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DISTRIBUTION OF POLYPRENOLS AND DOLICHOLS IN SOYBEAN PLANT

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Key Word Index—*Glycine max*; soybean; polyprenol; dolichol; ficaprenol; betulaprenol; glycinoprenol.

Abstract—Using a convenient two-plate thin layer chromatography method, polyprenols and dolichols in soybean plant were analysed. These polyisoprenoid alcohols were found to occur in different quantities from tissue to tissue. The leaves contained ficaprenols (C_{50}, C_{55}, C_{60}), glycinoprenols (C_{45}, C_{50}, C_{55}) and dolichols (C_{80}, C_{85}) with the major being ficaprenols, while the shoots, roots and seeds contained only dolichols. Analysis of the subcellular distribution of these compounds in the leaves demonstrated that ficaprenols, glycinoprenols and dolichols were mostly located in the chloroplast. When the chloroplast fraction was incubated with a combination of [1-^{14}C]isopentenyl diphosphate and farnesyl diphosphate, radioactive polyprenols composed of C_{45}, C_{50}, C_{55} and C_{70}, C_{75} were formed, suggesting that biosynthesis site for these polyprenols is localized in the chloroplast. The shorter chain ficaprenols and the longer chain dolichols were also found in the leaves of spinach, perilla, parsley and evergreen magnolia, which are dicotyledonous plants. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Plants have been reported to produce large amounts of long-chain *cis*-polyprenols each having an *E,E*-farnesyl residue or an *E,E,E*-geranylgeranyl residue at the ω-end of their phenyl chains. The former type of polyprenols was initially isolated from *Betula verrucosa* (betulaprenol) and have been found in the needles or the leaves of gymnosperms, while the latter type of polyprenols was isolated from *Ficus elastica* (ficaprenol) and have been found in the leaves of angiosperms [1]. The role of these polyprenols has not been elucidated. In a recent work, plants have also been reported to produce dolichol [2–4]. Dolichol is a family of *E,E*-farnesyl-*cis*-polyprenols having a saturated isoprene unit at the α-terminal, and dolichols with various chain lengths have been found in mammals, yeast and fungi [5]. The phosphorylated form, dolichyl phosphate, is known to function as a carrier lipid of monosaccharide and oligosaccharide group during the biosynthesis of *N*-linked glycoproteins and glycosylphosphatidyl inositol anchored proteins [6].

The soybean plant has been well studied in regard to polyprenols and dolichols. Dolichols, dolichyl phosphates and dolichyl esters were detected in the seeds and seedlings of the plant [7–9]. The contents of dolichols were at most 0.01% of the dry seed [7] and 0.002% of the fresh seedling [10]. Ravi *et al.* [10] reported a simultaneous decrease in dolichol content and increase in dolichyl phosphate content during the germination of the soybean, suggesting that the biosynthesis of dolichyl phosphate from dolichol might be required in germinating seeds for the synthesis of asparagine-linked glycosylated proteins. On the other hand, ficaprenol-type polyprenols and glycinoprenols, unusual *cis*-polyprenols having a phytyl residue at their ω-terminal were found in the methanol extracts of soybean leaves. It has been reported that the contents of these polyprenols in the leaves remarkably increased from 10 weeks after its germination up to *ca* 0.1–0.2% (w/w) of the fresh leaves [11].

Ficarprenol-type and betulaprenol-type polyprenols are very similar to dolichols in their polyprenyl structures, but the biosynthesis of the former two polyprenols seems to be different from that of the latter. Not only soybean but also other plants have been reported to massively accumulate polyprenols in tissues and to vary in their quantities depending on their age [11, 12] or seasons [1, 4]. In order to examine the relationship between the biosyntheses of these polyprenols and dolichols, it is desirable to establish a convenient method of separation of these compounds into each component to analyse them in detail. Recently, we have developed a two-plate TLC method by which dolichols and α-unsaturated polyprenols can be detected at the same time [13]. We applied this method for analysis of polyisoprenoid alcohols in soybean plants. As a result, ficaprenols, glycinoprenols and dolichols in soybean plants were completely separated from one another. By using this method, we investigated the location of

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accumulated amounts of ficaprenols, glycinoprenols and dolichols in mature soybean plant. The variation of these polyisoprenoid alcohols were also studied in other dicotyledonous plants.

