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On the resilience of nitrogen assimilation by intact roots under starvation, as revealed by isotopic and metabolomic techniques†

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The response of root metabolism to variations in carbon source availability is critical for whole-plant nitrogen (N) assimilation and growth. However, the effect of changes in the carbohydrate input to intact roots is currently not well understood and, for example, both smaller and larger values of root:shoot ratios or root N uptake have been observed so far under elevated CO_2. In addition, previous studies on sugar starvation mainly focused on senescent or excised organs while an increasing body of data suggests that intact roots may behave differently with, for example, little protein remobilization. Here, we investigated the carbon and nitrogen primary metabolism in intact roots of French bean (*Phaseolus vulgaris* L.) plants maintained under continuous darkness for 4 days. We combined natural isotopic $^{15}N/^{14}N$ measurements, metabolomic and ^{13}C-labeling data and show that intact roots continued nitrate assimilation to glutamate for at least 3 days while the respiration rate decreased. The activity of the tricarboxylic acid cycle diminished so that glutamate synthesis was sustained by the anaplerotic phospho*enol*pyruvate carboxylase fixation. Presumably, the pentose phosphate pathway contributed to provide reducing power for nitrate reduction. All the biosynthetic metabolic fluxes were nevertheless down-regulated and, consequently, the concentration of all amino acids decreased. This is the case of asparagine, strongly suggesting that, as opposed to excised root tips, protein remobilization in intact roots remained very low for 3 days in spite of the restriction of respiratory substrates. Copyright © 2009 John Wiley & Sons, Ltd.

Intense efforts are currently devoted to understand how plants respond to their environment in such a way as to optimize their resource use. Recent studies aimed at elucidating the rules of primary production improvements under elevated CO_2 showed that larger amounts of carbon entering root systems resulted in greater nitrogen uptake.[1] That in turn correlated with higher fine-root-to-shoot N ratios and an optimized leaf or canopy CO_2 assimilation.[2,3] In other words, the involvement of root nitrogen content and uptake efficiency is now thought to be a fundamental control parameter of plant productivity and carbon fixation.[1]

However, the metabolic basis of root nitrogen assimilation and uptake under increasing carbon resources has not yet been extensively studied while integrative techniques (metabolomics and genomics) have been developed on leaves.[4,5] Similarly, few studies have focused on changes in both C and N metabolisms induced by carbon restriction (i.e. starvation) in roots. Root metabolism under carbon source limitation appears close to the metabolisms of senescent leaves or cultured cells (described by Baysdorfer *et al*.[6] and Genix *et al*.[7]). In fact, excised maize (*Zea mays*) root tips show a rapid decline of both the carbohydrate content and the respiration rate, followed by a remobilization of lipids and proteins to feed the tricarboxylic acid cycle (TCA). Ammonium ions thus released are transiently stored as asparagine (Asn) and then expelled from cells until the root tips die after about 4 days.[8–10]

Experimental data on intact plant roots suggest that the metabolic acclimation of roots to sugar starvation varies between species, especially regarding protein-recycling. In maize plants subjected to darkness for 48 h, a slight decrease of the protein content was observed in roots as well as an increase in Asn concentration.[11] By contrast, in intact tomato plants (*Solanum esculentum*) maintained in darkness, the decline of the root protein content started later,[12] and no significant variation occurred in Asn concentration for 4 days.[13] As already hypothesized for secondary leaves and

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stems of pearl millet,[6] it is plausible that roots may show starvation tolerance in some species, as revealed by delayed protein degradation under continuous darkness.

In our previous study on French bean (*Phaseolus vulgaris*), we used ^{13}C labeling and magnetic nuclear resonance (NMR) analyses and showed a significant flux of nitrogen assimilation into glutamate (Glu) in the intact roots of plants subjected to continuous darkness for 4 days.[14] In fact, Glu is the key amino acid linking carbon and nitrogen metabolism, as ammonium assimilation proceeds through the GS/GOGAT cycle to form Glu from TCA-derived α-ketoglutarate; Glu is then used as an NH_2-donor for many transamination reactions (see Forde and Lea[15] for a recent review). ^{13}C-enrichment data strongly suggest that anaplerotic CO_2-fixation by the phospho*enol*pyruvate carboxylase (PEPc) feeds the TCA with oxaloacetate molecules (OAA) to sustain Glu synthesis in starved roots.[14]

^{15}N-tracer studies have shown that a large part (up to 70%) of absorbed ^{15}N-nitrate ions absorbed by roots during the night remains unreduced in soybean (*Glycine max*).[16] Maize seedlings exposed to a CO_2-depleted atmosphere exhibit lower ^{15}N-nitrate absorption and reduction rates.[17] In addition, under typical (non-starved) conditions in NO_3^- media, the root respiratory quotient (the ratio of evolved CO_2 to consumed O_2) is believed to be larger than 1, suggesting that the reductive power generated by the TCA and the reductive pentose phosphate pathway is also used for nitrate reduction in addition to respiratory O_2 reduction.[18] It thus may be assumed that the decrease of respiration upon carbon source restriction limits the rate of nitrate reduction in roots. Nevertheless, no direct evidence of N-reduction and N-partitioning have been carried out so far under these conditions and, thus, the origin of the N atoms (protein recycling versus absorbed nitrate ions) that are (re)assimilated remains unsure.

Here, we investigated the primary nitrogen assimilation and metabolism and the relationships with carbon metabolism in intact roots of *Phaseolus vulgaris* under starvation (continuous darkness). We took advantage of the natural ^{15}N-isotope fractionation that accompanies nitrate reduction,[19] thereby enriching in ^{15}N the nitrate ions left behind. In other words, we measured the natural nitrogen isotope composition ($\delta^{15}N$) and the nitrogen content in different biochemical fractions (total organic matter, soluble and insoluble fraction) and interpreted the ^{15}N-depletion or -enrichment observed upon starvation. We also analyzed the nitrate content and the root metabolome, and combined the latter data (metabolite relative concentrations) with the results of previous ^{13}C-labeling and NMR analysis[14] to clarify the metabolic origin of the concentration changes occurring under starvation.

The results strongly suggest that nitrate reduction and assimilation continue for at least 3 days under continuous darkness, and that they are accompanied by nitrate export to shoots or loss in exudates or efflux. The general decrease in amino acid concentrations results from a decrease of the metabolic commitment toward the synthesis of these compounds through a reduction of the TCA activity, while the relative contribution of PEPc activity to Glu synthesis increases. We conclude that protein remobilization is substantially delayed in intact roots under continuous darkness.

