Metabolism of Ferulic Acid Sucrose Esters in Anthers of *Tulipa* cv. Apeldoorn: I. The Accumulation of Esters and Free Sugars

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The authors of *Tulipa* cv. Apeldoorn accumulate a large amount of ferulic acid sucrose esters. In addition to the well-known diferuloyl-(DFS) and triferuloyl sucrose esters (TFS), a new phenylpropanoid characterized as a monoester of ferulic acid and sucrose is described. Like TFS and DFS, this ester exhibits an accumulation maximum in the early stages of pollen development.

Numerous free sugars have been extracted from *Tulipa* anthers. Three of them were identified as sucrose, glucose and fructose. Sucrose as the main component is accumulated in extremely high amounts during specific developmental stages (= 20% of the dry weight).

After separation of the anthers into an anther wall fraction (AWF) and pollentapetum (PTF), each fraction shows a different accumulation kinetic of the free sugars and the ferulic acid esters. A correlation could be observed between the occurrence of the esters and sucrose in the pollentapetum fraction during the anther development.

Introduction

Higher plants are optimized in the ability to synthesize and accumulate hydroxycinnamic acid conjugates [1]. Up to now an extreme variety of conjugates in form of esters, glycosides and amides is described [2–4, 8, 12]. Sugar esters of hydroxycinnamic acids are widely distributed in the plant kingdom [5–7, 16]. Most of the hydroxycinnamic sugar esters are composed of a monosaccharide and one or more acyl moieties. The occurrence of esters with the disaccharide sucrose known so far is restricted to several plant systems: di-sinapoylsucrose in cotyledons of *Raphanus sativus* [9], di- and triferuloylsucrose in anthers of many species of the Liliaceae [10, 11], tri-*p*-coumaroylsucrose in roots of *Polygonum hydropiper* [13] and diesters of sucrose with sinapoylferuloyl and acetylester moiety in aerial parts of *Polygala chamaebuxus* [14]. These sucrose esters differ extremely in the kind of acyl-moiety and the sites of esterification. It is remarkable that all hydroxycinnamic sucrose esters described as yet are accumulated as di- or triester. To our knowledge, there is no information about occurrence of a monoester of sucrose and a hydroxycinnamic acid.

This paper reports for the first time the isolation and characterization of a monoferuloylsucrose from extracts of tulip anthers. In order to elucidate the metabolism of the ferulic acid sucrose esters, the accumulation kinetics of the three ferulic acid esters were measured during the whole period of pollen differentiation and ripening.

Previous studies have shown that extracts from loculus material of young tulip anthers contain large amounts of ferulic acid esters [15], but the free ferulic acid could not be detected. We have now continued our earlier studies and focussed our interest on the occurrence of free sucrose, the conjugation partner of ferulic acid, as well as on the appearance of glucose and fructose, intermediates in the biosynthesis of sucrose. At present, only little data is available on the accumulation of free sugars in anthers. This is the first report on the isolation, identification and quantitative determination of sucrose, glucose and fructose during anther development.

Abbreviations: HPLC, high performance liquid chromatography; TLC, thin-layer chromatography; GC, gas-chromatography; PTF, pollen-tapetum fraction; AWF, anther wall fraction; DFS, diferuloylsucrose; TFS, triferuloylsucrose; MSTFA, N-methyl-N-trimethylsilyltrifluoracetamid.

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Material and Methods

Plant material

Tulip bulbs ("Apeldoorn") purchased from Nebelung (Münster, F.R.G.) were cultivated in the Botanical Garden of Münster. The anthers were harvested at defined days during a complete period of

anther development (October to April). Anthers were separated into two fractions, the anther-wall fraction (AWF) and the pollen-tapetum fraction (PTF)*. Immediately after separation the plant material was collected in fluid N_2. The PTF and AWF were ground in a pre-cooled mortar. The resulting powders were freeze-dried and stored at −70 °C until use.

