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Abstract: Carbon (C) and nitrogen (N) nutrient sources are essential elements for metabolism, and their availability must be tightly coordinated for the optimal growth and development in plants. Plants are able to sense and respond to different C/N conditions via specific partitioning of C and N sources and the regulation of a complex cellular metabolic activity. We studied how the interaction between C and N signaling could affect carbohydrate metabolism, soluble sugar levels, photochemical efficiency of photosystem II (PSII) and the ability to drive the excess energy in Arabidopsis seedlings under moderated and disrupted C/N-nutrient conditions. Invertase and sucrose synthase activity and localization were markedly affected by C/N-nutrient status depending on the phosphorylation status, suggesting that these enzymes may necessarily be modulated by their direct phosphorylation or phosphorylation of proteins that form complex with them in response to C/N stress. In addition, the enzymatic activity of these enzymes was also correlated with the amount of sugars, which not only act as substrate but also as signaling compounds. Analysis of chlorophyll fluorescence in plants under disrupted C/N condition suggested a reduction of electron transport rate at PSII level associated with a higher capacity for non-radiative energy dissipation in comparison with plants under mild C/N condition. In conclusion, the tight coordination between C and N not only affects the carbohydrate metabolism and their concentration within plant tissues, but also the partitioning of the excitation energy at PSII level between radiative (electron transport) and non-radiative (heat) dissipation pathways.

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Via Mariscoglio, 34 – 56124 Pisa, Italy

Pisa, March 4th, 2016

Prof. Mario De Tullio
Editor
Plant Physiology and Biochemistry

Dear Prof. De Tullio, enclosed please find a manuscript submitted for possible publication in Plant Physiology and Biochemistry.

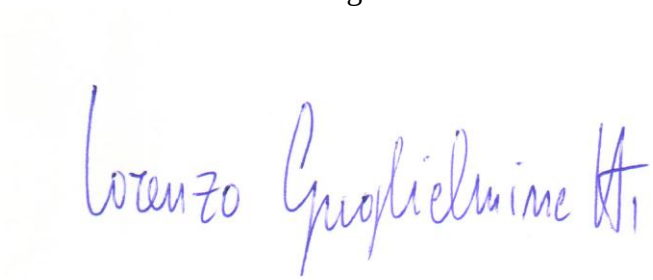
The manuscript is entitled as follows: "Effect of Carbon/Nitrogen ratio on carbohydrate metabolism and light energy dissipation mechanisms in *Arabidopsis thaliana*" by Thais Huarancca Reyes, Andrea Scartazza, Yu Lu, Junji Yamaguchi and Lorenzo Guglielminetti.

The present manuscript reports unpublished work, it has not been published and currently is not under consideration for publication elsewhere. Moreover, the authors have carefully read and are fully aware of the Plant Physiology and Biochemistry policy.

Hoping the manuscript will meet the criteria for its publication in Plant Physiology and Biochemistry,

Yours sincerely,

Lorenzo Guglielminetti



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Effect of Carbon/Nitrogen ratio on carbohydrate metabolism and light energy dissipation mechanisms in *Arabidopsis thaliana*

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Abstract

Carbon (C) and nitrogen (N) nutrient sources are essential elements for metabolism, and their availability must be tightly coordinated for the optimal growth and development in plants. Plants are able to sense and respond to different C/N conditions via specific partitioning of C and N sources and the regulation of a complex cellular metabolic activity. We studied how the interaction between C and N signaling could affect carbohydrate metabolism, soluble sugar levels, photochemical efficiency of photosystem II (PSII) and the ability to drive the excess energy in Arabidopsis seedlings under moderated and disrupted C/N-nutrient conditions. Invertase and sucrose synthase activity and localization were markedly affected by C/N-nutrient status depending on the phosphorylation status, suggesting that these enzymes may necessarily be modulated by their direct phosphorylation or phosphorylation of proteins that form complex with them in response to C/N stress. In addition, the enzymatic activity of these enzymes was also correlated with the amount of sugars, which not only act as substrate but also as signaling compounds. Analysis of chlorophyll fluorescence in plants under disrupted C/N condition suggested a reduction of electron transport rate at PSII level associated with a higher capacity for non-radiative energy dissipation in comparison with plants under mild C/N condition. In conclusion, the tight coordination between C and N not only affects the carbohydrate metabolism and their concentration within plant tissues, but also the partitioning of the excitation energy at PSII level between radiative (electron transport) and non-radiative (heat) dissipation pathways.

Keywords C/N-nutrient, phosphorylation state, chlorophyll fluorescence, invertase, soluble sugars, sucrose synthase

1. Introduction

Plants are sessile living organisms that have developed sophisticated mechanisms to perceive external signals and optimally respond to environmental conditions. Among many nutrients, carbon (C) and nitrogen (N) are essential elements for plant metabolism and their availabilities in natural condition are modulated by environmental stimuli such as atmospheric CO_2, light availability, diurnal cycles, seasonal effects, rainfall, cold and biotic stresses (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 coordinated and its ratio, named as C/N balance, is also important for the regulation of plant growth and development (Coruzzi and Zhou, 2001; Martin et al., 2002). Moreover, plants are able to adapt to different C/N conditions via specific partitioning of C and N sources and fine-tuning of complex cellular metabolic activity (Sato et al., 2011b; Sulpice et al., 2013). Disrupted C/N-nutrient response is also observed in phenotypically analysis. Arabidopsis seedlings that were grown under high C and limited N condition showed purple pigmentation in cotyledons and arrested post-germinative growth (Martin et al., 2002; Sato et al., 2009). The Arabidopsis Tóxicos en Levadura 31 (ATL31) is a member of the plant-specific E3 ubiquitin ligase ATL family (Aguilar-Hernandez et al., 2011). The E3 ubiquitin ligase ATL31 has been reported to be involved in the C/N response during post-germinative growth, developmental processes and defense response (Sato et al., 2009; Maekawa et al., 2012; Aoyama et al., 2014; Maekawa et al., 2014; Huarancca Reyes et al., 2015; Maekawa et al., 2015). Detailed studies demonstrated that ATL31 targets 14-3-3 proteins for ubiquitination in response to C/N-nutrient availability (Sato et al., 2011a; Yasuda et al., 2014). 14-3-3 proteins bind to phosphorylated motifs to regulate the activity of proteins involved in multiple developmental processes (Mackintosh, 2004; Chevalier et al., 2009). A 14-3-3 proteomic analysis using barley revealed that several enzymes of carbohydrate metabolism such as sucrose synthase and invertases are targets of 14-3-3

proteins (Alexander and Morris, 2006). Sucrose synthase reversibly catalyzes the synthesis and cleavage of sucrose, which is the main form of assimilated C, regulating sucrose flux and cellular location depending on the metabolic environment to participate in cellulose, callose, and starch biosynthesis through its interactions with membranes, organelles and cytoskeletal actin (Zheng et al., 2011; Tiessen and Padilla-Chacon, 2013). Another important enzyme that regulates the level of hexoses is invertase, which catalyzes sucrose hydrolysis in different subcellular compartments and thus regulates carbohydrate partitioning, developmental processes, hormone responses and biotic and abiotic interactions (Roitsch and Gonzales, 2004; Tiessen and Padilla-Chacon, 2013). In addition, other factors are modulating by the C/N-nutrient availability in plants as in other adaptive responses where more than one trigger is involved. For instance, C/N balance also plays a key role on determining a feedback control of photosynthesis (Paul and Pellny, 2003). Whilst it is commonly known that sugar-sensing mechanisms enable plants to turn off photosynthesis when C-skeleton is elevated due to the repression of photosynthetic gene transcription and Rubisco activity (Krapp and Stitt, 1995; Cheng et al., 1998; Coruzzi and Zhou, 2001), previous research showed that high sugar concentrations or elevated CO_2 do not show loss of photosynthetic gene expression or photosynthetic capacity when N availability is increased (Martin et al., 2002). Nonetheless, the molecular mechanisms responsible for the regulation of C/N sensing and signaling still remain unclear.

