PERGAMON

Phytochemistry 53 (2000) 433–440

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

L-Ascorbic acid and L-galactose are sources for oxalic acid and calcium oxalate in *Pistia stratiotes*

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Received 16 April 1999; received in revised form 7 July 1999

Abstract

Axenic *Pistia stratiotes* L. plants were pulse-chase labeled with [^{14}C]oxalic acid, L-[1-^{14}C]ascorbic acid, L-[6-^{14}C]ascorbic acid, D-[1-^{14}C]erythorbic acid, L-[1-^{14}C]galactose, or [1-^{14}C]glycolate. Specific radioactivities of L-ascorbic acid (AsA), free oxalic acid (OxA) and calcium oxalate (CaOx) in labeled plants were compared. Samples of leaf tissue were fixed for microautoradiography and examined by confocal microscopy. Results demonstrate a biosynthetic role for AsA as precursor of OxA and its crystalline deposition product, CaOx, in idioblast cells of *P. stratiotes* and support the recent discovery of Wheeler, Jones and Smirnoff (Wheeler, G.L., Jones M.A., & Smirnoff, N. (1998). The biosynthetic pathway of vitamin C in higher plants. *Nature*, 393, 365–369) that L-galactose is a key intermediate in the conversion of D-glucose to AsA in plants. D-[1-^{14}C]Erythorbic acid (a diastereomeric analog of AsA) is utilized also by *P. stratiotes* as a precursor of OxA and its calcium salt deposition product in idioblasts. Labeled OxA is rapidly incorporated into CaOx in idioblasts, but microautoradiography shows there is also significant incorporation of carbon from OxA into other components of growing cells, contrary to the dogma that OxA is a relatively stable end product of metabolism. Glycolate is a poor substrate for synthesis of OxA and CaOx formation, further establishing AsA as the immediate precursor in the synthesis of OxA used for calcium precipitation in crystal idioblasts.

Keywords: *Pistia stratiotes*; Araceae; L-ascorbic acid; Calcium oxalate; Crystal idioblast cells; D-erythorbic acid; L-galactose; Glycolate; Oxalic acid

1. Introduction

Historically, studies on oxalic acid (OxA) biosynthesis in plants have focused on the involvement of intermediary compounds; oxaloacetate, glycolate, and glyoxylate, as well as plant constituents such as citrate which give rise to these organic acids (Hodgkinson, 1977). The discovery that L-ascorbic acid (AsA) is cleaved at C2/C3 in *Pelargonium* leaves to yield OxA and L-(+)-tartaric acid (Wagner & Loewus, 1973) stimulated fresh interest in AsA metabolism and its relationship to OxA formation in plants (Franceschi & Loewus, 1995; Loewus, 1999). Earlier studies (Loewus, Jang & Seegmiller, 1956) showed that D-glucose is converted to AsA *without carbon-chain inversion* over a pathway that conserves the hydroxymethyl group as C6 of AsA, involves an epimerization at C5, and requires oxidations of C1 and C2 (or C3). Implicit is the conclusion that C1 + C2 of D-glucose provides the carbon source of plant-synthesized OxA.

For years, attempts to ferret out biochemical details to explain the D to L transition at C5 during AsA biosynthesis in plants were frustrated by three simple facts: exogenously-supplied L-galactono-1,4-lactone was effectively oxidized to AsA by plants (Isherwood, Chen & Mapson, 1954; Jackson, Wood & Prosser,

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0031-9422/00/$ - see front matter
PII: S0031-9422(99)00448-3

