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DIFFERENTIAL REGULATION AND DISTRIBUTION OF ACRIDONE SYNTHASE IN *RUTA GRAVEOLENS*

IN HONOUR OF PROFESSOR G. H. NEIL TOWERS 75TH BIRTHDAY

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Key Word Index—*Ruta graveolens L.*; *Rutaceae*; acridone alkaloids; acridone synthase; elicitor induction; plant disease resistance; preformed defense.

Abstract—Cell suspension cultures of *Ruta graveolens* L. accumulate polyketide metabolites such as acridone alkaloids and flavonoid pigments. Whereas flavonoid synthesis is induced by light, the production of alkaloids can be enhanced in dark-cultured cells by treatment with fungal elicitors. Acridone synthase (ACS) catalyzes the committed condensing reaction of acridone biosynthesis yielding 1,3-dihydroxy-*N*-methylacridone from *N*-methylanthraniloyl- and malonyl-CoAs. The reaction proceeds in a manner analogous to that of chalcone synthase (CHS) which catalyzes the first committed step in flavonoid biosynthesis and cDNA and protein sequences of *Ruta* ACS possess a high degree of sequence homology to heterologous CHSs. ACS transcript abundance and specific activity were monitored in cultured *R. graveolens* cells irradiated either continuously with white light or treated with fungal elicitor over a period of 24 h and found to increase transiently upon elicitor treatment and to decrease upon light irradiation. Immunodetection with a rabbit polyclonal ACS antiserum revealed that the amounts of ACS polypeptide decreased slightly in light-irradiated cells but increased in elicitor-treated *Ruta* cells. Fluorescence microscopy and tissue print hybridizations were employed to aid in localizing the sites of storage and biosynthesis of acridone alkaloids in *Ruta* plants. Yellow fluorescing alkaloids were detected particularly in root tissue adjacent to the rhizodermis, but also in the endodermis and vascular tissue of the hypocotyl. ACS transcript abundance *in situ* followed the same spatial pattern, indicating that the synthesis of acridones likely proceeds at all sites of deposition rather than exclusively in the root. Expression *in planta* and the induction response of ACS suggest that the alkaloids serve as phytoanticipins or phytoalexins in the defense of *Ruta* particularly to soil-borne pathogens or as feeding deterrents.

INTRODUCTION

The common rue (*Ruta graveolens* L.) has been the object of numerous studies on the biosynthesis of secondary metabolites abundant in the *Rutaceae*. Among these are linear furanocoumarins and furoquinolines [1] as well as acridone alkaloids, a class of products that appears to be unique to the *Rutaceae* family [2–4]. Acridones accumulate in specialized cells, the idioblasts, and their distribution as bright-yellow fluorescing droplets in vacuoles can be easily monitored by fluorescence microscopy. Examination of individual *Ruta* organs indicated that the loaded idioblasts are distributed mostly in the root and root hairs and, to a lesser extent, in the stems and leaves [5]. This suggested that the root is the primary site of acridone synthesis, as was proposed for other alkaloids [6, 7], with subsequent translocation to the shoot. The formation of idioblasts, however, is independent of the stage of tissue differentiation since an identical pattern of deposition of fluorescent acridone droplets was also observed in *Ruta* cell suspension cultures [8].

Acridone alkaloids have attracted attention because of their mutagenic [9], antiviral [10] and antiplasmodial activities [11]. Their mode of action is

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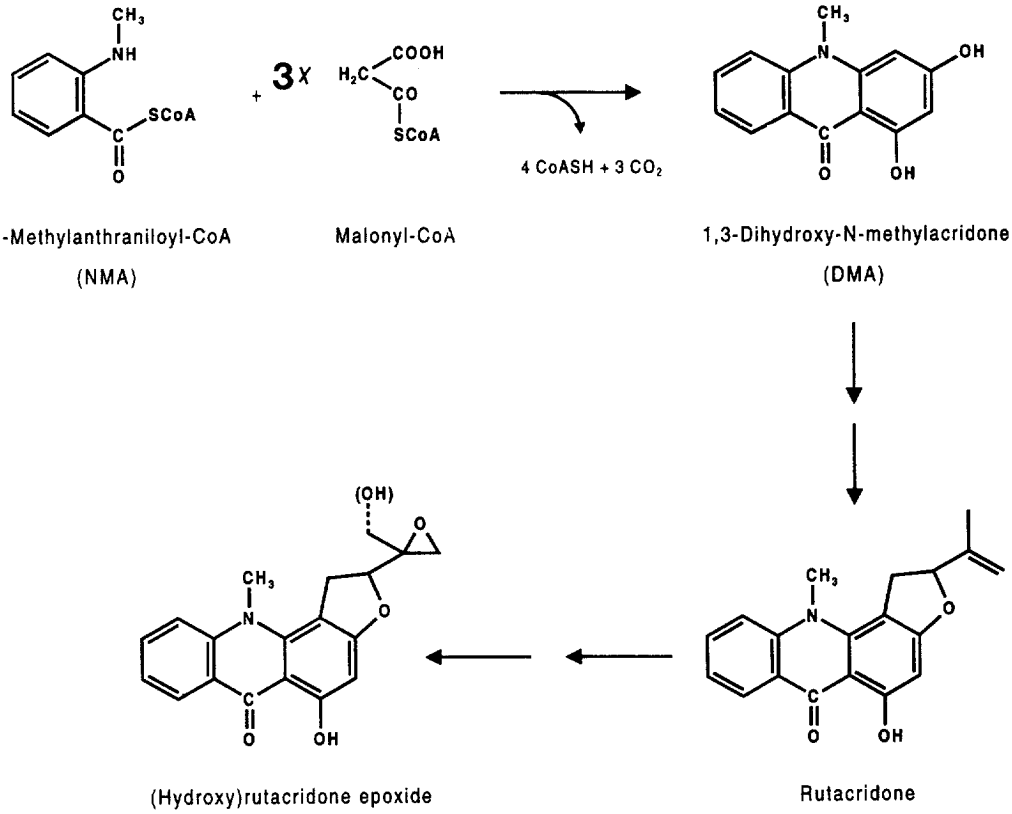

