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Nitrate Reductase Modulation in Response to Changes in C/N Balance and Nitrogen Source in Arabidopsis

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Regular Paper

Environmental cues modulate the balance of carbon (C) and nitrogen (N) which are essential elements for plant metabolism and growth. In Arabidopsis, photochemical efficiency of PSII, phosphorylation status and localization of many enzymes, and the level of total soluble sugars were affected by an unbalanced C/N ratio. Since differences in C/N affect these parameters, here we checked whether different sources of N have different effects when a high C/N ratio is imposed. NO_3^- and NH_4^+ were separately provided in C/N medium. We investigated the effects on photochemical efficiency of PSII, the level of total soluble sugars and nitrate reductase activity under stressful C/N conditions compared with control conditions. We found that treated plants accumulated more total soluble sugars when compared with control. Photochemical efficiency of PSII did not show significant differences between the two sources of nitrogen after 24 h. The actual nitrate reductase activity was the result of a combination of activity, activation state and protein level. This activity constantly decreased starting from time zero in control conditions; in contrast, the actual nitrate reductase activity showed a peak at 2 h after treatment with NO_3^-, and at 30 min with NH_4^+. This, according to the level of total soluble sugars, can be explained by the existence of a cross-talk between the sugars in excess and low nitrate in the medium that blocks the activity of nitrate reductase in stressful sugar conditions until the plant is adapted to the stress.

Keywords: Ammonium • ATL family • Enzyme phosphorylation • Nitrate • 14-3-3 proteins.

Abbreviations: NPQ, non-photochemical quenching; NR, nitrate reductase; PPFD, photosynthetic photon flux density; Rubisco, ribulose-1,5-bisphosphate carboxylase-oxygenase; TSS, total soluble sugar.

Introduction

Carbon (C) and nitrogen (N) are essential elements for plant metabolism and growth. Environmental conditions, such as CO_2, light availability, diurnal and seasonal cycles and, more in general, biotic or abiotic stresses, modulate the availability of C and N (Gibon et al. 2004, Klotke et al. 2004, Roitsch and Gonzalez 2004, Miller et al. 2007, Smith and Stitt 2007).

C and N metabolites are tightly co-ordinated, and their ratio, referred as the 'C/N balance' (Coruzzi and Zhou 2001, Martin et al. 2002), is central for the regulation of plant growth and development. Co-ordination between C and N metabolism, and therefore regulation of the C/N ratio, occurs at different levels such as photosynthesis and amino acid synthesis (Zheng 2009).

The C/N balance sensing and signaling mechanisms have been largely unknown for years; however, recently this has started to be clarified. In particular, the E3 ubiquitin ligases ATL31 and ATL6 have been reported to be involved in the C/N response during several development stages, such as post-germinative growth, developmental processes and defense response (Sato et al. 2009, Sato et al. 2011b, Maekawa et al. 2012, Aoyama et al. 2014, Huarancca Reyes et al. 2015, Maekawa et al. 2015). ATL31/6 are members of the ATL family, a large group of plant-specific RING-type E3 ubiquitin ligases with the domain structures conserved in both mono- and dicotyledon plants (Salinas-Mondragon et al. 1999, Takai et al. 2001). E3 ubiquitin ligases are enzymes attaching ubiquitin to target proteins by means of association with E1 and E2 ubiquitin ligase (Streich and Lima 2014). Detailed studies demonstrated that ATL31/6 targets 14-3-3 proteins for ubiquitination and promotes 14-3-3 protein degradation by the 26S proteasome in response to C/N nutrient availability (Sato et al. 2011a, Yasuda et al. 2014).

