Combined intracellular nitrate and NIT2 effects on storage carbohydrate metabolism in *Chlamydomonas*

C. Remacle¹, G. Eppe², N. Coosemans¹, E. Fernandez³ and H. Vigeolas¹,*

¹ University of Liege, Institute of Botany, B22, Genetics of Microorganisms, 4000 Liege, Belgium
² University of Liege, Inorganic Analytical Chemistry, LSM-CART, Allée de la Chimie B6c, 4000 Liege, Belgium
³ Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias, Universidad de Cordoba, Campus de Rabanales, 14071 Cordoba, Spain

* To whom correspondence should be addressed. Email: hvigeolas@ulg.ac.be

Received 19 July 2013; Revised 27 August 2013; Accepted 12 September 2013

Abstract

Microalgae are receiving increasing attention as alternative production systems for renewable energy such as biofuel. The photosynthetic alga *Chlamydomonas reinhardtii* is widely recognized as the model system to study all aspects of algal physiology, including the molecular mechanisms underlying the accumulation of starch and triacylglycerol (TAG), which are the precursors of biofuel. All of these pathways not only require a carbon (C) supply but also are strongly dependent on a source of nitrogen (N) to sustain optimal growth rate and biomass production. In order to gain a better understanding of the regulation of C and N metabolisms and the accumulation of storage carbohydrates, the effect of different N sources (NH₄NO₃ and NH₄⁺) on primary metabolism using various mutants impaired in either NIA1, NIT2 or both loci was performed by metabolic analyses. The data demonstrated that, using NH₄NO₃, nia1 strain displayed the most striking phenotype, including an inhibition of growth, accumulation of intracellular nitrate, and strong starch and TAG accumulation. The measurements of the different C and N intermediate levels (amino, organic, and fatty acids), together with the determination of acetate and NH₄⁺ remaining in the medium, clearly excluded the hypothesis of a slower NH₄⁺ and acetate assimilation in this mutant in the presence of NH₄NO₃. The results provide evidence of the implication of intracellular nitrate and NIT2 in the control of C partitioning into different storage carbohydrates under mixotrophic conditions in *Chlamydomonas*. The underlying mechanisms and implications for strategies to increase biomass yield and storage product composition in oleaginous algae are discussed.

Key words: Biomass, *Chlamydomonas*, fatty acid, nitrate, nitrogen, oil, starch.

Introduction

For photosynthetic organisms, such as higher plants and green microalgae, inorganic nitrogen (N) is not only one of the essential nutrients but also the most limiting mineral element for growth and yield (Kropat et al., 2011). Ammonium (NH₄⁺) and nitrate (NO₃⁻) are the major primary sources of N in higher plants and microorganisms, and their respective use is strongly dependent on the species and the environmental conditions.

Assimilation of inorganic N into amino acids and proteins requires both energy and organic carbon skeletons, leading to strong interactions between N and C assimilation (Huppe et al., 1994). NO₃⁻ uptake and assimilation involved two ‘transport’ steps (NO₃⁻ transport into the cells and nitrite transport into the chloroplasts) and two ‘reduction’ steps (NO₃⁻ and nitrite reductases, NR and NiR, respectively), leading to NH₄⁺, which is directly incorporated into central...
C metabolism. In *Chlamydomonas*, nitrate reduction is catalysed by a homodimeric NAD(P)H–NR complex containing two activities: NAD(P)H-cytochrome c reductase (diphorase, EC 1.6.6.1-3) and reduced benzyl viologen NRs (terminal NR) (Kalakoutskii and Fernández, 1995).

In *Chlamydomonas*, the predominant route of NH$_4$\(^+\) assimilation is the glutamine synthetase/glutamate synthase cycle (GS/GOGAT cycle). GS (EC 6.3.1.2) catalyses the transfer of NH$_4$\(^+\) to glutamate, leading to the formation of glutamine. Subsequently, GOGAT (EC 1.4.1.4) catalyses the formation of two molecules of glutamate from one molecule of glutamine and one molecule of α-ketoglutarate. Other sources of intracellular NH$_4$\(^+\) are photospiration, protein turnover, and nucleic acid catabolism. Indeed, it has been shown that as much as 50% of C in algae is integrally coupled with N metabolism (Vanlnerbergh et al., 1991), suggesting that biomass composition of algae is strongly affected by variation in both C and N partitioning within cells. This C/N balance is supported by several studies in many microalgae, including *Chlamydomonas*, that demonstrate the enhancement of oil and starch accumulation under N-deficient conditions (Work et al., 2010). Unfortunately, the resulting high oil and starch contents per cell are also accompanied by a slower growth rate leading to decreased biomass productivity, which is not sustainable for commercial, biotechnological applications.

Biomass is composed mainly of proteins, carbohydrates (starch), and lipids, the proportion of each depending on the strain and culture condition (Liang et al., 2009). Many microalgae are able to produce a large amount of oil, which has been widely considered as a promising source of renewable production of biodiesel to petroleum fuels (Wijffels and Barbosa, 2010). Lipids are synthesized via a complex set of pathways involving cooperation between plastidial and cytosolic metabolism. The polysaccharide starch, which is the dominant storage C product in *Chlamydomonas*, is produced within plastids. ADP-glucose pyrophosphorylase (AGPase) is the key enzyme in the regulation of starch biosynthesis in higher plants and green algae (Van den Koornhuysse et al., 1996; Zabawinski et al., 2001; Vigeolas et al., 2004). The synthesis of fatty acids (FAs) and starch occurs in the same compartments and requires the same precursors, suggesting a competition of the two pathways for the shared substrates or at least an interaction in higher plants (Zabawinski et al., 2001; Vigeolas et al. 2004; Li et al., 2010a,b). In green algae, this notion is supported by recent data showing that a *Chlamydomonas* starchless mutant with a large decrease in AGPase activity displays TAG accumulation under specific stress conditions (Li et al., 2010a,b).