RESULTS AND DISCUSSION

We have developed a two-plate TLC analytical method [13] for the analysis of polyprenols and dolichols and have now applied it to the separation of various isoprenoids including glycinoprenols as well as polyprenols and dolichols. As shown in Fig. 1, the separation of dolichols, betulaprenols, ficaprenols, glycinoprenols and all-*trans*-polyprenols (geraniol, farnesol, geranylgeraniol, solanesol and decaprenol) from one another was achieved with respect to the carbon chain length. Ubiquinone, plastoquinone, squalene and cholesterol could also be analysed by this method. However, it was difficult to separate C_{55}-dolichol from glycinoprenols completely since the former migrated between C_{50}- and C_{55}-glycinoprenols. As previously reported [13], this two-plate TLC method was not effective in distinguishing an α-unsaturated polyprenol with three *trans*-double bonds (ficaprenol-type polyprenol) from the stereoisomeric polyprenol with two *trans*-double bonds (betulaprenol-type polyprenol) having the same chain length. Although dolichyl fatty acyl esters extracted from soybean embryo were not separated satisfactorily under these conditions, the separation was markedly improved by using an appropriate solvent system. Four major family components of the esters were separated from one another with respect to the carbon chain length when the two-plate TLC was performed with a combination of the solvent system of hexane–diethyl ether (5:1) for silica gel TLC and acetone (100%) for LKC-18 reverse-phase TLC (data not shown).

Having established that this method is applicable to the separation of dolichols and various polyprenols including ficaprenols and glycinoprenols, we further examined the distribution of these polyisoprenoid alcohols occurring in soybean plants. Leaves, shoots and roots of a mature soybean plant (17-week-old) were treated with potassium hydroxide at 85° for 1 hr and extracted with chloroform–methanol (2:1). The non-saponifiable lipids were analysed by two-plate TLC. Larger amounts of ficaprenols and glycinoprenols were found in the leaves, and a small but significant amount of dolichols was also detected (Fig. 2(A)). On the other hand, a very small amount of ficaprenols was detected in the shoots, and neither ficaprenols nor glycinoprenols were found in the roots. These tissues, however, contained a small quantity of dolichols as observed in the leaves (Fig. 2(B) and (C)). We also confirmed that dolichols were the only long-chain polyisoprenoid alcohols in the seeds (Fig. 2(D)). The chain lengths of dolichols in the leaves, shoots and roots were C_{80} and C_{85}, but the dolichols of the seeds had longer chain lengths by one isoprene unit (C_{85} and C_{90}). The chain lengths of these dolichols were much longer than those of ficaprenols (C_{50}, C_{55}, C_{60}) or glycinoprenols (C_{45}, C_{50}, C_{55}) found in the leaves. These results indicate that the soybean plant contains ficaprenols and glycinoprenols in the leaves and much longer dolichols in other tissues.

Next, we investigated the subcellular distribution of ficaprenols, glycinoprenols and dolichols in the leaves. Ficaprenols and glycinoprenols were mainly localized

