = 6$ for all), and the S:R of WT plants were 3.53 ± 1.08, 2.37 ± 0.50 and 5.81 ± 1.67, respectively (n = 6 for all). But at a specific applied NO_3^--N level, no significant difference was found between the S:R of D1o3-3#47 and WT plants.

Shoot H_2O% increased with increasing applied NO_3^--N in D1o3-3#47 and WT plants up to 8 mM and then flattened off ($p < 0.01$). No significant differences were found between shoot H_2O% of D1o3-3#47 and WT plants at ≥ 2mM applied NO_3^--N (Fig 3.2 C).

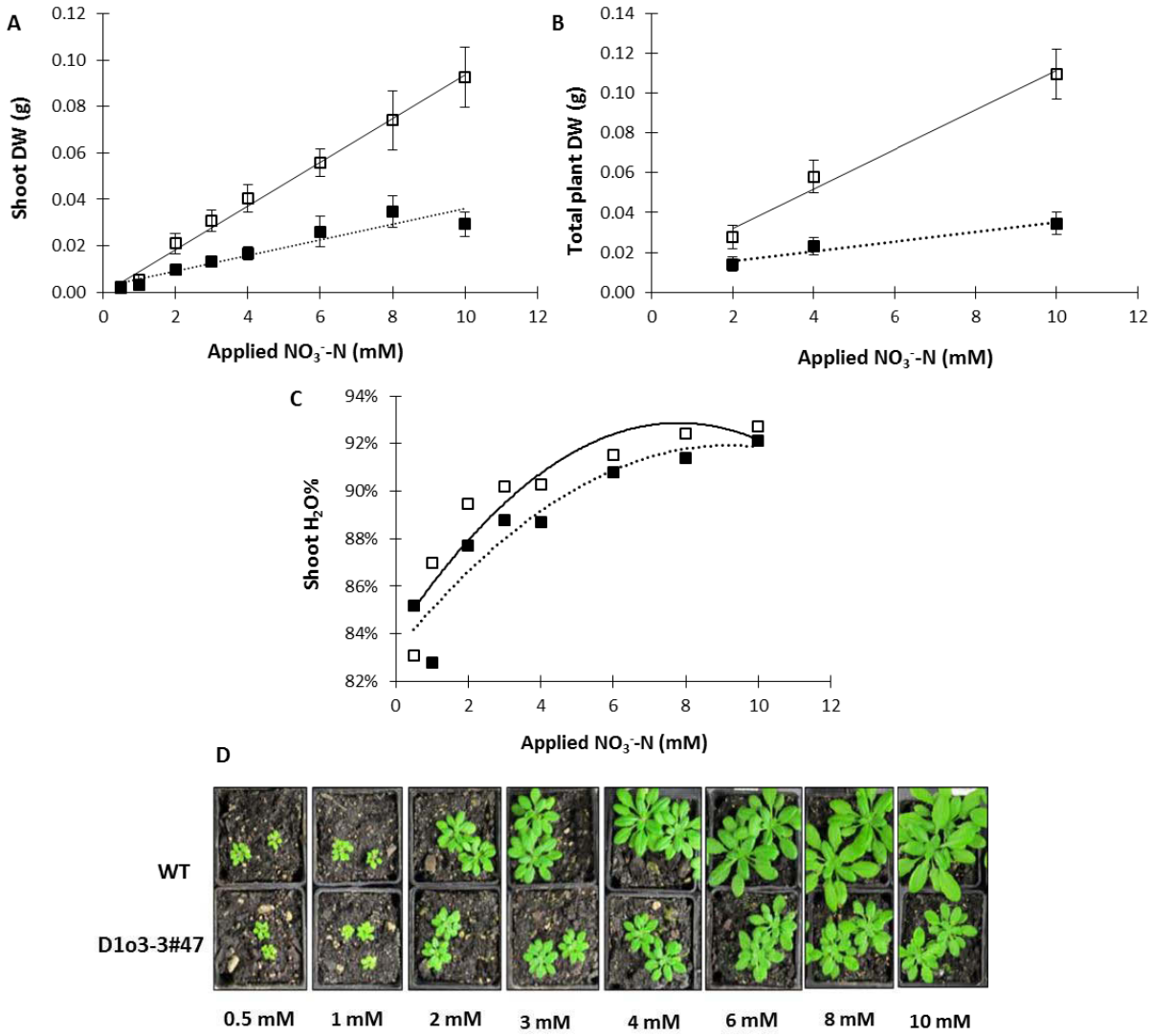

Figure 3.2 (A) Shoot dry weight (DW), (B) Total plant DW and (C) Shoot H_2O% of D1o3-3#47 (■) and wild type (WT) (□) plants under a range of applied NO_3^--N concentrations (mM) in experiment 1. Each point represents the mean of six replicates. Error bars represent the standard deviation (SD). Regression equations for the fitted lines are reported with R^2 values in section 3.3.1. (D) Photographs of the 5 weeks old D1o3-3#47 and WT plants.

The regression equations of fitted lines for the relationships between applied NO_3^--N and shoot DW, plant total DW and shoot H_2O% of D1o3-3#47 and WT plants are presented below. All equations were constructed using the mean values (n = 6) (app. N = applied NO_3^- treatment).

Fig. 3.2 A	WT	Shoot DW (g) = [0.009 * app. N (mM)] - 3 x 10^{-4}	R^2 = 0.99
	D1o3-3#47	Shoot DW (g) = [0.005 * app. N (mM)] - 0.005	R^2 = 0.93
Fig. 3.2 B	WT	Total plant DW (g) = [0.010 * app. N (mM)] + 0.013	R^2 = 0.98
	D1o3-3#47	Total plant DW (g) = [0.002 * app. N (mM)] + 0.011	R^2 = 0.95
Fig. 3.2 C	WT	Shoot H_2O% = [-0.146 * app. N^2 (mM)] + [2.280 * app. N (mM)] + 83.959	R^2 = 0.87
	D1o3-3#47	Shoot H_2O% = [-0.103 * app. N^2 (mM)] + [1.885 * app. N (mM)] + 83.261	R^2 = 0.88

3.3.2 Growth and nitrate (NO_3^-) assimilation in D1o3-3#47 Arabidopsis under a selected NO_3^- concentration

In experiment (exp.) 2 and 3, D1o3-3#47 and WT plants were supplied with 8 mM NO_3^--N, the NO_3^- concentration selected based on the results of experiment 1. Experiment 2 was carried out under 50-70 µmol photons m^{-2} sec^{-1} of light and experiment 3 was carried out under 250-350 µmol photons m^{-2} sec^{-1} of light.

Shoot DW, total plant DW and shoot H_2O% results were consistent across experiments 2 and 3 and similar to that of experiment 1. D1o3-3#47 plants showed lower shoot DW and total plant DW ($p < 0.01$ for both) as compared to WT plants and similar shoot H_2O% to WT plants. Shoot DW of D1o3-3#47 plants was 54% (exp. 2) and 59% (exp. 3) of shoot DW of WT plants (Fig 3.3 A and 3.4 A). Total plant DW of D1o3-3#47 plants was 53% (exp. 2) and 60% (exp. 3) of total plant DW of WT plants (Fig. 3.3 B and 3.4 B).

Shoot to root DW ratio (S:R) was lower in D1o3-3#47 plants (M = 2.52 ± 0.45, $n = 6$) than WT plants (M = 4.35 ± 0.45, $n = 6$) in initial experiment 2, but greater in D1o3-3#47 plants (M = 3.09 ± 0.23, $n = 6$) than WT plants (M = 2.40 ± 0.21, $n = 6$) in repeat experiment 2 ($p < 0.01$ for both). In the initial and repeat experiments 3, no significant differences were found between the S:R of D1o3-3#47 and WT plants

Shoot N% (of DW) of D1o3-3#47 and WT plants was similar in initial experiments 2 and 3, but approximately 20% and 40% greater in D1o3-3#47 plants than WT plants in repeat experiments 2 and 3, respectively ($p < 0.01$ for both) (Table 3.1).

Shoot total-N (mg) was 80% (initial) and 53% (repeat) lower in D1o3-3#47 plants than WT plants in experiment 2, and 37% (initial) and 40% (repeat) lower in D1o3-3#47 plants than WT in experiment 3 (Table 3.1).

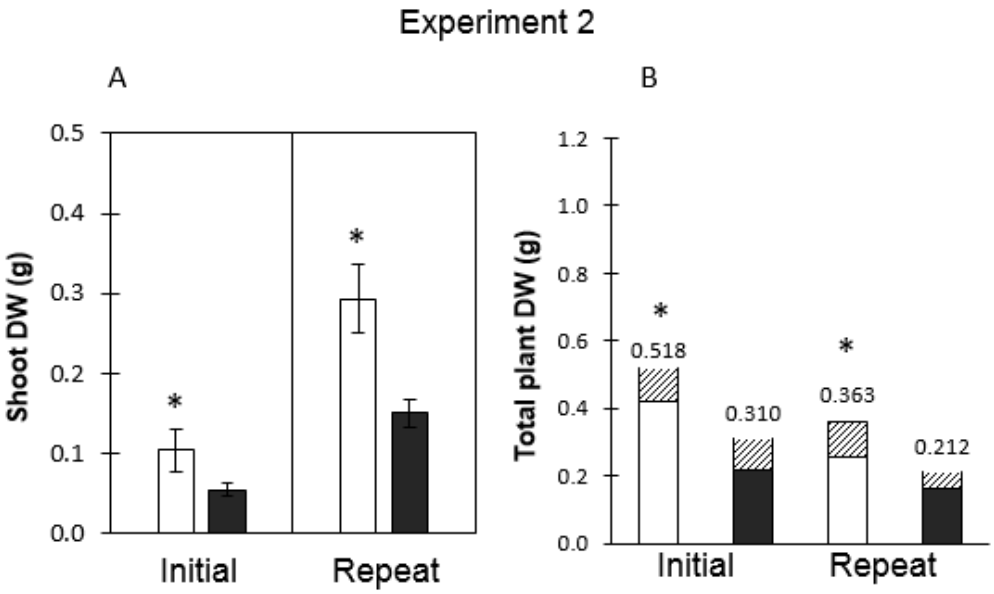

Figure 3.3 (A) Shoot dry weight (DW) and (B) Total plant DW of D1o3-3#47 (filled bars) and WT plants (unfilled bars) in experiment 2. Each bar represents the mean value (*n* = 15 for shoot DW and *n* = 6 for total plant DW). Error bars represent the standard deviation (SD). Pattern filled sections in (B) represent the mean of root DW. Asterisk represents a statistically significant difference between the two genotypes, as calculated by student's independent sample *t*-test with 95% confidence interval. Plants were 6 weeks (initial exp.) and 6.4 weeks (repeat exp.) old for the shoot DW measurement. Plants were 8 weeks (initial exp.) and 6.4 weeks (repeat exp.) old for the total plant DW measurement.