Extraction of the hydroxycinnamic acid derivates and free sugars

In order to optimize the quantitative determination, the plant material was freeze-dried once more prior to extraction. 40 mg of the PTF and AWF were extracted with 1 ml distilled water by shaking (Vibromix) for 1 min. After centrifugation (8000 × *g*, 5 min), the clear supernatants were used for the qualitative and quantitative analyses of sugars. Isolation of the hydroxycinnamic acid derivatives followed the same procedure, but the water was replaced by methanol (HPLC grade).

Chromatography

HPLC: High performance liquid chromatography was carried out on a Abimed system (Düsseldorf, F.R.G.), incorporating a Gilford UV/VIS detector-250 (Oberlin, Ohio 44074) and a computer integrator (R-C3A, Shimadzu, Kyoto, Japan). The methanolic extracts were analyzed by reversed-phase chromatography. The chromatographic column (250 × 4 mm) was packed with Nucleosil C-18 5 μm particle size (Macherey-Nagel, Düren, F.R.G.). Solvents for reversed-phase HPLC: Solvent A: acetic acid (1% in distilled water), solvent B: acetonitrile (85% in distilled water). Separations were accomplished by gradient elution: time (min) 0 10% solvent B in A, time (min) 15 50% solvent B in A, subsequently for 5 min kept at 50% solvent B in A (isocratic), time (min) 21 100% solvent B in A, time (min) 23 100% solvent B in A, time (min) 25 10% solvent B in A, subsequently for 20 min kept at 10% solvent B in A (equilibrating); flow rate 1 ml/min. Injection was performed *via* a Rheodyne rotary valve (Rheodyne Inc., Cotati, Cal., U.S.A.) with a 100 μl loop. In general, ferulic acid esters were detected at 320 nm.

GC: Sugars were chromatographed on a OV 101 capillary column (Macherey-Nagel, Düren, F.R.G.) using a Shimadzu system (model GC-9A, Düsseldorf, F.R.G.). Detector FID; detector- and injector temperature 280 °C; temperature gradient: time (min) 0 150 °C − time (min) 30 280 °C. Retention times were recorded with a computer integrator (R-C3A, Shimadzu, Düsseldorf, F.R.G.).

TLC: Hydroxycinnamic acid derivates were chromatographed in acetic acid (3% SSI) and butanol–acetic acid–water (4:1:5, v/v/v, organic phase; SSII) on microcristalline cellulose (Merck, Darmstadt, F.R.G.). Sugars were separated on silica gel plates (Merck, Darmstadt, F.R.G.) in chloroform–acetone–ethanol–water (37:37:23:3, v/v/v/v) and butanol–acetone–water (4:5:1, v/v/v).

Identification of free sugars and hydroxycinnamic acid derivatives by GC, TLC and HPLC

Sugars: For the qualitative analyses of the crude extracts 20 mg of freeze-dried powder of the PTF and AWF were extracted with 250 μl pyridin (1 min, Vibromix). After centrifugation (8000 × *g*, 1 min) aliquots of the supernatants were used for TLC or were mixed with MSTFA in a ratio of 1:1 (v/v). These solutions were stored for about 24 h at 4 °C in darkness. In general, 1 μl was submitted to GC. For the spot (TLC) and peak (GC) identification reference sugars were co-chromatographed (reference sugars: sucrose, glucose, fructose, xylose, rhamnose, stachyose, mannose, maltose). Sugars were available from commercial sources. Detection of sugars was achieved by spraying chromatograms with anthrone and heating at 100 °C until spots become visible. Regions of different sugars were cut off from a comparable chromatogram, compounds were eluted with pyridin and prepared for GC analyses.

Ferulic acid sucrose ester: Methanolic crude extracts of PTF and AWF were injected into the HPLC column. DFS and TFS were identified by direct chromatographic comparison (TLC, HPLC) with samples of these esters which had been prepared in a previous study [17]. Unknown products were collected separately and analyzed in the range of 200–500 nm (UV 810, Kontron, Düsseldorf, F.R.G.) to determine spectroscopic data including shift behaviour on acid and alkali treatment. Behaviour in UV light (UV 350 nm) was examined with

* The material is composed for the most part of meiocytes and/or pollen, with the rest being made up of tapetum cells and the nutrient solution which surrounds the pollen.

and without NH_3 vapour. For further identification, products were hydrolyzed: 1 N HCl for 30 min or 1 N NaOH for 5 min at 100 °C. Aliquots of hydrolyzed mixtures were used directly for the HPLC, TLC and the sucrose/glucose/fructose UV-test (Boehringer, Mannheim, F.R.G.) to identify and quantify reaction products.

