

Formation of Flavonol 3-O-Diglycosides and Flavonol 3-O-Triglycosides by Enzyme Extracts from Anthers of *Tulipa* cv. Apeldoorn

Characterization and Activity of Three Different O-Glycosyltransferases during Anther Development

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Three distinct glycosyltransferases have been isolated and partially purified from anthers of *Tulipa* cv. Apeldoorn. The following designations are proposed: UDP-glucose: flavonol 3-O-glucosyltransferase (GT-I), UDP-rhamnose: flavonol 3-O-glucoside rhamnosyltransferase (GT-II) and UDP-xylose: flavonol 3-glycoside xylosyltransferase (GT-III). The three enzymes exhibited an identical pH optimum within the range of 8.5–9.0. The estimated molecular weight of GT-I and GT-II was about 40 000, GT-III showed a molecular weight of 30 000. GT-III required ions like NH_4^+ or Ca^{2+} whereas these ions have almost no influence on GT-I and GT-II activity. The enzymes have a slight requirement for SH-reagents, particularly DTE. As opposed to GT-II activity of GT-I and GT-III is significantly influenced by SH reagents and PCMB. Sucrose enhanced GT-III activity but only slightly GT-I activity; GT-II activity is not influenced.

Flavonol aglycones can function as glycosyl acceptor for the GT-I, whereas flavonol 3-O-glycosides, luteolin, dihydroquercetin, naringenin, cyanidin, *p*-coumaric acid and some other phenols were inactive as acceptor. The best acceptors were isorhamnetin and quercetin (K_m : 0.9×10^{-6} M). GT-II did not accept aglycones as substrates. For this enzyme flavonol 3-O-glucosides were the most attractive substrates. GT-III did not have any affinity towards aglycones, too. This enzyme exhibited a high specificity for flavonol 3-O-glucosides as well as flavonol 3-O-galactosides. Both enzymes, the GT-II and GT-III, were able to glycosylate flavonol 3-O-diglycosides forming triglycosides. UDP-glucose ($K_m = 1.0 \times 10^{-4}$ M), UDP-rhamnose and UDP-xylose where shown to be the best glycosyl donors for GT-I, GT-II or GT-III respectively. The glycosyl transfer catalysed by the GT-I was shown to be a freely reversible reaction.

In the whole anthers, highest specific activities of GT-I and GT-II were found during late stages of anther development. Similar results were obtained using the contents of anthers or the tapetum fraction. In contrary, high GT-III activity can be detected already in young stages of anther development. The highest activities of the three glycosyltransferases were found in the tapetum fraction, whereas the pollen fraction exhibited only poor activities.

Introduction

The surface of pollen of *Tulipa* cv. Apeldoorn contains major amounts of flavonol glycosides. From this source, rhamnosyl glucosides of quercetin, of isorhamnetin, of kaempferol and triglycosides like kaempferol 3-O-xylosylrhamnosylglucoside and isorhamnetin 3-O-xylosylrhamnosylglucoside could be isolated and identified [1]. Accumulation of these compounds occurs during final phases of pollen ripening [2, 3].

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Abbreviations: DTE, Dithioerythritol; PCMB, *p*-Chloromercuribenzoate.

* Note added in proof: After submission of this manuscript Jourdan and Mansell [32] reported on the isolation and partial characterization of three glucosyl transferases involved in the biosynthesis of flavonol triglycosides.

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The pollen grains develop, differentiate and ripe in the loculus of anthers which is surrounded from tapetal cells. There are some reports about the importance of this tissue, regarding nutritional functions for developing pollen [4–6]. Likewise, highest specific activities of enzymes involved in phenylpropanoid metabolism were detected in the tapetum fraction after fractionation of the contents of anthers [7, 8]. These results supported the hypothesis that the tapetum may play a significant role in the regulation of phenylpropane and flavonoid metabolism within the loculus of anthers [9].

A number of publications deal with the transfer of glucose to distinct flavonoid acceptor molecules [10–16], but only poor information on the enzymic synthesis of flavonol 3-O-triglycosides [17] is available, and nothing is known about the enzymes catalyzing this reaction*. Therefore, investigations to

evaluate these open question were performed using the system of tulip anthers. In particular attention was paid to the isolation, separation, characterization, localization and activity of the glycosyltransferases as related to the development of the anthers.

Some of the results were presented at the symposium of the Gesellschaft für Biologische Chemie, 9.–12. March 1980, Münster, Bundesrepublik Deutschland.

Materials and Methods

Plants. Tulip bulbs (cv. Apeldoorn) were purchased from Nebelung, Münster. They were grown in the Botanical Garden of the University of Münster. For the isolation and purification of the enzymes anthers were used with pollen in middle and/or late post-meiotic developmental stages.

Chemicals: UDP-D-[U-¹⁴C]glucose, UDP-D-[U-¹⁴C]galactose and UDP-D-[U-¹⁴C]glucuronic acid were purchased from Amersham Buchler, Braunschweig, UDP-D-[U-¹⁴C]xylose from N.E.N., Dreieich, UDP-D-glucose, UDP-D-galactose, UDP-D-glucuronic acid, UDP, NAD, NADH, NADPH and reference proteins were obtained from Boehringer, Mannheim, and UDP-xylose and ADP-glucose from Sigma, München. Catechin, coniferyl alcohol, *p*-coumaric acid, ferulic acid, cyanidin, delphinidin, dihydroquercetin, naringenin, kaempferol, quercetin and rutin were purchased from Roth, Karlsruhe. We gratefully acknowledge a gift of quercetin 3-O-glucoside from Prof. Zinsmeister, Saarbrücken. We also thank Prof. Wagner, München, for a sample of quercetin 3-O-galactoside. Other flavonoid compounds were from our laboratory collection. Ampholine carrier ampholytes were obtained from LKB.

Buffer solutions

The following buffer solutions were used:

- A: 0.2 M glycine/NaOH, pH 8.75, containing DTE (11 mM);
B: 0.025 M Tris/HCl, pH 7.5, containing 2-mercaptoethanol (10 mM).

Enzyme assays

- a. Standard assay for the enzymatic formation of flavonol 3-O-monoglucosides.

The assay mixture contained 33.5 nmol of quercetin (dissolved in 10 µl ethylene glycol monomethyl ether), 100 nmol of UDP-D-[U-¹⁴C]glucose (either 0.025 µCi or 0.05 µCi), 45.8 µmol of glycine (buffered with NaOH to pH 8.75) and 2.5 µmol of DTE in a total volume of 240 µl. The reaction was started by addition of 100 µl of the enzyme preparation, containing up to 0.1 mg of protein. The assay was incubated for 60 min at 30 °C. The reaction was stopped by addition of 10 µl glacial acetic acid and the mixture chromatographed on Whatman 3 MM paper using 15% acetic acid.

