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Expression of a stilbene synthase gene in *Nicotiana tabacum* results in synthesis of the phytoalexin resveratrol

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Abstract

A gene from groundnut *(Arachis hypogaea)* coding for stilbene synthase was transferred together with a chimaeric kanamycin resistance gene. It was found to be rapidly expressed after induction with UV light and elicitor in tobacco cells *(Nicotiana tabacum)*. Comparative studies of stilbene synthase mRNA synthesis in groundnut and transgenic tobacco suspension cultures revealed the same kinetics of gene expression. Stilbene synthase specific mRNA was detectable 30 minutes after elicitor induction and 10 minutes after UV irradiation. The maximum of mRNA accumulation was between 2 and 8 hours post induction. 24 hours after induction stilbene synthase mRNA accumulation ceased. Furthermore, in transgenic tobacco plants, the gene was found to be inducible in sterile roots, stems and leaves. Stilbene synthase was demonstrated in crude protein extracts from transgenic tobacco cell cultures using specific antibodies. Resveratrol, the product of stilbene synthase, was identified by HPLC and antisera raised against resveratrol.

Introduction

A large proportion of the world's potential yield of crop plants is continually being destroyed by pests, plant diseases and weeds. Besides weed and pest control there is an urgent need to exploit all possibilities which are suitable for preventing or reducing the attack of crop plants by fungal pathogens.

As a result of multiple defence reactions, either constitutive or inducible, plants can be resistant against plant diseases [for review see 1, 26]. It is believed that induced defence reactions play an important role in resistance. Several genes are rapidly activated in plants during the complex interactions of pathogen and plant upon infection [3, 19].

The rapidly induced synthesis of 'pathogen-related (PR)' proteins, for example hydrolytic enzymes like chitinases or glucanases [11], and the pathogen-induced synthesis of phytoalexins in plants [for review see 4] are thought to play a role in the early defence mechanisms [21, 10]. One possibility to enhance desirable resistance in plants therefore might be the transfer of additional genes coding for resistance reactions.

Stilbenes, a group of secondary plant products with phytoalexin properties and antifungal activities [7], are synthesized in a limited number of plant families [5]. The formation of stilbenes is considered to be a part of the general defence mechanisms of these plants [for review see 7].

In *Vitis vinifera* a correlation of resveratrol concentration and resistance to the fungus *Botrytis cinerea* has been demonstrated [27] and it has also been shown that in Sitka spruce *(Picea sitchensis)* stilbenes are involved in the defence against fungal challenge [32]. The synthesis of stilbenes is either constitutive or induced by fungal attack and other environmental stress conditions. In groundnut cell cultures the synthesis of stilbenes can be induced by UV irradiation, stress, or fungal elicitor [9, 23, 29]. Stilbene synthase, the key enzyme in the biosynthesis of stilbenes in groundnuts *(Arachis hypogaea)*, has been described [18].

It converts one molecule of p-coumaroyl-CoA and three molecules of malonyl-CoA into 3,4′,5-trihydroxystilbene [9] commonly known as resveratrol (Fig. 1).

There is no stilbene synthase in tobacco, nevertheless the substrates necessary for the synthesis of resveratrol are present and are used by chalcone synthase (CHS). Therefore, the synthesis of resveratrol should be possible in tobacco plants provided they are complemented with a stilbene synthase gene.

For this reason, a genomic stilbene synthase clone [24] has been transferred to *N. tabacum*. The expression of the foreign gene has been studied at the level of mRNA, protein and product. The induction of the stilbene synthase mRNA synthesis by fungal elicitor and UV irradiation in tobacco callus was analysed and the time course of mRNA accumulation in transgenic tobacco and groundnut cultures was monitored. Stilbene synthase protein was detected in tobacco cells. The synthesis of resveratrol and the kinetics of resveratrol accumulation was determined in trangenic tobacco cell suspensions.

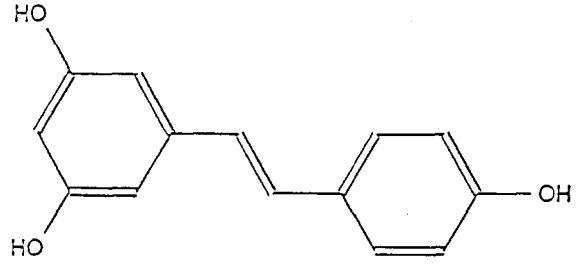


Fig. 1. Chemical structure of resveratrol.

Materials and methods

Plasmids

The plasmid pGS828.1 (EP A 0309862) contains a stilbene synthase gene isolated from groundnut [24, 25]. pLGVneo2103 contains a chimaeric kanamycin resistance gene [6].

Plant material and transformation procedure

The suspension culture of groundnut has been described [16]. It was subcultured each week. Groundnut callus was established by plating suspension cells on solid SH medium [22]. *Nicotiana tabacum* Petit Havana SR1 [13] protoplasts were isolated from sterile shoot cultures and transformed by direct DNA uptake [6]. For cotransformation 20 μg of linearized pGS828.1 and 10 μg of pLGVneo2103 cut with *Eco* RI and *Hin*d III were cotransformed to SR1 protoplasts. Transformants were selected on 75 μg/ml of kanamycin sulphate according to Hain *et al.* [6]. Tobacco suspension cultures were established on liquid MS medium [15] containing 1 mg/l NAA and 0.2 mg/l Kinetin.

