THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 23, Issue of June 6, pp. 20731–20737, 2003

Printed in U.S.A.

The Folate Precursor *p*-Aminobenzoate Is Reversibly Converted to Its Glucose Ester in the Plant Cytosol*

Received for publication, March 21, 2003, and in revised form, March 28, 2003
Published, JBC Papers in Press, March 31, 2003, DOI 10.1074/jbc.M302894200

Eoin P. Quinlivan‡, Sanja Roje§, Gilles Basset§, Yair Shachar-Hill¶, Jesse F. Gregory III‡, and Andrew D. Hanson§‖

From the ‡Food Science and Human Nutrition Department and the §Horticultural Sciences Department, University of Florida, Gainesville, Florida 32611 and the ¶Plant Biology Department, Michigan State University, East Lansing, Michigan 48824

Plants synthesize *p*-aminobenzoate (pABA) in chloroplasts and use it for folate synthesis in mitochondria. It has generally been supposed that pABA exists as the free acid in plant cells and that it moves between organelles in this form. Here we show that fruits and leaves of tomato and leaves of a diverse range of other plants have a high capacity to convert exogenously supplied pABA to its β-D-glucopyranosyl ester (pABA-Glc), whereas yeast and *Escherichia coli* do not. High performance liquid chromatography analysis indicated that much of the endogenous pABA in fruit and leaf tissues is esterified and that the total pool of pABA (free plus esterified) varies greatly between tissues (from 0.2 to 11 nmol g^{-1} of fresh weight). UDP-glucose:pABA glucosyltransferase activity was readily detected in fruit and leaf extracts, and the reaction was found to be freely reversible. *p*-Aminobenzoic acid β-D-glucopyranosyl ester esterase activity was also detected in extracts. Subcellular fractionation indicated that the glucosyltransferase and esterase activities are predominantly if not solely cytosolic. Taken together, these results show that reversible formation of pABA-Glc in the cytosol is interposed between pABA production in chloroplasts and pABA consumption in mitochondria. As pABA is a hydrophobic weak acid, its uncharged form is membrane-permeant, and its anion is consequently prone to distribute itself spontaneously among subcellular compartments according to their pH. Esterification of pABA may eliminate such errant behavior and provide a readily reclaimable storage form of pABA as well as a substrate for membrane transporters.

Tetrahydrofolate and its derivatives, collectively termed folates, are essential cofactors for one-carbon reactions in eukaryotes and most prokaryotes. Bacteria, fungi, and plants synthesize folates *de novo*, but humans and other higher animals do not and therefore need a dietary supply (1, 2). For humans, a major part of this supply comes from plants, and there is consequently interest in engineering food plants for enhanced folate content (3, 4). This engineering approach to human nutrition, termed biofortification, depends on understanding the synthesis, metabolism, and transport of folates and their precursors in plants.

The folate molecule is tripartite, comprising pteridine, glutamate, and *p*-aminobenzoate (pABA)[1] moieties (Fig. 1*A*). In plants, biochemical and genomic data indicate that the pteridine moiety is made in the cytosol, that the pABA moiety is made in the chloroplast, and that the two are coupled together and glutamylated in the mitochondrion (Fig. 1*B*) (reviewed in Refs. 5 and 6). In the course of folate synthesis, pABA must therefore exit chloroplasts, cross the cytosol, and enter mitochondria. This elaborate trajectory is not seen in bacteria and fungi, in which the entire pathway is cytosolic (7, 8).

Recent reviews of plant folate synthesis have tacitly assumed that pABA exists as the free acid in plant cells and that it moves between organelles in this form (5, 6). This assumption is questionable because there is no information about the endogenous level of free pABA in plants, but there are various reports of pABA conjugates. Thus, the β-D-glucopyranosyl ester of pABA (pABA-Glc) and *N*-acetyl and *N*-formyl pABA have been found as metabolites of supplied pABA in cell cultures of *Eucalyptus* and *Solanum* spp (9–11), and triterpenoid pABA esters have been identified in cucurbit seeds (12). The *N*-glucoside of pABA-Glc has been reported from plant cell cultures (9) but is probably a chemical artifact. Arylamines such as pABA and pABA-Glc readily condense with glucose and other aldoses (13), and this reaction can occur in culture media (14) and during alcohol extraction (15).

The possible presence of pABA esters or other conjugates raises two questions relating to the folate synthesis pathway and its engineering. First, are such derivatives the major form of pABA in plants? This is so for the pABA analog *p*-hydroxybenzoate, of which >98% accumulates as derivatives (16). Were this true of pABA, it would suggest that pABA derivatives could be reclaimable storage pools or substrates for membrane transport. Second, are the contents of pABA and its derivatives large as compared with the pABA demand for folate synthesis? If not, enhancing the capacity for pABA synthesis would need to be included in engineering strategies to raise plant folate levels.

To answer these questions, we used radiotracer methods to investigate pABA conjugate formation and developed HPLC procedures to quantify free and conjugated pABA. Having identified pABA-Glc as an important conjugate, we investigated the enzyme activities responsible for its formation and reconver-

* This work was supported in part by the Florida Agricultural Experimental Station, by an endowment from the C. V. Griffin, Sr. Foundation, and by Grant MCB-0129944 from the National Science Foundation, and approved for publication as Journal Series no. R-09397. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‖ To whom correspondence should be addressed: Horticultural Sciences Dept., University of Florida, P. O. Box 110690, Gainesville, FL 32611. Tel.: 352-392-1928 (ext. 334); Fax: 352-392-5653; E-mail: adha@mail.ifas.ufl.edu.

[1] The abbreviations used are: pABA, *p*-aminobenzoic acid; pABA-Glc, *p*-aminobenzoyl β-D-glucopyranoside; HPLC, high performance liquid chromatography; LC-MS, liqiud chromatography-mass spectrometry.

