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Dolichols of rubber plant, ginkgo and pine

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Abstract

Using a two-plate thin-layer chromatography method, we analyzed polyisoprenoid alcohols (dolichols and polyprenols) of the rubber plant *Hevea brasiliensis* (angiosperm), and of ginkgo *Ginkgo biloba* and pine *Pinus sylvestris* (gymnosperms). Special attention was paid to the occurrence of dolichol in various tissues of different plants. Dolichols were found to occur in all of the tissues examined except for flowers of the rubber plant. The chain length distributions of dolichols in seeds, young roots, young shoots, young leaves and old leaves of the rubber plant were C_{70}-C_{95}, C_{85}-C_{105}, C_{80}-C_{105}, C_{75}-C_{105} and C_{65}-C_{90}, respectively. In the case of ginkgo, the chain length distributions of dolichols in seeds, embryos, young and old leaves were C_{70}-C_{90}, C_{70}-C_{85}, C_{70}-C_{90} and C_{80}-C_{95}, respectively. Pine seeds were found to contain dolichols with the chain length distribution of C_{70}-C_{90}. Two kinds of polyprenol families were detected in leaves of the rubber plant and ginkgo. The longer chain polyprenol family was also detected in seeds of the rubber plant, in seeds and embryos of ginkgo and in seeds of pine. The chain length distributions of the polyprenols were not necessarily the same as those of dolichols occurring in the same tissues.

Keywords: Polyprenol; Dolichol; Ginkgo; *Ginkgo biloba*; Ginkgoaceae; Pine; *Pinus sylvestris*; Pinaceae; *Hevea brasiliensis*; Eupholbraceae; Rubber; Two-dimensional thin-layer chromatography

1. Introduction

Long chain *cis*-polyprenols have been reported to occur in various plants. These compounds include two types of polyprenols with respect to the stereochemistry. One is a betulaprenol-type polyprenol with an *E*,*E*-farnesyl residue at the ω-end of the prenyl chain, and the other is a ficaprenol-type polyprenol with an *E*,*E*,*E*-geranylgeranyl residue at the ω-end of the chain. The polyprenols contents of plants have been reported to show remarkable changes with age (Ibata, Mizuno, Takigawa, & Tanaka, 1983; Suga, Ohta, Nakai, & Munesada, 1989) and seasons (Swiezewska, Sasak, Mankowski, Jankowski, Vogtman, Krajewska, Hertel, Skoczylas, & Chojnacki, 1994; Jankowski, Kula-Swiezewska, Sasak, & Chojnacki, 1994), but the physiological significance and functions of polyprenols are not known. On the other hand, long chain dolichols have been reported to occur in animals, yeast and plants. These compounds have an *E*,*E*-farnesyl residue at the ω-end of their prenyl chains, but are different from other polyprenols in that the α-isoprene unit is saturated. Dolichol contents in animals and plants have been reported to increase with age (Ibata et al., 1983; Jankowski et al., 1994). The phosphorylated form, dolichyl phosphate, functions as a sugar-carrier lipid during the biosynthesis of *N*-linked glycoproteins and glycosylphosphatidyl inositol (GPI)-anchored proteins (Herscovics & Orlean, 1983). Ravi, Rip, & Carroll, 1986 described an increased formation of dolichyl phosphate during the germination in soybean (Ravi, Rip, & Carroll, 1986). They also described the occurrence of dolichols in various monocotyledon and dicotyledon seeds (Ravi, Rip, & Carroll, 1984). Jankowski et al., 1994 recently demonstrated the

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occurrence of trace amounts of dolichols in a number of angiosperm plants (Jankowski et al., 1994).

We developed a two-plate thin-layer chromatography method that effectively and efficiently separates mixtures of dolichols and polyprenols (Sagami, Kurisaki, Ogura, & Chojnacki, 1992). This method was used to show that dolichols and polyprenols occur in leaves of various dicotyledonous plants. In order to see whether dolichols also act as sugar-carrier lipids in glycoprotein biosynthesis in gymnosperms, it is important to establish whether gymnosperm plants also contain dolichols. For this purpose, we chose as one angiosperm the rubber plant (*Hevea brasiliensis*), which produces an abundance of rubber, and as one gymnosperm ginkgo (*Ginkgo biloba*), which is known to accumulate polyprenols in the leaves. Another gymnosperm, pine (*Pinus sylvestris*), was also studied. We report here the occurrence and tissue distribution of dolichols and polyprenols in these plants.