Induction of stilbene synthase mRNA synthesis in tobacco and groundnut by fungal elicitor and UV light

About 1 g of tobacco or groundnut callus as well as leaf, stem and root segments of tobacco were preincubated for 4–12 h in liquid LS medium [12] or SH medium [22] respectively. A crude water-soluble elicitor preparation of *Phytophthora mega-*

sperma (PMG elicitor) was then added to a final concentration of 25 μg/ml. Suspension cell cultures were induced four days after subculture by adding 25 μg/ml of elicitor. For UV light induction tobacco and groundnut calli were illuminated for 5–10 min on a transilluminator (305 nm).

RNA isolation and northern hybridization

RNA was isolated and purified as previously described [24] and separated on a denaturing 1.5% agarose/formaldehyde gel and transferred to nitrocellulose filters according to Maniatis *et al.* [14]. As probe a stilbene synthase cDNA fragment [24] was labelled by nick translation (Amersham Buchler) to a specific activity $>4 \times 10^8/\mu g$ DNA. Hybridization was for 24 h at 42 °C and washes at 50 °C in 0.1 SSC and 0.1% SDS. X-ray films were exposed for 2 h – 5 days at – 70 °C using intensifying screens.

In vitro *synthesis and analysis of radioactive protein*

Protein synthesis *in vitro* with a rabbit reticulocyte lysate was performed as recommended by the supplier (Amersham Buchler, Braunschweig, FRG) and immunoprecipitations with stilbene synthase specific antisera [23] were carried out according to Buchmann *et al.* [2].

Determination of resveratrol by HPLC and ELISA

Resveratrol was linked to bovine serum albumin (BSA, Sigma) using diazonium salt coupling [28] with diazotized p-amino-hipporyl BSA. The haptenization of BSA was performed as described [30]. The overall coupling ratio was determined to be approximately 12 molecules resveratrol per molecule of BSA. Another conjugate was made for coating ELISA plates during the immunoassay procedure. The synthesis was done as described above with p-aminobenzoyl derivatives from ovalbumin (Sigma).

Polyclonal antisera against resveratrol were raised in rabbits (strain: Chbb:HM, variety 'Kleiner Russe'). After 4 preimmunizations (intradermal application, weekly intervals), boost injection and serum sampling was performed in a 4-week cycle. Rabbit serum was collected over a period of one year. Serum fractions were pooled. The IgG fraction was prepared as described [8]. Freeze-dried immunoglobin was stored at – 20 °C.

Antibodies raised against resveratrol were conjugated to horseradish peroxidase (HRP) according to the perjodate method [31]. The ELISA test was based on a competitive antibody capture design. Microtitration plates were coated with

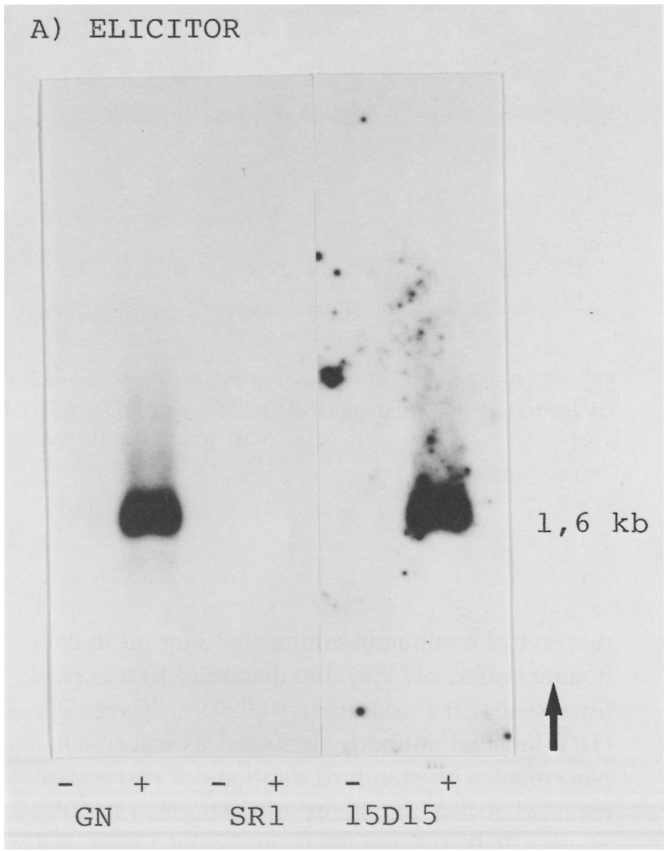


Fig. 2a. Northern blot analysis of 20 μg RNA isolated from elicitor-treated (+) and non-treated (–) calli. RNA of groundnut callus (GN), SR1 tobacco wild-type (SR1) were used as controls. 15D15 is a transgenic tobacco callus regenerated after cotransformation of pLGVneo2103 and pGS828.1 to SR1 tobacco protoplasts. Panels GN and SR1 were exposed for 1.5 h and the other panels for 60 h.