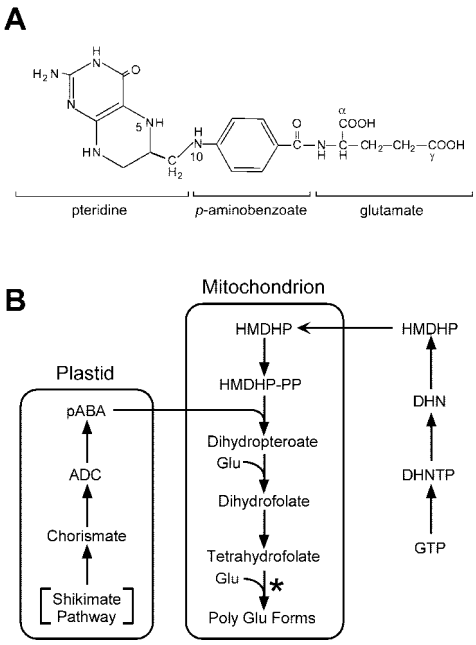

FIG. 1. **Folate structure and biosynthesis.** *A*, the chemical structure of tetrahydrofolate. One-carbon units are attached at the N^5 or N^{10} positions. Plant folates exist mainly as polyglutamylated forms in which a γ-linked chain of up to about eight glutamyl residues is added to the glutamate moiety. *B*, the folate synthesis pathway and its probable compartmentation in plant cells. The *asterisked* step occurs also in cytosol and plastids. *ADC*, aminodeoxychorismate; *DHN*, dihydroneopterin; *DHNTP*, dihydroneopterin triphosphate; *HMDHP*, hydroxymethyldihydropterin; PP, pyrophosphate.

sion to pABA and showed that these activities are located in the cytosol. We focused particularly on tomato fruit and pea leaves because folate synthesis has been studied in both systems and because tomatoes are our choice for engineering (5, 17).

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—[*Ring*-^{14}C]*p*-aminobenzoic acid (55 mCi $mmol^{-1}$) was purchased from Moravek Biochemicals (Brea, CA). Specific radioactivities were adjusted with unlabeled pABA (K^+ salt). [^{14}C]pABA-Glc (55 mCi $mmol^{-1}$) and *N*-glucosyl [^{14}C]pABA were isolated by preparative TLC from tomato pericarp disks incubated with [^{14}C]pABA, as described below. *N*-Glucosyl pABA was prepared by heating pABA with glucose in aqueous solution (13). All other biochemicals were from Sigma. AG-50W-X8 and AG 4-X4 ion-exchange resins were from Bio-Rad. The former was prepared just before use by treating 1-ml columns successively with 5 ml of 6 M NH_4OH, 10 ml of water, 5 ml of 6 M HCl, and 10 ml of water. Silica Gel 60 F_{254} TLC plates were from Merck.

Plants and Growth Conditions—Tomato (*Lycopersicon esculentum* cv. Micro-Tom), melon (*Cucumis melo*), and cotton (*Gossypium hirsutum*) were grown in potting soil in a chamber (16-h days, photosynthetic photon flux density 200 μmol of photons m^{-2} s^{-1}, 23 °C/8-h nights, 20 °C). Pea (*Pisum sativum* cv. Laxton's Progress 9 or Little Marvel) was grown in vermiculite at 20 °C in 12-h days (200 μmol of photons m^{-2} s^{-1}) for 10 days. *Arabidopsis thaliana* (ecotype Columbia) was grown in potting soil at 23–30 °C in 12-h days (200 μmol of photons m^{-2} s^{-1}). Maize (*Zea mays*) and bean (*Phaseolus vulgaris*) were greenhouse-grown. Lettuce (*Lactuca sativa*) and spinach (*Spinacia oleracea*) were purchased locally.

[^{14}C]pABA Metabolism by Plant Tissues—Disks (1-cm diameter) or squares (1 cm^2) were cut from all tissues except *Arabidopsis* leaves, which were used whole. The surface of the midrib was shaved from the abaxial surface of leaf tissues, and pericarp disks were given eight shallow radial cuts on the inner face. [^{14}C]pABA dissolved in 3–10 μl of water was applied to the cut surfaces. Tissues were incubated in Petri dishes for 6 h at 24 °C in light (200 μmol of photons m^{-2} s^{-1}), and then shaken (100 rpm) for 15 min in 5 ml of 0.1 mM pABA to remove non-absorbed label, which was quantified by scintillation counting. Tissues were extracted by grinding in 2 ml of semifrozen methanol. Aliquots (60 μl) of the methanol extract were separated by TLC on Silica Gel 60 F_{254} plates developed with ethyl acetate:methanol:water (solvent A, 77:13:10, v/v/v). Radioactive zones were detected by autoradiography and scraped from the plate for scintillation counting. pABA and its metabolites were visualized in UV light

[^{14}C]pABA Metabolism by Yeast and Escherichia coli—Saccharomyces cerevisiae strain 971/6c (*Mata ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1Δ2 can1*) (18) was cultured at 30 °C in minimal medium with appropriate supplements as described (17). *E. coli* strain K12 was cultured at 37 °C in minimal medium (19). Exponentially growing 40-ml cultures received 3.7 μCi (yeast) or 1.8 μCi (*E. coli*) of [^{14}C]pABA (55 mCi $mmol^{-1}$) and were incubated until 28% (yeast) or 46% (*E. coli*) of the label had been taken up. Cells were then pelleted, extracted in methanol, and analyzed by TLC as above.

Preparation and Quantification of pABA-Glc—Batches (5 g) of 1-cm disks cut from mature green tomato pericarp were supplied as above with unlabeled pABA (K^+ salt, 1 μmol/disk) for 16 h, frozen in liquid N_2, and pulverized. The ground material was extracted successively with 25 and 10 ml of methanol. After centrifuging to clear, the pooled extracts were concentrated *in vacuo* at 28 °C to ~1 ml, and 0.5-ml samples were fractionated on a silica gel column (70–230 mesh, 2.5 × 18 cm) equilibrated in and developed with solvent A. Fractions containing pABA-Glc were identified by TLC, pooled, dried *in vacuo*, and redissolved in 2 ml of methanol; overall pABA-Glc yield was 51%. NMR analyses were carried out on this sample. pABA-Glc was further purified by HPLC on a 5-μm Discovery C_{18} column (250 × 4.6 mm, Supelco) eluted with methanol:water (70:30, v/v) at a flow rate of 1 ml min^{-1}. Peaks were detected by absorbance at 295 nm and by fluorescence (290-nm excitation, 340-nm emission). pABA-Glc was quantified spectrophotometrically either directly or as free pABA after hydrolysis in 0.1 M HCl at 80 °C for 3 h, drying *in vacuo*, and redissolving in 0.1 M potassium phosphate, pH 7.2. The molar extinction coefficient (ε) for pABA was taken as 15,000 at 267 nm (20). An ε value of 18,120 at 296 nm (in 0.1 M potassium phosphate, pH 7.2) was calculated for pABA-Glc from hydrolysis data.