EXPERIMENTAL

Plant material and growth conditions

Experiments were conducted on French bean (*Phaseolus vulgaris* L. cv contender, Vilmorin, la Verpillière, France). Seeds were sown directly in vermiculite, in individual 1-L pots. Plants were grown in a greenhouse with a 16-h photoperiod and a minimum photosynthetic photon flux density maintained at approximately 500 $\mu mol\,m^{-2}\,s^{-1}$ by supplementary lighting from high-pressure sodium lights. The temperature and vapour pressure deficit inside the greenhouse were maintained at approximately 25.5/18.5°C and 1.4/1.2 kPa day/night, respectively. Watering was carried out with a commercial nutrient solution (Hydrokani C2, Yara, Nanterre, France) twice during the photoperiod. The nitrogen isotope composition of the nutrient solution (containing NO_3^-, 0.4 $mol\,L^{-1}$) was $2.69 \pm 0.35‰$ ($n = 4$). The carbon isotope composition of CO_2 in the greenhouse was $-9.85 \pm 0.2‰$ ($n = 3$). Experiments were carried out on plants that had a mature first trifoliate leaf, that is, under our growth conditions, 3- to 4-week-old plants. In such plants, the root biomass was typically about 7.5 g FW per plant. For N content analyses, all plants were picked from the same cultivation line (July 2007). Under our culture conditions, the roots did not have any N_2-fixing nodules. For all experiments, plants were taken from the greenhouse after a 10-h photoperiod on day 0. The plants were not supplied with nutritive solution when under continuous darkness (MilliQ water only was used; Millipore, Molsheim, France).

Gas exchanges

Measurements of respiratory fluxes were taken from a previous study.[14] Briefly, intact washed roots were enclosed in a respiration chamber connected to a closed system. Initially, air flowed through soda lime columns to remove CO_2 until the CO_2 concentration reached equilibrium (15 to 20 min). The soda lime trap was then bypassed, and CO_2 was accumulated in the system. The concentration of CO_2 was continuously monitored with an infra-red gas analyser (BINOS, Leybold-Heraeus, Huerth, Germany), enabling calculation of the respiratory flux from the slope of the CO_2 increase.

Nitrogen isotope and nitrogen content analysis

The samples used to measure the N content and isotope composition of roots were taken from experiments previously carried out under continuous darkness to measure root respiration and the $\delta^{13}C$ of respired CO_2.[14] Experiments in continuous darkness started after a 10-h photoperiod. After each experiment, roots were rapidly frozen in liquid nitrogen, then freeze-dried and ground to powder. Powder samples (3 mg) were weighed in tin capsules for organic matter isotope analysis

The soluble fraction and the insoluble fraction were separated following the procedure of Tcherkez *et al.*[20] Briefly, about 150 mg of root powder was suspended with 1.5 mL of cold distilled water in an Eppendorf tube, placed on ice for 10 min and then centrifuged. The supernatant was collected and heated to denaturize proteins, which were removed by centrifugation to obtain the soluble fraction (SF). The first

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pellet containing the insoluble fraction (IF) was washed with 95% ethanol to remove pigments and SF contamination, and then freeze-dried. For both the SF and the IF, 3 mg samples were then weighed in tin capsules for $^{14}N/^{15}N$ analysis.

Nitrogen content and $\delta^{15}N$ analysis were carried out using a model NA-1500 elemental analyzer (Carlo-Erba, Milan, Italy) interfaced to a VG Optima mass spectrometer (Micromass, Villeurbanne, France). The nitrogen isotope compositions were calculated as a deviation of the nitrogen isotope ratio ($^{15}N/^{14}N$, called R) from the international standard (air): $\delta^{15}N = 10^3 \times [(R_{sample} - R_{standard})/R_{standard}]$. A laboratory standard (atropine) was measured every 12 samples in order to correct for any offset of the mass spectrometer. The precision of the $\delta^{15}N$ measurements was ±0.3‰. All the isotopic analyses were carried out at the isotopic facility structure *Platforme Metabolism-Metabolome* (Orsay, France).

Nitrate content measurements

The nitrate content was measured in the soluble fraction using a nitrate ion selective electrode (CI-6735; PASCO Scientific, Roseville, CA, USA). Before measurements, samples were diluted 50 times with distilled water in order to obtain a sufficient volume for the electrode to be entirely immersed. The electrode was calibrated with two nitrate solutions of known concentrations (0.2 and 2 mol L^{-1}) which correspond to the typical nitrate range found in our samples. During measurements, the samples were continuously agitated with a magnetic stirrer.

Metabolome

Equipment

Gas chromatography/time-of-flight mass spectrometry (GC/TOFMS) was performed on a LECO Pegasus III mass spectrometer (LECO, Garges-Les-Gonesses, France) with an Agilent 6890N GC system and an Agilent 7683 automatic liquid sampler (Agilent Technologies, Massy, France). The column was an RTX-5 w/integra-Guard (30 m × 0.25 mm i.d. + 10 m integrated guard column; Restek, Evry, France).

Extraction

Root samples (20 mg of powder from freeze-dried material) were ground in a mortar in liquid N_2, and then in 2 mL of methanol 80%, to which ribitol (100 μmol L^{-1}) was added as an internal standard. Extracts were transferred to 2-mL Eppendorf tubes, and centrifuged at 10 000 *g* and 4°C for 15 min. Supernatants were transferred to fresh tubes and centrifuged again. Several aliquots of each extract (0.1 mL, 3 × 0.2 mL and 0.4 mL) were spin-dried under vacuum and stored at −80°C until analysis.

Sample derivatization and analysis

Methoxyamine was dissolved in pyridine at 20 mg mL^{-1} and 50 μL of this mixture was used to dissolve the dry sample (from the 0.2-mL aliquot, see above). Following vigorous mixing, samples were incubated for 90 min at 30°C with shaking. Then 80 μL of *N*-methyl-*N*(trimethylsilyl)trifluoroacetamide (MSTFA) was added, and the mixture was vortexed, and incubated for 30 min at 37°C with shaking. The derivatization mixture was then incubated for 2 h at room temperature. Before loading into the GC autosampler a mix of a series of eight alkanes (chain lengths: C_{10} to C_{36}) was included.