The product zone was cut out and transferred to a toluene-based scintillation cocktail (0.4% of Premix 4 in toluene).

- b. Assays for the enzymatic formation of flavonol 3-O-rhamnosylglucoside

b1: During enzyme purification (after Sephadex G-200 fractionation, ion exchange chromatography and isoelectric focusing), or when the influence of different ions (Ca^{2+} , NH_4^+ , Cl^- , SO_4^{2-}) was studied the following assay method was employed:

The incubation mixture contained quercetin 3-O-glucoside (33.5 nmol in 10 µl ethylene glycol monomethyl ether) and UDP-[U-¹⁴C]rhamnose (30 nmol, about 7.7 nCi, produced by method d.). The volume was adjusted to 240 µl by addition of either 30 mM Tris-HCl buffer, pH 8.5, containing 2-mercaptoethanol (0.01 M) (for DEAE-fractionated protein), or buffer A (after gel chromatography or isoelectric focusing, respectively). The reaction was started after addition of a protein solution (100 µl), containing up to 0.1 mg of protein.

- b2: Enzymic synthesis of UDP-[U-¹⁴C]rhamnose in a preparation scale for routine experiments

The assay contained 2 µmol of UDP-D-[U-¹⁴C]glucose (0.5 µCi), 32 µmol of NADH, 2 ml of the respective protein solution and buffer A, yielding a total volume of 3 ml. After addition of the enzyme solution, the mixture was incubated for 3 h at 34 °C. The reaction was stopped by addition of 50 µl of 96% acetic acid. Precipitated protein was centrifuged off, and the supernatants were combined. After evaporation to dryness, the residue was dissolved with 1 ml of aq. dest. In this solution the UDP-[U-¹⁴C]rhamnose formed represented about 30% of total radioactive compounds. This mixture was used as UDP-[U-¹⁴C]rhamnose source in a glycosyltransferase as-

say under the same conditions as described above (b1).

c. Standard assay for the enzymatic formation of flavonol 3-O-xylosylglucoside of flavonol 3-O-xylosylrutinoside

The assay contained UDP-[U-¹⁴C]xylose (25 nmol, 0.025 μ Ci), quercetin 3-O-glucoside (35.5 nmol in 10 μ l ethylene glycol monomethyl ether) or rutin (35.5 nmol in 10 μ l ethylene glycol monomethyl ether) and 100 μ l of the enzyme preparation (up to 5 mg protein/ml). The volume was adjusted to 240 μ l by addition of buffer A.

Preliminary experiments had shown that this enzymatic reaction required NH_4^+ ions for full activity. For this reason, $(\text{NH}_4)_2\text{SO}_4$ (100 mM) was added to the reaction mixture when gel chromatographed protein was used as enzyme source. In this case, the pH of the mixture was corrected to 8.75 by addition of NaOH.

d. Synthesis of UDP-[U-¹⁴C]rhamnose

For the synthesis of [¹⁴C]-UDPR-rhamnose, the procedure performed by [31] was modified. The particular technique will be described elsewhere (Kleinhollenhorst, Strunk und Wiermann, in preparation).

Identification of reaction products

The identity of the reaction products was proved by paper chromatography and rechromatography with authentic reference substrates in five different solvent systems (No. VII–XIII, see below). After elution with appropriate solvents, the absorption maxima of the enzymatically formed flavonol glycosides showed the characteristic shifts on UV-spectroscopy, when treated with sodium methoxide (NaOMe), AlCl_3/HCl , sodium acetate (NaOAc) and sodium acetate/boric acid [18]. After acid hydrolysis, the identity of the products was confirmed by co-chromatography with authentic samples and by their behaviour against spray reagents.

Partial purification of the different O-glucosyltransferases

a. For the preparation of crude extracts anthers, buffer A, quartz sand and Polyclar AT were mixed in a ratio of 1:10:1:0.1 (w/v/w/w) and homogenized at 0 °C in a mortar. Insoluble residues were removed by centrifugation and filtration. The crude

extract was fractionated by $(\text{NH}_4)_2\text{SO}_4$ precipitation (sat. 60%), the precipitate dissolved in buffer A or B, respectively, and desalted on a Sephadex G-25 column (equilibrated with the resp. buffer). The protein so obtained was further fractionated on a Sephadex G-200 column, equilibrated with buffer A (without DTE).

For isoelectric focusing, fractions showing enzyme activity were combined and passed through a Sephadex G-15 column, equilibrated with 1% glycine. Isoelectric focusing was performed using a sucrose-stabilized LKB 110 ml column and Ampholine carriers in pH ranges of 3.5–7.0 and of 4.0–6.0, respectively.

For DEAE Sephacel fractionation, combined fractions (see above) were passed through a Sephadex G-25 column, equilibrated with 30 mM Tris/HCl buffer (pH 8.5) and applied to the exchanger column which was equilibrated with the same buffer. After washing the column with starting buffer, a NaCl gradient was applied (0–1 M). The NaCl concentration was further increased stepwise to 2 M and eventually 3 M.

b. Crude extracts from the contents of anthers, from the tapetum fraction and from the pollen fraction were obtained as described (Sütfeld and Wiermann, 1974); buffer A containing 0.4 M sucrose was used for extraction.

Chromatographic methods

Solvent systems as specified below were used for the separation of glyco-nucleotides (ZN), sugars (Z), flavonol aglycones, flavonol glycosides and other phenols (F) on Whatman 3 MM chromatography paper.

- I (ZN): ammonium acetate-ethanol-acetic acid (2:5:3). To avoid decomposition of UDP-glycosides, the gas phase was saturated with ethanol-water (80:20) [19]. To remove ammonium acetate from the chromatograms, a second run was performed using ethanol-water (80:20);
- II (Z): 1-butanol-acetic acid-water (6:2:2);
- III (Z): ethyl acetate-pyridine-water (12:5:4);
- IV (Z): pyridine-ethyl acetate-acetic acid-water (36:36:7:21);
- V (Z): isobutyric acid (water-saturated);
- VI (Z): methyl ethyl ketone-acetic acid-water (8:1:1);

- VII (F): 1-butanol-acetic acid-water
(4:1:5; upper phase);
VIII (F): isopropanol-water (22:78);
IX (F): chloroform-isobutanol-water (2:4:4);
X (F): ethyl acetate (water-saturated);
XI (F): acetic acid-water (15:85)
XII (F): acetic acid-water (3:97);
XIII (F): acetic acid-water-HCl (25%) (30:10:3).

Usually, solvent systems XI or XII were applied for the separation of enzymatically formed flavonol glycosides.

Molecular weight estimation

The molecular weight of proteins was estimated on calibrated Sephadex columns. Ferritin, aldolase, bovine serum albumin, hen egg albumin, chymotrypsinogen A and cytochrome c served as standard.

