Phytochemistry, 1980, Vol. 19, pp. 1355–1359. © Pergamon Press Ltd. Printed in England. 0031-9422/80/0701-1355 $02.00/0

SUBCELLULAR LOCALIZATION OF FLAVONOID SYNTHESIZING ENZYMES IN *PISUM, PHASEOLUS, BRASSICA* AND *SPINACIA* CULTIVARS

GÉZA HRAZDINA,* RUTH ALSCHER-HERMAN†‡ and VALERIE M. KISH*

Cornell University, *Department of Food Science and Technology, and †Department of Seed and Vegetable Sciences, New York State Agricultural Experiment Station, Geneva, NY 14456, U.S.A.

(*Revised received* 10 *November* 1979)

Key Word Index—*Pisum*; *Phaseolus*; *Brassica*; *Spinacia*; subcellular localization; chloroplast; enzymes; methyl transferases; flavanone synthase; glucosyltransferase.

Abstract—Functionally-intact chloroplasts were obtained from 11-day-old pea (*Pisum sativum* cv Midfreezer) seedlings. Enzyme-distribution studies with ribulose bisphosphate carboxylase and NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase showed that *ca* 2.1% of the total tissue chloroplasts were present in the chloroplast preparation. The rate of intactness of chloroplast preparations was 34–82%. SAM:caffeic acid methyltransferase, flavanone synthase, UDPG:flavonoid-3-*O*-glucosyltransferase and SAM:quercetin methyltransferase activities were measured in the homogenate, supernatant and chloroplast lysate fractions. Significant activities of the above four enzymes could only be detected in the homogenate and supernatant fractions. Similar experiments with 11-day-old seedlings of green beans (*Phaseolus vulgaris* cv Early Gallatin), red cabbage (*Brassica oleracea* cv Red Danish) and 6-week-old plants of spinach (*Spinacia oleracea* cv Bloomsdale) showed a similar distribution of the flavonoid synthesizing enzymes. We conclude that under the reported conditions chloroplasts are not involved in flavonoid biosynthesis.

INTRODUCTION

It is generally agreed that the main site of flavonoid accumulation is the vacuole. A number of reports have appeared concerning the occurrence of cinnamic-acid derivatives and flavonoids in plastids [1–7]. These reports led to the speculation that these compounds are synthesized in plastids and are subsequently transported to the vacuole [8]. The development of techniques for the detection and characterization of specific enzymes of flavonoid metabolism permitted investigations of the subcellular localization of these enzymes.

Fractional phenylalanine ammonia-lyase (PAL) and chalcone–flavanone isomerase activities were reported by Weissenboeck *et al.* [9] in intact plastids, and activities of PAL, flavanone synthase and hydroxycinnamyl-CoA:quinate hydroxycinnamyltransferase by Ranjeva *et al.* [10, 11] in functional chloroplasts. On the other hand, experiments with *Hippeastrum* and *Tulipa* protoplasts showed that flavanone synthase, chalcone–flavanone-isomerase and UDP-glucose: anthocyanidin-3-*O*-glucosyltransferase activities were chiefly located in the cytosol [12].

In the present paper we report the isolation of intact chloroplasts from peas (*Pisum sativum* L. cv Midfreezer), green beans (*Phaseolus vulgaris* cv Early Gallatin), red cabbage (*Brassica oleracea* cv Red Danish), and spinach (*Spinacia oleracea* cv Bloomsdale) and the subcellular localization of flavanone synthase, UDPG:flavonoid glucosyltransferase, SAM:caffeic acid methyltransferase and SAM:quercetin methyltransferase.

The major flavonoid constituents of pea seedlings are kaempferol and quercetin-3-sophorotriosides, 3-*p*-coumarylsophorotriosides and 3-ferulylsophorotriosides [13, 14]. The flavonoid constituents of green-bean seedlings are the 3-glucosides and 3-xylosylglucosides of kaempferol and quercetin [15, 16]. Red-cabbage seedlings contain chiefly the malonyl, *p*-coumaryl, ferulyl and sinapyl esters of cyanidin-3-sophoroside-5-glucoside [17]. Spinach does not contain flavonoids in significant quantities, and those present are of rather unusual substitution pattern, such as patuletin (3,5,7,3′,4′-pentahydroxy-6-methoxyflavone) spinacetin (3,5,7,4′-tetrahydroxy-6,3′-dimethoxy flavone) and 7-methoxyaxillarin (5,4,3′-trihydroxy-3,6,7-trimethoxyflavone) [1, 18]. In order to determine if flavonoid synthesis takes place at different subcellular sites in different genera, the localization of the above four enzymes (e.g. flavanone synthase, glucosyltransferase, caffeic acid methyltransferase and quercetin methyltransferase) was investigated in these four species.