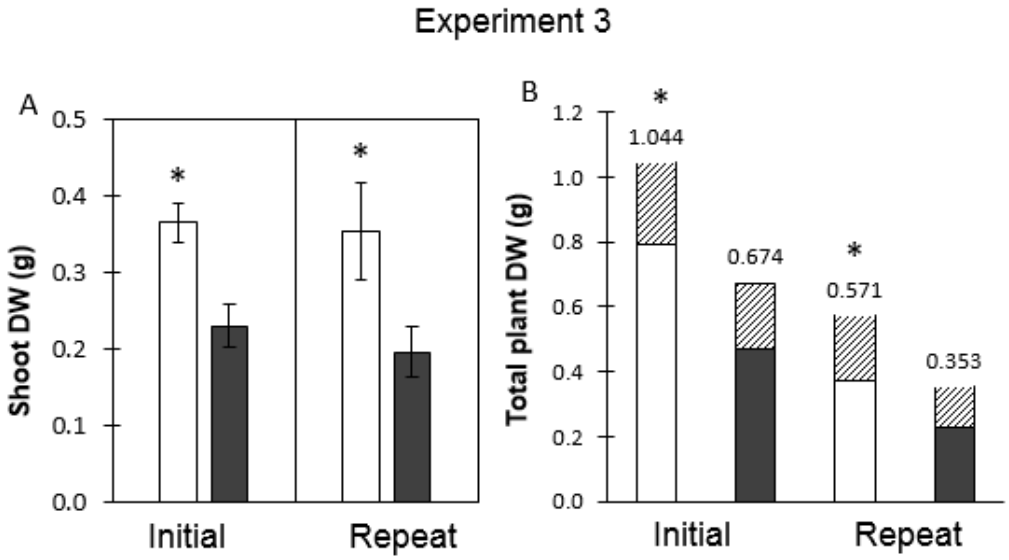

Figure 3.4 (A) Shoot dry weight (DW) and (B) Total plant DW of D1o3-3#47 (filled bars) and WT plants (unfilled bars) in experiment 3. Each bar represents the mean value (*n* = 15 for shoot DW and *n* = 6 for total plant DW). Error bars represent the standard deviation (SD). Pattern filled sections in (B) represent the mean of root DW. Asterisk represents a statistically significant difference between the two genotypes, as calculated by student's independent sample *t*-test with 95% confidence interval. Plants were 4 weeks old (initial and repeat exp.) for the shoot DW measurement. Plants were 5 weeks (initial exp.) and 4 weeks (repeat exp.) old for the total plant DW measurement.

Table 3.1 Shoot N components of D1o3-3#47 and wild type (WT) Arabidopsis plants in experiments 2 and 3.

Experiment	Genotype	N%	NO_3^--N	Reduced-N	Leaf soluble protein	Total-N (mg)
				(mg/g of DW)		
Exp. 2 (initial)	D1o3-3#47	6.29±0.10[a]	12.11±3.08[a]	50.77±3.04[a]	n/m	3.50±0.15[a]
	WT	6.18±0.14[a]	10.53±2.73[a]	51.23±3.82[a]	n/m	6.30±0.51[b]
Exp. 2 (repeat)	D1o3-3#47	5.92±0.07[a]	15.59±0.70[a]	43.56±0.99[a]	85.62±4.54[a]	9.19±0.91[a]
	WT	4.90±0.09[b]	10.05±0.83[b]	38.95±1.37[b]	55.15±7.61[b]	14.10±1.68[b]
Exp. 3 (initial)	D1o3-3#47	3.49±0.66[a]	6.49±0.64[a]	28.45±6.65[a]	n/m	8.72±1.66[a]
	WT	3.39±0.13[a]	2.56±0.34[b]	31.34±1.06[a]	n/m	11.98±0.81[b]
Exp. 3 (repeat)	D1o3-3#47	5.48±0.13[a]	9.99±1.57[a]	44.77±2.61[a]	74.31±7.98[a]	10.06±0.59[a]
	WT	3.87±0.41[b]	5.38±1.11[b]	33.27±3.41[b]	61.95±2.49[b]	14.03±1.59[b]

Values represent means ± standard deviation (SD) (n = 5 in experiment 2 and n = 6 in experiment 3). Within columns, means followed by the same alphabet superscript do not differ significantly as determined by student's independent sample t-test with 95% confidence interval. N/m means not measured.

Different shoot N components contributing to shoot N% were also measured for D1o3-3#47 and WT plants in experiments 2 and 3 (Table 3.1). Shoot NO_3^--N (mg/g DW) was 55% (repeat exp. 2), 153% (initial exp. 3) and 85% (repeat exp. 3) higher in D1o3-3#47 plants than WT plants ($p < 0.01$ for all). This shoot NO_3^--N accounted for 18-26% of shoot N% in D1o3-3#47 plants and 7-20% of shoot N% in WT plants (Table 3.1). D1o3-3#47 and WT plants had similar shoot reduced N in initial experiments 2 and 3, but approximately 12% and 34% greater shoot reduced N in D1o3-3#47 plants than WT plants in repeat experiments 2 and 3, respectively ($p < 0.01$ for both) (Table 3.1). This reduced N was contributing up to 72-81% and 79-92% in shoot N% for D1o3-3#47 and WT plants, respectively.

Leaf soluble protein content (mg/g DW), measured only in the repeat experiments 2 and 3 was 55% (exp. 2) and 20% (exp.3) greater in D1o3-3#47 plants than WT plants ($p < 0.01$ for both). In the repeat experiment 2, significant positive correlations were found between leaf soluble protein content and S:R ($R^2 = 0.90$, $p < 0.001$) and leaf soluble protein content and shoot N% ($R^2 = 0.94$, $p < 0.0001$). In repeat experiment 3, significant positive correlation was found between leaf soluble protein content and shoot N% ($R^2 = 0.76$, $p = 0.01$) but no correlation was found between leaf soluble protein and S:R.

In the repeat experiment 2 and the initial and repeat experiments 3, leaf NRA was greater for D1o3-3#47 plants than WT plants. This increase was approximately 30% in the repeat experiment 2, but around twice as great over the initial and repeat experiments 3.

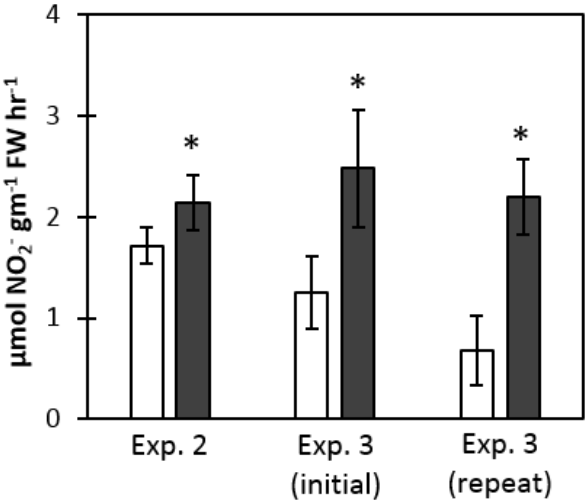

Figure 3.5 Leaf nitrate reductase activity (NRA) in D1o3-3#47 plants (filled bars) and wild type (WT) (unfilled bars) plants in experiments 2 and 3. Each bar represents the mean value (*n* = 6). Error bars represent the standard deviation (SD). Asterisk represents a statistically significant difference between the two genotypes, as calculated by student's independent sample *t*-test with 95% confidence interval.

3.3.3 Correlation between growth and leaf fatty acid content in different Arabidopsis lines

The aim of experiment 4 was to determine whether there is a correlation between growth and leaf fatty acid content across the Arabidopsis lines used in this study. Here, WT, BB1, DGAT1, D1o3-3#18, D1o3-3#41 and D1o3-3#47 lines were supplied with 10 mM NO_3^--N or 2 g/L of Thrive®.

In the initial experiment 4, cys oleosin lines (D1o3-3#18, #41 and #47) showed the highest leaf fatty acid content as compared to WT plants (For D1o3-3#18, #41 and #47, $p = 0.01$, $p = 0.01$ and $p < 0.01$, respectively) (Fig. 3.6 A). Likewise, in the repeat experiment 4, cys oleosin lines (D1o3-3#41 and #47) showed the highest leaf fatty acid contents than WT plants (For D1o3-3#41 and D1o3-3#47, $p < 0.01$ for both) (Fig. 3.6 B). These results were in agreement with Winichayakul et al. (2013).