Protein estimation

The protein content of crude enzyme preparations was measured by the biuret method. Protein from higher purified extracts was estimated by the Lowry method, modified by [20]. Bovine serum albumin was used as standard.

Results

a. Evidence for the existence of three different *o*-glycosyltransferases in anthers of *Tulipa cv. Apeldoorn*

When crude enzyme extracts were employed in preliminary experiments, reaction courses as listed

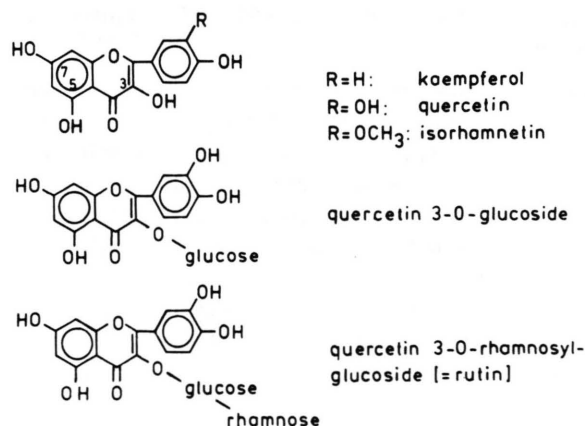


Fig. 1. Some of the flavonols and flavonol glycosides used as substrates for the different *O*-glycosyltransferase reactions (compare Table IV).

below could be observed using distinct flavonoid acceptors and nucleotid donors (compare Fig. 1):

- (I) UDP-glucose + quercetin → quercetin 3-O-glucoside + UDP;
 (II) UDP-rhamnose + quercetin 3-O-glucoside → quercetin 3-O-rhamnosylglucoside + UDP;
 (III) UDP-xylose + quercetin 3-O-glucoside → quercetin 3-O-xylosylglucoside + UDP;
 UDP-xylose + quercetin 3-O-rhamnosylglucoside → quercetin 3-O-xylosylrhamnosylglucoside + UDP.

The products formed could be identified unequivocally by paper chromatography and UV spectroscopy as described under "Methods". It was obvious to attempt the separation of the enzyme activities involved in the synthesis of these glycosides. The evidence for the existence of three specific transferases (GT-I, GT-II and GT-III, according to the reaction scheme above) was obtained from experiments in which gel chromatography, ion exchange chromatography and isoelectric focusing were employed.

On a Sephadex G-200 column, GT-III was separated distinctively from GT-I and GT-II which eluted simultaneously (Fig. 2). All glycosyltransferase activities were bound to DEAE Sephacel Cellulose; elution using a linear NaCl gradient resulted in a separation of GT-II from GT-I and GT-III (Fig. 3). Complete separation of the three enzymes could be successfully performed using isoelectric focusing (Fig. 4).

b. Properties of the glycosyltransferases

The occurrence of three different glycosyltransferases was also established during investigations of their biochemical properties:

b.1. pH optimum

The three enzymes exhibit an identical pH optimum within the range of 8.5–9.0.

b.2. Effect of inorganic ions and of EDTA

The results obtained are shown in Fig. 5a, b and Table Ia–c. Ca^{2+} and NH_4^+ , particularly, provoke considerable effects on the activity of GT-III whereas GT-I as well as GT-II were only weakly affected. Addition of Ca^{2+} together with NH_4^+ does not enhance enzyme activity to a higher extent. GT-III activity is influenced by addition of NH_4Cl as well as

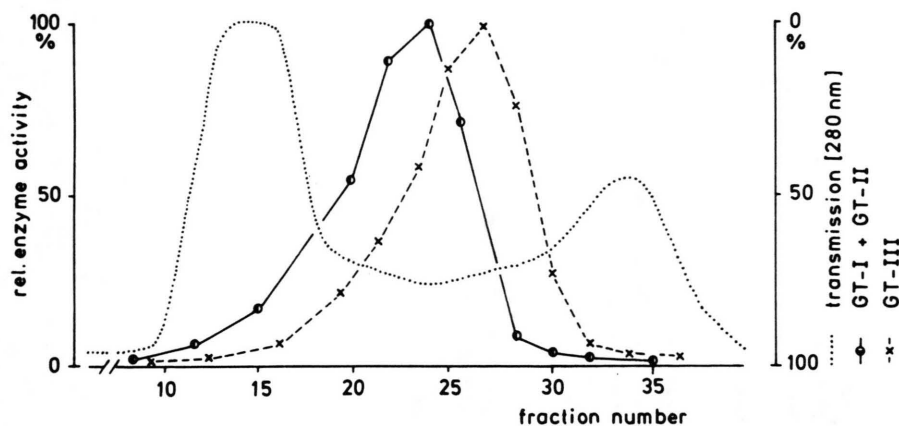


Fig. 2. Elution of the three glycosyltransferases from a Sephadex G-200 column.

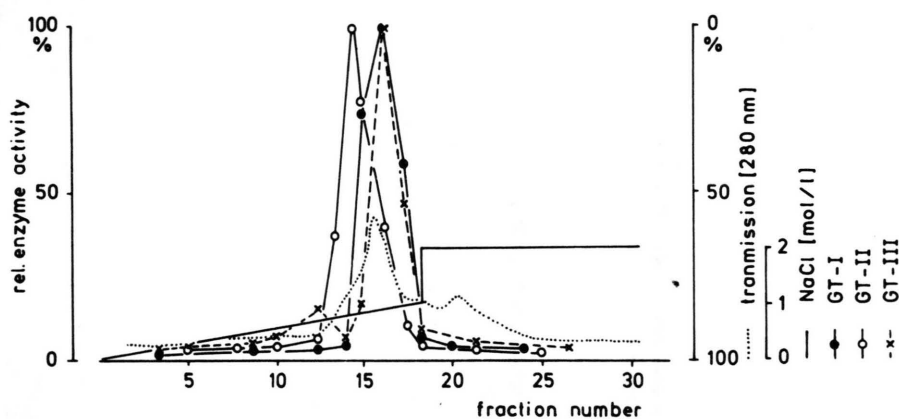


Fig. 3. Chromatography of the three glycosyltransferases on a DEAE-cellulose column.

