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STYRYLPYRONE BIOSYNTHESIS IN *EQUISETUM ARVENSE*

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Key Word Index—*Equisetum arvense*; Equisetaceae; horsetail; styrylpyrone synthase characterization; hydroxycinnamoyl-CoA; hispidin; bisnoryangonin.

Abstract—Styrylpyrone synthase was detected in cell free extracts from gametophytes of *Equisetum arvense*. This new enzyme catalyses the formation of styrylpyrones from malonyl-CoA and hydroxycinnamoyl-CoA precursors. A standard enzyme assay was established. The enzyme activity was characterized in partially purified protein extracts. *p*-Coumaroyl-CoA was accepted as substrate at pH 6.0–8.5 in various buffer systems with the formation of bisnoryangonin, and optimum enzyme activity was observed in potassium phosphate buffer at pH 7.5. Caffeoyl-CoA was accepted as substrate only in potassium phosphate buffer at pH 6.0–7.5 with formation of hispidin; optimum enzyme activity was observed at pH 7.0. The apparent K_m values were 220 μM for caffeoyl-CoA and 230 μM for *p*-coumaroyl-CoA. The temperature optimum of the enzyme activity was 37° for bisnoryangonin and 30° for hispidin formation. Molecular weight determination by FPLC indicated that this protein has a native molecular weight of *ca* 56–77 kDa. Styrylpyrones accumulate in rhizomes of sporophytes and gametophytes of *E. arvense* as major constitutive metabolites. In these organs no flavonoids could be detected. In green sprouts, styrylpyrone accumulation is only detected as a local response to mechanical wounding or microbial attack, and flavonoids are accumulated as major polyketide metabolites. Thus, chalcone synthase is active in the sporophytes and might have developed in the course of evolution from styrylpyrone synthase present in the more primitive gametophytes.

INTRODUCTION

In recent years, various flavonoid glycosides, caffeic acid esters and styrylpyrone glycosides have been isolated from the different organs of *Equisetum* species [1–7]. All compounds were found to accumulate in specific organs and their presence/absence to be dependent on the stage of organ development [8]. In gametophytes and rhizomes of *E. arvense* L., styrylpyrone glycosides (**3–6**) were accumulated as the major phenolic constituents in addition to several caffeic acid esters. In these organs no flavonoids were detected. Conversely, green sprouts contained a range of flavonoid glycosides and several caffeic acid esters, but no styrylpyrones. A marked switch from styrylpyrone biosynthesis in haploid gametophytes to flavonoid biosynthesis in green diploid sporophytes was observed [8].

Styrylpyrones are common constituents of fungi, mainly in the Hymenochaetaceae (Basidomycetes) [9, 10]. They also occur in the more primitive angiosperm families such as the Piperaceae, Lauraceae, Annonaceae, Ranuculaceae and Zingiberaceae [11]. In ferns only a single example, davallialactone, has been reported [12]. Phytochemical screening results indicate a more widespread occurrence of styrylpyrones in the pteridophytes (Veit, M., unpublished results). Due to distinctive accumulation of these compounds in rhizomes and gametophytes they might have been overlooked in the past because these organs were rarely investigated.

The formation of styrylpyrones in fungi from aromatic amino acids and acetate units was studied via feeding experiments with labelled precursors [13]. These data indicated a biosynthetic reaction similar to that of chalcone synthase (CHS) in the initial step of flavonoid biosynthesis [14]. Styrylpyrones were also observed as *in vitro* release products in CHS assays [15, 16]. Thus, both groups of compounds seemed to be biosynthesized by closely related enzymes. With styrylpyrone biosynthesis in *Equisetum* an alternative

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and possibly more primitive pathway is realized, in which acetate units are attached to hydroxycinnamate precursors. In this case the most primitive pathway occurs in *Psilotum,* where psilotins (arylpyrones, glucosylated at the aromatic ring) have been detected. Such compounds are derived from hydroxycinnamoyl-CoA and one molecule of malonyl-CoA [17, 18] (Fig. 1). We have examined the initial step of styrylpyrone

