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Chloroplast nitrite uptake is enhanced in *Arabidopsis* PII mutants

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Abstract In higher plants, the PII protein is a nuclear-encoded plastid protein that regulates the activity of a key enzyme of arginine biosynthesis. We have previously observed that *Arabidopsis* PII mutants are more sensitive to nitrite toxicity. Using intact chloroplasts isolated from *Arabidopsis* leaves and ^{15}N-labelled nitrite we show that a light-dependent nitrite uptake into chloroplasts is increased in PII knock-out mutants when compared to the wild-type. This leads to a higher incorporation of ^{15}N into ammonium and amino acids in the mutant chloroplasts. However, the uptake differences do not depend on GS/GOGAT activities. Our observations suggest that PII is involved in the regulation of nitrite uptake into higher plant chloroplasts.

Keywords: *Arabidopsis*; *AtGLB1* gene; Chloroplasts; NO_2^- uptake; PII protein; T-DNA insertion mutant

1. Introduction

PII is a highly conserved protein that plays a role in the sensing of carbon/nitrogen (C/N) balance and energy status in bacteria, cyanobacteria and plants. It has been shown to be involved in the regulation of inorganic nitrogen uptake and assimilation in both bacteria and cyanobacteria. The PII protein interacts with various target proteins that include signal transduction proteins, key metabolic enzymes and metabolite transporters (see [1–3] for review). In cyanobacteria, NtrC, a subunit of a nitrate/nitrite transporter is believed to be regulated by PII [4]. In all known cases, PII – target protein interactions depend on the Mg-ATP and 2-oxoglutarate (2-OG) levels in the cell [1]. In photosynthetic cyanobacteria and eubacteria, PII activity is also modified by phosphorylation or uridylylation, respectively [5].

In higher plants, a single PII homolog (*GLB1*) has been identified in *Arabidopsis*, rice, tomato, castor bean, alfalfa, and pine [6–8]. PII is a nuclear-encoded chloroplastic protein displaying 50% identity to bacterial PII proteins. As in bacterial systems, plant PII can bind 2-oxoglutarate in the presence of Mg-ATP [9]. However, there is no evidence for PII phosphorylation albeit the conservation of the phosphorylated serine residue found in *Synechococcus* PII [10]. To date, the only PII target protein discovered in plants is conserved between higher plants and cyanobacteria. This PII interacting protein is the chloroplastic *N*-acetyl glutamate kinase (NAGK) that is activated in vitro and shows a reduced retro-inhibition by arginine when the NAGK-PII complex is formed [11,12]. In the leaves of PII knock-out mutants grown under ammonium nutrition conditions, arginine, ornithine and citrulline levels were reduced by 50%, thus giving the first physiological evidence of the activation of NAGK by PII in planta [11].

The identification of new PII targets in plants is essential to understand the role of this protein and the use of PII mutants should be useful to decipher PII function. Indeed, *Arabidopsis* PII mutants appeared to be more sensitive to NO_2^- toxicity, as judged by the mortality of in vitro grown seedlings [13]. Interestingly, this observation suggests that PII might be involved in the regulation of NO_2^- metabolism. This function could be reminiscent of the role of PII in cyanobacteria where it has been shown to regulate a nitrate/nitrite transporter [4]. In higher plants, NO_2^- is synthesised in the cytosol by reduction of NO_3^- by nitrate reductase (NR). NO_2^- is then translocated to the chloroplast to be further reduced into ammonium by the nitrite reductase. It is noteworthy that NO_2^- does not accumulate in plant tissues. This could be partly due to the regulation of NR activity by chloroplastic redox poise and the oxaloacetate/malate shuttle that would deliver reductant for NR activity. In addition, an inhibition of NR activity by phosphorylation and subsequent binding of 14-3-3 proteins occurs in the dark, thus avoiding NO_2^- accumulation in the dark when NADH could be limiting [14]. However, the occurrence of a highly efficient transport system across the chloroplast envelope to avoid NO_2^- toxicity is conceivable. However, the molecular mechanism of NO_2^- uptake into chloroplasts is largely unknown in higher plants. It is possible that NO_2^- is transported across the plastid membrane by permeation of the nitrous acid [15] versus a NO_2^- channel or a NO_2^- transporter [16,17]. However the uptake of NO_2^- into intact pea chloroplasts shows saturable kinetics thus supporting the presence of a NO_2^- transport system [16,17]. In *Chlamydomonas*, *Nar1-1* encodes a chloroplast membrane protein involved in high affinity NO_2^- transport [18,19]. It is a member of the formate/nitrite transporter family [19], however no orthologs have been hitherto identified in the *Arabidopsis* genome. Recently, a NO_2^- transporter has been identified in higher plants [20]. It is a member of the proton-dependent oligopeptide transporter family but it displays little homology with the NO_2^- transporters described in cyanobacteria and *Chlamydomonas*.