2. Results and discussion

Non-saponifiable crude lipids were prepared from several tissues and analyzed using the two-plate thin-layer chromatography method as previously described (Sagami et al., 1992). Fig. 1 shows two-plate thin-layer chromatograms of polyisoprenoid alcohols from seeds, flowers, young roots, young shoots, young leaves and old leaves of rubber plant. Dolichols with chain lengths of C_{70}-C_{105}, C_{85}-C_{105}, C_{75}-C_{105}, C_{75}-C_{100}, and C_{65}-C_{90} were detected as major polyisoprenoid alcohols in seeds (A), young roots (C), young shoots (D), young leaves (E), and old leaves (F), respectively. In the case of flowers (B), no dolichols were detected. Polyprenols with C_{80}-C_{100} and C_{75}-C_{90} lengths in trace amounts were also detected in seeds and young leaves, respectively. Larger amounts of polyprenols (C_{80}-C_{120}) were detected in flowers, as compared to other tissues. We could detect trace amounts of heveaprenols (C_{50}-

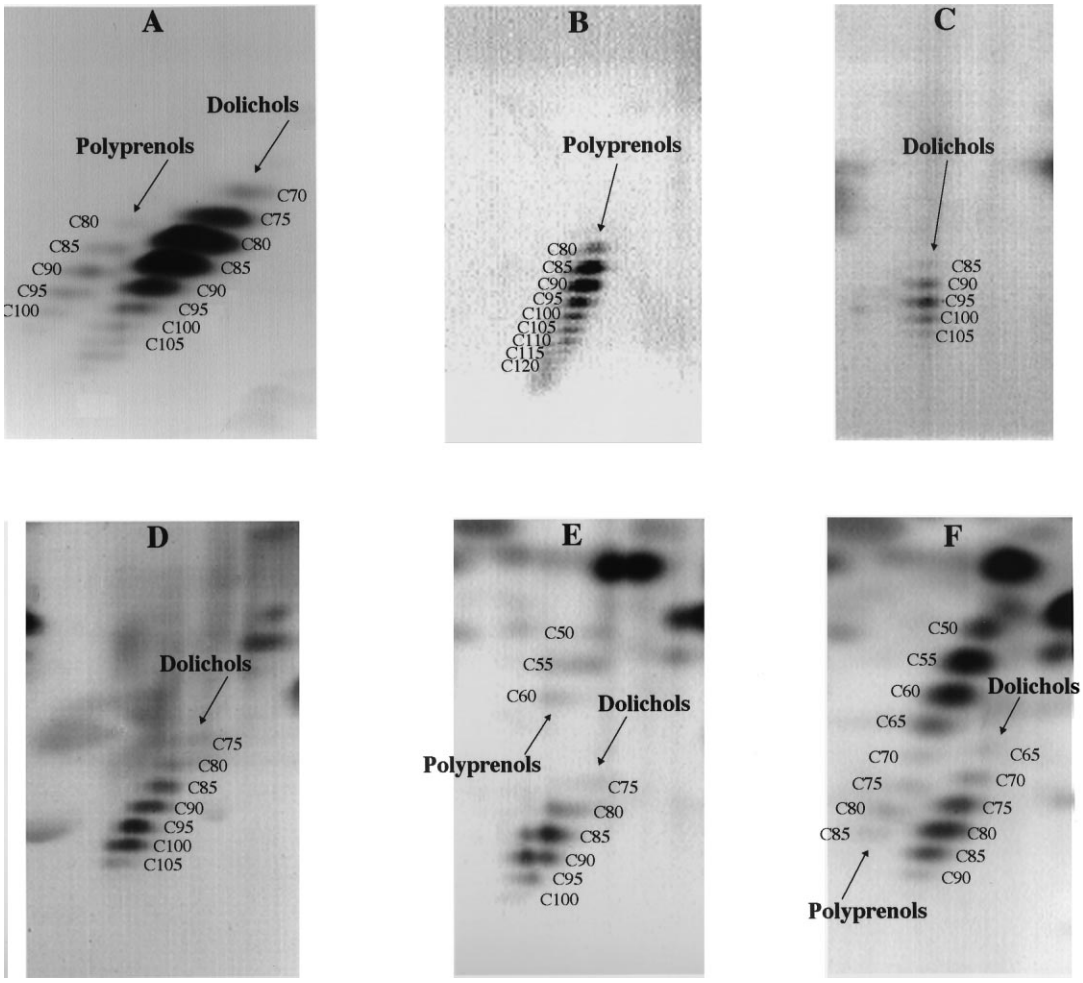

Fig. 1. Two-plate thin-layer chromatograms of polyisoprenoid alcohols from seeds (A), flowers (B), young roots (C), young shoots (D), young leaves (E) and old leaves (F) of rubber plant. The numbers refer to the carbon chain length of polyisoprenoid alcohols.

