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Chloroplasts and the Biosynthesis and Catabolism of Abscisic Acid

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Abstract. Chloroplast preparations from the mesocarp of *Persea gratissima* and from light-grown shoots of *Pisum sativum* were unable to synthesize abscisic acid (ABA) from mevalonolactone, mevalonic acid, or isopentenyl pyrophosphate. Similar plastid preparations transformed [2-^{14}C]ABA into acidic products that were chromatographically similar to those generated by *P. gratissima* mesocarp slices and excised shoots of *P. sativum*. Attempts to increase ABA catabolism in chloroplast preparations using sedimentation through Percoll to remove associated proteases also reduced the capacity for ABA catabolism, suggesting that such catabolism arose from contaminating, cytoplasmic enzymes. Both lincomycin and chloramphenicol inhibited the catabolism of ABA by excised shoots of *P. sativum* but had little effect on either ABA biosynthesis or ABA catabolism in mesocarp from *P. gratissima*. These processes were inhibited markedly by cycloheximide.

Abscisic acid (ABA) was first characterized in chloroplasts of *Pisum sativum* by Railton et al. (1974) using gas chromatography–mass spectrometry. Loveys (1977) subsequently demonstrated the presence of ABA in chloroplasts of *Spinacia oleracea* and showed that water stress resulted in large increases in ABA levels in plastids from wilted tissues of this plant. Chloroplasts from the mesocarp of fruits of *Persea gratissima* incorporated low amounts of label from [2-^{14}C]mevalonolactone (MVAL) into ABA, and Milborrow (1974a) suggested that chloroplasts could therefore be a major site of ABA biosynthesis.

Recently, however, considerable controversy has arisen concerning the role of chloroplasts in ABA metabolism. Hartung and co-workers have shown, using chloroplasts from leaves of *Spinacia oleracea*, that MVAL is not incorporated into ABA by intact or lysed plastids (Hartung et al. 1981) and that ABA is not catabolized significantly by these chloroplast preparations (Har-

tung et al. 1980). Hartung et al. (1982) concluded that chloroplasts are not involved in ABA metabolism.

Various factors could have contributed to the inability of Hartung et al. to demonstrate ABA biosynthesis and catabolism in chloroplasts of *S. oleracea*. These include the considerably higher amounts of chloroplasts used in the *S. oleracea* studies compared with those used by Milborrow (1974a) in *P. gratissima*. It is conceivable that enzyme dilution, which can increase the activity of certain terpenoid enzymes in tissue extracts (Shen-Miller and West 1982) and in chloroplast preparations from *Hordeum vulgare* and *Pisum sativum* (Railton and West, unpublished data) reduced the levels of substances interfering with enzyme activity in the *P. gratissima* study, thereby leading to detectable ABA biosynthesis. Interfering substances could include proteases, which have been reported to be associated with chloroplasts from several species (Hampp and De Filippis 1980, Waters et al. 1982), including *P. sativum* (Dahlmelm and Ficker 1982). The positive results for chloroplast-localized ABA biosynthesis obtained by Milborrow (1974a) were explained by Hartung et al. (1981) as arising from contaminating, cytoplasmic enzymes, although no attempt was made to substantiate this claim.

One surprising aspect of the studies by Milborrow (1974a) and Hartung et al. (1981) was the use of the unnatural substrate MVAL as a precursor of ABA. The natural precursor of ABA is 3-*R* mevalonic acid (MVA), and while the less polar lactone can be employed advantageously as an ABA precursor in whole-tissue feeds because of its ability to penetrate plant cells and presumably undergo hydrolysis (Milborrow and Robinson 1973), its use as a precursor in lysed organelles is undesirable, particularly when its rate of hydrolysis during incubation is unknown.

In view of the conflicting evidence regarding the role of chloroplasts in ABA biosynthesis and catabolism, we readdressed some of the aforementioned problems in the experimental system reported by Milborrow (1974a) and also examined ABA biosynthesis and catabolism in chloroplasts from *Pisum sativum* which actively synthesize the gibberellin intermediate *ent*-kaurene (Railton et al. 1984). In addition, the effects of inhibitors of chloroplast protein biosynthesis on ABA metabolism were tested in order to see whether or not plastid-synthesized proteins were involved in this process. Part of these studies were in progress when the publications of Hartung and co-workers appeared.