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Abbreviations: Col, Colombia ecotype; Chl, Chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; MSO, methionine sulfoximine; WS, Wassilewskija ecotype

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In this study we have tested the hypothesis that PII has a role in the regulation of NO_2^- uptake into higher plant chloroplasts, reminiscent of its role in cyanobacterial NO_2^- uptake [4]. Using intact chloroplasts isolated from *Arabidopsis* leaves and ^{15}N-labelled nitrite we show that a light-dependent NO_2^- uptake into chloroplasts is increased in PII knock-out mutants when compared to the wild-type.

2. Materials and methods

2.1. Plants and growth conditions

PIIV1 and PIIS2 mutant lines of *Arabidopsis* Wassilewskija ecotype (WS) and Colombia ecotype (Col) have been described previously and contain undetectable amounts of the PII protein [13]. Wild-type plants and PII mutants were grown on sterilised compost and watered daily with a complete nutrient solution containing 10 mM NO_3^- and 2 mM NH_4^+ [21]. Plants were grown in a growth chamber at 60% relative humidity, a light intensity of 150 μmol m^{-2} s^1 and a day–night regime of 8 h at 22 °C and 16 h at 20 °C, respectively. The total rosette of 6 week-old plants was used for intact chloroplast isolation. Plants were maintained in darkness during 24 h before being harvested in order to reduce chloroplastic starch levels.

2.2. Chloroplast purification

Chloroplasts were obtained from 40 g *Arabidopsis* leaves of wild-type and PII mutant plants. Leaves were washed in distilled water at 4 °C for 20 min and then ground in a blender for 2 s in a 20 mM Tricine KOH (pH 8) homogenization buffer maintained at 4 °C and containing 450 mM sorbitol, 10 mM EDTA, 10 mM $NaHCO_3$ and 0.1% BSA (100 ml/10 g of leaves). The homegates were immediately filtered through several layers of gauze and centrifuged at $700 \times g$ for 5 min at 4 °C. The pellets were resuspended in 3 ml buffer (RB) containing 20 mM Tricine KOH (pH 7.6), 300 mM sorbitol, 2.5 mM EDTA and 5 mM $MgCl_2$. The resulting solution was transferred to the top of a 40% percoll solution in RB (30 ml total volume) and then centrifuged for 3 min at $2500 \times g$ using a swing out centrifuge bucket at 4 °C. Intact chloroplasts were pelleted while broken chloroplasts and other membrane debris remained at the top of the percoll solution. The chloroplast suspensions were diluted (3-fold) in RB and centrifuged for 4 min at $5180 \times g$ at 4 °C and the pellets were resuspended in 1–2 ml of RB. The chlorophyll (Chl) content of the chloroplast suspension was calculated from the absorption at 652 nm after extraction of an aliquot (5 μl) in 80% acetone [22]. The chloroplast suspensions were stored at 4 °C in the dark until their use for NO_2^- uptake measurements.

Chloroplast intactness was determined by measuring the light-dependent oxygen production of 'intact' and osmotically broken chloroplasts using an O_2 electrode and ferricyanide (as an electron acceptor) [23]. The intactness was estimated by the ratio $[(A - B)/A \times 100]$ where A is the ferricyanide-dependent O_2 evolution measured after the osmotic shock and B the O_2 evolution measured before the osmotic shock.

