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INTRACELLULAR LOCALIZATION OF GERANYLPYROPHOSPHATE SYNTHASE FROM CELL CULTURES OF *LITHOSPERMUM ERYTHRORHIZON*

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Key Word Index—*Lithospermum erythrorhizon*; Boraginaceae; geranylpyrophosphate synthase; intracellular localization; shikonin.

Abstract—We have investigated the subcellular localization of the geranylpyrophosphate (GPP) synthase (EC 2.5.1.1) in *Lithospermum erythrorhizon* cell cultures using a cell fractionation procedure followed by comparison to marker enzyme activities and immunodetection in Western blots. The results demonstrate the localization of the enzyme within the cytosol. This contrasts to the plastidial localization of the GPP synthase in *Vitis vinifera*. This discrepancy is discussed in regard to the different metabolic pathways in which GPP is involved in these two plant species.

INTRODUCTION

Cell cultures of *Lithospermum erythrorhizon* Sieb. et Zucc. are able to produce derivatives of the red naphthoquinone pigment shikonin [1], which exert antibacterial, anti-inflammatory and wound-healing activities [2]. The two key precursors for the biosynthesis of shikonin are *p*-hydroxybenzoate (PHB), derived from the phenylpropanoid pathway, and geranylpyrophosphate (GPP), from the isoprenoid pathway. Geranylpyrophosphate: *p*-hydroxybenzoate geranyltransferase catalyses the condensation of PHB and GPP to form *m*-geranyl-*p*-hydroxybenzoic acid, an intermediate in the pathway leading to shikonin [3]. This enzyme is localized at the endoplasmatic reticulum (ER) [4]. Furthermore, it was shown by electronmicroscopy that the production of shikonin derivatives is linked to the formation of electron-dense vesicles from the ER which seem to transport these derivatives into the cell wall by exocytosis [5].

Different types of prenyltransferases exist which catalyse the condensation of isopentenylpyrophosphate (IPP) with an allylic substrate like dimethylallylpyrophosphate (DMAPP), geranylpyrophosphate (GPP), or pyrophosphates of higher isoprenoids [6]. Geranylgeranylpyrophosphate (GGPP) synthase (EC 2.5.1.29) is well characterized for many plant species [7, 8] and has been clearly localized within the plastids [9], where it synthesizes the precursors of carotenoids. Farnesylpyrophosphate (FPP) synthase (EC 2.5.1.10) is also well characterised for some plant species [10, 11, 12], and is known to reside in the cytosol and/or the ER [13] forming the precursors of sterols and sesquiterpenoids. The recently described GPP synthase is also well characterized but in only a few plant species [14–17]. Up to now, the localization of the GPP synthase has been described only in *Vitis vinifera* where it resides within the plastids [18]. This is in accordance with the assumption that plastids are the site of monoterpene biosynthesis [13, 19–21].

In the case of shikonin biosynthesis in *L. erythrorhizon*, GPP is needed as a substrate for the *p*-hydroxybenzoate geranyltransferase at the ER, so here GPP synthase may be localized in the cytosol and/or the microsomes in contrast to *Vitis vinifera*, where GPP synthase is localized within the plastids. The cytosolic localization of GPP synthase in *L. erythrorhizon* was demonstrated in the cell fractionation studies presented here.

RESULTS

A crude extract of suspension-cultured *L. erythrorhizon* cells was fractionated by differential centrifugation (see Experimental). This enabled us to distinguish three types of subcellular localization: cytosolic, microsomal and organelle. Due to their instability, handling of the plastids proved to be difficult; therefore, we did not attempt to separate the mitochondria, microbodies and plastids, but enriched these organelles all together in a single organelle fraction. The fractions were characterized using marker enzymes: alcohol dehydrogenase (EC 1.1.1.1) for the cytosol [22], shikimate dehydrogenase (EC 1.1.1.25) for the plastids [19], fumarase (EC 4.2.1.2) for the mitochondria [23], catalase (EC 1.11.1.6) for the microbodies [24]