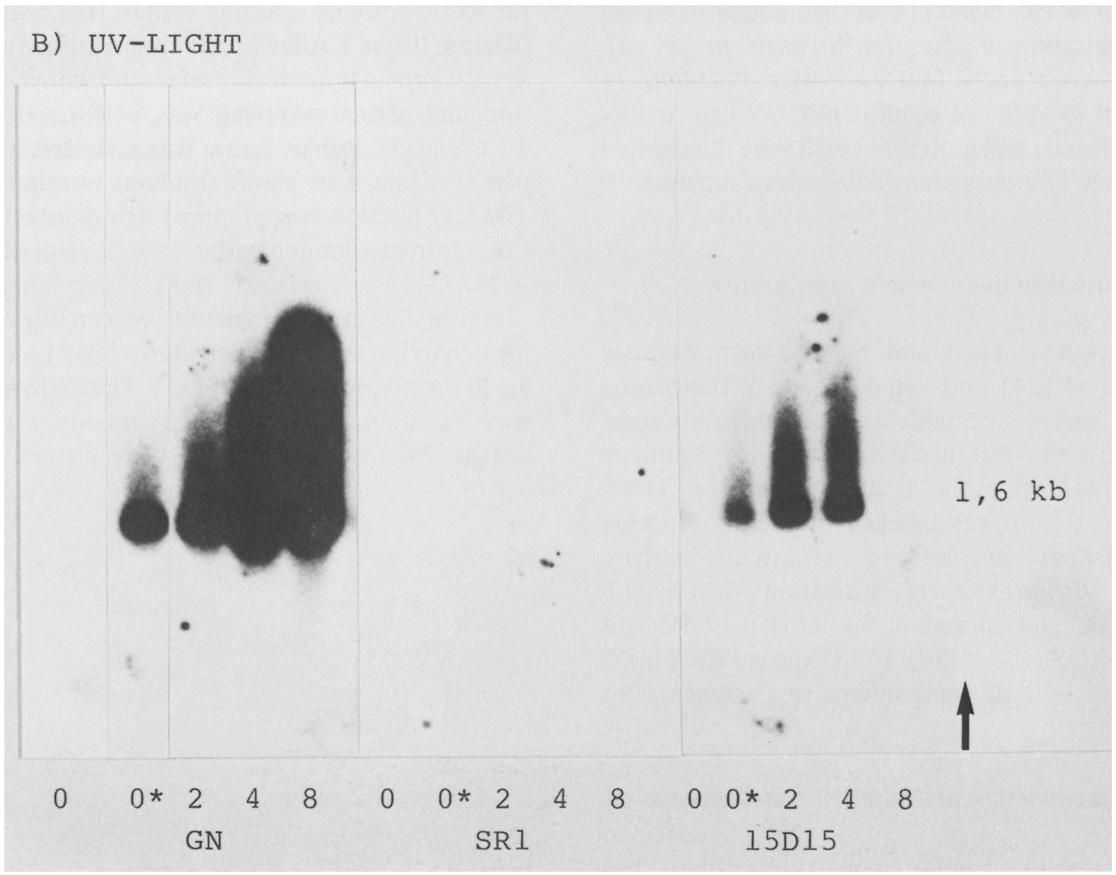


Fig. 2b. UV light-induced synthesis of stilbene synthase mRNA in tobacco callus. Northern blot analysis of RNA isolated from UV light-irradiated groundnut, SR1 tobacco and 15D15 callus. RNA was isolated before UV irradiation (time = 0), immediately after irradiation (0*) and 2, 4, 8 h after UV induction.

resveratrol-ovalbumin conjugate (3 μg/ml in carbonate buffer, pH 9.6). Incubation (3 h) was performed in PBS containing 0.0001% Tween 20. HRP-labelled antibody was used as tracer. Displacement with standard dilutions of resveratrol revealed a test sensitivity of 10 ng/ml (at 50% binding B/B_0). Samples from frozen tissue and plant material were homogenized in liquid nitrogen and extracted in chilled methanol (5 ml/g fresh weight). All samples were kept at −20 °C until final testing.

For determination of resveratrol by HPLC induced and non-induced cells of 15D15 and SR1 were treated with 1 ml methanol/g fresh weight for 24 h. Cell debris was removed by centrifugation and the supernatant was mixed with water and butyl-methyl-ether (1:2:1). After phase separation the ether phase was removed, dried under argon and redissolved in methanol. The content of resveratrol in such extracts was estimated by reversed-phase HPLC on a Nucleosil C18 column (Macherey-Nagel, 120 × 5 mm) using H_2O-acetonitrile as eluate. The retention time of 8 min for resveratrol was determined with chemically synthesized trans-resveratrol.