2.3. NO_2^- uptake by isolated chloroplasts

NO_2^- uptake by chloroplasts was measured in a "plexiglass cell" (maximum volume of 3 ml), with gentle agitation, at 20 °C and either a light intensity of 50 μmol m^{-2} s^{-1} or in darkness. The chlorophyll concentration of the chloroplast suspension in the "cell" was adjusted to 0.1 mg ml^{-1} in 2 ml of RB. Chloroplasts in the measuring cell were either illuminated 5 min before adding $^{15}NO_2Na$ (light treatment) or maintained in the darkness (dark treatment). The final concentration of $^{15}NO_2Na$ was 0.25 mM (98% ^{15}N enrichment) for the uptake studies or 0.1–2.5 mM for the determination of kinetic parameters as indicated in the text. Aliquots (300 μl) of the chloroplast suspension were collected at the times indicated in the text (from 0 to 30 min) and immediately centrifuged. The supernatant was stored at −20 °C until the measurement of nitrite. The pellets were rinsed in 300 μl of RB and centrifuged. The rinsed pellets were stored at −80 °C for ^{15}N total enrichment analysis. The inhibitor studies were carried out using 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (50 μM), methionine sulfoximine (MSO, 1 mM) and azaserine (1 mM). They were added 5 min before $^{15}NO_2Na$ addition. WS and PII mutant chloroplasts were measured simultaneously for each experiment. For the studies of the effect of the addition of NH_4 (1 mM) and αKG (1 mM), these metabolites were added 5 min before $^{15}NO_2$ addition (as described in [24]).

2.4. NO_2 measurement

NO_2 was measured colorimetrically at 540 nm using *N*-(1-Naphtyl) ethylene diamine dichlorhydrate as in [25].

2.5. ^{15}N labelling measurements

For the determination of total ^{15}N content, the pellets were dried at 70 °C for 48 h, weighed, and analyzed using a continuous-flow isotope ratio mass spectrometer coupled to a carbon/nitrogen elemental analyzer (model ANCA-MS, PDZ Europa, Crewe, UK), as described in [26]. For the ^{15}N labelling of NH_4 and amino acids in the pelleted chloroplasts, the amino acid extracts from (SSA extracts) were filtered and analysed as described in [27,28].

2.6. Transcriptome analyses

The transcriptome analyses were performed with CATMA arrays containing 24576 Gene Specific Tags from *Arabidopsis thaliana* as described in [11]. Two biological repetitions of the same experiment were performed and used for the calculations. The results of the microarray data analysis are shown as the ratio of the intensities for each gene in the two genotypes analysed (after the recalculation from the initial data expressed as a $\log_2$ ratio provided by the CATMA analysis).

3. Results

3.1. NO_2^- uptake into isolated chloroplasts is increased in PII mutants

Previous studies suggested PII to have a role in nitrite metabolism or transport due to the increased NO_2^- sensitivity observed in PII mutant seedlings grown in vitro on NO_2^- as the sole nitrogen source [13]. Since, cyanobacterial PII has been shown to modulate the activity of a NO_3^-/NO_2^- transporter [4], it was decided to investigate NO_2^- uptake into chloroplasts isolated from rosette leaves of PII mutants and wild-type plants. The yield of the isolated intact chloroplasts was verified by ferricyanide-dependant O_2 production measured using an oxygen electrode before (isolated chloroplasts) and after (broken chloroplasts) an osmotic shock. The intactness of the chloroplasts isolated from *Arabidopsis* rosette leaves was found to be between 50% and 80% for the wild-type ecotypes (WS and Col) and the PII mutant lines (PIIV1 and PIIS2) (data not shown). Broken chloroplasts did not exhibit any light-dependent nitrite uptake, and the measured transport rates appeared to be correlated with the degree of chloroplast intactness (data not shown).

NO_2^- uptake into isolated chloroplasts was estimated by measuring the disappearance of NO_2^- from the incubation medium or by ^{15}N (N total) labelling in the chloroplast pellets after different dark or light treatments. There was a good correlation between the two methods as seen in Fig. 1. Therefore, for further analyses it was decided to measure NO_2^- uptake by the disappearance of NO_2^- in the chloroplast incubation medium. The effect of NO_2^- concentration on the observed light-dependent NO_2^- uptake was measured between 0.1 and 2.5 mM $^{15}NO_2Na$. Uptake was saturated above 0.5 mM $^{15}NO_2Na$ for both the wild-type and PII mutant genotypes (Fig. 2) and a Km of approximately 200 μM NO_2^- was obtained in agreement with previous observations [16,20].

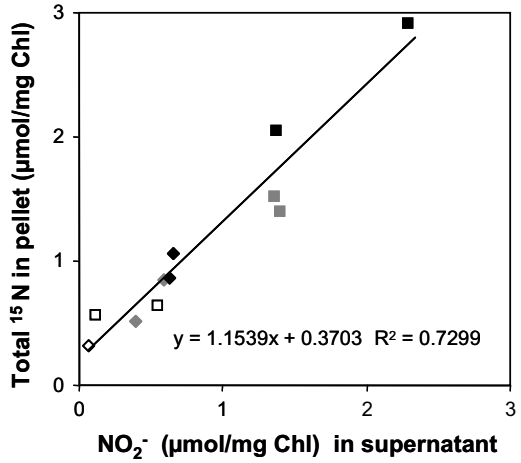

Fig. 1. Correlation between the two methods used to estimate NO_2^- uptake into intact *Arabidopsis* chloroplasts. Uptake was measured either by the increase in ^{15}N labelling (total N) of the chloroplast pellets or by the disappearance of NO_2^- from the incubation medium after either 5 (open symbol), 15 (grey symbol) or 30 min (closed symbol) in the light (■) or in the dark (◆).

