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ISOPENTENYL DIPHOSPHATE ISOMERASE AND PRENYLTRANSFERASE ACTIVITIES IN RUBIACEOUS AND APOCYNACEOUS CULTURES

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Key Word Index—*Cinchona robusta*; *Morinda citrifolia*; *Rubia tinctorum*; Rubiaceae; *Catharanthus roseus*; *Tabernaemontana divaricata*; Apocynaceae; anthraquinones; isopentenyl diphosphate isomerase; farnesyl diphosphate synthase.

Abstract—Isopentenyl diphosphate (IPP) isomerase (EC 5.3.3.2) and farnesyl diphosphate (FPP) synthase (EC 2.5.1.10) activities were studied in plant cell cultures of *Cinchona robusta*, *Morinda citrifolia*, *Rubia tinctorum*, all belonging to the Rubiaceae, and *Tabernaemontana divaricata* and *Catharanthus roseus*, both belonging to the Apocynaceae. The presence of isoforms of IPP isomerase was detected after hydroxyapatite chromatography and by Western blotting using anti-IPP isomerase antibodies. The Rubiaceae cultures are able to accumulate anthraquinones, and for their biosynthesis isoprene C_5-units are required. In a *M. citrifolia* cell line accumulating anthraquinones (AQ+), an almost two-fold higher IPP isomerase was observed compared with a cell line without accumulation of anthraquinones. In the Western blots three polypeptides were detected in the extracts from the AQ+ cells. In *C. robusta* cells the anthraquinone biosynthesis was induced by medium optimization and by elicitation. After both treatments, a specific isoform of IPP isomerase was induced. The *C. roseus* culture (not accumulating anthraquinones) expressed relatively low IPP isomerase activity, but two isoforms could be detected. The enzyme activities as observed in the different Rubiaceae cultures may indicate an efficient channelling of C_5-units into anthraquinone biosynthesis. © 1998 Published by Elsevier Science Ltd. All rights reserved

INTRODUCTION

Plant terpenoid biosynthesis results in the formation of a wide range of physiologically important and biologically active compounds. Some of them are universal, e.g. sterols, others are found only in a limited number of species, e.g. essential oils. Isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are central intermediates in this process; their isomerization is catalysed by the enzyme IPP isomerase (EC 5.3.3.2). The function and properties of this enzyme have been reviewed recently [1]. DMAPP is a reactive alkylating agent and is used for the formation of higher terpenoids, e.g. sterols, by chain elongation with IPP or it is used for the formation of meroterpenoid compounds as cytokinins and anthraquinones. The channelling of IPP and DMAPP (or C_5-units) could thus form an important instrument in the regulation mechanisms of the complex terpenoid biosynthesis. A major flux of C_5-units in plants into sterols and triterpenoids is necessary for cell membrane synthesis and functioning. A first intermediate in their biosynthesis is farnesyl diphosphate (FPP), formed from three C_5-units in a reaction catalysed by the enzyme FPP synthase (EC 2.5.1.10).

Anthraquinones are secondary metabolites, under certain conditions accumulating in relatively large amounts in plant cell cultures of some species in the Rubiaceae [2]. For the biosynthesis of these anthraquinones one C_5-unit is required [3]. In *Cinchona robusta* (Rubiaceae) cells, anthraquinone biosynthesis could be induced by addition of a homogenate of *Phytophthora cinnamomi* (*Pcin*) to the culture [4]. In such cells the activity of IPP isomerase is transiently induced, accompanied by a transient inhibition of FPP synthase activity. Recently, we reported on the purification of two isoforms of IPP isomerase from

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elicited *C. robusta* cells [5]. One isoform was specifically induced by elicitation; non-treated cells contained a low activity of this isoform.

To understand the possible role of IPP isomerase and FPP synthase in channelling C_5-units into primary and secondary metabolic pathways, the activities of these enzyme have been determined in cell cultures of *Morinda citrifolia*, *Rubia tinctorum* and *Cinchona robusta*, which accumulate anthraquinones, either constitutively or after induction. For comparison, cultures of *Catharanthus roseus* and *Tabernaemontana divaricata* (Apocynaceae) not capable of accumulating anthraquinones, were studied. IPP isomerases from the various sources were partially purified by hydroxyapatite chromatography.

RESULTS AND DISCUSSION

The activities of the enzymes IPP isomerase and FPP synthase, indicating the cellular biosynthetic capacity to form C_5 and C_{15} prenyl-units, were studied in several plant cell cultures. For *C. robusta*, the cells were cultured under three different conditions: 1) Standard culture conditions under which the accumulation of anthraquinones is suppressed by relatively high concentrations of 2,4-D, low concentrations of sucrose and light, 2) Cells growing on an induction medium developed to stimulate anthraquinone formation, and, 3) Cells, growing on standard medium, in which anthraquinone biosynthesis is induced by treatment with *Pcin*.

