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NITROGEN RECYCLING IN PHENYLPROPANOID METABOLISM

RAMON A. RAZAL, SHONA ELLIS,* SANTOKH SINGH,* NORMAN G. LEWIS†‡ and G. H. NEIL TOWERS*‡

University of Philippines, Los Banos College of Forestry, College, Laguna, Philippines 4031; *Department of Botany, University of British Columbia, Vancouver, B.C. Canada, V6T 1Z4; †Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340, U.S.A.

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Key Word Index—*Solanum tuberosum*; Solanaceae; ^{15}N NMR spectroscopy; nitrogen (ammonia) recycling; phenylpropanoid biosynthesis; amino acids; L-phenylalanine; L-glutamine.

Abstract—Approximately 30–45% of plant organic matter is derived from L-phenylalanine, and to a lesser extent from L-tyrosine, through the cinnamate pathway. In the initial step, mediated by phenylalanine ammonia lyase, cinnamic acid and an equimolar amount of ammonium ion is generated. To determine the metabolic fate of the ammonia formed, [^{15}N]-L-phenylalanine was administered to potato discs in the light under aseptic conditions. It was found, using ^{15}N NMR spectroscopic analyses, that this nitrogen is first incorporated into the amide nitrogen of glutamine and then into glutamate. When incubations were repeated in the presence of methionine-*S*-sulphoximine, a known inhibitor of glutamine synthetase, only resonances corresponding to $^{15}NH_4^+$ and [^{15}N]-L-Phe accumulated. It is proposed that during active phenylpropanoid metabolism, the ammonia released by phenylalanine ammonia lyase/tyrosine ammonia lyase is efficiently recycled back to Phe/Tyr with glutamate serving as aminoreceptor and donor. This is the first evidence for a novel nitrogen cycle in plants.

INTRODUCTION

Phenylpropanoid and phenylpropanoid-acetate pathways are major metabolic sinks for assimilated carbon in plants, e.g. affording the ubiquitous lignins, lignans, flavonoids, suberins and coumarins [1, 2]. Phenylpropanoid-derived constituents are primarily formed from phenylalanine, although small amounts of tyrosine may also be used in some species. Elaboration of this cinnamate or phenylpropanoid pathway distinguishes vascular plants from all other types of living organisms. If efficient ammonium ion recycling did not occur during active phenylpropanoid metabolism, then effects of nitrogen deficiencies would quickly be evident [3]. There is some evidence to suggest that the carbon : nitrogen balance of plants can be regulated or controlled by modulating activity of the pathway. For example, increasing the C : N ratio by addition of an exogenously provided carbon source, such as sucrose, or by lowering nitrogen availability, results in a general increase in phenylpropanoid metabolic activity [3–8]. In conjunction with our ongoing intensive efforts in defining the pathways to, and the regulation of lignan, lignin and suberin biosyntheses, it was important to ascertain the metabolic fate of the ammonium ion liberated in the first step of the process. Potato discs (*Solanum tuberosum*) were selected for study because of the active synthesis of chlorogenic acid [9] and the *de novo* synthesis of phenylalanine ammonia lyase in light [10] and ability to form suberin [11].

‡Authors to whom correspondence should be addressed.

RESULTS AND DISCUSSION

In the pre-aromatic pathway leading to Phe and Tyr, prephenate (Pph) is transaminated via glutamate to yield arogenate [12]. The latter is subsequently dehydrated and decarboxylated in the chloroplast to afford Phe or decarboxylated and/or oxidized to yield Tyr [12–14]. Phe and Tyr usually accumulate in low concentrations in plant cells as they are readily metabolized via three types of reactions: (a) incorporation into proteins (b) conversion into cinnamate/*p*-coumarate with concomitant release of ammonium ion; and (c) in some instances, used as precursors of aromatic alkaloids.