In this study, we carried out C/N response analyses using *Arabidopsis thaliana* seedlings under different growth medium containing moderated C/N (100 mM glucose and 30 mM N) and disrupted C/N (200 mM glucose and 0.3 mM N) ratios with the aim to clarify the interaction between C and N signaling on biochemical and physiological traits. For this purpose, the effect of different C/N ratios was monitored on the activity of several enzymes involved in carbohydrate metabolism including cell wall invertase, membrane bound invertase, soluble invertase, membrane bound sucrose synthase and soluble sucrose

synthase. Enzyme activities were assayed under conditions that the phosphorylation state of the proteins is maintained or not. In addition, we monitored the effect of C/N treatments on soluble sugars levels, efficiency of photosystem II (PSII) photochemistry and the ability to drive the excess energy through non-radiative dissipation mechanisms.

2. Material and Methods

2.1. Plant material and growth conditions

Wild-type *Arabidopsis thaliana* Columbia-0 was used in this study. Sterilized seeds were sown on modified MS medium containing 100 mM glucose and 30 mM N (10 mM NH_4NO_3 and 10 mM KNO_3) for 10 days after germination and transferred to C/N medium containing 100 mM glucose/30 mM N or 200 mM glucose/0.3 mM N. Plants were harvested at the indicated times after C/N treatment depending on the experiment.

2.2. Chemicals

All the reagents used were purchased from Sigma (St. Louis, MO, USA). The kit for protein quantification was purchased from BIO-RAD (Richmond, CA, USA) with BSA as a standard. The phosphatase inhibitor cocktail (PhosSTOP) was purchased from Roche (Basilea, Switzerland).

2.3. Enzymes extraction and assays

All the extractions and all the assays were conducted in the presence or in the absence of phosphatase inhibitor cocktail at the concentration suggested by the customer. Soluble sucrose synthase and soluble invertase were extracted and assayed, after desalting with micro Bio-Spin chromatography column (Bio-Rad) as reported previously (Guglielminetti et al., 1995). Membrane bound sucrose synthase and membrane bound invertase resulted from the pellet of soluble isoforms extraction were extracted in the same extraction buffer

with the addition of 0.1% Triton X-100 followed by 1 h incubation at 4ºC and final centrifugation at 20 000 g for 30 min in the case of invertase or 2 h at 100 000 g ultracentrifugation in the case of sucrose synthase. Membrane bound enzymes activity was measured in the respective supernatants. Cell wall invertase was extracted and assayed as described by Hirose et al. (2002).

2.4. Soluble carbohydrate quantification

Soluble carbohydrate was extracted from frozen homogenized vegetal material (100 mg FW) as described by Tobias et al. (1992). Samples were assayed with coupled enzymatic assay methods (Aoyama et al., 2014) measuring the increase in A_{340}. The accuracy of the method was tested using standards with known amounts of carbohydrates. Recovery experiments were carried out to evaluate losses during extraction. Two tests were performed for each metabolite by adding known amount of authentic standards to the samples before proceeding with the extraction. The concentrations of standards added were similar to those estimated to be present in the tissues in preliminary experiments. The percentage of recovery ranged between 93% and 106% depending on the sugar. The quantity of soluble carbohydrates was corrected on the basis of the recovery percentages for each sample, and expressed as µmoles hexose equivalents g^{-1} FW.

2.5. Chlorophyll Fluorescence

Chlorophyll fluorescence measurements were conducted using a miniaturized pulse-amplitude-modulated fluorometer (Mini-PAM; Heinz Walz GmbH, Effeltrich, Germany) on mono-layers leaf spot. The Photosynthetic Photon Flux Density (PPFD) of the saturation pulses to determine the maximal fluorescence emission in the presence (Fm') and in the absence (Fm) of actinic light was about 8000 µmol m^{-2} s^{-1}. Fluorescence parameters were determined at growing light intensity (100 µmol m^{-2} s^{-1}) and at increasing PPFD (from 50 to

150 400 μmol m^{-2} s^{-1}) at the indicated times after C/N treatment depending on the experiment.
151 The potential efficiency of PSII photochemistry was calculated on dark-adapted leaves as
152 Fv/Fm. The photon yield of PSII photochemistry (Φ_{PSII}) in the light was determined for each
153 PPFD value as Φ_{PSII} = (Fm'-F')/Fm' (Genty et al., 1989) when steady state was achieved.
154 Fm' represents the maximum fluorescence yield with all PSII reaction centres in the
155 reduced state obtained from superimposing a saturating light flash during exposition to
156 actinic light, while F' is the fluorescence at the actual state of PSII reaction centres during
157 actinic illumination. The fast-relaxing component of non-photochemical fluorescence
158 quenching (NPQ) was estimated according to Stern-Volmer equation as NPQ = Fm/Fm'-1
159 (Bilger and Björkman, 1990). Fluorescence nomenclature is according to van Kooten and
160 Snel (1990).
161
162 **3. Results**
163 *3.1. Enzymes activity*
164 Table 1 reports the activity of several enzymes after 0.5 h treatment with 100 mM
165 glucose/30 mM N (U) or 200 mM glucose/0.3 mM N (T). Activities were measured in the
166 presence (+) or not (-) of phosphatase inhibitor. The activities expressed as mU mg
167 $protein^{-1}$ is reported in the upper panel of the table, while the p-values to compare the
168 effect of C/N treatment and/or phosphatase inhibitor on the enzymes activities is reported
169 in the bottom panel. Cell wall invertase (CWI) was significantly, although weakly, affected
170 by C/N treatment. In fact, CWI activity increased more than 20% in T condition compared
171 with U condition; even that this effect is not correlated with phosphorylation state, as
172 demonstrated by U+ *vs* T+ p-value (Table 1). On the contrary, when sucrose synthase
173 (SS) activity was analyzed, the effect of phosphorylation state in relation to C/N treatment
174 was significant. The activity of soluble SS decreased significantly after C/N treatment in
175 presence of phosphatase inhibitor, while the activity of membrane bound SS increased

(U+ *vs* T+ p-value, Table 1). However, these patterns were not observed in the absence of phosphatase inhibitor (U *vs* T p-value, Table 1). A different situation was observed when soluble or membrane bound invertase activities were analyzed. Both invertases isoforms resulted positively affected by C/N treatment with or without phosphatase inhibitor showing significant differences (Table 1). In addition, activities of membrane bound SS and both invertase isoforms showed a significant increase by the effect of only phosphatase inhibitor, as demonstrated by the p-values of U *vs* U+ and T *vs* T+ (Table 1).

To better understand the effect of C/N stress on the enzymes activities obtained after 0.5 h treatment, we performed a time course experiment evaluating the activities of enzymes affected by phosphorylation state (Fig. 1 and 2). The invertase activities under C/N treatment with or without phosphatase inhibitor are showed in Fig. 1. Activity of soluble invertase without phosphatase under U condition did not show any change during different experimental time points, while under T condition the activity increased from 0.5 h to 2 h treatment and then it is maintained at the same level 24 h after treatment (Fig. 1, upper panel). The activity of membrane bound invertase without phosphatase under U condition increased continuously during the experimental time points, while under T condition the activity pattern increased similar to that of soluble invertase (Fig. 1, upper panel). In the presence of phosphatase inhibitor, invertase activities increased following similar pattern compared to the values obtained in samples without inhibitor with the exception of soluble invertase under U condition (Fig. 1, lower panel). In fact, when phosphorylation state is maintained, soluble invertase activity strongly increased under U condition during all experimental time points (Fig. 1, lower panel). The SS activities under C/N treatment with or without phosphatase inhibitor are showed in Fig. 2. In the absence of phosphatase inhibitor, soluble isoform activity under U or T conditions showed a significant increase from 0.5 h to 2 h and it is maintained stable until 24 h treatment (Fig. 2, upper panel). At the same conditions, different pattern was observed when membrane bound isoform was

analyzed. The activity of membrane bound SS under U condition transiently increased from 0.5 h to 2 h and then showed a decrease 24 h after treatment. On the contrary, the increment of activity under T condition was delayed resulting highest at 24 h after treatment (Fig. 2, upper panel). When phosphatase inhibitor was used, soluble SS activity under U condition was maintained during all experimental time points; while under T condition the increasing of the activity was delayed and raised to a high value at 24 h after treatment (Fig. 2, lower panel). As well as concern membrane bound SS isoform, its activity showed an increasing from 0.5 h to 2 h after treatment and then was maintained constant until 24 h after treatment under U condition. On the other hand, the enzyme activity under T condition was strongly decreased from 0.5 h to 2 h after treatment and just slightly increased at 24 h after treatment (Fig. 2, lower panel).

