Studies on Sporopollenin Biosynthesis in *Cucurbita maxima* I: The Substantial Labeling of Sporopollenin from *Cucurbita maxima* after Application of [^{14}C]Phenylalanine

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The results of tracer experiments performed with anthers of *Tulipa* cv. Apeldoorn have already shown that a high level of incorporation into the sporopollenin fraction was achieved, when [^{14}C]phenylalanine was applicated as a precursor. In order to investigate whether the substantial incorporation of [^{14}C]phenylalanine is a unique phenomenon restricted to *Tulipa*, tracer experiments were carried out on anthers of *Cucurbita maxima*.

The sporopollenin fraction was isolated and purified by a gentle method including extractions with various solvents, incubations with hydrolysing enzymes and fractionated saponifications. The remaining, as well as the released radioactivity, was determined after each purification step. After the application of [U-^{14}C]phenylalanine, a substantial incorporation into the sporopollenin fraction was determined. The values were clearly higher than those obtained with [1-^{14}C]glucose or those from corresponding experiments on *Tulipa* anthers.

After potash fusion of sporopollenin fractions labeled *via* [U-^{14}C]phenylalanine, *p*-hydroxybenzoic acid was shown to be the main component among the ether soluble acids; moreover it showed the highest level of radioactivity.

No radioactivity was detected in the degradation products oxalic acid, benzoic acid, phthalic acid and terephthalic acid or octanedioic acid- and decanedioic acid-dimethylester.

Introduction

To clarify the biosynthetic pathways leading to sporopollenin, a very resistant biopolymer of the pollen, tracer experiments are of fundamental significance. The first tracer experiments performed on sporopollenin of higher plants were carried out by Shaw [1] and Green [2]. The results of these experiments are difficult to interpret, due to the applied technique. Reliable results were obtained after an improved application technique with significantly reduced transport ways and incubation times was developed [3]. The results of tracer experiments performed with anthers of *Tulipa* cv. Apeldoorn have shown that a higher level of incorporation into the sporopollenin fraction was achieved when [^{14}C]phenylalanine was used as compared with precursors such as [^{14}C]glucose, -acetate, -malonate, -mevalonate, -tyrosine and -*p*-coumaric acid [4]. In the case of phenylalanine the substantial incorporation of radioactivity was independent of the position of labeling and not restricted to a specific stage of anther development [5]. Therefore it was assumed that the phenylpropane metabolism is involved in the sporopollenin biosynthesis.

In order to clarify the question whether the results received for *Tulipa* sporopollenin are unique or valid also for other systems, further tracer experiments were carried out with anthers of *Cucurbita maxima*.

Materials and Methods

Plant material

Experiments were carried out on anthers of *Cucurbita maxima* cultivated in a greenhouse of the Botanical Garden, Münster.

Chemicals

L-[U-^{14}C]phenylalanine (U-Phe) and D-[1-^{14}C]-glucose (1-Glc) were purchased from NEN research products, Dreieich, F.R.G.

The enzymes used for the enrichment of sporopollenin and their sources were described previously [4, 5].

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Application of radiolabeled substances

Androecia of *Cucurbita maxima* were prepared from flower buds directly before incubation and washed three times with 50 ml 0.01 mol/l K-phosphate buffer, pH 7.5. The application of radioactive precursors and trapping of the CO_2 released were carried out as described [3].

For the experiments P 1 and G 1 androecia from 2.5 to 8 mm length were incubated in a medium with the radioactive precursor. In case of the experiments P2, P3, G2 and P4 androecia from 4.5 up to 6 mm length were used (P: L-[U-^{14}C]-phenylalanine, G: D-[1-^{14}C]glucose).

Total and specific radioactivity applied for each experiment were: U-Phe: 341 (P1), 338 (P2), 328 (P3), 1055 (P4) kBq; 1-Glc: 372 (G1), 377 (G2) kBq; U-Phe: 68.3 (P1), 67.7 (P2), 65.8 (P3), 209.7 (P4) kBq/μmol; 1-Glc: 72.2 (G1), 73,0 (G2) KBq/μmol.