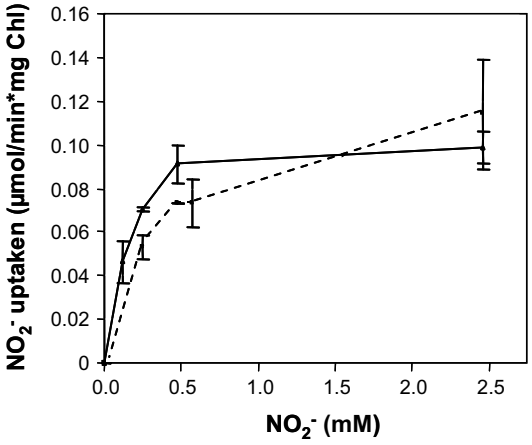

Fig. 2. Kinetics of $^{15}NO_2Na$ uptake in the light as a function of $^{15}NO_2Na$ concentration in the incubation medium for WS (dotted line) and the PIIV1 mutant (continuous line).

Taking these observations into account, all further measurements of NO_2^- uptake into the chloroplasts isolated from the rosette leaves of wild-type (WS and Col) and PII mutant lines (PIIV1 and PIIS2) lines were performed using 0.2 mM $^{15}NO_2Na$. Under our experimental conditions, NO_2^- uptake increased linearly during the investigated time course (between 5 min and 30 min) and required light, although a low and stable NO_2^- uptake was measured in the dark for both genotypes (Fig. 3A). Interestingly, a statistically significant and reproducible 30–40% increase in NO_2^- uptake was seen for intact chloroplasts of the PIIV1 mutant compared to WS in the light (Fig. 3A). No differences were observed in the dark between the two genotypes. As stated above, no NO_2^- uptake was detectable in the light using broken chloroplasts (by a 5 min osmotic shock) before the uptake measurements (data not shown). Similar trends were found when the PIIS2 and Col lines were analysed (data not shown).