Analyses were performed by injecting 1 μL in splitless mode at an injector temperature of 230°C. Chromatographic separation was performed with helium as the carrier gas at a flow rate of 1 mL min^{-1} in the constant flow mode and using a temperature ramp ranging from 80 to 330°C between minute 2 and 18, followed by 6 min at 330°C. The total run time per injection was 30 min. Electron ionization at 70 eV was used and the acquisition rate was 20 spectra s^{-1} over the m/z range 80–500, as previously described by Weckwerth *et al.*[21]

Peak identity was established by comparison of the fragmentation pattern with available MS databases (National Institute of Standards Technology database; supplied with LECO software), using a match cut-off criterion of 750/1000, and by retention index (RI) using the alkane series as retention standards.

Data processing and quantification

For GC/TOFMS, integration of peaks was performed using the LECO Pegasus software. Because automated peak integration was occasionally erroneous, integration was verified manually for each compound in all analyses.

This method has been shown to be quite reliable for the relative quantification of most amino acids,[22] and the main sugars (sucrose, glucose, fructose).[23]

NMR

The ^{13}C-NMR data used in Fig. 3 are taken from Bathellier *et al.*[14] In brief, whole intact root systems (still attached to shoots) were labeled for 2 h with 10 mM solutions of positionally ^{13}C-enriched substrates ([1-^{13}C]Glc, [3-^{13}C]Glc, [1-^{13}C]Pyr, [3-^{13}C]Pyr) just after a 10-h period of illumination (plants picked up from the greenhouse), or after 4 days of darkness. O_2 conditions were maintained by constant air bubbling. Perchloric acid extracts were then prepared from 5–10 g of frozen root material as described by Aubert *et al.*[24] for phloem cells. NMR spectra of the extracts were obtained on a NMR spectrometer (AMX 400, Bruker, Ettlingen, Germany) equipped with a 10-mm multinuclear probe tuned at 100.6 MHz for ^{13}C-NMR (see Bathellier *et al.*[14] for further details on the conditions of acquisition and data processing).

Graphical and statistical analyses

GC/TOFMS data were treated in order to correct for the large differences in concentration between metabolites (see Supporting Information) and thus to focus on the variations caused by continuous darkness. For that purpose, the metabolite contents of days 1 to 4 (denoted as C_i) were, for each metabolite, normalised with respect to the initial content at day 0 (denoted as C_0) as follows:

$$C_i^{norm} = \frac{C_i}{C_0} - 1 \quad (1)$$

where C_i^{norm} represents the normalized variation in the metabolite content on day *i*.

Normalized data were then drawn as a clustered metabolomic array using MeV 4.1 open source software.[25,26]

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The clustering was based on the Pearson correlation method. In this representation, the intensity of the green colour is proportional to the decrease in concentration relative to day 0, and, conversely, the intensity of the red colour is proportional to the increase in concentration relative to day 0. The black colour indicates that no relative change occurred. Such relative changes ('heat map') and the clustering analysis are shown on Fig. 2.

Figure 3 is a combination of metabolomic and NMR data. It represents the relative content variation between day 0 and day 4 on the *x* axis (as measured by GC/TOFMS) against the relative ^{13}C-enrichment change between day 0 and day 4 on the *y* axis (obtained from ^{13}C-NMR in four different labeling conditions). Each of the four panels of Fig. 3 is associated with a particular labeling condition ([1-^{13}C]Glc, [3-^{13}C]Glc, [1-^{13}C]Pyr, [3-^{13}C]Pyr). The relative content changes are the C_i^{norm} values calculated with Eqn. (1) at day 4. ^{13}C-enrichment data were taken from our previous study[14] (see the Experimental section in this reference). These data provide positional ^{13}C-enrichments on both days 0 and 4, for all the metabolites detected with NMR. For each metabolite represented in Fig. 3, the global ^{13}C enrichment of the molecule (denoted as *E* thereafter) was calculated as a mean of the positional ^{13}C-enrichments measured with NMR. Subsequently, relative ^{13}C-enrichment changes between day 0 and day 4 (E_{Rel}) were calculated with an equation similar to Eqn. (1):

$$E_{Rel} = \frac{E_4}{E_0} - 1 \qquad (2)$$

In such a representation, four areas (A to D, inset of Fig. 3) can be distinguished within each panel of Fig. 3. They correspond to four possible metabolic events:

Area A: the metabolite content decreased after 4 days in darkness, while the ^{13}C-content increased, indicating that the metabolic demand exceeded the biosynthesis rate.
Area B: both the content and the ^{13}C enrichment increased, which means that biosynthesis exceeded the demand.
Area C: the concentration increased while the ^{13}C-enrichment decreased, indicating remobilization (recycling of an unlabeled ^{13}C-depleted carbon source).
Area D: both the concentration and the ^{13}C-enrichment decreased, implying a decreased metabolic commitment to the metabolite of interest.

RESULTS

Nitrate and nitrogen contents under continuous darkness

Root respiration decreased almost continuously for 4 days in continuous darkness and reached about 40% of its initial rate on day 4 (Fig. 1(a), replotted from Bathellier *et al.*[14]). At the same time, the nitrate content in the soluble fraction (SF) rapidly fell, from 425 μmol NO_3^- g^{-1} DM to 25 μmol NO_3^- g^{-1} DM in 3 days (Fig. 1(a)). The trend observed in the nitrogen content of the SF (in mg N g^{-1} of dry weight of the fraction of interest) was very similar to that of the nitrate content (Fig. 1(b)) so that nitrate and SF nitrogen contents were strongly correlated ($r^2 = 0.97$, Fig. 1(a), inset). A slight and continuous decrease was also observed in the nitrogen content of the total organic matter (OM). Remarkably, such a decrease matched the quantity of nitrogen lost as nitrate: the N-decrease in OM represented 6.5 mg N g^{-1} on day 3 while the decrease in nitrate accounted for 5.6 mg N g^{-1}. The