1961; Baig, Kelly & Loewus, 1970; Leung & Loewus, 1985; Matsuda, Ishikawa, Takada & Shigeoka, 1995; Ôba, Ishikawa, Nishikawa, Mizuno & Yamamoto, 1995; Østergaardt, Persiau, Davey, Bauw & van Montagu, 1997); L-galactono-1,4-lactone was not found in plants; and an enzymic pathway from D-glucose or hexose phosphate to AsA that fully accommodated conditions described above was lacking (Loewus, Bedgar, Saito & Loewus, 1990). This dilemma was resolved by the discovery that D-mannose and L-galactose were key intermediates in the biosynthesis of L-ascorbic acid in plants (Wheeler, Jones and Smirnoff, 1998). D-Glucose-6-P was converted to GDP-D-mannose, presumably via D-glucose-6-P isomerase, D-mannose-6-isomerase, phosphomannomutase, and mannose-1-P guanyltransferase. Epimerization of GDP-D-mannose to GDP-L-galactose by GDP-D-mannose-3,5-epimerase and subsequent loss of phosphate yielded L-galactose. Oxidation of L-galactose to L-galactonic acid, lactonization and ultimately, oxidation of L-galactono-1,4-lactone gave AsA. Evidence for the role of GDP-D-mannose in this conversion has been strengthened by a complementary genetic approach using AsA-deficient (*vtc*) mutants of *Arabidopsis thaliana* (Conklin, Pallanca, Last & Smirnoff, 1997), wherein it was found that one of these mutants, *vtc1-1*, is defective in conversion of mannose to AsA, that GDP-mannose pyrophosphorylase is lower in this mutant than in wild-type plants, and that the VTC1 locus genetically maps to a region of genomic DNA encoding a GDP-mannose pyrophosphorylase homologue (Conklin, Norris, Wheeler, Williams, Smirnoff & Last, 1999). Steps in this pathway beyond L-galactose, and those leading to cleavage of AsA and release OxA, are shown here:

D-Glucose ⇢ L-Galactose → L-Galactono-1,4-lactone
→ L-Ascorbic acid → Oxalic acid + L-Threonic acid

To test the putative role of selected carbon sources including L-galactose as precursor of AsA and ultimately, of OxA, axenically cultured *Pistia stratiotes* L. (Aralaceae) (Tarlyn, Kostman, Nakata, Keates & Franceschi, 1998) was selected as the experimental model. *P. stratiotes* is a free-floating hydrophyte of particular value for study of OxA synthesis leading to calcium oxalate (CaOx) production (V.R. Franceschi, unpublished studies). CaOx is the end product of a high capacity calcium-regulation system in plants, but little is known concerning the source of OxA used for this important regulatory system. Six potential carbon sources for OxA biosynthesis, labeled with ^{14}C, were supplied to axenic cultures of *P. stratiotes*. Following