Quantitative determination of the free sugars and ferulic acid esters

Sucrose, glucose and fructose were measured quantitatively by the sucrose/glucose/fructose UV-test (Boehringer, Mannheim, F.R.G.).

The amount of ferulic acid sucrose esters was estimated by HPLC (HPLC conditions see above). Quantitative values were obtained using ferulic acid as the standard of UV detection. Differences in λ_{max} of ferulic acid and ferulic acid sucrose esters were taken into consideration. Calculation of peak areas were obtained by an integrator computer (R-C3A, Shimadzu, Kyoto, Japan).

Results

The occurrence of a monoferuloylsucrose ester

HPLC analyses confirmed the results of previous studies insofar as the major phenylpropanoid of early stages of pollen development are two ferulic acid esters, DFS and TFS (Fig. 1) [10].

As shown in the HPLC diagram, a further as yet unknown compound can be observed. This compound has an absorption spectrum before and after addition of HCl or NaOH, respectively, very similar to that of DFS and TFS, and exhibits an identical behaviour in UV light in comparison with DFS and TFS (see Table I).

The alkaline hydrolysis of the purified product (rechromatography by TLC and HPLC with various gradient elutions) results in the formation of ferulic acid and sucrose in a ratio of 1:1. Therefore, this substance must be characterized as a monoester of ferulic acid and sucrose (= feruloylsucrose, (FS)mono). At present the site of the esterification is unknown.

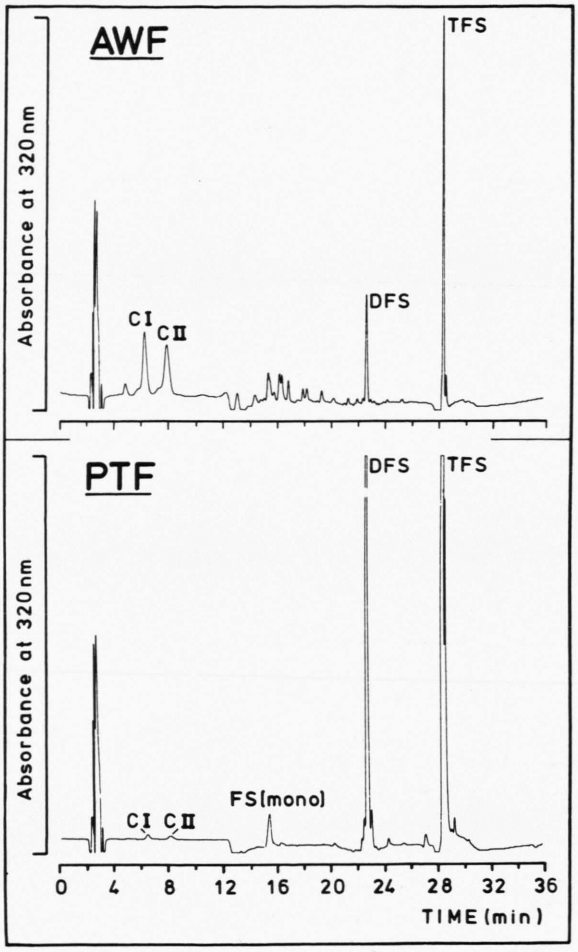

Fig. 1. HPLC-analyses of methanolic extracts from AWF and PTF (DFS = diferuloylsucrose, TFS = triferuloylsucrose, FS(mono) = feruloylsucrose (monoester), C I and C II = components of unknown structure with a hydroxycinnamic acid moiety).

Table I. Chromatographic and spectroscopic properties of TFS and DFS in comparison with FS(mono).