3.2. Chlorophyll fluorescence

Chlorophyll fluorescence represents a well-known non-invasive measurement of the quantum efficiency of PSII and heat dissipation of the light excess energy (Murchie and Lawson, 2013), and thus it represents a powerful tool to understand the effects of different C/N treatments on photosynthetic responses and energy dissipation mechanisms. Fig. 3 shows the photochemical efficiency of PSII after 0.5, 2 and 24 h C/N treatment. Dark-adapted seedlings under U and T conditions did not show any significant difference in the maximal photochemical efficiency of PSII (Fv/Fm). Conversely, the actual photon yield of PSII photochemistry in the light (Φ_{PSII}) was significantly affected by the C/N treatment (Fig. 3). Interestingly, although Φ_{PSII} decreased with increasing of the photosynthetic photon flux density (PPFD) under both C/N treatments, T condition showed lower Φ_{PSII} at each PPFD value than U condition after 2 and 24 h C/N treatment (Fig. 3). Fig. 4 shows the relation of Φ_{PSII} at different C/N treatment period respect to the time zero of treatment (T0) expressed as percentage. Seedlings under U and T conditions at 0.5 h treatment showed reduced

Φ_{PSII} compared with T0 with an abrupt decrease in plants under T condition in comparison to that of C condition (Fig. 4). From 0.5 h to 2 h of C/N treatment, the percentage of Φ_{PSII} in plants under T condition did not show any significant change whereas it was sharply increased in plants under U condition (Fig. 4). After 24 h C/N treatment, the percentage of Φ_{PSII} increased in plants under both U and T conditions, although U plants maintained a much higher photochemical efficiency than plants under T conditions (Fig. 4). Non-photochemical quenching (NPQ) in plants under U and T C/N treatments for 24 h was also analyzed (Fig. 5). NPQ was increased with the increasing of PPFD in plants under both C/N treatments and showed a significant difference only at PPFD of 400 μmol m^{-2} s^{-1} (Fig. 5).

3.3. Soluble carbohydrates

Fig. 6 reports the levels of glucose, fructose, sucrose and total soluble sugars expressed as hexose equivalents in seedlings under U and T C/N treatments at different time points. Levels of glucose in plants under T condition strongly increased after 0.5 h treatment rising to the highest value after 2 h treatment and then slowly decreased 24 h after C/N treatment. On the other hand, glucose level in plants under U condition remained almost stable for about 2 h after treatment and then slowly decreased at the end of the C/N treatment (Fig. 6). Levels of fructose and sucrose in plants under T condition remained stable and decreased 0.5 h after C/N treatment, respectively. Both values were restored and highly increased 2 h after treatment and then slowly decreased 24 h after C/N treatment (Fig. 6). Levels of sucrose and fructose levels in seedlings under U condition decreased 0.5 h after treatment, then slightly restored after 2 h and continuously decreased after 24 h C/N treatment (Fig. 6). Additionally, total soluble sugar content in plants under T condition showed an increasing pattern from the beginning of the experiment rising to the maximum level 2 h after C/N treatment, and then hexoses content

254 significantly decreased by about 25% after 24 h treatment (Fig. 6). In the case of plants
255 under U condition, total sugar content was decreased after 0.5 h treatment followed by a
256 slight increase 2 h after C/N treatment rising near to the value obtained at time zero, and
257 then a slow decrease was observed at the end of the experiment (Fig. 6).
258
259 **4. Discussion**
260 The change of the C/N balance can modulate the phosphorylation status of many
261 enzymes which may modify their activity, subcellular localization, stability or even signal
262 transduction. Our analysis showed that the activity and localization of invertase and SS
263 were markedly affected by C/N-nutrient status after 0.5 h treatment depending on the
264 phosphorylation status (Table 1), indicating that these enzymes may necessarily be
265 activated by their direct phosphorylation or phosphorylation of proteins that form complex
266 with them. A recent study unrevealed that SS structure comprised different
267 phosphorylation sites and its function is regulated by its post-translational modification
268 (Zheng et al., 2011). When C/N balance was disrupted and the phosphorylation status was
269 maintained, SS activity was markedly different according to its localization. Thus, soluble
270 SS activity decreased under C/N stress while the activity of its membrane bound isoform
271 increased, suggesting that C/N related phosphorylation status not only could modulate the
272 activity of this enzyme but also its subcellular localization. Interestingly, previous reports
273 showed that the post-translational modification of SS through its phosphorylation is
274 involved in its cellular distribution between cytosol and membranes in response to abiotic
275 stress (Winter et al., 1997; Subbaiah and Sachs, 2001). SS protein stability could be also
276 controlled by its phosphorylation and rapid degradation by the ubiquitin proteasome
277 system (Hardin and Huber, 2004; Alexander and Morris, 2006). Together with the
278 knowledge that ATL31 targets 14-3-3 proteins for ubiquitination in response to C/N-nutrient
279 availability (Sato et al., 2009; Yasuda et al., 2014), we also speculate that under high

C/low N stress condition 14-3-3 proteins are accumulated recruiting phosphorylated SS in the cytosol for its turnover via the ubiquitin proteasome system. 14-3-3 isoforms have a complex interaction network that is still unclear, thus they can be found in different/same subcellular localizations interacting with several proteins and having particular cellular functions (Paul et al., 2012). Plants possess different invertases (Roitsch and González, 2004) and this study showed that soluble and membrane bound invertases are highly activated after 0.5 h high C/low N condition maintaining the phosphorylation status. We postulate that invertase activity regulation in C/N stress condition is modulated in a different way than SS with a positive activation by phosphorylation in combination with 14-3-3 interaction, as previously reported (Alexander and Morris, 2006; Gao et al., 2014). However, a more detailed study is needed to elucidate the physiological specificity of 14-3-3 isoforms with carbohydrate metabolism enzymes in response to C/N nutrient availability. When C/N-stress treatment period is prolonged for 2 or 24 h, plants strategically regulate carbohydrate metabolism enzymes and the photochemical activity according to the availability of C and N nutrients in a tight coordination. At first, the activity of soluble and membrane bound invertase under long term mild C/N condition was higher in comparison with that observed after 0.5 h treatment when phosphorylation status was maintained (Fig. 1). Accordingly with this result, sucrose amount at long term mild C/N condition was higher than 0.5 h treatment (Fig. 6), indicating that invertase activity is activated by the regulation of the carbohydrate level in the plant. On the other hand, a different pattern was observed in the activity of SS isoforms according to their localization, where soluble SS activity was maintained constant under mild C/N condition during the time of the experiment while membrane bound SS activity increased (Fig. 2). These results indicate that phosphorylation status may induce SS to localize to specific membrane compartment for the correct delivery and balance of C source (Subbaiah and Sachs, 2001).

Second, when the mild C/N nutrient balance was disrupted for long term and the phosphorylation status was preserved, it was observed that the activities of soluble and membrane bound invertases dramatically increased in comparison with plants under C/N stress for 0.5 h (Fig. 1). The activation of invertase may be positively modulated by phosphorylation in combination with 14-3-3 interaction as previously reported (Alexander and Morris, 2006; Gao et al., 2014), and this effect correlates with the increasing amount of sugars which not only act as substrate but also as signaling compounds (Fig. 6). Results of SS activity at the same long term C/N stress condition maintaining the phosphorylation status showed that soluble SS increased its activity at long term while the membrane bound SS keeps low activity in comparison with plants under mild C/N condition (Fig. 2). This pattern of SS activity suggests that phosphorylation status after C/N stress could modulate not only SS activity according to its localization but also with the interaction of other proteins such as 14-3-3s, as described above. All these findings open new insights about the modulation of carbohydrate metabolism in response to C/N nutrient availability implicating the phosphorylation status.