Results

Transfer and expression of a stilbene synthase gene in tobacco cells

The plasmid clone pGS828.1 containing a genomic fragment coding for stilbene synthase [24, 25] was cotransferred with plasmid pLGVneo2103 [6] to allow kanamycin selection after gene transfer to SR1 tobacco protoplasts. Kanamycin-resistant calli were subsequently analysed for their elicitor-induced synthesis of stilbene synthase specific mRNA by northern blotting using a stilbene synthase cDNA probe. Of 21 calli analysed, 7 calli synthesized after induction stilbene synthase specific mRNA of the correct size of about 1.6 kb (Fig. 2a). Two transgenic lines (15D15 and 16D10) were further analysed.

In groundnut stilbene synthase mRNA can be induced by fungal elicitor and UV irradiation. In order to study the elicitor-induced mRNA accumulation in transgenic tobacco callus, we used a crude, water-soluble elicitor preparation of

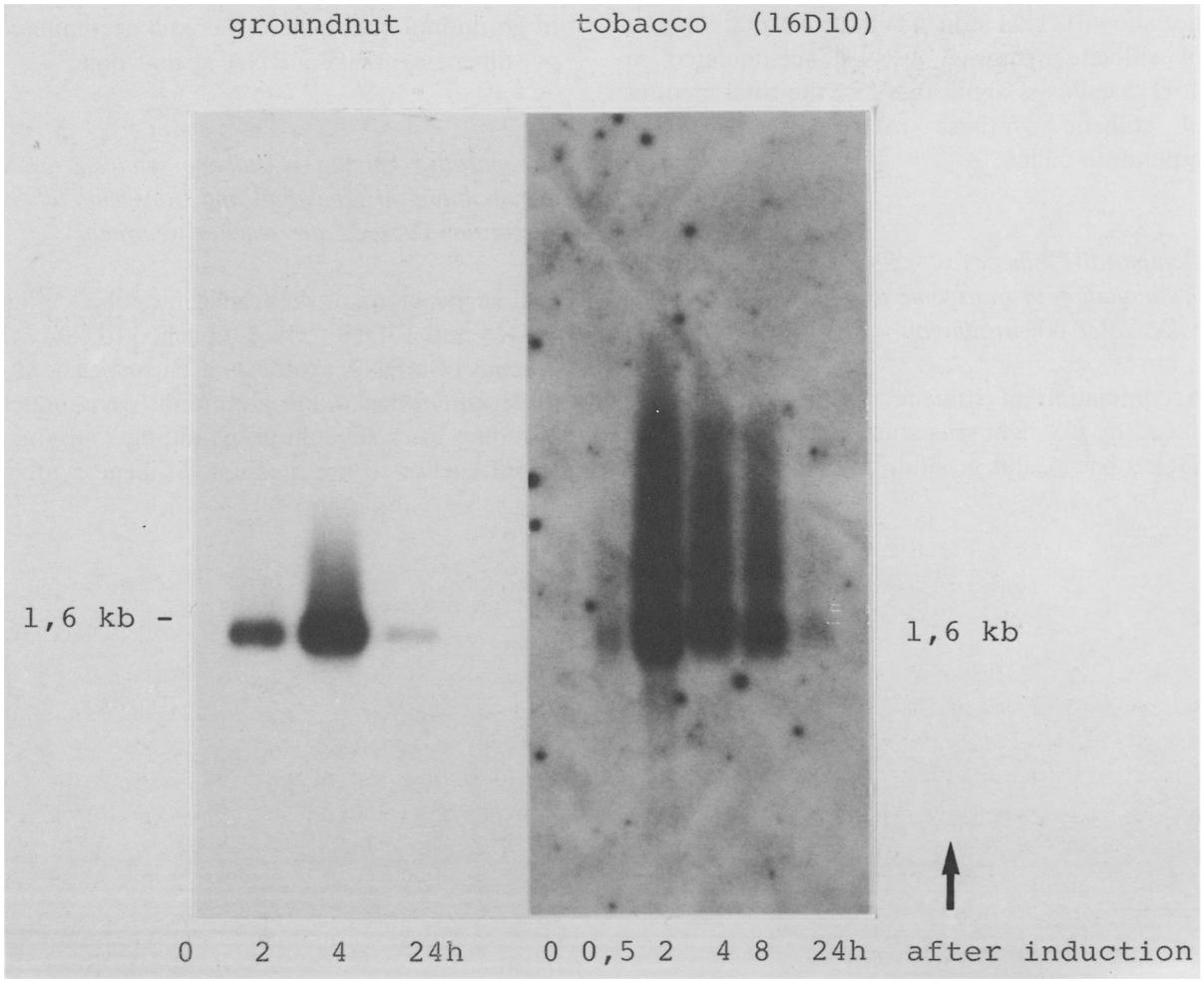


Fig. 3. Comparative kinetics of stilbene synthase mRNA accumulation in tobacco and groundnut. Transgenic tobacco and groundnut suspension cultures were treated with 25 μg/ml of PMG elicitor. RNA from groundnut cultures was isolated before addition (0), 2, 4 and 24 hours after addition of elicitor. In addition, tobacco (16D10) RNA was analysed 30 min (0,5) and 8 h after induction. Each panel contains 20 μg of total RNA. Films were exposed for 24 h (groundnut) and for 10 days in case of tobacco (16D10).