Ammonia is assimilated by plants via glutamine synthetase (GS) and glutamine-2-oxoglutarate amidotransferase (GOGAT). In the GS reaction, ammonium ion is transferred to form the amide group of L-glutamine (L-Gln). GOGAT subsequently catalyses transfer of this amide nitrogen to α-ketoglutarate, thereby generating two molecules of L-glutamate (L-Glu). Both conversions occur within the chloroplast, although GS activity has also been detected in the cytosol. The plastidic and cytosolic isozymes appear to have distinct metabolic functions [15]. Nevertheless, the L-Glu serves as amino acid donor for arogenate formation, a process which is considered to occur in the chloroplast [16].

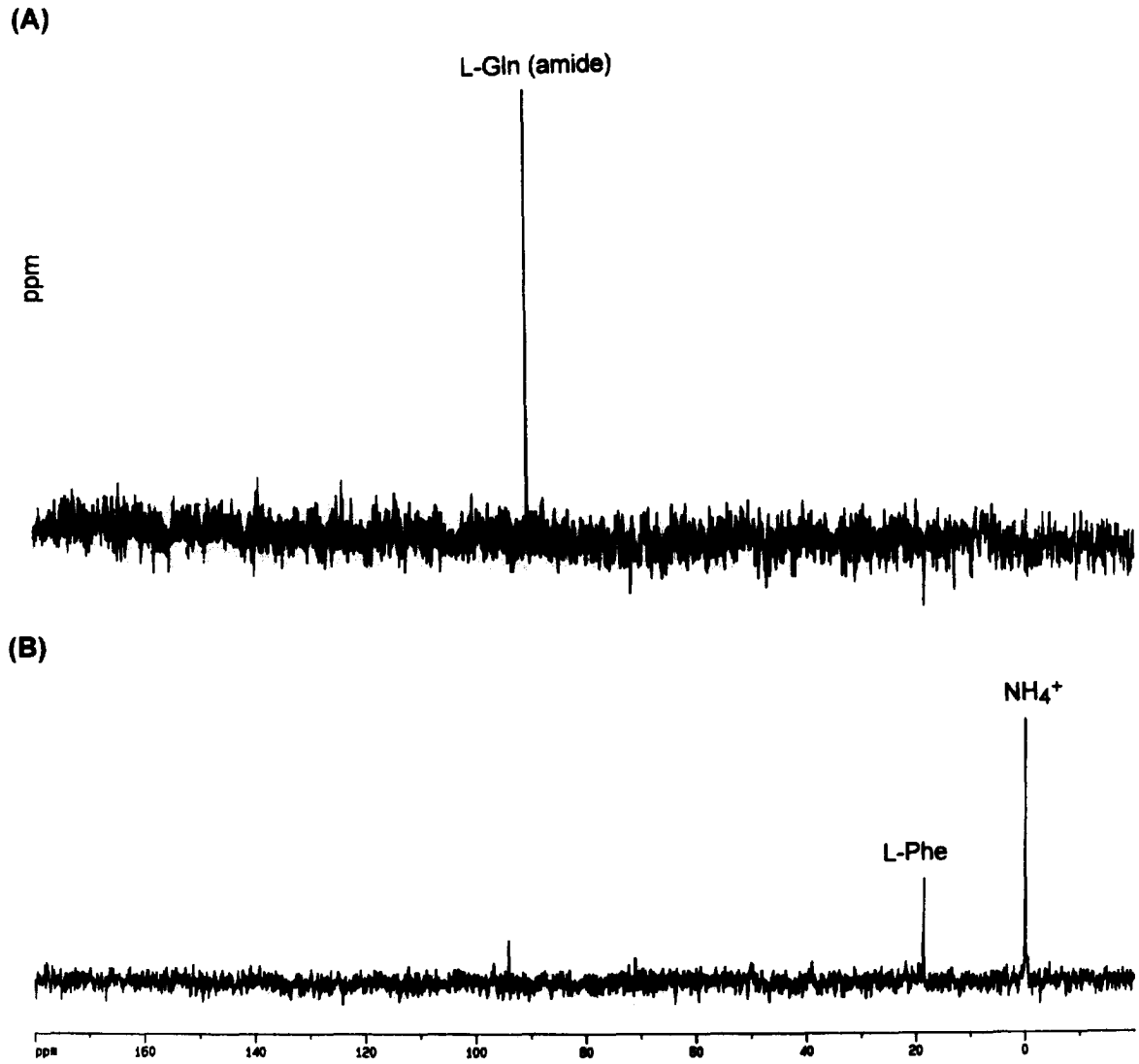

Fig. 1. ^{15}N NMR spectra of extracts prepared from potato discs fed for 24 hr with (A) [^{15}N]-L-Phe and (B) [^{15}N]-L-Phe in presence of MSO.

In this investigation, incubation of potato discs with 25 mM [^{15}N]-L-Phe for a 24-hr period revealed that active assimilation of the ammonium ion released during deamination had occurred. This was evidenced by a new resonance at δ 90.8 ppm, corresponding to the amide functionality of L-Gln, thus demonstrating participation of GS (Fig. 1A). This pathway was further verified by incubating discs with 25 mM [^{15}N]-Phe, but now in the presence of 5 mM methionine-S-sulphoximine (MSO), a known GS inhibitor [17]. As can be seen in Fig. 1B, only resonances due to L-Phe (δ 18.4 ppm) and ammonium ion (δ 0.14 ppm) were observed. Interestingly, administration of [^{15}N]-L-Tyr did not yield similar findings, i.e. no conversion into L-Gln was observed under the conditions employed.

When potato discs were next incubated with 25 mM [^{15}N]-L-Phe for extended intervals of time (48 hr), metabolism into both L-Gln (δ90.8 ppm) and L-Glu (δ18.5 ppm) was noted. Thus, the GS/GOGAT system effectively provides a mechanism whereby released ammonium ion is rapidly assimilated instead of rising to toxic levels within the cells. As expected, no evidence for accumulation of arogenic acid was observed. This is in agreement with previous observations that arogenate levels in plants are normally too low to be detected even at the picogram level [18].

Comparable results were obtained when potato discs were incubated with 20 mM [^{15}N]-NH_4Cl. Following a 24 hr period incubation, active assimilation of ammonia via GS was also observed as shown by the new resonance at δ 90.8 ppm [L-Gln, amide] (Fig. 2A). As before, addition of 5 mM MSO inhibited this conversion (Fig. 2B). Taken together, these experiments indicate that only ammonium ion assimilation via GS is operative, and not glutamate dehydrogenase (GDH) activity since it is not inhibited by MSO. (There is some controversy over