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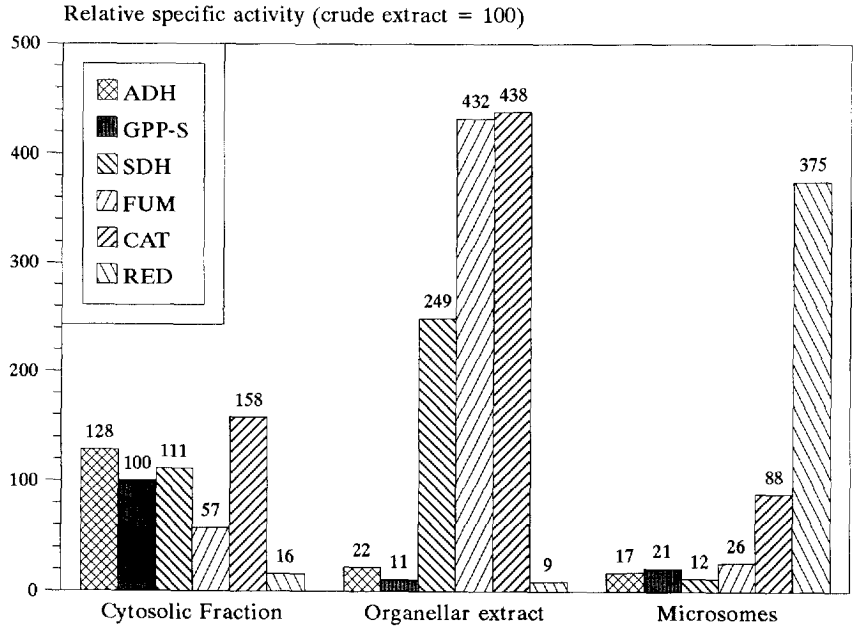
and NAD(P)H-dependent cytochrome *c* reductase (EC 1.6.99.3) for the microsomal fraction [24].

GPP synthase activity was measured using the usual prenyl transferase assay [25]. HPLC analysis of the products [25] clearly showed GPP, rather than FPP, to be the product of the reaction in the crude extract.

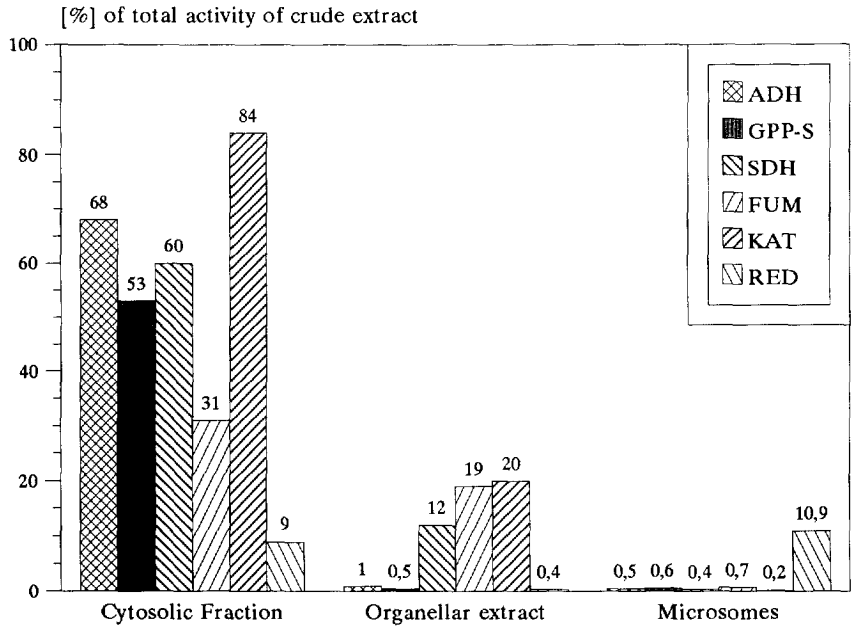
Comparing the specific activities of the marker enzymes with those of the GPP synthase (Fig. 1), we found a clear correlation of the GPP synthase with alcohol dehydrogenase. Both had maximal activities in the cytoplasmic fraction; whereas, in the organelle extract and the microsomal fraction, the activities decreased to 10–20% of the cytosolic value. In contrast to these two enzymes, the specific activities of the organelle markers (shikimate dehydrogenase, fumarase and catalase) increased in the organelle extract. By contrast, the NAD(P)H-dependent cytochrome *c* reductase was enriched to maximal specific activity in the microsomal fraction where GPP synthase and the other marker enzyme activities were hardly detectable (Fig. 1). Consequently, the co-fractionation of the specific activities of the GPP synthase and of the alcohol dehydrogenase indicated a cytosolic rather than plastid localization of the GPP synthase.