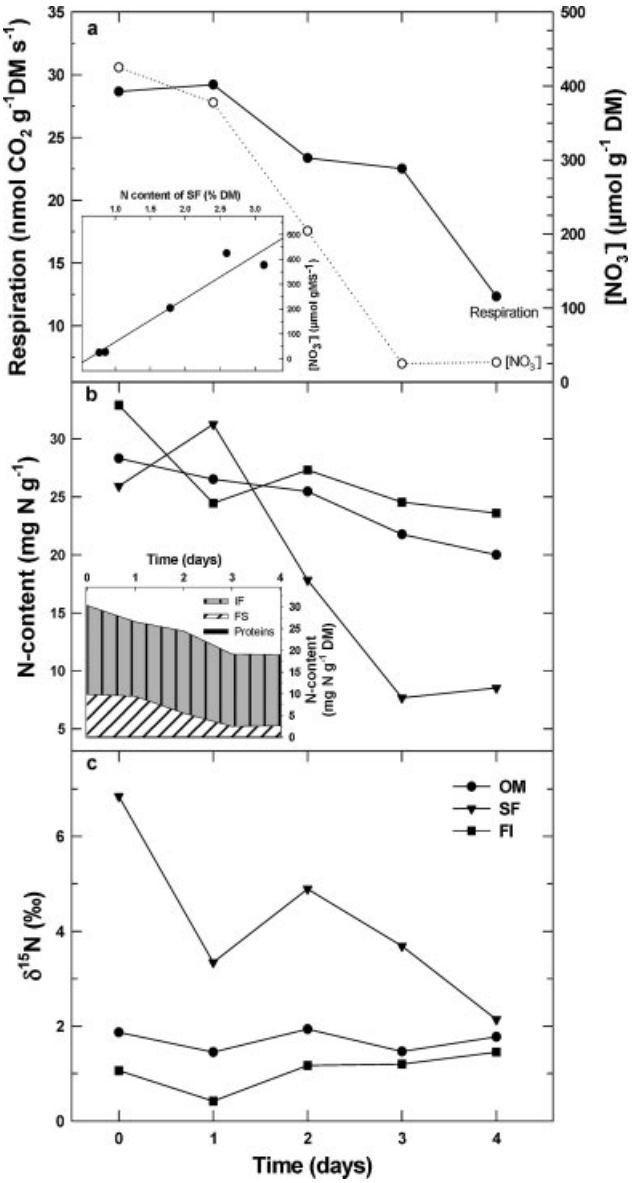

Figure 1. The time course of respiration and nitrogen amounts in intact roots of *Phaseolus vulgaris* under continuous darkness. (a) Respiration rate (left axis) and nitrate content (in μmol NO_3^- per g of total dry matter; right axis). *Inset*: relationship between the N content of the soluble fraction (SF) and the nitrate content. The continuous line is a linear regression which is significant ($r^2 = 0.97$, $p < 0.005$). (b) Nitrogen content (mg N per g of dry weight of the fraction of interest) in total organic matter (OM, circles), the soluble fraction (SF, triangles) and the insoluble fraction (IF, squares). *Inset*: contributions (in mg N g^{-1} of total dry matter) represented by the different fractions to the total N content. Soluble heat-precipitated proteins are indicated as 'proteins'. Their contribution is very small and can hardly be distinguished from the *x*-axis. (c) Natural nitrogen isotope composition (δ^{15}N) of total organic matter (OM, circles), the soluble fraction (SF, triangles) and the insoluble fraction (IF, squares). Data points are means over two to three replicates.

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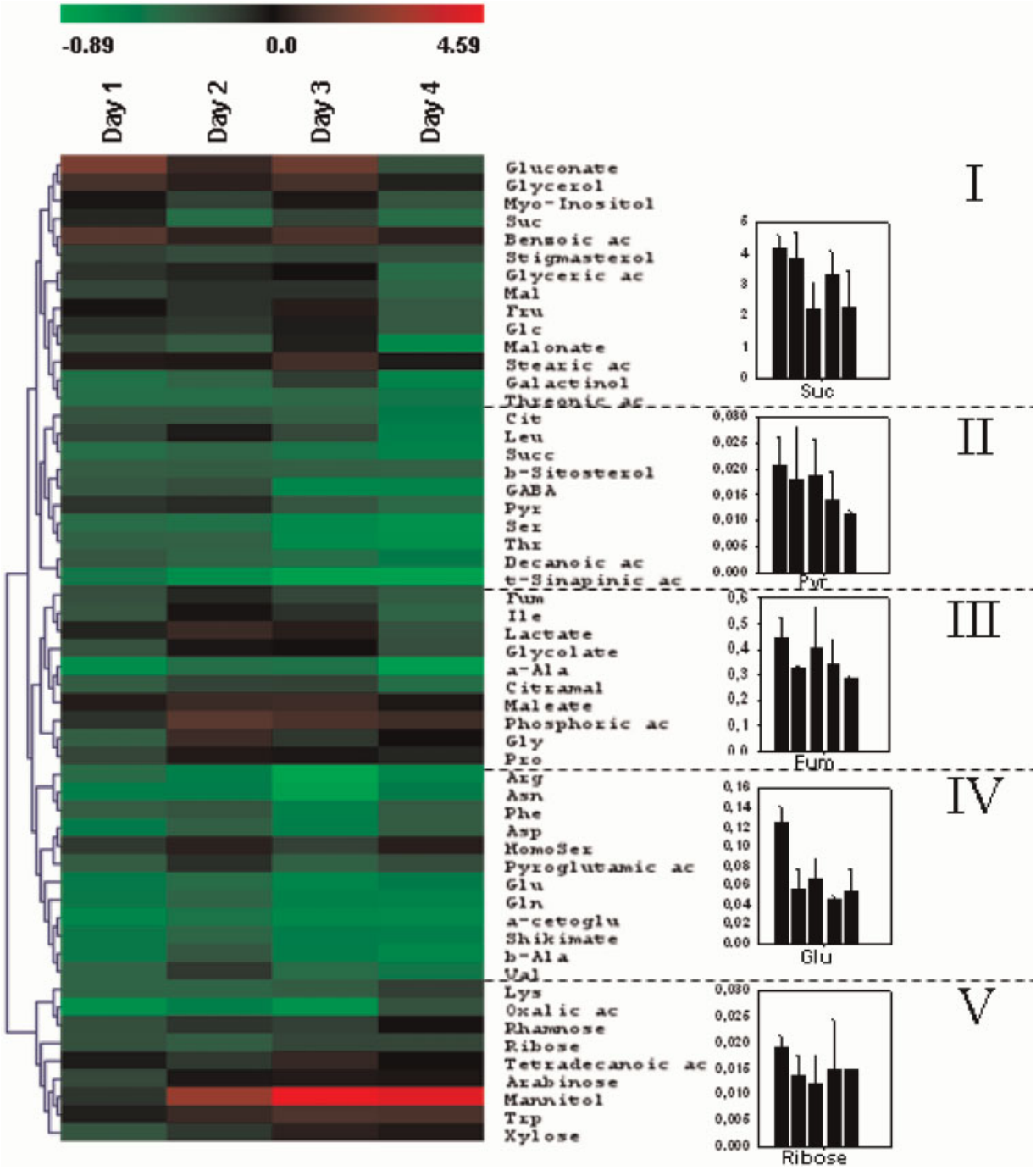