Fig. 1. Biosynthesis of acridone phytoalexins in *Ruta graveolens* L. The committed condensing reaction is catalyzed by acridone synthase.

likely based on DNA intercalation, which makes these compounds too toxic for therapeutic use. In plants, however, acridones may act as feeding deterrents, phytoalexins or phytoanticipins [12] and may significantly enhance resistance to microbial invasion. Considerable accumulation of acridone alkaloids in the root might be exploited to fight soil-borne pathogens which are difficult to control by other means.

The biosynthesis of acridones has been extensively studied in *Ruta* cell cultures and these cells have been important sources for the preparative isolation of the relevant enzymes (Fig. 1) [13–16]. The pivotal reaction of acridone formation is catalyzed by acridone synthase (ACS), a polyketide synthase which condenses *N*-methylanthraniloyl-CoA with three malonyl-CoAs to yield 1,3-dihydroxy-*N*-methylacridone (Fig. 1). Dark-cultured *Ruta* cells contain significant ACS activity, which can be enhanced by treatment of the cell cultures with a fungal elicitor causing an increased accumulation of acridones [17]. ACS cDNA was cloned from elicited *Ruta* cell cultures and the expression in *E. coli* yielded a catalytically highly active enzyme [18]. Although the sequences of DNA and translated polypeptide showed high homologies to those of heterologous chalcone (CHSs) and stilbene synthases (STSs), the *Ruta* ACS expressed in *E. coli* did not accept 4-coumaroyl-CoA as a substrate [18] nor was *N*-methylanthraniloyl-CoA accepted by heterologous CHSs or STSs (J. Schröder, Universität Freiburg, personal communication). Furthermore, in contrast to CHSs and STSs, which exist as catalytically active homodimers [19], ACS appeared to be active as a monomer. Thus, subtle differences in the primary polypeptide sequences are responsible for the diverse secondary structures and substrate specificities of known polyketide synthases. Furthermore, the transcriptional regulation of these three, closely related classes of polyketide synthases differs significantly, e.g. CHS is predominantly expressed during flower development and induced upon irradiation.

The close sequential interrelationship of taxonomically restricted ACS [4, 6] with the more abundant CHSs and STSs [20, 21], as well as its rather divergent substrate specificity and regulation of expression make ACS very attractive for molecular investigations. CHS and STS from a single plant have been extensively studied [19, 22, 23] and the comparison of ACS with the *Ruta* endogeneous CHS is a particularly appealing research goal. We describe in this report the differential induction characteristics of ACS and CHS in cultured *Ruta* cells as well as the localization of acridones and ACS transcripts in *R. graveolens* plants.