In the initial experiment 4, weak negative correlations were found between leaf fatty acid content and shoot DW and leaf fatty acid content and total plant DW ($p < 0.0005$ for both) (Fig 3.7 A and C). Shoot

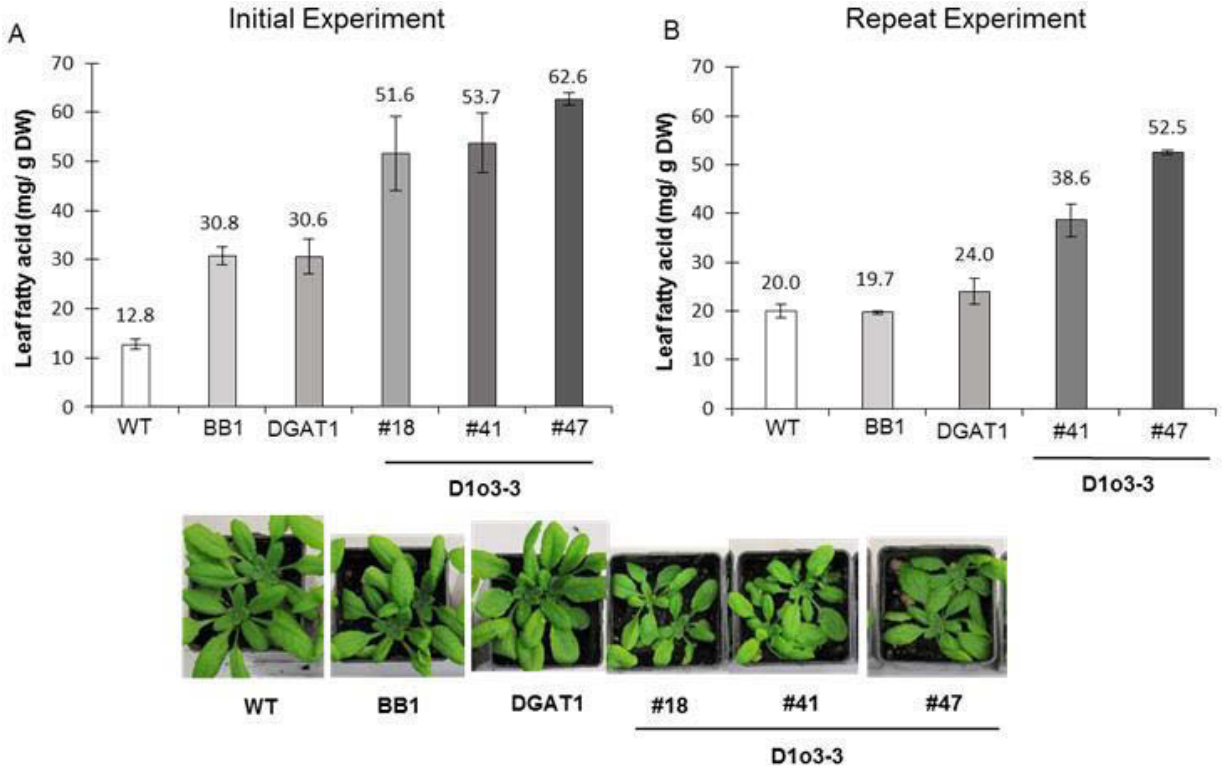

Figure 3.6 Leaf fatty acid content of the wild type (WT) and transgenic Arabidopsis lines BB1, DGAT1, D1o3-3#18, D1o3-3#41 and D1o3-3#47 in (A) initial and (B) repeat experiment 4. Each bar represents the mean value (n = 3) from replicated analysis. Photographs are of the 3 weeks old representative plants under 10 mM of applied NO_3^--N.

DW and total plant DW of the cys oleosin lines (D1o3-3#18, #41 and #47) were on average 44% and 45%, respectively, of shoot DW and total plant DW of the non-cys oleosin lines (WT, BB1 and DGAT1) ($p < 0.0001$ for both).

However, these correlations did not hold within the cys oleosin and non-cys oleosin groups. Within the cys oleosin group, shoot DW and total plant DW of the highest fatty acid containing D1o3-3#47 line were 21% and 22% greater, respectively, than shoot DW and total plant DW of D1o3-3#18 line ($p < 0.01$ for both), and similar to shoot DW and total plant DW of D1o3-3#41 line. Within the non-cys oleosin group, shoot DW and total plant DW of BB1 and DGAT1 lines were similar and on average 10% and 22% greater, respectively, than shoot DW and total plant DW of WT plants ($p = 0.01$ for both).

Similarly, in the repeat experiment 4, weak negative correlations were found between leaf fatty acid content and shoot DW and leaf fatty acid content and total plant DW ($p < 0.0005$ for both) (Fig. 3.7 B and D).

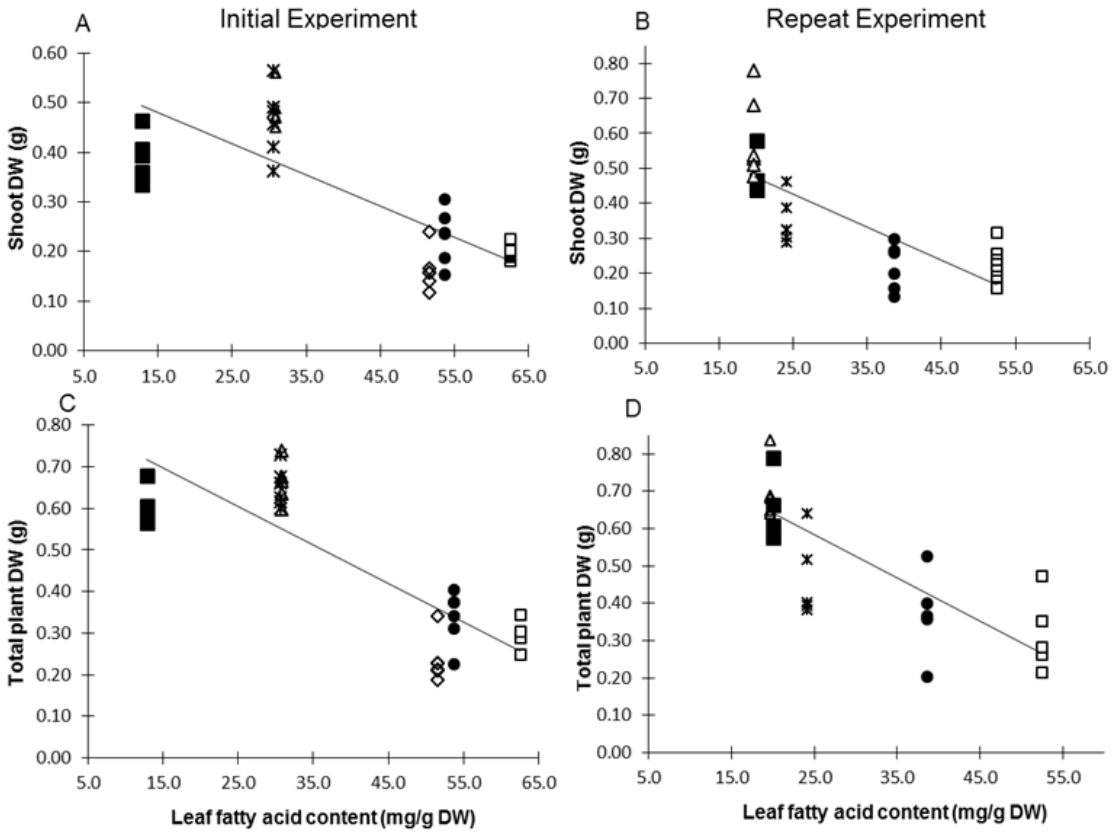

Figure 3.7 Relationship between leaf fatty acid content and shoot dry weight (DW) and total plant DW of wildtype (WT) (■), BB1, (✳), DGAT1 (△), D1o3-3#18 (◊), D1o3-3#41(●) and D1o3-3#47 (□) plants in initial (A and C) and repeat (B and D) experiments 4. Each point represents a single replicate. Regression equations for the fitted lines are reported with R^2 values in section 3.3.3.

Shoot DW and total plant DW of cys oleosin lines (D1o3-3#41 and #47) were on average 47% and 55%, respectively, of shoot DW and total plant DW of non-cys oleosin lines (WT, BB1 and DGAT1) ($p < 0.0001$ and $p < 0.01$, respectively). The correlations did not hold within the cys oleosin group, as the shoot DW and total plant DW of D1o3-3#47 line were similar to D1o3-3#41 line. The correlations hold within the non-cys oleosin group, as the DGAT1 line, which accumulated relatively higher leaf fatty acid content than WT and BB1 lines, showed 34% and 70% lower shoot DW than WT and BB1 lines, respectively ($p < 0.01$ for both).

The regression equations of fitted lines for the relationships between leaf fatty acid content and shoot DW and leaf fatty acid and total plant DW in initial and repeat experiment 1, are presented below. All equations were constructed using individual replicate values (DW = dry weight, LFAC = Leaf fatty acid content).

Fig. 3.7 A	LFCA (mg/g DW) = [-0.006 * shoot DW (g)] + 0.58	$R^2 = 0.59$
Fig. 3.7 B	LFCA (mg/g DW) = [-0.009 * total plant DW (g)] + 0.84	$R^2 = 0.70$
Fig. 3.7 C	LFCA (mg/g DW) = [-0.009 * shoot DW (g)] + 0.66	$R^2 = 0.58$
Fig. 3.7 D	LFCA (mg/g DW) = [-0.011 * total plant DW (g)] + 0.87	$R^2 = 0.56$

In the initial experiment 4, all Thrive® treated plants died before the completion of the experiment, possibly due to the application of three times the prescribed (2 g/L) concentration of Thrive®, and hence this N treatment could not be pursued further.

In repeat experiment 4, in which Thrive® treated plants survived, shoot DW and total plant DW of all lines were compared initially under NO_3^- and then under Thrive® treatments. Under NO_3^- treatment, shoot DW and total plant DW of the D1o3-3#47 plants were approximately 48% of shoot DW and total plant DW of the WT plants ($p < 0.01$ for both). Under Thrive® treatment, shoot DW and total plant DW of the D1o3-3#47 plants were approximately 30% of shoot DW and total plant DW of the WT plants ($p < 0.01$ for both) (Fig. 3.8 A and B).

At three weeks old, all lines seem to have smaller rosettes under Thrive® than under NO_3^- treatment (Fig. 3.8 C). However, at harvest (36 DAS), no significant growth differences were found between NO_3^- and Thrive® treatments (Fig. 3.8 A and B). Except in the case of BB1 line, which showed significantly lower shoot DW and total plant DW under Thrive® than under NO_3^- nutrition ($p = 0.01$ for both) (Fig. 3.8 A and B). Shoot DW and total plant DW were affected by both the genotypes and the N treatments (Thrive® or NO_3^-) applied ($p < 0.01$ for both), but the effect size of the genotype (partial squared eta, η^2) was two to three times greater than the effect size of the N treatment.