The stability of 14-3-3 proteins increased as the C/N ratio changed from mild to high C/N stress in Arabidopsis seedlings, and the overexpression of 14-3-3 resulted in a hypersensitive C/N stress response. ATL31-mediated degradation of 14-3-3 is reduced under high C/N stress (Sato et al. 2009, Sato et al. 2011a). When an overaccumulation of 14-3-3 proteins occurs, plant growth was compromised or arrested. 14-3-3 proteins bind to phosphorylated motifs to regulate the activity, stability and localization of proteins involved in multiple developmental processes (Mackintosh 2004). 14-3-3 proteomic analysis using barley and tomato, among other analyses, revealed that several enzymes of carbohydrate metabolism such as sucrose synthase and invertase are targets of 14-3-3 proteins (Alexander and

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Morris 2006, Lu et al. 2016). These enzymes regulate the level of sucrose and hexoses, playing a key role in carbohydrate metabolism and partitioning, developmental processes and hormone responses (Roitsch and Gonzalez 2004, Zheng et al. 2011, Tiessen and Padilla-Chacon 2013).

Both sucrose and glucose can induce changes in gene regulation (Coruzzi and Zhou 2001) that is reflected in C/N sensing and signaling. Together with ATL31/6 targeting the mechanism of 14-3-3 proteins, sugars are finally being recognized as important regulatory molecules with signaling functions in plants. In general, source activities such as photosynthesis, nutrient mobilization and export are up-regulated under low sugar conditions, whereas sink activities such as growth and storage are up-regulated when C sources are abundantly available (Rolland et al. 2006). It is known that sugar-sensing mechanisms enable plants to turn off photosynthesis when the C skeleton is increaed due to the repression of photosynthetic gene transcription and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity (Krapp and Stitt 1995, Cheng et al. 1998, Coruzzi and Zhou 2001). These regulatory mechanisms involve numerous signals that arise from C and N metabolism; hence, the C/N balance plays a key role in determining a feedback control on photosynthesis (Paul and Pellny 2003).

Nitrate is the major source of N for plants and, before its incorporation into organic metabolites, it must be reduced by means of the enzyme nitrate reductase (NR). NR is located mostly in roots and shoots, and appears to be the rate-limiting step in N acquisition (Campbell 1999, Tischner 2000). NR is positively regulated by nitrate, light and carbohydrates; its regulation can occur at the transcription level, which allows long-term regulation (days), and at the post-transduction level, allowing short-term regulation (Yanagisawa 2014). NR mRNA can be rapidly accumulated in response to environmental factors, despite protein synthesis being generally slower. NR mRNA is substrate inducible, therefore nitrate plays a central role as a signal for NR transcription, as well as other environmental stimuli such as light, CO_2, reduced C (particularly sucrose), N metabolites (particularly glutamine) and well-functioning chloroplasts in leaves. At the post-transduction level, a serine residue in hinge 1 is phosphorylated through a Ca^{2+}-dependent kinase followed by Mg^{2+}- or Ca^{2+}-independent binding of a 14-3-3 protein. This mechanism allows a rapid and reversible inhibition of NR activity under unfavorable nitrate assimilation conditions (e.g. limiting light or CO_2) (Yanagisawa 2014).

Among all the regulatory factors of NR, light plays a crucial role. Most of the energy for nitrate assimilation in a cell derives from photosynthesis, and it is also involved in the light regulation of NR activity and gene expression. When plants are exposed at a high and stressful irradiance level, part of the excess energy is diverted as reducing power towards nitrate reduction. In addition, light affects plant circadian rhythm, which has been proposed to influence the cyclic accumulation of NR transcript in anticipation of daylight, and a corresponding decrease as night approaches (Lillo and Ruoff 1989, Deng et al. 1990, Stitt et al. 2002).

Since previous studies demonstrated that stressful C/N conditions affect many aspects of plant metabolism (Sato et al. 2011a, Sato et al 2011b, Maekawa et al. 2012, Aoyama et al. 2014, Yasuda et al. 2014, Huarancca Reyes et al. 2016), here we investigate whether different N sources (NO_3^- and NH_4^+) have different effects on the photochemical efficiency of PSII (Φ_{PSII}), the level of total soluble sugars (TSSs) and NR activity under stressful C/N conditions.