In cells grown under conditions not favouring anthraquinone biosynthesis, IPP isomerase and FPP synthase activities increased during the exponential phase of growth cycle, reaching a maximum at day 6 (Fig. 1A, Fig. 1B). When these were transferred to the induction medium, a slight reduction in biomass production was observed (Fig. 1C), while IPP isomerase activity increased almost 2-fold, compared with the maximum activity found in non-producing cells (Fig. 1D). Highest activity was found when the cells reached the stationary phase of the growth cycle. Anthraquinone accumulation gradually increased from day 4. FPP synthase activity was not affected by the transfer of the cells to induction medium. Analysis by radio-HPLC of the products formed by the prenyltransferase reactions from the two cell cultures showed that the radioactivity was recovered from geraniol and farnesol; the radioactivity recovered from the former was always less than the 7% of the total. No radioactivity was incorporated into geranylgeraniol. The major activity of prenyltransferases thus corresponds to farnesyl diphosphate synthase activity.

The induction medium was composed in such a way that factors inhibiting anthraquinone formation were omitted; 2,4-D is an important factor in this respect [6–8]. The standard medium contained a relatively high concentration of 2,4-D and a relatively low concentration of sucrose. Furthermore, the cells were grown in the light, another factor reducing anthraquinone formation [7]. Under these conditions the cells on standard medium are free of anthraquinones. By replacing the standard medium with induction medium, a gradual dilution of inhibitory factors will occur and the anthraquinone biosynthesis will thus be induced slowly. A rapid induction of anthraquinone biosynthesis was observed after addition of *Pcin* to a 4-day-old *C. robusta* culture [4]; within 72 hr after elicitation a maximum of 12 μmol anthraquinone/g DW was reached and the growth ceased (Fig. 1E). During the first 24 hr after elicitation, IPP isomerase was transiently induced with maximum activity at 12 hr after addition, followed by a period of reduced enzyme activity (Fig. 1F). Shortly after elicitation prenyltransferase activity was reduced to about 50% of the activity present before addition of the elicitor. This may have resulted in a shortage of sterols, causing an inhibition of culture growth. Both enzyme activities had recovered 72 hr after elicitation. As compared by TLC, identical anthraquinones were formed by the cells after elicitation and on induction medium.

Similar experiments were performed with two cell lines of *M. citrifolia*, one (AQ+) accumulating anthraquinones, the other (AQ−) devoid of anthraquinones. In cell line AQ+, a 1.3 fold higher IPP isomerase activity was observed as compared with AQ−, while the growth curves and FPP synthase activity showed similar profiles in both cultures (Fig. 2A–D). Anthraquinone accumulation started in the growth phase, the maximum in IPP isomerase activity preceded the maximum level of anthraquinone accumulation. In a *Rubia tinctorum* cell line, accumulating anthraquinones, both IPP isomerase and FPP synthase activities were relatively high (Fig. 2E,F). Similar high enzyme activities were observed in a *Tabernaemontana divaricata* culture, a culture not accumulating anthraquinones, but known for its capacity to produce triterpenoids (Fig. 3B). A culture of another Apocynaceous species, *Catharanthus roseus*, showed relatively low activities of both enzymes (Fig. 3A).

It has been indicated that IPP isomerase occurs in three subcellular compartments (reviewed in [1]). Isoforms of IPP isomerase, although of unknown origin, have been separated by hydroxyapatite chromatography [5, 10]. Partially purified extracts of the various plant cell cultures used in this study were subjected to hydroxyapatite chromatography and the elution profiles of IPP isomerase and FPP synthase activities were monitored. From extracts of elicitor-treated *C. robusta* cells, harvested 12 hr after *Pcin* addition, IPP isomerase activity eluted in two peaks of relatively high activity (Fig. 4A), while only little prenyltransferase activity was detected. From untreated cells of the same age, only one IPP isomerase isoform (Fig. 4A), but high FPP synthase, was obtained (not shown). Apparently, in *C. robusta* an additional isoform of IPP isomerase was induced after elicitation, which may be related to the induction of