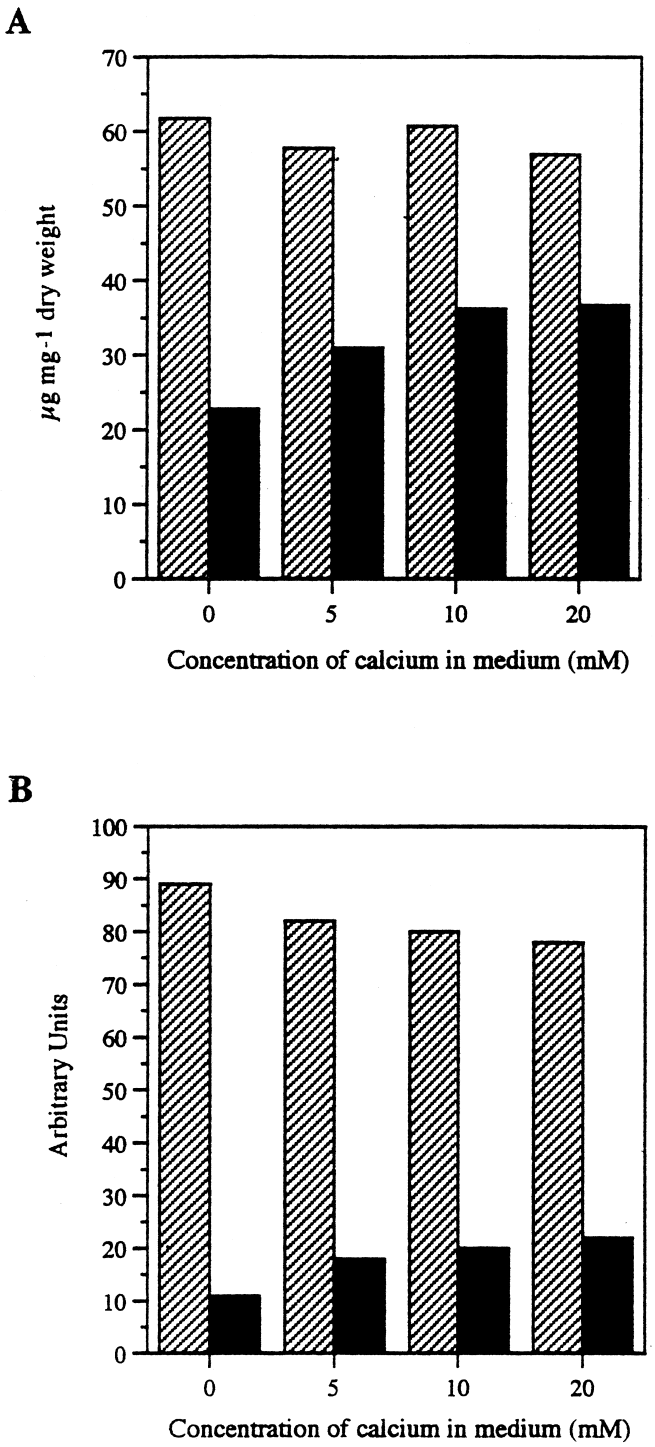

Fig. 1. Effect of calcium concentration on oxalate biosynthesis from L-[1-^{14}C]ascorbic acid. (A) Concentration (μg mg^{-1} dry weight) of oxalic acid (cross-hatched bar) and calcium oxalate (solid bar) in *Pistia stratiotes* L. grown on E medium containing 0, 5, 10 or 20 mM calcium. (B) A comparison of the specific radioactivity of oxalic acid (cross-hatched bar) to that of calcium oxalate (solid bar) in *P. stratiotes* grown on E medium containing 0, 5, 10 or 20 mM calcium. Typical data from a single experimental run are presented.

an appropriate period of labeling, tissues were analyzed by high performance liquid chromatography (HPLC) and liquid scintillation counting (LSC) for specific radioactivity in AsA, OxA, and CaOx, and microautoradiographs of tissue sections were examined to determine if any of these labeled sources was incorporated into CaOx.

2. Results and discussion

2.1. Effect of calcium concentration on oxalate biosynthesis from L-[1-^{14}C]AsA

Increasing the Ca^{2+} level in reset *P. stratiotes* cultures (see Section 3 for details) from nominally Ca^{2+} free to 20 mM Ca^{2+} increased insoluble CaOx deposition from 22 to 37 μg mg^{-1}dry weight over a 12 h period (Fig. 1A). In addition, there was a decrease in specific radioactivity in free OxA compared to that in CaOx over this same period (Fig. 1B). Based on these results, plants were routinely transferred to E medium containing 20 mM Ca^{2+}, 5 h prior to the start of pulse-chase experiments to induce maximum synthesis and deposition of oxalate from labeled substrates.

2.2. Pulse-chase experiments

Since the amount of ^{14}C-labeled substrate taken up by *P. stratiotes* plants varied depending on the chemical nature of the substrate, no attempt was made to establish net uptake during the 12 h pulse and subsequent chase. As described in Section 3, labeled tissues were separated into soluble and insoluble fractions. AsA and soluble OxA were recovered from the soluble fraction by HPLC, while CaOx was recovered from the insoluble fraction, solubilized, and then analyzed by HPLC. Specific radioactivities of AsA, OxA, and insoluble oxalate (CaOx) were then calculated, and comparisons were made among these three constituents based upon their specific radioactivities.

2.2.1. [^{14}C]Oxalic acid

In plants pulsed with [^{14}C]OxA, the highest specific radioactivity after the chase was in CaOx (Fig. 2, labeled compound A). Very little ^{14}C appeared in AsA and this labeling probably resulted from photosynthetic fixation of trace amounts of $^{14}CO_2$ produced during oxidation of [^{14}C]OxA by oxalate oxidase. Using laser scanning confocal microscopy reflected/transmitted overlap of the microautoradiographs (Fig. 3), location of ^{14}C was indicated by latent images over areas of radioactive emission. In Fig. 3A, some ^{14}C was localized in idioblasts (arrows) but there was also abundant ^{14}C in surrounding mesophyll cells, particularly in meristematic tissues, suggesting active metabolic cycling of [^{14}C]OxA in the plant.