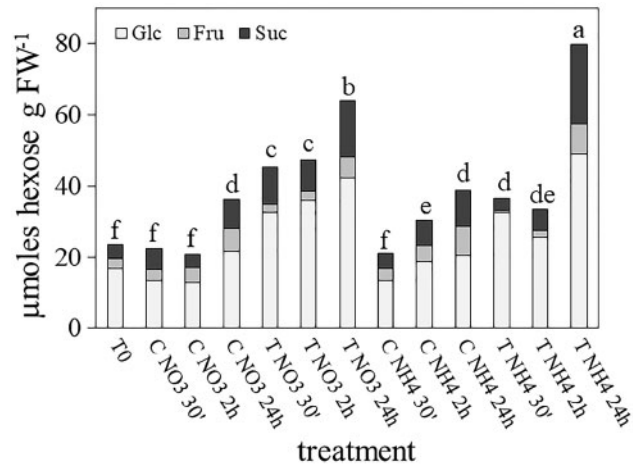

Fig. 1 Effect of glucose and NH_4^+ or NO_3^- on total soluble sugars (TSSs). Control condition (C) = 30 mM N and 100 mM glucose; treated condition (T) = 0.3 mM N and 200 mM glucose. Letters indicate significant differences within a treatment ($P < 0.05$) determined by analysis of variance (ANOVA), and the mean values were compared by using Tukey's test. Glc, glucose; Fru, fructose; Suc, sucrose.

Results

Total soluble sugars

Soluble sugars were extracted from *Arabidopsis thaliana* seedlings harvested 30 min, 2 h and 24 h after transferring them to C/N medium containing different N sources. In general, we found higher sugar concentrations in treated samples if compared with their respective controls. Under control conditions (30 mM N and 100 mM glucose) with NO_3^- as the N source (C NO3), TSSs maintained the same level as at time zero (T0) up to 2 h after treatment, although after 24 h the TSS level significantly increased. Conversely, using NH_4^+ as the N source under control conditions (C NH4), the TSS level started to increase after 2 h of treatment, maintaining this trend up to 24 h of treatment when values reached a similar level to that of the respective C NO3. In treated conditions (0.3 mM N and 200 mM glucose) with NO_3^- (T NO3), the TSS level was significantly higher than at T0 starting from 30 min, increasing up to 24 h of treatment. A similar pattern was observed with NH_4^+ (T NH4), starting with a lower TSS concentration than T NO3 and reaching the highest TSS concentration 24 h after treatment (**Fig. 1**).

Chl fluorescence

The photochemical efficiency of PSII was measured in both control and treatment conditions and in the presence of the two different N sources (NO_3^- or NH_4^+). Chl *a* fluorescence was

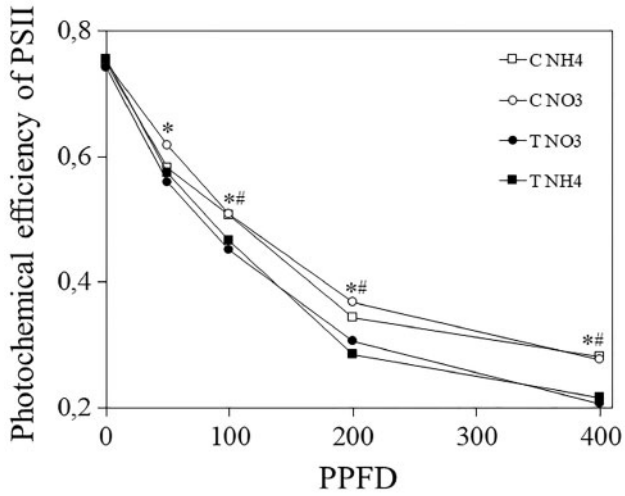

Fig. 2 Effect of glucose and NH_4^+ or NO_3^- on the light response curve of Φ_{PSII} at different light intensities after 24 h of treatment. Control condition (C) = 30 mM N and 100 mM glucose; treated condition (T) = 0.3 mM N and 200 mM glucose. * indicates significant differences within C NO3 and T NO3, and indicates significant differences between C NH4 and T NH4 ($P < 0.05$) determined by analysis of variance (ANOVA), and the mean values were compared by using Tukey's test. PPFD, photosynthetic photon flux density (μmol photons m^{-2} s^{-1}).