The total activities of the enzymes in the various fractions also showed that the distribution of GPP synthase and alcohol dehydrogenase were similar to each other; whereas, the distribution of the other marker enzymes for the organelles and the microsomes were different from that of GPP synthase (Fig. 2). The high total activities of the organelle markers (fumarase, shikimat dehydrogenase and catalase) in the cytosolic fraction were a result of the unavoidable destruction of the organelles during the fractionation procedure. In order to rule out the association of GPP synthase with the normally discarded membranes of the organelles, we measured the specific activity of the 100 000 *g* pellet of the crude organelle fraction. Here the specific GPP synthase activity was only 5% of the cytosolic fraction (data not shown). This is even lower than in the organelle extract. Additionally, we performed a quantification of the GPP synthase by immunodetection with specific antibodies prepared against GPP synthase from *Lithospermum erythrorhizon* cell cultures (see Experimental). Equal amounts of protein of each subcellular fraction were separated by a denaturing PAGE, blotted onto nitrocellulose, and the GPP synthase detected by chemolumin-



Enzyme	Crude extract	Cytosolic fraction	Organelle extract	Microsomes
ADH (nkat mg^{-1})	16.6 ± 1.4	21.2 ± 0.4	3.65 ± 0.05	2.75 ± 0 05
GPP-S (pkat mg^{-1})	68 ± 5.1	68 ± 6	7.5 ± 1.5	14.5 ± 0.5
SDH (nkat mg^{-1})	4.5 ± 0.1	5 ± 0.2	11.2 ± 0.2	0.53 ± 0.035
Fumarase (nkat mg^{-1})	9.95 ± 0.15	5.7 ± 0.5	43 ± 1	2.6 ± 0.1
Catalase (μkat mg^{-1})	0.96 ± 0.03	1.51 ± 0.03	4.19 ± 0.05	0.85 ± 0.05
Reductase (nkat mg^{-1})	1.2 ± 0.18	0.19 ± 0.01	0.11 ± 0.01	4.5 ± 0.2

Fig. 1 Specific activities of GPP synthase (GPP-S) and marker enzymes in different cell fractions: alcohol dehydrogenase (ADH, cytosolic marker), shikimate dehydrogenase (SDH, plastidial marker), fumarase (FUM, mitochondrial marker), NAD(P)H–cytochrome *c*-reductase (RED, microsomal marker). The diagram shows the specific activities normalized for each enzyme with the crude extract set as 100. In the table, the activities are shown with the standard deviation.



Enzyme	Crude extract	Cytosolic Fraction	Organelle extract	Microsomes
ADH (nkat)	759 ± 63	521 ± 210	8 ± 0.1	3.7 ± 0.1
GPP-S (pkat)	3111 ± 5.233	1670 ± 147	16 ± 3	20 ± 1
SDH (nkat)	206 ± 5	123 ± 5	24 ± 0.4	0.72 ± 0.05
Fumarase (nkat)	455 ± 6	140 ± 5	90 ± 2	3.5 ± 0.1
Catalase (μkat)	44 ± 1	37 ± 1	9 ± 0.1	0.1 ± 0.01
Reductase (nkat)	55 ± 8	5 ± 0.3	0.23 ± 0.02	6 ± 0.3

Fig. 2. Total activities of GPP synthase (GPP-S) and marker enzymes of the different cell fractions: alcohol dehydrogenase (ADH, cytosolic marker), shikimate dehydrogenase (SDH, plastidial marker), fumarase (FUM, mitochondrial marker), NAD(P)H–cytochrome *c*–reductase (RED, microsomal marker). The diagram shows the total activities normalized for each enzyme with the crude extract set as 100. In the table, the activities are shown with the standard deviation.

escence. The antiserum against GPP synthase recognized a specific band at *ca* 42 kDa in the four different fractions (Fig. 3), as well as in a control of partially purified GPP synthase [14] (not shown). Quantification of the four lanes, by a laser-densitometer, showed maximal amounts of GPP synthase in the cytosolic fraction (set as 100%) and in the crude extract (94%). Much less GPP synthase was detectable in the organellar extract (13%) and none in the microsomal fraction.