Materials and Methods

Plant Material

Ripe fruits of avocado (*Persea gratissima*) were purchased locally. Seeds of *Pisum sativum* L., cv. black-eyed Susan, were imbibed in running tap water for 6–8 h and grown in vermiculite under long days at 25°C until seedings were 10 days old.

Chloroplast Isolation

Chloroplasts were isolated by a modification of the method of Heber and San-

tarius (1970) as described by Railton et al. (1984) using 30 g f.w. of leaves of *P. sativum* or 30 g f.w. of mesocarp of *P. gratissima*. Chlorophyll was determined using a nomogram (Kirk 1968). Where specified, chloroplasts were further purified through Percoll (Mills and Joy 1980).

Incubation of Plastid Lysates with Radiolabeled Substrates

(±)[2-^{14}C]ABA, marketed as DL-*cis, trans*, [2-^{14}C]ABA (sp. act. 947 MBq/mmol), DL-[2-^{14}C]MVAL (sp. act. 1.96 GBq/mmol), R-[2-^{14}C]MVAL (sp. act. 1.96 GBq/mmol), and [1-^{14}C]isopentenyl pyrophosphate (IPP) (sp. act 1.96 GBq/mmol) were obtained from Amersham International, U.K. [2-^{14}C]MVAL was hydrolyzed to [2-^{14}C]MVA in 5 mM NaOH at 30°C for 30 min.

For studies on ABA biosynthesis, [2-^{14}C]MVAL (111 KBq), [2-^{14}C]MVA (111 KBq) or [1-^{14}C]IPP (74 KBq) were incubated with chloroplasts (15.2 μg chlorophyll) of *P. sativum* or *P. gratissima* lysed in 0.1 M K_2HPO_4/KH_2PO_4 buffer at pH 7.2 to which aqueous solns of ATP, $MgCl_2$, C_2H_5SH, and a mixture of FAD, FMN, NAD, NADH, NADP, and NADPH were added in a final volume of 1 ml in exactly the same way as described by Milborrow (1974a). Reaction mixtures were incubated for 17 h in a water bath at 28°C with constant illumination and were stopped with ice-cold, absolute methanol. Controls were prepared by heating the enzyme soln in a boiling H_2O bath and cooling prior to addition of the substrate and cofactors.

ABA catabolism in the same chloroplast preparations was followed by incubating [2-^{14}C]ABA (3.7 KBq) with chloroplasts (500 μg chlorophyll) lysed in incubation medium (Tricine, 50 mM; $MgCl_2$ $6H_2O$, 15 mM; KCl, 100 mM; cysteine, 0.01 M at pH 7.6) containing 5 μmol NADPH in a final volume of 1 ml and maintained at 30°C for 24 h under continous illumination with shaking. Heated controls were prepared as above. Reactions were routinely terminated with ice-cold methanol.

Application of Radiochemicals and Inhibitors to Plant Tissues

Turgid shoots of *P. sativum* (2.0 g fresh weight) were excised under H_2O and placed with their cut ends in a soln of [2-^{14}C]ABA (3.7 KBq) in 0.5 ml, 10 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.5. Similar treatments were also set up containing cycloheximide (1 mg/ml), chloramphenicol (1 mg/ml), or lincomycin (0.1 mg/ml) along with [2-^{14}C]ABA (2.8 KBq). Uptake was achieved via the transpiration stream under continuous illumination, and catabolism was allowed to proceed for 24 h under continuous illumination at 25°C.