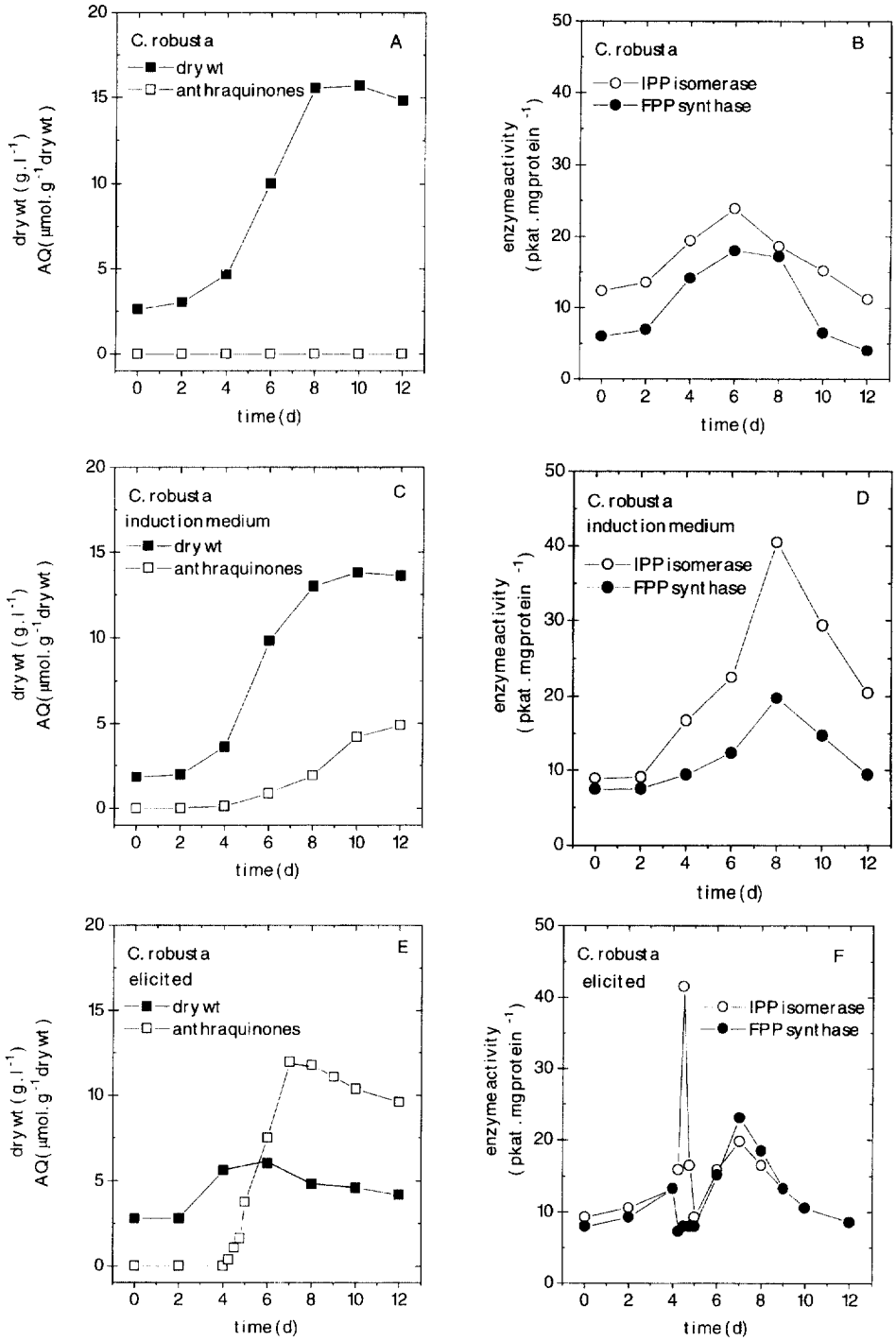

Fig. 1. Accumulation of biomass and anthraquinones, and IPP isomerase and FPP synthase activities in *Cinchona robusta* cells, cultured under standard conditions (A and B), after transfer to an induction medium for anthraquinone biosynthesis (C and D), and after addition of a *Phytophthora cinnamomi* elicitor preparation (E and F).

the anthraquinone biosynthesis. Simultaneously, after elicitation FPP synthase was inhibited, reducing the number of isoprene units channelled into sterol biosynthesis. Similarly, from *C. robusta* cells on the anthraquinone induction medium, two forms of IPP isomerase were separated (not shown).

Quite different hydroxyapatite-chromatographic profiles were obtained from the *M. citrifolia* AQ− and AQ+ cell lines, IPP isomerase activity eluted in three fractions (Fig. 4C,D). The total IPP isomerase activity of the cell line AQ+ was higher than in AQ−, largely due to the increase in one isoform eluting at