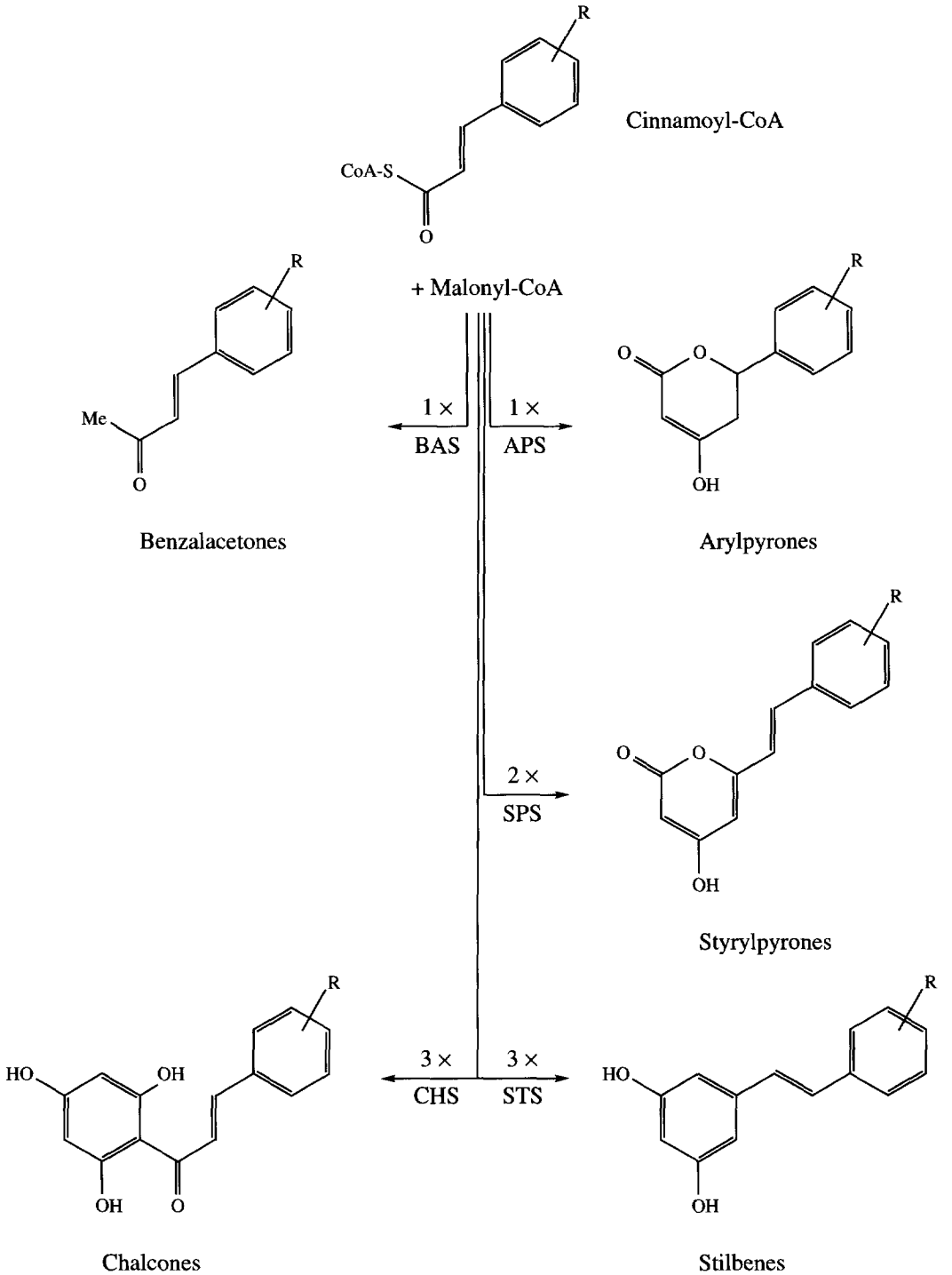


Fig. 1. Polyketide synthase reactions reported from plants [13–20] with hydroxycinnamoyl-CoA esters as starter molecules. BAS = benzalacetone synthase; APS = arylpyrone synthase; SPS = styrylpyrone synthase; CHS = chalcone synthase; STS = stilbene synthase.

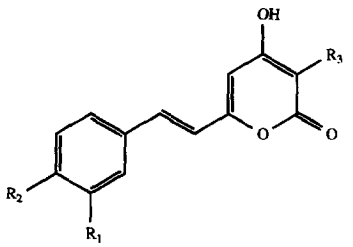
biosynthesis in gametophytes of *E. arvense* in order to compare it with the corresponding step in flavonoid biosynthesis in sporophytes.

RESULTS

Constitutive localization and induction of styrylpyrone accumulation

Equisetum arvense gametophytes in axenic cultures on sucrose media accumulated styrylpyrone glycosides. The major components **3–6** were identified, and they were present between 1 and 12 mg g^{-1} dry wt, together with mono- and di-caffeoyl-*meso*-tartaric acid and 5-*O*-caffeoylshikimic acid occurring in the same tissue up to 1.5–3.4 mg g^{-1} dry wt. Peak levels for styrylpyrones were reached after 4 weeks of subcultivation while caffeic acid esters reached their maximum levels after 6 weeks. Plants cultivated on sucrose-free media accumulated lower levels of all compounds described.