NMR and LC-MS Analyses—For NMR analysis, the sample was dried under nitrogen and redissolved in 0.5 ml of CD_3OD. One-dimensional ^{1}H NMR spectra were acquired at 500 MHz on a Varian Unity spectrometer (Palo Alto, CA) using a 5-mm probe. Acquisition parameters were such as to give close to fully relaxed spectra for integration of signal intensities (90° pulses, 15-s recycle time). LC-MS analysis was conducted using a Thermo Finnigan TSQ 7000 triple quadrupole mass spectrometer with APCI interface in selective reactions monitoring MS2 mode. N_2 pressure was 80 p.s.i. for the sheath and 10 p.s.i. auxiliary. The vaporizer was held at 500 °C, and the heated capillary was held at 200 °C. The corona current was set to 3 kA, maintaining a voltage of ~4 kV. Argon was used as a collision cell gas at a pressure of ~2.5 mTorr and a collision energy of 10 V. The 5-μm Synergi Polar-RP column (50 × 4.6 mm, Phenomenex) was eluted with 5 mM ammonium formate, 0.1% (v/v) acetic acid using a linear methanol gradient (2–85% in 5 min) at a flow rate of 0.5 ml min^{-1}.

Estimation of pABA and Folates—For pABA analyses, tissue (0.5 g of fresh weight) was ground in 5 ml of semifrozen methanol; the supernatant was removed, and the residue was re-extracted by shaking with another 2 ml of methanol for 5 min at 22 °C. Two samples of the pooled extracts were dried *in vacuo*; one (for free pABA estimation) was dissolved in 2 ml of water, and the other (for conjugated pABA estimation) was dissolved in 2 ml of 0.1 M HCl and heated at 80 °C for 2–3 h. Tests with [^{14}C]pABA-Glc, *N*-glucosyl [^{14}C]pABA, and *N*-(*p*-aminobenzoyl)-L-glutamate established that these conditions give essentially full hydrolysis of pABA-Glc or *N*-glucosyl pABA but negligible (~2%) cleavage of the amide bond between pABA and glutamate and so do not release significant pABA from folates. The extracts were then applied to 1-ml AG 50 (H^+) columns, which were washed with 15 ml of water (for free pABA) or 5 ml of 1 M HCl followed by 15 ml of water (for conjugated pABA). The columns were eluted with 5 ml of 1 M NH_4OH, and the eluates were lyophilized, redissolved in 0.2 ml of 0.1 M sodium citrate, pH 3.7, and partitioned against 0.6 ml of ethyl acetate. pABA was recovered from the organic phase by back extraction with 0.18 ml of 50 mM NaOH, which was neutralized with acetic acid. Samples of the neutralized extract were injected onto a 5-μm Discovery C_{18} column (250 × 4.6 mm, Supelco) and eluted with 0.5% acetic acid:methanol (80:20, v/v) at a flow rate of 1 ml min^{-1}. Peaks were detected by fluorescence (290-nm excitation, 340-nm emission). The pABA peak was quantified relative to standards. Data were corrected for the recovery of pABA spikes added to plant samples at the start of extraction; recoveries were 28–64% and 37–44% for unhydrolyzed and hydrolyzed samples, respectively. Free pABA values were further corrected for the proportion of pABA-Glc spikes (11–29%) that broke down to pABA

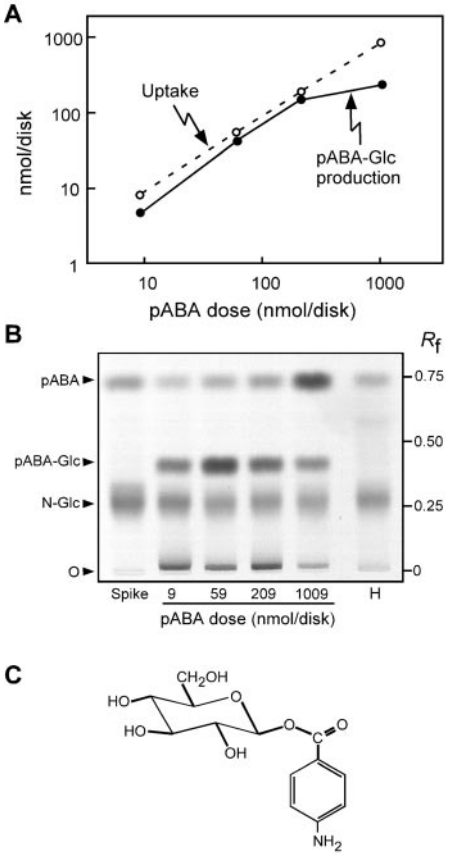

FIG. 2. **Uptake and metabolism of [^{14}C]*p*-aminobenzoate by tomato fruit tissue.** Pericarp disks (0.16 ± 0.1 g of fresh weight) cut from mature green fruits were supplied with the indicated doses of [^{14}C]pABA and incubated at 24 °C for 6 h in the light. Control samples received [^{14}C]pABA at the start of extraction (*Spike*) or were killed by heat (80 °C, 10 min) before incubation (*H*). *A*, uptake of [^{14}C]pABA and its incorporation into pABA-Glc. Data are means of three replicate experiments; S.E. bars are omitted because they are smaller than the symbols. *B*, autoradiograph of TLC fractionations of methanol extracts from a representative experiment. The origin (*O*) and running positions of pABA, pABA-Glc, and *N*-glucosyl *p*-aminobenzoate (*N-Glc*) are marked. *C*, chemical structure of pABA-Glc.

during processing. Tests with [^{14}C]pABA-Glc showed that ~10% breakdown occurred during the AG 50 step alone. Similar tests with *N*-glucosyl [^{14}C]pABA indicated ~30% breakdown during this step. Folates in *Arabidopsis* leaves were extracted according to Ref. 21 and then isolated by affinity chromatography, conjugase-treated, and determined by HPLC essentially as described (22). The folates were quantified using fluorescence detection at 295 and 356 nm for excitation and emission, respectively (23).