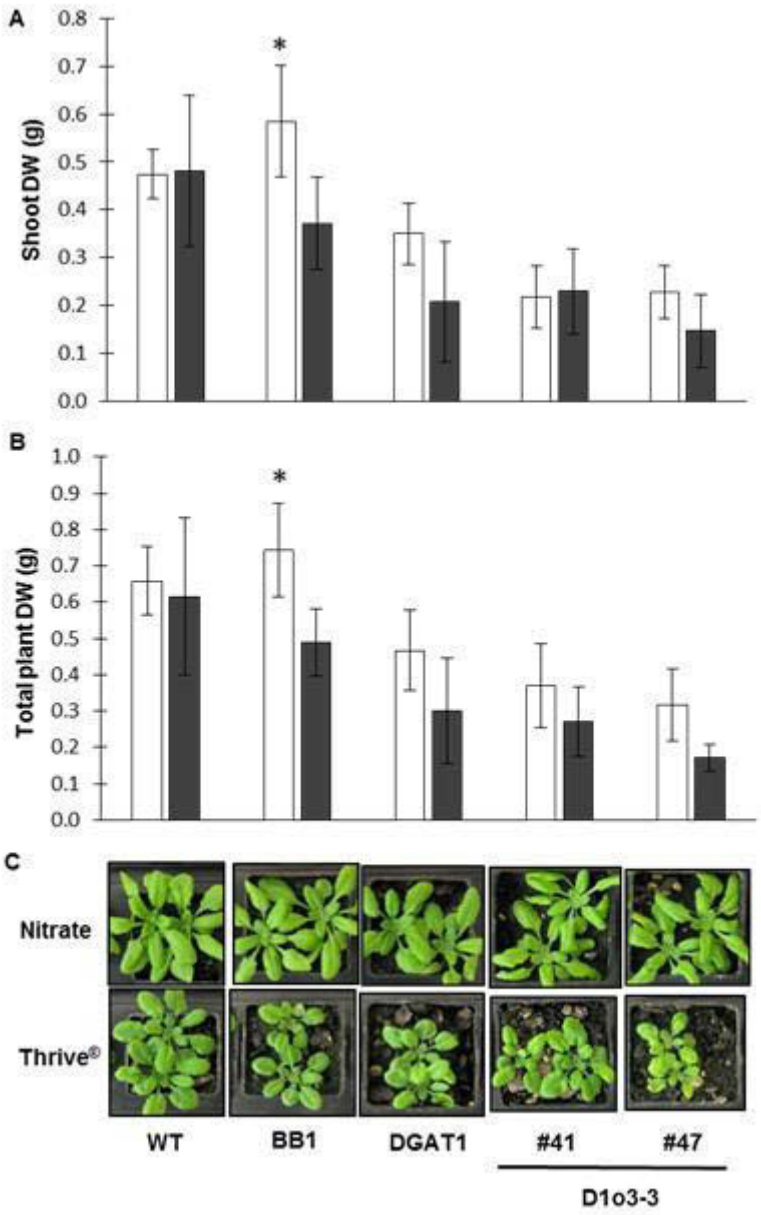

Figure 3.8 (A) Shoot dry weight (DW) and (B) Total plant DW of WT (wild type), BB1, DGAT1, D1o3-3#41 and D1o3-3#47 Arabidopsis lines under 10 mM nitrate (unfilled bars) and 2 g/L of Thrive® (filled bars) treatments in the repeat experiment 4. Each bar represents the mean value (*n* = 6). Error bars represent the standard deviation (SD). Asterisk represents a statistically significant difference between the two N treatments, as calculated by student's independent sample *t*-test with 95% confidence interval. (C) Photographs of the 3 weeks old representative plants.

3.4 Discussion

A transgenic Arabidopsis line, named 'D1o3-3#47', with elevated TAG content in its vegetative tissues, showed limited growth as compared to WT plants under NO_3^- nutrition but not under Thrive® (a commercial plant fertilizer) in a preliminary study at Agresearch Grasslands (N. Roberts, Agresearch Palmerston North, personal communication, May 2015). Previously, in some cases, biotechnological strategies to enhance TAG content in plants have not caused any negative impacts at all (Kim et al., 2015; Li et al., 2015). However, a variety of studies have reported physiological changes, such as reduced growth and decreased starch levels and photosynthetic capacity in plants genetically modified to accumulate excess oil in their seeds or foliar biomass (Andrianov et al., 2010; Cernac and Benning, 2004; Kelly et al., 2013; Liu et al., 2016; Sanjaya et al., 2011; Vanhercke et al., 2014 & Yang et al., 2015). Whether NO_3^-, as the sole N source, contributes to these effects has never been investigated. Also, it has not been exclusively investigated whether there is any link between the growth of high lipid transgenic plants and their leaf lipid content. Hence, to gain a greater understanding of the reason(s) for any growth limitation in D1o3-3#47 plants under NO_3^- nutrition, this chapter examined their growth and NO_3^- assimilation and determined whether any correlation exists between growth and leaf fatty acid content.

In experiment 1, D1o3-3#47 plants consistently showed only 50% of the growth of WT plants across all applied NO_3^--N concentrations, except 0.5 mM and 1 mM. Similar growth results were obtained in experiment 2, 3 and 4. These results have not only confirmed the earlier report of Agresearch but have also shown that growth reduction in D1o3-3#47 plants occurs at higher applied NO_3^--N, typically at ≥ 2 mM. Both D1o3-3#47 and WT plants showed increased in growth with increasing applied NO_3^--N levels in the range of 2 to 10 mM, which has been a well-documented response in species like common bean (*Phaseolus vulgaris*), pea (*Pisum sativum*) and temperate cereals and grasses (Andrews et al., 1992, 1999 & 2001).

In experiment 1, the shoot to root DW ratio (S:R) of D1o3-3#47 and WT plants increased with increasing applied NO_3^--N, which is often partially due to the effect of increase in growth with increasing N supply (Andrews et al., 1999 & 2006). However, at a particular applied NO_3^--N (2, 4 or 10 mM) no differences in S:R were found between D1o3-3#47 and WT plants. Also, in experiments 2 and 3, S:R for D1o3-3#47 and WT plants did not differ in two out of four experiments. These results indicated that both shoots and roots of D1o3-3#47 plants show growth limitation, and because of this their S:R is similar to that of WT plants.

In experiments 2 and 3, D1o3-3#47 and WT plants were supplied with 8 mM NO_3^--N treatment, selected on the basis of experiment 1 results. Experiment 2 was carried out under 50-70 µmol photons m^{-2} sec^{-1} of light and experiment 3 was carried out under 250-350 µmol photons m^{-2} sec^{-1} of light. Regardless of the light level, growth results in experiment 2 and 3 were similar to those in experiment 1. Specifically, shoot DW and total plant DW of D1o3-3#47 plants were approximately 50% lower than that of WT plants, and there was no consistent difference between the two genotypes on S:R.

In relation to plant N, shoot N% was either similar with D1o3-3#47 and WT plants or slightly greater in D1o3-3#47 plants. Thus, as shoot (and total plant) DW was approximately twice as great for WT plants as for D1o3-3#47 plants, then on average across experiments, approximately 50% less N was taken up by D1o3-3#47 plants in comparison to WT plants. However, the reduced N content was at least as great in D1o3-3#47 plants as in WT plants, while leaf soluble protein content was greater in D1o3-3#47 plants. Thus, although the uptake of NO_3^-/N was reduced per plant, there did not appear to be a restriction on the ability of D1o3-3#47 plants to assimilate the taken up NO_3^-.

Nitrate reductase activity (NRA) was greater in D1o3-3#47 plants than in WT plants, which is likely to be related to the higher NO_3^- concentration in D1o3-3#47 plants (Andrews et al., 1992, 2004 & 2013). Also, the finding that NRA was higher in D1o3-3#47 plants indicates that this enzyme is not limiting NO_3^- assimilation in this genotype. The NRA assay carried out was an *in vivo* assay which relies on the endogenous reductant (NADH), hence the results indicate that the NADH supply is also not limiting the assimilation of NO_3^- in D1o3-3#47 plants.

Previous studies that have reported growth reduction in high lipid transgenic plants have not specifically investigated the presence of any correlation between growth and leaf fatty acid content (Kelly et al., 2013). In experiment 4, WT and transgenic Arabidopsis lines with varying levels of leaf TAG content were used for this purpose. A weak negative correlation was found between growth and leaf fatty acid content. Kelly et al. (2013) concluded that a reduction in the growth of high lipid plants could be a consequence of the shift in C-partitioning from structural and storage C compounds to fatty acid metabolism. In the current chapter, even though a weak correlation was found between growth and leaf lipid content, which mostly did not hold within the cys oleosin modified (D1o3-3#18, #41, #47) and non-cys oleosin (WT, BB1, DGAT1) groups, it did suggest that the growth reduction in D1o3-3#47 plants could be related to their high leaf TAG content.

Interestingly, growth limitation in D1o3-3#47 plants as compared to WT plants was not only observed under NO_3^- but also under Thrive® treatment (Fig. 3.8), which contradicted the earlier report of Agresearch that D1o3-3#47 plants show reduced growth only under NO_3^- but not under Thrive®

nutrition. The reason(s) for this limited growth in D1o3-3#47 plants under Thrive® nutrition is (are) unknown and require further analysis. However, such results prompted the question, whether D1o3-3#47 plants show better growth than WT plants under any other form(s) of N, which was investigated in chapter 4.