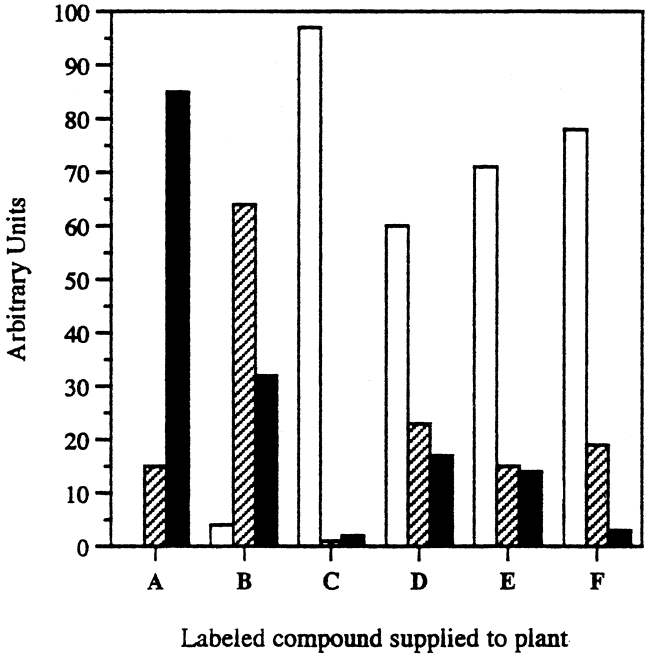

Fig. 2. Comparison of the specific radioactivities of L-ascorbic acid (clear bar), oxalic acid (cross-hatched bar) and calcium oxalate (solid bar) in plants pulse-chase labeled with: (A) [^{14}C]OxA, (B) [1-^{14}C]AsA, (C) [6-^{14}C]AsA, (D) D-[1-^{14}C]erythorbic acid, (E) L-[1-^{14}C]-galactose, or (F) calcium [1-^{14}C] glycolate.

2.2.2. L-[1-^{14}C]Ascorbic acid

In plants pulsed with [1-^{14}C]AsA, comparative specific radioactivities of the three constituents of interest were: OxA > CaOx ≫ AsA (Fig. 2, labeled compound B). This indicates that deposition of CaOx occurs at a slower rate than release of OxA or that there is an initial lag before CaOx accumulates during AsA cleavage. This pattern of labeling in OxA/CaOx supports C2/C3 cleavage of AsA. A microautoradiograph showed dense accumulation of ^{14}C over CaOx crystals within idioblasts (Fig. 3B, arrows). Sparse labeling in surrounding cells indicated some recycling of ^{14}C-labeled metabolic products likely via exchange or fixation of $^{14}CO_2$ stemming from oxalate oxidase activity.

2.2.3. L-[6-^{14}C]Ascorbic acid

Use of [6-^{14}C]AsA as a negative control confirmed the specific nature of AsA C2/C3 cleavage for OxA synthesis. As shown in previous studies, the C_4 fragment, most likely L-threonic acid (Loewus & Helsper,