Subcellular Fractionation—Pea (cv. Laxton's Progress 9) mitochondria and chloroplasts were isolated and purified on Percoll gradients as described (24, 25), resuspended in 25 mM Tris-HCl, pH 7.5, containing 10 mM β-mercaptoethanol (buffer A), and broken by 4–5 cycles of freezing and thawing. Organellar extracts were cleared by centrifugation (15 min, 16,000 × *g*), and glycerol was added to give a final concentration of 10% (v/v). To prepare a cytosol-enriched fraction, 15 g of pea leaves were homogenized in a Waring blender in 100 ml of potassium phosphate buffer, pH 7.2, containing 0.33 M sorbitol, 5 mM sodium ascorbate, 0.1% polyvinylpyrrolidone-40, and 10 mM β-mercaptoethanol, using three 3-s pulses at setting 3. Chloroplasts and mitochondria were pelleted by centrifugation (3 min at 500 × *g* and 20 min at 18,000 × *g*, respectively), and the resulting supernatant was desalted on a PD-10 column (Amersham Biosciences) equilibrated in buffer A containing 10% glycerol. The chloroplast stromal marker NADP-linked glyceraldehyde-3-phosphate dehydrogenase, the mitochondrial matrix marker fumarase, and the cytosol marker 5,10-methylenetetrahydrofolate reductase were assayed as described (26, 27). Fumarase was inactivated by freezing and thus was assayed in fresh samples. Other activities were measured after freezing in liquid N_2 and storing at −80 °C.

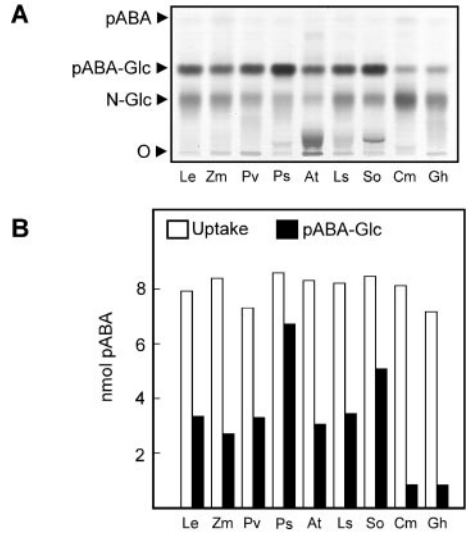

FIG. 3. **Uptake and metabolism of [^{14}C]p-aminobenzoate by leaf tissue of various plant species.** Four to six leaf disks or segments or whole *Arabidopsis* leaves (0.13–0.20 g of total fresh weight) were supplied with a total of 8.6 nmol (475 nCi) of [^{14}C]pABA and incubated at 24 °C for 6 h in the light. *A*, autoradiograph of a TLC separation of methanol extracts. The origin (*O*) and running positions of pABA, pABA-Glc, and *N*-glucosyl *p*-aminobenzoate (*N-Glc*) are marked. *B*, pABA uptake and conversion to pABA-Glc. *Open bars* show uptake, *solid bars* show pABA-Glc formation determined by duplicate TLC analyses. *Le*, *L. esculentum*; *Zm*, *Z. mays*; *Pv*, *P. vulgaris*; *Ps*, *P. satvum*; *At*, *A. thaliana*; *Ls*, *L. sativa*; *So*, *S. oleracea*; *Cm*, *C. melo*; *Gh*, *G. hirsutum*.

Assay of pABA Glucosyltransferase and pABA-Glc Esterase—Activities were measured at 30 °C; product formation was essentially linear with time. pABA glucosyltransferase was assayed in the forward (pABA-Glc synthesis) direction in 10- or 20-μl reaction mixtures containing 20 mM Tris-HCl, pH 7.5, 8 mM β-mercaptoethanol, 4 mM UDP-glucose, and the indicated amounts of [^{14}C]pABA and glycerol. After 1–3 h, [^{14}C]pABA-Glc was quantified by TLC as above using 5-μl aliquots of reaction mixtures. Alternatively, assay mixtures were applied to 1-ml columns of AG 4-X8 (OH^-) from which [^{14}C]pABA-Glc was eluted with water (5 ml) and counted. pABA-Glc esterase was assayed in 5-μl reaction mixtures containing 25 mM Tris-HCl, pH 7.5, 10 mM β-mercaptoethanol, and 20 nCi (0.36 nmol) of [^{14}C]pABA-Glc. [^{14}C]pABA release was quantified after 1–3 h by TLC. Alternatively, assay mixtures were applied to 1-ml columns of AG 4-X8 (OH^-); after washing with 20 ml of water, [^{14}C]pABA was eluted with 5 ml of 1 N HCl and counted. Reverse-direction pABA glucosyltransferase assays were the same except that they contained 20 mM UDP.

Partial Purification of pABA Glucosyltransferase from Pea Leaves—Pea (cv. Laxton's Progress 9) leaves (20 g) were pulverized in liquid N_2 and extracted in 60 ml of buffer containing 50 mM Tris-HCl, pH 8.0, 10 mM β-mercaptoethanol, 10 mM sodium ascorbate, and 3% (w/v) polyvinylpolypyrrolidone. After centrifuging to clear (18,000 × *g*, 20 min), 50% (w/v) polyethylene glycol 8000 was added to give a concentration of 30% (w/v), and the sample was held on ice for 30 min. After centrifuging (18,000 × *g*, 20 min), the pellet was discarded, and the supernatant was adjusted to 40% (w/v) polyethylene glycol, held on ice for 2 h, and recentrifuged (40,000 × *g*, 1 h). One-quarter of the resulting pellet (1.5 mg protein) was redissolved in 1.2 ml of 50 mM Tris-HCl, pH 8.0, 10 mM β-mercaptoethanol, and 20 mM NaCl and applied to a 1-ml Mono-Q column (Amersham Biosciences), which was developed with an NaCl gradient (20–410 mM), collecting 0.3-ml fractions. pABA glucosyltransferase activity eluted in one major peak at ~300 mM NaCl and in at least three minor peaks at lower NaCl concentration. The most active fraction (~100-fold enriched relative to the crude extract) was desalted on a 1-ml Sephadex G-25 column equilibrated with 25 mM Tris-HCl, pH 7.5, and 10 mM β-mercaptoethanol.

RESULTS

Massive Conversion of pABA to Its Glucose Ester by Tomato Fruit Tissue—When pericarp disks from mature green fruit were incubated with various doses of [^{14}C]pABA for 6 h, around 90% of the pABA was taken up regardless of the dose size (Fig.