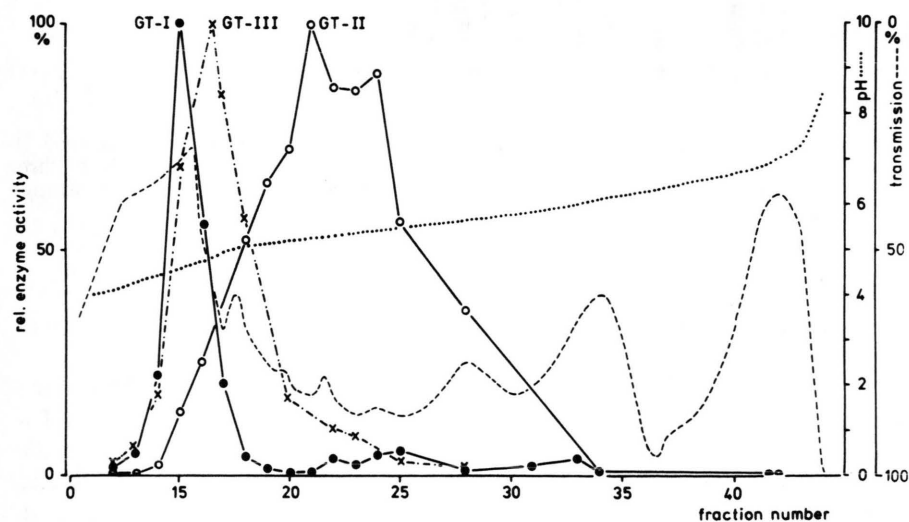


Fig. 4. Isoelectric focusing of the glycosyltransferases from Sephadex G-200 column protein.

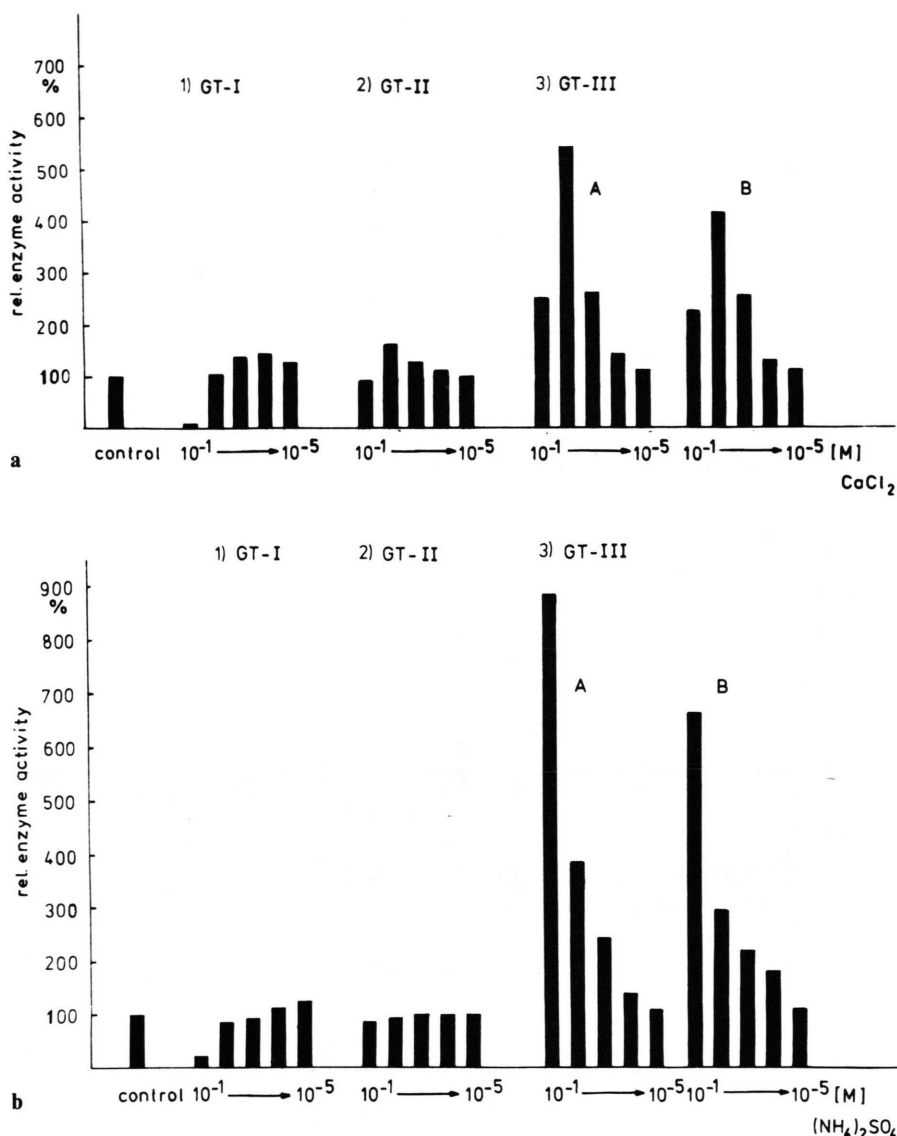


Fig. 5. a) The effect of Ca^{2+} ions on the activities of the three glycosyltransferases (A: GT-III activity with quercetin 3-O-glycoside as substrate; B: GT-III activity with rutin as substrate). b) The effect of NH_4^+ ions on the activities of the three glycosyltransferases (A, B: see 4a). Regarding GT-I and GT-II the effect is shown in percent of control without addition of ions; GT-III: no activity without addition of the ions.

of $(\text{NH}_4)_2\text{SO}_4$ employed in the same range. Addition of MgCl_2 does not cause any effect. These observations show clearly that anions like Cl^- and SO_4^{2-} do not affect glycosyltransferase activity at all (Table Ic). Furthermore, the data obtained show that bivalent heavy metal ions like Cu^{2+} , Mn^{2+} and Zn^{2+} did affect the reaction strongly when applied in higher concentrations.

b.3. Influence of SH reagents and of PCMB

The influence of SH reagents and of PCMB on the activity of the glycosyltransferases is shown in Table II. SH reagents, particularly DTE, enhance the activity of the enzymes slightly. SH reagents and PCMB hardly affect GT-II activity as opposed to GT-I and GT-III activities.

Table Ia. The influence of inorganic ions and EDTA on GT-I activity (effect in percent of control without addition of ions).

| Reagents | Concentration [M] | | | |
|-------------------|--------------------|--------------------|--------------------|--------------------|
| | 5×10^{-2} | 5×10^{-3} | 5×10^{-4} | 5×10^{-5} |
| MgSO ₄ | 18 | 80 | 92 | 97 |
| ZnSO ₄ | 0 | 8 | 68 | 90 |
| CuSO ₄ | 0 | 13 | 78 | 86 |
| MgCl ₂ | 29 | 90 | 94 | 95 |
| MnCl ₂ | 0 | 0 | 11 | 39 |
| EDTA | 31 | 87 | 94 | 111 |

Table Ib. The influence of inorganic ions and EDTA on GT-II activity (effect in percent of control without addition of ions).