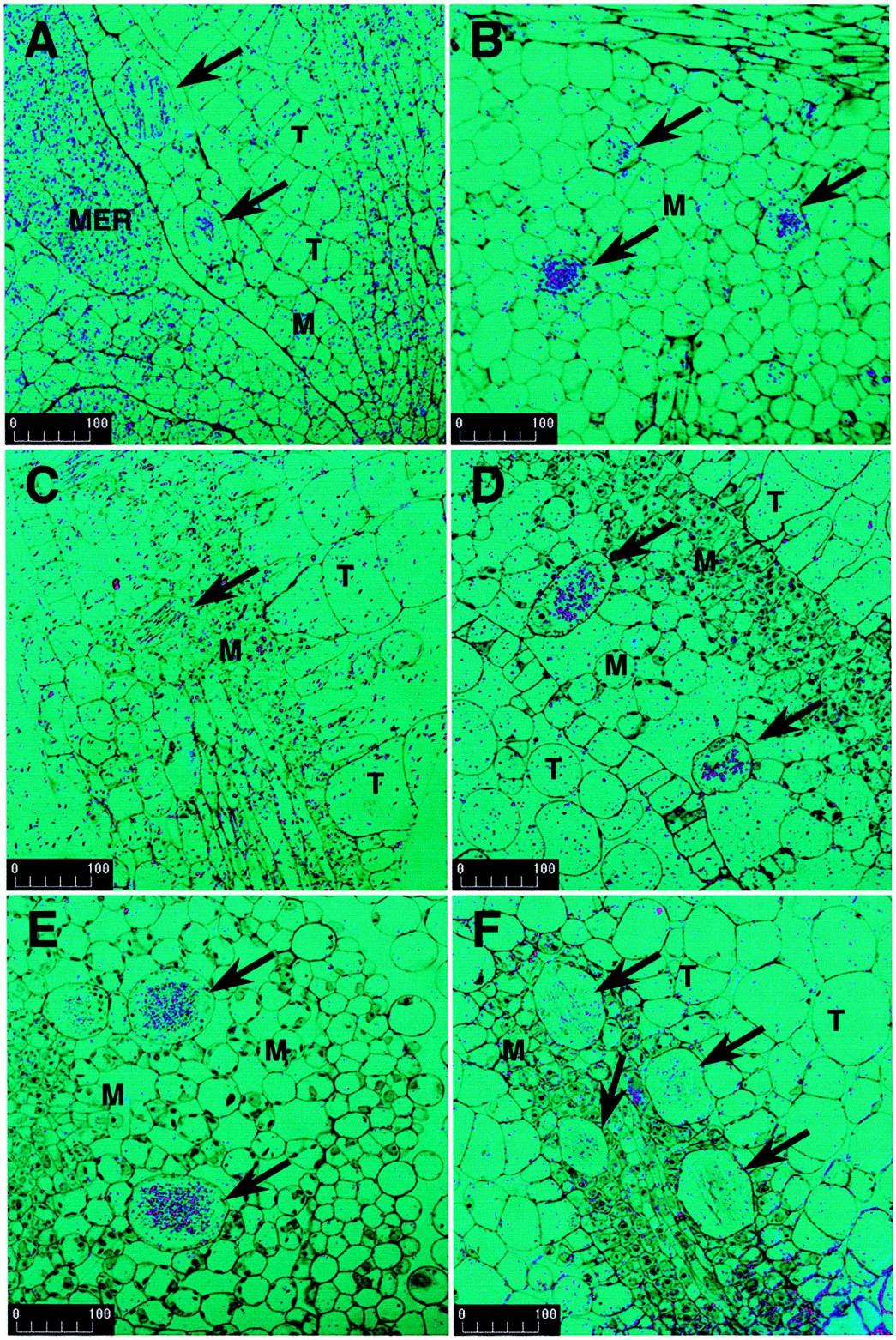

Fig. 3. Microautoradiographs of the distribution of ^{14}C in *Pistia stratiotes* tissue pulse-chase labeled with selected ^{14}C-tagged compounds. Images were collected with a confocal microscope in two channels with the silver grains from ^{14}C decay appearing as magenta point dots on the blue transmitted image of the tissue section. Arrows indicate developing calcium oxalate (CaOx) crystal idioblasts. M: mesophyll cell, T: trichome. Bar units are in μm. (A) Label from [^{14}C]OxA is incorporated into CaOx crystal idioblasts and also into insoluble products in the meristematic tissues (MER). (B) Label from L-[1-^{14}C]ascorbic acid is heavily incorporated into CaOx crystals in the vacuoles of developing idioblasts. (C) Label from L-[6-^{14}C]ascorbic acid shows general incorporation into mesophyll cell components but little incorporation into CaOx crystals. (D) Label from D-[1-^{14}C]erythorbic acid produces very strong labeling of CaOx crystals in idioblasts. (E) Label from L-[1-^{14}C]galactose is selectively incorporated into CaOx crystals. (F) Calcium [1-^{14}C]glycolate is not a good precursor of oxalic acid in crystal idioblasts, but is incorporated into insoluble products in developing mesophyll cells.

1982), probably recycled, and negligible ^{14}C appeared in either OxA or CaOx (Fig. 2, labeled compound C). A microautoradiograph (Fig. 3C) revealed random distribution of ^{14}C throughout the tissue with no specific accumulation in Ox crystals within idioblasts.

2.2.4. D-[1-^{14}C]Erythorbic acid

This diastereomer (D-*erythro*-hex-2-enono-1,4-lactone) of AsA also served as a ^{14}C-labeled carbon source of OxA/CaOx (Fig. 2, labeled compound D) but with less efficiency than L-[1-^{14}C]AsA (L-*threo*-hex-2-enono-1,4-lactone) (Fig. 2, labeled compound B). Accumulation of ^{14}C in CaOx crystals of idioblasts was very evident (Fig. 3D, arrows) confirming utilization of carbon 1 (and by inference, carbon 2) of D-erythorbic acid for OxA synthesis by *P. stratiotes*. Since HPLC protocol did not fully resolve AsA and D-erythrobic acid peaks, the bar for 'AsA' (Fig. 2, labeled compound D) represents a mixture of AsA and D-erythorbic acid. There have been few studies on D-erythorbic acid metabolism in plants, since it is not a natural plant constituent.