In order to efficiently modulate N assimilation and its allocation, cells developed signalling mechanisms to sense N and induce gene expression. The tight control of C/N metabolism involves, besides sugar sensing and its signalling pathways, signals produced from NO$_3$–, NH$_4$\(^+\), and other N metabolites such as glutamate, glutamine, and aspartate (Stitt and Krapp, 1999; Coruzzi and Zhou, 2001; Miller et al., 2008). NO$_3$–, for example, is not only an essential nutrient but also a key N signalling molecule, regulating the expression of genes involved in N assimilation and primary metabolism, as well as cellular and developmental processes (Scheible et al., 1997, 2004; Zhang and Forde, 1998; Wang et al., 2003; Gerin et al., 2010).

In plants, several potential regulatory NO$_3$ genes have been isolated and their role in NO$_3$ signalling has been studied (Daniel-Vedele et al., 1998). For example, the *Arabidopsis* NLP7 (Nin-like protein 7) modulates NO$_3$ signalling and metabolism (Castaings et al., 2009) and shows conservation with the *Chlamydomonas* NIT2 protein, both of which are RWP-RK transcriptional factors (Camargo et al., 2007; Konishi and Yanagisawa, 2013). Hormones, such as cytokinin, that respond to the N supply, clearly interact with N regulators, and regulate metabolism and development (Coruzzi and Zhou, 2001; Sakakibara, 2006; Argueso et al., 2009).

The availability of a large collection of mutants affected in most of the steps of NO$_3$ uptake and assimilation makes *Chlamydomonas* an interesting model to study N signalling in green algae. Indeed, negative (NRG1-4, FAR1, CYG56) and positive (NIT2) regulatory loci for N uptake and metabolism, which participate in N signalling have already been characterized in *Chlamydomonas* (Gonzalez-Ballesta et al., 2005; Fernandez and Galvan, 2007; de Montaigu et al., 2010). Based on expression studies, a number of genes involved in NO$_3$ assimilation have been shown to be positively regulated by NIT2, such as genes involved in NH$_4$\(^+\) uptake repression and NO$_3$– induction, including NIA1 and NIR1, encoding NR and NiR, respectively (Fernandez et al., 1989; Quesada et al., 1998a) and genes involved in N transport, such as NRT2;1, NRT2;2, NRT2;3 NAR2, and NAR1 encoding NO$_3$–/nitrite transporters (Quesada et al., 1998b; Rexach et al., 2000). Despite the role of NO$_3$– on its own metabolism, it has also been demonstrated that the latter is involved in NH$_4$\(^+\) uptake and acetate assimilation by repressing NIA1 gene expression, and inducing acetyl-CoA synthetase, respectively (Llamas et al., 2002; Gerin et al., 2010).

In this work, the potential regulatory effects of NIA1 and NIT2, encoding NR and the regulatory protein for nitrate assimilation, respectively, on primary C metabolism was investigated in four different NR-deficient strains displaying a mutation in NIA1, NIT2 (nitr2.1 and nitr2.2 strains) or both loci (Fernandez and Matagne, 1986). The study of the role of NIA1 and NIT2 in NO$_3$ signalling and the effect on primary metabolism is partly complicated by the fact that none of these different NR-deficient mutants can grow on NO$_3$– as the sole N source (Fernandez and Matagne 1986). For this purpose, all strains were grown under mixotrophic condition in the presence of either NH$_4$\(^+\) or NH$_4$NO$_3$. Acetate, which is rapidly incorporated into tricarboxylic acid cycle intermediates, via acetyl-CoA synthetase, required for NH$_4$\(^+\) incorporation into primary C metabolism, was chosen as organic carbon source. Metabolomic approaches were employed to identify biochemical changes that may be linked directly or indirectly to these two loci. It was shown that intracellular NO$_3$ and NIT2 participate in the control of C partitioning into different C storage pools under mixotrophic conditions in *Chlamydomonas*.
Materials and methods

Strains and culture conditions

The wild-type 21gr strain and the mutants nia1 (305), nit2.1 (nit2), nit2.2 (203) and nia1nit2 (137c) have been characterized previously (Fernández and Matagne, 1984). Strains were grown at 25 °C in Tris-acetate-phosphate (NH₄ medium 7 mM) or Tris-acetate-phosphate-ammonium nitrate 7 mM (NH₄NO₃ medium) liquid or solid (1.5% agar) medium, under continuous light (50 μE m⁻² s⁻¹) as described by Harris (1989). Cell counts were assessed using a Beckman Z2 Coulter cell and particle counter (Beckman Coulter).

Mass spectroscopy analyses of FA methyl esters (FAMEs)

Lipids were extracted and derivatized from liquid culture. Briefly, 1.0 ml of methanol saturated with 1 M HCl was added to 1 ml of culture and heated in tightly sealed vials at 80 °C for 90 min, resulting in cell lysis and lipid saponification. FAMES were then extracted into 2 ml of 1:1 hexane in 0.9% NaCl via gentle inversion. Hexane extracts, containing FAMES, were measured using a Trace GC2000-PolarisQ ion trap mass spectrometer (Thermo-Scientific, Waltham, MA, USA) equipped with a CTC Combi-Pal autosampler (CTC Analytics, Zwingen, Switzerland), using the GC column (SP2331, 30 m×0.25 mm×0.20 μm film thickness; Supelco Bellefonte USA). Pentadecanoic acid (C15:0) was also used as an internal standard for quantification.

Thin layer chromatography (TLC) of the neutral lipid fraction

Using freeze-dried cells (50 ml of algal culture), lipids were extracted according to the method of Bligh and Dyer (1959). Chloroform extracts corresponding to 10° cells were fractionated on TLC, as described by Stobart et al. (1997). The staining of the TLC plate was done with iodine vapour.

Determination of cellular dry weight, starch, and protein levels

Dry weight, starch, and protein contents were measured as described by Vigeolais et al. (2012).

Determination of total free amino acid, nitrate, and malic and fumaric acid contents

Metabolites were extracted twice with 80% ethanol and one with 50% ethanol. Total free amino acids were assayed according to Bantan-Polak et al. (2001). Malate, fumarate, and nitrate levels were measured as described by Tschoep et al. (2009).