DISCUSSION

The present study strongly suggests that GPP synthase in *L. erythrorhizon* is localized in the cytosol. This is plausible, because, in the biosynthesis of shikonin in *L. erythrorhizon*, GPP is one of the substrates of the PHB geranyltransferase which is localized at the ER [4]. Thus, the substrate GPP must be available in the appropriate cellular compartment. Because C_5-isoprenoids but not C_{10}-isoprenoids [26, 27] can be translocated across the plastid envelope, one expects GPP to be synthesized in the cytosol or at the ER of *Lithospermum* cells. Our results confirm this, demonstrating that the GPP synthase, both by measurement of the activity and by immunodetection, is localized in the cytosol.

From our cell fractionation data, we cannot exclude the possibility of a vacuolar localization of the GPP synthase; however, this seems to be highly unlikely for two reasons: First the low pH value of the vacuole (pH 4–5 [28]) would result in only low enzyme activities (pH-optimum for GPP synthase is 6.75 [14]); and second it would require a transport mechanism for both substrates, IPP and DMAPP, as well as for the product GPP across the tonoplast.

In the Western blot, the antiserum recognized a band at *ca* 42 kDa. The M_r determination of the GPP synthase, by gel exclusion chromatography under native conditions, revealed a size of *ca* 73 kDa [14]. This suggests the GPP synthase to be a dimer. This has also been shown for other prenyltransferases like GGPP synthase [8, 29] and FPP synthase [11], but not for the GPP synthase of *Vitis vinifera*, which seems to be a monomer [16]. The latter enzyme also seems to be different in its subcellular localization, as it has been shown to reside within the

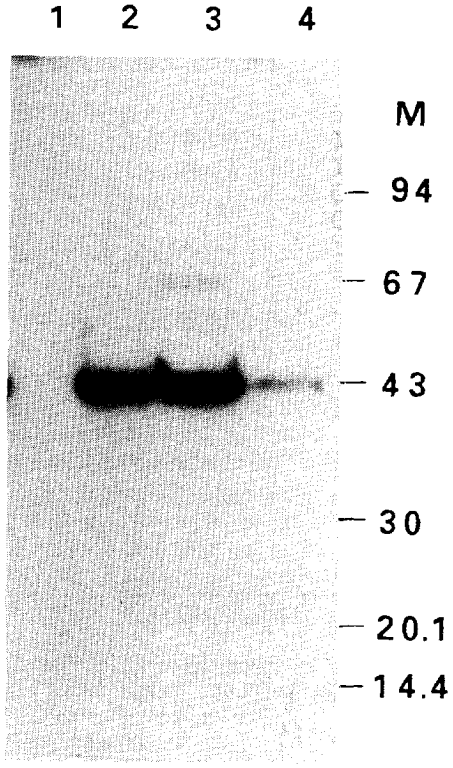


Fig. 3 Immunodetection of the GPP synthase in the different fractions after SDS-PAGE. 5μg protein from each fraction were loaded on each lane, respectively: (1) microsomal fraction, (2) cytosolic fraction, (3) crude extract, (4) organellar extract. M represents the molecular weight standard in kDa.

plastids. This difference could be expected because the GPP, in *Vitis*, is most likely used to produce monoterpenes via a metabolic pathway localized in the plastids [19–21]. Since, as mentioned above, it is unlikely that C_{10}-isoprenoids cross these membranes [27, 29], GPP as a precursor of monoterpenes, must be synthesized within the plastids.

We cannot absolutely exclude the possiblity of an additional plastid GPP synthase in *L. erythrorhizon*, but, because no monoterpene synthesis has been observed in this species, there is no need to postulate an additional isozyme. Generally, these results support the view that enzymes with identical activities involved in the biosynthesis of terpenoid secondary metabolites may be localized in different compartments according to the further steps of the biosynthetic pathway.

EXPERIMENTAL

Radiochemicals. [1-^{14}C]IPP (2,1 GBq/mmol) was obtained from Amersham.

Cell cultures. Cell culture M18 of *L. erythrorhizon* Sieb. et Zucc. [30] was maintained in LS liquid medium containing 10^{-6} M IAA and 10^{-5} M kinetin. The cultures were incubated in the dark at 25° and agitated on a shaker at 92 rpm.

