

1 **Tomato roots exhibit *in vivo* glutamate dehydrogenase aminating capacity in response to excess**
2 **ammonium supply**

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Summary

In higher plants ammonium (NH_4^+) assimilation occurs mainly through the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway. Nevertheless, when plants are exposed to stress conditions, such as excess of ammonium, the contribution of alternative routes of ammonium assimilation such as glutamate dehydrogenase (GDH) and asparagine synthetase (AS) activities might serve as detoxification mechanisms. In this work, the *in vivo* functions of these pathways were studied after supplying an excess of ammonium to tomato (cv. Agora Hybrid F1) roots previously adapted to grow under either nitrate or ammonium nutrition. The short-term incorporation of labelled ammonium ($^{15}\text{NH}_4^+$) into the main amino acids was determined by GC-MS in the presence or absence of methionine sulfoximine (MSX) and azaserine (AZA), inhibitors of GS and GOGAT activities, respectively. Tomato roots were able to respond rapidly to excess ammonium by enhancing ammonium assimilation regardless of the previous nutritional regime to which the plant was adapted to grow. The assimilation of $^{15}\text{NH}_4^+$ could take place through pathways other than GS/GOGAT, since the inhibition of GS and GOGAT did not completely impede the incorporation of the labelled nitrogen into major amino acids. The *in vivo* formation of Asn by AS was shown to be exclusively Gln-dependent since the root was unable to incorporate $^{15}\text{NH}_4^+$ directly into Asn. On the other hand, an *in vivo* aminating capacity was revealed for GDH, since newly labelled Glu synthesis occurred even when GS and/or GOGAT activities were inhibited. The aminating GDH activity in tomato roots responded to an excess ammonium supply independently of the previous nutritional regime to which the plant had been subjected.

Keywords: Asparagine synthetase; glutamate dehydrogenase; ammonium nutrition; methionine sulfoximine; azaserine; isotope labelling

Abbreviations: AAT: aspartate aminotransferase; AS: asparagine synthetase; AZA: azaserine; GDH: glutamate dehydrogenase; GS: glutamine synthetase; MSX: methionine sulfoximine

INTRODUCTION

Nitrogen (N) is the primary nutrient for plant growth and its absorption by roots occurs predominantly in the form of nitrate (NO_3^-) or ammonium (NH_4^+) (Marschner, 2012). Irrespective of the N source, NH_4^+ ion is the final form of inorganic N assimilated by the plant cell and the precursor of all organic N compounds. Under normal growth conditions, the glutamine synthetase/glutamate synthase (GS/GOGAT; EC 6.3.1.2/EC 1.4.1.14) cycle is widely accepted as the primary ammonium assimilation pathway in higher plants (Lea and Miflin, 2010). Due to the reduced state of N, the energy consumed for NH_4^+ assimilation is less than that needed for NO_3^- assimilation. However, NH_4^+ is a paradoxical metabolite since it becomes toxic for the plant when it is present as sole N source, especially at high concentrations (Bittsánszky et al., 2015; Esteban et al., 2016). Common toxicity symptoms include ion imbalance, leaf chlorosis, hormone deregulation, cytosolic pH disturbance, decrease in net photosynthesis and growth, changes in levels of metabolites such as amino acids, organic acids and carbohydrates, and decreased resistance to pathogen infection (Britto and Kronzucker, 2002; Gupta et al., 2013). So, it is imperative that the plant cell controls ammonium homeostasis to avoid its excessive accumulation. To meet this goal, it may be necessary to increase the NH_4^+ assimilation capacity, either by enhancing the principal assimilatory route (GS/GOGAT) or by activating other accessory pathways, such as those involving the enzymes asparagine synthetase (AS) and glutamate dehydrogenase (GDH).

Asparagine synthetase (AS, EC 6.3.5.4.) catalyses the synthesis of asparagine (Asn) from glutamine (Gln) and aspartate (Asp). However, it has been suggested that AS can also directly incorporate free NH_4^+ into Asn under certain types of stress (Gaufichon et al., 2016) acting thus as a complement to the GS/GOGAT pathway in primary N assimilation. In fact, substantial accumulation of Asn was observed in response to NH_4^+ supply in roots of rice (Kawachi et al., 2002), wheat (Setién et al., 2013) and tomato (Vega-Mas et al., 2017) as well as the overexpression of AS genes in rice (Ohashi et al., 2015) and Arabidopsis (Wong et al., 2004), strongly suggesting that AS may contribute to NH_4^+ assimilation.

In vitro kinetic analysis of the main N assimilating enzyme activities has been widely studied (Lea and Miflin, 2010; Gaufichon et al., 2016), but elucidating the *in vivo* functioning of the different pathways can be more complicated. This is especially true for enzymes that function reversibly or are linked to others, thus making it difficult to assign a definite metabolic role. The GDH enzyme (EC 1.4.1.2) is a classic example of a long-lasting controversy regarding its physiological function in carbon (C) and N metabolism. GDH catalyses *in vitro* both the reductive amination of 2-oxoglutarate and the oxidative deamination of Glu. It is a hexameric enzyme formed by the association of two different subunits, α and β , that results in seven different GDH isoenzymes (Turano et al., 1997). The two subunits are encoded by different genes, whose quantity is variable for different species. In the tomato genome four genes that encode for GDH have been reported by Ferraro et al. (2012), three for the α -subunit (slgdh -NAD;A1–3) and one for the β -subunit (slgdh -NAD;B1). In the case of Arabidopsis, a third γ subunit, encoded by *gdh3*, has been identified (Marchi et al., 2013). The GDH enzyme shows differential regulation depending on the developmental stage, organ or tissue type, nutritional conditions or abiotic agents (Melo-Oliveira et al. 1996; Restivo, 2004; Miyashita and Good, 2008; Lehmann et al., 2011; Ferraro et al., 2012). In particular, the enhancement of GDH expression and/or *in vitro* activity has been widely described under excess NH_4^+ accumulation (Dubois et al., 2003; Frechilla et al., 2002; Tercé-Laforgue et al., 2004; Setién et al., 2014; Sarasketa et al., 2016), suggesting an aminating *in vivo* role for GDH under these conditions.