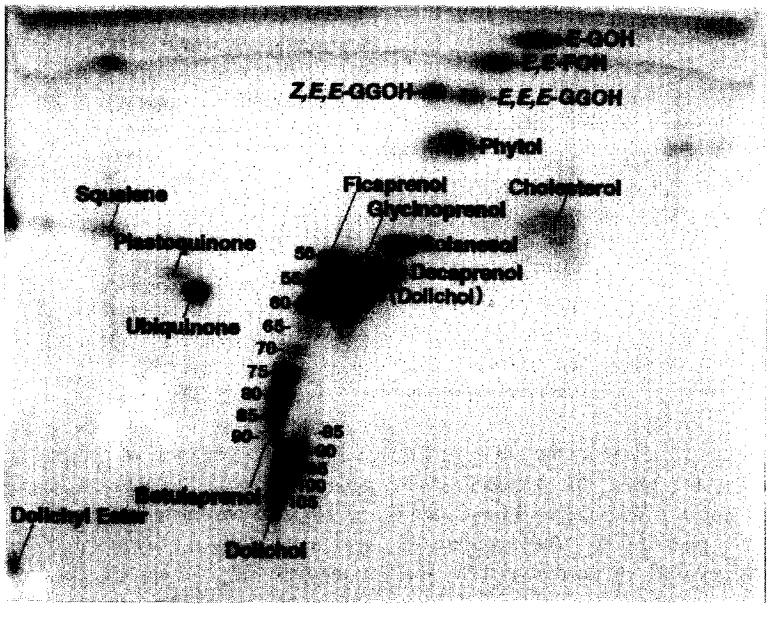

Fig. 1. Separation of various isoprenoids by two-plate TLC. The numbers refer to the carbon chain length of polyisoprenoid alcohols. The positions of authentic standards were visualized with iodine vapour. GOH, geraniol; FOH, farnesol; GGOH, geranylgeraniol.

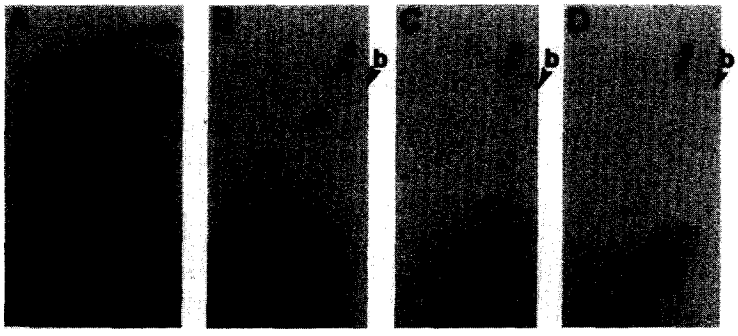

Fig. 2. Analysis of polyisoprenoid alcohols extracted from soybean plants by two-plate TLC. Polyisoprenoid alcohols extracted from 2 g (fr. wt) each of (A) leaves, (B) shoots, (C) roots and (D) seeds of the soybean plants were applied to the TLC plates. The positions of polyisoprenoid alcohols were visualized with iodine vapour. The arrowheads indicate the family of (a) ficaprenols, (b) glycinoprenols and (c) dolichols. The number refer to the carbon chain length of polyisoprenoid alcohols.

in the chloroplast fraction, and dolichols were also detected in this fraction (Fig. 3). As shown in Fig. 3(C), a considerable amount of ficaprenols was detected in the 105 000 *g* pellet. Since the content of chlorophyll in this pellet was estimated to be 39% of that of the chloroplast fraction, the ficaprenols detected in the 105 000 *g* pellet might be due to contamination of the chloroplast fraction.

Suga *et al.* [11] reported that ficaprenols and glycinoprenols in the leaves were mainly found from 10 weeks after germination and that their contents remarkably increased until 20 weeks. This suggested that the biosynthesis of ficaprenols and glycinoprenols started from 10 weeks after germination. We examined enzyme activities in 17-week-old soybean leaves responsible for the formation of ficaprenols or glycinoprenols. The chloroplast fraction was incubated with a combination of *E,E*-farnesyl diphosphate and [1-^{14}C]isopentenyl diphosphate, and the products were analysed. As a result, two groups of radioactive products were cochromatographed with C_{45}, C_{50}, C_{55}- and C_{70}, C_{75}-polyprenols on reverse-phase TLC. On silica gel TLC, they cochromatographed with solanesol or migrated a little slower than C_{85}, C_{90}, C_{95}- or C_{55}, C_{60}-polyprenols (Fig. 4). These results indicate that the chloroplast fraction from soybean leaves has the ability to synthesize solanesol (C_{45}) and two kinds of long chain *cis*-polyprenols, C_{50}, C_{55}-polyprenols and C_{70}, C_{75}-polyprenols, from farnesyl diphosphate and isopentenyl diphosphate. The polyprenols (C_{70}, C_{75}) were a little shorter than the naturally occurring dolichols (C_{80}, C_{85}). This might be due to a property of the long-chain *cis*-prenyltransferase since the chain length of the *in vitro* enzymatic products is known to shift downward with the increase of the concentration of detergents in the enzyme assay [14].