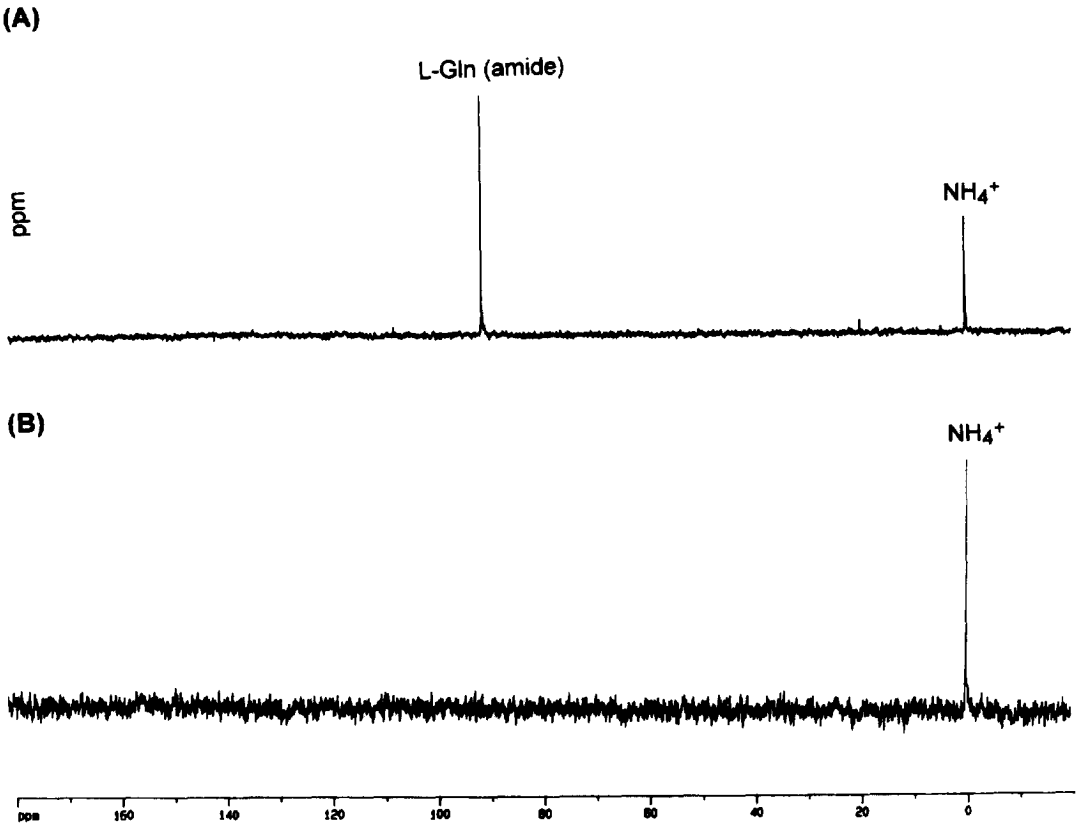

Fig. 2. ^{15}N NMR spectra of extracts prepared from potato discs fed for 24 hr with (A) $^{15}NH_4Cl$ and (B) $^{15}NH_4Cl$ in presence of MSO.

the role of GDH which for the most part is believed to be catabolic [19], although an anabolic function has been demonstrated in the green alga, *Chlorella fusca* [20]. Nevertheless, the absence of any [^{15}N]-L-Gln resonance in the MSO experiments suggests that GDH is not an important factor and that the primary assimilation route for PAL-generated ammonium ion is via GS.)

In summary, our findings with potato discs demonstrate the metabolic fate of ammonium ions released during active phenylpropanoid biosynthesis. It helps to explain why lignifying cells or tissues, which are undergoing massive phenolic synthesis, do not manifest metabolic stress due to nitrogen deficiency, i.e. because the active GS/GOGAT system rapidly assimilates the ammonium ion generated, thereby providing L-Glu as nitrogen donor for aromatic amino acid biosynthesis, leading to arogenate, phenylalanine and tyrosine. A proposed mechanism for recycling the liberated ammonium ion back to L-Phe for further phenylpropanoid metabolism is shown in Fig. 3.

Interestingly, these results indicate that the active ammonium ion assimilation process (i.e. GS/GOGAT) is the same as that observed for non-lignifying tissues from maize (*Zea mays*) seedling roots [21] and white spruce (*Picea glauca*) buds [22].

EXPERIMENTAL

Plant material. Tubers of *S. tuberosum* (var. Russet) were obtained from a local produce store.

Reagents. $CHCl_3$, HCl (Fisher Scientific, Chicago, IL, USA), EtOH (Commercial Alcohols, Mississauga, Canada) were reagent grade. [^{15}N]-L-Phenylalanine (98% ^{15}N), $^{15}NH_4Cl$ (98% ^{15}N), [^{15}N]-L-glutamic acid (95–99% ^{15}N), and [^{15}N]-L-glutamine (amide ^{15}N) (99% ^{15}N) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). MSO was purchased from Sigma.