RESULTS

The chloroplasts used for the study of the localization of enzymic activities appeared under the phase-

‡ Present address: Boyce-Thompson Institute, Ithaca, NY 14850, U.S.A.

Table 1. Specific activities of the ribulose-1,5-bisphosphate-carboxylase and NADPH-dependent glyceraldehyde-3-P-dehydrogenase in subcellular fractions of *Pisum* per preparation

	Homogenate	Supernatant	Chloroplast preparation	Shocked chloroplasts Stroma	Membrane
Chlorophyll (mg)	4.52	2.50	0.70		0.56
Protein (mg)	195	168	—	1.05	—
RUBP-carboxylase total activity (nmol/min)	740	706	10.3	15.5	—
Specific activity (nmol/min/mg protein)	3.8	4.2	—	14.8	—
G-3-P-dehydrogenase (total activity nmol/min)	64.3	51	0.16	1.4	—
Specific activity (μmol/min/mg protein)	0.33	0.30	—	1.3	

contrast microscope as mostly shiny, highly refractive and haloed, suggesting that their envelope remained intact during the rapid isolation technique of Avron and Gibbs [19].

Analysis of the subcellular distribution of enzymes known to be specifically located in chloroplasts such as ribulose-1,5-bisphosphate carboxylase and NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase showed that 2.1% of the total tissue chloroplasts were recovered in the chloroplast preparation. Using mechanical isolation in aqueous media, the yield of partially or wholly functional chloroplasts in multiple experiments could not be improved beyond 3%. After osmotic shock there was a significant increase in the activities of both marker enzymes. Based on this activity increase, 34–82% of the isolated chloroplasts retained their stromal contents (Table 1).

Parallel to the distribution of the above chloroplast enzymes the activities of phenylpropanoid (e.g. SAM:caffeic acid methyltransferase) and specific flavonoid pathyway enzymes (e.g. flavanone synthase, UDPG: flavonoid-3-*O*-glucosyltransferase and SAM: quercetin methyltransferase) were monitored. Analyses of tissue homogenate, supernatant, chloroplasts, stroma, and membrane demonstrated that only the homogenate and supernatant fractions contained measurable activities for SAM:caffeic acid methyltransferase, flavanone synthase, SAM:quercetin methyltransferase and UDP-glucose: flavonoid-3-*O*-glucosyltransferase. No phenylpropanoid or flavonoid path enzymic activity was associated with the intact chloroplast preparation or stromal or membrane fractions of pea seedlings (Table 2). Essentially the same data were found with seedlings of green bean (*Phaseolus vulgaris* cv Early Gallatin), red cabbage (*Brassica oleracea* cv Red Danish), and spinach (*Spinacia oleracea* cv Bloomsdale).

Comparison of the relative enzyme activities of the supernatant fractions in pea seedlings showed that SAM:caffeic acid methyltransferase and SAM:quercetin methyltransferase were present at approximately two orders of magnitude higher activity levels than flavanone synthase and UDPG-flavonoid-3-*O*-glucosyltransferase. The same holds true for seedlings of green beans, red cabbage and spinach (Table 3). However, spinach seedlings contained approximately 10-fold lower activity of the above four enzymes than the other plant species investigated. This is in agreement with the low flavonoid content of this genus.

DISCUSSION

The four investigated enzymes, e.g. SAM:caffeic acid methyltransferase, flavanone synthase, SAM:quercetin methyltransferase and UDPG:flavonoid-3-*O*-glucosyltransferase represent enzymes of the general phenylpropanoid and specific flavonoid metabolism. SAM:caffeic acid methyltransferase is responsible for the production of ferulic acid, which is

Table 2. % Distribution of enzyme activities in *Pisum* subcellular fractions

Enzymes	Homogenate	Supernatant	Chloroplast preparation	Shocked chloroplasts Stroma	Membrane
Ribulose-1,5-bisphosphate-carboxylase	100	95.3	1.4	2.1	—
NADPH-dependent glyceraldehyde-3-P-dehydrogenase	100	100	0.25	2.1	—
SAM:caffeic acid methyl transferase	100	98.3	0	0	0
Flavanone synthase	100	100	0	0	0
SAM:quercetin methyl transferase	100	98.1	0	0	0
UDPG:flavonoid-3-glucosyl transferase	100	100	0	0	0

Table 3. Relative enzyme activities of supernatant fractions from peas, green beans, red cabbage and spinach