Third, our results showed that C/N treatments did not affect the potential efficiency of PSII photochemistry since there was not significance difference in the values of Fv/Fm on dark-adapted seedlings under mild or disrupted C/N-nutrient condition at different time points (Fig. 3), suggesting that photoinhibition did not occur and that PSII reaction centers were not damaged by the different C/N conditions tested in this work. Conversely, the proportion of light absorbed by chlorophylls that is used in photochemistry in light-adapted seedlings results negatively affected by C/N stress and different light conditions, as indicated by the light response curves of Φ_{PSII} (Fig. 3) and the variation of Φ_{PSII} during the treatment period (Fig. 4). This fluorescence parameter gives an estimate of the rate of linear electron transport and so an indication of the overall photosynthesis (Genty et al., 1989), but alternative sinks for electrons other than carbon fixation, such as photorespiration,

reduction of molecular oxygen and nitrate assimilation can also affect Φ_{PSII}. In general, the Φ_{PSII} values observed in this study indicate that the electron transport rate of plants under high C/low N condition was lower than that subjected to mild C/N balance, suggesting that the excess of glucose and the limiting N trigger this feedback control on photosynthesis. In accordance with our results, previous reports showed that key components of the photosynthetic pathway such as Rubisco are transcriptionally down regulated in plants grown under high C/low N condition altering C and N metabolisms in the plant (Sheen, 1990; Krapp and Stitt, 1995; Cheng et al., 1998; Martin et al., 2002; Sato et al., 2009). At the same time, the light response curves of NPQ in plants after 24 h C/N treatment indicate that plants under high C/low N need to dissipate a higher proportion of light through non-radiative energy dissipation mechanisms than plants under mild C/N balance (Fig. 5). Together with the knowledge that NPQ is linearly related to heat dissipation in the light-harvesting antenna of PSII (Bilger and Björkman, 1990; Tikkanen and Aro, 2012), our results suggest that plants under high C/low N reduce the electron transport rate at PSII level and thus the excess of energy must be dissipated through alternative pathways. These mechanisms could safely dissipate the excess of excitation energy at PSII avoiding photo-damage processes, as indicated by the Fv/Fm values of seedling adapted to the dark (Fig. 3). Therefore, plants under high C/low N turn off photosynthesis by a convergent sensing mechanism of C and N, showing that C to N balance is more important rather than each nutrient *per se* (Coruzzi and Zhou, 2001; Paul and Pellny, 2003; Sang et al., 2012). Taken together, we conclude that C/N-nutrient availability may control the phosphorylation status on SS and invertase and thus their activities via specific subcellular delivery and/or its interaction with 14-3-3 proteins. Moreover, the tight coordination between C and N not only affects the carbohydrate metabolism but also the partitioning of the excitation energy at PSII level between radiative (electron transport) and non-radiative (heat) dissipation pathways. Further research should focus on the nutrient metabolism and photochemistry

regulation in plants in order to understand the complex protein network in response to C/N-nutrient availability.

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References

Aguilar-Hernández, V., Aguilar-Henonin, L., Guzmán, P., 2011. Diversity in the architecture of ATLs, a family of plant ubiquitin-ligases, leads to recognition and targeting of substrates in different cellular environments. *PLoS ONE*, *6*(8). http://doi.org/10.1371/journal.pone.0023934

Alexander, R. D., Morris, P. C., 2006. A proteomic analysis of 14-3-3 binding proteins from developing barley grains. *Proteomics*, *6*(6), 1886–1896. http://doi.org/10.1002/pmic.200500548

Aoyama, S., Huarancca Reyes, T., Guglielminetti, L., Lu, Y., Morita, Y., Sato, T., Yamaguchi, J., 2014. Ubiquitin ligase ATL31 functions in leaf senescence in response to the balance between atmospheric CO2 and nitrogen availability in arabidopsis. *Plant and Cell Physiology*, *55*(2), 293–305. http://doi.org/10.1093/pcp/pcu002

Bilger, W., Björkman, O., 1990. Role of the xanthophyll cycle in photoprotection elucidated by measurements of light-induced absorbance changes, fluorescence and photosynthesis in leaves of Hedera canariensis. *Photosynthesis Research*, *25*(3), 173–185. http://doi.org/10.1007/BF00033159

Cheng, S. H., Moore, B., Seemann, J. R., 1998. Effects of short- and long-term elevated

CO2 on the expression of ribulose-1,5-bisphosphate carboxylase/oxygenase genes and carbohydrate accumulation in leaves of Arabidopsis thaliana (L.) Heynh. *Plant Physiology*, *116*(2), 715–723. http://doi.org/10.1104/pp.116.2.715

Chevalier, D., Morris, E. R., Walker, J. C., 2009. 14-3-3 and FHA domains mediate phosphoprotein interactions. *Annual Reviews of Plant Biology*. 60: 67–91.

Coruzzi, G. M., Zhou, L., 2001. Carbon and nitrogen sensing and signaling in plants: Emerging “matrix effects.” *Current Opinion in Plant Biology*, *4*(3), 247–253. http://doi.org/10.1016/S1369-5266(00)00168-0

Gao, J., Van Kleeff, P. J. M., Oecking, C., Li, K. W., Erban, A., Kopka, J., Hincha, D. K., De Boer, A. H., 2014. Light modulated activity of root alkaline/neutral invertase involves the interaction with 14-3-3 proteins. *Plant Journal*, *80*(5), 785–796. http://doi.org/10.1111/tpj.12677

Genty, B., Briantais, J.-M., Baker, N. R., 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta*, *990*(1), 87–92. http://doi.org/10.1016/S0304-4165(89)80016-9

Gibon, Y., Bläsing, O. E., Palacios-Rojas, N., Pankovic, D., Hendriks, J. H. M., Fisahn, J., Höhne, M., Günther, M., Stitt, M., 2004. Adjustment of diurnal starch turnover to short days: Depletion of sugar during the night leads to a temporary inhibition of carbohydrate utilization, accumulation of sugars and post-translational activation of ADP-glucose pyrophosphorylase in the followin. *Plant Journal*, *39*(6), 847–862. http://doi.org/10.1111/j.1365-313X.2004.02173.x

Guglielminetti, L., Perata, P., Alpi, A., 1995. Effect of Anoxia on Carbohydrate Metabolism in Rice Seedlings. *Plant Physiology*, *108*(2), 735–741.

http://doi.org/10.1104/pp.108.2.735

Hardin, S. C., Huber, S. C., 2004. Proteasome activity and the post-translational control of sucrose synthase stability in maize leaves. *Plant Physiology and Biochemistry*, *42*(3), 197–208. http://doi.org/10.1016/j.plaphy.2003.12.004

Hirose, T., Takano, M., Terao, T., 2002. Cell wall invertase in developing rice caryopsis: molecular cloning of OsCIN1 and analysis of its expression in relation to its role in grain filling. *Plant and Cell Physiology*, *43*(4), 452–459. http://doi.org/10.1093/pcp/pcf055

Huarancca Reyes, T., Maekawa, S., Sato, T., Yamaguchi, J., 2015. The Arabidopsis ubiquitin ligase ATL31 is transcriptionally controlled by WRKY33 transcription factor in response to pathogen attack. *Plant Biotechnology*, *32*(1), 11–19. http://doi.org/10.5511/plantbiotechnology.14.1201b

Klotke, J., Kopka, J., Gatzke, N., Heyer, A. G., 2004. Impact of soluble sugar concentrations on the acquisition of freezing tolerance in accessions of Arabidopsis thaliana with contrasting cold adaptation - Evidence for a role of raffinose in cold acclimation. *Plant, Cell and Environment*, *27*(11), 1395–1404. http://doi.org/10.1111/j.1365-3040.2004.01242.x

Krapp, A., Stitt, M., 1995. An evaluation of direct and indirect mechanisms for the “sink-regulation” of photosynthesis in spinach: Changes in gas exchange, carbohydrates, metabolites, enzyme activities and steady-state transcript levels after cold-girdling source leaves. *Planta*, *195*(3), 313–323. http://doi.org/10.1007/BF00202587

Mackintosh, C., 2004. Dynamic interactions between 14-3-3 proteins and phosphoproteins regulate diverse cellular processes. *The Biochemical Journal*, *381*, 329–342. http://doi.org/10.1042/BJ20031332

Maekawa, S., Inada, N., Yasuda, S., Fukao, Y., Fujiwara, M., Sato, T., Yamaguchi, J., 2014. The carbon/nitrogen regulator ARABIDOPSIS TOXICOS EN LEVADURA31 controls papilla formation in response to powdery mildew fungi penetration by interacting with SYNTAXIN OF PLANTS121 in Arabidopsis. *Plant Physiology*, *164*(2), 879–887. http://doi.org/10.1104/pp.113.230995

Maekawa, S., Takabayashi, A., Huarancca Reyes, T., Yamamoto, H., Tanaka, A., Sato, T., Yamaguchi, J., 2015. Pale-green phenotype of atl31atl6 double mutant leaves is caused by disruption of 5-aminolevulinic acid biosynthesis in Arabidopsis thaliana. *PloS One*, *10*(2), e0117662. http://doi.org/10.1371/journal.pone.0117662