measured at 0 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD; potential photochemical efficiency or F_v/F_m) and at increasing light of 50, 100, 200 and 400 μmol m^{-2} s^{-1} PPFD (actual photochemical efficiency of PSII or Φ_{PSII}) after 24 h of C/N treatment in both control and treated conditions (**Fig. 2**). No significant differences were found between the two N sources contained in the C/N medium, either in control or in treated plants. The comparison between control and treated plants showed that both NO_3^- and NH_4^+ in treated C/N medium (T NO3 and T NH4) induced a lower Φ_{PSII} than the control C/N medium (C NO3 and T NH4); however, significant differences were observed starting from 100 μmol m^{-2} s^{-1} PPFD for NH_4^+ and from 50 μmol m^{-2} s^{-1} PPFD for NO_3^-. Non-photochemical quenching (NPQ) increased from 0.22 ± 0.04 at 50 μmol m^{-2} s^{-1} PPFD to 0.93 ± 0.19 at 400 μmol m^{-2} s^{-1} PPFD, without any significant difference among treatments (data not shown).

Nitrate reductase activity

To check how C/N stress conditions affect the ability of Arabidopsis seedlings to assimilate N in the presence of NO_3^- or NH_4^+, the potential NR activity (assayed without a phosphatase inhibitor) was investigated (**Fig. 3**). From time zero (T0) up to 2 h of treatment in different C/N conditions, the activity of NR remained almost unaltered for all conditions, with the exception of C NO3 in which it strongly increased after 2 h. After 24 h, the activity decreased for all C/N conditions, with significant differences between them. In detail, both treated conditions (T NO3 and T NH4) showed the lowest NR activity, while C NH4 had a quite significant higher activity and C NO3 had the highest NR activity of all C/N conditions.

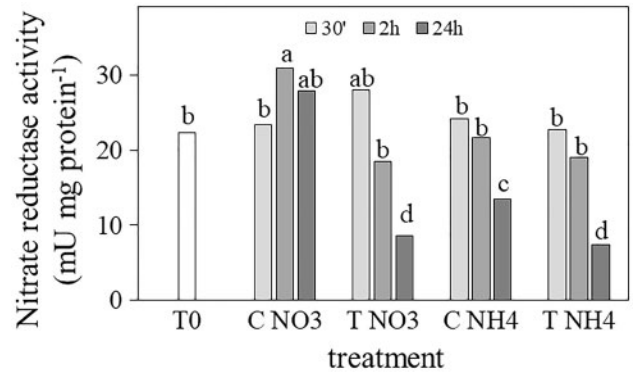

Fig. 3 Effect of glucose and NH_4^+ or NO_3 on potential nitrate reductase activity. Control condition (C) = 30 mM N and 100 mM glucose; treated condition (T) = 0.3 mM N and 200 mM glucose. Since the assay was conducted without phosphatase inhibitors, these values represent the maximum enzymatic activity. Letters indicate significant differences within a treatment ($P < 0.05$) determined by analysis of variance (ANOVA), and the mean values were compared by using Tukey's test.