Plant material	Relative enzyme activity (nmol/hr/100 g tissue)			
	SAM:caffeic acid methyltransferase	Flavanone synthase	SAM:quercetin methyltransferase	UDP-glucose:flavonoid glucosyltransferase
Pisum sativum (cv Midfreezer)	15 880	296	8477	21
Phaseolus vulgaris (cv Early Gallatin)	10 208	124	9790	128
Brassica oleracea (cv Red Danish)	9372	785	8477	31
Spinacia oleracea (cv. Bloomsdale)	1313	22	3283	2.2

further transformed to produce lignin [20]. Flavanone synthase is the first enzyme responsible for the establishment of the C_{15} flavonoid skeleton, SAM:quercetin methyltransferase represents a central enzyme of the metabolic process, and UDPG:flavonoid glucosyltransferase can be looked upon as the last enzyme involved in flavonoid biosynthesis.

A report by Oettmeier and Heupel [1] on the localization of *p*-coumaryltartaric acid and several flavonoid glycosides yielding methoxy and methylenedioxy derivatives of a trihydroxyflavone in spinach chloroplasts implied that these compounds could be synthesized there. Further reports on isolation of flavonoids from chloroplast preparations of *Hordeum* [2], *Impatiens* [4], *Avena* [6] and *Kalanchoe* [21] suggested that chloroplasts could be capable of synthesizing flavonoids. The detection of phenylalanine ammonia-lyase, the first enzyme of phenylpropanoid metabolism in chloroplast preparations of *Oenothera* hybrids [22], *Hordeum vulgare* [2] and from *Petunia* [9] further supported this view.

The enzymic synthesis of naringenin closely resembles the mechanism of fatty acid biosynthesis by the fatty acid synthase complex [23]. Fatty acid synthases are known to be localized in both the cytoplasm and chloroplasts [24, 25] and it seemed possible that flavanone synthase could be present in both subcellular compartments. Data on its localization were equivocal with reports on its occurrence in the cytosol [11] and in chloroplasts [10].

The four enzymes of the phenylpropanoid and flavonoid pathway investigated showed high activity in the supernatant fractions of the homogenates in all four plants. Chloroplast preparations, their stroma and membrane fractions were devoid of any activity. It is difficult to accurately determine the distribution of enzyme activities in chloroplast preparations where a large degree of breakage occurs during the isolation by mechanical means. However, within the limits of accuracy of the enzyme assays used in the experiments, a 15–20% distribution of the enzymes between cytoplasm and chloroplasts could have easily been detected. The absence of any activity of the phenylpropanoid and flavonoid path enzymes in the chloroplast, stroma and membrane preparations of the four plant species investigated suggest that chloroplasts are not involved in their synthesis.

EXPERIMENTAL

Plants. Pea (*Pisum sativum* L. cv Midfreezer) and green bean (*Phaseolus vulgaris* cv Early Gallatin) seeds were germinated in sand at 21° day and 18° night temps, illuminated for 16 hr daily with equal number of Cool White and Grow Lux fluorescent lights (200 μ Einstein—m^2/sec) and harvested after 11 and 16 days, respectively. Prior to harvest the seedlings were placed in the dark for 12 hr to minimize starch.

Red cabbage. (*Brassica oleracea* cv Red Danish) seeds were sterilized with 6% NaClO soln, soaked overnight in H_2O and germinated in two layers of moist Whatman No. 1 filter paper in clear plastic boxes in the dark in a thermostated growth chamber. The seedlings were illuminated continuously (200 μ Einstein/m^2/sec) as described elsewhere [25].

Spinach. (*Spinacia oleracea* cv Bloomsdale—germinated as above at 21° day and 15° night temps and grown for 6 weeks under 16 hr daily illumination (200 μ Einstein/m^2 sec).

Materials. *p*-Coumaryl-CoA was synthesized as described previously [26]. *S*-Adenosyl-L-methionine-[methyl-^{14}C] (SAM; sp. act. 59.7 mCi/mmol), uridinediphosphateglucose-[^{14}C]-[D-glucose-^{14}C(U)] (UDPG) sp. act. 240 mCi/mmol, malonyl-CoA-[2-^{14}C] (sp. act. 32.1 mCi/mmol) and sodium bicarbonate-[^{14}C] (sp. act. 7.7 mCi/mmol) were purchased from New England Nuclear, Boston, MA. Naringenin, D-ribulose-1,5-diphosphoric acid and ATP (Na salt) were obtained from Sigma, sorbitol, HEPES, NADPH and phosphoglycerate were obtained from Calbiochem. Caffeic acid, malvidin, malvidin-3-glucoside and quercetin were from our laboratory collection.

Methods. All experiments were carried out at least in triplicate.