Isotopic labelling of substrates is a convenient method for probing metabolic pathways *in vivo*, and the use of ^{15}N -Glu and $^{15}\text{NH}_4^+$ allows an assessment of the *in vivo* activity of GDH (Mesnard and Ratcliffe, 2005). The detection of the ^{15}N stable isotope is generally performed by gas chromatography-mass spectrometry (GC-MS) and/or nuclear magnetic resonance (NMR) (Ratcliffe and Shachar-Hill, 2001), both of which permit the direct determination of ^{15}N incorporation into amino acids. Both techniques have been widely applied in the physiological characterization of GDH enzyme and other ammonium assimilating pathways, often in conjunction with specific inhibitors for N assimilatory pathways and/or mutant plants lacking or overexpressing particular enzyme activities. Several labelling studies have shown that under standard growing conditions GDH operates in the deaminating direction, Glu catabolism being its primary role rather than being

involved in the assimilation of NH_4^+ (Aubert et al., 2001; Glevarec et al., 2004; Purnell and Botella, 2007; Labboun et al., 2009; Watanabe et al., 2011; Tercé-Laforgue et al., 2013). On the other hand, evidence for an *in vivo* aminating GDH activity has also been obtained by isotopic labelling of amino acids under conditions favouring NH_4^+ accumulation (Skopelitis et al., 2006). Nevertheless, in a later work on the anodic homohexamer of tobacco plants grown under mixed nutrition, only low *in vivo* aminating activity was detected for GDH-isoenzyme 7, while it strongly deaminated Glu (Skopelitis et al., 2007).

A wide range of plant species grown with ammonium as sole N source have shown a more active N assimilatory metabolism with respect to plants grown under nitrate nutrition (Lasa et al., 2002; Setién et al., 2013, 2014; Vega-Mas et al., 2015; 2017; Sarasketa et al., 2016). Previous studies on tomato plants (cv. Agora Hybrid F1, Vega-Mas et al., 2017) have shown that the huge NH_4^+ accumulation in roots under high external ammonium supply (15 mM) was accompanied by enhanced amino acid contents. In this regard, the root plays a primary role as the first ammonium-responsive organ, as it is faced with the challenge of either sequestering the ion in the vacuole or promoting its fixation into organic compounds (Britto and Kronzucker 2002). Interestingly, roots often show higher GDH activities than shoots (Frechilla et al., 2002; Skopelitis et al., 2007; Martínez-Andújar et al., 2013; Setien et al., 2013, 2014; Vega-Mas et al., 2017), suggesting that this organ may be the main candidate for a detoxifying GDH role. Surprisingly, the N source had no effect on the *in vitro* activities of GS and GDH in tomato roots (Vega-Mas et al., 2017), indicating the need for *in vivo* studies to elucidate N metabolism in tomato roots exposed to a high NH_4^+ supply.

Based on previous results, we hypothesized (1) that an enhanced *in vivo* aminating response of GDH would occur in the roots of plants grown under ammonium nutrition, while such a role for GDH would not be expected in a nitrate-based nutrition; and (2) that the detoxifying response of ammonium-grown tomato roots might be accompanied by the participation of AS enzyme in the direct assimilation of NH_4^+ into Asn. The objective of this investigation was to elucidate the *in vivo* function of these potential accessory ammonium assimilatory pathways (GDH and AS) under high ammonium supply conditions in roots of tomato plants previously adapted to grow on either nitrate or ammonium nutritional regimes. For this purpose, an experiment was performed to determine the incorporation of $^{15}\text{NH}_4^+$ into the main amino acids in tomato roots grown under

nitrate or ammonium nutrition, using an experimental approach where GS and/or GOGAT enzymatic activities were inhibited by adding specific metabolic inhibitors. Thus, under these conditions, the contribution of the potential alternative routes for ammonium assimilation was analyzed, as well as their dependence on the previous nutritional status to which the plant was adapted (nitrate or ammonium nutrition).

MATERIALS AND METHODS

Growth conditions

Tomato plants (*Solanum lycopersicum* cv. Agora Hybrid F1) were grown in a controlled glasshouse under a 16-h light (24°C) and 8-h darkness (21°C) regime. After germination, seedlings were transferred to 1-L pots with perlite:vermiculite (1:2; v:v) and irrigated three times per week with nutrient solution containing macronutrients (1 mM KH₂PO₄, 1 mM MgSO₄, 0.25 mM CaCl₂, 50 µM KCl, 5 mM CaCO₃, 0.1 mM FeEDTA) and micronutrients (0.1 mM H₃BO₃, 1.5 µM CuSO₄, 10 µM MnSO₄, 0.075 µM Na₂MoO₄, 0.1 mM Na₂O₃Si, 2 µM ZnSO₄). The pH of the nutrient solution was adjusted to 5.8. Plants were grown with two different nitrogen sources, nitrate or ammonium (as Ca(NO₃)₂ or (NH₄)₂SO₄, respectively), at a concentration of 15 mM N. To equilibrate the Ca concentration in ammonium-fed plants 7.5 mM CaSO₄ were supplied. After 28 days of growing under the two nitrogen regimes, 15 plants per treatment were harvested 3 h after the onset of the light period. Leaves, stem and roots were separated and individually weighed. Roots were carefully washed with deionized water in order to remove any substrate trace. Roots from three plants were immediately frozen in liquid nitrogen and stored at -80°C for later ammonium content and enzyme analyses. The rest of the roots was used for further ¹⁵N-labelling experiments.

¹⁵N-labelling experiments

The lower young part of each tomato root was cut off into several 1 cm segments, and a pool of root segments was established from 12 plants for each nutrition. Immediately, 1 g FW of root tissue from the ammonium- and nitrate-fed plants was transferred to 50 mL flasks and submerged into 10 mL of a buffer containing 50 mM glucose and 5 mM CaCO₃, keeping the vials open and with continuous shaking to ensure a

proper oxygenation of the root tissue. The roots segments were subjected to a 1 h pre-incubation either with the addition or absence of the metabolic inhibitors methionine sulfoximine (MSX) and azaserine (AZA), in order to specifically inhibit GS and GOGAT activities, respectively. A preliminary experiment was carried out to establish the optimal MSX concentration (2 or 5 mM). In view of the only slightly greater inhibitory effects of 5 mM MSX, 2 mM MSX was chosen for the labelling experiment, and the concentration of AZA was established at 1.75 mM. As a result, a total of four different pre-incubation treatments were applied to root segments of either ammonium- or nitrate-fed plants as follows (Supplementary Fig. S1): (1) control incubation without inhibitors; (2) incubation with 2 mM MSX; (3) incubation with 1.75 mM AZA; (4) incubation with 2 mM MSX and 1.75 mM AZA. After this 1 h pre-incubation, 15 mM labelled ammonium was added to all the incubation solutions in the form of $(^{15}\text{NH}_4)_2\text{SO}_4$ (99% ^{15}N) to evaluate the root ammonium assimilation capacity. Root segments were collected before the labelled substrate addition (time 0) and after 30 and 180 min following the addition of $^{15}\text{NH}_4^+$; samples were kept at -80°C until analysis.