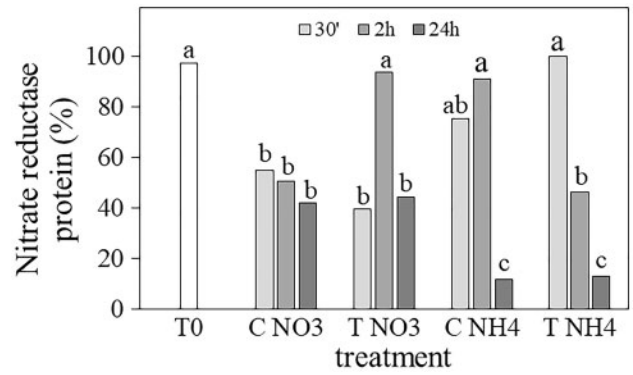

Fig. 4 Effect of glucose and NH_4^+ or NO_3^- on the relative nitrate reductase protein level. Control condition (C) = 30 mM N and 100 mM glucose; treated condition (T) = 0.3 mM N and 200 mM glucose. Results are shown as percentages relative to the highest band intensity (in this case T NH4 after 30 min of treatment) that is assumed to be 100%. Letters indicate significant differences within a treatment ($P < 0.05$) determined by analysis of variance (ANOVA), and the mean values were compared by using Tukey's test.

We also compared the potential NR activity results with the NR protein level (measured by immunoblotting) for each C/N condition, thus obtaining the relative NR protein level (**Fig. 4**; Supplementary Fig. S1). At 30 min of treatment, the relative protein level decreased when NO_3^- (C NO3 and T NO3) was used and remained constant when NH_4^+ (C NH4 and T NH4) was used in comparison with T0. At 2 h, the relative protein level under T NO3 and C NH4 conditions returned to the same level as T0, while it remained low in C NO3 and significantly decreased in T NH4. At 24 h, the relative NR protein level remained unchanged for C NO3, while in all the other conditions it significantly decreased, particularly when NH_4^+ was present in the C/N medium.

The NR activation state (i.e. comparison between the relative activity treated with and without phosphatase inhibitor) was investigated to evaluate its post-transcriptional regulation

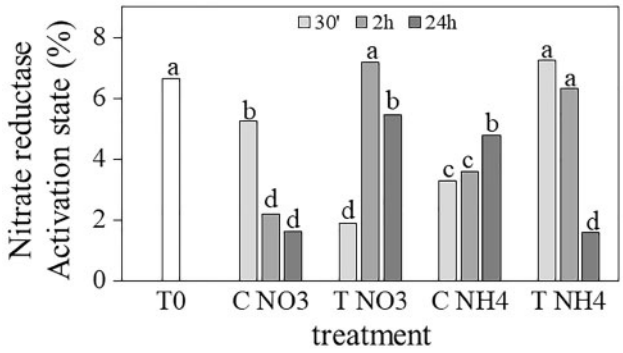

Fig. 5 Effect of glucose and NH_4^+ or NO_3^- on nitrate reductase activation state. Control condition (C) = 30 mM N and 100 mM glucose; treated condition (T) = 0.3 mM N and 200 mM glucose. The activation state is expressed as the percentage enzymatic activity with a phosphatase inhibitor of that without an inhibitor in each sample. Letters indicate significant differences within a treatment ($P < 0.05$) determined by analysis of variance (ANOVA), and the mean values were compared by using Tukey's test.

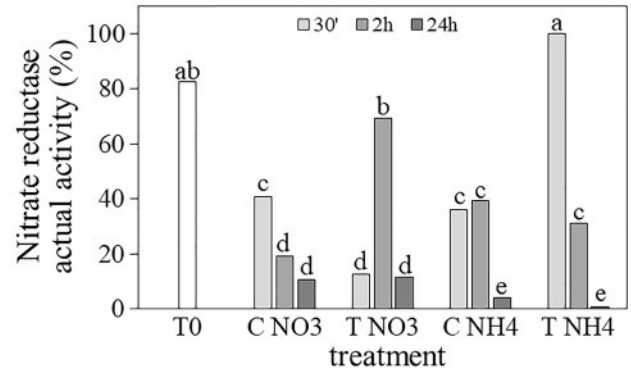

Fig. 6 Effect of glucose and NH_4^+ or NO_3^- on the actual nitrate reductase activity. Control condition (C) = 30 mM N and 100 mM glucose; treated condition (T) = 0.3 mM N and 200 mM glucose. Results are shown as percentages relative to the highest activity (in this case T NH4 after 30 min of treatment) that is assumed to be 100%. Letters indicate significant differences within a treatment ($P < 0.05$) determined by analysis of variance (ANOVA), and the mean values were compared by using Tukey's test.