Equisetum arvense sporophytes showed high levels of styrylpyrones only in underground parts of the plant. These methanol-extractable strylpyrones **3–6** accumulated with total levels ranging from 2 to 6 mg g^{-1} dry wt while caffeic acid esters were accumulated with levels ranging from 0.3 to 1.4 mg g^{-1} dry wt. Intermediate segments between the black rhizomes and the barren sprouts contained decreasing levels of styrylpyrones, but increasing levels of flavonoids (Fig. 2), which were exclusively detected in the green tissue. In the barren sprouts, styrylpyrone accumulation was only observed in response to mechanical wounding or microbial attack and then only in tissue surrounding local necrotic areas (Fig. 3).



	R_1	R_2	R_3	
1	H	OH	H	Bisnoryangonin
2	OH	OH	H	Hispidin
3	OH	OH	OGlc	Equisetumpyrone
4	OH	OMe	OGlc	
5	H	OH	OGlc	
6	OH	OH	OSoph	

Rhizomes also contained cell-wall-bound styrylpyrones, which were liberated from cell wall preparations via acidolysis with dioxane–HCl. The absorption spectrum of one such compound showed bands at 255 and 370 nm typical of styrylpyrones. A molecular ion at m/z 523 $[M + H]^+$ was obtained by HPLC–atmospheric pressure chemical ionization (APCI) mass spectrometry.

Enzyme preparation and assay

The protein was extracted in the presence of Polyclar AT (50%), which was used to remove interfering phenolics. The best recovery of enzyme activity was achieved using potassium phosphate (K-Pi) buffer at pH 6.8 containing 2-mercaptoethanol, Na ascorbate, EDTA and phenylmethylsulfonylphluoride (PMSF). 2-Mercaptoethanol could be substituted by dithiothreitol (DTT). Enzyme activity could not be detected in crude extracts, but was recovered after purification by $NH_4(SO_4)_2$ precipitation (40–65% saturation), desalting on Sephadex G 25 and concentrating of the eluate by ultrafiltration. The enzyme activity was stabilized by addition of 10% (v/v) glycerol. Storage at −80° did not affect the activity. Standard enzyme assays were performed in 0.5 M K-Pi buffer, at pH 6.8 with caffeoyl-CoA or pH 8.3 with *p*-coumaroyl-CoA as substrates.

Properties of styrylpyrone synthase

The enzyme activity was completely recovered from the supernatant of 100 000 *g* centrifugation, indicating the presence of a soluble enzyme protein. The pH optima of enzyme reactions were determined in three different buffer systems (Fig. 4). The pH optimum for bisnoryangonin (**1**) formation from *p*-coumaroyl-CoA was at pH 7.5 in Tris–HCl, K-Pi and imidazole buffers. The optimum for the formation of hispidin (**2**) from caffeoyl-CoA was pH 7.0 in K-Pi buffer. No enzyme activity with this substrate was obtained in actetate, bicine, K–Mes, Tris–HCl, imidazole–HCl, Na citrate and glycine buffers. Maximum enzyme activity was found at 37° for **1** and at 30° for **2** formation, and the reaction was linear with time for up to 90 min and with protein up to 50 μg per assay.

Molecular weight

In order to facilitate and speed up product identification for styrylpyrone synthase (SPS) activity measurements during protein purification, a radioactive enzyme assay with [2-^{14}C]malonyl-CoA was used. The molecular weight of native SPS from gametophytes of *E. arvense* was determined with protein extracts from the 40–65% $(NH_4)_2SO_4$ saturation precipitate. The elution profile of the size-exclusion chromatography is shown in Fig. 5. SPS activity eluted as a single peak corresponding to a molecular weight of *ca* 56–77 kDa.