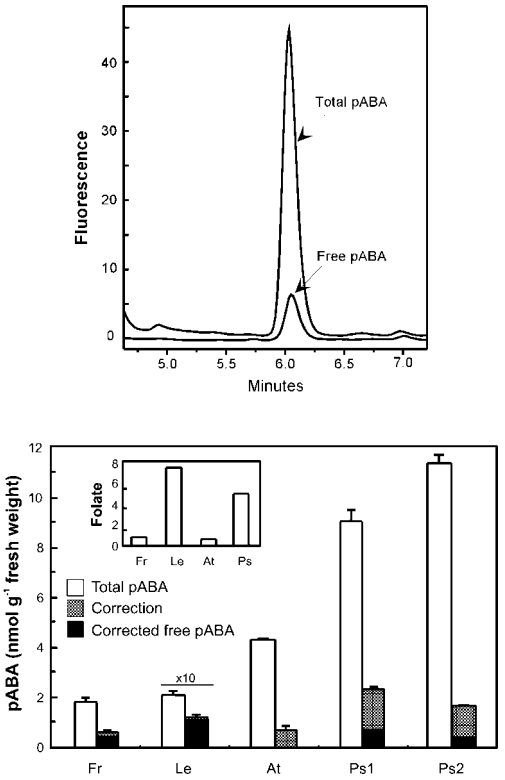

FIG. 4. **Estimation of free and total *p*-aminobenzoate in fruit and leaf tissues.** *A*, HPLC fluorescence analysis of *Arabidopsis* leaf for free pABA and total pABA obtained after acid hydrolysis. Note that pABA-Glc breakdown during sample workup contributes to the free pABA peak. *B*, total pABA and free pABA contents of the pericarp of mature green tomato fruit (*Fr*) and of leaves of tomato (*Le*), *Arabidopsis* (*At*), and pea cultivars Little Marvel (*Ps1*) and Laxton's Progress 9 (*Ps2*). Data are means of three replicates and S.E. Note that the values for tomato leaves are multiplied ×10. *Open bars* are total pABA values. *Black bars* are free pABA values after correction for pABA-Glc hydrolysis during sample processing, and the superimposed *gray bars* are the size of the correction. *Inset* shows folate contents (nmol g^{-1} of fresh weight) taken from the literature (17, 42, 43) or measured in this study (*Arabidopsis* leaves).

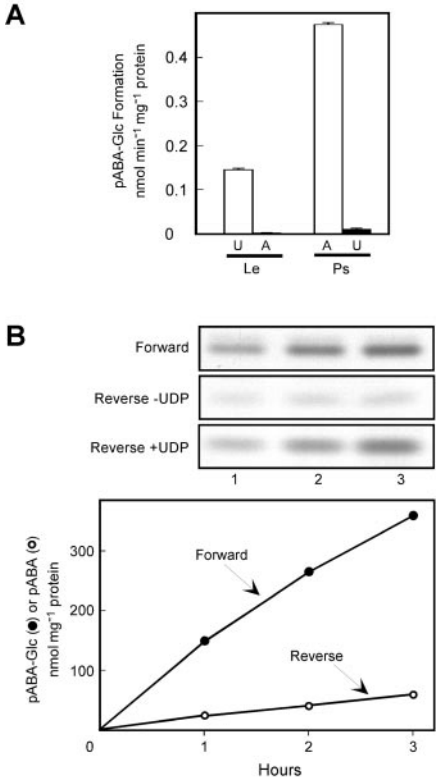

FIG. 5. **Measurement of UDP-glucose:*p*-aminobenzoate glucosyltransferase activity.** *A*, pABA glucosyltransferase activities in desalted extracts of mature green tomato pericarp (*Le*) and pea leaves (*Ps*). Assays contained 4.2 mM [^{14}C]pABA and 4 mM UDP-glucose (*U*) or ADP-glucose (*A*); these concentrations were shown to be saturating or nearly so. Data are means of three replicates and S.E. The activities with ADP-glucose were ≤2% of those with UDP-glucose. The UDP-glucose-dependent activities were at least as high as *in vivo* rates of pABA-Glc synthesis (Figs. 2 and 3), assuming a soluble protein content of 10 mg g^{-1} of fresh weight. *B*, progress curves for the forward and reverse reactions of pABA glucosyltransferase, obtained with ~100-fold purified enzyme. Forward assays contained 72 μM [^{14}C]pABA and 4 mM UDP-Glc. Reverse assays contained 72 μM [^{14}C]pABA-Glc and 20 mM UDP; the reaction was measured as UDP-dependent formation of [^{14}C]pABA. Data are means of three replicates; S.E. values were no larger than the symbols. The *upper three frames* show autoradiographs of TLC separations of the products of the forward reaction and the reverse reaction plus and minus UDP. The faint [^{14}C]pABA band seen in the absence of UDP is attributable to a trace (0.6%) of [^{14}C]pABA present in the [^{14}C]pABA-Glc substrate and to slight esterase activity remaining after the purification process.

2*A*), suggesting a non-saturable uptake mechanism. The absorbed pABA was in large part converted to a product with the TLC mobility (R_f ~0.4) reported for pABA-Glc (11), the conversion being about 50% of uptake at the three lower doses and 21% at the highest (Fig. 2, *A* and *B*). The product accumulation rate at the highest dose (~1 μmol g^{-1} of fresh weight in 6 h) is close to the pABA-Glc formation rates in *Solanum* cells grown in pABA-rich media (10, 11).

The product migrating at R_f ~0.4 was confirmed to be pABA-Glc, the β-D-glucopyranosyl ester of pABA (Fig. 2*C*), by UV spectroscopy (λ_{max}, = 299 nm), by release of pABA upon mild acid hydrolysis, by LC-MS ([M+NH_4^+] = 317), and by ^{1}H NMR. The positions and coupling constants of characteristic ^{1}H NMR aromatic and glucosyl resonances of the product (3.69 ppm, 1H, *dd*, J = 11 Hz, 5 Hz, H′-6a; 3.85 ppm, 1H, *dd*, J = 11 Hz, ~1Hz, H′-6b; 5.65 ppm, 1H, *d*, J = 8 Hz; 6.63 ppm, 2H, *d*, J = 8 Hz, H-4 and H-6; 7.81 ppm, 2H, *d*, J = 8 Hz, H-3 and H-7) matched the previously reported NMR signals of pABA-Glc but not those of other plant metabolic derivatives of pABA (9, 11). The quality of the spectra was sufficient to rule out the presence of other pABA derivatives at levels exceeding 10% of the pABA-Glc.