(**Fig. 5**). Under C NO3 conditions, the activation state after 30 min of treatment significantly decreased in comparison with T0, followed by a sharp decline at 2 h of treatment. Under T NO3, the activation state at 30 min of treatment was strongly reduced, reaching the lowest level, then a further increase was observed at 2 h similar to the T0 state and a slight but significant decrease was seen at 24 h. The NR activity state under C NH4 was lower at 30 min of treatment in comparison with T0; this level was maintained up to 2 h and slightly increased at 24 h, but still was lower than at T0. The opposite pattern was observed under T NH4. The activation state at 30 min and 2 h of treatment was similar to that at T0, followed by a sharp decline.

In order to estimate the actual activity of NR, we combined the potential activity, activation state and the protein level in each experimental condition (**Fig. 6**). These integrated results are reported as a relative percentage of the highest recorded activity (i.e. that found in T NH4 after 30 min). The result of this analysis showed how much nitrate reduction is occurring in each C/N condition, giving an insight into the interaction between NR translation and its real activity. Generally, we observed that actual NR activity constantly decreased during the time course in comparison with T0. In C NO3, this trend was maintained until 24 h, while in T NO3 the activity strongly decreased at 30 min after treatment, but at 2 h returned to a level similar to that at T0, and then decreased again at 24 h. In C NH4, the trend was similar to C NO3, even if no differences were recorded between 30 min and 2 h of treatment. Finally, in T NH4 at 30 min of treatment, the activity was maintained at the same level as T0, followed by a sharp decrease until 24 h.

Discussion

Nitrogen triggers several metabolic pathways depending either on the availability of C and N and their relative ratio in the medium, or on the form of the source in the substrate (Zheng 2009). In our experiments, the presence of NO_3^- or NH_4^+ in control conditions (30 mM N and 100 mM glucose) did not show any significant effects on accumulation of soluble sugars. Conversely, in treated conditions (0.3 mM N and 200 mM glucose), both NO_3^- and NH_4^+ showed an accumulation of sugars. While NO_3^- treatment rapidly induced sugar accumulation at earlier stages, NH_4^+ treatment stimulated significantly higher sugar accumulation at 24 h only, possibly due to higher energy needed for NO_3^- reduction (Stitt et al. 2002). Interestingly, sucrose accumulation was only observed in treated plants, probably due to the necessity of the cells to contrast an over accumulation of glucose (**Fig. 1**).

The photochemical efficiency of PSII was lower when plants were transferred to high C/N conditions for 24 h, independent of the N source and especially with an increase in light intensity. This indicates that under a high C/N ratio, plants are less tolerant to high irradiance levels. An excess of sugars also negatively affects the photosynthetic rate (Krapp and Stitt 1995, Cheng et al. 1998, Coruzzi and Zhou 2001, Paul and Pellny 2003). This regulatory mechanism enables plants to turn off photosynthesis when C skeleton availability in the growth medium is elevated, confirming that the C/N balance plays a key role in determining feedback control on photosynthesis. Photosynthesis is affected by nitrate availability (Paul and Pellny 2003), and NR represents an important channel for dissipation of excess energy from PSII, by means of transferring reducing power to NR to reduce NO_3^- (Gniazdowska-Skoczek 1998, Lillo and Appenroth 2001). Consequently, when N is low in the growth medium, NR is substrate limited, and the electron transport rate is negatively affected because the excess energy cannot be dissipated through this alternative pathway. These facts agree with the reduced Φ_{PSII} in low N treatments. On the other hand, our data also showed that the photochemical efficiency in control