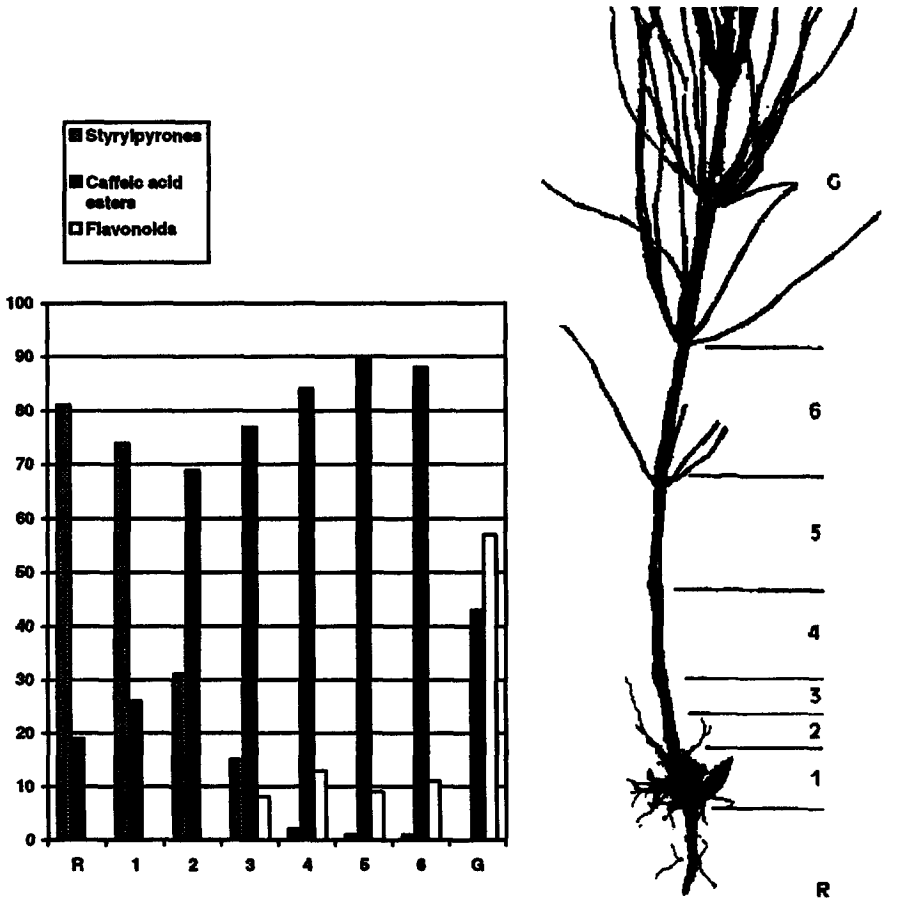


Fig. 2. Relative contents (%) of soluble styrylpyrones, caffeic acid esters and flavonoids (100% represents the sum (mg g^{-1} dry wt) of all three groups) in the intermediate zones between the black rhizomes (R) to the green barren sprouts (G) of the sporophyte of *E. arvense*.

Substrate specificity

At the present stage it is not clear whether *p*-coumaroyl-CoA or caffeoyl-CoA acts as the main physiological substrate for styrylpyrone formation in the plants. However, with the partially purified protein extracts in standard enzyme assays with K-Pi buffers higher product formation was found with *p*-coumaroyl-CoA as substrate (Fig. 4). From the data obtained using the standard enzyme assays an apparent K_m of 220 μM for caffeoyl-CoA, 230 μM for *p*-coumaroyl-CoA, 80 μM for malonyl-CoA (with caffeoyl-CoA) and 215 μM for malonyl-CoA (with *p*-coumaroyl-CoA) were calculated.

Identification of reaction products

In standard enzyme assays identification of the reaction products was achieved by HPLC analysis. Peak identities of **1** and **2** were proved by comparison with authentic samples. The ^{14}C-labelled products with [2-^{14}C]malonyl-CoA as substrate were detected on TLC plates and were identified by TLC in comparison with the non-labelled reference compounds.

DISCUSSION

Equisetum arvense constitutively accumulates styrylpyrones and flavonoids in distinct organs. The haploid gametophytes contain only styrylpyrones and no flavonoids. Polyketide synthase activity in cell free extracts prepared from gametophytes yields styrylpyrones, which appear to be the only polyketides in these tissues. We never detected products of other polyketide synthase reactions in these organs *in vivo*, and in *in vitro* enzyme assays no chalcone or stilbene formation was observed. Thus, a distinct enzyme (SPS) seems to be responsible for styrylpyrone formation in *Equisetum*.

The diploid sporophyte in contrast accumulates flavonoids and styrylpyrones, but the occurrence of these metabolites varied between organs, and their constitutive accumulation was strictly tissue-specific. In particular, styrylpyrones were detected in the rhizomes and flavonoids in the barren sprouts (Fig. 2). All organs contain considerable amounts of caffeic acid esters. Styrylpyrones as well as caffeic acid derivatives are known to have antimicrobial activity [22]. Thus, both groups of compounds together may act as effective resistance factors. In rhizomes the accumulation of these compounds could be either constitutive or in-