In conclusion, this chapter has confirmed the earlier report of Agresearch by persistently showing that D1o3-3#47 plants exhibit limited growth as compared to WT plants under NO_3^- nutrition. Decreased shoot and total plant DW in D1o3-3#47 plants under NO_3^- nutrition was associated with reduced N/NO_3^- uptake. However, shoot reduced N, leaf soluble protein content (concentration) and leaf NRA were at least as great with D1o3-3#47 plants as WT plants indicating that there was no restriction in the ability of D1o3-3#47 plants to assimilate the NO_3^- taken up. A weak negative correlation was found between growth and leaf fatty acid content, suggesting a probable link of the growth problem to leaf lipid content in D1o3-3#47 plants. Moreover, it was found that the D1o3-3#47 plants not only show limited growth under NO_3^- nutrition but also under Thrive® nutrition.

Chapter 4

Growth of high lipid Arabidopsis under different nitrogen forms and concentrations

4.1 Introduction

Nitrogen (N) is an essential macronutrient that is available to plants in a variety of inorganic and organic forms, depending upon soil physiochemical characteristics and environmental conditions (Miller and Cramer, 2004; Miller, 2010). As mentioned in chapter 2 and 3, nitrate (NO_3^-) is the most abundantly available inorganic N form taken up and utilized by plants in cultivated soils (Andrews et al., 2013). Following uptake, NO_3^- must be reduced to ammonium (NH_4^+) before being assimilated first into glutamine and then into other amino acids through the transaminase reactions (Miller et al., 2007; Xu et al., 2012). Ammonium and amino acids can also be taken up and under certain conditions are important sources of N (Näsholm et al., 2009; Xu et al., 2012). Another important N source for plants is urea (CH_4N_2O), which enters agricultural soils primarily as N fertiliser and animal waste, and can be directly taken up and utilized by all plants (Merigout et al., 2008; Kraiser et al., 2011, Witte, 2011).

Soil concentrations of the N forms can vary greatly from micromolar (µM) to millimolar (mM) levels, depending upon environmental factors; such as water supply, temperature and microbial activity (Miller, 2010). Nitrate concentrations usually range between 1 to 20 mM in well-aerated/cultivated soils (Andrews et al., 2013). Ammonium concentrations can vary between 20 to 200 µM in agricultural soils and up to 2 mM in undisturbed soils and/or acidic soils (Jones et al., 2002; Miller and Cramer, 2004). Free amino acids are most abundant near the surface of organic-rich uncultivated soils and in the range of 1 to 100 µM in agricultural soils (Owen and Jones, 2001). To cope with this heterogeneous availability of N in soil, plants have evolved several transport systems and assimilatory pathways to take up N in various forms and concentrations and ultimately incorporating it into organic amino acids, proteins and other N containing compounds (Kraiser et al., 2011; Krapp, 2015).

In chapter 3, the report of Agresearch that D1o3-3 #47 plants show limited growth as compared to WT plants under NO_3^- nutrition was confirmed. Decreased shoot and total growth of D1o3-3#47 plants under NO_3^- nutrition was associated with reduced N/NO_3^- uptake. However, shoot reduced N, leaf soluble protein content and leaf NRA were at least as great with D1o3-3#47 plants as with WT plants, indicating that there was no restriction in the ability of D1o3-3#47 plants to assimilate the taken up NO_3^-. A weak positive correlation was present between growth and leaf lipid content of different WT and transgenic Arabidopsis lines. Also, it was found that D1o3-3#47 plants not only show limited growth under NO_3^- but also under Thrive® nutrition. Therefore, the next step was to investigate the

growth response of D1o3-3#47 plants in comparison to WT plants under other N forms and their concentrations.

This chapter described a single 'initial' experiment (Experiment 5) that was aimed to determine 'whether D1o3-3#47 plants show better growth than WT plants under any other N form(s)?' This experiment mainly assessed the growth of D1o3-3#47 and WT plants under a range of concentrations of NO_3^-, NH_4^+, urea and glutamine. Additionally, S:R, shoot N% and shoot total-N of D1o3-3#47 and WT plants were examined under selected concentrations of N forms. A relatively small 'repeat' experiment was also carried out to validate the growth results of the initial experiment under selected concentrations of NO_3^-, NH_4^+, urea and glutamine.

4.2 Materials and methods

4.2.1 Plant material

Seeds for *Arabidopsis thaliana* (Arabidopsis) ecotype Columbia (Col-0) wild type (WT) and high lipid transgenic line 'D1o3-3#47' (T_4 generation) were provided by Dr Somrutai Winichayakul, AgResearch Grasslands, Palmerston North.

4.2.2 General methods

Experiment 5 (initial and repeat) was carried out at the PC2 level containment facility at Lincoln University, New Zealand; under 16 hrs day-length, 20-22 °C temperature and 60-70% humidity level. Pots, trays, potting mix and methods for seed stratification and sowing were as described in chapter 3 (section 3.2.2). Seeds were sown over the soil beds pre-moistened with the respective N treatment (N treatment = a single N form and its concentration). All solutions were prepared using double distilled water (ddH_2O). Seedlings were thinned down to two per pot once they were one week old.

4.2.3 Experiments

In the initial experiment 5, D1o3-3#47 and WT plants were supplied with 2.5, 5, 7.5, 10, 15 or 20 mM N treatments of NO_3^-, NH_4^+, urea or glutamine flushed through with 50 ml in each pot every two days. Nitrate, as potassium nitrate (KNO_3), ammonium as ammonium sulphate $(NH_4)_2SO_4$, urea and glutamine (L-glutamine), with stock solutions concentrations of 1 M, 0.5 M, 0.5 M and 0.1 M respectively, were prepared fresh every week. These stock solutions were used to prepare N working solutions/treatments of different concentrations. Potassium concentration was balanced at 20 mM in all N treatments by adding calculated volumes of 1 M KCl stock, but Cl^- concentration was not balanced. There were six replicate pots of D1o3-3#47 or WT plants per N treatment, distributed amongst six blocks (one replicate per block). These six blocks, were kept under six slightly different light intensities; block # 1 was under 180-210 µmol photons m^{-2} sec^{-1}, block # 2 was under 220-280 µmol µmol photons m^{-2} sec^{-1}, block # 3 was under 250-300 µmol photons m^{-2} sec^{-1}, block # 4 was under 250-320 µmol photons m^{-2} sec^{-1}, block # 5 was under 260-350 µmol photons m^{-2} sec^{-1} and block # 6 was under 290-365 µmol photons m^{-2} sec^{-1} of light. All plants were harvested within a period of four days, with each block harvested on the same day, and their shoot and root FW and DW and shoot H_2O% were measured. Shoot N%, shoot total-N and S:R were measured only at 10 mM N treatments.

In the repeat experiment 5, D1o3-3#47 and WT plants were supplied with 10 mM N from NO_3^-, urea, glutamine and 5 mM N from NH_4^+ treatments, flushed through with 50 ml in each pot every other day. Potassium concentration was balanced at 10 mM in all working solutions by adding calculated volumes of 1 M KCl stock, but Cl^- concentration was not balanced. There were total four blocks kept under four slightly different light intensities; block # 1 was under 180-320 µmol photons m^{-2} sec^{-1}, block # 2 was

under 220-280 µmol photons m^{-2} sec^{-1}, block # 3 was under 250-320 µmol photons m^{-2} sec^{-1} and block # 4 was under 260-350 µmol photons m^{-2} sec^{-1} of light. Each block contained three replicates of D1o3-3#47 or WT plants per N treatment. All plants were harvested within a period of two days, with each block harvested on the same day, and their shoot FW and DW and shoot H_2O% were measured.

4.2.4 Fresh and dry weights measurements

Harvested shoots were weighed for FW (g), dried in an oven at 65 °C for three days and re-weighed for DW (g). Roots from selected treatments were washed to remove soil particles and proceeded with the same method as shoots for their FW and DW measurements. S:R was calculated from shoot and root DW data. Shoot H_2O% was calculated from the difference of shoot FW and DW.

4.2.5 Shoot N measurements

Approximately 0.2 g (± 0.001) of dried and powdered shoot material, pooled from 1 to 3 replicates of 10 mM NO_3^-, NH_4^+, urea and glutamine treatments, was used to measure shoot N% via the Vario Max CN elemental analyser (Elementar GmbH, Hanau, Germany). Briefly, each sample was combusted at 900°C and the resultant gases were detected by a thermoconductivity detector. Shoot total-N (mg) was calculated from shoot N% and DW data.

4.2.6 Experimental design and statistical analysis

In the initial experiment 5, there were six blocks, each containing one replicate of each N treatment. For each N form, a two-way ANOVA was carried out to determine whether shoot DW or shoot H_2O% were affected by the genotype (D1o3-3#47 and WT), applied N concentration (2.5-20 mM) or their interaction. Student's independent sample *t*-tests were carried out to compare S:R, shoot N%, shoot total-N data from D1o3-3#47 and WT plants under each N form. The repeat experiment 5 consisted of four blocks, each containing triplicates of an N treatment. Mean shoot DW values of the triplicates were used for data analysis per block. Student's independent sample *t*-tests were carried out to compare the shoot DW of D1o3-3#47 and WT plants at each N form per block.

All significant effects had *p*-values < 0.05. The two-way ANOVAs and *t*-tests were carried out using IBM SPSS 22. Microsoft Excel 2013 was used for plotting graphs and fitting regression models.

4.3 Results

Initial experiment 5 consisted of six blocks, each kept under a specific light intensity (see section 4.2.3). All blocks were harvested within a period of four days. As no correlations were found between the shoot DW (at 10 mM N) and light intensity, and the shoot DW (at 10 mM N) and harvest dates across all blocks (results not shown), the whole experiment was considered as a single block.

Under NO_3^- treatments, shoot DWs increased with increasing applied NO_3^--N in D1o3-3#47 and WT plants ($p < 0.01$), however, the magnitude of increment was greater in WT plants than in D1o3-3#47 plants ($p < 0.01$) (Fig. 4.1 A). D1o3-3#47 and WT plants had similar shoot DW at 2.5 mM NO_3^--N, but as applied NO_3^--N was increased the shoot DWs of the two genotypes diverged significantly. At 10 mM NO_3^--N, shoot DW of D1o3-3#47 plants was 54% of shoot DW of WT plants. These findings were similar to the shoot DW results from experiment 1 of chapter 3 (Fig 3.2 A).