Ammonium content and enzyme activities

Ammonium content was determined by using the colorimetric method based on the phenol hypochlorite assay (Berthelot reaction) in aqueous extractions from 25 mg of frozen root powder as Vega-Mas et al. (2015). The activities of the enzymes GS, NADH-GOGAT, NADH-GDH, NAD-GDH and NADP-ICDH were spectrophotometrically determined from 50 mg of frozen root powder as described by Sarasketa et al. (2016).

RNA extraction and qPCR

RNA was extracted from 50 mg of frozen roots with the Nucleospin RNA plant kit (Macherey-Nagel), which includes DNase treatment, and 1.5 μg of RNA was retro-transcribed into cDNA (PrimeScriptTM RT; Takara Bio Inc.). qPCR was performed using SYBR Premix ExTaqTM (Takara Bio Inc.) in a Step One Plus Real Time PCR System (Applied Biosystems). The primers and qPCR conditions for the amplification of Slgdh-NAD;B1, Slgdh-NAD;A1-A3 genes as well as the housekeeping gene and Rpl2 (encoding the ribosomal

protein large subunit 2) were described by Ferraro et al., (2012). Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ equation proposed by Livak and Schmittgen (2001).

Amino acid extraction

Aliquots of 50 mg FW frozen root were extracted in 500 μ L methanol to which 0.043 μ M norvaline was added as internal reference for amino acid determination by GC-MS. Homogenates were centrifuged for 10 min at 16,000 g and 200 μ L of supernatant were taken to evaporation in a SpeedVac. For the amino acid derivatization with tert-butyldimethylsilyl (TBDMS) the resulting pellet was resuspended with 25 μ L pyridine and incubated at 37°C, shaking at 900 rpm for 30 min. Then, 35 μ L TBDMS were added and the mixture was incubated at 65°C, shaking at 900 rpm for 30 min. The supernatant was transferred to new glass vials to carry out the amino acid determination by GC-MS.

Amino acid and ^{15}N -labelling quantification

The results obtained with GC-MS were first analysed with the ChemStation (Agilent) chromatography software after normalization with the MetAlign program (<http://www.MetAlign.nl>). The complete amino acid profile was quantified in root samples incubated without inhibitors and harvested just before the addition of labelled ammonium (time 0). For the quantification of label incorporation, the intensity of ionization fragments for each amino acid was determined using a macro designed for that purpose, and MSCorr was used to correct for the natural abundance of the isotopes of elements other than N (Wahl et al., 2004).

When amino acids are not labelled, their quantity is given by the molecular peak ($m+0$). When amino acids are labelled, the quantity is given by ($m+1$) isotope peak for a single labelled N atom and by ($m+2$) for two labelled N atoms. In this case, ($m+1$) and ($m+2$) are mass isotopomers, and fractional enrichment values were calculated as follows:

For single labelled amino acids: ^{15}N -amino acid (%) = $(m+1) / [(m+0)+(m+1)] \times 100$

For double labelled amino acids: ^{15}N -amino acid (%) = $(m+2) / [(m+0)+(m+1)+(m+2)] \times 100$

Since the amides Gln and Asn have two N atoms, they can be single (m+1) or double labelled (m+2); so, for both amides, the total enrichment is given by the sum of (m+1) and (m+2) isotope peaks.

Statistical analysis

Data analyses were carried out using IBM SPSS 20.0 software (Armonk, NY: IBM Corp.). Comparison of means was carried out by *t*-student analysis. Details of statistical analyses and significance levels are presented in the figure legends.

RESULTS

Plant growth and internal ammonium content in response to N nutrition

Tomato plants grown under ammonium nutrition showed a decreased biomass production for leaves, stem and roots compared to nitrate nutrition (Supplemental Fig. 2A), reflecting the growth impairment caused by ammonium stress conditions. In agreement with this, the internal ammonium content in the root increased under ammonium nutrition (Supplemental Fig. 2B). In roots subjected to the labelling assay, the 1 h pre-incubation with the metabolic inhibitors MSX and AZA did not caused any significant change in the internal ammonium contents with respect to the control samples, with a mean value of $1.57 \pm 0.06 \mu\text{mol NH}_4^+ \text{g}^{-1} \text{FW}$ and $2.44 \pm 0.07 \mu\text{mol NH}_4^+ \text{g}^{-1} \text{FW}$ for nitrate and ammonium nutrition, respectively. The addition of 15 mM $^{15}\text{NH}_4^+$ equalled the internal ammonium contents for both N regimens, showing a mean value of $8.49 \pm 0.12 \mu\text{mol NH}_4^+ \text{g}^{-1} \text{FW}$ at 30 min and $18.41 \pm 0.34 \mu\text{mol NH}_4^+ \text{g}^{-1} \text{FW}$ at 180 min after the addition of labelled substrate.

Enzyme activities in response to metabolic inhibitors.

The activities of the main ammonium assimilation pathways were determined in root segments subjected to the incubation assay (Supplemental Fig. 3). After the 1 h pre-incubation the presence of the MSX decreased GS activity by 86-90% and AZA inhibited GOGAT activity by 82-100%. The inhibition was

maintained over the whole incubation period. In contrast, NADH-GDH, NAD-GDH and NADP-ICDH were barely affected by the presence of the inhibitors or by the previous N regimen of the root.

Amino acid profile in nitrate and ammonium-fed roots

The individual amino acid profile was analysed in control roots segments collected immediately prior to the addition of ^{15}N labelling. The most abundant amino acids were the amides Asn and Gln, and their contents were 4.4 and 8 times higher, respectively, for ammonium nutrition (with values of $12.5 \mu\text{mol g}^{-1}$ FW and $7.7 \mu\text{mol g}^{-1}$ FW, respectively) relative to nitrate nutrition (Fig. 1). Glu, Pro, Thr and Ser were the next most abundant amino acids, ranging between 263.7 and 928.8 nmol g^{-1} FW. Among these four, only the Glu content showed a significant difference between ammonium and nitrate nutrition, being 2.6-times higher for ammonium-fed roots. No differences between both N nutrition types were observed in the content of the other less abundant amino acids, whose contents ranged between 49.8 and 303.6 nmol g^{-1} FW (Fig. 1). The only exception was Asp, whose content in ammonium-fed roots was double relative to nitrate nutrition.