Blocks (20 g fresh weight) of softening mesocarp of *P. gratissima*, excised from skinned fruits, were sliced with a razor, and either [2-^{14}C]MVAL (111 KBq), [2-^{14}C]MVA (111 KBq), or [2-^{14}C]ABA (3.7 KBq) in 200 μl Tween 20-acetone-H_2O (1:1:8 v/v) was infiltrated into the cuts, which were then closed, and metabolism allowed to proceed for 24 h in an H_2O saturated environment under constant illumination at 25°C (Milborrow and Robinson 1973). For studies with protein synthesis inhibitors, [2-^{14}C]ABA (4.2 KBq) or R-[2-^{14}C]-MVAL (90 KBq) were supplied to mesocarp tissue along with either cy-

cloheximide, chloramphenicol, or lincomycin at the above concentrations. The inhibitor concentrations employed were predetermined to be the most effective for modifying ABA metabolism in the tissues used but were, nevertheless, in the case of chloramphenicol and cycloheximide, higher than those reported to inhibit protein biosynthesis in some systems (Galling 1982).

Extraction and Purification of ABA and Its Catabolites

Excised *P. sativum* shoots and *P. gratissima* mesocarp were ground in a pre-cooled mortar with acid-washed sand and ice-cold methanol-ethyl acetate (50:50 v/v) which also contained butylated hydroxy toluene (BHT) (10 mg/l) as an antioxidant. The residues were washed until colorless, and the filtrates along with the methanolic chloroplast incubates were reduced to small aqueous volumes *in vacuo* at 35°C. Following the addition of phosphate buffer (0.5 M, pH 8.5) to all aqueous phases, they were partitioned against diethyl ether (4×) to remove pigments and then, after adjusting the pH to 2.5 with HCl, were further partitioned against either diethyl ether to extract ABA generated in the MVAL, MVA, and IPP feeds, or ethyl acetate to extract supplied ABA and its acidic catabolites. The organic phases were dried by freezing out the H_2O and were then reduced to a small volume *in vacuo* at 35°C. Samples of high dry weight were purified on C_{18} Sep-Pak cartridges (Waters) eluted with 6 ml 32% methanol in K_2HPO_4/KH_2PO_4 buffer (20 mM, pH 8.0). Samples were separated by TLC on silica gel GF_{254} (Merck) in the solvent system, toluene:ethyl acetate:acetic acid (50:30:4 v/v) which contained BHT (10 mg/l), by developing the chromatograms 2× to 15 cm. Chromatograms were dried overnight, and then each was divided into 30 strips, eluted with 0.5 ml methanol in scintillation vials, and counted in a cocktail (2,5 diphenyl oxazole in toluene, 5 g/l) in a Beckman LS 3150T liquid scintillation spectrometer with a counting efficiency for ^{14}C of 94.9%. Radioactivity remaining in aqueous fractions, following partitioning, was counted in Bray's scintillant (Bray 1960).

Identification of Biosynthetically Generated ABA

This was done according to the criteria of Milborrow (1974a). The putative ABA, separated from precursor MVAL or MVA by TLC in toluene:ethyl acetate:acetic acid (50:30:4 v/v) was eluted with H_2O-saturated ethyl acetate and methylated with ethereal diazomethane (generated at room temperature, without codistillation, by the hydrolysis of N-nitroso N′ methyl urea) and then rechromatographed by TLC in hexane:ethyl acetate (3:1 v/v). Following re-elution with ether, it was reduced to an equal mixture of the 1′,4′ *trans*- and 1′,4′ *cis* diols of methyl abscisate (ABAMe) using $NaBH_4$ in aqueous methanol at 0°C which was then separated on TLC in hexane:ethyl acetate (1:1 v/v).

Preparation of Standard Compounds

Standards of the methylated diols of ABA were prepared by reducing ABAMe

(4 mg) with $NaBH_4$ (4 mg) in 2 ml of H_2O-methanol (1:2 v/v) at 0°C. The reaction was complete in ca. 5 min, and the mixture was extracted (3×) with an equal volume of ethyl acetate after removing the methanol under N_2. The products were separated on TLC (silica gel GF_{254}) in benzene:ethyl acetate:acetic acid (25:3:4 v/v), and the 1′,4′-*trans* diol (Rf 0.56) and 1′,4′-*cis* diol (Rf 0.40) of ABAMe were eluted with H_2O-saturated ethyl acetate.