Determination of NH₄⁺ and acetate contents

NH₄⁺ and acetate contents were measured by using Megazyme assay K-AMIAR and K-ACETAK kits, respectively (Megazyme, Wicklow, Ireland).

Chemicals

Unless stated otherwise, chemicals were obtained from Sigma (Taufkirchen, Germany) or Merck (Darmstadt, Germany).

Results

Growth rate is strongly affected in a nia1 mutant in the presence of NH₄NO₃ as the N source

Growth rate strongly depends on the nutrient availability in the medium, with N and C being the most important macronutrients. As NR-deficient strains are not able to grow on nitrate as the sole source of N, analyses of the nia1, nit2, and nia1nit2 strains were performed under two different mixotrophic mediums, containing either NH₄⁺ or NH₄NO₃. Using only NH₄⁺ as a source of N, all the NR-deficient and wild-type strains displayed similar growth rates (Fig. 1), suggesting that NH₄⁺ assimilation is unaffected in the mutant strains. In NH₄NO₃ medium, the wild-type strain, the nia1nit2 double mutant, and both nit2 mutant strains displayed unchanged growth rates compared with pure NH₄⁺ nutrition. In contrast, the nia1 strain grew significantly slower using NH₄NO₃ compared with NH₄⁺ as the source of N in the medium. These values were consistent with data published previously (Fernandez and Cardenas, 1982).

The nia1 mutant displays stimulation of acetate and NH₄⁺ uptake under NH₄NO₃

In order to investigate whether the differences in growth rate in the nia1 mutant could be explained by an alteration of C and N uptake, the levels of acetate and NH₄⁺ in the medium were determined during the exponential growth phase (Figs 2 and 3). Levels of NH₄⁺ and acetate progressively decreased during exponential growth, while the concentration of algal cells increased in all mutants and the wild type under both N regimes. In either condition, extracellular acetate content reached approximately 0.6 g l⁻¹ in all the strains, including wild type, at the middle of the exponential phase, which corresponded to a total quantitative uptake of approximately 40% of the initial amount of acetate supplied in the medium. In all strains, about 0.2 g l⁻¹ of acetate remained in the

Fig. 1. Chlamydomonas growth on NH₄⁺ and NH₄NO₃. Cells of wild-type (WT) and NR-deficient strains were grown in acetate medium containing NH₄⁺ (black bars) or NH₄NO₃ (grey bars). Values are means±SE (n=3–6). Asterisks represent values significantly different from the wild type; + represents a significantly different value between NH₄⁺ and NH₄NO₃ cultures for each particular strain (based on Student’s t-test with Ps0.05).
medium at the end of the exponential growth phase (Figs 2 and 3). Interestingly, the nia1 mutant showed levels of NH₄⁺ and acetate consumption similar to all other strains, while displaying a strongly reduced growth rate under NH₄NO₃ conditions, suggesting a stimulation of acetate and NH₄⁺ uptake in the nia1 mutant. The impaired growth of the nia1 strain, together with an unchanged respiratory rate under NH₄NO₃ fertilization (13.6 ± 1.1 and 13.0 ± 0.6 nmoles O₂ min⁻¹ per 10⁷ cells in wild-type and nia1 strains, respectively), is consistent with previous data demonstrating that total respiratory rate is not affected by the source of N (Baurain et al., 2003).

**Intracellular NO₃ accumulation in the nia1 mutant under NH₄NO₃ nutrition**

Due to the low NO₃⁻ uptake in all NR-deficient lines analysed in this study (Fernandez and Cardenas, 1982), uptake was evaluated by measuring the intracellular NO₃⁻ levels (Fig. 4). No intracellular NO₃⁻ was detectable in strains grown on NH₄⁺ (data not shown). Under NH₄NO₃ nutrition, the low level of NO₃⁻ in the wild type was probably due to a decreased uptake of NO₃⁻ governed by NH₄⁺ repression of NO₃⁻ uptake and/or direct assimilation of NO₃. Whereas the nit2.1, nit2.2, and nia1nit2 mutants displayed the same low amount of NO₃⁻ as the wild type, the nia1 mutant line accumulated up to 3.4-fold more NO₃⁻ (Fig. 4). This observation is consistent with data already published, demonstrating that NIT2 is required for the expression of NO₃⁻ transporters (Quesada et al., 1993; Camargo et al., 2007).

**Induction of organic acid biosynthesis in the nia1 mutant under NH₄NO₃**

N assimilation into amino acids and proteins requires the synthesis of organic acids in the tricarboxylic acid cycle, which serve as acceptors for amino groups. The effects of both N regimes (NH₄⁺ and NH₄NO₃) on N assimilation into amino acids were studied by measuring the total level of free amino acids per cell and malate and fumarate levels in all the different strains (Table 1). In the presence of NH₄⁺, total free amino acid content was similar in NR-deficient and
wild-type strains, consistent with the fact that NH$_4^+$ assimilation was not affected in the mutants. In the presence of NH$_4$NO$_3$, the nit2.2 and nia1 strains displayed up to 1.5–1.8-fold higher free amino acid content on a per-cell basis compared with the wild type and the nit2.1 and nia1nit2 mutants (Table 1). The increased NH$_4^+$ uptake and elevated free amino acid content together with an unchanged protein level in the nia1 strain suggested either stimulation of de novo amino acid biosynthesis or inhibition of amino acid incorporation into the protein fraction (Fig. 5A).

In NH$_4^+$ medium, the wild-type and nia1nit2 strains contained similar levels of malate and fumarate, whereas the nia1 mutant displayed slightly increased levels of both of these organic acids. Although the amount of malate was lower in the nit2.1 and nit2.2 strains than in the wild type, only the nit2.1 strain displayed a significantly decreased fumarate level (Table 1). Compared with the wild type, only the nia1-deficient line accumulated up to 4-fold more malate and 10-fold more fumarate under NH$_4$NO$_3$ nutrition (Table 1), suggesting induction of organic acid biosynthesis for de novo synthesis of amino acids (Scheible et al., 1997).