C/N conditions is affected by the N source only at the lowest PPFD value of the light response curves (50 µmol photons $m^{-2} s^{-1}$), with C NO3 showing significantly higher Φ_{PSII} than C NH4 ($P < 0.05$). Moreover, at 50 µmol photons $m^{-2} s^{-1}$ PPFD, Φ_{PSII} was significantly higher in C NO3 than in T NO3, probably due to the efficient energy dissipation driven by NR activity, while C NH4 and T NH4 did not show any significant difference. Conversely, when PPFD was increased, the excess energy at PSII caused a similar reduction of Φ_{PSII} in both T NO3 and T NH4 with respect to their relative controls, suggesting a predominant role for sugars in this feedback regulation mechanism.

The activity of NR is finely regulated at both the phosphorylation and transcriptional level in higher plants (Yanagisawa 2014). The actual activity of NR is determined by an interaction among potential activity, activation state and protein level. In this study, seedlings at T0 showed a relatively high actual NR activity probably due to the complete consumption of the N source in the growth C/N medium (100 mM glucose and 30 mM NH_4NO_3) where seeds were sown. In control conditions, plants could sense both N sources, modulating the activity of NR as N was assimilated. During the time course, NO_3^- acted as a signal leading to a progressive reduction of NR activity, while NH_4^+ showed a slower but similar effect possibly due to the missing nitrate signal (Stitt et al. 2002). In T NO3 at 30 min, NR activity collapsed when compared with T0, probably associated with the high excess of sugars that seemed to inhibit nitrate assimilation (Stitt et al. 2002). When plants were adapted to this stress condition, NR activity peaked at 2 h probably due to the low availability of nitrate in the treated medium, and then it strongly decreased. On the other hand, when NH_4^+ was used in the treated medium, NR activity remained stable at 30 min and then it decreased. These different trends could be explained assuming that nitrate availability and sugar accumulation trigger a cross-talk that affects other cellular activities, allowing plants to dispose of excess sugar (Stitt et al. 2002). Therefore, when nitrate is missing, sugar excess could not be promptly sensed, resulting in higher accumulation and increasing the inhibitory effect of sugars.

In conclusion, our results highlight that the cross-talk between C and N sensing finely regulates NR at both the transcriptional and translational levels by an interaction between the C/N ratio and nitrogen sources. In particular, our data suggest a time-dependent regulation mechanism of NR activity, due to a combination of sugar accumulation and nitrate availability: (i) nitrate availability seems to play a key role in short-term NR modulation (2 h), while (ii) sugar accumulation (especially sucrose and glucose, as known in other plant systems) could play the major role in long-term NR modulation (24 h). Both factors can affect the actual activity of NR by the control of protein turnover and the phosphorylation state probably mediated by 14-3-3 proteins. Further research should be focused on understanding the origin of this cross-talk in order to better clarify the complex protein network in response to C/N availability.

Materials and Methods

Plant material and growth conditions

Wild-type *A. thaliana* Columbia-0 was used in this study. Sterilized seeds were sown on modified Murashige and Skoog (MS) medium containing 100 mM glucose and 30 mM N (10 mM NH_4NO_3 and 10 mM KNO_3) and grown for 10 d after germination with a 16/8 h light/dark (long-day) photoperiod under constant temperature (23°C). Plants were then transferred to C/N media with different concentrations of glucose and N: 100 mM glucose and 30 mM N; 200 mM glucose and 0.3 mM N, where the N source was nitrate as KNO_3 or ammonium as NH_4Cl for each medium. Each condition was produced in triplicate. Plants were harvested 30 min, 2 h and 24 h after transfer to the various C/N conditions.