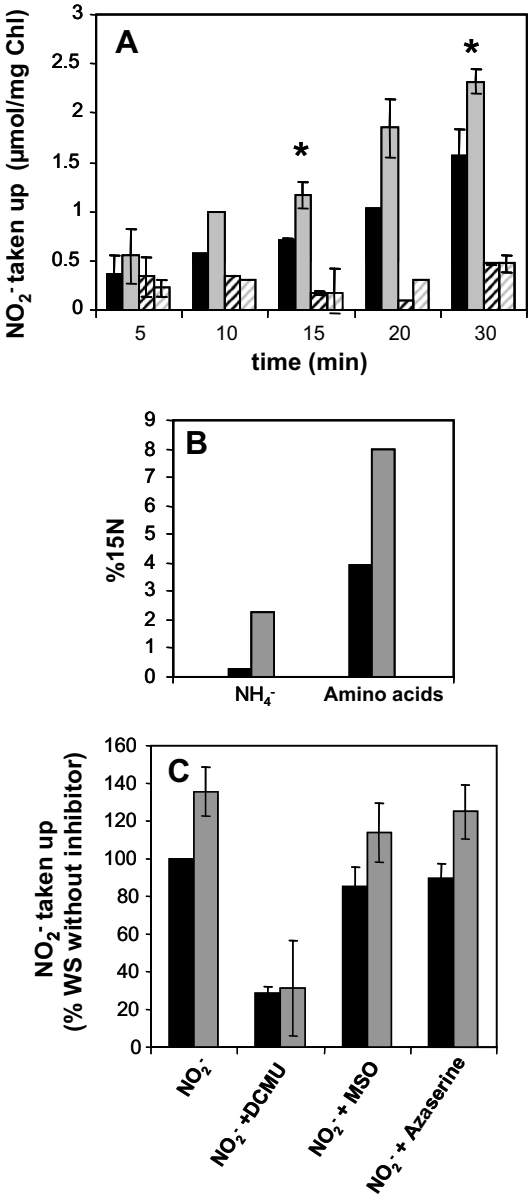

Fig. 3. (A) Kinetics of $^{15}NO_2Na$ uptake in the light (full bars) and in the dark (lined bars) into chloroplasts isolated from WS (dark bar) and from the PIIV1 mutant (grey bar) as a function of the time of incubation. The values are means of four independent replicates. (B) ^{15}N Labelling of NH_4^+ and free soluble amino acids pools in isolated chloroplasts after 30 min of incubation in $^{15}NO_2Na$ from WS (dark bar) and from the PIIV1 mutant (grey bar). (C) Effect of inhibitors (50 µM DCMU, or 1 mM azaserine, or 1 mM MSO) on the light-dependent $^{15}NO_2Na$ uptake into chloroplasts isolated from WS (dark bar) and the PIIV1 mutant (grey bar) after a 30 min incubation. Results are given as a percentage of the WS uptake in the absence of inhibitor.The concentration of $^{15}NO_2Na$ was 0.2 mM for all experiments (A, B and C).

In order to assess that $^{15}NO_2^-$ was assimilated after being up taken into the chloroplast, ^{15}N labelling was analysed in the NH_4^+ and in the amino acids present in the chloroplast pellets after 30 min of $^{15}NO_2^-$ incubation in the light. Interestingly, the % ^{15}N labelling was higher in both the NH_4^+ and the total free amino acids of the PII mutant chloroplasts compared to the wild-type (Fig. 3B).

Table 1
Expression analyses of At1g68570 in the PIIV1 mutant

Function	Sequence ID	Experiment 1		Experiment 2	
		Ratio PIIV1/WS	*P*-value	Ratio PIIV1/WS	*P*-value
PII protein	AT4G01900	0.17 ± 0.006	0	0.235 ± 0.034	0
POT family protein (NO_2^- transporter)	AT1G68570	1.075 ± 0.123	1	1.108 ± 0.025	1

Two biological repetitions of one experiment of CATMA microarrays were used to compare the expression of the NO_2^- transporter between the PII mutant and wild-type plants. The differential expression ratios are given and the *P*-value after the Bonferroni correction was used to determine which genes were statistically differentially expressed. A *P*-value of 0 indicates that the difference in gene expression was statistically significant ($P < 10^6$). *P*-value of 1 indicates that the difference in gene expression is not statistically significant.

3.2. NO_2^- uptake into isolated chloroplasts was not modified in the presence of inhibitors of photosynthesis and nitrogen metabolism

In order to better understand the observed NO_2^- uptake into isolated chloroplasts, the effect of some specific inhibitors was investigated. The addition of DCMU (an inhibitor of photosynthetic photosystem II electron transfer) significantly reduced the light-dependent NO_2^- uptake in both wild-type and PII mutant chloroplasts (Fig. 3C). Indeed, DCMU led to uptake values equivalent to dark-treated chloroplasts. In order to assess whether the higher NO_2^- uptake was related to NO_2^- assimilation by the isolated chloroplasts of the PII mutants, the effect of N assimilation inhibitors on light-dependent nitrite uptake was studied. The addition of MSO, or azaserine (inhibitors of glutamine synthetase and ferredoxin-dependent glutamate synthase, respectively) did not significantly reduce the difference in nitrite uptake between the PII mutant and WS chloroplasts (Fig. 3C).

The effects of NH_4^+ and α-ketoglutarate (αKG) addition on NO_2^- uptake were also studied since NH_4^+ has been described as an inhibitor of NO_3^- and NO_2^- uptake in *Synechocystis* and *Synechococcus* [29,30]. In cyanobacteria, the inhibitory effect of NH_4^+ on NO_2^- uptake is mediated via the PII protein that interacts and inhibits the NO_3^-/ NO_2^- transporter under low αKG conditions. However, in agreement with the previous observations of [16] no effect of NH_4^+ and αKG was observed on the light-dependent NO_2^- uptake by *Arabidopsis* chloroplasts and this was the case for both the wild-type and PII mutant genotypes (data not shown).

3.3. The expression of a high affinity NO_2^- transporter is not affected in PII mutants

Recently, the *Arabidopsis* gene At1g68570 has been shown to encode a chloroplastic nitrite transporter [20]. To examine if the observed increase in nitrite uptake in the PII mutants could be due to an increased level of this transporter, At1g68570 expression was analysed. The transcriptomic analyses of the PIIV1 mutant compared to the WS control indicated that the expression of the nitrite transporter was not altered in the mutant (Table 1). These observations were obtained from plants grown under different N-regimes as described in [11]. An unaltered At1g68570 expression in PII mutant rosette leaves was confirmed by RT-PCR (data not shown).