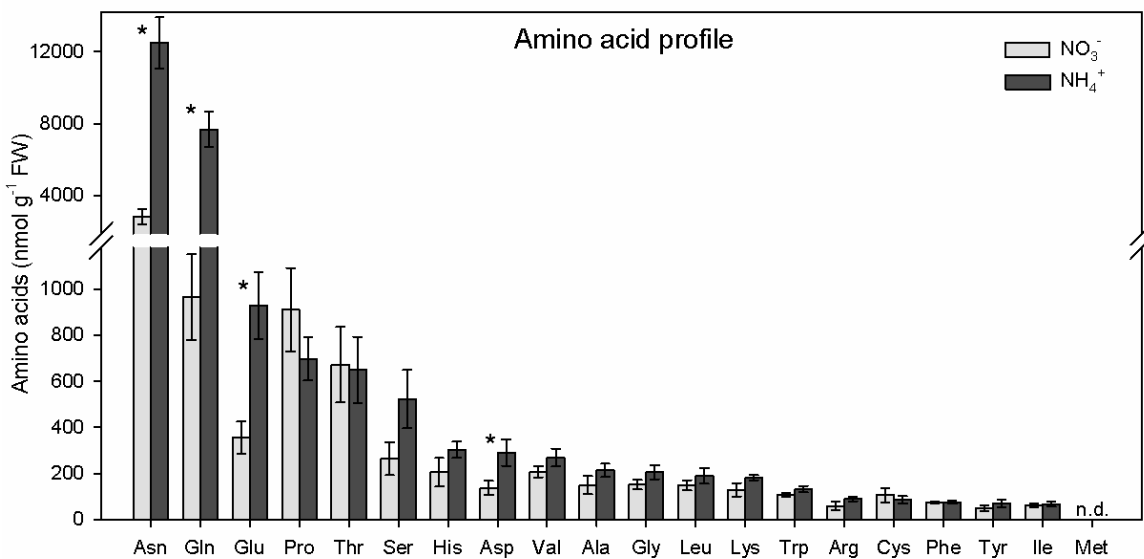


Figure 1. Individual amino acid contents in roots of tomato plants grown under nitrate (grey) or ammonium (black) nutrition. Significant differences between N sources ($p < 0.05$) are indicated by an asterisk (*). Values represent mean \pm SE ($n=6$).

Expression analyses of GDH genes.

The expression patterns for the four GDH genes encoding for α -subunit (slgdh-NAD;A1, slgdh-NAD;A2, slgdh-NAD;A3) and β -subunit (slgdh-NAD;B1) were determined in tomato roots (Fig. 2). The expression of the Slgdh-NAD;B1 gene was predominant in the root and was induced by the addition 15 mM $^{15}\text{NH}_4^+$. The expression of Slgdh-NAD;A1 was higher in roots of plants grown under ammonium nutrition compared to nitrate nutrition (Fig. 2), but neither Slgdh-NAD;A1 nor Slgdh-NAD;A2 genes were affected by $^{15}\text{NH}_4^+$ supply. As previously described by Ferraro et al., (2012), slgdh-NAD;A3 gene expression was not detected in tomato roots.

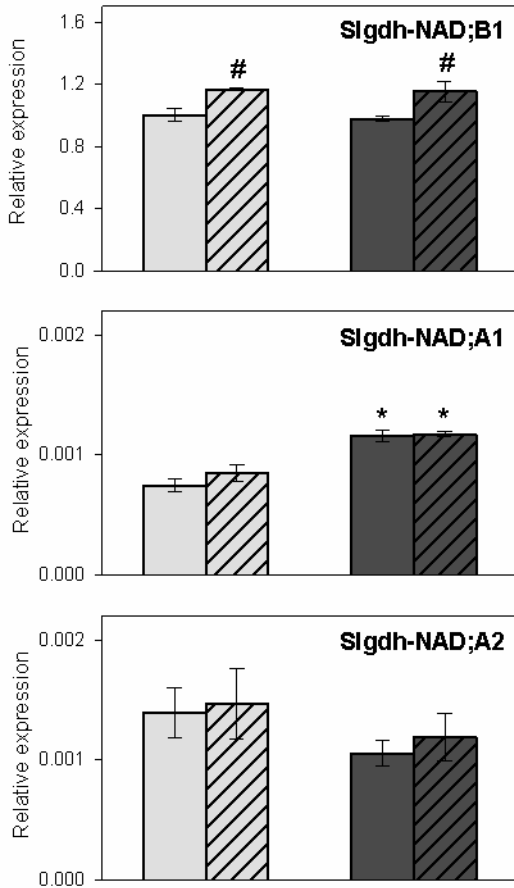


Figure 2. Relative expressions of Slgdh-NAD;B1, Slgdh-NAD;A1, and Slgdh-NAD;A2 genes in roots of tomato plants grown under nitrate (grey) or ammonium (black) nutrition. The striped bar indicates the relative expression 30 min after the addition of 15 mM $^{15}\text{NH}_4^+$ to the incubation medium. The mean value of Slgdh-NAD;B1 for nitrate-fed roots was used as the control sample for the relative gene expression calculation. Significant differences ($p < 0.05$) between N sources are indicated by asterisk (*), while significant differences after $^{15}\text{NH}_4^+$ supply are indicated by hashtag (#). Values represent mean \pm SE (n=6).

283 *Kinetic analysis of $^{15}\text{NH}_4^+$ incorporation into amino acids*

284 In order to study the first steps of ammonium assimilation, the incorporation of externally supplied
285 $^{15}\text{NH}_4^+$ into Glu, Asp, Gln and Asn was analysed, since these were the only amino acids that showed different
286 contents between the two N nutrition types. The incorporation of the label into each of these amino acids was
287 expressed as fractional enrichment (Fig. 3). Positive and negative enrichment values lower than 1% were
288 below the detection limit of the technique and so they were not considered to be significant. Higher enrichment
289 values were observed in roots of plants grown under nitrate nutrition. However, since the enrichment values
290 are dependent on the individual amino acid contents of the root, and since the initial amino acid pools have
291 shown to be very different for nitrate or ammonium nutrition, this fact must be taken into account when
292 comparing the two nutritional conditions.