Analysis of the products was carried out by capillary gas chromatography–mass spectrometry (GCMS) using a 50-m^{-1} fused-silica column (ID 0.3 mm) coated with OV-1 (Hewlett-Packard) and temperature programmed (start temp. 180°C, final temp. 190°C at 2°C/min) in a Varian Aerograph series 2700 GC coupled to a Varian MAT 311A mass spectrometer and Varian SS-100 MS data system.

A single mass peak for the 1′,4′-*cis* diol of ABAMe gave a weak molecular ion at M^+280 and a fragmentation pattern that was consistent with the structure of this diol. The more labile 1′,4′-*trans* diol of ABAMe, on the other hand, decomposed on GC to yield a major mass peak at shorter retention time than ABAMe with a molecular ion at M^+262. This compound was characterized as desoxy ABAMe (Milborrow 1983). Therefore, the identity of the 1′,4′-*trans* diol was sought by direct probe analysis where a weak molecular ion at M^+280 was detected using a Varian MAT 212 mass spectrometer. The fragmentation pattern of this compound was consistent with the structure of the 1′,4′-*trans* diol of ABAMe. The free acid diols were generated following hydrolysis of the corresponding esters with 2 N KOH/ethanol (1:2 v/v) for 45 min at 22°C.

Phaseic acid (PA) and dihydrophaseic acid (DPA) were prepared biosynthetically using either bean shoots (Zeevaart and Milborrow 1976) or barley leaves and were characterized by capillary GC-MS as above.

Protease Assays

These were carried out using acid-denatured haemoglobin (Drivdahl and Thimann 1977, Hampp and De Filippis 1980). The protease inhibitors leupeptin, pepstatin, phenyl-methyl sulphonyl fluoride (PMSF) and trypsin inhibitor were dissolved in the incubation buffer at the concentrations described in Results and Discussion.

Results and Discussion

Initially, the competence of *P. gratissima* mesocarp to biosynthesize ABA was studied in conjunction with the competence of vegetative shoots of both *P. sativum* and *P. gratissima* mesocarp to catabolize applied [2-^{14}C]ABA, before undertaking similar studies with chloroplasts isolated from these tissues. The results of ABA catabolism by shoots of *P. sativum* and *P. gratissima* mesocarp are shown in Fig. 1a,b, and the biosynthesis and identification of ABA from [2-^{14}C]MVAL by *P. gratissima* mesocarp are shown in Table 1.

In agreement with the findings of Dörffling et al. (1974) and Teitz et al. (1979), shoots of *P. sativum* catabolized [2-^{14}C]ABA into two main, acidic products

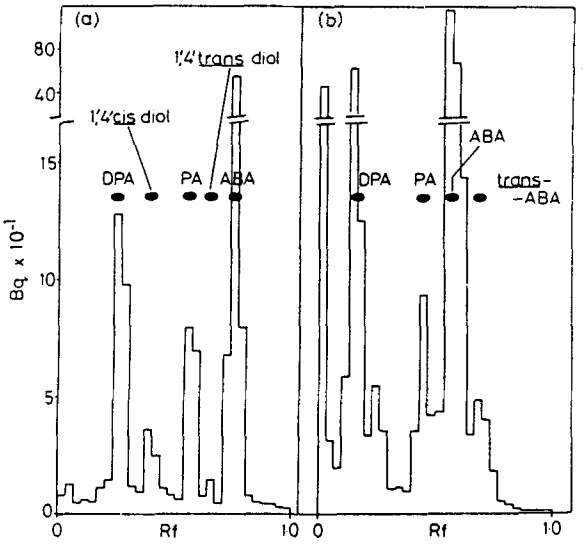

Fig. 1. Distribution of radioactive, acidic products on TLC (silica gel GF_{254}; toluene:ethyl acetate:acetic acid 50:30:4, v/v) from feeds of [2-^{14}C]ABA to (a) light-grown shoots of *P. sativum*, (b) *P. gratissima* mesocarp.