	λ_{max} [nm]			Behaviour in UV-light (350 nm)		Chromatographic properties		
	Methanol	Methanol + NaOH	Methanol + HCl	with NH_3 vapour	without NH_3 vapour	TLC ($R_f \times 100$) SS I	SS II	HPLC
TFS	326	380	326	green	blue	10	90	see
DFS	326	380	326	green	blue	30	80	Fig. 1
FS(mono)	322	378	322	green	blue	65	50	

The tissue-specific localization of the esters

The three ferulic acid esters are predominantly accumulated in the PTF of young anthers. The methanolic and water extracts are nearly free of any other phenylpropanoid compound (Fig. 1). This was confirmed by HPLC with various gradients and analyses at different wave-lengths.

In comparison to these results, the extracts of the AWF contain only a small amount of DFS and TFS. But additionally, two further polar compounds of unknown structure occur (see Fig. 1, AWF: CI and CII). Studies to identify these components are underway.

The accumulation of the three ferulic acid esters during anther development

An important prerequisite for studies on enzymes involved in the metabolism of the ferulic acid esters is the knowledge of the accumulation of the compounds during anther development. The accumulation kinetics are shown in Fig. 2 separately for AWF and PTF. As far as the PTF is concerned, an intensive accumulation of all three esters takes place during early stages of pollen differentiation. The highest values were reached at the same developmental stage.

Compared to the PTF, the AWF contains only small amounts of DFS and TFS without any remarkable characteristics in the kinetics. We assume that the occurrence of DFS and TFS in the AWF is reducible to a contamination by a rest-material of the PTF.

The occurrence of sucrose as the conjugation partner of the ferulic acid esters

The anthers of *Tulipa* represent a site of considerable high sugar accumulation. Among the numerous sugars, sucrose, glucose and fructose could be identified by TLC (data not shown) and GC. The identification of these sugars was performed by direct comparison of R_f-values of reference sugars and by the aid of co-chromatography. Sucrose was proved to be the main sugar component in the AWF and PTF (see Fig. 3).

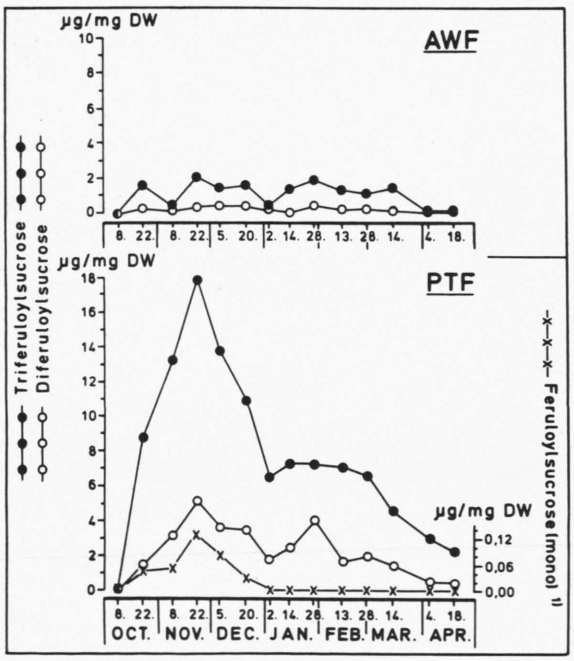

Fig. 2. The accumulation of ferulic acid sucrose esters isolated from AWF and PTF during anther development.