Isolation of the exine fraction

After 24 h of incubation, the anthers were washed three times as shown above. The anther wall fraction and loculus material (= pollen/tapetum fraction, for definition see [4]) were gained as follows: Thin cross sections were cut from each androecium with a razor blade and placed on a supporting net over a funnel. Loculus material was washed out of these sections into a centrifugation tube by means of a sharp jet of buffer. The process was controlled microscopically. The material was collected by centrifugations. In case of the experiments P 1 and G 1 the loculus material of the younger anthers (2.5 to 4 mm = P1T, G1T) was separated from that of the older anthers (5–8 mm = P1P, G1P). The younger anthers showed developmental stages between meiosis and pollen tetrads (T), the older anthers showed microspores and developing pollen (P). The material remaining on the net represents the anther wall fraction.

Purification steps

The pollen/tapetum fraction was homogenized for 90 min in methanol using a magnetic stirrer and the sporopollenin was enriched by subsequent extractions with solvents followed by enzymatic and alkaline hydrolyses. The radioactivity of the loculus material was determined both after the incubation and after each purification step, that of the anther wall fraction was measured after the incubation. Measurements of soluble and insoluble radioactive substances were done according to [3]. A quenching correction was carried out, using ESR techniques.

Extractions were done as before [5]. The same solvent was used until the radioactivity of the supernatant corresponded to the radioactive background level of an unlabeled control.

Incubations with enzymes were repeated until no release of radioactivity could be detected in the supernatants. The enzymes were added in the following order: pronase, cellulase/pectinase, amylase Type VI a/amyloglucosidase, lipase and esterase. Amylase (240 min/incubation), lipase (240 min/incubation) and pronase (35 min/incubation) were applied according to [5]. As determination of sugars was not intended, the pollen/tapetum fraction was incubated directly with the supernatant of the cellulase/pectinase suspension described in [5]. Alkaline hydrolyses, potash fusions and determination of radioactivity of ether soluble acids were performed as before [4, 5].

Results

Uptake of labeled substances into the anther and loculus material of Cucurbita

After 24 h 30 to 40% of the applied radioactivity was detected in the anthers (= loculus material and anther wall fraction) of *Cucurbita*. During the harvest of the loculus material out of cross sections with a jet of buffer a certain amount of labeled substances was already dissolved. Only between 0.1% (after incubating young anthers with [U-^{14}C]phenylalanine) and 2.5% of the applied radioactivity (after incubation with [1-^{14}C]glucose) was detected in the loculus material (see Fig. 1). Radioactivity was always more readyly incorporated into the anther wall fraction than into the loculus material, which is in accordance with results of experiments using *Tulipa* anthers. After application of [1-^{14}C]glucose, a substantially higher amount of the applied radioactivity was found in CO_2 (33.7%) than after application of phenylalanine (3.4%).

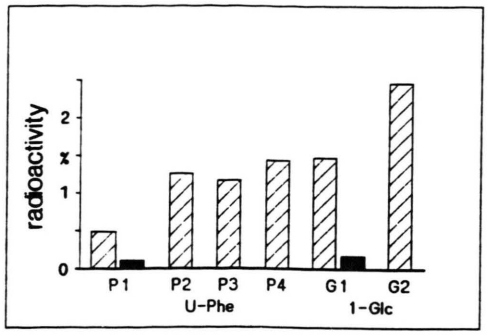

Fig. 1. Radioactivity (% of the originally applicated radioactivity) determined in the loculus material after incubation with [U-^{14}C]phenylalanine (U-Phe) or with [1-^{14}C]glucose (1-GLC). The amount of radioactivity of the loculus material obtained from the younger anthers (length of the anthers: 2.5–4 mm) in case of the experiments P 1 and G 1 is presented by black columns. For abbreviations see Material and Methods.

Localization of the radioactivity during the purification of the sporopollenin fractions

Treatment with solvents

In particular through extractions using methanol a high portion of the labeled substances was removed. Table I shows the radioactivity of the loculus material after the complete extraction procedure. The radioactivity before and after the extraction of the loculus material is compared (labeling after incubation = 100%); also the incorporation (pmol/mg) into the extracted loculus material is given.