We have confirmed that the leaves of soybean plant, in fact, contain three types of long chain *cis*-polyprenols, that is, dolichols, ficaprenols, and glycinoprenols, To learn whether these polyisoprenoid alcohols also occur in the leaves of other dicotyledonous plants, we examined spinach, perilla and parsley. As for a sample of woody plants, we also tested evergreen magnolia (*Magnolia grandiflora*), which is known to contain a smaller amount of polyprenols in the leaves (0.083% fresh weight) compared with other known woody plants [1]. As shown in Fig. 5(A), spinach contained three kinds of polyisoprenoid alcohols in the leaves. On the other hand, perilla and parsley had a relatively small amount of ficaprenols and no glycinoprenol in their

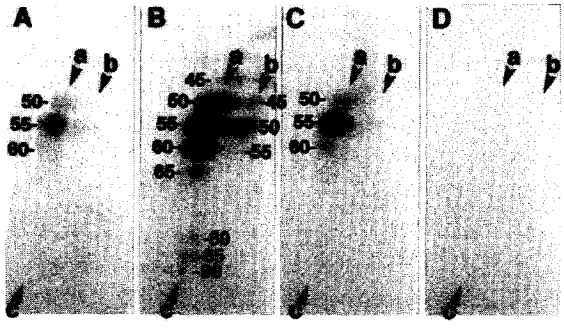

Fig. 3. Analysis of polyisoprenoid alcohols extracted from subcellular fractions of soybean leaves by two-plate TLC. Polyisoprenoid alcohols extracted from each sample containing 20 mg of proteins: (a) homogenates of the leaves, (B) chloroplast fraction, (C) 105 000 *g* pellet and (D) cytosol, were applied to the TLC plates. The positions of polyisoprenoid alcohols were visualized with iodine vapour. The arrowheads indicate the family of (a) ficaprenols, (b) glycinoprenols and (c) dolichols. The numbers refer to the carbon chain lengths of polyisoprenoid alcohols.

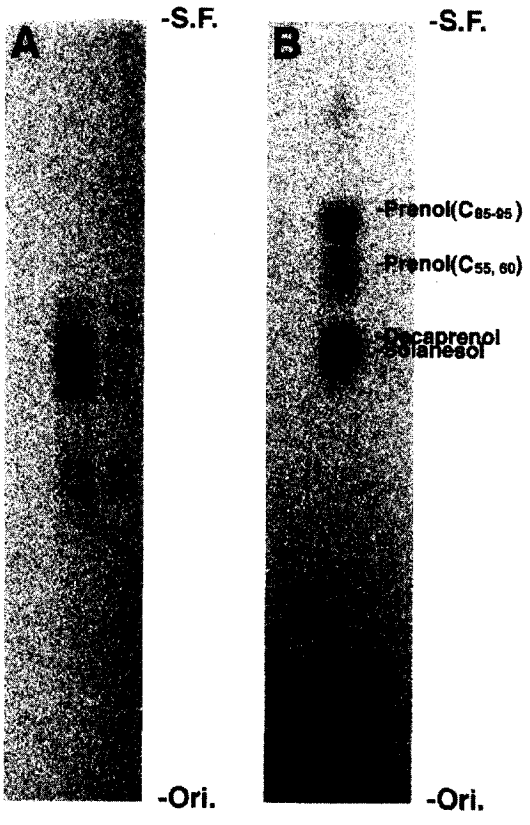

Fig. 4. Enzymatic formation of polyprenyl compounds by the chloroplast fraction. The assay was carried out as described in the Experimental. Autoradiography of radioactive products developed on (A) reverse-phase LKC-18 TLC with a solvent system of Me_2CO–MeOH (9:1) and on (B) silica gel TLC with a solvent system of toluene–EtOAc (9:1) are shown. The numbers in (A) refer to the carbon chain length of polyisoprenoid alcohols. Authentic compounds used in (B) were polyprenols (C_{85}–C_{95}) from *Ginkgo biloba* and those from silkworm faeces (C_{55}, C_{60}). Ori., origin; S.F., solvent front.