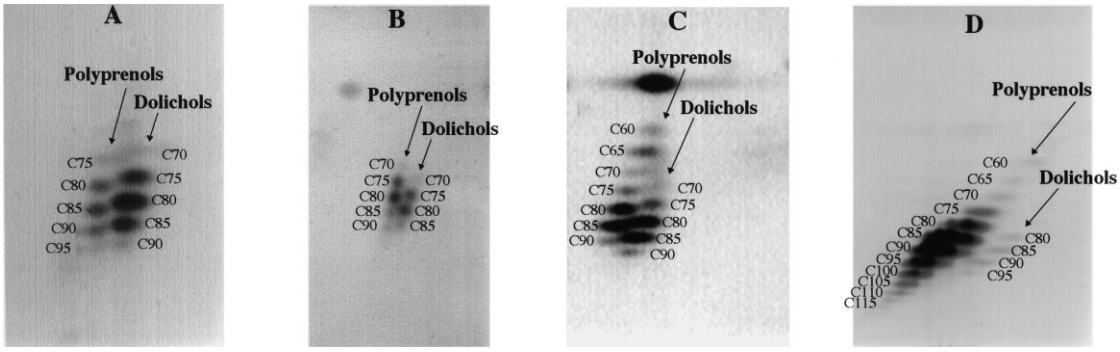

Fig. 2. Two-plate thin-layer chromatograms of polyisoprenoid alcohols from seeds (A), embryos (B), young leaves (C) and old leaves (D) of ginkgo. The numbers refer to the carbon chain length of polyisoprenoid alcohols.

C_{60}) in young leaves, the occurrence of which has been reported in rubber leaves by Dunphy, Kerr, Pennock, Whittle, Feeney, 1967. Assuming that heveaprenols may be accumulated with aging of leaves, we prepared crude lipids from old leaves and analyzed the polyisoprenoid alcohols. As shown in Fig. 1(F), heveaprenols were easily detected in larger amounts than those of dolichols. However, both young and old leaves showed somewhat similar occurrence of the dolichols. Fig. 2 shows two-plate thin-layer chromatograms of polyisoprenoid alcohols from seeds (A), embryos (B) and young leaves (C) of ginkgo. Dolichols (C_{70}-C_{90}, C_{70}-C_{85} and C_{70}-C_{90}) as well as polyprenols (C_{75}-C_{95}, C_{70}-C_{90} and C_{60}-C_{90}) were detected in all of these samples, respectively. Since Ibata et al., 1983 reported the age-dependent accumulation of polyprenols (C_{80}-C_{95}) in ginkgo leaves, we analyzed polyisoprenoid alcohols from old green leaves. As shown in Fig. 2(D), polyprenols (C_{80}-C_{95}) were in fact the major polyisoprenoid alcohols in the old leaves. Only trace amounts of dolichols (C_{80}-C_{95}) were detected in the old leaves as compared to their pronounced presence in the young leaves. In the case of pine seeds, dolichols (C_{70}-C_{90}) were detected together with the polyprenols (C_{70}-C_{90}). Trace amounts of C_{55}-C_{65} polyprenols were also detected (data not shown).

Table 1 summarizes the chain-length distribution of polyprenols and dolichols found in several tissues of rubber plant, ginkgo and pine. Dolichols were found in all seeds and tissues except for the flower of rubber plant. The distribution of chain lengths of dolichols varied from tissue to tissue. However, the dolichols examined seem to form distinct families in each tissue. On the other hand, polyprenols were also found in seeds and tissues in the rubber plant. These polyprenols occur as one or two polyprenol families, depending on the plants and tissues. Occurrence of two polyprenol families was observed in the rubber plant leaves, one with a dominating length of C_{55} and the other with a dominating length of C_{80}. Two polyprenol

Table 1
Chain lengths distribution of polyprenols and dolichols occurring in the rubber plant, ginkgo and pine*