2.2.5. L-[1-^{14}C]Galactose

L-Galactose has been shown to be an intermediate in the conversion of D-glucose to AsA in plants (Wheeler et al., 1998). When *P. stratiotes* plants were pulse-labeled with L-[1-^{14}C]galactose, comparative specific radioactivities of the three constituents of interest were: AsA $\gg$ OxA > CaOx (Fig. 2, labeled compound E), a pattern anticipated by the biosynthetic pathway, wherein AsA is intermediate to L-galactose and OxA/CaOx, the cleavage product of AsA. A microautoradiograph of tissues labeled with L-[1-^{14}C]galactose confirmed the analytical evidence (Fig. 3E) with abundant ^{14}C accumulating in CaOx crystals in idioblasts, a localization pattern very similar to that of [1-^{14}C]AsA (compare Fig. 3B and E).

2.2.6. Calcium [1-^{14}C] glycolate

Although this photorespiratory product has been generally regarded as a possible precursor of OxA (Hodgkinson, 1977), the pulse-chase experiment indicated that ^{14}C from this compound recycled into hexose phosphate metabolism and that AsA was the initial compound of interest to become labeled, followed by its cleavage product, OxA and to a lesser extent CaOx (Fig. 2, labeled compound F). This was amply confirmed by microautoradiography of the labeled tissues (Fig. 3F) where it was found that ^{14}C distribution was greatly randomized and minor ^{14}C labeling in idioblasts was probably the result of metabolic recycling of [1-^{14}C]glycolate.

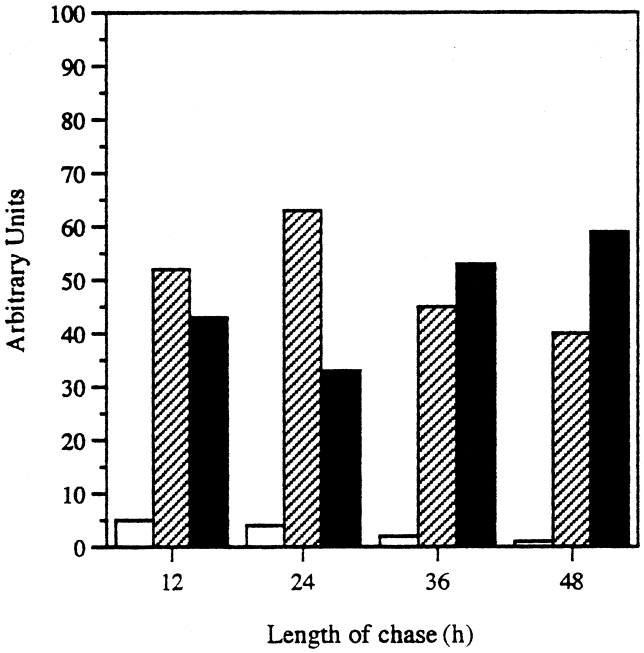

Fig. 4. Comparison of the effect of the length of chase (*h*) to specific radioactivities of L-ascorbic acid (clear bar), oxalic acid (cross-hatched bar) and calcium oxalate (solid bar) from plants supplied with L-[1-^{14}C]ascorbic acid.

2.3. Length of chase and its effect on incorporation of ^{14}C from [1-^{14}C]AsA into OxA/CaOx

In *P. stratiotes* pulsed with [1-^{14}C]AsA, the specific radioactivities of AsA and OxA decreased while the specific radioactivity in CaOx increased when the duration of chase was slightly increased from 12 to 48 h (Fig. 4). The initial increase in CaOx at 12 h probably represents a scavenging effect of OxA on available Ca^{2+} stores in the plant. Results suggest that a longer chase with unlabeled AsA may lead to a more complete utilization of [1-^{14}C]AsA and deposition of CaOx.