Soluble carbohydrate quantification

Soluble carbohydrates (sucrose, glucose and fructose) were extracted and assayed as reported in Pompeiano et al. (2017). The accuracy of the method was tested using standards with known amounts of carbohydrates. Recovery experiments were carried out to evaluate losses during extraction. The concentrations of standards added were similar to those estimated to be present in the tissues in preliminary experiments. The recovery ranged between 97% and 104%. The quantity of soluble carbohydrates was expressed as µmol hexose equivalents g FW^{-1}.

Chl fluorescence

Chl fluorescence measurements were conducted using a miniaturized pulse amplitude-modulated fluorometer (Mini-PAM) (Heinz Walz GmbH) on mono-layer leaf spots, as previously described (Huarancca Reyes et al. 2016). The PPFD of the saturation pulse to determine the maximal fluorescence emission in the presence (F_m') and in the absence (F_m) of actinic light was about 8,000 µmol photons $m^{-2} s^{-1}$. Fluorescence parameters were determined at growth light intensity (100 µmol photons $m^{-2} s^{-1}$) and at increasing PPFDs (from 50 to 400 µmol photons $m^{-2} s^{-1}$) at the indicated times after C/N treatment depending on the experiment. The potential efficiency of PSII photochemistry was calculated on dark-adapted leaves as F_v/F_m. The actual photochemical PSII efficiency (Φ_{PSII}) and NPQ in the light were determined for each PPFD value as $\Phi_{PSII} = (F_m' - F_0)/F_m'$ (Genty et al. 1989) and $NPQ = F_m/F_m' - 1$, respectively, when steady state was achieved. F_m' represents the maximum fluorescence yield with all PSII reaction centers in the reduced state obtained from superimposing a saturating light flash during exposure to actinic light, while F_0 is the minimum yield of fluorescence in dark-adapted samples.

Nitrate reductase activity

Samples (0.2-0.5 g FW) were extracted in 5 vols. of 50 mM MOPS-NaOH, pH 7.5, 1 mM EDTA, 15 mM $MgCl_2$, 2.5 mM dithiothreitol (DTT) and 0.1% Triton X-100, in the presence or absence (potential activity) of one tablet of phosphatase inhibitor (PhosSTOP by Roche®) solubilized in 10 mL of extraction solution. Extracts were centrifuged at 20,000×*g* for 15 min at 4°C, and the resulting

supernatants were used for the enzymatic assays after desalting. The desalting column was equilibrated with the extraction buffer without Triton X-100. Bio-Rad Protein Assay, based on the method of Bradford, was used to quantify the protein concentration in the resulting samples (Bradford 1976), and bovine serum albumin were used as standard.

Assays of NR activity were performed as previously described (Redinbaugh and Campbell 1983, Smarrelli and Campbell 1983), in the presence or absence of one tablet of phosphatase inhibitor (PhosSTOP by Roche®) each 10 ml, except for the reactions that were stopped by the addition of sulfanilamide/HCl solution instead of zinc acetate (Redinbaugh and Campbell 1985).

Nitrate reductase protein level

Plant material was gr#ound to a fine powder in liquid nitrogen and weighed. Extraction was conducted without a phosphatase inhibitor. Bio-Rad Protein Assay, based on the method of Bradford, was used to quantify the protein concentration in the resulting samples (Bradford 1976). A 10 μg aliquot of total protein for each sample was subjected to SDS-PAGE. Western blot was carried out with polyclonal anti-NR antibody (AS08310, Agrisera) at a 1:2,000 dilution as the primary antibody followed by polyclonal goat anti-rabbit IgG (AS09602, Agrisera) at a 1:25,000 dilution as secondary antibody. Illuminance was determined with chemiluminescent horseradish peroxidase substrate.

Statistical analysis

Values presented are means of three replicates. Data were subjected to analysis of variance (ANOVA), and the mean values were compared by using Tukey's test. Significant differences for all statistical tests were evaluated at the level of $P < 0.05$. For all parameters, the assumption of normality distribution was confirmed by Shapiro-Wilks test.

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Supplementary Data

Supplementary data are available at PCP online.

Disclosures

The authors have no conflicts of interest to declare.

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