leaves. Moreover, a small amount of α-unsaturated polyprenols having the same chain lengths as those of dolichols was detected in addition to dolichols (Fig. 5(B) and (C)). Evergreen magnolia leaves, however, contained ficaprenols, glycinoprenols, dolichols and α-unsaturated polyprenols (Fig. 5(D)). We also examined the occurrence of these polyisoprenoid alcohols in other tissues of these plants. Ficaprenols were detected only in the leaves of these plants. Glycinoprenols were detected in leaves of spinach and evergreen magnolia but not in other tissues of these plants. Dolichols were detected in every tissue of these plants with similar contents. α-Unsaturated polyprenols, however, were detected in every tissue of perilla, parsley and evergreen magnolia and in the seeds of spinach (data not shown). The α-unsaturated polyprenols detected in these experiments might be precursors in the biosynthesis of dolichols [15].

In this paper, we have shown that ficaprenols and glycinoprenols are found only in leaves of soybean and some other dicotyledonous plants, and that these polyprenols are localized in the chloroplast fraction. As for spinach leaves, Swiezewska *et al.* [16] obtained similar results that ficaprenol (C_{55}) was localized in the chloroplast. We have also shown that the chloroplast fraction of soybean leaves has the ability to synthesize polyprenols from isopentenyl diphosphate and farnesyl diphosphate with similar chain lengths to those of naturally occurring ficaprenols and dolichols. Soll *et al.* [17] reported that phytol and geranylgeraniol were biosynthesized in chloroplasts to give the precursors of chlorophyll. Phytol and geranylgeraniol might be also used as the precursors for the biosyntheses of ficaprenols and glycinoprenols in this organelle. On the other hand, it is still unknown why these polyprenols accumulate in the leaves at a certain stage of their maturation. In the experiments using tomato and pepper chloroplasts, Goodwin [18] has shown that carotenoids are massively synthesized in chloroplasts during the

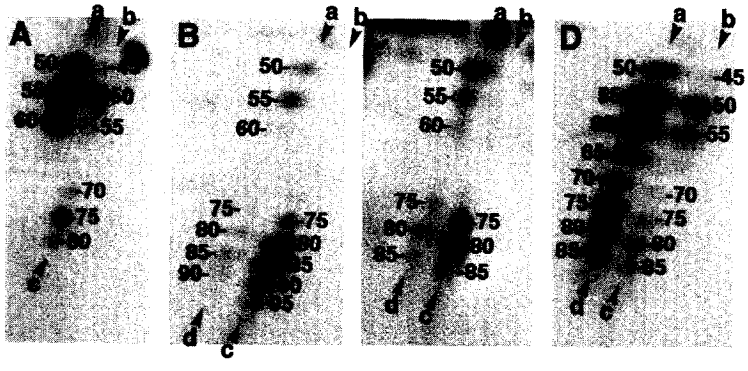

Fig. 5. Analysis of polyisoprenoid alcohols extracted from leaves of some dicotyledonous plants by two-plate TLC. Polyisoprenoid alcohols extracted from leaves of (A) spinach (7 g), (B) perilla (7 g), (C) parsley (4 g) and (D) evergreen magnolia (1 g) were applied to the TLC plates. The positions of polyisoprenoid alcohols were visualized with iodine vapour. The arrowheads indicate the families of (a) ficaprenols, (b) glycinoprenols, (c) dolichols and (d) α-unsaturated polyprenols. The numbers refer to the carbon chain length of polyisoprenoid alcohols.

ageing of this organelle. As for the soybean plant, neither ficaprenols nor glycinoprenols are synthesized for at least 10 weeks after germination or budding of leaves, whereas outstanding accumulation of polyprenols occurs for the next 10 weeks [11]. This may suggest that accumulation of these polyprenols in the chloroplast is related to the ageing of the chloroplast. On the other hand, dolichols were ubiquitously distributed in plant tissues, suggesting that dolichols are utilized in plants for the biosynthesis of glycoproteins or GPI-anchored proteins as is known to be the case in mammals, yeast and fungi.