Figure 2. Clustered metabolomic array representing the time course of the content in all root metabolites (detected by GC/TOFMS) for 4 days under continuous darkness. Metabolite contents are relative to their initial content (see Experimental section for further details on data treatments). Each cell represents the mean of three independent measurements. The green and red colours indicate a decrease or an increase of the concentration relative to day 0, respectively. The black colour indicates no variation. The clustering was done with the Pearson correlation method. Clusters are numbered (from I to V) and, for each of them, a histogram of a metabolically relevant compound is represented as an illustration of the pattern of variation. Values in the histogram are dimensionless relative concentration values, expressed relative to the internal standard, i.e. ribitol (see Experimental section for details). Cit: citrate; Fum: fumarate; GABA: γ-aminobutyric acid; HomoSer: homoserine; Mal: malate; Succ: succinate. Other abbreviations correspond to the international three-letter code.

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nitrogen content in the insoluble fraction (IF) almost paralleled that in the OM, except between day 0 and day 1. The decrease in the nitrogen content in the IF at day 1 corresponded to an increase in the SF (Fig. 1(b)) while the nitrate content diminished only slightly (Fig. 1(a)). It can thus be hypothesised that a transient N transfer from the IF to the SF occurred on day 1. However, we cannot rule out that this pattern is an artefact stemming from the variability between plants, which were different for each day.

Nitrogen isotope composition of OM, IF and SF

The nitrogen isotope composition ($\delta^{15}N$) of the nutritive solution used to grow plants was $+2.69 \pm 0.35‰$ ($n = 4$). Root OM and IF were ^{15}N-depleted compared with the nutritive solution by about 0.8 and 1.6‰ on day 0, respectively (Fig. 1(c)). Conversely, the SF was substantially ^{15}N-enriched by 4.15‰ on day 0, suggesting that a discrimination against ^{15}N did occur during nitrate reduction in roots thereby enriching the NO_3^- ions left behind in the soluble fraction (see the Discussion below). Under continuous darkness, the $\delta^{15}N$ of the SF decreased strongly, almost reaching the $\delta^{15}N$ value of the OM (day 4, Fig. 1(c)). The $\delta^{15}N$ of the SF was, however, unexpectedly low on day 1, and this corresponds to the transient increase in the SF nitrogen content highlighted above (Fig. 1(b)). The $\delta^{15}N$ in both the OM and the IF was nearly constant during the experiment, although the difference between these two fractions tended to vanish from day 3 due to a slight decrease in the $\delta^{15}N$ value of the OM. Again, values for the OM and the IF were slightly lower on day 1, and this might indicate that the growing conditions were not perfectly homogeneous, slightly impacting on the nitrate assimilation feature in day 1 plants (see above).

Root metabolome under continuous darkness

Metabolomic GC/TOFMS analyses were carried out on roots to follow the time course of metabolite contents under continuous darkness (see Supporting Information). The results are shown as a clustered metabolomic array (Fig. 2) representing the content of metabolites relative to their initial concentration (Eqn. (1)).

The content of most metabolites decreased under the four consecutive days of darkness (prevalence of the green colour in Fig. 2) with a few exceptions such as mannitol, which increased substantially from day 2. Nevertheless, the kinetics of the decrease differed slightly between metabolites so that the statistical analysis allowed us to identify five distinct clusters of compounds: (i) major soluble carbohydrates (Suc, Glc, Fru) showed a slight though progressive decrease; the presence of gluconate, glycerate and malate together within this cluster is consistent with the view (supported by labeling patterns; see the section on ^{13}C-labeling below) that Glc entered the pentose phosphate pathway and phospho*enol*-pyruvate (PEP) formed by glycolysis sustained malate synthesis via the PEPc. (ii) pyruvate (Pyr) clustered with several of its derivatives (citrate and amino acids derived from Pyr metabolism: Leu, Ser) and their concentration decreased more rapidly than that of the soluble sugars. GABA and all the major intermediates of the GABA shunt as well as succinate also belong to this cluster. (iii) The third cluster does not contain any major metabolite of either carbon or nitrogen metabolism, except fumarate that is found here isolated from both succinate and malate. (iv) The fourth cluster contains Asp, Glu, 2-oxoglutarate and some of their derivatives (Asn, HomoSer, Gln) the concentration of which dropped quite rapidly. Other amino acids are close (Ala, Val, Phe), emphasizing the central role of Glu for transamination reactions. (v) The fifth cluster gathers several pentoses (ribose, xylose, arabinose). Their concentration did not vary much with time, suggesting the persistence of the pentose phosphate pathway or their structural role.

It is worth noting that almost all the amino acids detected by GC/TOFMS showed a decreasing pattern. However, the content of some amino acids, such as Asp, Asn and Arg, increased slightly at the end of the experiment (day 4). In addition, the Trp content increased continuously, although always very low under our conditions (10-fold lower than Gln).

The relationship between metabolite contents and ^{13}C-enrichment in root metabolites

In order to gain an insight into the dynamics of concentration changes in key metabolites (linked to both carbon and nitrogen primary metabolism), GC/TOFMS data were combined with the ^{13}C-enrichment data (^{13}C-Glc- or ^{13}C-Pyr-labeling experiments followed by NMR analysis; see Bathellier *et al.*[14]) into a bidimensional representation. The representation is given in Fig. 3 which shows the relative change in ^{13}C enrichment between day 0 and day 4 (y-axis) as a function of the relative change in concentration (x-axis), for four different labeling conditions.

With ^{13}C-Glc-labeling, several metabolites, especially those downstream of glycolysis, were never found labeled, on either day 0 or day 4. Subsequently, their relative enrichment change is zero. Under [1-^{13}C]Glc-labeling conditions, Suc and Glc showed a very low enrichment change; in fact, they were substantially but similarly labeled on days 0 and 4. This suggests that the diluting effect of endogenous ^{12}C-Glc was low and the commitment to Suc synthesis from Glc was proportionally constant, that is, adjusted to the demand. The decrease of the ^{13}C-enrichment in succinate (Succ) and Glu supports the view of a reduced commitment of ^{13}C-Glc molecules to the TCA after 4 days, or of an increased contribution of non-labeled acetyl-CoA to the TCA from remobilisation.