P. megasperma (PMG) at a concentration of 25 μg/ml. Transgenic callus 15D15 was pre-incubated in liquid LS medium for 12 hours and then treated with PMG elicitor for 4 to 8 hours. Groundnut callus and wild-type SR1 tobacco callus were used as controls. 20 μg of total RNA isolated from induced and non-induced material were analysed by northern blotting.

The elicitor-induced accumulation of stilbene synthase mRNA was detected in callus 15D15 with a groundnut cDNA probe (Fig. 2a). Signals of similar intensity were obtained when 100 ng of total RNA from groundnut and 20 μg of 15D15 RNA were analysed in parallel experiments (data not shown). This indicates that the total amount of stilbene synthase mRNA accumulated in 15D15 callus is about 0.5% of the total amount of stilbene synthase mRNA detectable in groundnut callus.

Comparative kinetics of stilbene synthase MRNA accumulation in transgenic tobacco and groundnut callus after UV irradiation

Accumulation of stilbene synthase mRNA induced by UV light was studied in 15D15 callus. SR1 tobacco and groundnut calli were used as controls. Callus material was irradiated for 10 min on a transilluminator (305 nm). RNA was isolated before illumination ($t = 0$), immediately after illumination ($t = 0^*$), and 2, 4, 8 h after irradiation ($t = 2, 4, 8$) and analysed by northern blotting using the cDNA probe. Analysis of each 20 μg of total RNA isolated from 15D15 callus showed the transferred stilbene synthase gene to be induced by UV irradiation. Immediately after the illumination ($t = 0^* = 10$ min) accumulation of stilbene synthase mRNA could be detected. The accumulation of mRNA reached its maximum between 2 and 4 hours and ceased after 8 hours (Fig. 2b) in 15D15 tobacco callus, whereas in groundnut callus there was still accumulation of stilbene synthase mRNA at that time.

Comparative kinetics of stilbene synthase mRNA accumulation in groundnut and transgenic tobacco suspension cultures after elicitor treatment

Cell suspensions of the transgenic tobacco lines 15D15 and 16D10 were established to study the kinetics of mRNA synthesis. Cell suspensions of transgenic tobacco and groundnut were induced 3–4 days after subculture by adding 25 μg/ml of PMG elicitor to the medium. Stilbene synthase

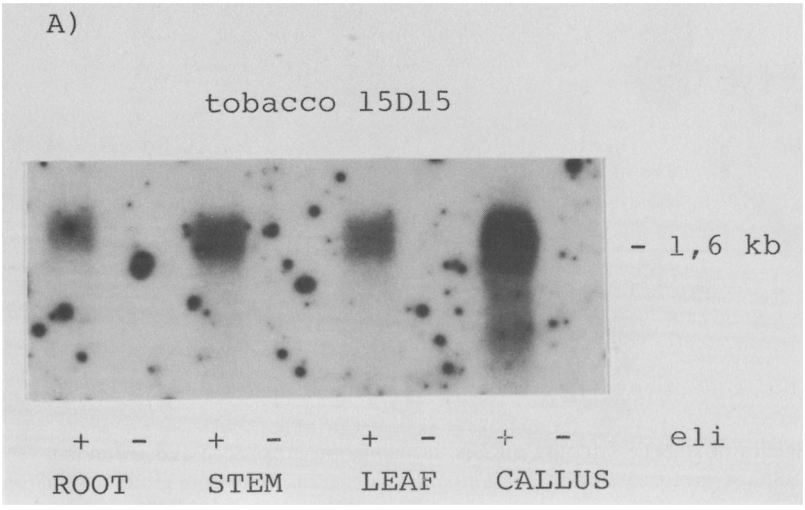


Fig. 4a. Accumulation of stilbene synthase mRNA in transgenic tobacco plants. 20 μg of total RNA isolated from elicitor-treated (+ eli) and non-treated (– eli) from leaf, stem, root and callus of 15D15 grown under sterile conditions were analysed.

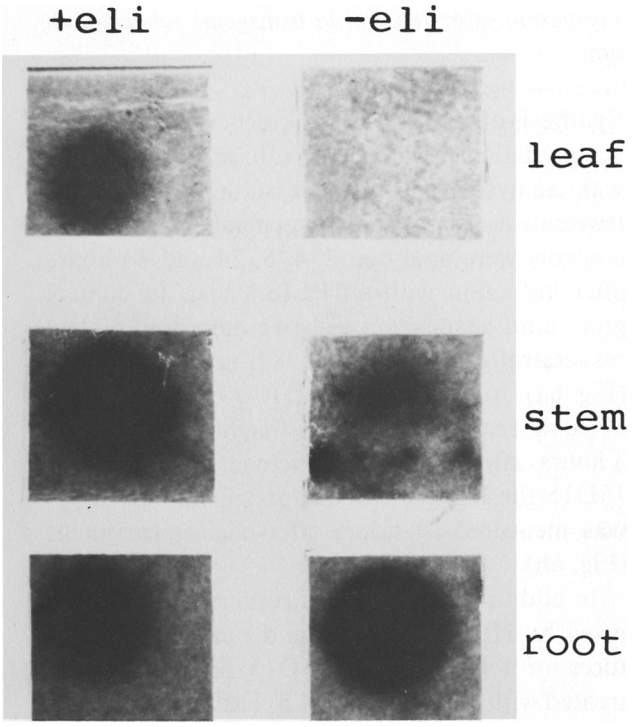


Fig. 4b. Accumulation of stilbene synthase mRNA in 15D15 plants grown in the greenhouse. Dot blot analysis of 20 μg total RNA isolated from elicitor-treated (+eli) and non-treated (−eli) leaf, stem, and root segments of 15D15.

mRNA was detected already 30 min after elicitor addition in the suspension cultures and reached a maximum after 2–8 h. 24 hours after addition of elicitor only low amounts of stilbene synthase mRNA could be found in transgenic tobacco and groundnut cultures (Fig. 3).