EXPERIMENTAL

Materials. Glycinoprenols were isolated from leaves of mature soybean plants according to the method of ref. [11]. Polyprenols (C_{85}–C_{95}) from *Ginkgo biloba* and *Z,E,E*-geranylgeraniol were given by Kuraray Corp. Polyprenols (C_{55}, C_{60}) from silkworm faeces were given by Takasago Perfumery Company. Dolichyl fatty acyl esters from soybean embryo were generously donated from Dr Masataka Ishinaga of Hiroshima Women's University [9]. *E,E*-Farnesyl diphosphate was prepd according to the method of ref. [19]. Mature plants of a soybean (*Glycine max*) and leaves and shoots of a evergreen magnolia (*Magnolia grandiflora*) were collected locally at the beginning of September. Seeds of the soybean were purchased locally. Spinach, parsley and perilla were purchased in a supermarket. All other chemicals were of reagent grade.

Extraction of polyisoprenoid alcohols from plants. Procedures for extraction of tissue polyisoprenoid alcohols were basically the same as described in the previous paper [13]. Leaves (20 g) were homogenized in 40 ml of H_2O or finely ground shoots, roots or seeds (20 g) were mixed with 40 ml of H_2O and then saponified at 85° for 1 hr with supplements of 20 ml of EtOH, 20 g of KOH and 1 g of pyrogallol. Nonsaponifiable lipids were extracted with $CHCl_3$–MeOH (2:1), and the extracts (*ca* 0.2–1.0 g) were applied to a 2 mm-thick silica-gel prep. TLC plate. The plate was developed in a solvent system of toluene–EtOAc (9:1). The silica gel corresponding to polyisoprenoid alcohols (R_f 0.6–0.9) was scraped from the plate, and the polyisoprenoid alcohols were extracted with Et_2O. The partially purified polyisoprenoid alcohols were applied to a RP-18 Sep-Pak column (Waters) equilibrated with MeOH–H_2O (19:1). The column was washed with MeOH–H_2O (19:1), and polyisoprenoid alcohols were eluted with hexane. The hexane eluent fr. was used as the sample for two-plate TLC analysis.

Analysis by the two-plate TLC method. All procedures were the same as described previously [13] except that the solvent system for LKC-18 was Me_2CO–MeOH (4:1).

Subcellular fractionation of soybean leaves. All further steps were performed in cold room at 4°. Washed and deribbed 17-week-old leaves (73 g) were homogenized using a Polytron at high speed for 2 × 4 sec in an ice-cold grinding buffer (730 ml) containing 50 mM HEPES (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulphonic acid)–KOH (pH 7.6), 330 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 0.2% bovine serum albumin. The homogenates were filtered through six layers of cheese cloth and then through two layers of nylon net (20 μm). Chloroplasts were obtained at 6500 *g* for 10 min. The pellet was homogenized in the lysis buffer containing 10 mM HEPES–KOH (pH 7.6), 5 mM DTT and 1 mM EDTA, and used as the chloroplast fr. The supernatant fr. was centrifuged at 105 000 *g* for 1 hr. The pellet was resuspended in the lysis buffer, and used as 105 000 *g* pellet. Proteins were determined with a Bio-Rad protein assay kit. The amounts of proteins in homogenates, chloroplasts, 105 000 *g* pellet cytosol were 3575, 262.8, 100.0 and 3213 mg, respectively. The chlorophyll content was determined by the method of ref. [20]. The contents of chlorophyll in homogenates, chloroplasts, 105 000 *g* pellet and cytosol were 4.89, 57.6, 22.5 and 0.0392 mg mg^{-1} protein, respectively.

Extraction of polyisoprenoid alcohols from subcellular frs. Each fr. (10 ml) was saponified at 85° for 1 hr with the supplement of 5 ml of EtOH, 5 g of KOH, and 0.25 g of pyrogallol. The following procedures were the same as described earlier.