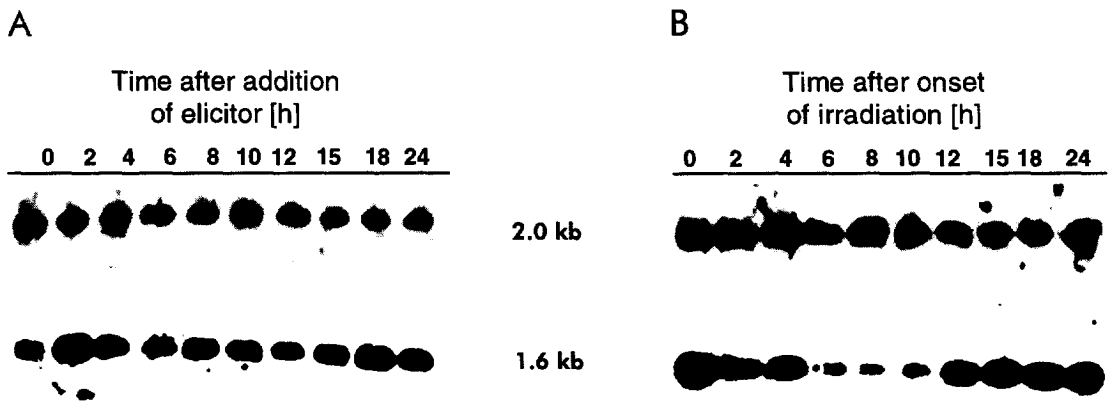

Fig. 2. Northern blot hybridizations of acridone synthase transcripts from elicitor-treated or light-irradiated *Ruta* cells. Poly(A)$^+$ RNA (2 μg/lane) from dark-cultured *Ruta* cells that had been induced for various time intervals with (A) *Pmg* elicitor or (B) white light irradiation was separated on a 1.2% agarose gel, blotted onto nylon filters and the blots were hybridized with ^{32}P-labeled ACS cDNA. The transient induction of ACS transcript amounts (1.6 kb) in *Ruta* cells had been recorded previously upon the addition of elicitor [18]. The autoradiographic signal intensities were compared by densitometric evaluation and equal loading of the gel was confirmed by rehybridisation of the blots with ^{32}P-labeled ubiquitin cDNA (2.0 kb).

RESULTS

Transcriptional regulation of ACS upon irradiation or elicitor treatment

The accumulation of acridone alkaloids in dark-grown *Ruta graveolens* cell cultures is stimulated upon elicitation and the ACS cDNA had been cloned from the cells treated for 6 h with *Pmg* elicitor [18]. This cDNA did not hybridize with CHS transcripts under stringent conditions [18]. The kinetics of ACS transcript abundance in *Ruta* cell cultures was monitored in response to continuous irradiation with white light or treatment with *Pmg* elicitor by Northern hybridization of the poly(A)$^+$ RNA from cells harvested at various intervals of treatment over a period of 24 h (Fig. 2). Calibrated equivalent amounts of mRNA were used in these studies and the blots were rehybridized with a ubiquitin cDNA probe for additional standardization. Hybridization and densitometric scanning revealed that ACS mRNA (1.6 kb) was present in non-treated cells and reached a transient maximum of approximately 3-fold intensity within 2–4 h of elicitation [Fig. 2(A)]. Thereafter, the specific mRNA decreased rapidly to the background level. When *Ruta* cultures were irradiated with continuous white light for 24 h, the response differed significantly. Under these conditions, the amounts of mRNA decreased rapidly to a minimum at 8 h after onset of the irradiation and increased again to the initial level after 12 to 15 h [Fig. 2(B)].

ACS protein levels after irradiation or elicitation

The polypeptide sequence translated from the ACS cDNA [18] revealed a high degree of homology to heterologous CHSs and STSs and polyclonal CHS antibodies crosshybridized with the *Ruta* ACS polypeptide [18]. Conceivably, extensive homology to the *Ruta* indigenous CHS is also to be expected. A stretch of nine amino acids with negligible sequence homology to CHSs and STSs was thus chosen from the ACS polypeptide. This nonapeptide was synthesized, coupled to ovalbumin and an ACS-specific polyclonal antiserum was generated in rabbit. The crude antiserum showed minor cross-reactivities with a few additional proteins in *E. coli* and plant extracts, and, therefore, the ACS-specific IgG fraction was isolated using a blot-affinity procedure [24]. The purified antibodies showed narrow specificity for ACS, which was documented by Western blotting experiments employing crude and purified ACS from *Ruta*, CHS from Scotch pine (G. Schröder, Freiburg) or crude extracts from parsley cells containing CHS (data not shown). The specificity was furthermore confirmed by immunotitration experiments in which ACS and CHS activities were quantified (Fig. 3).

The purified antibodies were used to measure the relative amounts of ACS protein in crude cell extracts of cultured *Ruta* cells after exposure to either continuous irradiation or elicitor treatment. Significant amounts of ACS enzyme are present in non-induced *Ruta* cells, which nevertheless increased continuously from 6 to 24 h following the addition of elicitor [Fig. 4(A)]. In contrast, continuous light irradiation of the cells induced a very subtle decrease in the amount of ACS protein over the entire duration of the experiment [Fig. 4(B)]. The divergent induction patterns were verified in these experiments by densitometric evaluation of the film signal intensities produced by the ECL™ detection system.

Regulation of ACS activity upon irradiation or elicitation

The ACS activity in crude extracts of elicitor-treated *Ruta* cells followed the observed time course of

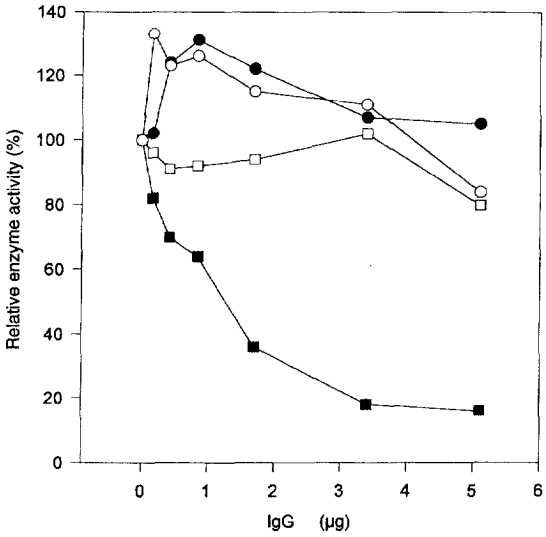

Fig. 3. Immunotitration of ACS and CHS synthase activities with anti-ACS IgGs. The enzyme assays were carried out with crude extracts from *Ruta* cells that had been treated with elicitor (ACS activity •, ■) or irradiated with white light for 15 h (CHS activity ○, □). The extracts (50 µg protein) were incubated for 1 h in the presence of anti-ACS (□, ■) or preimmune IgGs (○, •) (100 µl total volume), and the activities were determined from the supernatant following the centrifugation. The immunotitrations were carried out in triplicate and the relative enzyme activity of 100% corresponded to 3.7 µkat/kg.