**Starch and triacylglycerol content are strongly affected in the nia1 mutant under NH$_4$NO$_3$ nutrition**

To investigate whether the changes in C/N balance in NR-deficient lines under NH$_4$NO$_3$ nutrition were accompanied by an alteration in C partitioning into protein, total lipid, starch levels, and cellular dry weight were determined in the different strains under both N regimes (Fig. 5A–D). The total protein fraction was approximately 18 pg per cell in the wild type and was similar to all the mutant strains under the NH$_4^+$ regime. The switch from NH$_4^+$ to NH$_4$NO$_3$ medium did not lead to a significant change in total protein level among the different strains (Fig. 5A).

Under N-replete conditions, most of the FAs were incorporated into polar lipids in *Chlamydomonas* cells (Fig. 6, lanes 1; Siaut et al., 2011). To determine the global changes...
in FA biosynthesis, the total cellular lipid levels were measured by quantifying the total FAMEs using gas chromatography–mass spectrometry (GC-MS) analysis (Fig. 5B). In the presence of NH₄⁺ in the medium, the amounts of total FAs were similar in the wild-type, nit2.1, nia1, and nia1nit2 strains (14 ± 0.2, 15 ± 3, 16 ± 0.3, and 14 ± 1 pg per cell, respectively), and showed a slight increase in the nit2.2 strain (19 ± 1.2 pg per cell). These values were consistent with data published previously (Moellering and Benning, 2010). As chain lengths and degrees of FA saturation also strongly influence the properties and quality of algae lipids, FA composition was also investigated by GC/MS analysis of FAMEs (Supplementary Tables S1 and S2 at JXB online).

The unchanged cellular lipid level under NH₄⁺ nutrition (Fig. 5B) was accompanied by a similar FA composition (Supplementary Table S1) in most of the NR-deficient strains compared with the wild type, except in the nit2.2 mutant. Indeed the latter showed a significant 3.5-fold and 2.3-fold increase in the relative amounts of monounsaturated C16:1 (oleic acid) and C18:1 (palmitoleic acid), respectively, together with a slightly decreased proportion of polyunsaturated C18:3 (linolenic acid), suggesting a change in FA desaturation (Supplementary Table S1).

In our study, the FA composition in NH₄⁺ medium was similar to those published previously (EL-Sheekh, 1993; Work et al., 2010). The absence of the polyunsaturated FA C16:4, could be explained by different culture conditions such as irradiance and C source.

While the total cellular FA content in wild-type, nit2.1, and nit2.2 strains and the double mutant nialnit2 remained unchanged under both N regimes (24.4 µg per cell), the nial strain showed a 1.5-fold increased total FA content under NH₄NO₃ compared with the NH₄⁺ regime, corresponding to 24.4 pg per cell (Fig. 5B). Under NH₄NO₃, the nial and nit2.2 strains also displayed an increase in C18:2 linoleic acid (1.8- and 1.5-fold, respectively) and a slight decrease in C18:3 linolenic acid (Supplementary Table S2), suggesting inhibition of either the plastidic isoform ω3-desaturase FAD7 and/or the
membrane-bound linoleate desaturase FAD3 located at the endoplasmic reticulum (Riekhof et al., 2005).

TLC analysis of the neutral lipid profile clearly demonstrated that growth on NH4NO3, but not on NH4+, led to the accumulation of TAGs in all NR-deficient strains but not in the wild type (Fig. 6). The strongest accumulation of TAG was observed in the nia1 strain, which also contained a generally higher FA content.

A strong interaction between starch and lipid biosynthesis pathways has been described previously in different organisms such as higher plants (Vigeolas et al., 2004), Chlorella pyrenoidosa (Ramazanov and Ramazanov, 2006), and Chlamydomonas (Li et al., 2010a,b; Zabawinski et al., 2001). Whereas starch levels were similar in all strains under NH4+ nutrition, there was a 2-fold increase in starch in the nia1-deficient strain compared with the wild type when grown with NH4NO3 as the source of N (Fig. 5C). In contrast, the two nit2-deficient strains and the nia1nit2 double mutant displayed similar levels of this component compared with the wild type.

Growth on NH4NO3, but not on NH4+, leads to changes in biomass composition in the nia1 mutant

Cell dry weight was also determined in all conditions in order to investigate whether the changes in storage carbohydrate contents on a per cell basis, especially for the nia1 strain, were due to changes in C partitioning into biomass compounds or a general change in dry biomass productivity (Fig. 5D). In NH4 medium, wild-type, nia1 and the double mutants showed a similar cell dry weight, while both nit2-deficient strains (nit2.2, nit2.1) displayed a 1.5-fold and 2.3-fold increase in dry weight per cell, respectively. In NH4NO3, all the cellular dry weights were similar to those in NH4+ medium, indicating that the proportions of total lipids, TAGs, and starch in relation to the other biomass compounds within cells were higher in the nia1-deficient strain.

Discussion

In Chlamydomonas, biochemical and genetic analyses have allowed the identification and characterization of most of the different components involved in NO3 transport and assimilation, with some participating in NO3 signalling pathways, including the structural gene NIA1 encoding NR (EC. 1.6.6.2) and the NIT2 regulator, which is considered a central regulatory gene required for NO3 signalling. Besides NO3, several N components have also been suggested to act as signals to regulate C and N metabolism, such as nitric oxide, glutamate, glutamine, and aspartate (Stitt and Krapp, 1999; Coruzzi and Zhou, 2001; Miller et al., 2008; de Montaigu et al., 2010). These data strongly suggest the presence of other potential regulatory effectors for NO3 signalling, further downstream of NO3 assimilation.