Accumulation of stilbene synthase mRNA in tobacco plants

For the analysis of stilbene synthase gene expression in tobacco plants leaves, stem segments and roots of 15D15 were treated with elicitor for 4 hours and compared to untreated material. In all three organs the gene had to be activated by elicitor (Fig. 4a). However, tobacco roots from greenhouse-grown plants exhibit stilbene synthase gene expression with and without elicitor treatment. In stems and leaves of these plants the gene was inducible (Fig. 4b).

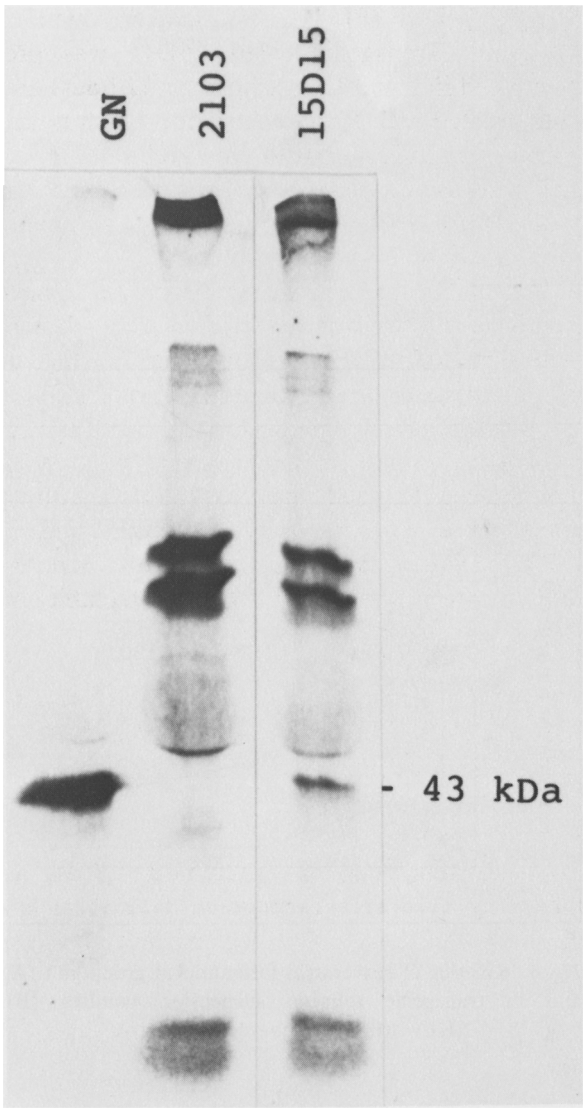
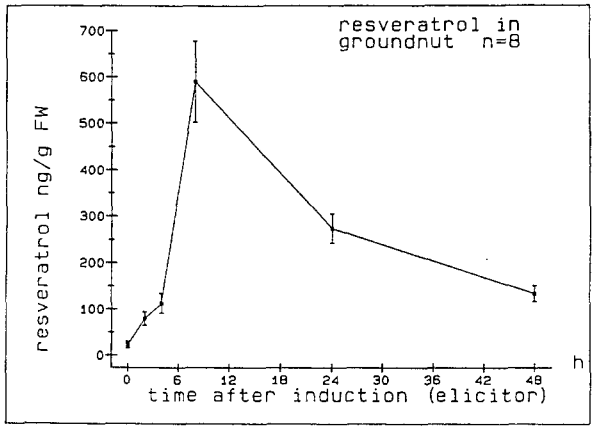


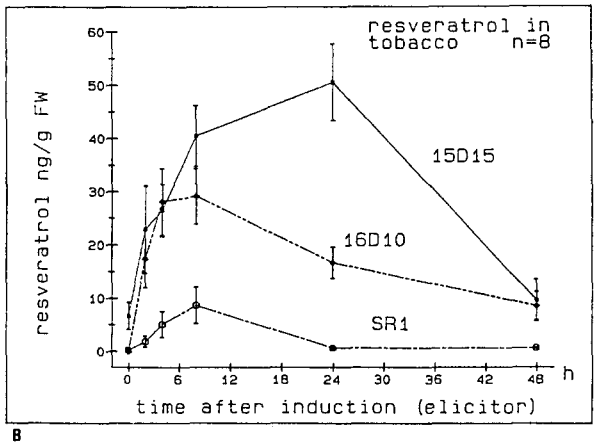
Fig. 5. In vitro translation of tobacco and groundnut RNA. 20 μg of total RNA, isolated from elicitor-treated calli, were translated *in vitro* (rabbit reticulocyte lysate) and labelled with ^{35}S-methionine. Stilbene synthase was precipitated with specific antisera and analysed by SDS-polyacrylamide gel electrophoresis. Autoradiography of the gel was for 10 days. GN = groundnut, 2103 = kanamycin-resistant tobacco [6], 15D15 = transgenic line accumulating stilbene synthase mRNA.