Under NH_4^+ treatments, shoot DWs of D1o3-3#47 and WT plants increased with increasing applied NH_4^+-N ($p < 0.01$), with the magnitude of increment greater in WT plants than D1o3-3#47 plants ($p < 0.01$) (Fig. 4.1 B). D1o3-3#47 and WT plants had similar shoot DW at 2.5 mM NH_4^+-N, which diverged significantly as applied NH_4^+-N was increased. At 10 mM applied NH_4^+-N, shoot DW of D1o3-3#47 plants was 53% of shoot DW of WT plants. As most WT plants died at 20 mM NH_4^+-N possibly due to NH_4^+ toxicity (Britto & Kronzucker, 2002), this treatment was discarded from the analysis.

Under urea treatments, shoot DWs of D1o3-3#47 and WT plants increased with increasing applied urea-N ($p < 0.01$), however, the magnitude of increment was greater in WT plants than D1o3-3#47 plants ($p < 0.01$) (Fig. 4.1 C). D1o3-3#47 and WT plants had similar shoot DW at 2.5 mM urea-N, but as applied urea-N was increased the shoot DW of the two genotypes diverged significantly. At 10 mM applied urea-N, shoot DW of D1o3-3#47 plants was 57% of shoot DW of WT plants.

Under glutamine treatments, shoot DWs of D1o3-3#47 and WT plants increased with increasing applied glutamine-N ($p < 0.01$), but the magnitude of increment was greater in WT plants than D1o3-3#47 plants ($p < 0.01$) (Fig. 4.1 D). None of the plants from 2.5 mM glutamine-N treatment survived. D1o3-3#47 and WT plants had similar shoot DW at 5 mM, but as glutamine-N was increased the shoot DWs of the two genotypes diverged significantly. At 10 mM applied glutamine-N, shoot DW of D1o3-3#47 plants was 59% of shoot DW of WT plants. D1o3-3#47 plants showed the highest shoot DW at 15 mM urea-N, which was greater than the shoot DW of only those WT plants that were supplied with 2.5 mM NO_3^-, 5 mM NH_4^+, 5 mM of urea and 5 mM of glutamine-N treatments. In summary, shoot DW of D1o3-3#47 plants were 53-59% of shoot DWs of WT plants under all N forms (at 10 mM N) (Fig. 4.1).

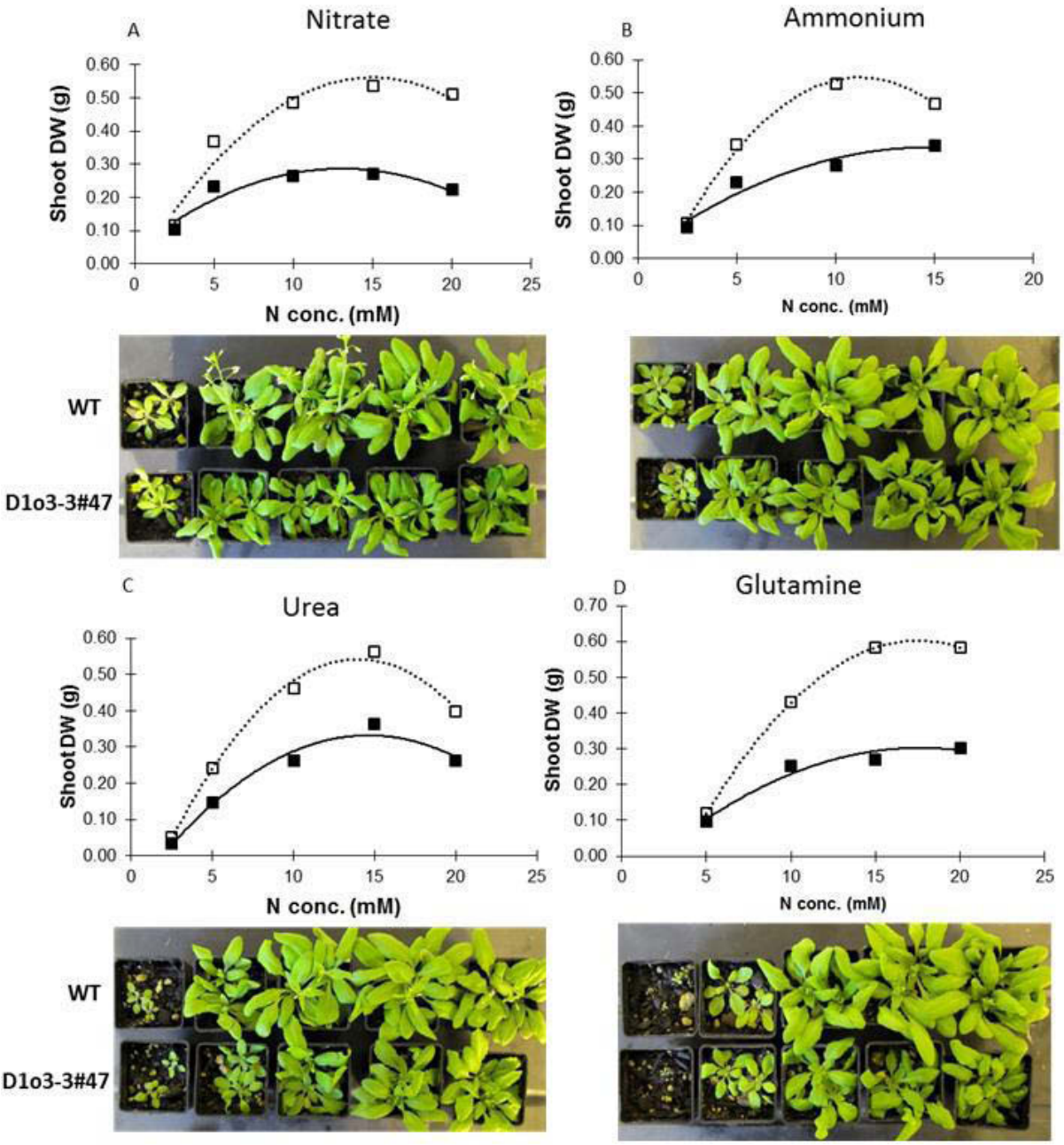

Figure 4.1 Effects of applied N concentrations of (A) Nitrate, (B) Ammonium, (C) Urea and (D) Glutamine on shoot DW of D1o3-3#47 (■) and wild type (WT) (□) Arabidopsis plants in initial experiment 5. Each point represents the mean value of six replicates. Trend lines are fitted using polynomial curves in all cases. Regression equations for the fitted lines are reported with R^2 values in section 4.3. Photographs are of the 4 weeks old representative plants.

Despite having smaller rosettes than WT plants, the D1o3-3#47 plants did not show any damage or inter veinal-chlorosis across all N form treatments (Fig. 4.1 photographs).

The regression equations of the fitted curves for the relationships between shoot DW and applied N concentrations of NO_3^-, NH_4^+, urea or glutamine are presented below. All equations were constructed using the mean values (n = 6) (SDW = shoot dry weight, app. N = applied N concentration):

Fig. 4.1 A	WT	SDW (g) = [-0.002 * app. N (mM)]^2 + [0.077 * app. N (mM)] - 0.020	R^2 = 0.94
	D1o3-3#47	SDW (g) = [-0.001 * app. N (mM)]^2 + [0.038 * app. N (mM)] + 0.042	R^2 = 0.87
Fig. 4.1 B	WT	SDW (g) = [-0.006 * app. N (mM)]^2 + [0.127 * app. N (mM)] - 0.167	R^2 = 0.99
	D1o3-3#47	SDW (g) = [-0.001 * app. N (mM)]^2 + [0.043 * app. N (mM)] + 0.014	R^2 = 0.93
Fig. 4.1 C	WT	SDW (g) = [-0.004 * app. N (mM)]^2 + [0.105 * app. N (mM)] - 0.192	R^2 = 0.99
	D1o3-3#47	SDW (g) = [-0.002 * app. N (mM)]^2 + [0.060 * app. N (mM)] - 0.107	R^2 = 0.97
Fig. 4.1 D	WT	SDW (g) = [-0.003 * app. N (mM)]^2 + [0.108 * app. N (mM)] - 0.342	R^2 = 1
	D1o3-3#47	SDW (g) = [-0.001 * app. N (mM)]^2 + [0.044 * app. N (mM)] - 0.085	R^2 = 0.95

Under NO_3^- treatments, shoot H_2O% increased with increasing applied NO_3^--N level (p < 0.01) but did not differ between the D1o3-3#47 and WT plants. These results were consistent with the shoot H_2O% results from experiment 1, 2 and 3 of chapter 3 (Fig. 3.2 C). Under NH_4^+ treatments, shoot H_2O% also increased with increasing applied NH_4^+-N (p < 0.01), but was greater in WT plants than D1o3-3#7 plants (p = 0.02). Under, urea and glutamine treatments, shoot H_2O% did not change with the concentration applied and were similar for D1o3-3#47 and WT plants across all N levels.

No significant differences were found between the S:R of D1o3-3#47 and WT plants under 10 mM N of any of the applied N forms (Table 4.1).

Under NO_3^- nutrition, no significant differences were found in shoot N% and shoot total-N of D1o3-3#47 and WT plants (Table 4.1). Under NH_4^+ nutrition, shoot N% of D1o3-3#47 plants was 27% greater and shoot total-N was 46% lower than WT plants (p < 0.01 for both). Under urea treatment, shoot N% was similar between the two genotypes but shoot total-N was 41% lower in D1o3-3#47 plants than WT plants (p < 0.01). Under glutamine nutrition, shoot N% of D1o3-3#47 plants was 22% greater and shoot total-N was 34% lower than WT plants (p < 0.01 for both).

Table 4.1 Shoot N%, shoot total-N and S:R in D1o3-3#47 and WT plants at 10 mM of N forms.