Enzyme assay. Long-chain *cis*-prenyltransferase activity was assayed according to the procedure of ref. [21] with some modifications. The standard assay mixt. contained, in a final volume of 0.5 ml, 50 mM Tris–HCl (pH 7.7), 2 mM dithiothreitol, 50 mM KF, 1 mM $MgCl_2$, 1.0% (w/v) Triton X-100, 100 μM *E,E*-farnesyl diphosphate, 5 μM [1-^{14}C]isopentenyl diphosphate (56 Ci mol^{-1}) and *ca* 4 mg of chloroplast fr. The mixt. was incubated at 30° for 11 hr. The reaction products were extracted with 1 ml of *n*-BuOH satd with H_2O, and the *n*-BuOH extracts were washed twice with 0.5 ml of satd NaCl. The *n*-BuOH extracts were treated with wheat germ acid phosphatase (Sigma Type 1) according to the method of ref. [22]. After incubation at 37° for 12 hr, the hydrolysates were extracted with pentane. Since pentane extracts contained large amounts of chlorophyll which interfere the migration of polyisoprenoid alcohols on the TLC plates, the extracts were saponified at 85° for 1 hr with 0.5 ml of 0.25% (w/v) pyrogallol in MeOH and 0.25 ml of 60% (w/v) KOH. The mixts were extracted with pentane, and the extracts were washed 2 × with satd NaCl. The radioactive polyisoprenoid alcohols in the pentane extracts were analysed by reverse-phase LKC-18 TLC and silica gel TLC with a solvent system of Me_2CO–MeOH (9:1) and toluene–EtOAc (9:1), respectively. The positions of authentic standards were visualized with iodine vapour. The radioactivity of polyisoprenoid alcohols developed on TLC were determined with a Fuji Bio-Image Analyser BAS 1000.

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REFERENCES

1. Swiezewska, E., Sasak, W., Mankowski, T., Jankowski, W., Vogtman, T., Krajewska, I., Hertel, J., Skoczylas, E. and Chojnacki, T. (1994) *Acta Biochim. Pol.* **41**, 221.
2. Ravi, K., Rip, J. W. and Carroll, K. (1983) *Biochem. J.* **213**, 513.
3. Rip, J. W., Rupar, C. A., Ravi, K. and Carroll, K. K. (1985) *Prog. Lipid Res.* **24**, 269.
4. Jankowski, W., Kula-Swiezewska, E., Sasak, W. and Chojnacki, T. (1994) *J. Plant Phys.* **143**, 448.
5. Dunphy, P. J., Kerr, J. D., Pennock, J. F., Whittle, K. J. and Feeney, J. (1967) *Biochim. Biophys. Acta* **136**, 136.
6. Herscovics, A. and Orlean, P. (1983) *FASEB J.* **7**, 540.
7. Ravi, K., Rip, J. W. and Carroll, K. K. (1984) *Lipids* **19**, 401.
8. Rip, J. W. and Carroll, K. K. (1987) *Analyt. Biochem.* **160**, 350.
9. Ishinaga, M., Mukai, K. and Tanibe, C. (1990) *Agric. Biol. Chem.* **54**, 943.
10. Ravi, K., Rip, J. W. and Carroll, K. K. (1986) *Biochem. Biophys. Acta* **875**, 626.
11. Suga, T., Ohta, S., Nakai, A. and Munesada, K. (1989) *J. Org. Chem.* **54**, 3390.
12. Ibata, K., Mizuno, M., Takigawa, T. and Tanaka, Y. (1983) *Biochem. J.* **213**, 305.
13. Sagami, H., Kurisaki, A., Ogura, K. and Chojnacki, T. (1992) *J. Lipid Res.* **33**, 1857.
14. Matsuoka, S., Sagami, H., Kurisaki, A. and Ogura, K. (1991) *J. Biol. Chem.* **266**, 3464.
15. Sagami, H., Kurisaki, A. and Ogura, K. (1993) *J. Biol. Chem.* **268**, 10109.
16. Swiezewska, E., Dallner, G., Andersson, B. and Ernster, L. (1993) *J. Biol. Chem.* **268**, 1494.
17. Soll, J., Schult, G., Rüdiger, W. and Benz, J. (1983) *Plant Physiol.* **71**, 849.
18. Goodwin, T. W. (1993) *Methods Enzymol.* **214**, 330.
19. Davisson, V. J., Woodside, A. B. and Poulter, C. D. (1985) *Methods Enzymol.* **110**, 130.
20. Arnon, D. I. (1949) *Plant Physiol.* **24**, 1.
21. Sagami, H., Matsuoka, S. and Ogura, K. (1991) *J. Biol. Chem.* **266**, 3458.
22. Yasugi, E. and Oshima, M. (1994) *Biochim. Biophys. Acta* **1211**, 107.