Incubation of potato discs with ^{15}N-labelled precursors. Potato tubers were scrubbed, thoroughly washed with tap water, peeled and soaked in 30% bleach solution (with 0.1% Tween-20) for 20 min under sterile conditions and then rinsed with sterile distilled water (8 ×). Potato discs (1.2 cm diameter and *ca* 1 mm thick) were cut from cylinders obtained with a sterile cork borer (no. 6). The discs were rinsed in sterile distilled water (3 × 50 ml), and blotted on sterile filter paper; 5 g (21 discs) of potato were incubated with ^{15}N-labelled precursors (dissolved in 5 ml 10 mM HEPES buffer, pH 7.2) in prepn dishes (150 × 60 mm). ^{15}N-labelled precursors were used either separately or in combination with MSO (5 mM) in the following concns: 25 mM

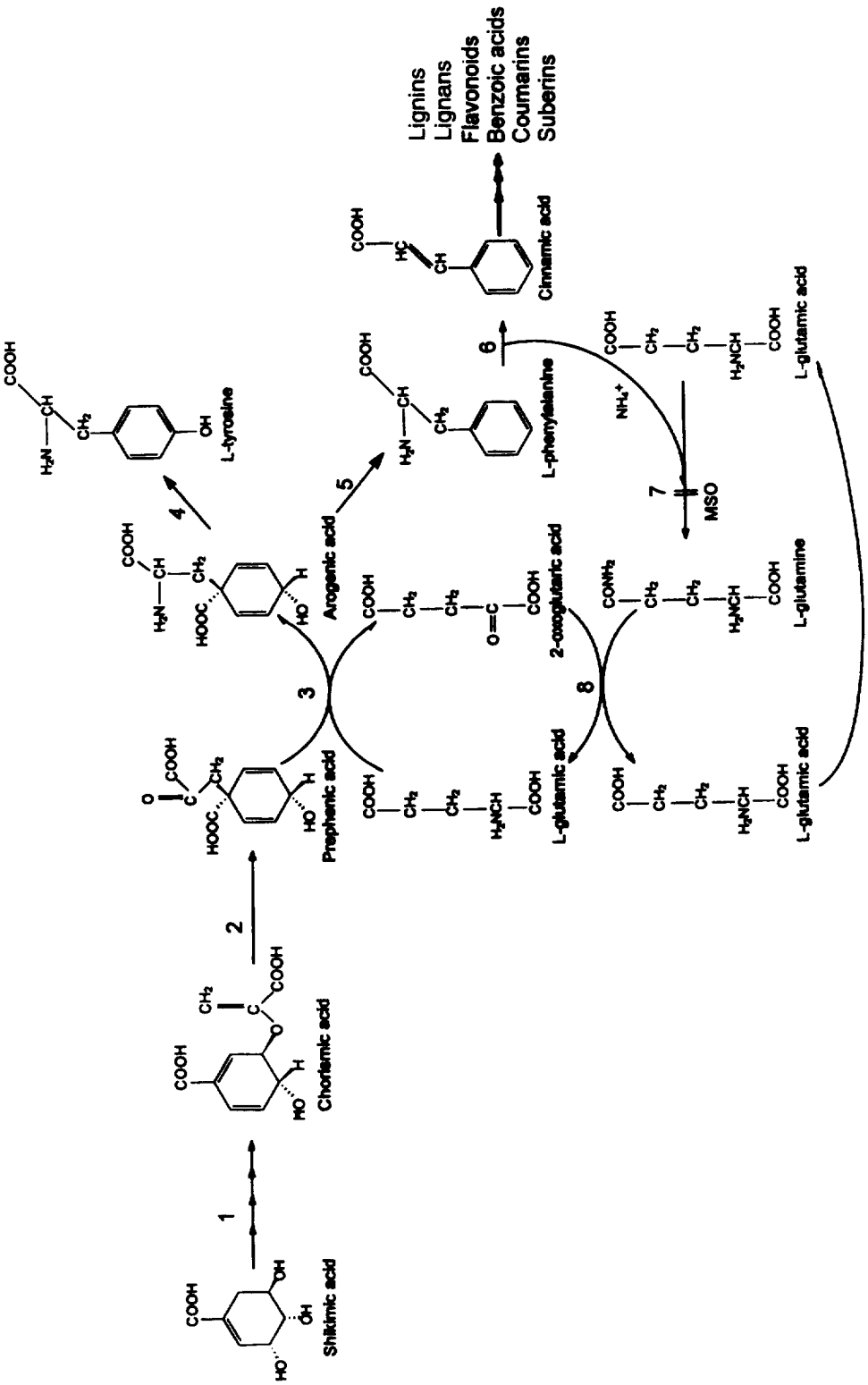

Fig. 3. Proposed scheme for nitrogen recycling in phenylpropanoid metabolism. Numbers indicate enzymes: 1, shikimate–chorismate pathway enzymes; 2, chorismate mutase; 3, prephenate:glutamate aminotransferase; 4, arogenate dehydrogenase; 5, arogenate dehydratase; 6, phenylalanine ammonia lyase; 7, glutamine synthetase (GS); 8, glutamine: 2-oxo-glutarate amidotransferase (GOGAT).

[^{15}N]-L-phenylalanine, 25 mM [^{15}N]-L-glutamine, 25 mM [^{15}N]-L-glutamic acid and 20 mM $^{15}NH_4Cl$. The prepn dishes were sealed with parafilm and transferred to a shaker in a growth chamber. Incubation was at 25°, under fluorescent and incandescent light with shaking at 100 oscillations min^{-1} for 24 or 48 hr.

Extraction of amino acids of the soluble pool from the potato discs. Following each incubation, the precursor soln was aseptically removed and an aliquot plated onto Meuller–Hinton agar medium to test for microbial contamination. The discs were washed with cold distilled H_2O (4 × 50 ml), wrapped in aluminium foil, frozen (liquid N_2), and stored at − 70° until needed.