| Reagents | Concentration [M] | | | |
|-------------------|--------------------|--------------------|--------------------|--------------------|
| | 5×10^{-2} | 5×10^{-3} | 5×10^{-4} | 5×10^{-5} |
| MgSO ₄ | 174 | 136 | 116 | 108 |
| ZnSO ₄ | 2 | 18 | 91 | 94 |
| CuSO ₄ | 0 | 48 | 83 | 90 |
| MgCl ₂ | 127 | 137 | 116 | 97 |
| MnCl ₂ | 100 | 106 | 111 | 104 |
| EDTA | 70 | 97 | 100 | 102 |

Table Ic. The influence of inorganic ions and EDTA on GT-III activity (effect in percent of control without addition of ions).

| Reagents | Concentration [M] | | | |
|--|--------------------|--------------------|--------------------|--------------------|
| | 5×10^{-2} | 5×10^{-3} | 5×10^{-4} | 5×10^{-5} |
| MgSO ₄ | 95 | 98 | 100 | 100 |
| ZnSO ₄ | 32 | 40 | 74 | 88 |
| CuSO ₄ | 56 | 85 | 95 | 100 |
| MgCl ₂ | 93 | 97 | 100 | 100 |
| MnCl ₂ | 36 | 49 | 80 | 92 |
| NH ₄ Cl | 774 | 320 | 219 | 121 |
| NH ₄ Cl + CaCl ₂ | 798 | 345 | 249 | 134 |
| EDTA | 91 | 98 | 100 | 100 |

b.4. Influence of sucrose and of BSA

Because of the presence of sucrose in the extraction medium using the contents of anthers as enzyme source, a possible effect of sucrose on the activity of glycosyltransferases was investigated. As shown in Table III, sucrose enhances GT-III activity but only slightly GT-I activity. The GT-II activity is not influenced. Addition of BSA causes no effect on glycosyltransferase activity.

b.5. Substrate specificity

b.5.1. Acceptor specificity

Various aglycones, mono- and diglycosides were employed for the evaluation of the substrate specificity of the three glycosyltransferases. As shown in Table IV (compare Fig. 1), GT-I exhibits a major affinity towards flavonol aglycones; the best acceptors were isorhamnetin and quercetin. GT-II does

Table II. The effects of SH-reagents and *p*-chloromercuribenzoate (PCMB) on the activities of the different glycosyltransferases (control: without addition of a reagent = 100%). Maximum values are labelled.

| Reagents [M] | GT-I | GT-II | GT-III |
|------------------------|------------|------------|------------|
| <i>control</i> | 100 | 100 | 100 |
| <i>DTE</i> | | | |
| 10^{-1} | 109 | 110 | 109 |
| 10^{-2} | 115 | 110 | 134 |
| 10^{-3} | 124 | 101 | 135 |
| 10^{-4} | 109 | 97 | 106 |
| 10^{-5} | 103 | 97 | 102 |
| <i>glutathion</i> | | | |
| 10^{-1} | 91 | 103 | 94 |
| 10^{-2} | 112 | 107 | 112 |
| 10^{-3} | 118 | 104 | 103 |
| 10^{-4} | 112 | 106 | 101 |
| 10^{-5} | 106 | 106 | 100 |
| <i>mercantoethanol</i> | | | |
| 10^{-1} | 89 | 111 | 90 |
| 10^{-2} | 105 | 110 | 110 |
| 10^{-3} | 120 | 103 | 122 |
| 10^{-4} | 110 | 100 | 108 |
| 10^{-5} | 102 | 100 | 101 |
| <i>PCMB</i> | | | |
| 10^{-4} | 28 | 81 | 24 |
| 10^{-5} | 30 | 88 | 37 |
| 10^{-6} | 97 | 99 | 92 |

Table III. The effect of sucrose and bovine serum albumin on the different glycosyltransferase activities.

| Reagents | | Activity, in percent of control without addition of a reagent | | |
|----------|--------|---|-------|--------|
| | | GT-I | GT-II | GT-III |
| sucrose | 5% | 123 | 98 | 109 |
| | 25% | 107 | 101 | 121 |
| | 50% | 108 | 96 | 149 |
| BSA | 10 µg | 104 | 106 | 100 |
| | 50 µg | 109 | 106 | 100 |
| | 100 µg | 106 | 112 | 98 |
| | 500 µg | 107 | 114 | 95 |

Table IV. Acceptor specificity of the three glycosyltransferases (compare Fig. 1).

| Substrate | Relative activity [%] ^a | | |
|--|------------------------------------|--------------------|---------------------|
| | GT-I ^b | GT-II ^c | GT-III ^d |
| Quercetin | 93 | — | — |
| Kaempferol | 45 | — | — |
| Isorhamnetin | 100 | — | — |
| Kaempferid | 13 | n.d. | n.d. |
| Luteolin | — | n.d. | — |
| Dihydroquercetin | — | — | n.d. |
| Naringenin | — | — | — |
| 2',4,4',6'-tetrahydroxychalcone | — | n.d. | — |
| Cyanidin | — | — | — |
| Catechin | — | n.d. | n.d. |
| Coniferyl alcohol | — | — | — |
| <i>p</i> -Coumaric acid | — | — | — |
| Ferulic acid | — | — | — |
| Quercetin 3-O-glucoside | — | 100 | 65 |
| Kaempferol 3-O-glucoside | n.d. | 96 | 100 |
| Isorhamnetin 3-O-glucoside | n.d. | 93 | 42 |
| Quercetin 3-O-galactoside | n.d. | 32 | 111 |
| Quercetin 3-O-rhamnoside | — | 23 | 11 |
| Kaempferol 3-O-rhamnoside | n.d. | 21 | 13 |
| Myricetin 3-O-rhamnoside | n.d. | 9 | 6 |
| 2',4,4',6'-tetrahydroxychalcone 4'-O-glucoside | — | — | — |
| Apigenin 7-O-glucoside | n.d. | — | — |
| Eriodictyol 7-O-glucoside | n.d. | — | — |
| Rutin | 4 | 45 | 13 |
| Isorhamnetin 3,7-O-glucoside | n.d. | — | 9 |
| Quercetin 3-O-arabinoglucoside | n.d. | — | 8 |

^a Activities were compared to the reaction rate determined with isorhamnetin = 100% (GT-I), quercetin 3-O-glucoside = 100% (GT-II), kaempferol 3-O-glucoside = 100% (GT-III). Enzyme source: anthers at the stage of middle postmeiotic pollen ripening.

^b Donor: UDP-glucose.

^c Donor: UDP-rhamnose.