Synthesis of stilbene synthase in tobacco suspension culture

20 μg of total RNA isolated from 15D15 callus were translated *in vitro* and produced a protein of



A



B

Fig. 6. Kinetics of resveratrol formation in groundnut (A) and in transgenic tobacco suspension cultures (B). SR1 = wild-type tobacco as control.

43 kDa, which could be precipitated with an antiserum [23] raised against stilbene synthase (Fig. 5). With control RNA isolated from SR1 and kanamycin-resistant SR1 tobacco callus [6] no protein of 43 kDa was produced.

In order to demonstrate stilbene synthase *in vivo*, crude protein extracts of 15D15 suspension culture cells were isolated at 2-hour intervals after induction with PMG elicitor and analysed by western blotting using the same antiserum. 30 hours after induction a signal corresponding to a 43 kDa protein could clearly be detected in this experiment.

Formation of resveratrol in transgenic tobacco cultures

Synthesis of resveratrol and kinetics of resveratrol accumulation in transgenic tobacco cell cultures was analysed with an antiserum specific for resveratrol. Transgenic suspension cultures and controls were analysed 2, 4, 8, 24 and 48 hours after induction with an ELISA test. In control groundnut suspension cells we measured 600 ng resveratrol/g fresh weight 8 h after induction (Fig. 6a). In suspension 16D10 a maximum level of 30 ng resveratrol/g fresh weight was detected 8 hours after induction whereas in suspension 15D15 the highest amount of stilbene (50 ng/g) was measured 24 hours after elicitor treatment (Fig. 6b).

In addition, formation of resveratrol was analysed by HPLC. Induced and non-induced cultures of tobacco lines 15D15 and SR1 were treated with methanol for 24 h. Extracted material was transferred to an ether phase, concentrated, and subjected to reversed-phase chromatography (Fig. 7). In cell line 15D15 a component was identified as trans-resveratrol by retention time and comparison to synthetic resveratrol. No such compound was detectable in the control SR1 under any condition.

Discussion

Phytoalexins are believed to be involved in defence reactions of plants. Therefore it can be envisioned that providing a plant with an additional phytoalexin will improve its resistance to pathogen infection. Trans-resveratrol is a phytoalexin synthesized by a single enzyme in certain plant species using substrates present in all plant cells. Since a gene coding for stilbene synthase has been isolated recently from groundnut [24, 25] it is now possible to test this hypothesis.

We transferred the gene from groundnut by direct gene transfer to protoplasts of tobacco, a species devoid of stilbene synthase. Transformants were screened for the inducible synthesis of stilbene synthase mRNA and those accumulating