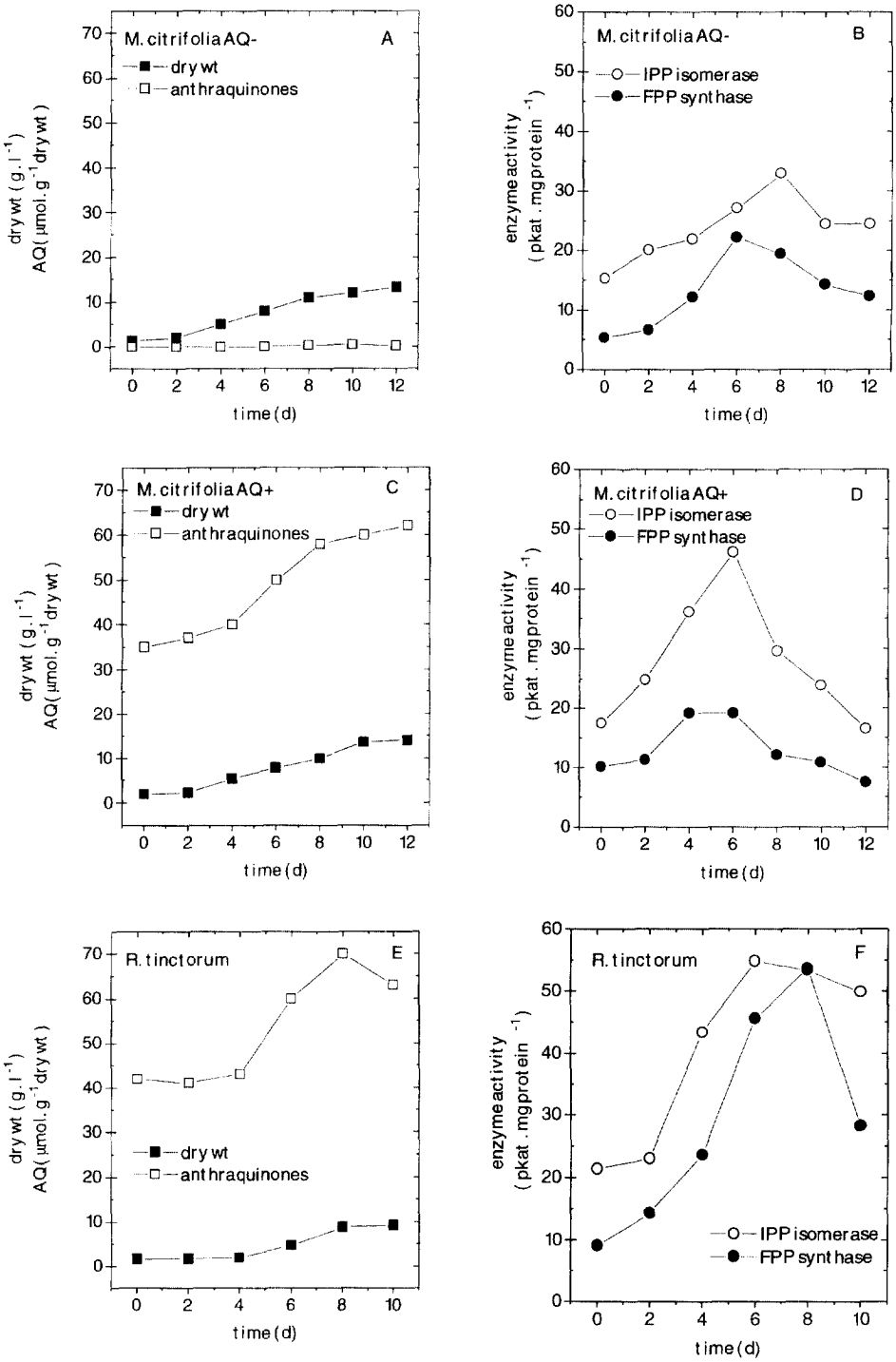

Fig. 2. Accumulation of biomass and anthraquinones, and IPP isomerase and FPP synthase activities in *Morinda citrifolia* (AQ−) cells (not accumulating anthraquinones, A and B), in *Morinda citrifolia* (AQ+) cells (accumulating anthraquinones, C and D), and in *Rubia tinctorum* cells (E and F).

high phosphate concentrations (83 mM). In the *R. tinctorum* culture, with high production of anthraquinones, two forms of IPP isomerase activity were found (Fig. 4B), accompanied by relatively low FPP synthase activity (not shown).

The *C. roseus* and the *T. divaricata* cultures produced small amounts of terpenoid indole alkaloids. It is expected, as the cultures were both free of chlorophyll, that IPP isomerase is mainly involved in sterol and triterpenoid formation. By hydroxyapatite chro-

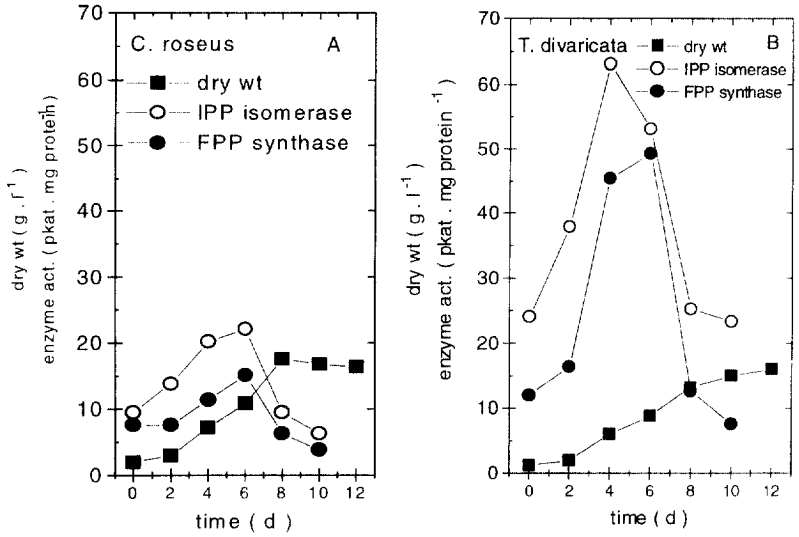

Fig. 3. Accumulation of biomass and IPP isomerase and FPP synthase activities in cell suspension cultures of *Catharanthus roseus* (A) and *Tabernaemontana divaricata* (B).

matography, FPP synthase from *T. divaricata* and *C. roseus* were not separated from the single form of IPP isomerase present (not shown).