Treatment with hydrolytic enzymes

The strongest effect was reached through incubations with protease (this was the first enzyme applied in the sequence), and by the following applications of cellulase/pectinase: After incubation of anthers labeled *via* [U-^{14}C]phenylalanine more than 90% of the radioactivity removed by treatment with enzymes was found in the supernatants of protease, compared to less than 5% after applications of cellulase/pectinase. Cellulase hydrolysed more than 20% of the labeled substances when loculus material had been labeled *via* [1-^{14}C]glucose. Only little effect was observed when amylase/amyloglucosidase and lipase were used, esterase showed no effect at all. The incorporations that were measured after extractions with solvents and after enzymatic hydrolyses of the loculus material are compared in Fig. 2.

Alkaline hydrolyses

Due to alkaline hydrolyses the radioactivity of the loculus material was reduced to 40–50% of the radioactivity determined after enzymatic hydrolyses. This was independent of the applied precursor or developmental stage of the anthers. The material obtained after alkaline hydrolyses represents the sporopollenin fraction. The incorporation data given in Table II demonstrate that a good incorporation of phenylalanine is achieved compared to glucose in case of *Cucurbita,* too. The incorporation was significantly higher than that measured after corresponding experiments with *Tulipa* anthers (at the best 162 pmol/mg [5]). The identification of radioactive products released in course of the alkaline hydrolyses was not achieved, but several unlabeled substances could be identified in the fraction of the ether soluble acids. *p*-Hydroxybenzoic acid, caffeic acid, ferulic acid, vanillic acid and vanillin were demonstrated by means of HPLC. Parallel to the related investiga-

Table I. Radioactivity (% of the radioactivity present before solvent extraction), specific radioactivity (dpm/mg dry weight) and incorporation (pmol/mg dry weight) after the extraction procedure. (P 1–P 4: [U-^{14}C]phenylalanine, G 1, G 2 [1–^{14}C]glucose applied. P 1 T, G 1 T: loculus material from younger anthers showing developmental stages up to tetrades; P 1 P, G 1 P: loculus material from older anthers showing microspores and pollen, see also Materials and Methods.)

Experiments →	P1P	P1T	P2	P3	P4	G1P	G1T	G2
Radioactivity of the loculus-material: % of radioactivity	81.3%	66.0%	79.2%	76.8%	78.0%	44.9%	36.5%	55.3%
Specific radioactivity [dpm/mg]	9233	–	8828	8915	28776	14988	–	14261
Incorporation [pmol/mg]	2254	–	2172	2259	2288	3461	–	3254

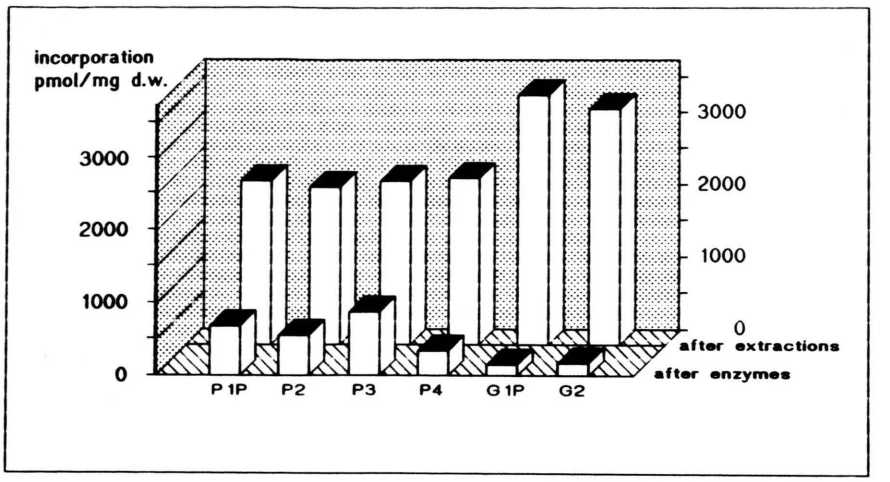

Fig. 2. Incorporation of labeled U-Phe and 1-Glc into the loculus material determined after the partial purification of this material by extractions with solvents and by treatments with hydrolyzing enzymes.

tions on *Tulipa* anthers ferulic acid was partly identified as its methylester.