Maekawa, S., Sato, T., Asada, Y., Yasuda, S., Yoshida, M., Chiba, Y., Yamaguchi, J., 2012. The Arabidopsis ubiquitin ligases ATL31 and ATL6 control the defense response as well as the carbon/nitrogen response. *Plant Molecular Biology*, *79*(3), 217–227. http://doi.org/10.1007/s11103-012-9907-0

Martin, T., Oswald, O., Graham, I. A., 2002. Arabidopsis Seedling Growth, Storage Lipid Mobilization, and Photosynthetic Gene Expression Are Regulated by Carbon:Nitrogen Availability. *Plant Physiology*, *128*(2), 472–481. http://doi.org/10.1104/pp.010475

Miller, A. J., Fan, X., Orsel, M., Smith, S. J., Wells, D. M., 2007. Nitrate transport and signalling. *Journal of Experimental Botany*, *58*(9), 2297–2306. http://doi.org/10.1093/jxb/erm066

Murchie, E. H., Lawson, T., 2013. Chlorophyll fluorescence analysis: A guide to good practice and understanding some new applications. *Journal of Experimental Botany*, *64*(13), 3983–3998. http://doi.org/10.1093/jxb/ert208

Paul, A.-L., Denison, F. C., Schultz, E. R., Zupanska, A. K., Ferl, R. J., 2012. 14-3-3 Phosphoprotein Interaction Networks – Does Isoform Diversity Present Functional

453 Interaction Specification? *Frontiers in Plant Science*, *3*, 1–14.
454 http://doi.org/10.3389/fpls.2012.00190

455 Paul, M. J., Pellny, T. K., 2003. Carbon metabolite feedback regulation of leaf
456 photosynthesis and development. *Journal of Experimental Botany*, *54*(382), 539–547.
457 http://doi.org/10.1093/jxb/erg052

458 Roitsch, T., González, M. C., 2004. Function and regulation of plant invertases: Sweet
459 sensations. *Trends in Plant Science*, *9*(12), 606–613.
460 http://doi.org/10.1016/j.tplants.2004.10.009

461 Sang, Y., Sun, W., Yang, Z., 2012. Signaling mechanisms integrating carbon and nitrogen
462 utilization in plants. *Frontiers in Biology*, *7*(6), 548–556. http://doi.org/10.1007/s11515-
463 012-1249-4

464 Sato, T., Maekawa, S., Yasuda, S., Domeki, Y., Sueyoshi, K., Fujiwara, M., Fukao, Y.,
465 Goto, D. B., Yamaguchi, J., 2011a. Identification of 14-3-3 proteins as a target of
466 ATL31 ubiquitin ligase, a regulator of the C/N response in Arabidopsis. *Plant Journal*,
467 *68*(1), 137–146. http://doi.org/10.1111/j.1365-313X.2011.04673.x

468 Sato, T., Maekawa, S., Yasuda, S., Sonoda, Y., Katoh, E., Ichikawa, T., Nakazawa, M.,
469 Seki, M., Shinozaki, K., Matsui, M., Goto, D. B., Ikeda, A., Yamaguchi, J., 2009.
470 CNI1/ATL31, a RING-type ubiquitin ligase that functions in the carbon/nitrogen
471 response for growth phase transition in Arabidopsis seedlings. *Plant Journal*, *60*(5),
472 852–864. http://doi.org/10.1111/j.1365-313X.2009.04006.x

473 Sato, T., Maekawa, S., Yasuda, S., Yamaguchi, J., 2011b. Carbon and nitrogen
474 metabolism regulated by the ubiquitin-proteasome system. *Plant Signaling and*
475 *Behavior*, *6*(10), 1465–1468. http://doi.org/10.4161/psb.6.10.17343

476 Sheen, J., 1990. Metabolic repression of transcription in higher plants. *The Plant Cell*,
477 *2*(10), 1027–1038. http://doi.org/10.1105/tpc.2.10.1027

478 Smith, A. M., Stitt, M., 2007. Coordination of carbon supply and plant growth. *Plant, Cell*
479 *and Environment*, *30*(9), 1126–1149. http://doi.org/10.1111/j.1365-3040.2007.01708.x

480 Subbaiah, C. C., Sachs, M. M., 2001. Altered patterns of sucrose synthase
481 phosphorylation and localization precede callose induction and root tip death in anoxic
482 maize seedlings. *Plant Physiology*, *125*(2), 585–94.
483 http://doi.org/10.1104/pp.125.2.585

484 Sulpice, R., Nikoloski, Z., Tschoep, H., Antonio, C., Kleessen, S., Larhlimi, A., Selbig, J.,
485 Ishihara, H., Gibon, Y., Fernie, A. R., Stitt, M., 2013. Impact of the carbon and
486 nitrogen supply on relationships and connectivity between metabolism and biomass in
487 a broad panel of Arabidopsis accessions. *Plant Physiology*, *162*(1), 347–63.
488 http://doi.org/10.1104/pp.112.210104

489 Tiessen, A., Padilla-Chacon, D., 2013. Subcellular compartmentation of sugar signaling:
490 links among carbon cellular status, route of sucrolysis, sink-source allocation, and
491 metabolic partitioning. *Frontiers in Plant Science*, *3*, 306.
492 http://doi.org/10.3389/fpls.2012.00306

493 Tikkanen, M., Aro, E. M., 2012. Thylakoid protein phosphorylation in dynamic regulation of
494 photosystem II in higher plants. *Biochimica et Biophysica Acta*, *1817*(1), 232–238.
495 http://doi.org/10.1016/j.bbabio.2011.05.005

496 Tobias, R. B., Boyer, C. D., Shannon, J. C., 1992. Alterations in Carbohydrate
497 Intermediates in the Endosperm of Starch-Deficient Maize (Zea mays L.) Genotypes.
498 *Plant Physiology*, *99*(1), 146–152. http://doi.org/10.1104/pp.99.1.146

van Kooten, O., Snel, J. F. H., 1990. The use of chlorophyll fluorescence nomenclature in plant stress physiology. *Photosynthesis Research*, *25*(3), 147–150. http://doi.org/10.1007/BF00033156

Winter, H., Huber, J. L., Huber, S. C., 1997. Membrane association of sucrose synthase: Changes during the graviresponse and possible control by protein phosphorylation. *FEBS Letters*, *420*(2-3), 151–155. http://doi.org/10.1016/S0014-5793(97)01506-8

Yasuda, S., Sato, T., Maekawa, S., Aoyama, S., Fukao, Y., Yamaguchi, J., 2014. Phosphorylation of arabidopsis ubiquitin ligase ATL31 is critical for plant carbon/nitrogen nutrient balance response and controls the stability of 14-3-3 proteins. *Journal of Biological Chemistry*, *289*(22), 15179–15193. http://doi.org/10.1074/jbc.M113.533133

Zheng, Y., Anderson, S., Zhang, Y., Garavito, R. M., 2011. The structure of sucrose synthase-1 from arabidopsis thaliana and its functional implications. *Journal of Biological Chemistry*, *286*(41), 36108–36118. http://doi.org/10.1074/jbc.M111.275974

Figure legends

Fig. 1. Effect of C/N stress and phosphorylation status on the activity of soluble and membrane bound isoforms of invertase. Enzyme activity of Arabidopsis seedlings was analyzed in triplicate with or without phosphatase inhibitor. Enzyme extraction was obtained from whole plants 0.5, 2 and 24 h after transfer to C/N medium containing 100 mM glucose/30 mM N (control medium, U) or 200 mM glucose/0.3 mM N (stress medium, T) from control medium. Activities are expressed as mU mg protein^{-1}. Means ± standard

error are shown. Letters indicate significant differences within treatment ($p<0.05$) determined by Fisher's protected LSD.

Fig. 2. Effect of C/N stress and phosphorylation status on the activity of soluble and membrane bound isoforms of sucrose synthase. Enzyme activity of Arabidopsis seedlings was analyzed in triplicate with or without of phosphatase inhibitor. Enzyme extraction was obtained from whole plants 0.5, 2 and 24 h after transfer to C/N medium containing 100 mM glucose/30 mM N (control medium, U) or 200 mM glucose/0.3 mM N (stress medium, T) from control medium. Activities are expressed as mU mg protein^{-1}. Means ± standard error are shown. Letters indicate significant differences within treatment ($p<0.05$) determined by Fisher's protected LSD.