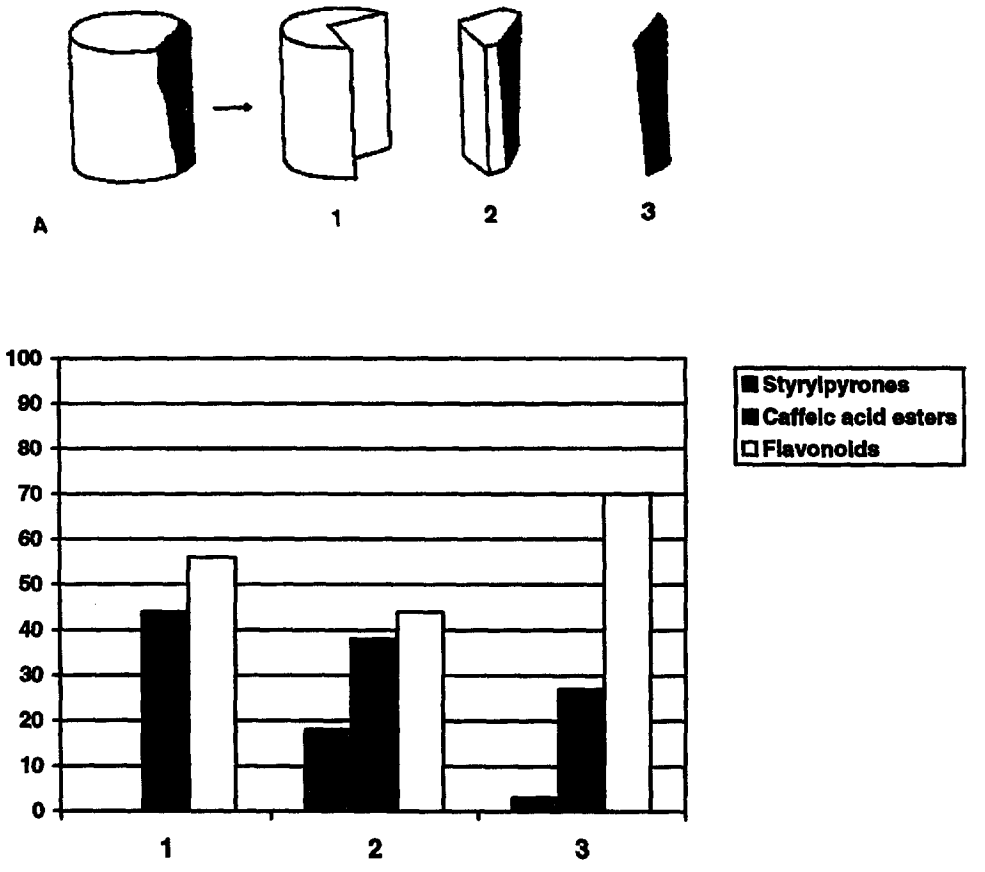


Fig. 3. Relative contents (%) of soluble styrylpyrones, caffeic acid esters and flavonoids (100% represents the sum (mg g^{-1} dry wt) of all three groups) in a stem section near a local necrotic area (black coloured) initiated by microbial infection at barren sprouts *E. arvense*.

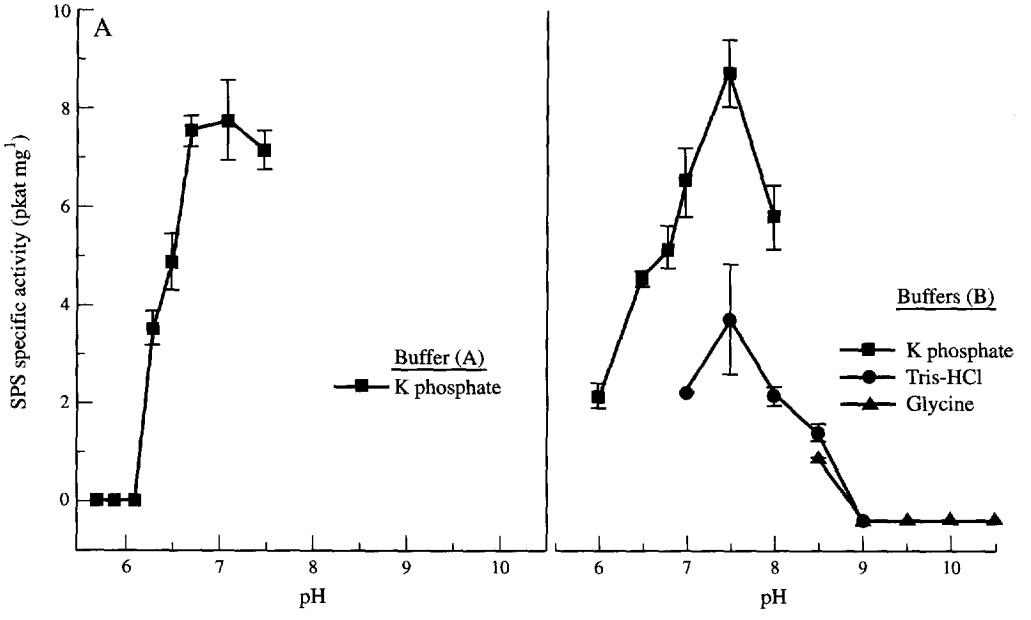


Fig. 4. pH dependence of SPS activities in selected buffers with different substrates determined in partially purified protein extracts from *E. arvense* gametophytes. Substrates: caffeoyl-CoA (A) and coumaroyl-CoA (B), respectively.