N form	Genotype	N (%)	Total-N (mg)	S:R
Nitrate	D1o3-3#47	5.31±0.56[a]	13.50±2.31[a]	3.24±0.51[a]
	WT	4.53±0.89[a]	18.84±4.64[a]	3.25±0.68[a]
Ammonium	D1o3-3#47	6.06±0.43[a]	16.11±3.36[a]	3.15±0.40[a]
	WT	4.77±0.80[b]	23.56±3.30[b]	3.15±0.55[a]
Urea	D1o3-3#47	4.39±0.50[a]	11.16±1.24[a]	2.71±0.63[a]
	WT	3.67±0.50[a]	15.81±1.52[b]	2.87±1.00[a]
Glutamine	D1o3-3#47	5.09±0.47[a]	13.01±1.45[a]	2.42±0.39[a]
	WT	4.19±0.51[b]	17.49±2.52[b]	2.23±0.41[a]

Values represent means ± standard deviation (SD) (n = 6). Within columns, means followed by the same alphabet superscript do not differ significantly as determined by student's independent sample t-test with 95% confidence interval.

The repeat experiment 5 consisted of four blocks, each kept under a specific light intensity (see section 4.2.3). All blocks were harvested within a period of two days. As for the initial experiment 5, no significant correlations were found between shoot DW and light intensity, and shoot DW and harvest dates across all blocks (results not shown). Shoot DW data was analysed on a per block basis.

Shoot DW of D1o3-3#47 plants was overall either lower or, in some cases, similar to WT plants across all N forms in each block (Fig. 4.3). The differences of shoot DW of D1o3-3#47 and WT plants under each N form were variable across the blocks. Under NO_3^- treatment, shoot DWs of D1o3-3#47 plants were 37%, 63%, 45% and 61% (average 51%) of shoot DWs of WT plants in block # 1, 2, 3 and 4, respectively ($p < 0.01$ for all).

Under NH_4^+ treatment, shoot DW of D1o3-3#47 plants was 47% of shoot DW of WT plants in block # 1 ($p < 0.01$), and similar to shoot DW of WT plants in block # 4. All NH_4^+ treated replicates of D1o3-3#47 and WT plants died in blocks # 2 and 3.

Under urea treatment, shoot DW of D1o3-3#47 plants were around 54% and 64% of shoot DW of WT plants in block # 2 and 3 ($p < 0.01$ for both), respectively, and similar to shoot DW of WT plants in block # 1 and 4.

Under glutamine treatment, shoot DW of D1o3-3#47 plants were 48% and 31% of shoot DW of WT plants in block # 2 and 4 ($p < 0.01$ for both), respectively, and similar to shoot DW of WT plants in block # 1 and 3.

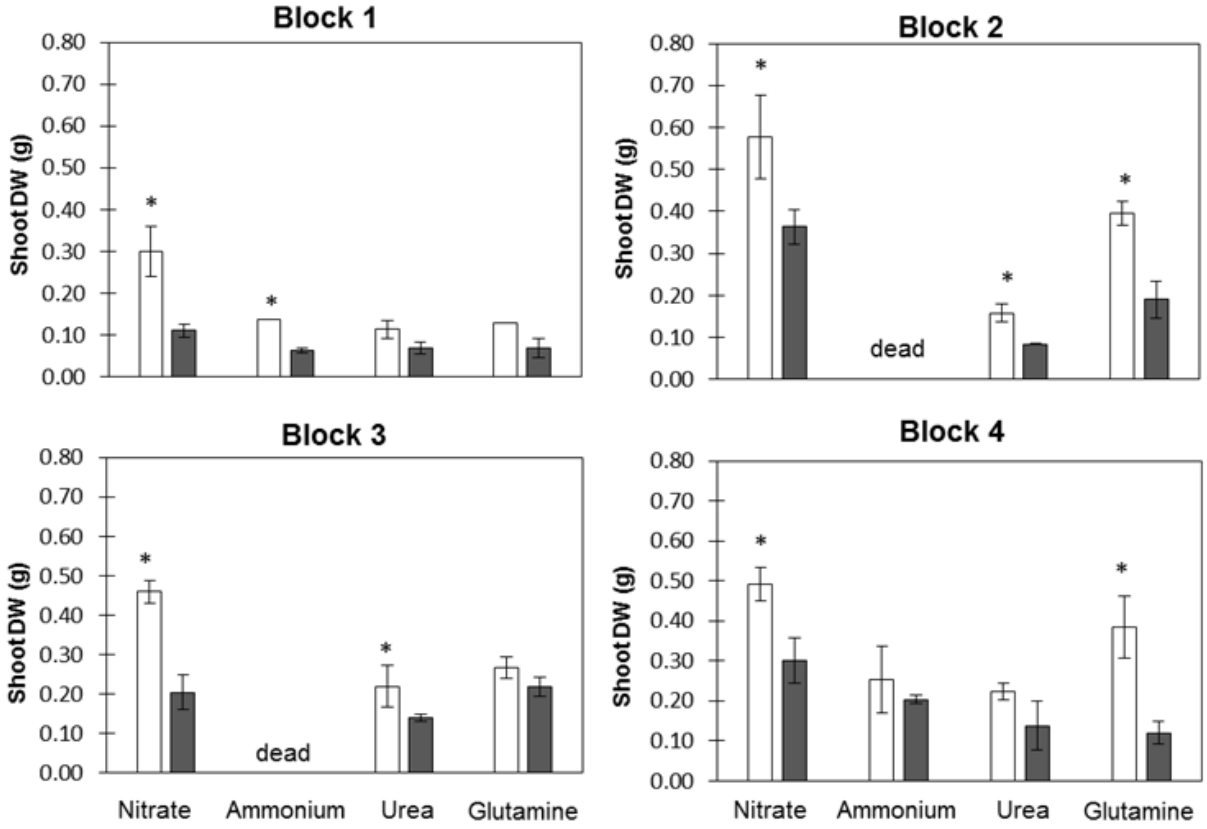

Figure 4.2 Shoot DW of D1o3-3#47 (filled bars) and wild type (WT) (unfilled bars) Arabidopsis under 10 mM NO_3^-, urea and glutamine-N and 5 mM NH_4^+-N in four blocks of repeat experiment 5. Each bar represents mean of triplicates (*n* = 3). Error bars represent the standard deviation (SD). Asterisk represents a statistically significant difference between the two genotypes, as calculated by student's independent sample *t*-test with 95% confidence interval.

4.4 Discussion

Plants can take up and utilize N in a variety of different inorganic and organic forms; mainly NO_3^-, NH_4^+, urea and amino acids (Miller and Cramer, 2004; Miller, 2010). Chapter 3 has already examined and compared growth and NO_3^- assimilation-related aspects of D1o3-3#47 plants and WT plants under NO_3^- nutrition and also determined the correlation between growth and leaf lipid content. It was found that shoot and total plant growth were substantially lower for D1o3-3#47 plants in comparison with WT plants. This decreased growth was associated with reduced N/NO_3^- uptake, but shoot reduced N, leaf soluble protein and leaf NRA were at least as great with D1o3-3#47 plants as WT plants, indicating that there was no restriction in the ability of D1o3-3#47 plants to assimilate the taken up NO_3^-. A weak correlation was found between growth and leaf fatty acid content, suggesting a probable link of the growth problem to leaf lipid content in D1o3-3#47 plants. Growth was also lower for D1o3-3#47 plants than WT plants with Thrive® as N source. This chapter investigated whether D1o3-3#47 plants show better growth than WT plants under any other N form(s). Growth (shoot DW) of D1o3-3#47 and WT plants was initially assessed under a range of concentrations of NO_3^-, NH_4^+, urea and glutamine, and then under a selected N concentration of these N forms. Additionally, S:R, shoot N% and shoot total-N of D1o3-3#47 and WT plants were analysed in selected N treatments.

In the initial experiment 5, D1o3-3#47 plants did not show better growth than their WT counterparts under any of the applied N forms and concentrations (Fig. 4.1). Growth of D1o3-3#47 and WT plants was similar at lower N levels (2.5 mM of NO_3^-, NH_4^+ and urea and 5 mM of glutamine) but increased and diverged with increasing N supply (Fig. 4.1). Growth of D1o3-3#47 plants was 53-59% of the growth of WT plants across all N forms at 10 mM N level.

Shoot to root DW ratio (S:R) did not differ between D1o3-3#47 and WT plants under any of the N forms applied. These results were consistent with the results from experiments 1-3 of chapter 3 and confirmed that both roots and shoots of D1o3-3#47 plants equally showed limited growth across all N forms.

Increasing N concentration increased shoot H_2O% under NO_3^- and NH_4^+ treatments, but not under urea and glutamine treatments. At similar N concentration, no differences were observed between shoot H_2O% of D1o3-3#47 and WT plants under NO_3^-, urea or glutamine treatments; except in the case of NH_4^+ treatment, the reason for which is not clear. Water is one of the key factors that can limit leaf expansion, and hence can affect plant growth and productivity (Pantin et al., 2012). As shoot H_2O% was similar for D1o3-3#47 and WT plants across most N forms and concentrations, there is no indication that H_2O limitation is a factor in the reduced growth of D1o3-3#47 plants in comparison with WT plants under any of the N forms.

Across all N forms, shoot N% of D1o3-3#47 plants was either higher than or comparable to WT plants, but the shoot total-N (mg) of D1o3-3#47 plants was generally lower than WT plants (Table 4.1). These results were similar to the shoot N% and shoot total-N results from experiments 2 and 3 of chapter 3 (Table 3.1). Thus, regardless of the N form supplied, the decreased growth of D1o3-3#47 plants in comparison to WT plants was associated with reduced N uptake, but that there was no restriction in the ability of D1o3-3#47 plants to utilize the taken up N.

In conclusion, D1o3-3#47 plants did not show better growth than WT plants under any of the applied N forms and concentrations. Nitrate was the prefered N form for growth by D1o3-3#47 and WT plants at ≤ 10 mM N level, however, urea and glutamine gained importance at > 10 mM N levels. Shoot H_2O% was similar in D1o3-3#47 and WT plants regardless of the N forms. S:R was also similar between D1o3-3#47 and WT plants, which indicated that root DW is equally reduced as shoot DW in D1o3-3#47 plants. The finding that D1o3-3#47 plants had either higher or comparable shoot N% and either lower or comparable shoot total-N than WT plants, were consistent with the results of chapter 3, and indicated that the reduced growth of D1o3-3#47 plants was associated with reduced N uptake, but the taken up N could be utilized.