293 In the roots incubated without inhibitors (control samples), the highest fractional enrichments were
294 shown by ^{15}N -Glu and ^{15}N -Asp under both nutritional conditions, followed by ^{15}N -Gln. Enrichment values
295 increased up to the 180 min for ^{15}N -Glu, ^{15}N -Asp and ^{15}N -Gln, although to a lower extent from 30 to 180 min
296 with respect to the first 30 min (Fig. 3). In the case of Asn, only non-significant background enrichment values
297 were detected at 30 min, but the enrichment of ^{15}N -Asn increased by 180 min under both nutritional conditions.
298 Both nitrate and ammonium-grown roots responded to the inhibitors treatments in a similar way, especially in
299 the first period of incubation (Fig. 3). The addition of MSX completely prevented the incorporation of ^{15}N -label
300 into Gln and Asn in the first 30 min, indicating that GS activity was completely inactive. At 180-min, the
301 absence of ^{15}N -Gln and ^{15}N -Asn formation was maintained in presence of MSX, with only background labelling
302 values that were higher under nitrate nutrition. With MSX+AZA, both nitrate and ammonium-fed roots showed
303 low background values for ^{15}N -Gln and ^{15}N -Asn in the whole incubation period. In contrast, when AZA was
304 added alone, ^{15}N -Gln enrichment at 30 min was equal to the control samples, although depleted at 180 min.
305 The addition of any inhibitor reduced the enrichment values of ^{15}N -Glu to a half at 30 min. After this time point,
306 low ^{15}N label incorporation occurred into ^{15}N -Glu. So, at 180 min, the reduction in the enrichment with respect
307 to the control was maintained, except when MSX was added alone to ammonium-grown roots that led to even

lower enrichment values. The formation of ^{15}N -Asp in presence of inhibitors showed a similar trend, with more than halfway reduction in its enrichment with respect to the control.

Double labelling of amides

To explore the distribution of the label into the amides, single and doubly labelled ^{15}N -Gln or ^{15}N -Asn were separately analyzed. The fraction of single (corresponding to $m+1$) or double (corresponding to $m+2$) labelled amine is presented in Table 1, together with the ratio between $m+2$ and $m+1$.

Under both N nutrition types, a third of ^{15}N -Gln appeared to be double labelled in the control roots after 30 min, but the $m+2/m+1$ ratio increased by 180 min (Table 1). For Asn, the double labelled isotopomer was only detected at 180 min. Enrichment values were higher for double labelled Asn with respect to single labelled under nitrate nutrition, while the enrichment was equally distributed under ammonium nutrition. After the addition of MSX, neither single nor double labelled amides were significantly detected in ammonium-grown roots (Table 1). In contrast, in nitrate-grown roots both single and double labelled Gln and Asn could be detected at 180 min (Table 1). In the presence of AZA, the ratio between double and single labelled Gln was lower than in the control samples under both types of nutrition across the whole incubation period.

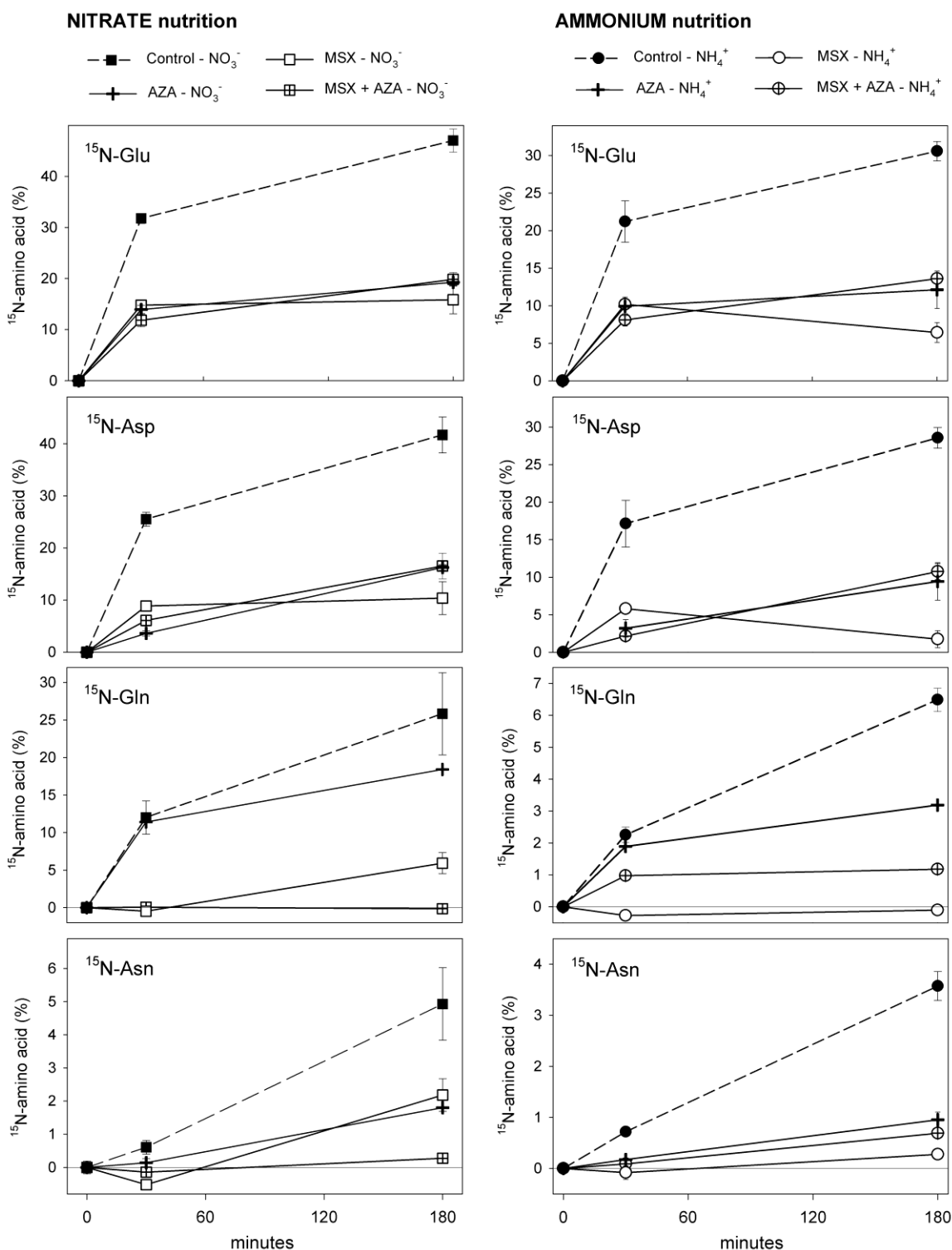


Figure 3. Kinetic analysis of $^{15}\text{NH}_4^+$ incorporation into amino acids, expressed as fractional enrichment, in roots of tomato plants grown under nitrate (squares) or ammonium (circles) nutrition. After 1 h pre-incubation without or with inhibitors (MSX, AZA or both), 15 mM $^{15}\text{NH}_4^+$ was added and root samples were taken after 0, 30 or 180 min. Values represent mean \pm SE (n=3).