^d UDP-xylose.

not react with aglycones. For this enzyme, flavonol 3-O-glucosides were most attractive substrates whereas flavonol 3-O-diglycosides were glycosylated to a minor extent. In a similar way, GT-III also did not have any affinity towards aglycones; this enzyme exhibits a high specificity for flavonol 3-O-glucosides as well as flavonol 3-O-galactosides. Both enzymes, the GT-II and GT-III were able to glycosylate a flavonol 3-O-diglycoside forming triglycosides.

b.5.2. Donor specificity

For GT-I, UDP-glucose was demonstrated to be a better donor than UDP-galactose whereas UDP-rhamnose, UDP-glucuronic acid, UDP-xylose and ADP-glucose were not effective (Table V). By contrast, UDP-rhamnose turned out to be a very attractive donor for GT-II as did UDP-xylose for GT-III. With GT-II, a distinct activity was observed also when UDP-xylose was used as a donor. This may be

due to contamination by GT-III protein because for this assays, only partially purified enzyme preparations were used. The value measured for UDP-glucuronic acid with rutin as substrate seems to be surprisingly high in so far as, in tulip anthers, the existence of glucuronic acid derivatives could not be demonstrated as yet.

b.5.3. K_m values

In this investigation, only a GT-I preparation was used for K_m determination. For this enzyme, UDP-glucose as a donor exhibited a K_m of 1.0×10^{-4} M with quercetin ($K_m = 0.9 \times 10^{-6}$ M) as an acceptor.

b.6. Molecular weight

Chromatography on Sephadex G-200 showed a MW of 40 000 for each GT-I and GT-II and 30 000 for GT-III

Table V. Donor specificity of the three glycosyltransferases.

| Substrate | Relative activity [%] ^a | | | |
|---------------------|------------------------------------|--------------------|---------------------|---------------------|
| | GT-I ^b | GT-II ^c | GT-III ^d | GT-III ^e |
| UDP-glucose | 100 | 18 | 38 | 10 |
| UDP-galactose | 45 | 20 | 48 | 9 |
| UDP-rhamnose | — | 100 | 29 | 50 |
| UDP-glucuronic acid | — | — | 55 | 11 |
| UDP-xylose | — | 50 | 100 | 100 |
| ADP-glucose | — | — | — | — |

^a Activities were compared to the reaction rate determined with UDP-glucose = 100% (GT-I). UDP-rhamnose = 100% (GT-II), UDP-xylose = 100% (GT-III).

^b Acceptor: Isorhamnetin.

^c Acceptor: quercetin 3-O-glucoside.

^d Acceptor: rutin.

^e Acceptor: Quercetin 3-O-glucoside.

c. Free reversibility of the UDP-glucose flavonol 3-O-glycosyltransferase reaction

Using a partially purified enzyme preparation and quercetin 3-O-[U-¹⁴C]glucoside and UDP as substrates, the glucose moiety was transferred to UDP, forming UDP-glucose. Control experiments without UDP showed that deglycosylation of the quercetin glucoside took place only in the presence of UDP in the reaction mixture.

d. Development of the three glycosyltransferases during microsporogenesis

During the microsporogenesis, the development of glycosyltransferase activities was pursued on enzyme extracts from anthers (GT-I, II and III), and from the contents of anthers as well as from the tapetum and pollen fraction (GT-I and II; Fig. 6a, b). In whole anthers, highest specific activities of GT-I and GT-II were found during late stages of the postmeiotic microsporogenesis which are characterized by an intensive accumulation of flavonol glycosides [3]. Similar results were obtained using the contents of anthers or the tapetum fraction, respectively, as enzyme source: Also in this case, glycosyltransferase activity develops during pollen ripening. It reaches a maximum during the time of the beginning accumulation of flavonol glycosides. In contrast, high GT-III activity can be detected already in younger stages. Moreover, its activity varies during the development of anthers; a correlation between GT-III activity and flavonol glycoside accumulation cannot be given (Fig. 6c)

e. Localization of the glycosyltransferases

The data obtained after fractionation of the contents of anthers suggest that highest activity of the three glycosyltransferases is localized in the tapetum fraction (Fig. 7). A part of GT-III activity seems to be associated with the pollen fraction when compared to GT-I and GT-II activity.

Discussion

The results presented here show for the first time the enzymatic formation of flavonol 3-O-mono, di- and triglycosides performed by three different O-glycosyltransferases in anthers of *Tulipa* cv. Apeldoorn (see footnote on page 587). According to their substrate specificity, GT-I catalyzes the glucosylation of flavonol aglycones at the 3-hydroxy-position; the second (GT-II) catalyses the transfer of rhamnose to the glucose bound in 3 position to form a rutinoside; the third (GT-III) specifically adds xylose to flavonol 3-O-glucosides or to the flavonol 3-O-rutinoside yielding the corresponding di- or triglycoside. The results obtained show that the formation of flavonol 3-O-xylosyl rhamnosylglucosides proceeds in a sequential manner.

The investigations reported here are closely related to others performed on different systems such as intact plants and cell cultures [10–13, 17]. The enzymatic formation of quercetin 3-O-rhamnosylglucoside was already demonstrated by [10], using enzyme preparations from seedlings of *Phaseolus aureus*. However, no efforts were made to detect different