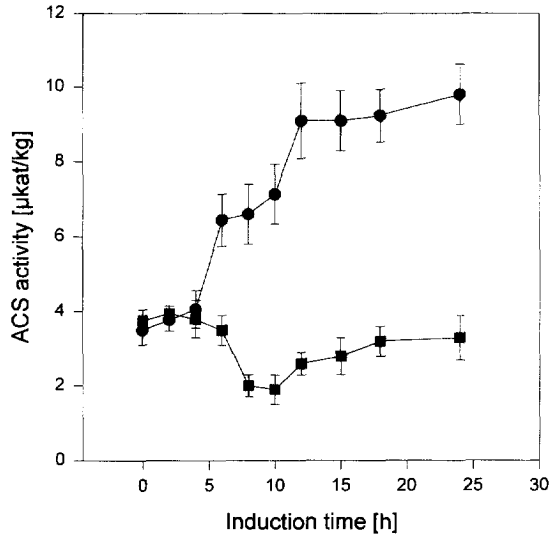

Fig. 5. ACS activity of crude extracts from elicitor- or light-treated *Ruta* cells. The extracts were prepared from dark-cultured *Ruta* cells that had been induced for various time intervals with (•) *Pmg* elicitor or (■) white light irradiation. Values are the average of five independent induction assays.

ACS enzyme protein levels. Significant activity was present in non-induced cells, which, after a lag of about 5 h, increased continuously to an approximately 3-fold level within 24 h following the addition of elicitor (Fig. 5). The opposite effect was observed on light irradiation, which suppressed the ACS activity to minimal values of roughly 50% after 8 to 10 h but the activity returned to a near-normal level within 24 h (Fig. 5).

ACS expression and distribution of acridones in Ruta plant tissues

The detrimental effect of light on acridone biosynthesis might suggest that acridone alkaloids are synthesized exclusively in the roots of *Ruta* plants, from where they are translocated to the shoot. This would be compatible with reports which ascribed the highest storage of *Ruta* acridones to the root [5]. Acridone alkaloids emit strong yellow fluorescence upon excitation at 330–500 nm and are readily detected in tissue sections by fluorescence microscopy [5]. Their spatial distribution can be correlated with the probable sites of acridone biosynthesis, if the ACS mRNAs are spotted in the same sections by tissue print hybridization employing an antisense RNA probe [25].

The expression of ACS mRNAs was observed in the root as well as in the hypocotyl and in the seed coat of *Ruta* plants. In the root, strong hybridization signals corresponded to the cell layers bordering the rhizodermis [Fig. 6(A)] and high ACS transcript abundance prevailed also in the hypodermis and vascular tissue of the hypocotyl [Fig. 6(B)] as well as in the seed coat [Fig. 6(C)]. The observed patterns faithfully

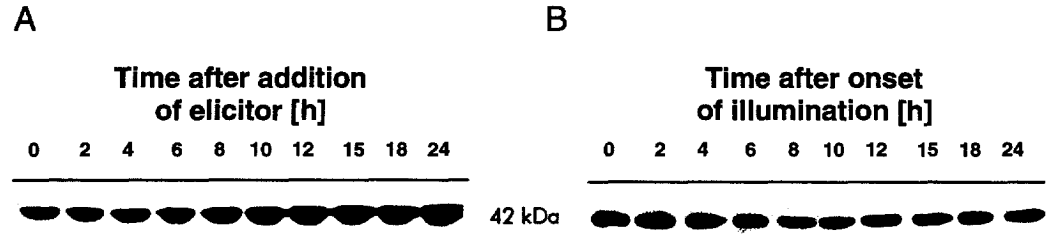

Fig. 4. Evaluation of ACS protein amounts by Western blotting employing anti-ACS IgGs. Crude extracts (10 µg protein) were prepared from dark-cultured *Ruta* cells that had been induced for various time intervals with (A) *Pmg* elicitor or (B) white light irradiation. The proteins were separated by 12% SDS-PAGE and blotted subsequently to nitrocellulose filters for crossreaction with the anti-ACS-IgGs. The crossreacting band was visualized by the ECL™ detection system, the Mr was compared to standard protein size markers and the relative signal intensities were evaluated by densitometric scanning.