The frozen potato discs were ground in a chilled mortar in 5 ml 95% EtOH and the resulting slurry transferred, with 2 rinses (2.5 ml 95% EtOH each) to a stainless steel Sorvall Omnimixer canister. The mixture was blended (100 sec) and the resulting suspension centrifuged for 10 min (9000 rpm, 4°) in a Sorval Superspeed RC2-B Centrifuge. The supernatant so obtained was decanted, the pellet collected and resuspended in 2 ml 95% EtOH and centrifuged for 10 min as before (2 ×). The supernatants were combined and dried under reduced pressure.

Prepn of amino acid extract for ^{15}N NMR. Distilled deionized water (2.5 ml) was added to each dried EtOH extract. The resulting suspension was transferred to a separatory funnel (25 ml). The flask was rinsed with 2.5 ml distilled water, and washings (2.5 ml) were combined and extracted with $CHCl_3$ (5 ml). The resulting aq. fr. was removed, centrifuged for 10 min (9000 rpm, 4°) and the supernatant containing the amino acids, frozen (liquid N_2), lyophilized and dissolved in 0.1 M HCl (1 ml). D_2O (50 μl) was added as int. standard and the soln filtered into a NMR (5 mm) tube and subjected to NMR analysis.

^{15}N NMR spectroscopy. ^{15}N NMR spectroscopic measurements were performed on a Bruker AMX 500 spectrometer operating at 50.68 MHz at 298 K, with broad band coupling, employing a Waltz-16 composite pulse sequence. Chemical shifts are relative to the $^{15}NH_4^+$ resonance at 0 ppm obtained using $^{15}NH_4Cl$ (250 mM) as ext. standard. Assignment of resonances in each sample was made by comparison with authentic ^{15}N-labelled amino acids. Spectra were also re-run using [^{15}N]-L-Phe and [^{15}N]-L-Glu as int. standards.

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