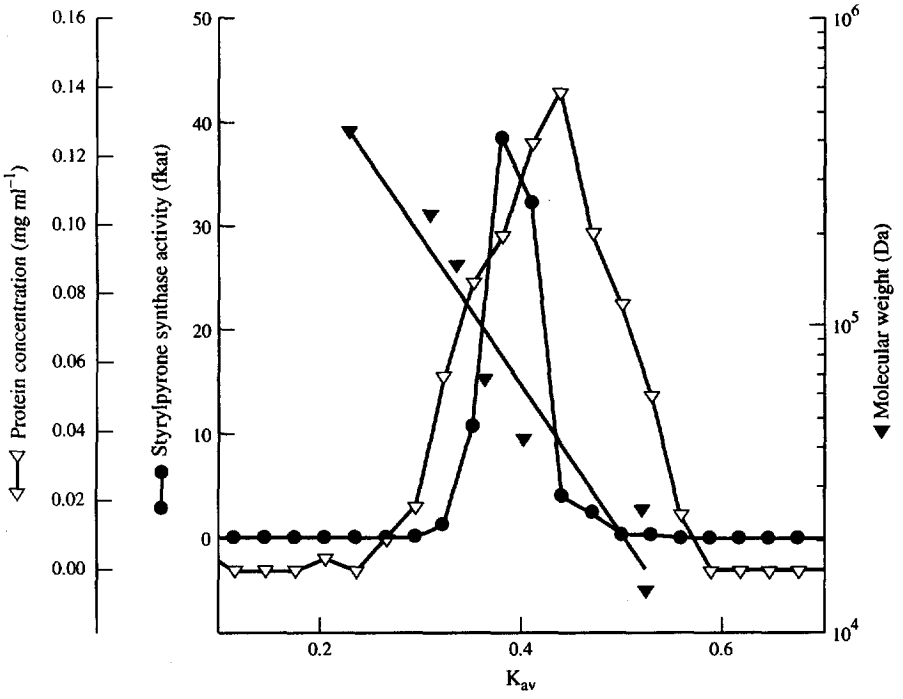


Fig. 5. Molecular weight determination of styrylpyrone synthase by FPLC on Superose-12™. Protein determination was performed according to ref. [21].

duced by the abundant presence of soil microorganisms. In the above-ground parts caffeoyl esters, however, appear to be a constitutive resistance factor for the plant, in particular prior to the formation of the main physical infection barrier, i.e. cell wall silica deposition [23]. However, SPS activity could be induced in this tissue in response to microbial attack or mechanical wounding. At the same time the concentration of caffeic acid esters in areas surrounding the site-localized infection remained unaffected (Fig. 3). This apparent defence mechanism was observed mainly towards the end of the vegetation period when the barren sprouts of *Equisetum* showed necrotic spots on their surfaces indicating local infections. Preliminary data on cell-wall-bound styrylpyrone dimers in necrotic areas of the barren sprouts and the cortical tissue of rhizomes suggest that they play an important role in the primary reinforcement of the cell wall elicited on exposure to microbial challenge or other stress. This response seems to be similar to that reported for cell wall conjugation of hydroxycinnamic acids reported for some other plants [24]. The compound liberated from cell wall preparations of *E. arvense* by dioxane/HCl acidolysis seemed to be a **2** dimer as it was revealed to have a similar molecular weight as a bishispidinyl moiety released from a so-called 'fungal lignin' isolated from fruiting bodies of Basidiomycetes [25]. The gametophytes also seem to depend on high constitutive levels of caffeic acid esters and styrylpyrones to ensure defence against pathogens. The possible role and regulation of the defence mechanisms in *Equisetum* will be the subject of future work.

While the enzymology of caffeic acid accumulation in *Equisetum* gametophytes has been studied extensively [23] little is so far known on the formation of styrylpyrones. Although the biosynthesis of **1** has been reported in fungi [25], its *in vivo* formation in higher plants has only been postulated [26]. Compound **1** has, however been described as an *in vitro* release product in chalcone synthase assays in the presence of SH compounds [15, 16] in cell free extracts from *Petroselinum* suspension cultures. In *Equisetum* gametophytes, the SPS catalysed formation of **1** and **2** seems to be the first step of a biosynthetic sequence leading, after further hydroxylation and subsequent glucosylation, to equisetumpyrone (**3**), the main styrylpyrone constituent in *E. arvense*.