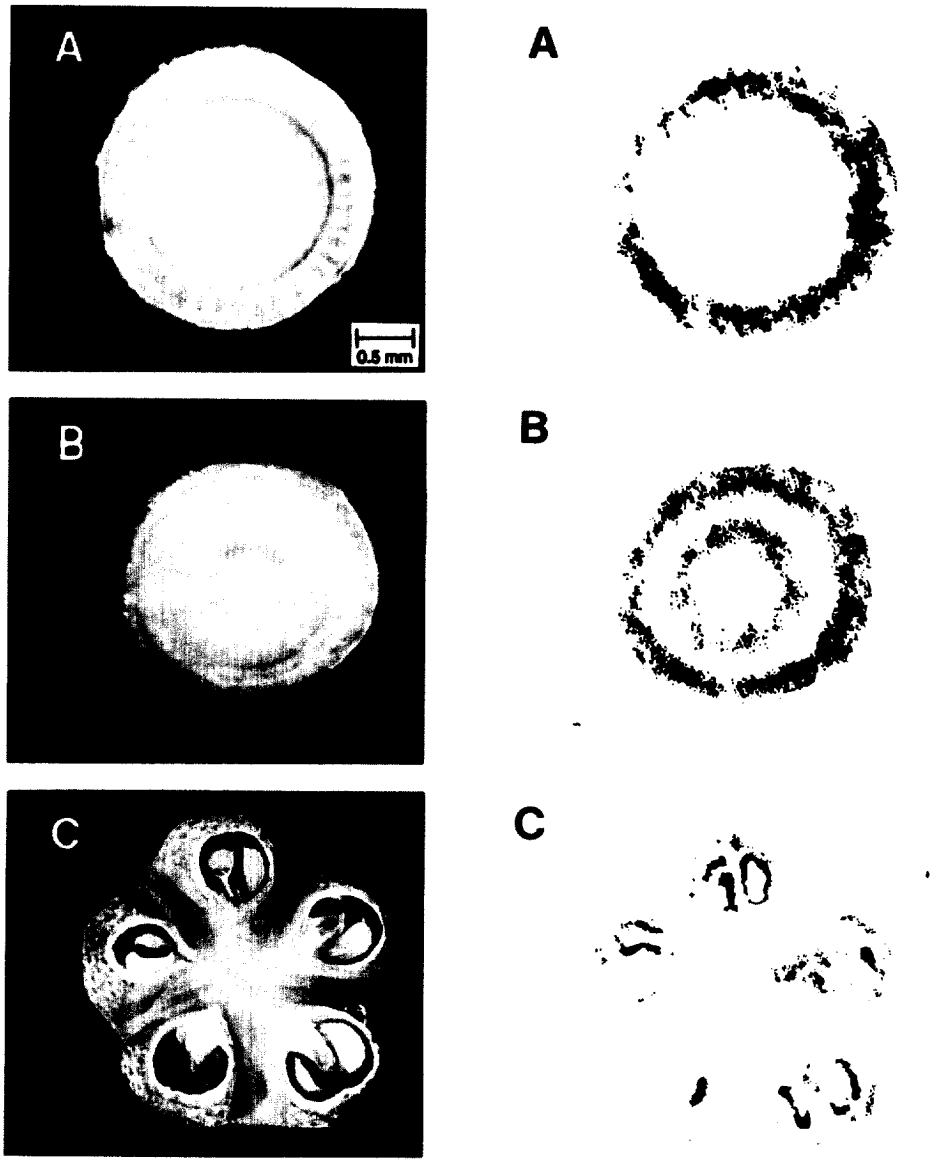

Fig. 6. Tissue-specific expression of ACS in *Ruta graveolens* L. plants. Hand-cut sections from the root, stem and fruit were printed onto nylon membranes and the spatial relative abundance of ACS transcripts was monitored by hybridization with the ^{35}S-labeled ACS antisense RNA probe using the tissue print hybridization technique [25]. Control hybridizations with labeled ACS sense RNA yielded scattered, weak background signals only (data not shown). Left column: unstained tissue sections of (A) the root, (B) the hypocotyl and (C) the fruit of *R. graveolens*. Right column: the spatial distribution of ACS mRNAs in the tissue sections detected by tissue print hybridizations.

represented the ACS mRNA distribution, since control hybridizations with the sense RNA probe failed to reveal discrete signals (data not shown). The fluorescence of acridone alkaloids was most prominent in the root tissue, where it was primarily present in idioblasts and in the parenchymatic cells of the protective tissue (kalyptra and rhizodermis or hypodermis) [Fig. 7(A)]. In the hypocotyl and leaf base, acridones accumulated in the vascular tissue as well as directly beneath the epidermis [Fig. 7(B) and (C)].

DISCUSSION

ACS catalyzes a polyketide condensing reaction very similar to those of CHS or STS but requiring an aminobenzoyl- rather than (hydroxy)cinnamoyl-CoA starter substrates (Fig. 1). The marginal differences in the substrate specificities of CHS and STS have long caught the attention and stimulated numerous mutational studies [19, 22, 23], whereas ACS was cloned only recently and shown to share a high degree of homology at both the DNA and polypeptide level