Table 1. Biosynthesis of ABA from MVA and MVAL by mesocarp slices from fruits of *Persea gratissima*.

	Incorporation[a] of radioactivity into ABA							
	ABA		methyl ABA		1',4'-*cis* diol of methyl ABA		1',4'-*trans* diol of methyl ABA	
Substrate	Bq	(%)	Bq	(%)	Bq	(%)	Bq	(%)
[2-^{14}C]MVAL	62.0	(0.057)	62.0	(0.057)	12.0	(0.011)	16.5	(0.015)
[2-^{14}C]MVA	17.6	(0.016)		—		—		—

[a] Blocks of softening mesocarp (20 g f.w.) were supplied with 111 kBq of either [2-^{14}C]MVAL or [2-^{14}C]MVA.

(Fig. 1a) identified as PA and DPA by cochromatography. In addition, two other acidic catabolites were present that cochromatographed with authentic markers of the 1',4'-*trans* diol and 1',4'-*cis* diol of ABA (Milborrow 1983), although the *trans*-diol was apparently generated in much smaller amounts in the nonwilted *P. sativum* seedlings used here compared with the wilted and rewatered seedlings used by Milborrow (1983).

Mesocarp of *P. gratissima* transformed [2-^{14}C]ABA into three main acidic products (Fig. 1b), the one at Rf 0.10–0.20 cochromatographing with DPA and that at Rf 0.4–0.5 with PA. Although no comprehensive work has been published on ABA catabolism by fruit of *P. gratissima*, Milborrow (1974b) reported the incorporation from [2-^{3}H]MVAL into ABA and its catabolism, albeit in low yield, to PA and DPA.

Illuminated *P. gratissima* mesocarp synthesized ABA from [2-^{14}C]MVAL

Table 2. Effect of protein synthesis inhibitors on ABA catabolism in excised shoots of light-grown seedlings of *Pisum sativum* L.

	Incorporation of radioactivity[a] into catabolites of ABA					
	PA		1',4'-*cis* diol		DPA	
Treatment	Bq	(%)	Bq	(%)	Bq	(%)
H_2O	47.0	(3.6)	24.0	(1.7)	113.0	(8.4)
Lincomycin (0.1 mg/ml)	29.0	(2.1)	30.0	(2.2)	10.0	(0.76)
Chloramphenicol (1.0 mg/ml)	58.8	(4.2)	26.8	(1.9)	71.8	(5.1)
Cycloheximide (1.0 mg/ml)	3.7	(0.29)	4.4	(0.34)	5.8	(0.45)

[a] [2-^{14}C]ABA (2.78 kBq) was supplied to excised shoots (2.0 g f.w.) of *P. sativum* via the transpiration stream.

Table 3. Effect of protein synthesis inhibitors on ABA biosynthesis in illuminated, excised blocks of mesocarp of *Persea gratissima* L.

	Incorporation of radioactivity[a] into ABA from MVAL	
Treatment	Bq	(%)
H_2O	97.4	(0.108)
Lincomycin (0.1 mg/ml)	72.3	(0.080)
Chloramphenicol (1 mg/ml)	81.3	(0.090)
Cycloheximide (1 mg/ml)	22.2	(0.025)

[a] Mesocarp treated with 90 kBq R-[2^{14}C]MVAL.

(Table 1) as reported before (Milborrow and Robinson 1973), but this is the first time these results have been corroborated in another study. Applied [2-^{14}C]MVA was a less efficient precursor of ABA than [2-^{14}C]MVAL (Table 1) (Milborrow 1974b).

If chloroplasts are involved in ABA metabolism in plants, it is reasonable to assume that plastid-synthesized enzymes could be implicated in catalyzing some of the steps in this process. It might be possible to detect alterations in ABA metabolism using inhibitors of plastid protein synthesis. Therefore, two such inhibitors, chloramphenicol and lincomycin, which affect translation on 70s plastid ribosomes (Ellis 1982) were supplied to either illuminated, light-grown shoots of *Pisum sativum* along with [2-^{14}C]ABA or mesocarp slices from *Persea gratissima*, together with either R-[2-^{14}C]MVAL or [2-^{14}C]ABA, conditions under which ABA is known to enter chloroplasts via a gradient in pH (Heilmann et al. 1980).