These results indicate that in the case of anthraquinone accumulation, IPP isomerase activity is relatively higher. For *C. robusta* and *M. citrifolia* this is largely due to a specific induction of one of the isoforms, as shown by the hydroxyapatite-chromatographic profiles.

Purified polyclonal antibodies raised against IPP isomerase isolated from *Capsicum annuum* chloroplasts [5] were used to further evaluate the presence of IPP isomerase isoforms. By Western blot analysis after native PAGE and immunodetection, in the extracts from induced and elicited *C. robusta* cells two proteins were detected, while in the cells grown on standard medium one form was clearly detected, the second protein was present in very low concentrations (Fig. 5A). In the immmunoblots of the *M. citrifolia* AQ+ extract, at least 3 proteins were recognized by the antibodies, one protein showing a relatively high mobility. Noteworthy are the differences observed for *T. divaricata* and *C. roseus*, the latter expressing low IPP isomerase activity but two clearly detected isoforms. For *T. divaricata* the opposite was observed. The strong correlation between the number of isoforms obtained by hydroxyapatite chromatography and as detected by immunoblotting in crude extracts, indicate that the different forms are not chromatographic artifacts. After SDS-PAGE and immunodetection, polypeptides of similar sizes between 30 and 34 KDa were detected, which appear not to be well separated and rather broad from anthraquinone acccumulating cell cultures (Fig. 5B). For the IPP isomerase from flower-plastids of *Clarkia breweri*, which gene has been cloned, a M_r of 32 970 was calculated [11]. Furthermore we observed that the total amount of protein extracted from the Apocynaceae was about 2 times higher than extracted from the Rubiaceous cultures.

These experiments indicate that IPP isomerase and FPP synthase activities are modified when there is an urgent need for substrates, e.g. for the accumulation of anthraquinones after induction or elicitation. These changes in activity may result in channeling C_5-units into different metabolic pathways. The exact regulatory roles of these enzymes can only be understood when the subcellular localisation of the enzymes and the biosynthesis of IPP is known. Also the site of biosynthesis of anthraquinones is unknown, but cells accumulating large amounts of anthraquinones showed highly elongated ER membranes and irregular or distorted plastids [12]. An inducible isoform of IPP isomerase might play a role in anthraquinone biosynthesis.

EXPERIMENTAL

Plant material and culture methods

Cell cultures of *C. robusta*, initiated from stem explants of *C. robusta* How. in 1985, were maintained on B5 medium [13] containing 50 mg/l L-cysteine, 0.2 mg/l kinetin, 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 20 g/l sucrose (standard medium). Under these conditions the cells do not accumulate anthraquinones. For induction of anthraquinone biosynthesis 7-day-old cells were harvested by filtration and suction. Subsequently 5 g fr. wt was inoculated in 50 ml induction medium (B5 medium 1 : 4 diluted, supplemented with 0.1 mg/l naphthyleneacetic acid (NAA), 10 mg/l 6-benzylaminopurine (BAP), 40 g/l sucrose and 500 mg/l casein hydrolysate).