Fig. 3. Effect of C/N stress on the photochemical efficiency of photosystem II (PSII). PSII efficiency was obtained in leaves of Arabidopsis seedlings 0.5, 2 and 24 h after transfer to C/N medium containing 100 mM glucose/30 mM N (control medium, U) or 200 mM glucose/0.3 mM N (stress medium, T) from control medium. PSII efficiency was evaluated under the photosynthetic photon flux densities (PPFD) of 0, 50, 100, 200 and 400 μmol m^{-2} s^{-1}. Means ± standard error were calculated from three independent experiment replications.

Fig. 4. Time course of C/N-nutrient availability effect in the actual (Φ_{PSII}) photochemical PSII efficiency under 100 μmol m^{-2} s^{-1} light intensity. Φ_{PSII} was obtained in leaves of Arabidopsis seedlings 0.5, 2 and 24 h after transfer to C/N medium containing 100 mM glucose/30 mM N (control medium, U) or 200 mM glucose/0.3 mM N (stress medium, T) from control medium. Data are expressed as percentage comparing each point with the beginning of C/N treatment (T0) which was set as 100%. Dashed line represents T0 activity level. Means ± standard error were calculated from three independent experiment replications.

Fig. 5. Effect of C/N stress on the non-photochemical quenching (NPQ). NPQ was obtained in leaves of Arabidopsis seedlings 24 h after transfer to C/N medium containing 100 mM glucose/30 mM N (control medium, U) or 200 mM glucose/0.3 mM N (stress medium, T) from control medium. NPQ was evaluated under the photosynthetic photon flux densities (PPFD) of 50, 100, 200 and 400 μmol m^{-2} s^{-1}. Means ± standard error were calculated from three independent experiment replications.

Fig. 6. Effect of C/N stress on soluble sugars content. Glucose, fructose, sucrose and total soluble sugars content was obtained from whole Arabidopsis seedlings 0, 0.5, 2 and 24 h after transfer to C/N medium containing 100 mM glucose/30 mM N (control medium, U) or 200 mM glucose/0.3 mM N (stress medium, T) from control medium. Sugar content was expressed as μmol hexose equivalent g FW^{-1}. Means ± standard error were calculated from three independent experiment replications.

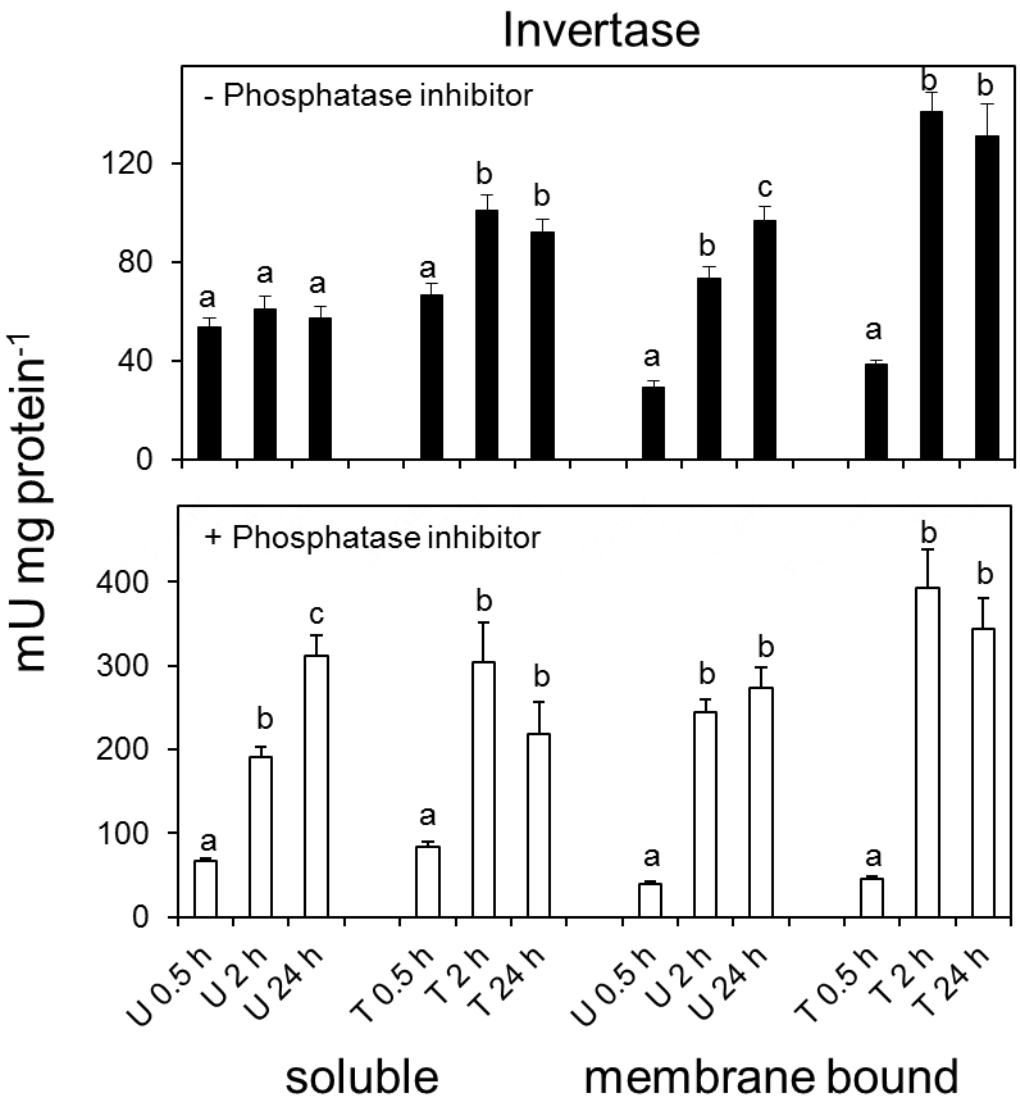
Invertase
- Phosphatase inhibitor
+ Phosphatase inhibitor
mU mg protein-1
0
40
80
120
0
100
200
300
400
a
a
a
a
b
b
a
b
c
a
b
b
a
b
c
a
b
b
a
b
b
a
b
b
a
b
b
a
b
b
U 0.5 h
U 2 h
U 24 h
T 0.5 h
T 2 h
T 24 h
U 0.5 h
U 2 h
U 24 h
T 0.5 h
T 2 h
T 24 h
soluble
membrane bound

Figure 1

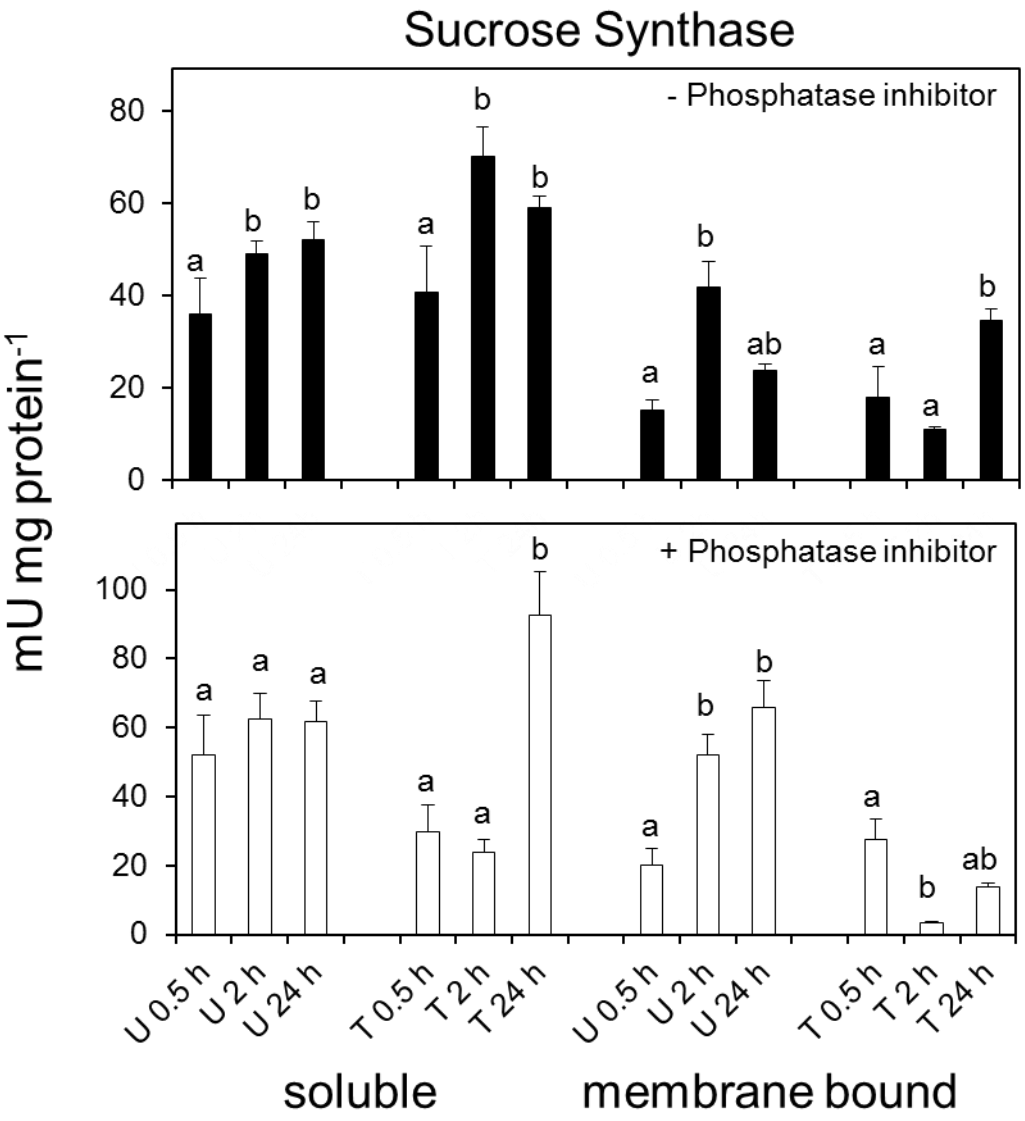
Sucrose Synthase
- Phosphatase inhibitor
+ Phosphatase inhibitor
mU mg protein-1
0
20
40
60
80
100
a
b
ab
U 0.5 h
U 2 h
U 24 h
T 0.5 h
T 2 h
T 24 h
soluble
membrane bound