In addition to the pABA-Glc band, TLC showed a diffuse labeled band at R_f ~0.25, which is characteristic of the *N*-glucoside of pABA. Since this band was present in controls that received no [^{14}C]pABA until extraction or that were heat-killed before incubation (Fig. 2*B*), and since the *N*-glucoside forms spontaneously and easily (13, 14), the R_f ~0.25 band was taken to be *N*-glucoside of non-enzymatic and most likely postmortem origin and was not further analyzed.

Diverse Plants Accumulate pABA Glucose Ester but Bacteria and Yeast Do Not—To find whether pABA-Glc formation occurs in leaves as well as fruits and whether it is common among angiosperms, leaf samples of tomato and eight other species were given [^{14}C]pABA for 6 h. In all cases, [^{14}C]pABA uptake was nearly complete, the average being 93%, and label accumulated in pABA-Glc (Fig. 3, *A* and *B*). The proportion of absorbed pABA converted to pABA-Glc was in most species between 32 and 78%, which is similar to tomato fruit; only melon and cotton showed a lower conversion (10–12%). As with tomato fruit, TLC analysis showed a labeled band in the pABA *N*-glucoside position in all samples. Two species (*Arabidopsis* and spinach) also had prominent low mobility labeled bands, presumably representing additional pABA metabolites (Fig. 3*A*). These bands were shown not to correspond to *N*-acetyl

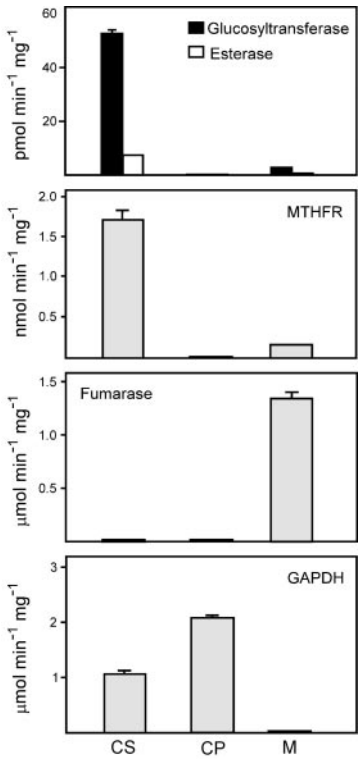

FIG. 6. **Subcellular distribution of pABA glucosyltransferase and pABA-Glc esterase activities in pea leaves.** Glucosyltransferase, esterase, and marker enzyme activities were assayed in a cytosol-enriched fraction (*CS*), in the stromal fraction of Percoll-purified chloroplasts (*CP*), and in the matrix fraction of Percoll-purified mitochondria (*M*). Glucosyltransferase activity was assayed in the forward direction using 180 μM [^{14}C]pABA, in buffer containing 8% glycerol. Esterase activity was assayed using 72 μM [^{14}C]pABA-Glc. Data are means of three replicates and S.E. *MTHFR*, 5,10-methylenetetrahydrofolate reductase; *GAPDH*, NADP-linked glyceraldehyde-3- phosphate dehydrogenase.

pABA (R_f, 0.62), *p*-hydroxybenzoate (R_f, 0.72), or folates ($R_f \sim 0$) but were not further investigated in this study.

To test whether pABA-Glc accumulates in other folate-synthesizing organisms, growing cultures of *E. coli* and yeast were given doses of [^{14}C]pABA calculated to be well in excess of the pABA requirement for folate synthesis and incubated until >25% of the dose had been taken up. TLC analysis of methanol extracts of the harvested cells showed that ≤0.1% of the absorbed label was present as pABA-Glc. This negative result is consistent with the lack of reports of pABA-Glc in *E. coli*, whose pABA metabolism has been thoroughly studied (14, 28).

Much of the Endogenous pABA in Plants Is in Conjugated Form—That plant tissues rapidly convert exogenously supplied pABA to pABA-Glc implies that much of their endogenous pABA may exist in this form. To investigate this point, we analyzed free and conjugated forms of pABA by a protocol entailing methanol extraction, cation-exchange chromatography, ethyl acetate partitioning, and HPLC with fluorescence detection. A mild acid hydrolysis step was inserted after methanol extraction to release pABA from esters and other labile conjugates. The hydrolysis conditions (0.1 M HCl at 80 °C for 2–3 h) were selected to obtain essentially complete pABA release from pABA-Glc (and *N*-glucosyl pABA) without significant release of pABA from folates and without major losses of pABA via Maillard reactions with contaminating sugars. Since pABA-Glc undergoes some spontaneous hydrolysis during extraction and chromatography, representative samples were spiked with purified pABA-Glc to enable correction for such breakdown.

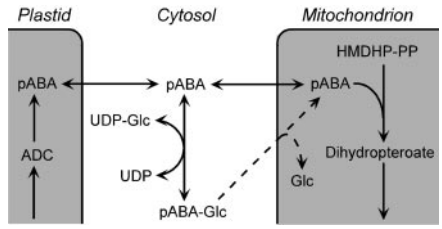

FIG. 7. **Scheme showing the probable compartmentation of pABA-Glc formation in relation to folate synthesis and to the traffic in pABA moieties between plastids and mitochondria.** The *dotted arrow* indicates a hypothetical way for pABA moieties to enter mitochondria as pABA-Glc.

Fig. 4*A* is an illustrative HPLC fluorescence trace showing that the pABA peak is symmetrical, well resolved from other peaks, and substantially larger when the sample is hydrolyzed. Data of this type for tomato pericarp and for leaves of tomato, *Arabidopsis*, and two pea cultivars are summarized in Fig. 4*B*. For free pABA, both the corrected value and the correction, often large, applied for pABA-Glc breakdown are indicated (Fig. 4*B*). Folate contents are included for comparison (Fig. 4*B*, *inset*). Two sets of generalizations can be made from these data. First, the pABA contents after hydrolysis (hereafter termed total pABA) cover a wide span (from 0.2 to 11 nmol g^{-1} of fresh weight) and vary from about 3–600% of the folate content. Second, in all cases except tomato leaves (whose total pABA content is exceptionally low), the corrected free pABA values are ≤20% of total pABA, suggesting that ≥80% of pABA is in conjugated form *in vivo*.