Under [3-^{13}C]Glc-labeling conditions, Suc and Glc showed a substantial positive enrichment-change. This effect stems from an artifact due to similar NMR chemical shifts between some carbon positions of Suc or Glc and various pentoses (ribose, lyxose, xylose, arabinose; see Bathellier *et al.*[14] for specific details). In other words, the ^{13}C-enrichment in the corresponding C atom positions in Glc or Suc may be over-estimated on day 4 because *de novo* synthesis of pentoses occurred. Xylose appears in the area denoted as B (Fig. 3) indicating the increase of both xylose concentration and ^{13}C-enrichment after 4 days. This effect was not observed under the [1-^{13}C]Glc-labeling condition as the labeled position (C-1) was decarboxylated by the pentose phosphate pathway and, thus, the ^{13}C-label could not be recovered in pentoses.

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Under [3-^{13}C]Pyr-labeling conditions, the label was directly incorporated into TCA intermediates. Both their concentration and their ^{13}C-enrichment decreased, indicating the reduced activity of the TCA on day 4 and the possible entry of recycled, unlabeled carbon. Notable exceptions are Ala, GABA and to a lesser extent Asp, that showed a positive enrichment change, that is, an increased commitment from ^{13}C-Pyr.

The results obtained under [1-^{13}C]Pyr-labeling conditions give some pieces of information about CO_2 refixation processes, since the ^{13}C-label is in that case rapidly decarboxylated by the pyruvate dehydrogenase and can subsequently be refixed by the PEPc. Thus, the increase in relative enrichment observed in Glu and fumarate while no effect is visible in succinate suggests an increased contribution of the PEPc to the production of oxalo-acetate, which is in turn converted into fumarate. Parenthetically, the observed enrichment levels were relatively low under such a labeling condition because $^{13}CO_2$ evolved from ^{13}C-Pyr is isotopically diluted by natural respiratory $^{12}CO_2$. In addition, the ^{13}C-enrichment is weak in both malate and citrate since their concentration was large in bean roots (about 1.5 and 0.75 mol L^{-1}, respectively) thereby diluting the ^{13}C-label. It should also be noted that a substantial part of the label was recovered in Glu C-3 while the most straightforward labeling from PEPc refixation of $^{13}CO_2$ would be in both the Glu C-1 and the C-4 positions.[14] Some metabolic reactions certainly then occurred and redistributed the ^{13}C-label. Several plausible pathways might explain such a ^{13}C-enrichment in Glu C-3, such as the so-called 'C_5 acid metabolism', that is, the production and recycling of malonate or citramalate. These two compounds were indeed detected by GC/TOFMS in this study (Fig. 2).

DISCUSSION

In a previous study, we showed that Glu synthesis was maintained in bean roots after four consecutive days of darkness although respiratory metabolism was notably reduced.[14] However, the origin of N atoms incorporated into Glu and the relationship between the TCA and the nitrogen primary metabolism under starvation remained unclear. Here, the nitrogen metabolism of intact bean roots maintained under continuous darkness for 4 days was investigated by carrying out nitrogen content and δ^{15}N measurements as well as GC/TOFMS analyses of root metabolome. ^{13}C-labeling data previously obtained with NMR were also used to clarify metabolite variations in terms of metabolic commitments.

The fate of nitrate ions

There is compelling evidence that N assimilation occurred in roots of starved plants. In fact, ^{13}C-Pyr-feeding experiments on bean roots showed that Glu synthesis continued in plants subjected to 4 days of continuous darkness.[14] Here, we further observed a rapid decline (within 3 days) of the nitrate content in the SF and this strongly correlated to the decrease in N content in total organic matter (Fig. 1). In addition, this decrease matched most of the N loss measured in the OM during the first 3 days (5.6 mg N g^{-1} lost as nitrate vs. 6.5 mg N g^{-1} lost in the OM). Plausibly then, nitrate assimilation occurred and contributed to the exhaustion of the nitrate pool for 3 days. Nitrate reduction under low carbohydrate availability has been previously reported in tobacco (*Nicotiana tabacum*) and ryegrass (*Lolium perenne*) subjected to about 40 h of continuous darkness and, furthermore, this was accompanied by a severe reduction of nitrate uptake[27,28] thereby depleting the internal nitrate pool.

We nevertheless recognize that nitrate assimilation to Glu synthesis is not solely responsible for the collapse of the nitrate content. Assuming that nitrate influx is negligible, the loss of 5.6 mg N g^{-1} of nitrate within 3 days represented an average assimilation rate of about 1.5 nmol g^{-1} s^{-1}. The rate of Glu synthesis estimated from the ^{13}C-enrichment values (measured with NMR analyses) decreased from about 1 nmol g^{-1} s^{-1} on day 0 to about 0.5 nmol g^{-1} s^{-1} on day 4 (data not shown). Such a drop is apparent on Fig. 3, with a smaller commitment of ^{13}C-Pyr toward TCA-derived amino acids after 4 days. Consequently, nitrate loss exceeded Glu production by up to 1 nmol g^{-1} s^{-1} at the end of the experiment. We conclude that other processes consumed nitrate ions in roots, such as nitrate efflux[29] and nitrate export to leaves.[16] Under continuous darkness, the persistence of a rhythmic circadian pattern of $^{15}NO_3^-$ translocation to the shoot was observed in tobacco[27] and an increased nitrate efflux from roots was reported in ryegrass.[28]

Such a scenario also agrees with the δ^{15}N values measured here. In fact, if N assimilation occurs in roots, nitrate ions are expected to be ^{15}N-enriched because of the discrimination against ^{15}N during nitrate reduction by nitrate reductase (NR) (see Tcherkez and Hodges[30] for a review). It is especially true for plants supplied with high nitrate concentrations (nitrate excess) as nitrate reduction does not consume all the nitrate ions (incomplete process) thereby allowing the NR to fractionate between N isotopes.[31] This was the case in the present study as the initial nitrate concentration in roots was very large (up to 0.4 mmol L^{-1}, Fig. 1). Unsurprisingly then, the δ^{15}N value of the SF decreased (Fig. 1), indicating a fractionation against ^{15}N and the efflux or translocation (to shoots) of ^{15}N-enriched nitrate ions left behind.