2.4. Conclusions

The use of axenic *P. stratiotes* cultures for pulse-chase studies with selected compounds bearing ^{14}C at specific sites has allowed for confirmation of a general pathway of synthesis of AsA and OxA in situ. HPLC/LSC analysis demonstated that L-galactose is a precursor of AsA in *P. stratiotes* and that AsA formed via this pathway is further metabolised to OxA. The *P. stratiotes* system is particularly useful for these studies as whole plants can be analyzed and it is possible to induce an increase in OxA synthesis by manipulation

of calcium nutrition to the plant. The microautoradiography technique clearly demonstrates that OxA synthesized by this pathway is used to produce CaOx in idioblasts, and also indicates that there is significant metabolism of OxA in other cells that do not accumulate CaOx. This observation of active metabolism of OxA by the plant requires further study, as the dogma concerning OxA has been that it is an end product of metabolism with little possibility of re-entry into the carbon cycle of the plant (Franceschi & Horner, 1980). *P. stratiotes* produces abundant CaOx, and these studies show that AsA is a primary precursor in the synthesis of OxA used for CaOx precipitation in situ. Since deposition of CaOx occurs in specialized cells and it is unlikely that OxA is transported from adjacent cells (Li & Franceschi, 1990), the idioblasts must be enriched in the biochemical pathway for OxA synthesis via AsA. Furthermore, there may be a selectivity in terms of substrate usage at the idioblast level, as suggested by the reduced specific radioactivity in CaOx when plants are supplied with labeled glycolate rather than labeled L-galactose or AsA/D-erythorbic acid. Directed study of this cell type could provide information about the enzyme(s) involved in the C2/C3 cleavage.

An even broader question raised by this study concerns the metabolic fate of OxA/CaOx, as it undergoes re-entry into basic carbon metabolism. One possibility, photosynthetic recovery of oxalate oxidase-generated CO_2 in plants that normally store OxA and/or CaOx, has been proposed (Loewus, 1999).

3. Experimental

3.1. Labeled compounds

L-[1-^{14}C]Galactose and calcium [1-^{14}C]glycolate were obtained from American Radiolabeled Chemicals, St. Louis, MO. [^{14}C]Oxalic acid was obtained from New England Nuclear Corp., Boston, MA. [1-^{14}C]AsA, [6-^{14}C]AsA, and D-[1-^{14}C]erythorbic acid were provided by Professor Bert M. Tolbert and repurified prior to use by cation/anion exchange resin chromatography (Wagner & Loewus, 1973).

3.2. Pulse-chase experiments

Axenic cultures of *P. stratiotes* L. (Tarlyn et al., 1998) were maintained on E medium (Cleland & Briggs, 1967) containing 5 mM Ca^{2+}. Plants were grown in GA7 Magenta vessels (approximately 10 plants and 125 ml of medium per vessel) under wide-spectrum fluorescent lamps using a 16 h photoperiod. The plants used for pulse-chase experiments were of uniform developmental age and averaged 2.5 cm in diameter. Plants were transferred to fresh medium (5 mM Ca^{2+}) for 48 h. After 48 h on fresh medium, plants were transferred to E medium minus Ca^{2+} for 72 h (*reset*). To study the effect of Ca^{2+} concentration on the rate of CaOx synthesis and to determine the concentration at which maximum CaOx synthesis was induced, an initial experiment was run in which reset plants were transferred to E medium containing 0, 5, 10, or 20 mM Ca^{2+} for 5 h. Plants that had been trimmed of all mature leaves were then transferred to a six-well culture dish holding 4 ml well^{-1} of fresh E medium containing 1 μCi ml^{-1} of [1-^{14}C]AsA (200 μg of AsA ml^{-1} of medium).

For all other pulse-chase experiments, reset plants were transferred to E medium containing 20 mM Ca^{2+} 5 h prior to addition of ^{14}C-labeled substrate. The procedure for labeling followed that used to determine optimal Ca^{2+} concentration. Plants were incubated on labeled medium for 12 h (*pulse*) in 4 ml of medium using six-well culture plates. In all experiments, approximately 1 μCi ml^{-1}of ^{14}C-labeled substrate (200 μg of substrate ml^{-1} of medium) was used per well. Following the pulse, labeled medium was removed, tissues were rinsed 3× with dH_2O, and then transferred to a clean well holding 4 ml of E medium containing 20 mM Ca^{2+} and non-labeled substrate at a concentration equivalent to that used in the pulse (200 μg of substrate ml^{-1} of medium) for an additional 12 h (*chase*). In a separate experiment designed to investigate the effect of chase duration on accumulation of labeled CaOx, plants were incubated on 20 mM Ca^{2+} in E medium with non-labeled AsA for 12, 24, 36, and 48 h. Following the chase, the medium was removed and plants were rinsed in 5 mM dithiothreitol (DTT), blotted dry, frozen in liquid N_2, lyophilized, and stored with desiccant at −20°C. A sub-sample of plants from each experiment was fixed for microautoradiography (see below).