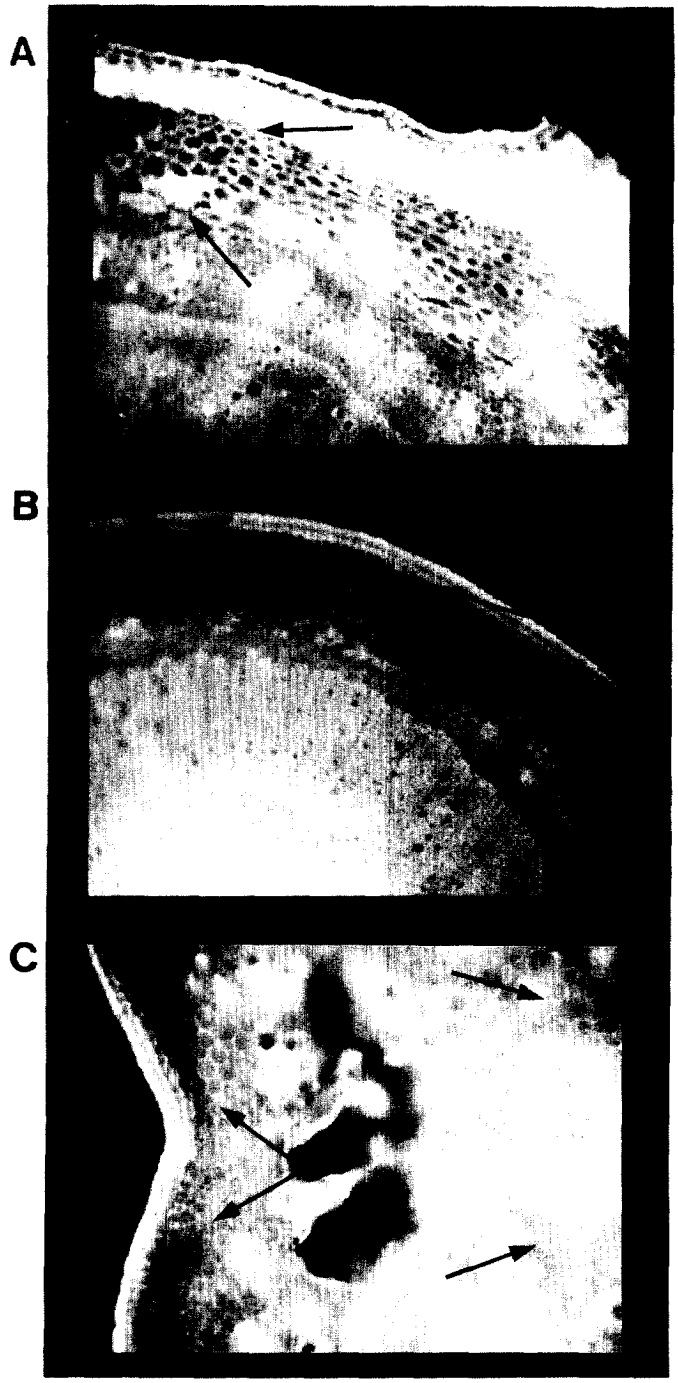

Fig. 7. Tissue distribution of acridone alkaloids in *Ruta graveolens* L. plants. The acridones were recognized under the microscope by their yellow fluorescence (330 nm excitation, 500 nm cut-off filter) and the sites of acridone deposition were denoted by arrows in hand-cut sections of (A) the root, (B) the hypocotyl and (C) the leaf base (magnification 185-fold).

with heterologous CHSs and STSs [18]. Nevertheless, ACS cDNA did not hybridize with CHS cDNAs from *Pinus sylvestris* (J. Schröder, Freiburg) or *Sinapis alba* (A. Batschauer, Freiburg) under stringent conditions, which was the basis for selective *de novo* induction studies. The cloning of *Ruta* endogenous CHS is now in progress and significant sequence differences to ACS are to be expected in the 4-coumaroyl-CoA binding site [19] and other DNA regions to account for the lack of cross-hybridization; these differences might reveal further information on the reaction mechanism.

Analogous to other enzymes of the plants' defense [26, 27], ACS mRNA abundance was transiently induced upon the elicitation of *Ruta* cells [Fig. 2(A)],

whereas the transcript level decreased upon light irradiation to a transient minimum at 6–8 h [Fig. 2(B)] and the differential mode of induction was basically confirmed by Western blotting experiments which revealed a steady 24 h-increase of ACS polypeptide levels in the elicited *Ruta* cells [Fig. 4(A)] while the amounts decreased slightly upon irradiation [Fig. 4(B)]. Such a pattern was to be expected, if the half-life of the enzyme exceeds 15 to 20 h. The determination of ACS activities again confirmed the significantly different effects of the elicitation vs light irradiation of *Ruta* cell cultures (Fig. 5). CHS activity is known to be stimulated by light irradiation in various plant cell cultures [23, 27] and, for comparison, the CHS activity of the *Ruta* cells, which remained negligible in dark-grown control cells, also reached a transient maximum at 12–15 h under these conditions (unpublished).