Cell fractionation. Harvested cells (15 g) were suspended in 15 ml of 0.1 M K-Pi buffer (pH 6.5) containing 10 mM DTT and 5% PVPP. The cells were disrupted with an Ultra-Turrax (2 sec × 3). The brew was filtered through two layers of nylon gauze (40 μm). The filtrate was centrifuged at 800 *g* for 1 min. A 1 ml-aliquot of the supernatant was homogenized in a Potter homogenizer to give a crude extract fraction. The residual 14 ml of the supernatant were centrifuged at 20000 *g* for 15 min to give an organelle pellet, which was resuspended in 3 ml 0.1 M K-Pi buffer (pH 6.5) by means of a Potter homogenizer and sonication giving the crude organelle fraction. The suspension was centrifuged at 100000 *g* for 1 hr to remove the membranes (pellet) resulting in the organelle extract (supernatant). The 20000 *g* supernatant was also centrifuged at 100000 *g* for 1 hr resulting in the cytosolic fraction (supernatant) and the microsomal fraction, obtained from the 100000 *g* pellet by resuspension in 1 ml 0.1 M K-Pi buffer (pH 6.5). For investigations of the enzyme activities, 0.5 ml aliquots of each fraction were eluted from a Sephadex G25 column (NAP5, Pharmacia) with 1 ml of 0.1 M K-Pi buffer (pH 6.5).

Enzyme assays. GPP synthase activity was assayed according to ref. [25]. A correction for the IPP isomerase activity was made by omitting the substrate DMAPP in the assay of [25] and substracting of the isomerase activity from the total activity. Additionally, the product was identified using the HPLC assay of [25]. Alcohol dehydrogenase was assayed photometrically at 37° using a wavelength of 340 nm in an incubation mix containing 80 mM K-Pi buffer (pH 7.5), 170 mM ethanol, 0.58 mM NAD^+ and 30 μg protein (modified after [31]). Shikimate dehydrogenase was assayed photometrically with a wavelength of 340 nm at 37° in an incubation mix containing 66 mM glycin buffer (pH 10.1), 1 mM shikimate, 0.16 mM $NADP^+$ and 30 μg protein (modified after [32]). Fumarase was assayed photometrically at 240 nm in an incubation mix containing 90 mM K-Pi buffer (pH 7.5), 50 mM malate and 30 μg protein at 37° (modified after [33]). NAD(P)H-dependent cytochrome *c* reductase was assayed photometrically at 550 nm in an incubation mix containing 15.8 mM K-Pi buffer (pH 7.2), 0.2 mM NADH, 0.2 mM cytochrome *C*, 1 mM KCN, 1 μM Antimycin A and 15 μg protein at 37° (modified after [34]). Catalase was assayed photometrically at 240 nm at 37° in an incubation mix containing 42.5 mM K-Pi buffer (pH 6.75), 17.6 mM H_2O_2 and 30 μg protein (modified after ref. [35]).

Protein determination. The protein concentration of the cell fractions was determined by the method of ref. [36].

Preparation of antibodies. Purification of GPP synthase from *L. erythrorhizon* cell cultures and preparation of antibodies was carried out as described previously for GGPP synthase [37].

Western blotting and immunodetection. SDS-PAGE was performed according to Laemmli [38] (on 12% polyacrylamide gel). Proteins were transferred onto nitrocellulose in an electrophoretic semidry transfer cell (Bio-Rad). The nitrocellulose was incubated for 20 hr at 4° with blocking reagent (Boehringer Mannheim), and sub-

sequently with an antiserum against the GPP synthase of *L. erythrorhizon* [1:1000 diluted in TBS (50 mM Tris, pH 7.5, 150 mM NaCl) with 0.5% blocking reagent] for 1 hr. After four additional washes with TBST [TBS with 0.1% (w/v) Tween 20], it was incubated with anti-rabbit/goat $F(ab')_2$ antibody conjugated to horseradish peroxidase (Sigma) (1:2000 diluted in TBS with 0.5% blocking reagent). After washing four times with TBST, the nitrocellulose sheet was covered with detection solution (ECL, Boehringer Mannheim) and exposed to Kodak XAR5 film for 10 sec to visualize the immunodetected proteins.

Densitometry. The bands on the X-ray films were measured with a laser densitometer (LKB, Ultroscan, modell 2202).