pABA Glucose Ester Is Formed by a Reversible UDP-glucosyltransferase Reaction—UDP-glucose:pABA glucosyltransferase activity was readily detected in desalted extracts of mature green tomato pericarp and pea leaves, and the activities measured at near saturating substrate concentrations were sufficient to account for the observed *in vivo* rates of pABA-Glc formation (Fig. 5*A*). Almost no activity was detected when ADP-glucose replaced UDP-glucose (Fig. 5*A*). Using an enzyme preparation from pea leaves (~100-fold enriched relative to the crude extract), the UDP-glucosyltransferase reaction was shown to be reversible (Fig. 5*B*), as has been reported for glucosyltransferases that produce other carboxyl esters (29, 30). While investigating the reverse reaction, we also detected modest pABA-Glc esterase activity in desalted crude extracts. The partially purified enzyme used to study the reverse reaction contained only a trace of this hydrolytic activity (Fig. 5*B*).

pABA Glucosyltransferase and pABA-glucose Esterase Activities Are Cytosolic—The subcellular locations of pABA glucosyltransferase and pABA-Glc esterase activities were investigated in pea leaves, which are well suited for recovery of intact organelles (25, 31). Chloroplasts and mitochondria were purified on Percoll gradients and showed little contamination from other fractions (Fig. 6). The preferred method for preparing minimally contaminated cytosol, via protoplast rupture, was unsuitable because of high pABA-Glc esterase activity in the commercial enzymes (cellulase, macerase, and pectinase) used in protoplast isolation. A cytosol-enriched fraction was therefore prepared by gentle mechanical grinding, centrifugation, and concentration. Such fractions are inevitably contaminated by chloroplastic enzymes, but this did not complicate data interpretation because glucosyltransferase and esterase activities were essentially absent from chloroplasts (Fig. 6). By far the highest specific activities of both these enzymes was in the cytosol fraction, and the activities in the mitochondrial fraction were low enough to be ascribed to cytosolic contamination (Fig. 6). We therefore conclude that the glucosyltransferase and esterase activities are both cytosolic, subject to the caveats that

the cytosol fraction includes vacuolar enzymes and that a few percentages of both activities could be mitochondrial.

DISCUSSION

It is central to the interpretation of our data that pABA is a hydrophobic weak acid, having a log K_{OW} (*n*-octanol/water partition coefficient) of ~0.75 (32, 33) and a pK_a of 4.9 for the carboxyl group (20). (The pK_a of the amino group is so low (2.4) that it is essentially uncharged in most physiological conditions.) Hydrophobic weak acids pass by simple diffusion from apoplast to symplast, where they accumulate by anion trapping due to the higher pH (34). This is most probably so for pABA, for we failed to saturate uptake with pABA doses as high as ~5000 times the endogenous content. Furthermore, a close analog of pABA, *o*-aminobenzoic acid, is known to cross membranes by passive diffusion (35).

Once inside plant cells, the anions of hydrophobic weak acids distribute themselves spontaneously among subcellular compartments according to pH, and therefore, flow in and out of chloroplasts, and consequently, other organelles, in response to the stromal pH changes that accompany light-dark transitions (36). Such light-driven redistribution within the cell may be undesirable for a crucial metabolic intermediate such as pABA. Another negative feature of hydrophobic weak acids is that they can act as uncouplers by shuttling across membranes and collapsing proton electrochemical gradients (37). A third drawback, specific to pABA, is that it is a substrate inhibitor of dihydropteroate synthase, the enzyme that couples it to a pterin moiety during folate synthesis. This inhibition occurs at μM levels of pABA for the bacterial enzyme (38) and apparently also for the plant enzyme (39). There are thus various *a priori* reasons for expecting plants to store pABA in a readily accessible form rather than as the free acid.

We propose that pABA-Glc formation serves this "accessible storage" function, based on four lines of evidence. First, pABA-Glc accumulation is widespread among plants and perhaps special to them as it was found in eight diverse angiosperm families but not in *E. coli* or yeast. Second, both radiotracer data and HPLC analyses indicate that the pABA-Glc pool is typically much larger than the pABA pool. Third, biochemical data show that pABA-Glc formation is reversible in two ways, via the bidirectionality of the synthetic reaction itself and via an independent hydrolytic activity. Last, subcellular distribution data show that pABA-Glc synthesis and reconversion to pABA take place in the cytosol, a plausible depot site for pABA *en route* between chloroplasts and mitochondria. This proposal for the role of pABA-Glc formation is summarized schematically in Fig. 7.

The presence of pABA-Glc in plant cells invites the question of whether it is a substrate for membrane transporters. The tonoplast is an obvious potential site for a pABA-Glc transporter since plant vacuoles are known to import other aromatic glucose esters (40), and indirect evidence suggests that not all pABA-Glc is cytosolic. Thus, the highest pABA-Glc level reported for plant cells, ~25 μmol g^{-1} of fresh weight (10), would, if confined to the cytosol, produce the improbably high cytosolic concentration of ~250 mM. The mitochondrial inner membrane is also a possible site for a pABA-Glc transporter (Fig. 7). If the cytosolic concentration of free pABA is extremely low, it might not drive enough pABA diffusion into mitochondria to sustain folate synthesis. Such a situation might be rectified by carrier-mediated pABA-Glc import into mitochondria, with concurrent or subsequent hydrolysis. This scenario might seem far-fetched in the light of HPLC data indicating significant levels of free pABA. However, it should be noted that the free pABA data are in most cases imprecise because they involve such large corrections for pABA-Glc breakdown. Moreover, the estimates of free pABA entail the assumption that *all* conjugated pABA is in the form of pABA-Glc. If some pABA exists *in vivo* as the *N*-glucoside (which our data cannot exclude), then because the *N*-glucoside breaks down more extensively during sample processing than pABA-Glc, free pABA would be overestimated. In short, although free pABA values are plainly in most cases low relative to total pABA, exactly how low is uncertain. In one case at least (*Arabidopsis* leaves), they are so low as to be undetectable against the background of spontaneous pABA-Glc breakdown.

Lastly, the data on pABA content of tomato fruit relate to the engineering of enhanced folate content. The total pABA content of mature green fruit is 1.8 nmol g^{-1} of fresh weight (Fig. 4*B*) as compared with a folate content of 1 nmol g^{-1} of fresh weight (17). The standing pool of pABA moieties is therefore not enough to support more than a 180% increase in folate content, which is less than the increase desired. Thus, if pABA synthesis in plants, as in bacteria (41), is not feedback regulated by pABA, it may need to be boosted as part of the engineering process.