In this study, the effects of NO3 as a signalling molecule and the resulting changes in primary C metabolism were investigated in NR-deficient strains affected in either the catalytic subunit of NR (NIA1) or the regulatory locus (NIT2). Growth on NH4NO3, but not on NH4+, leads to a stimulation of N and acetate assimilation into primary C metabolism in the nia1 strain

Under NH4NO3, only the nia1 strain displayed a growth reduction, whereas all the strains affected in the NIT2 gene (nit2.1, nit2.2, and nia1nit2) displayed a similar growth pattern to the wild type (Fig. 1). This inhibition was accompanied by a stimulation of acetate uptake and an unchanged dark respiration, except that the observed growth inhibition was due to slower acetate assimilation via the respiratory chain. The stimulation of acetate uptake and assimilation in the nia1 strain was supported by previous comparative proteomic data analysis of wild-type strains demonstrating that acetyl-coA synthases, key steps in the assimilation of acetate, are upregulated in NO3-grown compared with NH4+-grown cells (Gerin et al., 2010).

The extracellular levels of NH4+ and NO3 during the exponential phase were consistent with the preferential use of NH4+ under the NH4NO3 regime. This might be due to a lower energy cost for the cells to assimilate NH4+ directly rather than from NO3 via NR, and due to the presence of a more efficient uptake and transport system (Florence, 1983; Harris, 1989). This was also supported by a higher free amino acid content under an NH4+ regime compared with a NH4NO3 regime in the wild type, which is probably due to a rapid incorporation of NH4+ into amino acids to avoid NH4+ toxicity. This efficient process has been described in several organisms such as higher plants, and provides a mechanism to allow cells to cope with elevated internal free NH4+ levels that would otherwise increase the intracellular pH leading to toxicity (Gerendás et al., 1997).

Interestingly, compared with the wild-type and nit2-deficient lines displaying similar intracellular nitrate levels (2 fmol per cell), the nia1-deficient strain accumulated up to 2.5-fold more intracellular NO3. These data support the suggestion that the NIT2 gene is involved in the control of NO3 transports in the presence of intracellular NO3 (Camargo et al., 2007). The role of NIT2 in the regulation of NH4+ and NO3 transports in the presence of intracellular NO3 has been already described in the nia1 strain under phototrophic conditions by transferring cells grown on NH4+ into NO3 medium (Gonzalez-Ballester et al., 2004; Camargo et al., 2007). Based on previous studies, demonstrating that the high-affinity nitrate/nitrite transporters I, II, and III were blocked by NH4+, and that system IV is insensitive to NH4+, the accumulation of intracellular NO3 under NH4NO3 in the nia1 strain was probably due to stimulation or induction of the transport system IV (Llamas et al., 2002).

Under NH4NO3, the accumulation of organic acids and intracellular NO3 in the nia1 strain supports the idea that both NO3 and NIT2 are involved in a signalling cascade that induces organic acid biosynthesis and initiates co-ordinated changes in C and N metabolism in Chlamydomonas (Zioni et al., 1971; Purvis et al., 1974). It has been demonstrated previously that NO3 is a signal molecule in plants that has been shown to induce several thousand genes and promote diverse transcriptional responses in Arabidopsis (Wang et al., 2000, 2003).
Under an NH₄NO₃ regime, starch and FA contents are strongly affected in the nia1 mutant

Despite an increased de novo fatty acid synthesis suggested by a higher level of total FA content, TLC analysis of the neutral lipid fraction clearly showed that the nia1 line displayed accumulation of TAG and free FAs, which was not observed in the wild type. Moreover, these increases were also accompanied by changes in total FA composition, such as higher C18:1/C18:3 ratios, which was observed in TAG under N starvation (Siaut et al., 2011) and which is consistent with a higher TAG content. More detailed analysis of the different classes of lipids would be required to investigate the effects of lipid metabolism under NH₄NO₃. It is noteworthy, that all NR strains displayed a slight increased TAG and free FA content on a per-cell basis in the presence of NH₄NO₃, suggesting that the lack of NR itself led to changes in lipid composition. Interestingly, the increased total FA level, including TAGs, was accompanied by an accumulation of starch in the nia1-deficient strain (Fig. 2). The accumulation of both storage carbohydrates has already been observed in the earlier phases of N and sulfur deficiency studies in Chlamydomonas (Matthew et al., 2009; Moellering and Benning, 2010), which is not the case in the present study. Indeed, several lines of evidence indicate that the phenotype of the nia1 line was not due to N deprivation. First, the nia1 strain did not turn yellow during growth, which is typical of N-starved cells (data not shown). Secondly, no evidence for a reduced NH₄⁺ availability such as changes in protein and free amino acid levels under NH₄NO₃ compared with NH₄⁺ nutrition was found.

Interestingly, the nia1 line preferentially accumulated starch rather than oil under NH₄NO₃. This is consistent with recent studies demonstrating that C channeling into storage lipid also occurred either when the maximal rate of starch biosynthesis was reached or blocked, or when the C source was in excess over that required for N metabolism (Work et al., 2010; Fan et al., 2012). The differential effect on starch and TAG synthesis could also be linked to the different energy requirements of the two biosynthetic processes. Based on theoretical considerations of the stoichiometry of the reaction pathways, addition of a six-carbon unit would cost one ATP in the case of starch and three ATPs in the case of lipid synthesis.

It is noteworthy that, while growth rates of both nit2 mutants were similar under both N regimes, C metabolism was differentially affected in the nit2.1 and nit2.2 mutants under NH₄NO₃ nutrition. Compared with the nit2.1 mutant, nit2.2 contained higher levels of total FAs and free amino acids (Table 1, Fig. 5B). The stronger phenotype observed in nit2.2 is likely to be related to two mutations in the NIT2 gene, which are located in the third glutamine-rich region containing Ala repeats, and this domain is of crucial importance to NIT2 function (Camargo et al., 2007). In contrast, mutation in nit2.1 occurs in the last exon of NIT2 resulting in a stop codon within the RWP-RK domain. The latter has been shown to be the DNA-binding site of the homologue of the Arabidopsis transcription factor NLP (Konishi and Yanagisawa, 2013). The molecular nature of these two nit2 mutants might result in a different strength of the nit2 mutation and explain the slightly different data obtained with the two mutants.