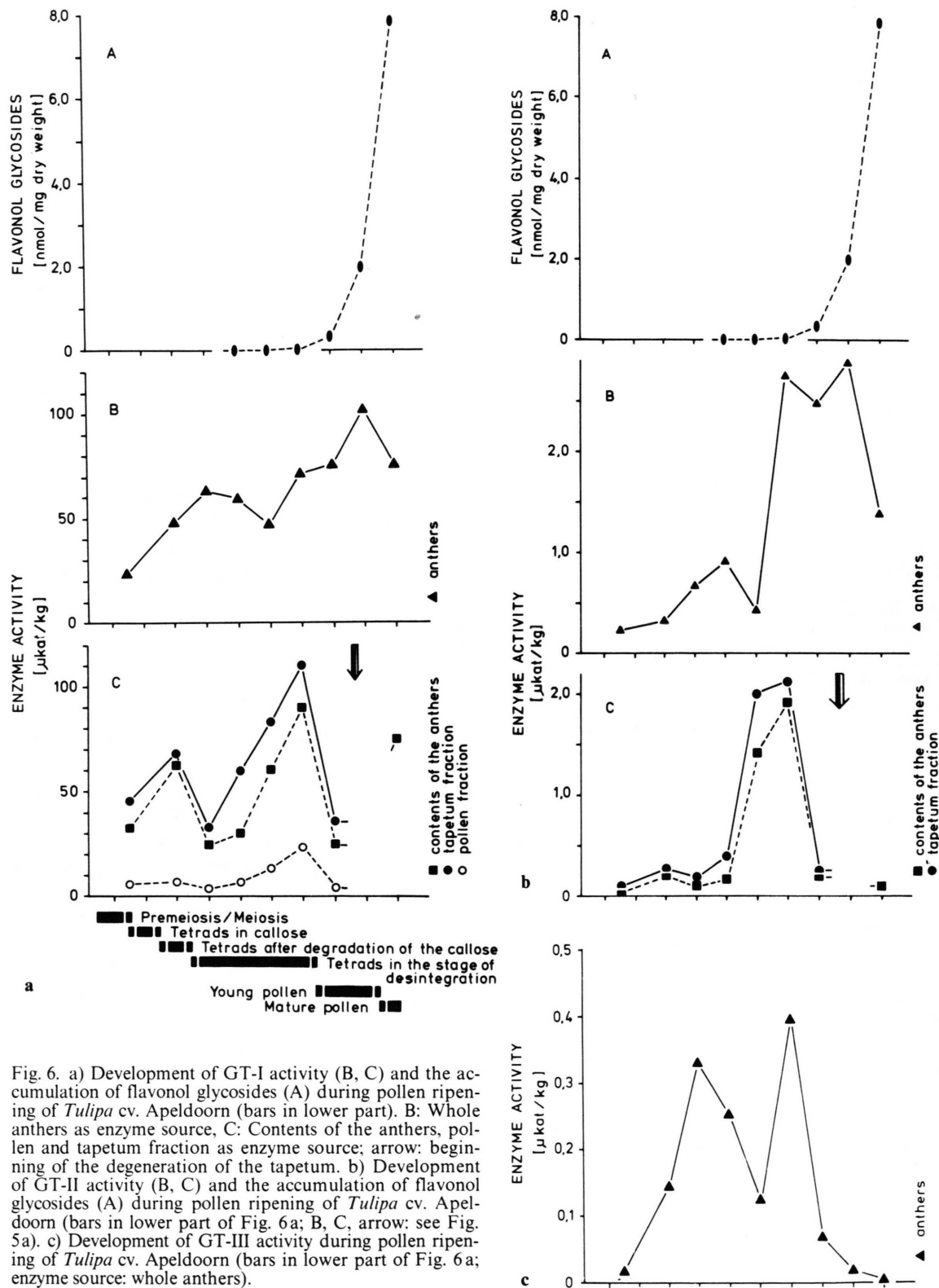


Fig. 6. a) Development of GT-I activity (B, C) and the accumulation of flavonol glycosides (A) during pollen ripening of *Tulipa* cv. Apeldoorn (bars in lower part). B: Whole anthers as enzyme source, C: Contents of the anthers, pollen and tapetum fraction as enzyme source; arrow: beginning of the degeneration of the tapetum. b) Development of GT-II activity (B, C) and the accumulation of flavonol glycosides (A) during pollen ripening of *Tulipa* cv. Apeldoorn (bars in lower part of Fig. 6a; B, C, arrow: see Fig. 5a). c) Development of GT-III activity during pollen ripening of *Tulipa* cv. Apeldoorn (bars in lower part of Fig. 6a; enzyme source: whole anthers).

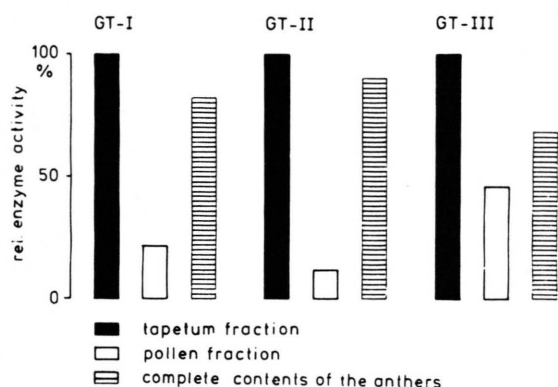


Fig. 7. Distribution of the glycosyltransferase activities in the separated tapetum and pollen fraction and in the contents of tulip anthers (values in % of highest specific activities).

glycosyltransferases, and only a few quantitative data were available from this work. Similarly, Shute *et al.* [17] described the formation of quercetin 3-O-triglycoside in an enzyme preparation from *Pisum sativum* seedlings. These authors, however, did not report on the separation and biochemical characterization of the enzymes involved.

Most properties of the glycosyltransferases from tulip anthers are in accordance to those reported from the literature: pH optima are within a range of 7.5 to 8.5 [13, 16, 21]. The tulip enzymes are ranging slightly higher (pH 8.7 to 9.0). These results agree with data published by [15]. Cations like Mg^{2+} , NH_4^+ and Ca^{2+} as well as EDTA did not or only poorly affect GT-I and GT-II activity which corresponds with results reported by [11, 22, 23], on the contrary, GT-III activity is enhanced by Ca^{2+} and NH_4^+ ions in an extraordinary manner. This result agrees with data reported by [24]. The activity of the isovitexin 6-C-glucosylglucosyltransferase from petals of *Melandrium album* is also clearly stimulated by the addition of Ca^{2+} ions [21]. In accordance with other studies [11], bovine serum albumin slightly enhances GT-I and GT-II activity, whereas no effect of BSA could be observed on GT-III. SH-reagents, particularly DTE, show a stimulating effect on the glycosyltransferase activities. This effect corresponds well with previous observations [22–24]. The MW of 30 000–40 000 is in the range of values reported for various glycosyltransferases from other plant systems [16].

With regard to their substrate specificity, GT-I predominantly glycosylates flavonols particularly

flavonol aglycones like quercetin, kaempferol and isorhamnetin whose derivatives occur in the natural system [1–3]. No activity could be detected with tetrahydroxychalcone, naringenin, dihydroquercetin, luteolin, cyanidin or with flavonol 3-O-glycosides as substrates. The enzyme acts specifically in the 3-position; no 7-O-glucosyltransferase activity as described for enzyme preparations from parsley cell suspension cultures could be detected [11, 12, 15]. UDP-glucose was the most effective donor (compare Sutter *et al.*, 1972). The apparent K_m value for UDP-glucose is 1.0×10^{-4} M and for quercetin is 0.9×10^{-6} M, respectively. These data are within the range reported previously [11, 13, 16, 24].

The glycosyl transfer catalyzed by the UDP-glucose: flavonol 3-O-glucosyltransferase turned out to be a freely reversible reaction. A free reversibility was also described for a similar transferase from parsley cell cultures [25].

As described for GT-I, GT-II also exhibits a strict position specificity. For GT-II flavonol 3-O-glucosides are the effective substrates. Lower activity was observed with rutin, quercetin 3-O-galactoside or flavonol 3-O-rhamnosides as acceptors. The introduction of a second glycoside moiety takes place in subsequent steps [compare 26–29]. With respect to the donor specificity, UDP-rhamnose turned out to be the most effective substrate. For GT-III, a flavonol 3-O-glucoside or flavonol 3-O-galactoside acts as main acceptor, and rutin to a minor extent only (due to a lack of suitable compounds other flavonol 3-O-rutinosides could not be tested as substrates). In the course of the present investigations it could not be clarified whether xylosylation of rutin involves a transfer of xylose to the glucose or to the rhamnose moiety. It can be assumed, however, that the transfer takes place to the glucose moiety because the flavonol 3-O-glucoside was shown to be a very attractive substrate.