In addition, separate tissues were also treated with cycloheximide, a potent inhibitor of protein biosynthesis on cytoplasmic 80s ribosomes (Galling 1982) in the presence of either [2-^{14}C]ABA or R-[2-^{14}C]MVAL as above.

The results in Tables 2, 3, and 4 show that whereas ABA biosynthesis and catabolism in *P. gratissima* mesocarp were not markedly affected by the 70s

Table 4. Effect of protein synthesis inhibitors on ABA catabolism in illuminated, excised blocks of mesocarp of *Persea gratissima*.

	Incorporation[a] of radioactivity into catabolites of ABA					
	PA		unknown (Rf 0.26–0.30)		DPA	
Treatment	Bq	(%)	Bq	(%)	Bq	(%)
H_2O	70.4	(1.7)	25.5	(0.61)	357.3	(8.5)
Lincomycin (0.1 mg/ml)	68.8	(1.6)	21.4	(0.51)	357.9	(8.5)
Chloramphenicol (1 mg/ml)	103.5	(2.4)	23.3	(0.55)	355.5	(8.4)
Cycloheximide (1 mg/ml)	38.4	(0.91)	11.3	(0.27)	23.2	(0.55)

[a] Mesocarp treated with 4.2 kBq [2-^{14}C]ABA.

ribosome inhibitors, ABA catabolism in shoots of *P. sativum* was altered significantly. In particular, incorporation of label into both PA and DPA was inhibited by lincomycin, and incorporation into DPA inhibited by chloramphenicol. Both ABA biosynthesis and catabolism were inhibited markedly by cycloheximide. Though the difficulties associated with interpreting data from experiments involving protein synthesis inhibitors are well known (Galling 1982), the limited effect of 70s ribosome inhibitors on ABA biosynthesis in mesocarp of *P. gratissima* suggests that plastid-synthesized proteins are probably not involved in this process.

Recently, Quarrie and Lister (1984) reported on the effects of various protein synthesis inhibitors on the wilt-induced increase in endogenous ABA in leaves of *Triticum aestivum*. They observed that inhibitors of chloroplast protein synthesis were ineffective in preventing such an ABA increase, whereas cycloheximide inhibited this process completely. Unfortunately there is only limited, circumstantial evidence to suggest that wilt-induced increases in ABA levels involve changes in the rate of ABA biosynthesis (Milborrow and Noddle 1970, Pierce and Raschke 1981, Zeevaart 1980) and so the significance of these findings is not completely clear.

The present results, employing radiolabeled substrates, on the other hand, suggest that both biosynthesis and catabolism of ABA involve the participation of labile, cytoplasmically synthesized proteins. Furthermore, ABA catabolism in shoots of *P. sativum* appears to require active, plastid protein synthesis. Several plastid-localized enzymes are, however, known to the synthesized on cytoplasmic ribosomes and transported into the chloroplast (Bottomley and Bohnert 1982), so inhibition of ABA metabolism by cycloheximide but a lack of effect by lincomycin and chloramphenol as in the mesocarp of *P. gratissima* does not necessarily exclude a role for chloroplasts in ABA biosynthesis and catabolism. To try to clarify this aspect further, it was necessary to examine the contribution of isolated chloroplasts to ABA metabolism. Initially, the catabolism of ABA was investigated by incubating [2-^{14}C]ABA with plastid lysates; the results are depicted in Table 5.

Despite the fact that Hartung et al. (1980) were unable to demonstrate ca-

Table 5. Conversion of [2-^{14}C]ABA into acidic products by chloroplast lysates from leaves of light-grown shoots of *Pisum sativum* and from mesocarp of *Persea gratissima*.