328 **Table 1.** Fractional enrichment (%) of single labelled (m+1) or double labelled (m+2) amides (Gln and Asn) at 30 and 180 min after the addition of $^{15}\text{NH}_4^+$ to roots of plants grown
329 under nitrate or ammonium nutrition incubated without (control) or with inhibitors (MSX and/or AZA). Ratios of (m+2) to (m+1) values are given when the sum of both fractional
330 enrichment value is above the confidence limit (>1%).

| | | NITRATE nutrition | | | | | | AMMONIUM nutrition | | | | | | | | | | | | | | | | | |
|-------|-----------|-------------------|----|--------------|-------------|------|---|--------------------|---------|--------------|-------------|------|---------|--------------|---|--------------|--|------|--|--------------|--|--------------|--|------|--|
| | | t = 30 min | | | t = 180 min | | | t = 30 min | | | t = 180 min | | | | | | | | | | | | | | |
| Amide | Treatment | m+1 | | m+2 | m+2/m+1 | m+1 | | m+2 | m+2/m+1 | m+1 | | m+2 | m+2/m+1 | | | | | | | | | | | | |
| | | % | SE | % | | SE | % | SE | | % | SE | % | | SE | % | SE | | | | | | | | | |
| Gln | Control | 8.24 ± 1.68 | | 3.77 ± 0.55 | | 0.46 | | 11.40 ± 2.33 | | 14.43 ± 3.14 | | 1.27 | | 1.57 ± 0.21 | | 0.69 ± 0.08 | | 0.44 | | 3.58 ± 0.20 | | 2.91 ± 0.29 | | 0.81 | |
| | MSX | -0.35 ± 0.18 | | -0.13 ± 0.09 | | - | | 3.80 ± 0.80 | | 2.06 ± 0.60 | | 0.54 | | -0.16 ± 0.05 | | -0.11 ± 0.01 | | - | | -0.02 ± 0.04 | | -0.07 ± 0.01 | | - | |
| | AZA | 10.20 ± 0.20 | | 0.96 ± 0.09 | | 0.09 | | 15.06 ± 0.03 | | 3.50 ± 0.01 | | 0.23 | | 1.56 ± 0.01 | | 0.25 ± 0.02 | | 0.16 | | 2.48 ± 0.03 | | 0.54 ± 0.05 | | 0.22 | |
| | MSX+AZA | 0.09 ± 0.11 | | -0.03 ± 0.05 | | - | | -0.04 ± 0.06 | | -0.09 ± 0.03 | | - | | 0.81 ± 0.01 | | 0.13 ± 0.02 | | - | | 0.97 ± 0.03 | | 0.13 ± 0.00 | | - | |
| Asn | Control | 0.45 ± 0.06 | | 0.34 ± 0.05 | | - | | 2.12 ± 0.41 | | 2.81 ± 0.68 | | 1.32 | | 0.43 ± 0.03 | | 0.29 ± 0.06 | | - | | 1.78 ± 0.12 | | 1.79 ± 0.18 | | 1.01 | |
| | MSX | -0.48 ± 0.09 | | -0.06 ± 0.04 | | - | | 1.26 ± 0.27 | | 0.83 ± 0.25 | | 0.66 | | 0.00 ± 0.11 | | -0.07 ± 0.03 | | - | | 0.37 ± 0.04 | | -0.09 ± 0.02 | | - | |
| | AZA | 0.11 ± 0.08 | | 0.03 ± 0.05 | | - | | 1.09 ± 0.01 | | 0.74 ± 0.02 | | 0.68 | | 0.13 ± 0.03 | | 0.04 ± 0.06 | | - | | 0.65 ± 0.05 | | 0.30 ± 0.10 | | 0.47 | |
| | MSX+AZA | -0.06 ± 0.04 | | -0.08 ± 0.06 | | - | | 0.22 ± 0.00 | | 0.06 ± 0.02 | | - | | 0.10 ± 0.03 | | -0.03 ± 0.03 | | - | | 0.66 ± 0.04 | | 0.03 ± 0.01 | | - | |

DISCUSSION

Tomato roots adapted to ammonium and nitrate nutrition display similar capacity to assimilate exogenous ammonium

The efficient assimilation of the high NH_4^+ accumulations when the plant is subjected to ammonium nutrition is crucial in order to maintain ammonium homeostasis in the cell (Britto and Kronzucker, 2002; Bittsánszky et al., 2015). Following primary ammonium assimilation through the GS/GOGAT pathway, Glu and Gln act as amine and amide group donors for the reactions performed by aspartate aminotransferase (AAT; EC 2.6.1.1) and AS, respectively. Moreover, in plants growing under large and continuous N supplies, Gln and Asn perform an important role as N storage and/or long-distance N transport because of their high N:C ratio (Lea and Mifflin, 2010). In this regard, the huge contents of these amides observed in ammonium-fed roots, and to a lesser extent Glu and Asp (Fig. 1), would indicate they contribute to the detoxification and storage of excess NH_4^+ in tomato roots (Ariz et al., 2011; Setién et al., 2013, 2014; Wang et al/Vega-Mas et al., 2017). Note that the well described fine regulation of Glu contents in plants (Forde and Lea, 2007) may have promoted its diversion to other routes such as the synthesis of γ -aminobutyric acid (GABA), a key signalling metabolite responding to abiotic and biotic stresses as well as changes in C:N metabolism (Bouche and Fromm, 2004).

The incubation of the roots with $^{15}\text{NH}_4^+$ was expected to be a challenge particularly for the plants grown under nitrate nutrition since, in principle, they are not adapted to the presence of a high ammonium content inside the cell (Vega-Mas et al., 2017). Nevertheless, isotopic ^{15}N labelling experiments showed that $^{15}\text{NH}_4^+$ was rapidly incorporated into the major amino acids when GS/GOGAT were active, with a similar labelling pattern for roots grown under both N nutrition types (Fig. 3). This indicates that the nitrate-adapted roots were as efficient as the ammonium-grown roots in the assimilation of externally supplied $^{15}\text{NH}_4^+$, and the capacity of tomato roots to assimilate exogenously provided NH_4^+ was independent of the adaptation to the previous nutritional regimen. Indeed, the internal ammonium content in the root became equal under both N regimens in response to 15 mM $^{15}\text{NH}_4^+$ supply. The $^{15}\text{NH}_4^+$ primarily incorporated into ^{15}N -Gln by GS would

have been rapidly transferred into the ^{15}N -Glu pool, and later transaminated to ^{15}N -Asp as they are highly enriched under both N conditions (Fig. 3). As expected, ^{15}N -Glu acted as further ammonium receptor to form double labelled ^{15}N -Gln; and both ^{15}N -Gln and ^{15}N -Asp led to double labelled ^{15}N -Asn formation after 3 h incubation (Table 1). Therefore, the primary ammonium assimilation pathways may be jointly acting in an efficient manner to avoid a toxic accumulation of ammonium in tomato roots regardless of the previous nutrition type. Nevertheless, the role of GDH in the synthesis of ^{15}N -Glu could not be completely discounted either in ammonium or nitrate-fed tomato roots, since Agora Hybrid F1 tomato roots have shown to present a high *in vitro* GDH activity under both nutritional conditions (Supplemental Fig. 3; Vega-Mas et al., 2017).