Most effective donors were UDP-glucose for GT-I UDP-rhamnose for GT-II and UDP-xylose for GT-III. This donor specificity corresponds well to the glycosyl moieties which participate in the synthesis of the different flavonol glycosides as found in the ripe pollen [1].

For the system of tulip anthers, the results reported here clearly establish the existence of three distinct glycosyltransferases which are involved in flavonoid biosynthesis. With regard to the acceptors in question, GT-I and GT-II show a remarkably

small substrate specificity whereas GT-III obviously exhibits a broader one. On the basis of the different data described we therefore propose the following designations for the enzymes.

GT-I: UDP-glucose: flavonol 3-O-glucosyltransferase;

GT-II: UDP-rhamnose: flavonol 3-O-glucoside rhamnosyltransferase and

GT-III: UDP-xylose: flavonol 3-O-glycoside xylosyltransferase.

In the course of microsporogenesis, the development of glycosyltransferase I and II activity correlates well with the kinetics of the accumulation of flavonols when the whole anthers are used as enzyme source. The presence of high enzyme activity during stages with high flavonol accumulation is plausible. On the other hand, no good explanation can be given for the development of GT-III. Regarding the localization of the glycosyltransferases, highest activities could be detected in the tapetum fraction; in the pollen fraction, only low enzyme activity was present. These results correspond well to those demonstrated recently [7]. Once more, they prove the important role of the anther tapetum in the regulation of phenylpropanoid metabolism within the loculus of anthers [30].

The course of GT-I and GT-II development during the microsporogenesis was investigated using both the contents of anthers as well as the tapetum fraction as enzyme source. In both cases maximum activity was found during late stages of pollen development. These stages are characterized by just a beginning accumulation of flavonol glycosides. At present there is no obvious explanation for the concomitant accumulation of flavonol glycosides and a drastic decrease of transferase activities. The degeneration of the tapetum, starting in this stages (as indicated by an arrow, Fig. 6 a, b), may be connected to this phenomenon.

The results presented here prove for the first time that a major part of the activity of these glycosyltransferases is localized in the tapetum fraction during the whole period of tapetum development until its degeneration.

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- [1] D. Strack, G. Sachs, and R. Wiermann, *Z. Pflanzenphysiol.* **103**, 291–296 (1981).
- [2] R. Wiermann, *Planta* **110**, 353–369 (1973).
- [3] L. Quast and R. Wiermann, *Experientia* **29**, 1165–1166 (1973).
- [4] H. F. Linskens, Pollen. In: *Encyclopedia of Plant Physiology*, (W. Ruhland, ed.), vol. **XVIII**, pp. 368–406, Springer, Berlin, Heidelberg, New York 1967.
- [5] I. K. Vasil, *Naturwissenschaften* **60**, 247–253 (1973).
- [6] J. P. Mascarenhas, *Bot. Rev.* **41**, 259–314 (1975).
- [7] E. Herdt, R. Sütfield, and R. Wiermann, *Cytobiologie* **17**, 433–441 (1978).
- [8] R. Sütfield, B. Kehrel, and R. Wiermann, *Z. Naturforsch.* **33c**, 841–846 (1978).
- [9] R. Sütfield and R. Wiermann, *Ber. Dtsch. Bot. Ges.* **87**, 167–174 (1974).
- [10] G. A. Barber, *Biochemistry* **1**, 463–468 (1962).
- [11] A. Sutter, R. Ortmann, and H. Grisebach, *Biochim. Biophys. Acta* **258**, 71–87 (1972).
- [12] A. Sutter and H. Grisebach, *Biochim. Biophys. Acta* **309**, 289–295 (1973).
- [13] J. E. Poulten and M. Kauer, *Planta* **136**, 53–59 (1977).
- [14] K. F. F. Kho, J. Kamsteeg, and J. van Brederode, *Z. Pflanzenphysiol.* **88**, 449–464 (1978).
- [15] J. Köster and W. Barz, *Arch. Biochem. Biophys.* **212**, 98–104 (1981).
- [16] W. Hösel, Glucosylation and Glucosidases. In: *The Biochemistry of Plants*, (E. E. Conn, ed.), vol. **7**, Secondary Plant Products, pp. 725–753, Academic Press, New York, London, Toronto, Sydney, San Francisco 1981.
- [17] J. L. Shute, P. S. Jourdan, and R. L. Mansell, *Z. Naturforsch.* **34c**, 738–741 (1979).
- [18] T. J. Mabry, K. R. Markham, and M. B. Thomas, *The systematic identification of flavonoids*. Springer, Berlin, Heidelberg, New York 1970.
- [19] K. C. Tovey and R. M. Roberts, *J. Chromatogr.* **47**, 287–290 (1970).
- [20] U. Phol, Untersuchungen zur Isolation von Vakuolen Höherer Pflanzen; Entwicklung von Methoden zur Isolation, zur biochemischen Reinheitsüberprüfung und zum Nachweis lytischer Enzyme. Dissertation, Universität Münster 1979.
- [21] J. van Brederode and G. van Nigtevecht (1974) *Biochem. Genet.* **11**, 65–81 (1974).
- [22] R. K. Ibrahim and H. Griesbach, *Arch. Biochem. Biophys.* **176**, 700–708 (1976).
- [23] R. K. Ibrahim, *Z. Pflanzenphysiol.* **85**, 253–262 (1977).
- [24] R. L. Larson and C. M. Lonergan, *Planta* **103**, 361–364 (1972).
- [25] A. Sutter and H. Grisebach, *Arch. Biochem. Biophys.* **167**, 444–447 (1975).

- [26] R. Ortmann, A. Sutter, and H. Grisebach, *Biochim. Biophys. Acta* **289**, 293–302 (1972).
- [27] J. van Brederode and G. van Nigtevecht, *Molec. Gen. Genet.* **122**, 215–229 (1973).
- [28] G. van Nigtevecht and J. van Brederode, *Heredity* **35**, 429 (1975).
- [29] J. Kamsteeg, J. van Brederode, and G. van Nigtevecht, *Biochem. Gen.* **16**, 1045–1058 (1978).
- [30] R. Wiermann, Stage-specific phenylpropanoid metabolism during pollen development. In: Regulation of secondary product and plant hormone metabolism, (M. Luckner and K. Schreiber, eds.), **vol. 55**, pp. 231–239, Pergamon press, Oxford, New York, 1979.
- [31] J. Kamsteeg, J. van Brederode, and G. van Nigtevecht, *FEBS Letters* **91**, 281–284 (1978).
- [32] P. S. Jourdan and R. L. Mansell, *Arch. Biochem. Biophys.* **213**, 434–443 (1982).