Species	Chlorophyll (μg)	Incorporation[a] of radioactivity into catabolites[b]									
		I (Rf 0.0–0.05)		II (Rf 0.1–0.15)		III (Rf 0.36–0.43)		IV (Rf 0.63–0.7)		ABA (Rf 0.74)	
		Bq	(%)	Bq	(%)	Bq	(%)	Bq	(%)	Bq	(%)
Pisum sativum	500	35.0	(0.95)	81.0	(2.2)	88.0	(2.4)	932.0	(25.2)	—	—
Persea gratissima	500	24.0	(0.65)	33.0	(0.9)	148.0	(4.0)	70.0	(1.9)	—	—

[a] Chloroplasts in a final volume of 1 ml were incubated with 3.7 kBq [2-^{14}C]ABA for 17 h at 28°C.

[b] Rf values in toluene:ethyl acetate:acetic acid (50:30:4, v/v), developed 2× to 15 cm.

Table 6. Effect of inhibitors on protease activity in chloroplast lysates[a] from *P. sativum*.

Treatment	Enzyme activity (% inhibition)
Control	0
Phenylmethylsulphonyl fluoride (0.01 M)	45
Pepstatin (0.01 M)	39.8
Trypsin inhibitor (1 mg/ml)	53
Leupeptin (0.002 M)	100

[a] 500 μg chlorophyll used per assay.

tabolism of [2-^{14}C]ABA in incubates of isolated chloroplasts of *S. oleracea* using high levels of chlorophyll (4.2 mg), catabolism of [2-^{14}C]ABA into more polar, acidic products was observed consistently by plastid preparations from *P. sativum* and *P. gratissima* where much lower amounts of chlorophyll (500 μg) were used. Some of these products were chromatographically similar to those obtained in feeds of [2-^{14}C]ABA to excised shoots of *P. sativum* and mesocarp slices of *P. gratissima*. Notably, catabolites II and III from *P. sativum* chloroplast feeds appeared similar to DPA and the 1′,4′-*cis* diol of ABA, respectively. Catabolite IV, which was always produced in highest amounts by chloroplast lysates from *P. sativum*, cochromatographed with the 1′,4′-*trans* diol of ABA.

Given the relatively small incorporation into some of the acidic catabolites by plastid lysates, it was possible that organelle-associated proteases might contribute to reductions in enzyme activity, as has been shown in other tissues (Alpi and Beevers 1981). This was thought likely in view of the relatively long incubation times with substrates in the present work and in other studies (Hartung et al. 1980).

In agreement with previous studies (Hampp and De Filippis 1980, Lin and Wittenbach 1981, Waters et al. 1982, Dahlmelm and Fisher 1982), significant protease activity was detected in chloroplast lysates, and several inhibitors of protease activity were examined in an attempt to reduce this possible source of inhibition of ABA catabolism; the results are shown in Table 6. In agreement with the findings of Alpi and Beevers (1981), leupeptin proved to be the most potent inhibitor of protease activity.

To determine whether such leupeptin-sensitive proteases represented plastid-localized enzymes or merely cytoplasmic contamination, attempts were made to purify chloroplasts further, prior to lysis, using Percoll (Mills and Joy 1980). Although chloroplasts from *P. gratissima* did not lend themselves to this technique because of the gelatinous nature of the pellets, which prevented passage through the Percoll layers, those from *P. sativum* yielded the results shown in Table 7, which demonstrated that a large percentage of the plastid-associated protease activity was probably cytoplasmic in nature.

The catabolism of [2-^{14}C]ABA was thus investigated in lysates of Percoll-purified *P. sativum* chloroplasts in which the levels of associated proteases had been reduced. The results are shown in Table 7. Percoll treatment of *P. sativum* plastids caused a marked decrease in the levels of acidic products

Table 7. Effect of Percoll purification on protease activity and ABA catabolism in chloroplast lysates[a] of *P. sativum*.

Treatment	Protease activity (% control)	Incorporation of radioactivity into acidic catabolites (total % control)
− Percoll	100	100
+ Percoll	32.6	31.9

[a] 500 μg chlorophyll used per assay.

generated from [2-^{14}C]ABA, suggesting that cytoplasmic enzymes adhering to plastid envelope membranes could be responsible for the ABA catabolism observed.