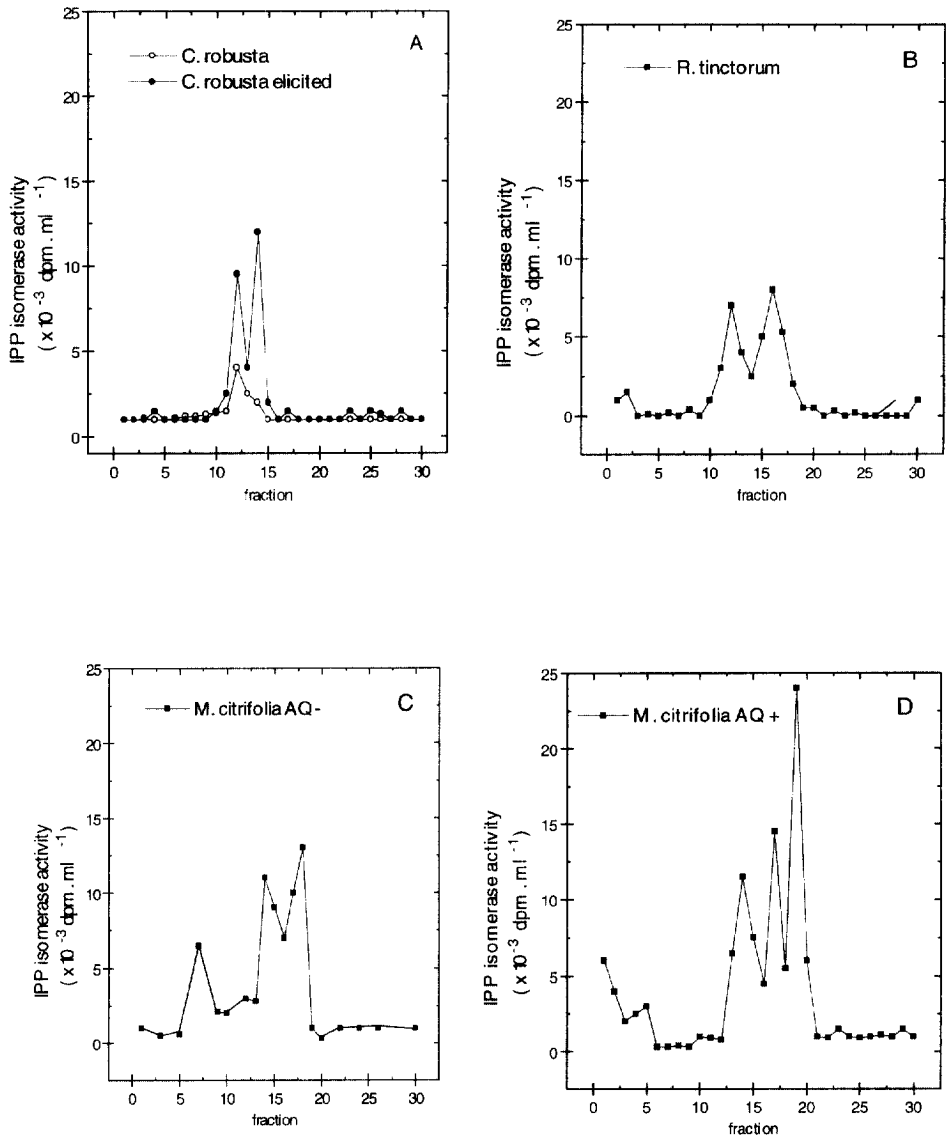

Fig. 4. Separation of IPP isomerase activities by hydroxyapatite chromatography from extracts of elicited and non-treated cells of *Cinchona robusta* (A), of *Rubia tinctorum* cells (B), of *Morinda citrifolia* cells, not accumlating (AQ−) (C) and accumulating anthraquinones (AQ+) (D).

Anthraquinone accumulation was also induced by elicitation with a *Phythophthora cinnamomi* (*Pcin*) preparation [4]. *Pcin* was added to 4-day-old *C. robusta* cell cultures, growing on standard medium. 40 mg of *Pcin* was suspended in 2 ml of H_2O, and after autoclaving, added to 50 ml suspension culture. Control (untreated cells) received 2 ml of sterile H_2O.

The *Morinda citrifolia* culture, accumulating anthraquinones (AQ+), was grown on a B5 medium [13] containing 1.86 mg/l NAA and 20 g/l sucrose. The non-anthraquinone-accumulating *M. citrifolia* cell line (AQ−) was grown in a B5 medium [13] containing 0.2 mg/l kinetin, 1 mg/l 2,4-D and 40 g/l sucrose. *Rubia tinctorum* cells were grown in B5 medium [13] supplemented with 2 mg/l 2,4-D, 0.2 mg/l kinetin, 0.5 mg/l NAA, 0.5 mg/l indole-3-acetic acid (IAA) and 20 g/l sucrose. The *Tabernaemontana divaricata* (L) R. Br. ex Roem et Schult. suspension culture (cell line 6 divBW13 [14]) was subcultured in an MS medium [15] containing 1 mg/ml kinetin, 1 mg/l 2,4-D and 30 g/l sucrose. The cell culture of *Catharanthus roseus* (L.) G. Don. was grown in LS medium [16] which contained 2 mg/l NAA, 0.2 mg/l kinetin and 30 g/l sucrose. All cell cultures were subcultured weekly by a 4 fold dilution, except *T. divaricata* which was subcultured every 14 days. The cultures were grown in the light (24 hr, 400–500 lux) at 25° on gyratory shakers at 120 rpm.