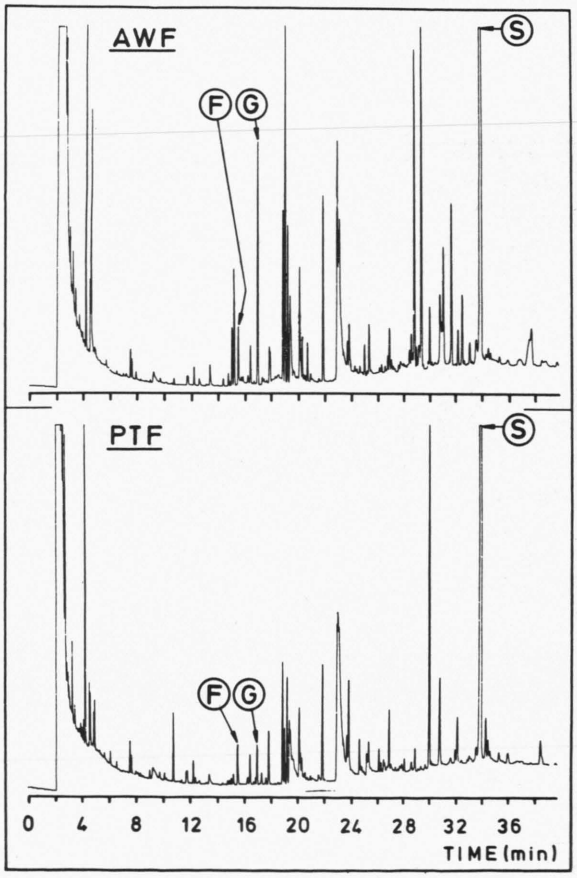

Fig. 3. GC-analyses of water extracts from AWF and PTF (F = fructose, G = glucose, S = sucrose).

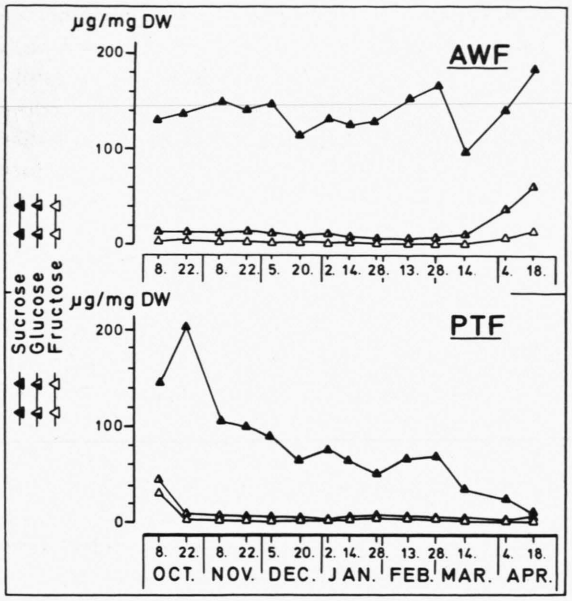

Fig. 4. The accumulation of sucrose, glucose and fructose in the AWF and PTF during anther development.

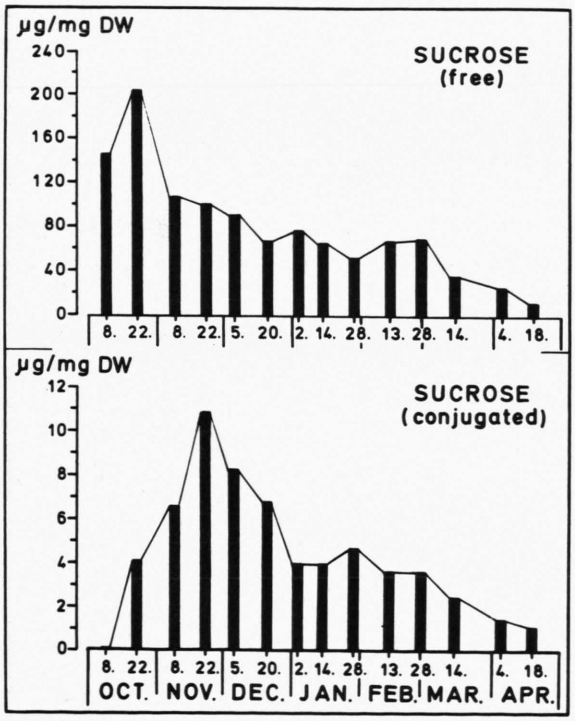

Fig. 5. Accumulation kinetics of free sucrose and conjugated sucrose in the PTF during pollen differentiation and ripening.

The quantitative values of sucrose, glucose and fructose obtained from the two anther fractions, AWF and PTF, over the whole period of anther development are summarized in Fig. 4. In the PTF, sucrose exhibits an accumulation maximum in very early stages of pollen differentiation and is metabolized during the following stages. Conversely, AWF is characterized by a more or less constant high level of sucrose with a slight increase at the end of pollen ripening.