Studies on ABA biosynthesis were initially carried out in an identical manner to that described by Milborrow (1974a), using plastid lysates from *P. gratissima*. In view of the use of the unnatural substrate MVAL as a precursor of ABA by both Milborrow (1974a) and Hartung et al. (1981), a comparison was made between the biosynthesis of ABA from both [2-^{14}C]MVAL and [2-^{14}C]MVA. The results in Table 8 show that incorporation into the *putative* ABA was higher from MVA than it was from MVAL, thereby casting doubt on the suitability of MVAL as an ABA precursor in chloroplast lysates.

However, when attempts were made to confirm the identity of ABA by microchemical methods, no radioactive ABA derivatives were observed. Without exception, we have never been able to demonstrate the production of ABA from either [2-^{14}C]MVAL or [2-^{14}C]MVA by *P. gratissima* plastid preparations, whereas similar experiments with mesocarp slices have always yielded positive results. So far, no attempts have been made to examine the biosynthesis of ABA from MVA by cell-free homogenates of *Persea gratissima* mesocarp, but these would be useful to support the idea that ABA biosynthesis occurs in the cytosol rather than in chloroplasts in this tissue.

Similar biosynthetic studies using chloroplast lysates from *P. sativum* yielded only poor incorporation into putative ABA from [2-^{14}C]MVA, and radioactivity was too low to enable confirmation of identity by derivatization. Furthermore, this radioactive product was markedly reduced when plastids were purified on Percoll (Table 8), suggesting that it arose as a result of the activity of cytoplasmic enzymes. The radioactive, acidic product was also not generated from later, phosphorylated terpenoid intermediates such as [1-^{14}C]IPP (Table 8), and its significance for ABA biosynthesis in *P. sativum* plastids is thus in doubt.

The results of this study indicate that chloroplasts from *Persea gratissima* mesocarp and from vegetative shoots of *Pisum sativum* do not play a significant part in the biosynthesis and catabolism of ABA and therefore agree with the findings of Hartung et al. (1980, 1981, 1982), using chloroplasts of *Spinacia oleracea*, but contradict the biosynthetic data obtained by Milborrow (1974a), who used plastids from the mesocarp of *P. gratissima*.

Despite these findings, caution should be exercised in discounting chloroplasts as a site of ABA biosynthesis and catabolism in plants until more species

Table 8. Biosynthesis of putative ABA from MVA and MVAL by chloroplast lysates from mesocarp of *P. gratissima* and from MVA and IPP by chloroplast lysates from *P. sativum* shoots.

			Incorporation of radioactivity into ABA[a]							
			ABA		methyl ABA		1',4'-*cis* diol of methyl ABA		1',4'-*trans* diol of methyl ABA	
Substrate	Species	Percoll purification	Bq	(%)	Bq	(%)	Bq	(%)	Bq	(%)
[2-^{14}C]MVAL	*P. gratissima*	–	2.2	(0.002)	0	(0)	0	(0)	0	(0)
[2-^{14}C]MVA	*P. gratissima*	–	20.0	(0.019)	0	(0)	0	(0)	0	(0)
[2-^{14}C]MVA	*P. sativum*	–	11.0	(0.010)	—		—		—	
	P. sativum	+	3.3	(0.003)	—		—		—	
[1-^{14}C]IPP	*P. sativum*	–	0.0	(0.0)	—		—		—	

[a] 15.2 μg chlorophyll used per incubate in a volume of 1 ml containing 111 kBq [2-^{14}C]MVAL or [2-^{14}C]MVA or 74 kBq [2-^{14}C]IPP.

have been screened. In addition, a role for chloroplast-localized violaxanthin in the biosynthesis of ABA in higher plants remains a distinct possibility (Moore and Smith 1985), and a detailed study of violaxanthin metabolism in relation to ABA biosynthesis in chloroplasts is long overdue.

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