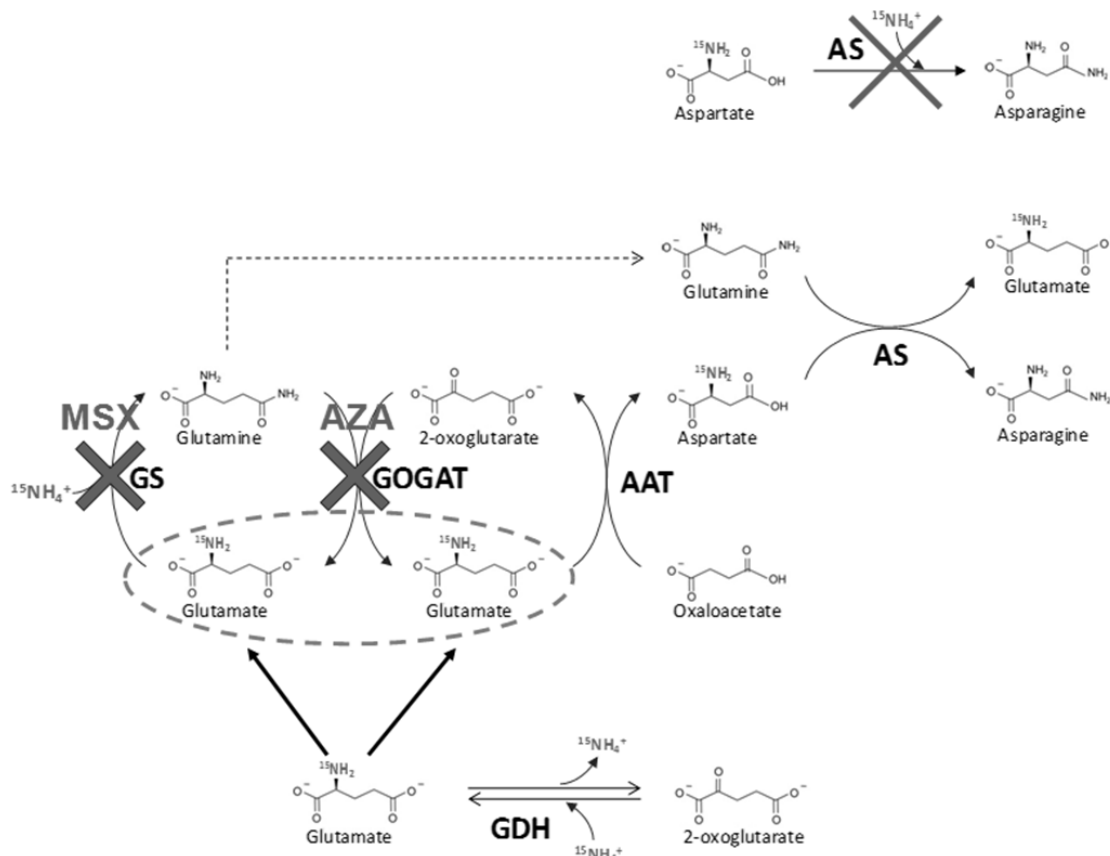


Figure 4. Tomato root $^{15}\text{N}_4^+$ assimilation in response to GS and GOGAT inhibition by MSX and AZA. The potential contribution of GDH enzyme to the synthesis of ^{15}N -Glu is indicated, while the ammonium-dependent AS activity is discarded.

The GDH isoenzyme pattern is known to be sensitive to the C/N status of plants and exposure to high ammonium (Masclaux-Daubresse et al. 2005; Melo-Oliveira et al. 1996; Skopelitis et al. 2006; Sarasketa et al., 2016). Here, the expression of Slgdh-NAD;A1 was up-regulated in roots of plants adapted to ammonium nutrition (Fig. 2), in line with results on tobacco where ammonium-based nutrition induced the expression of α -enriched isoenzymes (Skopelitis et al., 2006). The increased ammonium supply during the labelling experiment quickly induced Slgdh-NAD;B in contrast to the α -subunit encoding GDH induction previously described (Turano et al. 1997). However, since Slgdh-NAD;B is the dominant transcript in tomato roots (Fig. 2; Ferraro et al., 2012), it can be assumed this gene is the main driver of the ammonium-induced response in this tissue.

Is there an alternative $^{15}\text{NH}_4^+$ incorporation pathway into amino acids when GS is inhibited?

The application of the glutamate analogue methionine sulfoximine (MSX) specifically inhibits the activity of GS enzyme, so preventing any incorporation of $^{15}\text{NH}_4^+$ into Gln (Oaks et al., 1998). This fact was confirmed in our tomato roots in Supplemental Fig. 3 and is in agreement with similar studies (Glevarec et al., 2004; Skopelitis et al., 2006; Wang et al., 2016). Besides, no ^{15}N -Gln was detected after 30 min in the presence of MSX (Fig. 3), indicating that strong inhibition of GS activity had occurred. Additionally, the lack of labelled substrate (^{15}N -Gln) for GOGAT would have reduced its activity (Supplemental Fig. 3) and limited the formation of ^{15}N -Glu (Fig. 3). Indeed, several studies carried out in leaves suggest that GS catalyses the sole efficient ammonium entry as the incorporation of ^{15}N -label into amino acids was substantially decreased by MSX (Glevarec et al., 2004; Masclaux-Daubresse et al., 2006; Labboun et al., 2009; Watanabe et al., 2011). However, inhibition of GS by MSX was not an absolute impediment for tomato roots to incorporate $^{15}\text{NH}_4^+$ into Glu, and to a lesser extent into Asp (Fig. 3). This newly synthesized ^{15}N -Asp could be available as acceptor for the direct incorporation of $^{15}\text{NH}_4^+$ by AS. However, the absence of ^{15}N -Asn whenever Gln synthesis was inhibited by MSX (Fig. 3) indicates that no free $^{15}\text{NH}_4^+$ was fixed into Asn. Thereby, AS would behave as a Gln-dependent activity in Agora Hybrid F1 tomato roots (Fig. 4). In rice roots a similar conclusion was obtained by

Kawachi et al. (2002), as the inhibition of GS activity impaired the ammonium-inducible accumulation of AS protein.