REFERENCES

1. Bailey, L. B., and Gregory, J. F., III (1999) *J. Nutr.* **129,** 779–782
2. Cossins, E. A. (1987) in *The Biochemistry of Plants,* Vol. 11 (Davies, D. D., ed), pp. 317–353, Academic Press, New York
3. Bouis, H. E. (2002) *J. Nutr.* **132,** S491–S494
4. Scott, J., Rébeillé, F., and Fletcher, J. (2000) *J. Sci. Food Agric.* **80,** 795–824
5. Rébeillé, F., and Douce, R. (1999) in *Regulation of Primary Metabolic Pathways in Plants,* (Kruger, N. J., Hill, S. A., and Ratcliffe, R. G., eds), pp. 53–99, Kluwer, Dordrecht, The Netherlands
6. Hanson, A. D., and Gregory, J. F., III (2002) *Curr. Opin. Plant Biol.* **5,** 244–249
7. Cherest, H., Thomas, D., and Surdin-Kerjan, Y. (2000) *J. Biol. Chem.* **275,** 14056–14063
8. Green, J. M., Nichols, B. P., and Matthews, R. G. (1996) in *Escherichia coli and Salmonella. Cellular and Molecular Biology* (Neidhardt, F. C., ed) pp. 665–673, ASM Press, Washington, D. C.
9. Furuya, T., Asada, Y., Mizobata, S., Matsuura, Y., and Hamada, H. (1998) *Phytochemistry* **49,** 109–111
10. Panjaitan, T. S., Syahrani, A., and Indrayanto, G. (2000) *J. Planar Chromat. Mod. TLC* **13,** 114–118
11. Syahrani, A., Ratnasari, E., Indrayanto. G., and Wilkins. A. L. (1999) *Phytochemistry* **51,** 615–620
12. Appendino, G., Jakupovic, J., Belloro, E., and Marchesini, A. (2000) *Fitoterapia* **71,** 258–263
13. Ellis, G. P. (1966) *Chem. Ind.* **22,** 902–903
14. Bacher, A., Gilch, B., Rappold, H., and Lingens, F. (1973) *Z. Naturforsch. Section C Biosciences* **28,** 614–617
15. Kitamura, M., Nakao, M., and Yanagisawa, I. (1960) *J. Biochem. (Tokyo)* **47,** 60–68
16. Siebert, M., Sommer, S., Li, S. M., Wang, Z. X., Severin, K., and Heide, L. (1996) *Plant Physiol.* **112,** 811–819
17. Basset, G., Quinlivan, E. P., Ziemak, M. J., Diaz de la Garza, R., Fischer, M., Schiffmann, S., Bacher, A., Gregory, J. F., III, and Hanson, A. D. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 12489–12494
18. Nardese, V., Gutlich, M., Brambilla, A., and Carbone, M. L. (1996) *Biochem. Biophys. Res. Commun.* **218,** 273–279
19. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Appendix A.3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
20. Dawson, R. M. C., Elliott, D. C., Elliott, W. H., and Jones, K. M. (1986) *Data for Biochemical Research*, 3rd Ed., pp. 116–117, Clarendon Press, Oxford
21. Wilson, S. D., and Horne, D. W. (1984) *Anal. Biochem.* **142,** 529–535
22. Pfeiffer, C. M., Rogers, L. M., and Gregory, J. F. (1997) *J. Agric. Food Chem.* **45,** 407–413
23. Gregory, J. F., III, Manley, D. B., and Day, B. P. F. (1984) *J. Nutr.* **114,** 341–353
24. Douce, R., Bourguignon, J., Brouquisse, R., and Neuburger, M. (1987) *Methods Enzymol.* **148,** 403–415
25. Cline, K. (1986) *J. Biol. Chem.* **261,** 14804–14810
26. Roje, S., Wang, H., McNeil, S. D., Raymond, R. K., Appling, D. R., Shachar-Hill, Y., Bohnert, H. J., and Hanson, A. D. (1999) *J. Biol. Chem.* **274,** 36089–36096
27. Trossat, C., Nolte, K. D., and Hanson, A. D. (1996) *Plant Physiol.* **111,** 965–973
28. Rappold, H., and Bacher, A. (1976) *Z. Naturforsch. Section C Biosciences* **31,** 285–287
29. Leznicki, A. J., and Bandurski, R. S. (1988) *Plant Physiol.* **88,** 1481–1485
30. Mock, H. P., and Strack, D. (1993) *Phytochemistry* **32,** 575–579
31. Tissot, G., Douce, R., and Alban, C. (1997) *Biochem. J.* **323,** 179–188
32. Donlon, B. A., Razo-Flores, E., Field, J. A., and Lettinga, G. (1995) *Appl. Environ. Microbiol.* **61,** 3889–3893
33. Guanghua, L., Jie, T. Xing, Y., and Yuanhui, Z. (2001) *Chemical Journal on Internet* **3,** 74, http://www.chemistrymag.org/cji/2001/037034pe.htm
34. Ciucani, G., Trevisan, M., Sacchi, G. A., and Trapp, S. A. (2002) *Pest Manag. Sci.* **58,** 467–473
35. Fukui, S., Schwarcz, R., Rapoport, S. I., Takada, Y., and Smith, Q. R. (1991)

J. Neurochem. **56,** 2007–2017
36. Cowan, I. R., Raven, J. A., Hartung, W., and Farquhar, G. D. (1982) *Aust. J. Plant Physiol.* **9,** 489–498
37. Cohen, F. S., Eisenberg, M., and McLaughlin, S. (1977) *J. Membr. Biol.* **37,** 361–396
38. Hampele, I. C., D'Arcy, A., Dale, G. E., Kostrewa, D., Nielsen, J., Oefner, C., Page, M. G., Schonfeld, H. J., Stuber, D., and Then, R. L. (1997) *J. Mol. Biol.* **268,** 21–30
39. Rébeillé, F., Macherel, D., Mouillon, J. M., Garin, J., and Douce, R. (1997) *EMBO J.* **16,** 947–957
40. Wink, M. (1997) *Adv. Bot. Res.* **25,** 141–169
41. Viswanathan, V. K., Green, J. M., and Nichols, B. P. (1995) *J. Bacteriol.* **177,** 5918–5923
42. Cossins, E. A. (2000) *Can. J. Bot.* **78,** 691–708
43. Gambonnet, B., Jabrin, S., Ravanel, S., Karan, M., Douce, R., and Rébeillé, F. (2001) *J. Sci. Food Agric.* **81,** 835–841