Contribution of starch and NO₃ in the control of growth

The accumulation of storage carbohydrate compounds was expected when growth is decreased, but the reasons for this growth inhibition within the nia1 mutant remain elusive. The latter was not due to a reduction of energy processes such as respiration and photosynthesis, as dark respiration and the chlorophyll alb ratio remained unchanged in the nia1 strain compared with the wild type (data not shown; Kirst et al., 2012).

The first possible explanation would be related to the potential effects of NO₃ accumulation on growth in nia1-deficient line cells. It is commonly known that N acts as a signal to regulate and adjust growth rate in several tissues, such as roots in higher plants, and thus control C/N distribution at the whole-plant level (Stitt 1999; Wang et al., 2003; Scheible et al., 2004). In oilseed rape, starch metabolism has been demonstrated to be closely linked to cellular growth and differentiation (Vigeolas et al., 2004; Andriotis et al., 2010). Interestingly, the effects of NO₃ on starch biosynthesis are different from those observed in many higher plants such as tobacco and Arabidopsis where NO₃ represses the expression of AGS gene (Scheible et al., 1997), encoding the regulatory subunit of AGPase, which represents a key enzyme in starch biosynthesis.

In conclusion, our study clearly demonstrates that intracellular NO₃ plays a major role in the regulation of starch and TAG biosynthesis in Chlamydomonas. This mechanism involves NIT2 and is a NIA1-independent signalling pathway. Although the role of NIT2 in the NO₃ assimilation pathway is quite well documented, little is known about how internal NO₃ acts as signalling molecule and interacts with NIT2. Camargo et al. (2007) demonstrated that NO₃ is not essential to induce NIT2 expression, but its presence leads to the stabilization of NIT2 transcripts. Moreover, NIT2 is composed of several different domains, characteristic of transcription factors and co-activators in other organisms, but none appears to bind NO₃. One of these is a RWP-RK, showing conservation with the Arabidopsis NLP7. The latter has been shown to modulate NO₃ signalling and metabolism (Castaings et al., 2009; Konishi and Yanagisawa, 2013). The GAF domain is in the N-terminal fragment of the protein and has been shown to bind small molecules including oxoglutarate, nitric oxide, and cGMP, but not NO₃. NIT2 also contains glutamine-rich domains involved in protein–protein interactions and a nuclear export sequence that binds specifically to the NIA1 promoter regions, essential for the regulation of its expression (Camargo et al., 2007). Interestingly, neither nit2 mutant accumulated either NO₃ or storage compounds, indicating that this mechanism requires at least a functional RWP-RK domain and the third glutamine-rich region of the NIT2 protein.

The strong accumulation of starch and TAG in the nia1 mutant was remarkable. To our knowledge, this is the first report of a genetic approach leading to an increase in both
starch and TAG quantities of microalgae under repleted N conditions. In our point of view, due to the great economic importance and expanded use of microalgae as industrial and nutritional feedstock, this finding has obvious implications for the use of microalgae as alternative production systems for renewable energy such as biofuel. Unfortunately, its higher starch and TAG composition is also accompanied by growth inhibition. The reasons for the lower growth rate in the nial mutant are still unclear and could be due to different parameters, such as the C source or external NO3 concentration.

Further studies using the double mutant nial sta6, with STAB2 encoding the small catalytic subunit of AGPase, a key step for starch biosynthesis, will be required to distinguish and clarify the contribution of starch and NO3 in the control of growth.

**Supplementary data**

Supplementary data are available at *JXB* online.

**Supplementary Table S1.** Fatty acid composition of total cellular lipids from wild-type and NR-deficient strains in NH4NO3 medium.

**Supplementary Table S2.** Fatty acid composition of total cellular lipids from wild-type and NR-deficient strains in NH4NO3 medium.

**Acknowledgements**

This work was funded by a FP7-funded project (Sunbiopath, GA 245070) to CR, and FRFC (2.4567.11, 2.4597.11) and les ‘Fonds speciaux de l’Université de Liege’ to CR, HV and GE. EF is funded by ‘Ministerio de Economia y Competitividad’ (BFU2011-29338) with support from the European FEDER programme. We thank M. Radoux for expert technical assistance. HV is an FNRS Research Collaborator.

**References**

Andriotis VM, Pike MJ, Kular B, Rawsthorne S, Smith AM. 2010. Starch turnover in developing oilseed embryos. *New Phytologist* 187, 791–804.

Argueso JL, Carazzolle MF, Mieczkowski PA, et al. 2009. Genome structure of a *Saccharomyces cerevisiae* strain widely used in bioethanol production. *Genome Research* 19, 2258–2270.

Bantan-Polak T, Kassai M, Grant KB. 2001. A comparison of fluorescamine and naphthalene-2,3-dicarboxaldehyde fluorogenic reagents for microplate-based detection of amino acids. *Analytical Biochemistry* 297, 128–136.

Baurain D, Dinant M, Cocoemans N, Matagne RF. 2003. Regulation of the alternative oxidase Aox1 gene in *Chlamydomonas reinhardii*. Role of the nitrogen source on the expression of a reporter gene under the control of the Aox1 promoter. *Plant Physiology* 131, 1418–1430.

Bligh EG, Dyer WJ. 1959. A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* 37, 911–917.

Camargo A, Llamas A, Schnell RA, Higuera JJ, Gonzalez-Ballester D, Lefebvre PA, Fernandez E, Galvan A. 2007. Nitrate signaling by the regulatory gene NIT2 in *Chlamydomonas*. *Plant Cell* 19, 3491–3503.

Castaings L, Camargo A, Pocholle D, et al. 2009. The nodule inception-like protein 7 modulates nitrate sensing and metabolism in *Arabidopsis*. *The Plant Journal* 57, 426–435.

Coruzzi GM, Zhou L. 2001. Carbon and nitrogen sensing and signaling in plants: emerging ‘matrix effects’. *Current Opinion in Plant Biology* 4, 247–253.

Daniel-Vedele F, Filleur S, Caboche M. 1998. Nitrate transport: a key step in nitrate assimilation. *Current Opinion in Plant Biology* 1, 235–239.

de Montaigu A, Sanz-Luque E, Galvan A, Fernandez E. 2010. A soluble guanylate cyclase mediates negative signaling by ammonium on expression of nitrate reductase in *Chlamydomonas*. *Plant Cell* 22, 1532–1548.