Potash fusion of the sporopollenin fraction and analysis of radioactive degradation products

The sporopollenin fractions of the experiments P1P, P2, P3, P4, G1P and G2 were submitted to potash fusion at 265 °C (see Table III). Because of its high specific radioactivity (dpm/mg) the sporopollenin fraction of experiment P4 was especially useful for the identification of labeled degradation products. Up to 78% of the sporopollenin fraction (dry weight) were degraded by the potash fusion (see Table III). After the degradation procedure the highest amount of radioactivity was found in the fraction of the ether soluble acids, only very low values were measured in the acidified hexane extracts. Using TLC, several fluorescing substances were demonstrated in the ether soluble

Table II. Incorporation (pmol/mg dry weight) of labeled U-Phe and 1-Glc into the loculus material after the treatment by enzymatic as well as alkaline hydrolyses (after alkaline hydrolysis = sporopollenin fraction). (P1–P4: [U-^{14}C]phenylalanine, G1, G2 [1-^{14}C]glucose applied. P1T, G1T loculus material from younger anthers showing developmental stages up to tetrades, P1P, G1P loculus material from older anthers showing microspores and pollen, see also Materials and Methods.)

Experiment	P1P	P1T	P2	P3	P4	G1T	G1P	G2
Incorporation after incubation with enzymes [pmol/mg]	674.7	–	547.2	869.7	336.1	–	137.2	155.4
Incorporation after alkaline hydrolyses [pmol/mg]	307.3	–	268.7	473.4	206.0	–	68.8	83.7

Table III. Data of experiments before and after degradation of the sporopollenin fraction by potash fusion.

	Before degradation				After degradation			
	dry w.				loss of	ether		hexane
Experiment	mg	Bq	dpm/mg	pmol/mg	weight (%)	Bq	%	Bq
P1P	4.35	91	1259	307.3	53	14	15	–
P2	9.11	166	1092	268.7	72	51	31	6
P3	7.58	236	1868	473.4	78	137	58	5
P4	9.6	415	2592	206.0	71	213	51	11
G1P	4.29	21	298	68.8	70	1.4	7	–
G2	7.23	44	367	83.7	77	10.5	24	–

fraction. Among these *p*-hydroxybenzoic acid was identified by GC/MS analyses. It showed the highest radioactivity as is shown in Fig. 3. Up to 50% of the radioactivity applicated to analysis were eluted with *p*-hydroxybenzoic acid. Due to the low labeling and the small amount of substance left at this stage of the investigation, further labeled substances could not be demonstrated.

The specific radioactivity of the sporopollenin fractions submitted to potash fusions and of the insoluble residue after the degradation procedure as well as that of the main product *p*-hydroxybenzoic acid are compared in Table IV.

Table IV. The radioactivity of sporopollenin labeled *via* [U-^{14}C]phenylalanine, of insoluble residue and *p*-hydroxybenzoic acid obtained after degradation of the sporopollenin fraction by potash fusion.

Sporopollenin	dry weight radioactivity spec. radioactivity	9.6 mg (100%) 24900 dpm (100%) 2592 dpm/mg
Insoluble residue after degradation	dry weight radioactivity	2.81 mg (29.3%) –
p-Hydroxybenzoic acid	dry weight radioactivity spec. radioactivity	59 µg (0.61%) 3750 dpm (15.05%) 63559 dpm/mg
Isotope dilution factor		1.198×10^3

By GC/MS, oxalic acid, benzoic acid, phthalic acid and terephthalic acid were demonstrated as their TMS derivatives, furthermore octanedioic acid- and decanedioicacid-dimethylester were identified (see Fig. 4) in the fraction of ether soluble acids after potash fusions. Further degradation products could not be identified due to the small amount of substance.