Figure 2

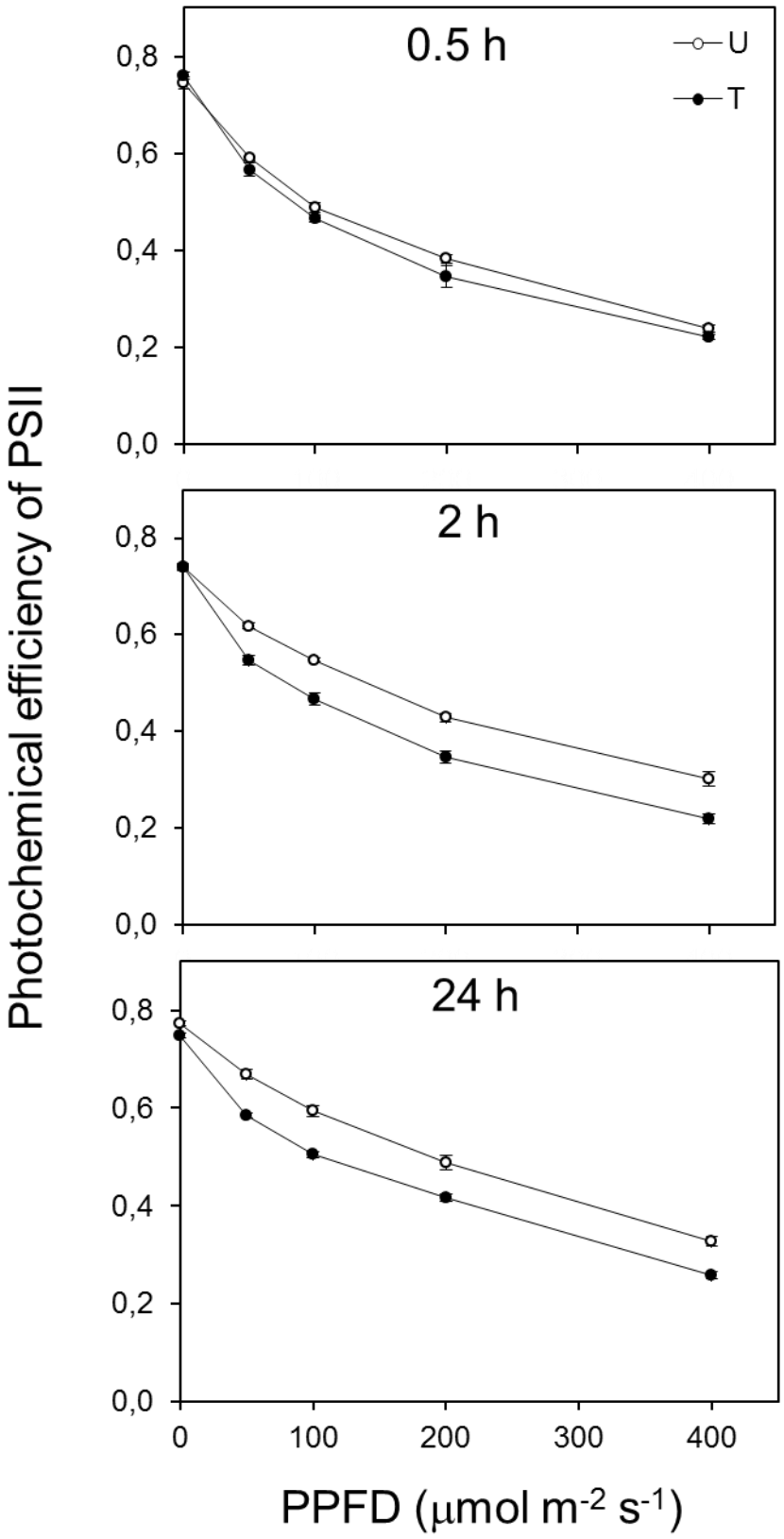
0.5 h
U
T
2 h
24 h
Photochemical efficiency of PSII
0,0
0,2
0,4
0,6
0,8
0
100
200
300
400
PPFD (μmol m-2 s-1)

Figure 3

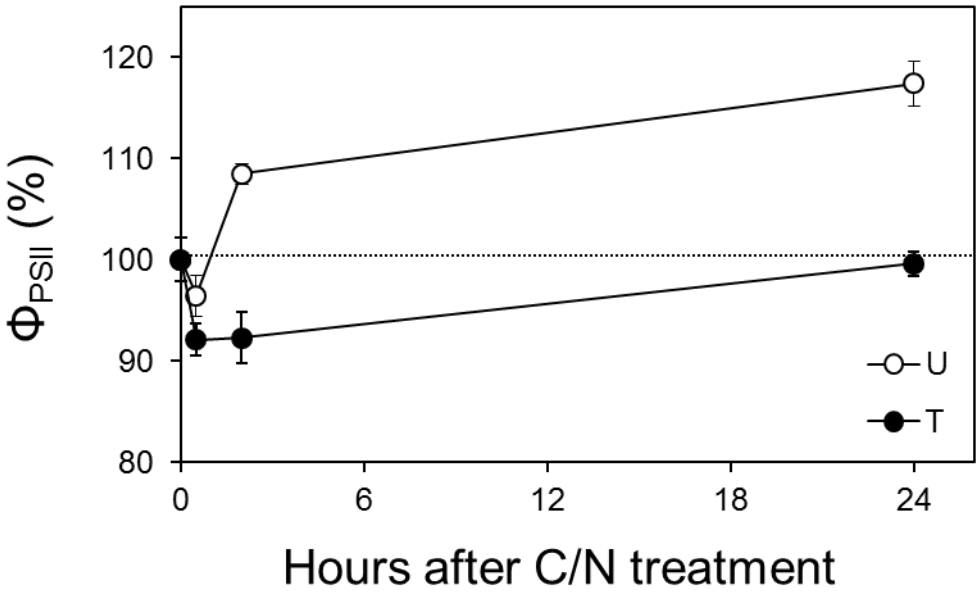
120
110
100
90
80
Φ_{PSII} (%)
0
6
12
18
24
Hours after C/N treatment
U
T