Isolation of chloroplasts. All fractionation procedures were carried out at 4° as described by Avron and Gibbs [19]. Plant material (12–25 g) was homogenized for 2 × 2 sec in a Waring blendor equipped with razor blades in 200 ml partially frozen buffer [50 mM HEPES, pH 6.8, 300 mM sorbitol, 2 mM EDTA, 20 mM KCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5 mM KH_2PO_4, 2 mg/ml BSA, 2 mM ascorbate (added immediately before use)]. The resulting homogenate was strained through 4 layers of cheesecloth and one layer of Miracloth and centrifuged immediately at 2000 g for 30 sec. The supernatant was decanted and the pellet taken up in 0.5–1.0 ml resuspension buffer (50 mM HEPES, pH 7.6, 400 mM sorbitol, 2mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$) [27]. The

integrity of the chloroplast preparation was monitored by phase-contrast microscopy, and by assaying its ribulose bisphosphate carboxylase [28] and NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase [29] activity.

Osmotic shocking of the chloroplasts. An aliquot of the isolated chloroplast fraction was mixed with 10 vol. of hypotonic buffer (50mM HEPES, pH, 7.6, 2mM EDTA, 1mM $MgCl_2$, 1 mM $MnCl_2$) and centrifuged at 20000 g for 20 min at 4°. The resulting supernatant is referred to as stroma, the pellet as the membrane fraction.

Determination of chloroplast related enzyme activities. All enzyme reactions, except where noted differently, were carried out at 30°.

NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase. This was determined by the method of Heber *et al.* [29] as modified by Poulton (pers. commun.). The reaction mixture contained 46 μmol Tris-HCl, pH 7.5, 10 μmol $MgSO_4$, 1.2 μmol dithiothreitol (DTT), 11.2 μmol phosphoglycerate, 1.4 μmol ATP, 0.16 μmol NADPH and 40 μl of either homogenate, supernatant, chloroplast or stroma fraction in a total vol of 1.0 ml.

Ribulose bisphosphate carboxylase assay. This was carried out according to Gibbs and Robinson [28]. The reaction mixture contained 0.29 μmol $NaH^{14}CO_3$ in 90 μl resuspension buffer (50mM HEPES, pH 7.6, 400mM sorbitol, 2 mM EDTA, 1mM $MgCl_2$, 1mM $MnCl_2$), 0.1mmol ribulose-1,5-bisphosphate and 25–40 μl of the appropriate cell fractions. All samples were assayed in duplicate. Aliquots (40 μl) were withdrawn from the incubation mixtures at 5 min intervals, placed on a 2.4 cm Whatman 3 MM filter paper discs and acidified immediately with 50 μl 5 N HCl. Discs were allowed to dry for at least 3 hr prior to liquid scintillation spectrometry in toluene (2.5 g PPO/l.).

Activities of enzymes of phenylpropanoid and flavonoid metabolism. For the determination of the activities of the phenylpropanoid (SAM:caffeic acid methyltransferase) and flavonoid enzymes (flavanone synthase, SAM:quercetin methyltransferase, UDPG:flavonoid-3-*O*-glucosyltransferase) aliquots of the cell fractions were treated for 30 min with Dowex 1×2 [24] and the filtrate centrifuged at 100 000 g for 30 min. All incubations were carried out for 20 min.

Flavanone synthase. Determined using a modified method of Kreuzaler and Hahlbrock [30]. The incubation mixture was composed of 50 μl 200mM K_2HPO_4/H_3PO_4 buffer (pH 8.0) containing 4 mg BSA/ml, 50 μl cell fraction, 5 μl *p*-coumaryl-CoA (1 nmol) and 5 μl malonyl-CoA-[2-^{14}C] (3 nmol) and 5 μmol ascorbate in 5 μl phosphate buffer of above. The products were separated by PC using authentic naringenin as carrier substance and counted by liquid scintillation spectrometry. To account for any interference from fatty acid synthase reactions, background activities of the different fractions were determined by omitting *p*-coumaryl-CoA from the reaction mixtures.

SAM:caffeic acid methyltransferase activity. Determined according to Poulton *et al.* [31] with 50 μl 200 mM K_2HPO_4/H_3PO_4 phosphate buffer (pH 8.0) containing 4 mg/ml BSA and 50 μl cell fractions.

SAM:quercetin methyltransferase activity. This was determined as above with quercetin as substrate. In the determination of both methyltransferase activities, backgrounds for the individual subcellular fractions were determined by omitting the phenolic substrate from the reaction mixtures.

UDP-glucose:flavonoid-3-O-glucosyltransferase activity. Determined as previously described [12] with malvidin as substrate and malvidin-3-glucoside as carrier substance. Background was determined by omitting malvidin from the reaction mixture.