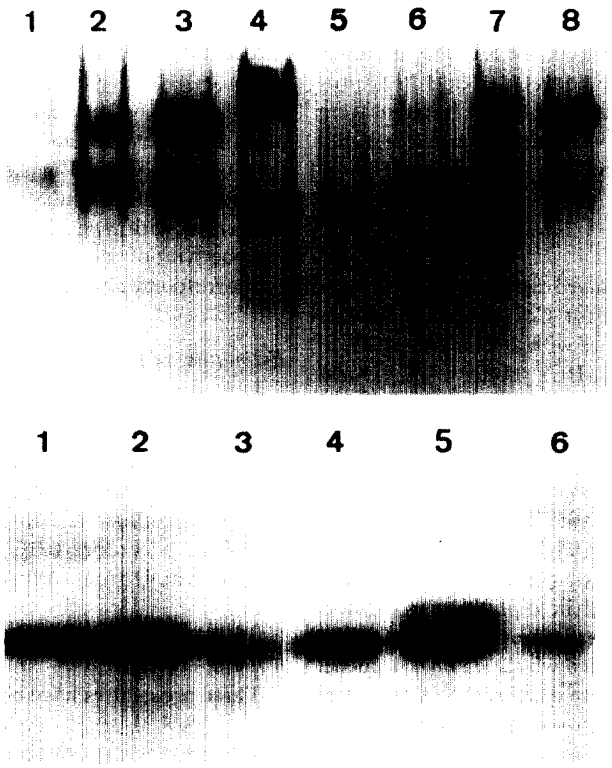

Fig. 5. Western blot of desalted protein extracts (25 μg protein per lane). After native PAGE proteins were transferred to a nitrocellulose membrane and subsequently incubated with anti-IPP isomerase antibodies. Detection via alkaline phosphatases coupled to secondary antibodies. 1. *T. divaricata* (6-day-old culture), 2 *R. tinctorum* (6-day-old culture), 3 *C. roseus* (6-day-old culture), 4 *M. citrifolia* AQ+ (8-day-old culture), 5 *M. citrifolia* AQ − (8-day-old culture), 6 *C. robusta* standard medium (4-day-old culture), 7 *C. robusta* induction medium (8-day-old culture), 8 *C. robusta* (4-day-old culture), *Pcin* treated for 12 hr (A, top). After SDS-PAGE. Lane 1. *C. robusta* (induction medium, 8-day-old culture), 2 *C. robusta* (*Pcin* treated for 12 hr, 4-day-old culture), 3 *C. robusta* (standard medium, 4-day-old culture), 4 *Rubia tinctorum* (6-day-old culture), 5 *M. citrifolia* AQ+ (6-day-old culture), 6 *T. divaricata* (6-day-old culture) (B, bottom).

Time course experiments

14 conical flasks of 250 ml, containing 50 ml of medium, were inoculated with an accurately weighed amount of cells (5 g fr. wt). 7-day-old cells (for *T. divaricata* 10-day-old cells) were used for inoculation. For each sampling point, cells of each culture were harvested (duplicated flask) and individually analysed except for the samples of the inoculation time (t = 0) and 2 days when 2 flasks were combined for one sample. Of each sample 5 g fr. wt. was used for the preparation of the protein extracts, the remainder was lyophilized. Culture growth and anthraquinone contents were determinated from aliquots of dried cells.

Crude protein extracts were prepared from homogenized (Waring blender) liquid N_2 frozen cells. Biomasses were thawed in the presence of: 0.15 g of polyvinylpolypyrrolidone (PVPP) and 1 ml extraction buffer (100 mM Tris-HCl pH 7.5, 2 mM EDTA, 2 mM dithiothreitol (DTT), 5% glycerol (= buffer A), 10 μM leupeptin) per g fr. wt. The homogenate was squeezed through Miracloth and centrifuged at 10 000 g for 30 min.

Chromatography of enzymes

3 250 ml flasks of each cell culture, inoculated as described above, were harvested 7 days after inoculation. 40 g fr. wt were used for protein extraction, as described above. Fractionations were performed by anion-exchange chromatography in a batchwise procedure at 4°. After the protein extraction, 30 ml of the supernatant were stirred slowly for 30 min with 10 ml of Q-Sepharose, which was first equilibrated with buffer A. The slurry was transferred to the column (3 × 5 cm) and after suction washed with 30 ml of buffer A. Proteins were eluted with 30 ml of buffer A containing 50 mM and 150 mM NaCl, respectively. The 150 mM NaCl fraction, containing IPP isomerase and FPP synthase activity, was concd to 7 ml by means of ultrafiltration (10 kDa membrane) and was subsequently desalted using PD-10 columns (Pharmacia). 2 ml of the concentrate was applied to hydroxyapatite-chromatography (Merck 75–5 column) using an FPLC system. The column was equilibrated with buffer A containing 5 mM KH_2PO_4. After injection, the column was washed with 9 ml of the equilibration

buffer. Bound proteins were eluted with a linear 19 ml K-Pi gradient from 0.005 to 0.2 M KH_2PO_4 in buffer A; fractions of 1 ml were collected. The FPLC procedures were performed at room temp., fractions were immediately stored at 4°. Active fractions were stored at −80° in the presence of 20% (v/v) glycerol.

Enzyme assays

IPP isomerase was assayed using the acid lability procedure [17] optimized for *C. robusta* cell cultures [4]. The incubation mixture with a total vol. of 200 μl contained 100 mM Tris-HCl buffer 7.5, 18 μM (10 μCi/μmol) ^{14}C-IPP, 1.5 mM $MnCl_2$, 1.5 mM $MgCl_2$, 2 mM DTT and 25 mM KF. The incubation was initiated by addition of 10 μl (10–20 μg protein) of crude extract enzyme preparation. After incubation for 10 min at 30° the enzyme reaction was stopped by addition of 200 μl MeOH-10 M HCl (4:1). The incubation was subsequently continued for 10 min at 37° for hydrolysing the allylic diphosphates formed. The incubation mixture was saturated with NaCl. The allylic prenols were extracted with 2 × 1 ml of toluene. The combined extracts were dried with Na_2SO_4. 1 ml of the toluene layer was removed and mixed with 10 ml of Opti-Fluor and the radioactivity was determinated by a liquid scintillation counter. After protein chromatography, the assay procedure was slightly modified by reducing the total vol. to 100 μl and the substrate conc. to 9 μM [^{14}C]-IPP (52 Ci/mol). The fractions obtained by HA chromatography were similarily assayed, except that KF was omitted from the incubation mixture. The reaction was stopped by addition of 200 μl MeOH-HCl (4:1). After addition of 100 μl of H_2O the mixture was subsequently incubated for 10 min. The prenols were extracted using 400 μl of $CHCl_3$. Of the organic layer 0.3 ml was mixed with 10 ml of scintillator (Emulsifier, Packard) and the radioactivity was determined (using quench correction) as described above.