Figure 4

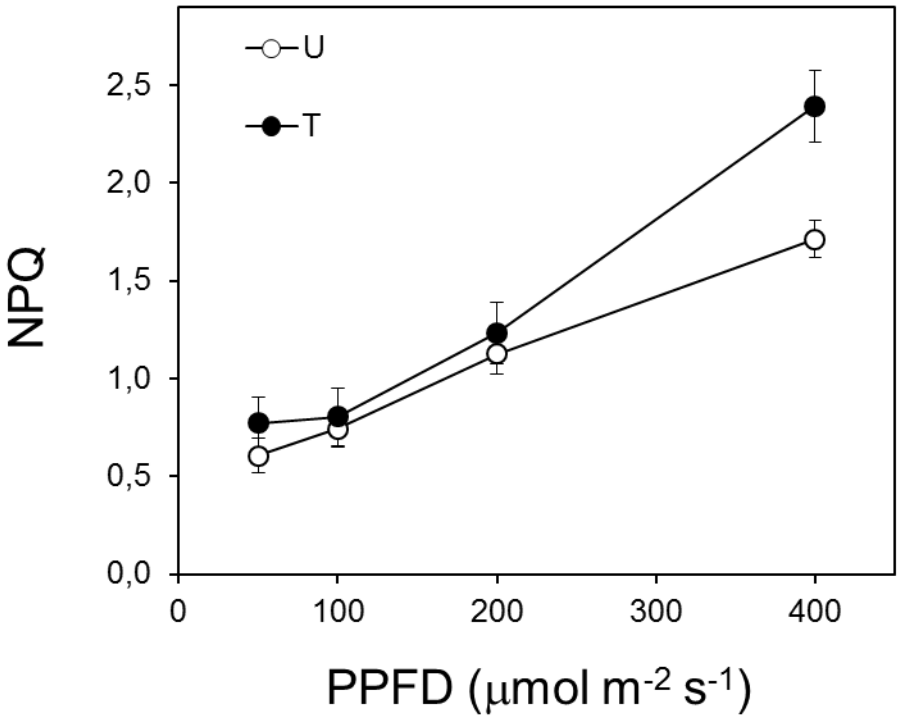

Figure 5

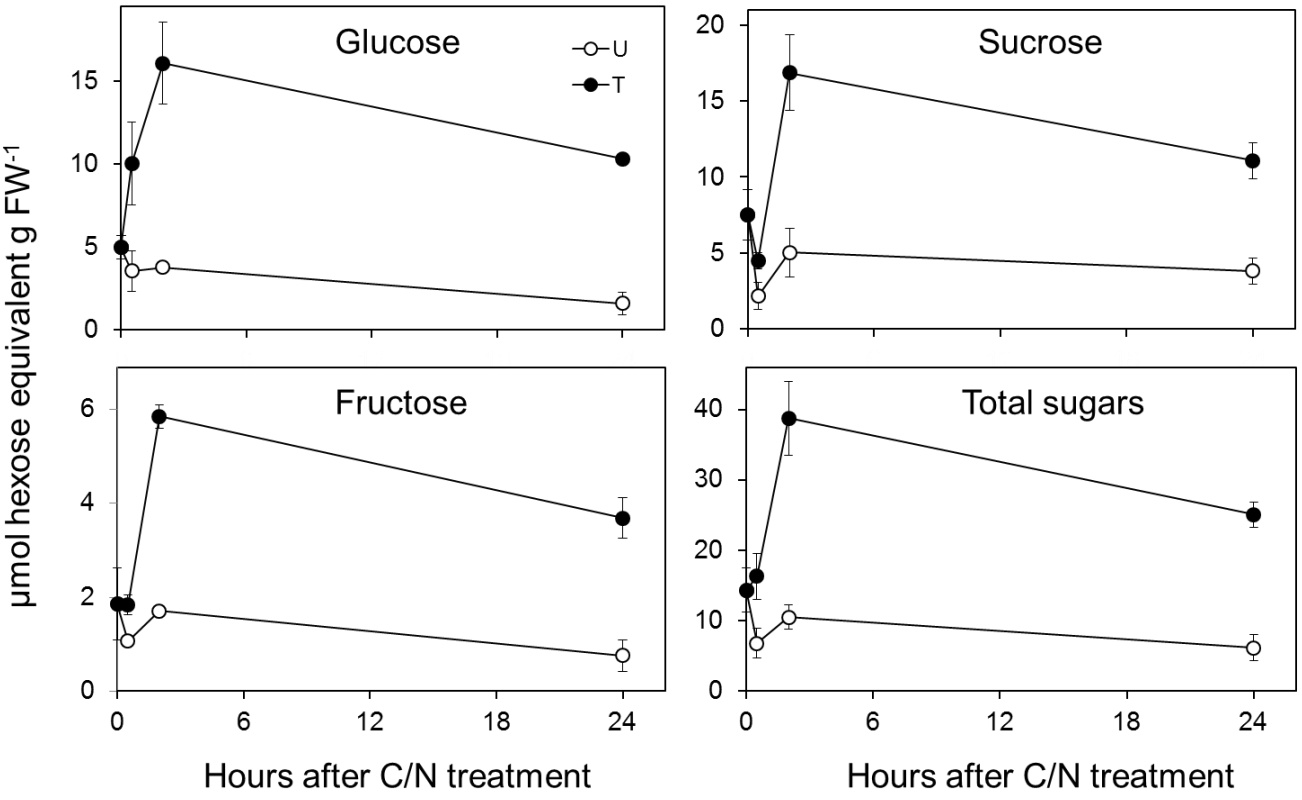
Glucose
U
T
Sucrose
Fructose
Total sugars
µmol hexose equivalent g FW-1
Hours after C/N treatment
Hours after C/N treatment

Figure 6

mU mg protein^{-1}	U	T	U+	T+
Cell Wall Invertase	15.0±2.4	18.5±1.9	13.9±0.9	14.3±1.8
Soluble Sucrose Synthase	36.0±7.8	40.6±10.1	52.1±11.3	29.8±7.7
Membrane Bound Sucrose Synthase	15.1±2.1	17.9±6.5	20.1±4.7	27.7±5.9
Soluble Invertase	53.7±3.4	66.6±4.4	66.2±3.1	83.2±6.3
Membrane Bound Invertase	29.2±2.6	38.7±1.3	39.3±3.0	44.9±2.9
***p*-value**	U vs.U+	T vs.T+	U vs.T	U+ vs.T+
Cell Wall Invertase	0.3969	**0.0175**	**0.0195**	0.4811
Soluble Sucrose Synthase	0.0579	0.1394	0.5015	**0.0170**
Membrane Bound Sucrose Synthase	**0.0402**	**0.0214**	0.3350	**0.0343**
Soluble Invertase	**0.0016**	**0.0051**	**0.0035**	**0.0029**
Membrane Bound Invertase	**0.0022**	**0.0082**	**0.0006**	**0.0349**

Table 1 Effect of C/N stress and phosphorylation status on the activity of invertase and sucrose synthase. Enzyme activity of Arabidopsis seedlings was analyzed in triplicate with (+) or without (-) of phosphatase inhibitor. Enzyme extraction was obtained from whole plants 0.5 h after transfer to C/N medium containing 100 mM glucose/30 mM N (control medium, U) or 200 mM glucose/0.3 mM N (stress medium, T) from control medium. Activities are expressed as mU mg protein^{-1}. Means ± standard error are shown (upper panel). Statistical analysis of C/N treatment (U vs. T), phosphorylation status (U vs.U+ and T vs. T+) and both parameters (U+ vs. T+) effects was done by Student's *t*-test ($p<0.05$, bottom panel). Significant differences are in bold.

*Contribution

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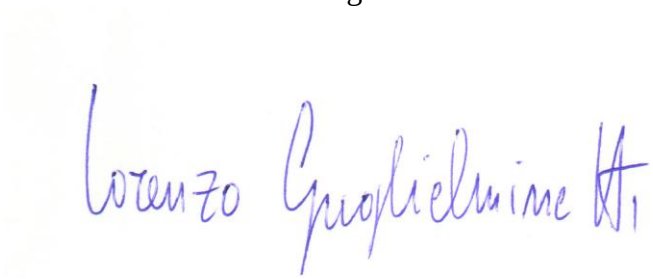
The author's contribution for the manuscript entitled: "Effect of Carbon/Nitrogen ratio on carbohydrate metabolism and light energy dissipation mechanisms in *Arabidopsis thaliana*" was equally distributed between Thais Huarancca Reyes, Andrea Scartazza, Yu Lu, Junji Yamaguchi and Lorenzo Guglielminetti.

Yours sincerely,

Lorenzo Guglielminetti



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