Thus, having ruled out the participation of AS in the direct assimilation of free NH_4^+ , the fact that label was incorporated into ^{15}N -Glu when GS was inhibited suggests that the early assimilation of high $^{15}\text{NH}_4^+$ contents must have occurred through some other accessory assimilatory pathway, such as a GDH aminating activity (Fig. 4). An *in vivo* aminating role for GDH in both leaves and roots has been previously demonstrated by GC-MS in tobacco and grapevine plants growing under physiological conditions that resulted in excess ammonium accumulation (Skopelitis et al., 2006) and in transgenic tobacco plants overexpressing the ammonium-responding *gdh* gene (Skopelitis et al., 2007). So, the potential GDH assimilating capacity would be expected to be more significant in plants grown under ammonium nutrition and hence, previously adapted to internal ammonium excess; while minimal or no labelling of amino acids might be expected in the absence of GS activity in nitrate-fed roots. Surprisingly, the early response in the synthesis of amino acids to the addition of $^{15}\text{NH}_4^+$ was equal in roots subjected to both nutrition types, with only 50% reduction in the labelling of ^{15}N -Glu as consequence of GS inhibition (Fig. 3). So, in the light of these results, a possible aminating capacity could be attributed to GDH in response to the high exogenous NH_4^+ supply, regardless of the previous nutrition type to which the plant was subjected.

It is known that an efficient assimilation of ammonium requires the provision of carbon skeletons, 2-oxoglutarate being a key metabolite for the synthesis of amino acids (Sweetlove et al., 2010). Indeed, under excess ammonium accumulating conditions, an enhancement of TCA cycle activities has been observed, together with anaplerotic activities, in the Agora Hybrid F1 tomato cultivar (Setien et al., 2014, Vega-Mas et al., 2017). In this regard, many of the previous ^{15}N -labeling studies that have provided evidence of a deaminating role of GDH were focused on C limiting conditions, where the provision of 2-oxoglutarate by GDH would be necessary to fuel mitochondrial respiration (Aubert et al., 2001, Masclaux-Daubresse et al., 2006). In our case, the equal provision of glucose to nitrate- and ammonium-fed roots would have ensured sufficient 2-

oxoglutarate availability via ICDH enzyme activity (Supplemental Fig. 3) for GDH to have the potential to assimilate ammonium regardless of the N regimen to which the plant is adapted.

Is there an alternative $^{15}\text{NH}_4^+$ incorporation pathway into amino acids when GS and GOGAT are inhibited?

In order to confirm that the ^{15}N -Glu formed in presence of MSX was not synthesized by GOGAT from any residual GS-synthesized ^{15}N -Gln, GOGAT activity was inhibited by the application of azaserine (AZA) (Supplemental Fig. 3), preventing thus the synthesis of labelled Glu from Gln (Oaks et al., 1998). Masclaux-Daubresse et al. (2006) reported that the addition of AZA highly reduced the formation of labelled ^{15}N -Glu and Glu-derived amino acids in tobacco leaves. In contrast, the detection of ^{15}N -Glu and ^{15}N -Asp in our tomato roots under AZA incubation, alone or in combination with MSX (Fig. 3), confirms that this Glu labelling occurred by an alternative pathway. Thus, the incorporation of the newly supplied $^{15}\text{NH}_4^+$ under conditions of GOGAT inhibition could be ascribed to the *in vivo* aminating capacity of GDH, as the only alternative pathway for $^{15}\text{NH}_4^+$ fixation (Fig. 4). And this GDH aminating activity would have similar *in vivo* kinetic properties in both ammonium- and nitrate-fed plants.

On the other hand, the inhibition of GOGAT did not impede the enrichment for ^{15}N -Gln after 30 min (Fig. 3), corroborating, as expected, that the synthesis of ^{15}N -Gln through GS was taking place to the same extent as in the control roots (Supplemental Fig. 3). However, the depletion in the ^{15}N -Glu turnover due to the lack of GOGAT activity would be responsible for a reduction in the double labelling of ^{15}N -Gln (Table 1), and later in the time course, also in the total ^{15}N -Gln enrichment (Fig. 3). Nonetheless, the fact that, although diminished, there was still double labelled ^{15}N -Gln formation under both types of nutrition would indicate that the ^{15}N -Glu supplied to GS originated through pathways other than GOGAT. So, both the double labelling pattern of Gln and the synthesis of ^{15}N -Glu when the GS/GOGAT pathway was interrupted support the existence of an *in vivo* aminating role for GDH, which would contribute to the detoxification of excessive amounts of NH_4^+ regardless of the previous N nutritional conditions.

Lastly, the enrichment of ^{15}N -Asn was also lowered in the late incubation period when the main assimilation pathway (GS/GOGAT) was impaired (Fig. 3). Moreover, the reduced ^{15}N -Gln and ^{15}N -Asp

provision at the last time point provoked a drop in the double labelled ^{15}N -Asn proportion (Table 1), corroborating again the Gln-dependent AS activity in tomato roots.

CONCLUSIONS

The use of stable isotope ^{15}N labelling, combined with metabolic inhibitors of the GS/GOGAT pathway, permitted an analysis of the *in vivo* function of the principal and accessory N assimilation pathways in tomato roots supplied with excess ammonium. The *in vivo* activity of AS was shown to be Gln-dependent in both nitrate- and ammonium-grown tomato roots, as demonstrated by their inability to directly incorporate labelled $^{15}\text{NH}_4^+$ into Asn whenever Gln synthesis was inhibited. Evidence for GDH-mediated *in vivo* ammonium assimilation capacity was obtained from the formation of labelled ^{15}N -Glu in tomato roots when GS and/or GOGAT activities were inhibited and from the double labelling pattern of Gln. This *in vivo* aminating capacity of GDH provides a complementary mechanism for detoxifying excess ammonium in Agora Hybrid F1 tomato roots. This mechanism can be applied for other crop species to increase ammonium tolerance. Additionally, this accessory route could allow a quick adaptation of the plant to the use of changing N sources.

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CONFLICT OF INTEREST

Authors declare no conflict of interest

APPENDIX A. Supplementary data

Supplementary Figure S1. Design of the labelling experiment.

Supplementary Figure S2. Plant biomass and root internal ammonium content

Supplementary Figure S3. GS, NADH-GOGAT, NADH-GDH, NAD-GDH and NADP-ICDH enzyme activities.

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