Total prenyltransferase activity was assayed using a similar procedure as described above for IPP isomerase except that 100 μM DMAPP or 40 μM geranyl diphosphate (GPP) was added to the reaction mixture as a second substrate [4]. Protein concns were estimated according to ref.[18].

Analysis of the enzyme reaction products

After the IPP isomerase or prenyltransferase incubations, the diphosphates in the incubation mixture were hydrolysed by addition of 100 μl of 200 mM Tris-HCl pH 9.5 and 10 μl (14U) of alkaline phosphatase suspension. Incubation was continued for 3 hr at 37°. The mixture was extracted with 2 × 200 μl of hexane and centrifuged for 5 min at 10 000 *g* to separate the H_2O and the organic layer. Extracts (100 μl) were analysed by HPLC on a Hypersil column (particle size 5 μm, 250 × 4.6 mm, Shandon) using a solvent system consisting of 1.5% *n*-BuOH in hexane at a flow rate of 0.5 ml/min [19]. The radioactive compounds were detected by a Packard FLO-ONE/B radioisotope detector). The products were identified by retention times of unlabelled standards of geraniol, dimethyl allylalcohol, isopentenol and farnesol which were detected at 210 nm. For simultaneous UV and radiodetection the incubation mixtures were supplemented with 8 μg of each unlabelled prenol. The radioactivity of each compound was calculated from the corresponding peak area.

Estimation of anthraquinone contents

Cells (0.2 g dry wt) were extracted with boiling EtOH (80%) until the cells were colorless. The extracts were pooled and diluted to an appropriate vol. Spectra of the extracts were recorded in the range of 650 to 350 nm. Quantification of the anthraquinone content was done according to [2], using the maximum *A* measured at 410 nm, with rubiadin as standard.

Electrophoresis and immunodetection

These procedures have been described before [4].

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REFERENCES

1. Ramos-Valdivia, A. C., van der Heijden, R. and Verpoorte, R., *Natural Products Reports*, 1997, **14**, 591.
2. Schulte, U., El Shagi, H. and Zenk, M. H., *Plant Cell Reports*, 1984, **3**, 51.
3. Wijnsma, R. and Verpoorte, R., *Progress in the Chemistry of Organic Natural Products*, 1986, **49**, 79.
4. Ramos-Valdivia, A. C., van der Heijden, R. and Verpoorte, R., *Planta*, 1997, **203**, 155.
5. Ramos-Valdivia, A. C., van der Heijden, R., Verpoorte, R. and Camara, B., *European Journal of Biochemistry*, 1997, **249**, 161.
6. Zenk, M. H., El-Shagi, H. and Schulte, U., *Planta Medica*, 1975, **28**(Suppl), 79.
7. Harkes, P. A. A., Krijbolder, L., Wijnsma, R., Nsegiyaremge, T. and Verpoorte, R., *Plant Cell Tissue Organ Culture*, 1985, **4**, 199.
8. Robins, R. J., Payne, J. and Rhodes, M. J. C., *Phytochemistry*, 1986, **25**, 2327.
9. Van der Heijden, R., Verheij, E. R., Schripsema, J., Baerheim Svendsen, A., Verpoorte, R. and

Harkes, P. A., *Plant Cell Reports*, 1988, **7**, 51.
10. Ogura, K., Nishino, T. and Koyama, T., Seto, S., *Phytochemistry*, 1971, **10**, 779.
11. Blanc, V. M. and Pichersky, E., *Plant Physiology*, 1995, **108**, 855.
12. Yamamoto, H., Tabata, M. and Leistner, E., *Plant Cell Reports*, 1987, **6**, 187.
13. Gamborg, O. L., Miller, R. A. and Ojima, K., *Experimental Cell Research*, 1968, **50**, 151.
14. Van der Heijden, R., Wijsma, R., Verpoorte, R., Harkes, P. A. A. and Baerheim Svendsen, A., *Fitoterapia*, 1986, **62**, 415.
15. Murashige, T. and Skoog, F., *Physiologia Plantarum*, 1962, **15**, 473.
16. Linsmaier, E. M. and Skoog, F., *Physiologia Plantarum*, 1965, **18**, 100.
17. Satterwhite, D. M., *Methods in Enzymology*, **110**, 92.
18. Peterson, G. L., *Analytical Biochemestry*, 1977, **83**, 346.
19. Suga, T. and Endo, T., *Phytochemistry*, 1991, **30**, 1757.