	Polyprenols	Dolichols
Angiosperm		
Rubber plant (*Hevea brasiliensis*		
Seeds	80 85 90 95 100	70 75 **80** **85** 90 95 100 105
Flowers	80 **85** **90** 95 100 105 110 115 120	
Young roots		85 90 95 100 105
Young shoots		80 85 90 **95** **100** 105
Young leaves	50 55 60	75 80 **85** **90** **95** 100
Old leaves	50 **55** **60** 65 70 75 80 85	65 70 **75** **80** **85** 90
Gymnosperm		
Ginkgo (*Ginkgo biloba*)		
Seeds	75 **80** **85** **90** 95	70 **75** **80** **85** 90
Embryo	70 **75** **80** 85 90	70 **75** **80** 85
Young leaves	60 **65** 70 75 **80** **85** 90	70 75 **80** **85** 90
Old leaves	60 65 70 75 80 **85** **90** 95 100 105 110 115	80 85 90 95
Pine (*Pinus sylvestris*)		
Seeds	55 60 65 70 **75** **80** **85** 90	70 **75** **80** **85** 90

*The numbers refer to the carbon chain length of polyisoprenoid alcohols. The chain lengths of the major polyisoprenoid alcohols found in each tissue are indicated in bold

Table 2
Polyprenols and dolichols contents in the rubber plant, ginkgo and pine*

Source	(A) Crude lipids mg/10 g dry weight	(B) Polyisoprenoid alcohols	(C) Polyprenols μg/10 g weight	(D) Dolichols	(B/A)×100 %	(D/A)×100 %	(D/B)×100 %
Rubber plant							
Seeds	15	1340	140	1200	9	8.1	90
Flowers	110	9900	9900	ND	9	0	0
Young roots	10	75	ND	75	1	1.0	100
Young shoots	10	160	ND	160	2	2.0	100
Young leaves	24	740	110	630	3	2.6	85
Old leaves	140	7500	5400	2100	5	1.4	28
Ginkgo							
Seeds	22	1460	460	1000	7	4.7	68
Embryo	40	3800	1900	1900	10	5.0	50
Young leaves	36	490	280	210	1	0.43	43
Old leaves	280	45,055	45,000	55	16	0.016	0.10
Pine							
Seeds	18	800	390	410	4	2.0	51

*All non-polar lipids containing dolichols and polyprenols were obtained as described under "Experimental".
Contents of these polyisoprenoids were evaluated with NIH image.
ND, not detected.

families were also observed in ginkgo leaves (one with a dominating length of C_{65} and the other with a dominating length of C_{85}) and in pine seeds (one with a dominating length of C_{55} and the other with a dominating length of C_{80}). The longer chain polyprenol family was commonly found in rubber plant seeds and flowers as well as in ginkgo seeds and embryos. Therefore, it would be reasonable to assume that there may be at least three different biosynthetic pathways in these plants responsible for the formation of shorter-chain polyprenols, longer-chain polyprenols and dolichols.

In a previous report (Matsuoka, Sagami, Kurisaki, & Ogura, 1991), we described that the chain length distribution of polyprenyl enzymatic products by rat liver microsomes was dependent on the concentration of substrates. Not only increasing the concentration of isopentenyl diphosphate but also decreasing farnesyl diphosphate concentration led to an increase of longer chain products. In a recent report (Sagami, Kurisaki, & Ogura, 1993), we have also described that dolichols are directly synthesized from polyprenols without isoprenyl chain length extension. The dolichols are formed by the action of microsomal NADPH-dependent reductase from the pre-existing polyprenols. With these combined findings, we suggest that shorter-chain polyprenyl products are synthesized by the action of a *cis*-prenyltransferase, and longer-chain polyprenyl products are synthesized by a different *cis*-prenyltransferase specific for longer-chain length. And, we suggest that dolichols are synthesized from the preformed longer-chain polyprenols by the action of an NADPH-dependent reductase.

Table 2 summarizes the quantitative analysis of polyprenols and dolichols contents in different tissues of rubber plant, ginkgo and pine. Variations of the contents in term of aging were also indicated, especially in young versus old leaves. The highest polyprenol contents were observed in rubber plant flowers and old ginkgo leaves. Accumulation of polyprenols with aging was remarkable for both rubber plant leaves and ginkgo leaves with 49-fold and 160-fold increases, respectively. The dolichol contents were the highest in old leaves of the rubber plant and in ginkgo embryos. In contrast, the old leaves of ginkgo contained a very small amount of dolichols, though a very high content of polyprenols was observed. Polyprenols and dolichols in rubber plant leaves showed parallel increases with aging, while the ginkgo leaves were opposite in that dolichols decreased with aging.