Discussion

A high level of incorporation into the sporopollenin fraction was achieved when [^{14}C]phenylalanine was applicated as a precursor to anthers of *Tulipa* [4]. This significant incorporation of phenylalanine was observed regardless of the position of labeling or the stage of anther and pollen development [5]. After the degradation of a sporopollenin fraction labeled *via* DL-[ring(U)-^{14}C]-phenylalanine by potash fusion labeled *p*-hydroxybenzoic acid was identified as a main product [5]. This proved the integral incorporation of the aromatic ring system originating from the phenylalanine, possibly *via* phenylpropane metabolism. It was the aim of this investigation to test whether the substantial incorporation of [^{14}C]phenylalanine is a unique phenomenon restricted to *Tulipa* or not. The results clearly demonstrate that in case of *Cucurbita* a better incorporation of radioactivity into the sporopollenin fraction is achieved after appli-

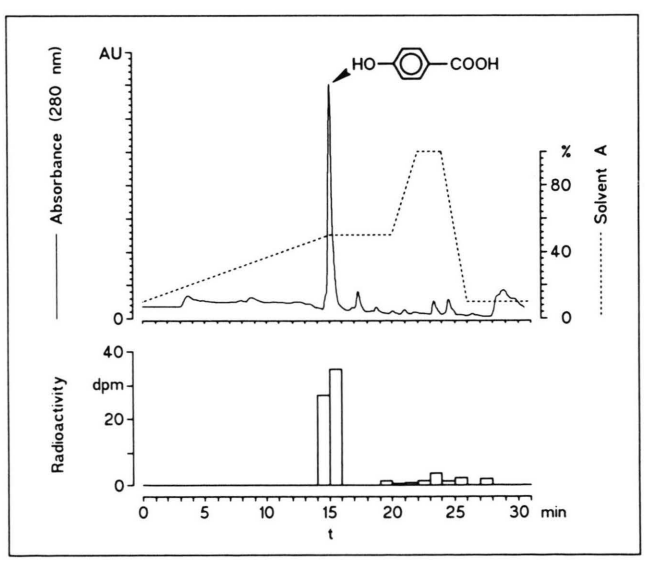

Fig. 3. HPLC of the acidified ether extract obtained after degradation of the sporopollenin fraction by potash fusion. Sporopollenin had been labeled by U-Phe. The radioactivity determined for the main degradation product *p*-hydroxybenzoic acid is shown.

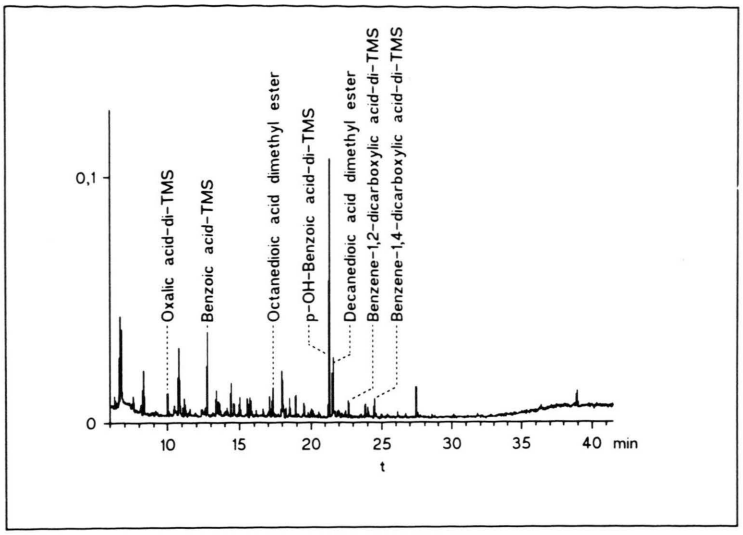

Fig. 4. GC-MS analysis of an acidified ether extract obtained after degradation of the sporopollenin fraction by potash fusion.

cation of [^{14}C]phenylalanine than after application of [^{14}C]glucose. After the degradation of a sporopollenin fraction labeled *via* [^{14}C]phenylalanine again *p*-hydroxybenzoic acid was identified as the main labeled product. These facts stress the importance of phenylpropane metabolism for the synthesis of sporopollenin. The results are in good accordance with earlier investigations [6–8]. Taking into account the results of solid state ^{13}C NMR spectroscopic analyses [9, 10], it has to be assumed at present that sporopollenin comprises different phenolic components of varying portion besides a high amount of long-chain aliphatic substances. Based on this model the occurrence of octanedioic acid – and decanedioic acid – dimethylester as degradation products after potash fusions might be explained. These compounds could be released from long-chain aliphatic compounds due to the harsh conditions of the degradation process. Hayatsu *et al.* [11] identified di-, tri-, tetra- and pentacarboxylic aromatic acids after oxidative degradation of sporopollenin.

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