3.3. Sample preparation and analysis

Lyophilized tissues were finely ground in a chilled mortar and suspended in 4 mM H_2SO_4 (HPLC mobile phase) containing 5 mM DTT and 0.01% insoluble polyvinylpolypyrrolidone at 10 mg ml^{-1}. Extracts were spun at 15,000 g for 30 min at 4°C to produce a clear supernatant. Soluble OxA was precipitated from the supernatant with Ca acetate as CaOx. The pellet was rinsed in dH_2O and recovered by centrifugation. The CaOx pellet was converted to free OxA by stirring it with a suspension of cation exchange resin (Dowex-

50Wx8, 200–400 mesh, H^+) for 30 min at 60°C. Resolubilized OxA was adjusted to original volume, the resin removed by centrifugation, and the supernatant analyzed for OxA.

The pellet of extracted tissue was suspended in dH_2O and re-precipitated. CaOx was recovered as free OxA by the procedure just described and analyzed for ^{14}C and amount of OxA.

Aliquots of supernatant (2 × 500 μl) were assayed for ^{14}C by liquid scintillation counting (LSC, Packard Tri-Carb Model 2100TR). An additional 300 μl of supernatant was filtered (0.45 μm) and analyzed for OxA and AsA by HPLC (Bio-Rad Aminex HPX-87H ion exclusion column, 300 × 7.8 mm, 0.6 ml min^{-1}, 35°C, 900 psi; variable wavelength UV detector, Thermal Separation Products SpectraChrom 100; and electrochemical detector, BioAnalytical Systems Model LC-4C). HPLC data were integrated and analyzed with Gilson 715 controller software v.1.21. OxA was measured at 210 nm (R_t = 7.0 min). AsA was measured by amperometric detection at 600 mV (R_t = 10.0 min). External standards at known concentrations were used to prepare calibration plots for assaying amounts of OxA and AsA. Samples from peaks of interest were collected manually and analyzed for ^{14}C by LSC.

3.4. Microautoradiography

Pulse-chase labeled tissues were rinsed, blotted dry, dissected into 2 mm^2 pieces under 50 mM PIPES buffer (piperazine-N,N′-bis[2-ethanesulfonic acid]; pH 7.2), and fixed in 2% paraformaldehyde/2.5% glutaraldehyde in 50 mM PIPES buffer for 16 h at 4°C. Following fixation, tissues were rinsed 3× in 50 mM PIPES buffer and then dehydrated in an acetone series [30%, 50%, 70%, 80%, 95%, 100% (3×); 15–30 min $step^{-1}$]. Tissues were infiltrated with Spurr's epoxy resin (Ted Pella) through graded changes of 100% acetone:resin [3:1, 2:1, 1:1, 1:2, 1:4, pure resin (3×); 24 h $step^{-1}$]. The samples were transferred to freshly prepared resin and polymerized at 60°C for 12–16 h. Embedded tissues were sectioned to a thickness of 1 μm (duPont Sorvall MT-5000 microtome). Sections were dried onto gelatin-coated microscope slides and coated with a 1:2 dilution of photographic emulsion (Ilford LP-4 nuclear tracking emulsion) prepared in dH_2O at 45°C. Slides were dried thoroughly (1–4 h) and exposed for 2–4 weeks at −20°C. Slides were developed for 5 min (undiluted Kodak D-19 developer), rinsed in a stopbath of dH_2O for 30 s, fixed for 15 min [Kodak Rapid-fixer; fixer:dH_2O (1:2)], and then gently rinsed with running dH_2O for an additional 30 min. Sections were stained with 0.05% aqueous Safranin O for 5 min at 20°C, dried completely on a slide warmer at 65°C, covered with type B immersion oil and coverslipped. Microautoradiographs were viewed with a Bio-Rad MRC 1024 laser-scanning confocal microscope. Reflected and transmitted images were collected simultaneously and false-colored, to produce the final image.

Acknowledgements

This study was supported in part by NIDDKD, NIH grant DK-45248 (FAL and VRF), grant NSF, MCB 9632027 (VRF), and Project 0266 (FAL) of the College of Agriculture and Home Economics Research Center, Washington State University, Pullman, WA 99164. We are deeply indebted to Professor B.M. Tolbert, University of Colorado for providing [1-^{14}C]AsA, [6-^{14}C]AsA and D-[1-^{14}C]erythorbic acid used in this study. Microscopy was done at the WSU Electron Microscopy Center.

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