Chlorophyll was determined according to Arnon [32], protein according to the method of ref. [33].

Acknowledgements—The authors are grateful to Dr. G. A. Marx and Miss Mary Ann Boettger for providing the pea, bean and spinach seedlings used in the experiments.

REFERENCES

1. Oettmeier, W. and Heupel, A. (1972) *Z. Naturforsch. Teil B* **27B,** 117.
2. Saunder, J. and McClure, J. W. (1972) *Am. J. Botany* **59,** 673.
3. Saunders, J. A., McClure, J. W. and Wallace, J. W. (1973) *Am. J. Botany* **60,** 29.
4. Weissenboeck, G., Tevini, M. and Reznick, H. (1971) *Z. Pflanzenphysiol.* **64,** 274.
5. Weissenboeck, G. (1972) *Hoppe-Seyler's Z. Physiol. Chem.* **353,** 136.
6. Weissenboeck, G. (1973) *Ber. Dtsch. Bot. Ges.* **86,** 351.
7. Weissenboeck, G. and Schneider, V. (1974) *Z. Pflanzenphysiol.* **72,** 2325.
8. McClure, J. W. (1975) in *The Flavonoids* (Harborne, J. B., Mabry, T. J. and Mabry, H. eds.) p. 1005. Chapman & Hall, London.
9. Weissenboeck, G., Plesser, A. and Trinks, K. (1976) *Ber. Dtsch. Bot. Ges.* **89,** 457.
10. Ranjeva, R., Alibert, G. and Boudet, A. M. (1977) *Plant Sci. Letters* **10,** 225.
11. Ranjeva, R., Alibert, G. and Boudet, A. M. (1977) *Plant Sci. Letters* **10,** 235.
12. Hrazdina, G., Wagner, G. J. and Siegelman, H. W. (1978) *Phytochemistry* **17,** 53.
13. Mumford, I. E., Smith, D. H. and Castle, J. E. (1961) *Plant Physiol.* **36,** 752.
14. Harborne, J. B. (1963) *Experientia* **19,** 7.
15. Harborne, J. B. (1965) *Phytochemistry* **4,** 107.
16. Feenstra, W. J. (1960) *Meded. Landbouwhogensch. Opzoekingsstn. Staat Gent* **60,** 1.
17. Hrazdina, G., Iredale, H. and Mattick, L. R. (1977) *Phytochemistry* **16,** 297.
18. Zane, A. and Wender, S. H. (1961) *J. Org. Chem.* **26,** 4718.
19. Avron, M. and Gibbs, M. (1974) *Plant Physiol.* **53,** 136.
20. Grisebach, H. and Hahlbrock, K. (1974) in *Recent Advances in Phytochemistry* (Runeckles, V. C. and Conn, E. E. eds.) Vol. 8, pp. 21–52. Academic Press, New York.
21. Gifford, E. M., Jr. and Stewart, K. D. (1968) *Am. J. Botany* **55,** 269.
22. Hachtel, W. and Schwemmle, B. (1972) *Z. Pflanzenphysiol.* **68,** 127.
23. Lynen, F. (1961) *Proc. Robert A. Welch Found. Conf. Chem. Res.* **5,** 293.
24. Brooks, J. L. and Stumpf, P. K. (1966) *Arch. Biochem. Biophys.* **116,** 108.
25. Stumpf, P. K., Brooks, J. L., Gaillard, T., Hawke, J. C. and Simoni, R. (1967) in *Biochemistry of Chloroplasts.* (Goodwin, T. W. ed.) Vol. II, pp. 213–240. Academic Press, London.

26. Hrazdina, G. and Creasy, L.L. (1979) *Phytochemistry* **18,** 581.
27. Hrazdina, G., Kreuzaler, F., Hahlbrock, K. and Grisebach, H. (1976) *Arch. Biochem. Biophys.* **175,** 392.
28. Gibbs, M. and Robinson, M. (1974) *Experimental Plant Physiology* (San Pietro, A. ed.) Mosby, St. Louis.
29. Heber, U., Pon, N. G. and Heber, M. (1963) *Plant Physiol.* **38,** 355.
30. Kreuzaler, F. and Hahlbrock, K. (1972) *FEBS Letters* **28,** 69.
31. Poulton, J. E., Hahlbrock, K. and Grisebach, H. (1977) *Arch. Biochem. Biophys.* **180,** 543.
32. Arnon, D. I. (1949) *Plant Physiol.* **24,** 1.
33. Schaffner, W. and Weissmann, C. (1973) *Analyt. Biochem.* **56,** 502.