In SPS enzyme assays with partially purified protein extracts from *Equisetum* gametophytes, *p*-coumaroyl-CoA was accepted as substrate at pH 6.0–9.0 in various buffer systems, whilst caffeoyl-CoA was only accepted in K-Pi buffers at pH values between 6.0 and 7.5. A similar dichotomy of the pH optima for these two substrates has been observed for CHS from various plant species [19, 28–30]. The lack of activity with caffeoyl-CoA under alkaline conditions might be the consequence of oxidation product formation, such as *o*-diquinones, which are known to strongly inhibit enzyme reactions by nucleophilic addition reactions of the protein. Regarding caffeic acid ester biosynthesis in *E. arvense* gametophytes it has been shown that these compounds are formed by the activity of hydroxycinnamoyl-CoA-dependent transferases, which mostly accept caffeoyl-CoA compared to *p*-coumaroyl-CoA

[23]. It therefore seems that caffeoyl-CoA may act as the main substrate for SPS also. Since 3′-hydroxylation of **1** and *p*-coumaroyl-CoA has been observed in the reaction assays the question of substrate specificity cannot be clearly answered at the present stage of enzyme purification. Phenolase-type enzyme activities seem to be involved since there is no evidence for cytochrome-P-450-dependent hydroxylation by monooxygenases in the partially purified enzyme fractions used in standard enzyme assays.

Several polyketide synthases have been found in plants that accept hydroxycinnamoyl-CoA with either one, two or three malonyl-CoA molecules as substrates (Fig. 1). Those enzymes using the lower number of malonyl-CoA units were found in more primitive plants, e.g. arylpyrones in *Psilotum* and styrylpyrones in *Equisetum*. The sequence: arylpyrones, styrylpyrones, flavonoids (see Fig. 1) might therefore represent an evolutionary trend in this type of polyketide biosynthesis. Other polyketide synthase reactions leading to benzalacetones and stilbenes as products (see Fig. 1) could be included in the sequence; however, the evolutionary relationship of the enzymes involved is still unknown. Recent work has provided evidence that stilbene synthase evolved from CHS, possibly more than once [31]. In *Equisetum* the more primitive gametophyte biosynthesizes only styrylpyrones, whereas the sporophyte is able to biosynthesize both styrylpyrones and flavonoids. Thus, SPS seems to catalyses putatively the more primitive reaction sequence (i.e. a two-step elongation with malonyl-CoA *versus* three-step elongation for CHS) in the more primitive organ. It is tempting to speculate, therefore, that CHS might have developed from SPS in the course of evolution. *Equisetum arvense* provides a suitable model system to study these closely related polyketide synthases and to answer key questions relating to phenylpropanoid metabolism.

EXPERIMENTAL

Plant material. Gametophytes of *E. arvense* were axenically cultivated on autoclaved MS medium [32] under illumination with light intensities between 1000 and 2500 lux. They were initially derived from single spore cultures in spring 1994 and were repeatedly subcultured for 6 weeks. The experiments were carried out with 1–2 year old cultures. Sporophytes were obtained from plants cultivated in the Botanical Garden of the University of Würzburg, Germany.

Protein extraction. The prepn of protein extracts was performed according to ref. [33]. All steps were carried out at 0–4°. The fresh gametophytes (30 g) were ground using a pestle and mortar in the presence of liquid N_2 and 50% (w/w) PVPP (Polyclar AT, Serva). The frozen powder was mixed with 100 ml 0.1 M K-Pi buffer (pH 6.8) containing 14 mM 2-mercaptoethanol, 40 mM Na ascorbate, 3 mM EDTA and 0.2 mM PMSF, which was flushed with N_2 prior to addition of Na ascorbate. After thawing, the mixt. was stirred for 30 min, then centrifuged (40 min at 28 000 *g*). The protein was pptd from the clear supernatant with $(NH_4)_2SO_4$ using a range from 40 to 65% satn. The pellet was dissolved in 2.5 ml 0.1 M K-Pi buffer (pH 6.8), 14 mM 2-mercaptoethanol, 40 mM Na ascorbate and 5% (w/v) trehalose and flushed with N_2 prior to addition of Na ascorbate. After desalting using a PD10 column (Sephadex G-25 M, Pharmacia) the protein was concd by ultrafiltration through an anisotrope membrane (Centricon-30, Amicon, Germany).

Substrates. Hydroxycinnamoyl-CoA thioesters were prepd by transesterification of hydroxycinnamoyl-*N*-hydroxysuccinimide esters with coenzyme A, as described elsewhere [34, 35]. [2-^{14}C]Malonyl-CoA (2.035 GBq mmol^{-1}) was from American Radiolabeled Chemicals, U.S.A. (distributed by Biotrend, Köln, Germany).

Standard styrylpyrone synthase assay. A slightly modified method of ref. [33] for CHS was used. The standard assay mixt. for measuring synthase activity contained 15 μl protein extract (2.5–8.0 mg ml^{-1} protein) and 70 μl 0.5 M K-Pi buffer (pH 6.8). The reaction was started by addition of 5 μl 20 mM malonyl-CoA and 10 μl 4 mM coumaroyl-CoA or caffeoyl-CoA. Incubation occurred at 30° for 30 min and was stopped by addition of 100 μl EtOH. After centrifugation the supernatant was used directly for HPLC analysis.