However, the δ^{15}N of the SF reaches a value close to that of the OM on day 4 only, while the nitrate ions are already nearly exhausted on day 3. Other processes may thus have contributed to the ^{15}N-depletion of the SF on day 4, such as the degradation of ^{15}N-depleted proteins into ammonium and amino acids. Accordingly, (i) there was a small increase in the SF nitrogen content while that of the IF decreased slightly (Fig. 1) and (ii) the concentration of free Asp, Asn and Arg slightly increased between days 3 and 4 (Fig. 2).

Under such a framework, the nitrogen isotope composition of nitrate can be calculated from the mass balance as follows:

$$\delta_{NO_3^-} = \frac{\delta_{SF} - \delta_{N_{org}}(1 - x)}{x} \quad (3)$$

where δ_{SF}, δ^-_{NO3} and δ_{Norg} are the δ^{15}N of SF, nitrate and organic nitrogen, respectively, and x is the fraction of N represented by NO_3^- in total SF nitrogen. With the nitrate

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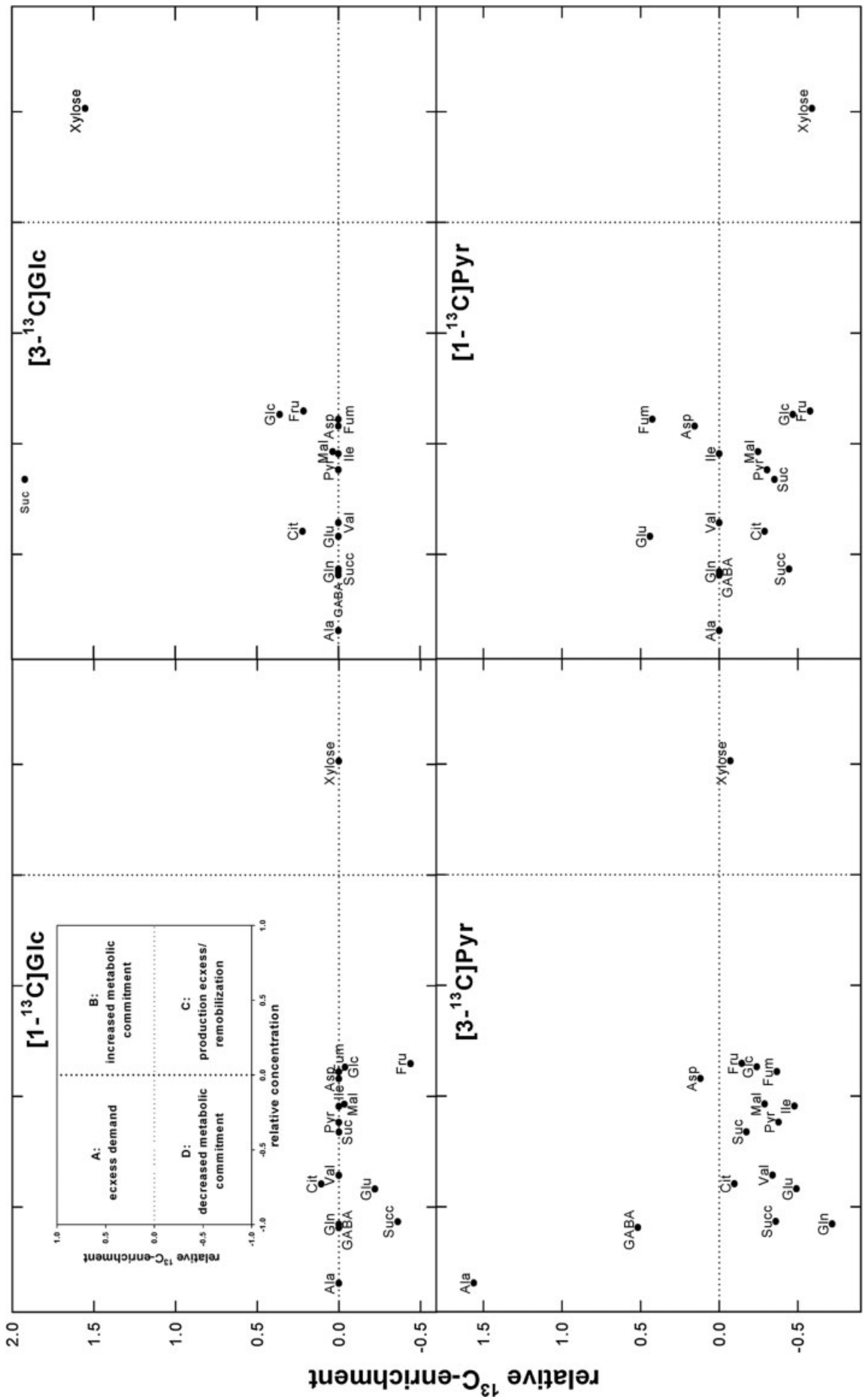

Figure 3. Relative variation in root metabolite contents between day 0 and day 4 in darkness as a function of the relative variation in the mean ^{13}C enrichment for the same time frame. ^{13}C enrichments were measured with NMR after four different ^{13}C-labeling treatments ([1-^{13}C]Glc, [3-^{13}C]Glc, [1-^{13}C]Pyr, [3-^{13}C]Pyr). Metabolite contents are derived from independent GC/TOFMS analyses. Each panel thus corresponds to a specific labeling condition while all panels refer to the same dataset of metabolite contents. The inset recalls the metabolic meaning of the four areas A to D that are visible in all panels and delimited by dotted lines. Cit: citrate; Fum: fumarate; GABA: γ-aminobutyric acid; Mal: malate; Succ: succinate. Other abbreviations correspond to the international three-letter code.

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concentration, the dry mass (about 300 mg g^{-1} DM) and the N content of the SF, we calculated an x value of about 0.75 on day 0, consistently decreasing under continuous darkness (0.56, 0.53, 0.15, 0.14 on day 1 to 4, respectively). Assuming that δ_{Norg} equals the $\delta^{15}N$ of the IF (as nitrogen contained by the IF mainly comes from parietal proteins), this gives a $\delta^{15}N$ value for root nitrate of $+9.2 \pm 2.1$‰, which is nearly 7‰ enriched compared with the OM. This value is close to, although lower than, that reported by Evans *et al.*[32] for hydroponically grown tomato (*Lycopersicon esculentum*) (12.9‰). Such a difference is probably caused by a larger fraction of reduced nitrate ions in bean roots (i.e., a smaller nitrate excess, see above) and nitrate loss by efflux and translocation, thereby depleting in ^{15}N the remaining N atoms in the SF.