In contrast to the *de novo* irradiation induction [27, 28], light-induced down-regulation has been reported in only a few instances [29–35] and may be accompanied by light-dependent degradation of enzyme proteins as has been shown for NADPH-dependent protochlorophyllide oxidoreductase during chloroplast development [29, 34]. The transient down-regulation of ACS may aid in channeling malonyl-CoA into the accumulation of UV-filtering flavonoid pigments [36], a phenomenon which has been shown to be induced by light treatment in many plants [27, 35]. In this sense, the expression of *Ruta* ACS and CHS is mutually competitive and is conceivably under the control of diverse and tissue-specific promoters [23]. CHS is distributed ubiquitously in higher plants, whereas ACS occurs exclusively in the evolutionary advanced family of the *Rutaceae*. Cladistic analysis revealed that ACS does not group readily with heterologous CHSs or STSs (unpublished) and the evolution of *Ruta* ACS may have branched early from common ancestral genes. Thus, the *Rutaceae* appear to have managed to exploit CHS homologous genes for an entirely different purpose, although the phylogenetic relationship of CHS and ACS remains to be more closely examined.

The accumulation of acridones in distinct plant tissues can result from localized gene expression and/or product translocation processes; and the large amount of acridones in the root might suggest that root-to-shoot translocation operates in *Ruta* as has been shown for tropane alkaloids in solanaceous plants [37] or nicotine and pyrrolizidine alkaloids in *Senecio vulgaris* [7]. Furthermore, whereas an accumulation of alkaloids in the peripheral stem and leaf tissues is a common phenomenon (cf.[7]) *Ruta* acridone alkaloids accumulate in idioblasts [Fig. 7(A)] as well as in the vascular tissue [Fig. 7(B) and (C)]. The signals observed in tissue print hybridizations [Fig. 6(A)–(C)] correlated closely with all the microscopic sites of acridone deposition, including the vascular tissue, which clearly demonstrates that the acridones are most likely synthesized at the site of storage and that translocation is not required in *Ruta* plants. Furthermore, the hybridization signals from hypocotyl sections did not correspond solely to idioblasts but rather spread evenly through the hypodermal cells and were also present to some extent in the vascular tissue [Fig. 6(B)]. Since the autoradiographic resolution was sufficiently high, these results indicate that the acridones may be transported into the idioblasts from neighboring cell layers. Acridones such as rutacridone and hydroxyrutacridone epoxide (Fig. 1) possess potent antimicrobial activities, and their concentration may increase up to 100-fold two days after fungal elicitation [38]. The tendency of acridones to accumulate predominantly in the peripheral cell layers of the root and the fact that ACS expression responds to fungal elicitation may thus be exploited for the transformation of other plants with the ACS gene(s) to confer resistance against soil-borne pathogens such as *Phytophthora* spp.

EXPERIMENTAL

Enzymes and materials

Biochemicals, vectors, *E. coli* host strains, restriction enzymes and DNA and RNA modifying enzymes were purchased from Boehringer-Mannheim, Pharmacia, BRL, Stratagene and USB. All reagents were of analytical grade.[^{35}S]UTPαS (approximately 37 TBq · mmol^{-1}) and [2-^{14}C]malonyl-CoA (1.89 GBq · mmol^{-1}) were from Amersham-Buchler and malonyl-CoA was purchased from Sigma. 4-Coumaroyl-CoA, *N*-methylanthraniloyl-CoA and 1,3-dihydroxy-*N*-methyl-acridone had been synthesized previously [16, 18].

Cell cultures

Cell suspension cultures of the common rue (*Ruta graveolens* L. strain R-20) [16]; were propagated in the dark [16] and the cells were induced by either the addition of crude elicitor (5 mg/40 ml culture) from the cell wall of *Phytophthora megasperma* f. sp. *glycinea* (*Pmg* elicitor) [39] syn. *Phytophthora sojae* or by irradiation with white light (18.4 W/m^2). The elicitor was routinely dissolved in hot fresh cell culture medium which by itself had no inducing effect.

Crude enzyme extracts and enzyme assays

Cell-free extracts for enzyme determination and mRNA isolation were prepared from cells harvested after various time intervals of induction, frozen immediately in liquid nitrogen and stored at −70°C until use. The frozen cells were extracted by grinding for 5 min with an equal volume of 100 mM sodium phosphate buffer pH 7.5, containing 20 mM sodium ascorbate, 1 mM DTT, 0.5 mM EDTA and 10% (v/v) glycerol. The extract was cleared by centrifugation (12,000 × *g*) and the supernatant was used immedi-

ately for enzyme assays or SDS-PAGE. ACS and CHS activity assays were carried out as described [16, 40].