SPS assay with radiolabelled substrate. The radioactive SPS assay was performed under standard conditions with 45 μl protein extract (0.5 mg ml^{-1} protein), 45 μl K-Pi buffer (pH 6.8), 5 μl [2^{14}C] malonyl-CoA (5.85 μM) and 10μl *p*-coumaroyl-CoA (0.4 mM) and 5 μl aq. BSA (10% w/v). Incubation occurred at 30 min at 37° and was terminated by extraction of reaction products with 200 μl EtOAc. Detection of radiolabelled products was performed by TLC according to ref. [36] under UV light (366 nm) and radioactivity with a TLC radio scanner (Berthold, Wildbad, Germany). FPLC frs were analysed by monitoring for radiolabelled SPS reaction products in 150 μl aliquots of the EtOAc extracts by liquid scintillation counting (Beckman LS 3801, Germany) using 10 ml scintillation fluid (Hydroluma, Baker, Germany).

pH optimum. After standard protein extraction the following buffer systems at 400 mM each were used to determine the pH optimum of synthase activity with *p*-coumaroyl-CoA as substrate: actetate (4.0–5.0), bicine (7.3), K–Mes (6.7–7.1), Tris–HCl (7.3–9.0), imidazole HCl (6.7), Na citrate (5.6), glycine (8.5–10.5.) and K-Pi (6.0–8.0) and with caffeoyl-CoA as substrate: K–Mes (5.5–7.1), Tris–HCl (7.5–9.1), imidazole–HCl (5.9–7.7) and K-Pi (6.8–7.5).

SPS product detection by TLC. TLC was performed on cellulose plates (0.1 mm, Merck, Germany) with 35% aq. HOAc (v/v). R*f* values were: 0.27 (**1**) and 0.18 (**2**). Spot detection with UV light (366 nm) was enhanced by fuming the plates with NH_3.

HPLC. HPLC was performed with a gradient system (Beckman) comprising pumps (type 126), detector

(type 168) and integration software ('GOLD' ver. 7.11). Chromatographic conditions: temp., 15° thermostated; analyt. column, Nucleosil 120-5-C_{18}, (125 × 4 mm); guard column, Nucleosil 120-5-C_{18} (11 × 4 mm) (Machery and Nagel); elution profile: solvent A = 1.5% (v/v) aq. H_3PO_4, solvent B = 80% (v/v) aq. MeOH; gradient: 0 min, 80% A, linear to 30 min, 0% A, isocratically to 35 min; flow rate: 1.0 ml min^{-1}; detection: UV (256 and 370 nm). Peak purity and product identity were checked by comparison of R_t, on-line UV spectra (diode array detector) and data from HPLC APCI MS [37] with those obtained from reference compounds. R_t [λ_{max} (nm)]: caffeic acid 10.5 (323), *p*-coumaric acid 13.0 (328), caffeoyl-CoA 14.1 (256, 345), *p*-coumaroyl-CoA 15.3 (256, 333), **2** 17.9 (370), **1** 19.7 (363). Calibration data were obtained from **2** for glycosylated styrylpyrones, from **2** for non-glycosylated styrylpyrones, from dicaffeoyl-*meso*-tartaric acid for caffeic acid derivatives, from quercetin-3-*O*-glucoside for flavonole glycosides, and luteolin-5-*O*-glucoside for flavone glycosides according to ref. [8].

FPLC size exclusion chromatography. The M_r of SPS protein was determined from frs of 40–65% $(NH_4)_2SO_4$ satn pptn following concn by ultrafiltration to a final concn of 2 mg ml^{-1}. Portions (200 μl) of these frs were applied to a Superose 12™ column (Pharmacia) and sepd by FPLC (Pharmacia) as reported previously [38]. The proteins were eluted in 50 mM K-Pi buffer containing 1 mM DTT, 20 mM Na ascorbate and 50 mM NaCl, at a flow rate of 0.5 ml min^{-1}. Elution of protein was monitored at 280 nm.

Protein determination. Protein determination was performed according to ref. [21], using BSA as standard.

Cell wall preparations. Cell walls were prepd and extracted according to ref. [39]. Acid hydrolysis of insoluble material was carried out with dioxane–2 M HCl (9:1) at 70° for 8 hr.

Chemicals. Malonyl-CoA and coenzyme A (Sigma). caffeoyl-CoA and *p*-coumaroyl-CoA were prepd according to refs. [33, 34] and **2** was obtained synthetically according to ref. [40]. All other chemicals were of analyt. grade and were obtained from Merck, Sigma or Serva.

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