Whereas the shorter-chain heveaprenols were increased about 49-fold in rubber plant leaves with aging, dolichols in the same tissues were only increased 3-fold as shown in Table 2. In ginkgo leaves, the polyprenols accumulated with aging are of the longer-chain types. These have been analyzed to be betulaprenol-type polyprenols with an *E*,*E*-farnesyl residue at the ω-end of their prenyl chains (Ibata et al., 1983). The longer-chain polyprenols in ginkgo leaves were about 160-fold increased with aging, which was very pronounced and remarkable. In contrast, the dolichols content in the same leaves was decreased to one-fourth

with aging (Table 2). The change in the dolichol content with aging in the case of the leaves of rubber plant was opposite to that in the case of ginkgo leaves. These observations suggest that the biosynthetic pathways of shorter-polyprenols, longer-polyprenols and dolichols are differently regulated in rubber plant and in ginkgo. Further experiments are necessary to clarify the reason why the flowers of rubber plant contain no dolichols. The other major question to be addressed is the basis of the difference between rubber plant and ginkgo leaves in the dolichol content changes with aging.

3. Experimental

3.1. Materials

Polyprenols (C_{90}-C_{100}) and dolichols (C_{75}-C_{85}) standard compounds were purchased from Sigma. Silica gel 60 TLC plates and reversed-phase silica gel HPTLC plates were obtained from Merck. The RP-18 Sep-Pak column was from Waters Associates, Inc. Young leaves of ginkgo (*Ginkgo biloba*) were collected in early April. Old leaves and seeds of ginkgo were collected around November–December. Embryos of ginkgo were obtained from the seeds that were collected in the previous year. Pine (*Pinus sylvestris*) seeds were purchased from a local market. Rubber plant (*Hevea brasiliensis*) tissues (leaves, seeds, shoots, roots and flowers) were obtained from Songkla Rubber Research Center (Kanchanavanich Road Hat-Yai, Songkla 90110, Thailand). The rubber seeds were collected around September–October and kept at −20°C. The young shoots and roots were collected during germinating season around November. Fresh samples were dipped in liquid N_2 for grinding into fine powder and products kept at −20°C. All other rubber plant tissues were likewise collected fresh, ground into fine powders and stored at −20°C until used. All other chemicals and solvents were of analytical or reagent grade.

3.2. Extraction of polyisoprenoid alcohols from plant tissues

Procedures were basically as previously described (Ibata et al., 1983; Sagami et al., 1992; Kurisaki, Sagami, & Ogura, 1997). Leaves, roots, shoots and flowers (10 g each, respectively) were dried for 1–2 days at 60°C. The coats of seeds were removed and discovered, then the seed tissues (10 g each) were dried for 1–2 days at 60°C. The dried tissues were crushed into small pieces or fine powders and immersed in 100 ml solvent of acetone:hexane (1:1) for one week. The lipid extracts of leaves and flowers were saponified at 70°C for 2 h in 20 ml of 50% methanol containing 2 M KOH and 0.5% pyrogallol. The lipid extracts of seeds, roots, shoots and embryos were saponified at 55°C for 3 h in 20 ml of 95% ethanol containing 15% (w/v) KOH and 0.5% pyrogallol. Non-saponifiable crude lipids of each tissue were extracted with hexane and the organic solvent was evaporated. Residues of each sample were dissolved in methanol and applied to an RP-18 Sep-Pak column equilibrated with methanol. The column was washed thoroughly with methanol and non-polar lipids containing polyisoprenoid alcohols were eluted with hexane.

3.3. Analysis by two-plate thin-layer chromatography

As previously described (Sagami et al., 1992), the first silica gel TLC and the second reversed phase C_{18} silica gel TLC were performed in the solvent systems, toluene:EtOAc (19:1) and acetone:methanol (19:1), respectively. Polyisoprenoid alcohols being separated and developed by two-plate silica-gel TLC were identified and visualized with iodine vapour. The developed chromatographic images were obtained and recorded with the EPSON GT-6500ART scanner. Quantitative analysis of the polyprenols and dolichols detected on TLC plates were evaluated using the NIH-Image 1.58 software (Youdo-sha, Co. Ltd.) with dolichols and polyprenols standards as references.

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