Polyclonal antisera

The peptide A–L–F–S–A–N–I–D–T with negligible homology to heterologous CHSs was chosen as a hapten from the translated ACS polypeptide sequence. The peptide was synthesized and coupled with BDB to ovalbumin (27 mol peptide/mol ovalbumin) (Neosystems). The conjugate (250 μg) in Freund's adjuvans was injected s.c. into a New Zealand White rabbit followed by four booster injections (500 μg each) at 4 week intervals. The serum used in this report was collected two weeks following the second booster injection. The ACS-specific IgG fraction was purified from the crude antiserum by blot-affinity purification [24]. Briefly, homogeneous ACS polypeptide was separated on a 12% SDS-PAGE gel and electroblotted to nitrocellulose. The protein band was visualized with Ponceau S, cut from the filter and blocked overnight with 1% BSA in TBS (50 mM Tris · Cl pH 8.1, 150 mM NaCl). The strips were then incubated overnight with the crude antiserum and washed subsequently 5 times with TBS containing 0.05% Nonidet P-40. Bound IgG was eluted from the filter strips in 1 ml 50 mM glycine pH 3.0 and immediately neutralized with 2 M Tris–HCl pH 9.0. Preimmune serum IgG was purified by chromatography on a Protein A Sepharose column (1.6 × 10 cm) (Pharmacia). The serum (5 ml) was applied to the column which had been equilibrated with 100 mM Tris–HCl buffer pH 7.2. The column was washed further with this buffer until the absorption of the eluate returned to baseline. The IgG fraction was eluted with 100 mM glycine–HCl buffer pH 3.0 and the eluate was immediately adjusted to pH 8 with 1 M Tris–HCl buffer pH 9. The IgG fraction was concentrated by ultrafiltration.

Immunotitration and Western blotting

Crude extract (10 μg protein in approximately 10 μl for Western blotting; 50 μg protein in 40 μl for immunotitration) from *Ruta* cells that had been induced for 15 h by treatment with elicitor or by irradiation with white light were incubated with either ACS or preimmune IgGs. After 1 h at room temperature, 20 μl of Pansorbin (Calbiochem) was added and the incubation was continued for an additional 30 min at room temperature. The Pansorbin precipitate was pelleted in a microfuge (15,000 × *g* for 15 min at 4°C) and ACS [16] and CHS [40] activities in the supernatant were determined. SDS-PAGE and Western blotting (equivalent protein amounts/lane) was carried out using 12% separation and 5% stacking gels. Fixing and staining was accomplished in 50% methanol, 10% acetic acid, 0.1% Serva Blue R (Serva, Heidelberg) and destaining in the same solution without Serva Blue R. For Western blotting, proteins were transferred to cellulose nitrate filters (0.45 μm, Schleicher and Schuell, Dassel) in transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol) and blocked with 1% BSA in TBS (50 mM Tris–HCl buffer pH 8.1, containing 150 mM NaCl). The blots were treated with diluted ACS IgGs in TBS (1:300, 1.3 ng/μl) and crossreacting proteins were visualized with the ECL™ Western blot detection system (Amersham-Buchler). The semi-quantitative determination of ACS polypeptide in the range of 0.1 to 5 μg had been confirmed with this detection system employing purified ACS expressed in *E. coli* [18]. Developed films were quantified by densitometric scanning (five scans per lane) using an LKB 2202 Ultroscan Laser Densitometer.

Isolation of mRNA and Northern blotting

Total RNA was isolated from deep-frozen cells using an RNeasy Midi Kit (QIAGEN) with a modified protocol. Frozen cells (10 g) were homogenized with quartz sand (1 g) in 10 ml warm (65°C) Buffer RLT and 10 μl 2-mercaptoethanol for 5 min using a mortar and pestle. The homogenate was filtered through nylon mesh (100 μm) and centrifuged (15,000 × *g* at 20°C for 10 min). The supernatant was passed 5 times through a 28-gauge needle and centrifuged (15,000 × *g* at 20°C for 10 min). Ethanol was added to the supernatant (final concentration, 42%), and the standard RNeasy procedure was continued. Poly $(A)^+$ RNA was isolated from total RNA using Oligotex mRNA Midi Kit (QIAGEN). Equivalent amounts of the mRNA (2 μg/lane) were separated by agarose gel electrophoresis [41] and blotted on Hybond-N^+ membranes for hybridization [42]. Blots were prehybridized overnight at 42°C with herring sperm DNA (10 μg/ml) in 6-fold SSC and 5-fold Denhardt's solution [42] containing 0.5% SDS. Hybridization was continued for an additional 24 h at 62°C after the addition of the labeled ACS cDNA [18] or ubiquitin probe (A. Batschauer, Universität Freiburg). The filters were washed twice in 6-fold SSC containing 0.1% SDS at room temperature for 20 min followed by one wash at 72°C for 5 min and the dried filters were subjected to autoradiography [42]. The relative ACS transcript abundance was compared to the amount of $poly(A)^+$ RNA determined spectroscopically (2 μg/lane) and equal loading of the gel was confirmed on the basis of the ubiquitin mRNA abundance monitored by rehybridization of the filters after washing in 0.1 SSPE. The film signal intensities were quantified by densitometric scanning (five scans per lane) using an LKB 2202 Ultroscan Laser Densitometer.

Tissue print hybridization and microscopy

Stems and roots of 8-month-old *Ruta* plants were hand-sectioned, printed onto Hybond-N membranes (Amersham) and cross-linked as described [25]. Tissue sections were photographed under a dissecting microscope. pBluescript SK containing the ACS cDNA [18]

was used for the synthesis of ^{35}S-labeled sense or antisense RNA probes [42]. The blotted filters were hybridized and washed using the conditions described for the Northern blotting experiments. Fluorescence microscopy and photography was carried out using an Axiophot microscope (Zeiss). The intensive yellow fluorescence upon excitation at 330 nm [8] identified the acridone alkaloids in the tissue sections.

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