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REFERENCES

1. Tabata, M. and Fujita, Y. (1985) in *Biotechnology in Plant Science* (Zaitlin, M., Day, P. and Hollaender, A., eds), p. 207. Academic Press, Orlando.
2. Tanaka, S., Tajima, M., Tsukada, M. and Tabata, M. (1986) *J. Nat. Prod.* **49**, 466.
3. Heide, L., Bechthold, A., Yazaki, K. and Tabata, M. (1992) in *Plant Tissue Culture and Gene Manipulation for Breeding and Formation of Phytochemicals* (Oono, K., Hirabayashi, T., Kikuchi, S., Handa, H., Kajiwara, K., eds), p. 245. National Institute of Agrobiological Resources, Tsukaba, Japan.
4. Yamaga, Y., Nakanishi, K., Fukui, H. and Tabata, M. (1993) *Phytochemistry* **32**, 633.
5. Tsukada, M. and Tabata, M. (1984) *Planta Med.* **50**, 338.
6. Poulter, C. D. and Rilling, H. C. (1981) in *Biosynthesis of Isoprenoid Compounds* Vol. 1 (Porter, J. W. and Spurgeon, L., eds), p. 161. John Wiley and Sons, New York.
7. Spurgeon, S. L. Sathyamoorty, N. and Porter, J. W. (1984) *Arch. Biochem. Biophys* **230**, 446.
8. Dogbo, O. and Camara, B. (1987) *Biochim. Biophys. Acta.* **920**, 140.
9. Cheniclet, C., Rafia, F., Saint-Guily, A., Verna, A. and Carde, J.-P. (199 2) *Biol. Cell* **75**, 145.
10. Hugueney, P. and Camara, B. (1990) *FEBS Letters* **273**, 235.
11. Rilling, H. C. (1985) *Meth. Enzymol.* **110**, 145.
12. Gray, J. C. (1987) *Adv. Bot. Res.* **14**, 25.
13. Kleinig, H. (1989) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40**, 39.
14. Heide, L. and Berger, U. (1989) *Arch. Biochem. Biophys.* **273**, 331.
15. Croteau, R. and Purkett, P. T. (1989) *Arch. Biochem. Biophys.* **271**, 524.
16. Clastre, M., Bantignies, B., Feron, G., Soler, E. and Ambid, C. (1993) *Plant Physiol.* **102**, 205.
17. Suga, T. and Endo, T. (1991) *Phytochemistry* **30**, 1757.
18. Soler, E., Feron, G., Clastre, M., Dargent, R., Gleizes, M. and Ambid, C. (1992) *Planta* **187**, 171.
19. Schulze-Siebert, D. Schultz, G. (1987) *Plant Physiol. Biochem.* **25**, 145.
20. Gleizes, M., Pauly, G., Carde, J.-P., Marpeau, A. and Bernard-Dagan, C. (1983) *Planta* **159**, 373.
21. Mettal, U., Boland, W., Beyer, P. and Kleinig, H. (1988) *Eur. J. Biochem.* **170**, 613.
22. Scandalios, J. G. (1977) in *Regulation of Enzyme Synthesis and Activity in Higher Plants* (Smith, H., ed.), p. 129. Academic Press, New York.
23. Hatch, M. D. (1987) *Anal. Biochem.* **85**, 271.
24. Quail, P. (1979) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **30**, 425.
25. Heide, L. (1988) *FEBS Letters* **237**, 159.
26. Soler, E., Clastre, M., Bantignies, B., Marigo, G. and Ambid, C. (1993) *Planta* **191**, 324.
27. Pérez, L. M., Pauly, G., Carde, J.-P., Belingheri, L. and Gleizes, M. (1990) *Plant Physiol. Biochem.* **28**, 221.
28. Mohr. H. and Schopfer, P. (1992), *Pflanzenphysiologie*, 4th Edn. springer, Berlin.
29. Lafferière, A. and Beyer, P. (1991) *Biochim. Biophys. Acta* **1077**, 167.
30. Mizukami, H., Konoshima, M. and Tabata, M. (1987) *Phytochemistry* **22**, 95.
31. Dolferus, R. and Jacobs, M. (1984) *Biochem. Genet.* **22**, 817.
32. Sanderson, G. W. (1966) *Biochem. J.* **98**, 248.
33. Massey, V. (1995) *Meth. Enzymol.* **1**, 729.
34. Lord, J. M., Kagawa, T., Moore, S. and Beevers, B. (1973) *J. Cell Biol.* **57**, 659.
35. Bergmeyer, H. U. (1974) *Methoden der enzymatischen Analyse*, 3rd Edn. Verlag Chemie, Weinheim.
36. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248.
37. Camara, B. (1993) *Methods Enzymol.* **214**, 352.
38. Laemmli, U. K. (1970) *Nature* **227**, 680.