4. Discussion

Since its discovery in plants in 1998 [6], the only PII function identified to date is the regulation of NAGK activity; a function that has been conserved between higher plants and cyanobacteria. However, the plant PII protein probably undertakes further functions as is the case for the different PII proteins found in eubacteria and cynaobacteria. To date all known bacterial PII partners have no orthologs in the sequenced higher plant genomes (except for NAGK). Interestingly, we had already observed an increased sensitivity to NO_2^- toxicity of *Arabidopsis* PII mutant seedlings when grown in vitro [13]. This could be an indication of a role for PII in NO_2^- metabolism and/or perhaps NO_2^- uptake, a function already described for PII in cyanobacteria [29,30]. However, there is no homology between the cyanobacterial ABC-type NO_2^-/ NO_3^- transporter and the recently identified *Arabidopsis* chloroplastic NO_2^- transporter [20] and no orthologs of the *Chlamydomonas* NO_2^- transporter (NAR1 family) have been found so far in higher plants [19].

In this work, we have characterised a light-dependent NO_2^- uptake into isolated chloroplasts from wild-type and PII mutant rosette leaves. The kinetic parameters of the observed NO_2^- uptake are similar to previously published data [16] and they are in agreement with the characteristics of the above-mentioned high affinity NO_2^- transporter [20]. The NO_2^- uptake was observed using external NO_2^- concentrations that are compatible with estimated cytosolic levels according to [31]. Interestingly, a 30–40% higher NO_2^- uptake into PII mutant chloroplasts was found when compared to wild-type chloroplasts (Fig. 3A). Our observations could explain the increased NO_2^- sensitivity of the PII mutants that was correlated to higher NO_2^- contents in the plantlets grown in vitro on NO_2^- as the only nitrogen source [13]. The accumulation of NO_2^- was observed in the PII mutants only when exogenously supplied with NO_2^-. Such drastic conditions do not normally occur although NO_2^- accumulation has been observed in anoxic roots where NR is fully active but where Nir activity is impaired [14]. Since NO_2^- uptake into isolated chloroplasts appears to be more active in the absence of PII and considering that in vitro measured nitrite reductase activity was not affected in the PII mutants [13], an accumulation of chloroplastic NO_2^- could explain the higher toxicity of NO_2^-.

Interestingly, chloroplastic NO_2^- was light-dependent and required PSII electron transfer since both DCMU and a dark treatment led to a severely reduced NO_2^- uptake. No differences were observed between the PII mutants and their controls under dark or DCMU/light conditions, indicating that PII was not involved in NO_2^- uptake in the dark. Therefore, PII appears to be required to fine-tune chloroplastic NO_2^- uptake with respect to the metabolic and/or energetic status of the chloroplasts in the light. The entry of NO_2^- into the chloroplasts should be tightly regulated in order to not exceed the reduction capacity of the chloroplasts in higher plants. It is noteworthy to underline that NO_2^- up-

take into *Chlamydomas* chloroplasts appears to be regulated only under certain conditions, i.e. limiting nitrogen and carbon supply [32].

The NO_2^- entering the chloroplasts seemed to be partially assimilated since ^{15}N labelling was measured in both NH_4^+ and amino acids present in the pelleted chloroplasts (Fig. 3B). Moreover, NO_2^- assimilation seemed to vary in the same way as the NO_2^- uptake since the ^{15}N labelling was higher in the PII mutant. Conversely, chloroplastic NO_2^- uptake appeared to be independent on its assimilation by the GS/GOGAT pathway under our experimental conditions since inhibitors such as MSO and azaserine had no effect on nitrite uptake in both genotypes.

Taken together, our observations suggest PII is involved in the down regulation of NO_2^- uptake into *Arabidopsis* rosette leaf chloroplasts in the light. This function is reminiscent of the role of PII in cyanobacteria where it has been shown to regulate a nitrate/nitrite transporter. However, the exact PII partner involved in NO_2^- uptake regulation remains to be identified. The only chloroplastic high affinity NO_2^- transporter identified to date in *Arabidopsis* (At1g68570) is a candidate since its kinetic characteristics are in agreement with the observations obtained with the isolated chloroplasts. Since the transcript level of this chloroplastic nitrite transporter was not changed in the PII mutant, this suggests that chloroplastic NO_2^- uptake could be post-transcriptionally regulated by PII. However, a molecular interaction between PII and the NO_2^- transporter awaits validation.

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