Chapter 5

Final Discussion

5.1 Final discussion and conclusions

The rising world population and industrial development have dramatically increased global energy requirements. The possibility of fossil fuels depletion and increasing atmospheric CO_2 concentration due to fossil fuels consumption have motivated researchers to find alternative energy options. Plant-derived oils can be sustainable and potentially 'environment-friendly' energy resources. The present global production of seed-derived oil is 194.3 million metric tonnes (Foreign Agricultural Service/United States Department of Agriculture, 2017). Most of the seed-derived oil is used for nutrition and only a small proportion, mainly coming from oil crops, is used for the biofuel production.

Lipids offer more than twice the energy density and more economical extraction methods than equivalent amounts of carbohydrates (Durrett et al., 2008; Chapman & Ohlrogge, 2012). The major form of lipid in seed-derived oils is triacylglycerol (TAG)—a neutral storage lipid synthesized mainly through the Kennedy pathway. It consists of three fatty acids esterified to a glycerol backbone (Lung & Weselake, 2006). Triacylglycerol is mainly stored in seeds, enclosed in discrete subcellular organelles called lipid droplets or oil bodies, as long-term energy deposits for germination and seedling development. Moreover, the role of TAG in maintaining lipid homeostasis by acting as a buffer for cytotoxic fatty acids has also been elucidated (Fan et al., 2013). The surface of an oil body is covered with a monolayer of phospholipids embedded with hydrophobic proteins. Oleosins are the major class of oil body-associated proteins in seeds, which stabilizes and prevents the oil bodies from coalescence during seed dehydration (Siloto et al., 2006).

Compared to seeds, vegetative tissues contain only small amounts of oil, which is generally used as a temporary storage intermediate during the turnover of membrane lipids. Vegetative tissues, specifically from energy crops like perennial grasses, have a greater proportion of harvestable biomass per hectare as compared to their seeds and fruits, and can potentially accumulate higher amounts of TAG (Chapman, Dyer & Mullen 2013). Thus, it is proposed that a substantial improvement in global oil yield can be achieved if terrestrial crops are engineered to produce oil in their vegetative tissues rather than only in seeds (Durrett et al., 2008). Such crops with elevated TAG in their vegetative organs could be of immense economic value in the biofuel and forage production, human nutrition and the nutraceutical industry (Durrett et al., 2008; Ohlrogge et al., 2009).

At Agresearch Grasslands, Nick Roberts and the Plant Biotechnology group have been involved in a research program to enhance the metabolizable energy of forage grasses. Part of this work involved

using *Arabidopsis thaliana* as the model plant species. Winichayakul et al. (2013) have shown that constitutive co-expression of DGAT1 and cys-oleosins (oleosin stabilized by strategically placed cysteines) results in long-term storage of oil bodies in Arabidopsis leaves, stem, and roots even after senescence. The study reported 2.1% and 6.5% TAGs of dry weight (DW) in the mature leaves and roots, respectively, of a transgenic line named 'D1o3-3#47'. It was also reported that the mature leaves of D1o3-3#47 plants fixed 24% more CO_2 m^{-2} s^{-1} causing a 50% increase in total leaf biomass per plant as compared to the WT Arabidopsis (when grown in a commercial potting mix) (Winichayakul et al., 2013). However, a preliminary study by the Plant Biotechnology group at Agresearch found that the 50% increase in total leaf biomass was observe only when the D1o3-3#47 Arabidopsis plants were treated with Thrive®, a commercial plant fertilizer containing urea and traces of NH_4^+, and not with a nutrient solution containing NO_3^- as the sole N source. In fact, they found that under NO_3^- nutrition, the D1o3-3#47 plants showed limited growth as compared to the WT plants. This was a concern because although NH_4^+ and urea are usually applied as crop fertilizers, NO_3^- is still the most abundantly available N form taken up and utilized by plants in cultivated agricultural soils (Andrews et al., 2013).

The overall aim of the project was to gain a greater understanding of the reason(s) behind any growth limitation in D1o3-3#47 Arabidopsis under NO_3^- nutrition. The objectives of this study were to; (1) confirm that D1o3-3#47 plants show limited growth as compared to WT plants under NO_3^- nutrition, as reported by Agresearch; (2) determine whether the growth of D1o3-3#47 plants is lower than WT plants across all NO_3^- concentrations; (3) examine NO_3^- assimilation in D1o3-3#47 and WT plants under a selected NO_3^- concentration; (4) determine whether there is any correlation between vegetative growth and leaf fatty acid content of the Arabidopsis lines studied; (5) assess whether the growth of high lipid D1o3-3#47 plants is better than WT plants with forms of N other than NO_3^-. The first four objectives were met in chapter 3 and the fifth objective was met in chapter 4.

In relation to objectives 1 and 2, shoot DW and total plant DW increased with increasing applied NO_3^--N in D1o3-3#47 and WT plants, however, the increment was significantly greater in WT plants than in D1o3-3#47 plants. D1o3-3#47 and WT plants had similar shoot and total plant DW at 0.5 and 1.0 mM applied NO_3^--N, but as applied NO_3^--N increased from 2-10 mM and the total plant DW of the two genotypes diverged, significantly. At 8 mM applied NO_3^--N, shoot and total plant DW was two to three times as great with WT than with D1o3-3#47 plants. However, although D1o3-3#47 plants had smaller rosettes than WT plants, they did not show any visible damage or inter venial-chlorosis.

There was a significant increase in S:R of both D1o3-3#47 and WT plants with increasing NO_3^--N supply, but at a specific applied NO_3^--N level, there was no significant difference between the S:R of D1o3-3#47 and WT plants, indicating that both shoots and roots show growth limitation. Shoot H_2O% increased with increased applied NO_3^--N in D1o3-3#47 and WT plants up to 8 mM and then flattened

off, but no significant differences were found between shoot H_2O% of D1o3-3#47 and WT plants. Thus there is no obvious involvement of H_2O in the growth difference between the two genotypes at high NO_3^- supply.

In relation to objective 3, comparing NO_3^- assimilation between the two genotypes, shoot N% was either similar with D1o3-3#47 and WT plants or slightly greater with D1o3-3#47 plants. Thus, as shoot (and total plant DW) were approximately twice as great for WT than for D1o3-3#47 plants, then on average across experiments approximately 50% less N was taken up by D1o3-3#47 plants as compared to WT plants. However, the reduced N content was at least as great with D1o3-3#47 plants as with WT plants, while leaf soluble protein content was greater with D1o3-3#47 plants. Thus, although NO_3^-/N uptake was reduced per plant, there did not appear to be a restriction on the ability of the D1o3-3#47 plants to assimilate the taken up NO_3^-.

Nitrate reductase activity was greater for D1o3-3#47 plants than WT plants, which is likely to be related to the higher NO_3^- concentration in D1o3-3#47 plants (Andrews et al., 1992, 2004 & 2013). The NRA assay carried out in this study was an *in vivo* assay, which relies on the endogenous supply of reductant (NADH). Hence, the finding of greater NRA in D1o3-3#47 plants also indicated that NADH is not limiting the assimilation of NO_3^- in this genotype.

Objective 4 was to determine if there is a correlation between vegetative growth and leaf fatty acid content of the Arabidopsis lines studied. A weak negative correlation was found between growth and leaf fatty acid content, which suggested that the growth reduction in D1o3-3#47 plants could be related to their high leaf TAG content. Kelly et al. (2013) also reported smaller rosettes and a 20-30% reduction in leaf and root biomass of the high lipid *sdp1* Arabidopsis mutants, and proposed that the growth limitation in high lipid plants could be a consequence of the shift in C-partitioning from structural and storage C compounds to fatty acid metabolism.

The final objective was to assess if the growth of high lipid D1o3-3#47 plants is better than WT plants with the forms of N other than NO_3^-. Interestingly, in experiment 4, the growth limitation in D1o3-3#47 plants as compared to WT plants was not only observed under NO_3^- but also under Thrive® treatment, which contradicted the earlier report of Agresearch that D1o3-3#47 plants show reduced growth only under NO_3^- but not under Thrive® nutrition. In initial experiment 5, D1o3-3#47 plants did not show better growth than their WT counterparts under any of the applied N forms and concentrations. Growth of D1o3-3#47 and WT plants was similar at lower N levels (2.5 mM of NO_3^-, NH_4^+, urea and 5 mM of glutamine), but increased and diverged with increasing N supply. Growth of D1o3-3#47 plants was 53-59% of the growth of WT plants across all N forms at 10 mM N level. Also, across all N forms, shoot N% of D1o3-3#47 plants was either higher than or comparable to WT plants, but shoot total-N

(mg) of D1o3-3#47 plants was generally lower than WT plants. These results were similar to the shoot N% and shoot total-N results from experiment 2 and 3 of chapter 3.

Thus, regardless of the N form supplied, the reduced growth of D1o3-3#47 plants in comparison to WT plants was associated with the reduced N uptake, and there was no restriction in the ability of D1o3-3#47 plants to utilize the uptaken N. Moreover, the earlier report of Agresearch that D1o3-3#47 plants show 50% increase in leaf biomass as compared with WT plants under Thrive® nutrition, did not hold in the current study. It could be that the inheritance of this growth-promoting effect of Thrive® on the D1o3-3#47 plants is unstable, and diminish after certain generations, as this study has used the T_4 generation seeds. Generally, a number of factors can effect the inheritance of transgenic traits; such as the nature of the host genome, the transformation method used and the transmission and integration of the transgene (Yin et al., 2004). On the contrary, if the growth limitation in D1o3-3#47 plants is related to their high leaf TAG content, as hinted in experiment 4, then in comparison to the earlier report of Agresearch the results of the current study are as expected.

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