Sucrose esters and free sucrose are accumulated in the anther loculus. In Fig. 5 the accumulation kinetics of free sucrose and of sucrose esterified with ferulic acid are compared. A correlation is seen between the decrease of the sucrose content and the beginning of the intensive accumulation of the sucrose ester. But it should be mentioned that the quantities of the free sucrose and the conjugated sucrose differ considerably.

Discussion

The anthers of *Tulipa* cv. Apeldoorn represent a system of an intensive phenylpropanoid metabolism. An intensive accumulation of ferulic acid esters takes place in early stages of pollen differentiation and decreases in advanced developmental stages [10]. In this paper, we describe for the first time an ester which has been characterized as monoester of ferulic acid and sucrose. Our results have shown that this new ferulic acid conjugate is accumulated more or less exclusively in the loculus of the anthers. Compared to the amount of the well-known diferuloyl- and triferuloylsucrose, the monoester is accumulated in very low concentrations. The occurrence of monoferuloylsucrose leads to the following consideration:

1. The ester may be considered to be an intermediate in the biosynthesis of DFS and TFS and may function as an acyl donor and/or as an acyl acceptor in an enzymatically acyl transfer [18–20]. The only trace amount might be explained by a rapid turnover of this compound.
2. The monoferuloylsucrose appears as a degradation product of DFS catalyzed by a high specific reaction ([17]; Bäumker, Arendt, and Wiermann, this volume). Thus, we cannot decide at present whether monoferuloylsucrose isolated from crude extracts is involved in biosynthesis or degradation of DFS and/or TFS, or in both pathways.

The ferulic acid are accumulated in considerable amounts in tulip anthers compared with similar hy-

droxycinnamic acid esters from other systems. 0.5% and 1.8% per dry weight could be measured for DFS and TFS respectively. (For comparison: 0.57% feruloylglucose from cell cultures of *Chenopodium rubrum* [16]; 0.26% chlorogenic acid from fruits of *Lycopersicum esculentum* [3]; 0.23% *p*-coumaroylmalat from *Rhaphanus sativus* [4]).

Our results have shown for the first time that the anther represents a centre of a remarkable high sugar accumulation. Among the sugars identified so far, sucrose, glucose and fructose, the sucrose is the predominant constituent of the PTF as well as of the AWF. According to the PTF the sucrose reaches the highest values (up to 20% of the dry weight) in very early stages of anther development. Thus, the conjugation partner of ferulic acid is formed in sufficient quantities in the PTF when the ferulic acid esters are formed. The accumulation kinetics have shown that the decrease of the sucrose content is well correlated with the formation of the ferulic acid esters. But it must be pointed out that the quantities of the free sucrose and an esterbound sucrose differ considerably.

This is expressed by the quantitative data: between 22. Oct. to 22. Nov., a portion of 100 µg/mg dry weight of free sucrose was metabolized, whereas in the same time only 7 µg/mg dry weight sucrose was bound as ferulic acid esters (see Fig. 5).

Nevertheless, we assume that increasing amounts of sucrose might function as an endogenous factor which induces the formation of the ferulic acid sucrose conjugates. Similar observations were made by Dahlbender and Strack [23] when investigating malat-metabolism in *Rhaphanus sativus*. Increasing amounts of malat induce the formation of the sinapoylmalat. The authors assume a permanent flow of carbon through pools of secondary constituents and postulate this for many other cases.

As for *Tulipa* anthers, the metabolic position of ferulic acid sucrose esters would seen to be multiple. Thus, the metabolically active esters may play a crucial role as a transient pool for hydroxycinnamic acid moieties leading, for example, to the formation of flavonoids, sporopollenin and so forth.

As discussed above, the accumulation of ferulic acid sucrose esters is obviously closely related to an intensive sucrose metabolism. The role of sucrose in the anther and the pollen development is not clear as yet. The sucrose might function as a carbohydrate pool generally involved in the pollen development. However, the extremely high sucrose content in the PTF and AWF is remarkable. In addition, therefore, osmotic effects of the massive sucrose accumulation should be taken into consideration.

Acknowledgements

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