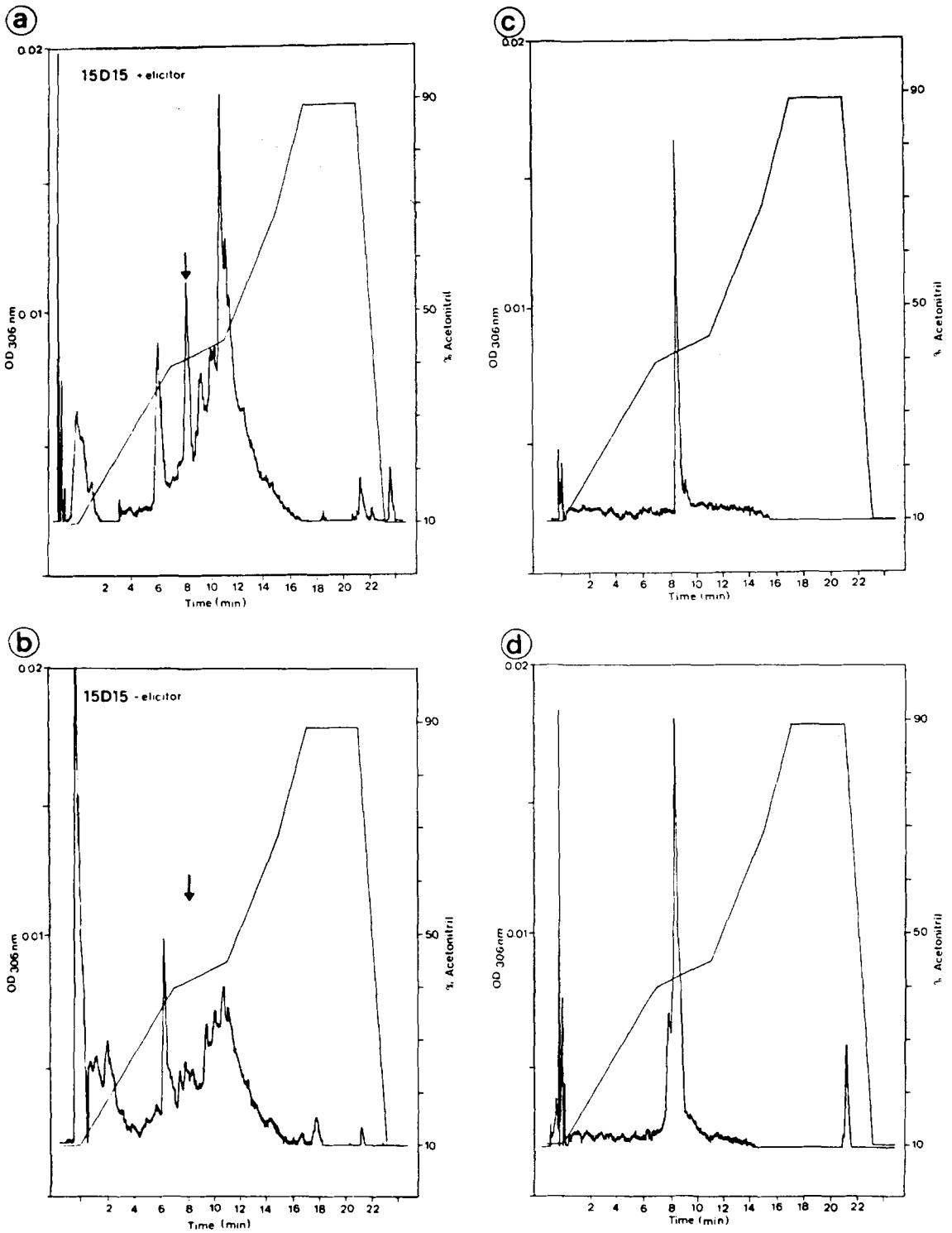


Fig. 7. Purification of trans-resveratrol from transgenic tobacco cells 15D15 by reversed-phase HPLC. Elution patterns of methanol-soluble material from cell line 15D15 after induction (a) and without induction (b) are shown. Samples applied, corresponding to 2 g of fresh weight, were concentrated by ether extraction, dried, and redissolved for chromatography. Chemically synthesized trans-resveratrol (c, 50 ng/50 μl MeOH) shows exactly the same retention time of 8 min as purified material from cell line 15D15 (d).

specific mRNA of the correct size were regenerated to plants.

Synthesis of stilbene synthase mRNA was found to be inducible either by fungal elicitors or UV irradiation in transgenic tobacco cells. Since the kinetics of stilbene synthase mRNA accumulations after induction are similar in tobacco and groundnut cell cultures, the chain of signals for induction of the stilbene synthase gene seems to be present also in tobacco. However, the amount of stilbene synthase specific mRNA synthesized from the transferred gene in tobacco is much lower than the overall synthesis of stilbene synthase mRNA in groundnut. The reason for this might be that there are many (at least four) different genes coding for stilbene synthase in groundnut [25] whereas one has been transferred to tobacco.

Accumulation of stilbene synthase mRNA was also analysed in different organs of sterile-grown tobacco plants and plants grown in the greenhouse. In sterile roots, stems and leaves the gene is activated by elicitor, whereas in roots of greenhouse plants the gene is constitutively active and treatment with elicitor does not lead to a higher expression level. In stems of these plants there was weak expression without induction, which could be increased by the addition of PMG elicitor. This implies that stilbene synthase gene is induced in transgenic tobacco grown in soil. This may occur by wounding or by slight infections with so-called minor pathogens [20] leading to gene expression at the sites of infection.

The presence of stilbene synthase protein in transgenic tobacco was demonstrated *in vitro* by translation assays performed with total RNA from induced groundnut cell cultures and transgenic line 15D15. Stilbene synthase was precipitated by specific antibodies [23] and a protein of the same size as stilbene synthase from groundnut was detected. In western blot experiments a faint band was observed 30 hours after induction with elicitor, representing very low amounts of protein.

We used an ELISA test with antisera raised against resveratrol to determine stilbene synthesis in transgenic tobacco suspension cell cultures. The polyclonal antisera raised against resveratrol showed a certain cross-reactivity to polyhydroxylated aromatic molecules (data not shown), which can explain the background signal obtained with wild-type tobacco SR1. However, the differences between transformed and non-transformed tobacco extracts in the ELISA quantifications were both significant and reproducible.

Nevertheless for further evidence we used HPLC analysis to demonstrate the synthesis of trans-resveratrol in transformed tobacco. In conclusion, using both methods resveratrol synthesis has been clearly demonstrated. Experiments will be performed to analyse the kinetics of local resveratrol accumulation in leaves after fungal infection and its correlation to disease resistance. The biological effects of the synthesis of resveratrol in tobacco on fungal pathogenesis will be published elsewhere.

Since in groundnut calli induced by elicitor about 100 times more resveratrol is formed compared to the amount observed in transgenic tobacco there is a chance to synthesize more resveratrol in tobacco by modifying the stilbene synthase gene for higher expression or by the transfer of other stilbene synthase genes which are expressed at a higher level in groundnut or grapevine.

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