EL-Sheekh MM. 1993. Lipid and FA composition of photautotrophically and heterotrophically grown *Chlamydomonas reinhardii*. *Biologia Plantarum* 35, 435–441.

Fan J, Yan C, Andre C, Shanklin J, Schwender J, Xu C. 2012. Oil accumulation is controlled by carbon precursor supply for fatty acid synthesis in *Chlamydomonas reinhardii*. *Plant and Cell Physiology* 53, 1380–1390.

Fernandez E, Cardenas J. 1982. Regulation of the nitrate-reducing system enzymes in wild-type and mutant strains of *Chlamydomonas reinhardii*. *Molecular Genetics and Genomics* 186, 164–169.

Fernandez E, Galvan A. 2007. Inorganic nitrogen assimilation in *Chlamydomonas*. *Journal of Experimental Botany* 58, 2279–2287.

Fernández E, Matagne R. 1984. Genetic analysis of nitrate reductase-deficient mutants in *Chlamydomonas reinhardii*. *Current Genetics* 8, 635–640.

Fernandez E, Matagne RF. 1986. In vivo complementation analysis of nitrate reductase-deficient mutants in *Chlamydomonas reinhardii*. *Current Genetics* 10, 397–403.

Fernandez E, Schnell R, Ranum LP, Hussey SC, Silflow CD, Lefebvre PA. 1989. Isolation and characterization of the nitrate reductase structural gene of *Chlamydomonas reinhardii*. *Proceedings of the National Academy of Sciences, USA* 86, 6449–6453.

Florencio FJ. 1983. Separation, purification, and characterization of two isoforms of glutamine synthetase from *Chlamydomonas*.* Zeitschrift für Naturforschung* 38c, 531–538.

Gerendás J, Zhu Z, Bendixen R, Ratcliffe RG, Sattelmacher B. 1997. Physiological and biochemical processes related to ammonium toxicity in higher plants. *Zeitschrift für Pflanzenzernährung und Bodenkunde* 160, 239–251.

Gerin S, Mathy G, Blomme A, Franck F, Sluse FE. 2010. Plasticity of the mitoproteome to nitrogen sources (nitrate and ammonium) in *Chlamydomonas reinhardii* the log of Aox1 gene localization. *Biochimica et Biophysica Acta* 1797, 994–1003.

Gonzalez-Ballester D, Camargo A, Fernandez E. 2004. Ammonium transporter genes in *Chlamydomonas*: the nitrate-specific regulatory gene NIT2 is involved in Amt1;1 expression. *Plant Molecular Biology* 56, 863–878.
Gonzalez-Ballestero, D, de Montaigu, A, Higuera, JJ, Galvan, A, Fernandez, E. 2005. Functional genomics of the regulation of the nitrate assimilation pathway in Chlamydomonas. Plant Physiology 137, 522–533.

Harris E. 1989. The Chlamydomonas sourcebook. San Diego, CA: Academic Press.

Huppe HC, Farr TJ, Turpin DH. 1994. Coordination of chloroplastic metabolism in N-limited Chlamydomonas reinhardtii by redox modulation. II. Redox modulation activates the oxidative pentose phosphate pathway during photosynthetic nitrate assimilation. Plant Physiology 105, 1043–1048.

Kalakoutski and Fernandez. 1995. Chlamydomonas reinhardtii nitrate reductase complex has 105kDa subunits in the wild-type strain and a structural mutant. Plant Science 105, 195–206.

Kirst H, Garcia-Cerdan JG, Zurbriggen A, Melis A. 2012. Assembly of the light-harvesting chlorophyll antenna in the green alga Chlamydomonas reinhardtii requires expression of the TLA2-CpFTSY gene. Plant Physiology 158, 930–945.

Konishi, M, and Yanagisawa, S. 2013. Arabidopsis NIN-like transcription factors have a central role in nitrate signalling. Nature Communications 19, 1617.

Kropat J, Hong-Hermesdorf A, Casero D, Ent P, Castruita M, Kalakoutski J, et al. 2009. The metabolome of Chlorella vulgaris L. reveals a novel ABC transport system involved in nitrite transport. Plant Physiology 151, 259–265.

Li Y, Han D, Hu G, Dauville D, Sommerfeld M, Ball S, Hu Q. 2010b. Inhibition of starch synthesis results in overproduction of lipids in Chlamydomonas reinhardtii. Biotechnology and Bioengineering 107, 770–780.

Li Y, Han D, Hu G, Sommerfeld M, Hu Q. 2010a. Chlamydomonas starchless mutant defective in ADP-glucose pyrophosphorylase hyper-accumulates triacylglycerol. Metabolic Engineering 12, 387–391.

Li Y, Han D, Hu G, Sommerfeld M, Hu Q. 2010. Nitrate reductase activity of the nitrate transport systems in Chlamydomonas. The Plant Journal 66, 770–780.

Matthew T, Zhou W, Rupprecht J, et al. 2009. The metabolome of Chlamydomonas reinhardtii following induction of anaerobic H2 production by sulfur depletion. Journal of Biological Chemistry 284, 23415–23425.

Miller AJ, Fan X, Shen Q, Smith SJ. 2008. Amino acids and nitrate as signals for the regulation of nitrogen acquisition. Journal of Experimental Botany 59, 111–119.

Moellering ER, Benning C. 2010. RNA interference silencing of a major lipid droplet protein affects lipid droplet size in Chlamydomonas reinhardtii. Eukaryotic Cell 9, 97–106.

Purvis AC, Peters DB, Hageman RH. 1974. Effect of carbon dioxide on nitrate accumulation and nitrate reductase induction in corn seedlings. Plant Physiology 53, 934–941.

Quesada A, Galvan A, Schnell RA, Lefebvre PA, Fernandez E. 1993. Five nitrate assimilation-related loci are clustered in Chlamydomonas reinhardtii. Molecular Genetics and Genomics 240, 387–394.