Carbon primary metabolism and nitrogen assimilation

Organic acids derived from the TCA cycle are well-recognized N acceptors for nitrogen assimilation. As such, it may be presumed that the C primary metabolism and the respiration rate follow a compromise between the depletion of the C source (carbohydrate pools) and the need to produce C skeletons to sustain N assimilation.[18] The metabolomic analysis indicates that the carbohydrate pools (especially Suc) decreased under continuous darkness and, similarly, the content in most of TCA intermediates and amino acids was also reduced (Fig. 2). In addition, [1-^{13}C]Glc-labeling demonstrates that the decrease in the carbohydrate content was accompanied by a lower ^{13}C-enrichment in Fru thereby suggesting a lower commitment of Glc to glycolysis (Fig. 3). Similarly, there was a reduced commitment of [3-^{13}C]Pyr to TCA intermediates (citrate, succinate, fumarate and malate) and Glu. In other words, there is an orchestrated down-regulation of glycolysis, the TCA cycle and CO_2 evolution (Fig. 1(a)).

However, while CO_2 evolution decreased more than 2-fold, the malate, succinate and citrate contents were less affected (Fig. 2), suggesting the input of a supplemental C source to organic acid production. Under [3-^{13}C]Pyr-labeling, Glu is less ^{13}C-enriched at day 4 while the reverse is visible under [1-^{13}C]Pyr-labeling (increased ^{13}C-enrichment at day 4 compared with day 0, Fig. 3). This contrasting pattern probably indicates the contribution of the phospho*enol*pyruvate carboxylase (PEPc) which enriched Glu molecules in ^{13}C through ^{13}C-enriched phospho*enol*pyruvate and the refixation of $^{13}CO_2$ decarboxylated from [1-^{13}C]Pyr by the pyruvate dehydrogenase. In other words, there was an increased contribution of the PEPc to sustain the synthesis of Glu (anaplerotic function). This agrees with the pattern seen in γ-aminobutyrate (GABA). In fact, GABA is more ^{13}C-enriched after 4 days with [3-^{13}C]Pyr-labeling than with [1-^{13}C]Pyr-labeling simply because, in the latter case, the ^{13}C-label brought by the PEPc-catalyzed refixation of $^{13}CO_2$ is lost during the conversion of Glu into GABA (Glu decarboxylase of the GABA shunt).

The larger labeling of several pentoses (such as xylose) with [3-^{13}C]Glc on day 4 suggests that the pentose phosphate pathway remained active and provided reducing power that might be used for nitrate assimilation.[33]

Did protein remobilization occur?

Both the ^{13}C-labeling and the $^{14}N/^{15}N$ natural abundances discussed above show that N reduction and assimilation continued in intact roots for 3 to 4 days in darkness. However, such a preserved capacity to assimilate nitrogen under low carbohydrate availability is generally not observed in excised roots or root tips.[8,9,12,34] In these tissues, the rapid remobilization of proteins is believed to play a key role in fuelling respiration as the carbohydrates concentration abruptly decreases and is accompanied by an increase of the ammonium content as well as of various amino acids. In particular, Asn is thought to be a transient N storage molecule avoiding large levels of free NH_4^+.[9]

By contrast, in the present work, the content in nearly all the amino acids decreased progressively and this is the case with Asn (Fig. 2). In addition, there is little decrease in the respiratory quotient (CO_2/O_2) from the initial value of 1[14] showing that the respiratory substrate did not shift to proteins. Furthermore, the strong correlation between the nitrate content and the N content of the SF suggests that no substantial increase in the ammonium pool occurred (Fig. 1, inset). Other authors have observed that the ammonium efflux was reduced in ryegrass roots under extended darkness.[28] However, on day 4, the Glu content increased slightly (Fig. 2) while the N content decreased (Fig. 1) suggesting that protein remobilization may have contributed to Glu production while N assimilation still took place at the very end of our experiment (day 4). Therefore, we conclude that protein remobilization was very low in bean roots for at least 3 days and was substantially delayed compared with that in excised root tips under our conditions.

Root excision probably leads to a more rapid depletion of carbohydrate pools than darkness treatments thereby triggering proteolysis. While starvation-induced proteolysis has been reported for intact roots in maize plantlets subjected to 48 h darkness,[11] the kinetics of the induction of protein degradation is quite different from that in root tips. Proteolysis only began after more than 20 h of darkness in intact roots whereas it was almost instantaneous in root tips.[9] Furthermore, recalculated data of Brouquisse *et al.*[9,11] show that the protein degradation rate was about 3 times higher in excised root tips than in intact roots (0.9 and 0.3 μg tip^{-1} h^{-1}, respectively). The interplay with shoots under starvation is thus presumably an important component controlling N metabolism response in roots, and this could account for the apparent contradictions reported in the literature. Further investigation focusing on C and N exchanges between above- and below-ground organs under starvation are now needed to disentangle the nature of such an interaction.

CONCLUSIONS

Our results show that root carbon primary metabolism maintains nitrogen assimilation under conditions of carbon source restriction (continuous darkness). Previous ^{15}N-labeling experiments followed by compound-specific isotopic analyses brought out consistent pieces of evidence, as a persistent nitrate-reducing activity in the roots of intact plant of tobacco and ryegrass under extended darkness has already been demonstrated with $^{15}NO_3^-$ and $^{13}NO_3^-$.[27,28]

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These data support the view that nitrogen assimilation in roots is not much affected by variations in the photosynthetic carbon input and, accordingly, nitrate reduction has been found constant or even increased during the night in contrast to leaves,[16,35–37] but see Pearson and Steer.[38] In addition, while nitrate assimilation clearly correlates to root respiration (this study and Granato et al.,[39] and references cited therein), the latter does not correlate to the root carbohydrate content.[40] Similarly, metabolomic studies on wild-type and respiratory mutants showed no correlation between Suc and Glu content in tomato roots.[41] Such a low sensitivity (high resilience) of root nitrogen metabolism to the carbon source availability contributes to explain why, under high CO_2 and fixed soil-N conditions, there is little increase in the root nitrogen uptake and reduction rates.[42] At the plant scale, it is then unsurprising that improved nitrogen assimilation may be reached only through an increased root:shoot ratio and N root content,[1] thereby allowing a higher absorption surface area as well as larger respiration rates and N-fixing enzymatic capacities.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

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