Quesada A, Gomez I, Fernandez E. 1998a. Clustering of the nitrile reductase gene and a light-regulated gene with nitrate assimilation loci in Chlamydomonas reinhardtii. Planta 206, 259–265.

Quesada A, Hidalgo J, Fernandez E. 1998b. Three Nrt2 genes are differentially regulated in Chlamydomonas reinhardtii. Molecular Genetics and Genomics 258, 373–377.

Ramazanov A, Ramazanov Z. 2006. Isolation and characterization of a starchless mutant of Chlorella pyrenoidosa STL-PI with a high growth rate, and high protein and polysaturated fatty acid content. Phycological Research 54, 255–259.

Rexach J, Fernandez E, Galvan A. 2000. The Chlamydomonas reinhardtii Nrt1 gene encodes a chloroplast membrane protein involved in nitrite transport. Plant Cell 12, 1441–1453.

Riekhof WR, Sears BB, Benning C. 2005. Annotation of genes involved in glycerolipid biosynthesis in Chlamydomonas reinhardtii: discovery of the betaine lipid synthase BTA1Cr. Eukaryotic Cell 4, 242–252.

Sakakibara H. 2006. Cytokinins: activity, biosynthesis, and translocation. Annual Review of Plant Biology 57, 431–449.

Scheible WR, Morcuende R, Czeckowski T, Fritz C, Osuna D, Palacios-Rojas N, Schindelbach D, Thimm O, Udvardi MK, Stitt M. 2004. Genome-wide reprogramming of primary and secondary metabolism, protein synthesis, cellular growth processes, and the regulatory infrastructure of Arabidopsis in response to nitrogen. Plant Physiology 136, 2483–2499.

Scheible WR, Gonzalez-Fontes A, Lauerer M, Muller-Rober B, Caboche M, Stitt M. 1997. Nitrate acts as a signal to induce organic acid metabolism and repress starch metabolism in tobacco. Plant Cell 9, 783–798.

Siaut M, Cuine S, Cagnon C, et al. 2011. Oil accumulation in the model green alga Chlamydomonas reinhardtii: characterization, variability between common laboratory strains and relationship with starch reserves. BMC Biotechnol 11, 7.

Stitt M, Krapp A. 1999. The interaction between elevated carbon dioxide and nitrogen nutrition: the physiological and molecular background. Plant, Cell & Environment 22, 583–621.

Stitt M. 1999. Nitrate regulation of metabolism and growth. Current Opinion in Plant Biology 2, 178–186.

Stobart K, Mancha M, Lenman M, Dahlqvist A, Stymne S. 1997. Triacylglycerols are synthesised and utilized by transacylation reactions in microsomal preparations of developing safflower (Carthamus tinctorius L) seeds. Planta 203, 58–66.

Tschoep H, Gibon Y, Carillo P, Armengaud P, Szczekwicka M, Nunes-Nesi A, Fernie AR, Koehl K, Stitt M. 2009. Adjustment of growth and central metabolism to a mild but sustained nitrogen-limitation in Arabidopsis. Plant, Cell & Environment 32, 300–318.

Van den Koornhuyse N, Libessart N, Delrue B, Zabawinski C, Decq A, Iglesias A, Carton A, Preiss J, Ball S. 1996. Control of starch composition and structure through substrate supply in the monocellular alga Chlamydomonas reinhardtii. Journal of Biological Chemistry 271, 16281–16287.
Vanlerberghe GC, Joy KW, Turpin DH. 1991. Anaerobic metabolism in the N-limited green alga Selenastrum minutum: III. Alanine is the product of anaerobic ammonium assimilation. Plant Physiology 95, 655–658.

Vigeolas H, Duby F, Kaymak E, Niessen G, Motte P, Franck F, Remacle C. 2012. Isolation and partial characterization of mutants with elevated lipid content in Chlorella sorokiniana and Scenedesmus obliquus. Journal of Biotechnology 162, 3–12.

Vigeolas H, Mohlmann T, Martini N, Neuhaus HE, Geigenberger P. 2004. Embryo-specific reduction of ADP-Glc pyrophosphorylase leads to an inhibition of starch synthesis and a delay in oil accumulation in developing seeds of oilseed rape. Plant Physiology 136, 2676–2686.

Wang R, Guegler K, LaBrie ST, Crawford NM. 2000. Genomic analysis of a nutrient response in Arabidopsis reveals diverse expression patterns and novel metabolic and potential regulatory genes induced by nitrate. Plant Cell 12, 1491–1509.

Wang R, Okamoto M, Xing X, Crawford NM. 2003. Microarray analysis of the nitrate response in Arabidopsis roots and shoots reveals over 1,000 rapidly responding genes and new linkages to glucose, trehalose-6-phosphate, iron, and sulfate metabolism. Plant Physiology 132, 556–567.

Wijffels RH, Barbosa MJ. 2010. An outlook on microalgal biofuels. Science 329, 796–799.

Work VH, Radakovits R, Jinkerson RE, Meuser JE, Elliott LG, Vinyard DJ, Laurens LM, Dismukes GC, Posewitz MC. 2010. Increased lipid accumulation in the Chlamydomonas reinhardtii sta7–10 starchless isoamylase mutant and increased carbohydrate synthesis in complemented strains. Eukaryotic Cell 9, 1251–1261.

Zabawinski C, Van Den Koornhuyse N, D’Hulst C, Schlichting R, Giersch C, Delrue B, Lacroix JM, Preiss J, Ball S. 2001. Starchless mutants of Chlamydomonas reinhardtii lack the small subunit of a heterotetrameric ADP-glucose pyrophosphorylase. Journal of Bacteriology 183, 1069–1077.

Zhang H, Forde BG. 1998. An Arabidopsis MADS box gene that controls nutrient-induced changes in root architecture. Science 279, 407–409.

Zioni AB, Vaadia Y, Lips SH. 1971. Nitrate uptake by roots as regulated by nitrate reduction products of the shoot. Physiologia Plantarum 24, 288–290.