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Gene expression of 5-*epi*-aristolochene synthase and formation of capsidiol in roots of *Nicotiana attenuata* and *N. sylvestris*

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Abstract

Three new copies of a sesquiterpenoid synthase gene encoding 5-*epi*-aristolochene synthase (*EAS*) were cloned as cDNAs from *Nicotiana attenuata* and functionally characterized after expression of the recombinant enzymes in *E. coli*. Differential patterns of *EAS* gene expression and formation of the antimicrobial sesquiterpenoid capsidiol were found in roots and shoots of two species of *Nicotiana*. *EAS* is expressed constitutively in roots of *N. attenuata* and *N. sylvestris* corresponding with constitutive capsidiol formation in roots. Constitutive expression of *EAS* and capsidiol accumulation were not detectable in shoots of rosette plants of *N. attenuata*, but accumulation of terpene synthase transcripts could be induced in shoots by feeding of the tobacco hornworm, *Manduca sexta*. Constitutive expression of *EAS* has not been previously reported from *N. tabacum*, where capsidiol is known as an elicitor- and pathogen-inducible phytoalexin. It is conceivable that capsidiol contributes not only to an inducible defense against pathogens, but also to a constitutive defense in an organ-specific manner in some species of *Nicotiana*.

Keywords: *Nicotiana attenuata*; *Nicotiana sylvestris*; Solanaceae; Gene expression; Terpene cyclase; 5-*epi*-Aristolochene; Capsidiol

1. Introduction

Plants form a diverse array of low molecular weight terpenoids (Buckingham, 1998), including monoterpenoids, sesquiterpenoids and diterpenoids. Many of these are thought or known to function in ecological interactions of plants with other organisms (Langenheim, 1994), for example as terpenoid components of floral scents (Knudsen et al., 1993; Dudareva and Pichersky, 2000), as airborne signals in plant-insect multitrophic interactions (Kessler and Baldwin 2001, Paré and Tumlinson, 1999), or as antimicrobial defense compounds (Kuć, 1995). The sesquiterpenoids and diterpenoids include well-known phytoalexins in genera such as *Nicotiana*, *Gossypium*, *Ricinus*, *Oryza* and others (Heinstein, 1985; Dudley et al., 1986; Ren and West, 1992; Kuć, 1995). One of these, the bicyclic sequiterpenoid phytoalexin capsidiol, has been described in *Nicotiana tabacum* and *Capsicum annuum* as a pathogen- and elicitor-inducible defense metabolite in intact plants and cell cultures (Bailey et al., 1975; Brooks et al, 1986; Vögeli and Chappell, 1988; Threllfall and Whitehead, 1988). Formation of capsidiol involves cyclization of farnesyl diphosphate (FPP) to the sesquiterpenoid hydrocarbon 5-*epi*-aristolochene catalyzed by a terpenoid synthase (TPS), 5-*epi*-aristolochene synthase (EAS) (Vögeli and Chappell, 1988; Facchini and Chappell, 1992), and two subsequent hydroxylation steps (Ralston et al., 2001) (Fig. 1). Elicitor-induction of *EAS* gene expression and EAS enzyme activity leads to formation and accumulation of capsidiol in cell cultures of *N. tabacum* (Vögeli and Chappell, 1988, 1990; Facchini

Abbreviations: bp(s), base pair(s); GC, gas chromatograph; MS, mass spectrum/spectrometry; nt(s), nucleotide(s); dNTP, deoxyribonucleotide triphosphate; ORF, open reading frame; PCR, polymerase chain reaction; SSC, saline sodium citrate; SSPE, saline sodium phosphate EDTA; Tris, tris(hydroxymethyl)aminomethane; Nucleotide sequences: The nucleotide sequences reported in this paper will appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence data banks under accession number AF484123, AF484124 and AF484125

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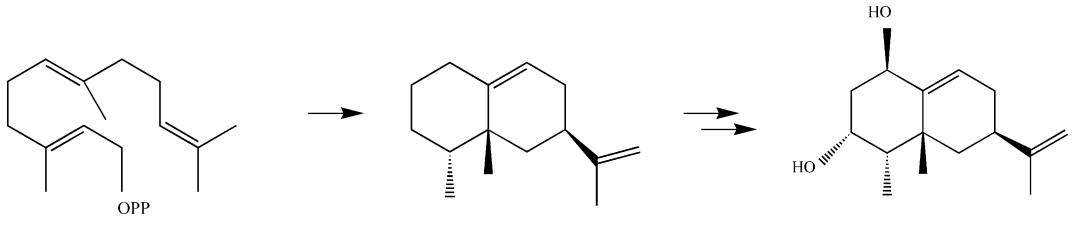

(1) (2) (3)

Fig. 1. Scheme for the conversion of farnesyl diphosphate (**1**) to 5-*epi*-aristolochene (**2**) by 5-*epi*-aristolochene synthase and subsequent formation of capsidiol (**3**).

and Chappell, 1992). Similarly, in intact plants of *N. tabacum*, elicitors and microbial pathogens induce accumulation of capsidiol in leaves (Keller et al., 1998) and expression of *EAS* in leaves and roots (Yin et al., 1997). In *N. tabacum*, the *EAS4* promoter was found to be strictly inducible in all tissues tested, including roots and leaves (Yin et al., 1997).

In the present study we have used the *EAS* gene from *N. tabacum* as a probe to isolate sequiterpenoid synthase cDNAs from *N. attenuata*, a wild relative of *N. tabacum*, as an initial step towards molecular characterization of terpenoids in a system that has been developed as a model for studies in chemical ecology (Baldwin, 1999). Cloning and characterization of recombinant *TPS* cDNAs identified three members of the *EAS* gene family of *N. attenuata*. *EAS* was constitutively expressed in roots of *N. attenuata* and *N. sylvestris* and the phytoalexin capsidiol was found constitutively in roots of *N. attenuata* and *N. sylvestris*, but was not detected in undamaged shoots. Weak induction of *EAS*-hybridizing transcript also occurred in some cases in shoots of *N. attenuata* after feeding by larvae of the tobacco hornworm, *Manduca sexta*.

2. Results and discussion

2.1. Isolation of terpenoid synthase cDNAs from *N. attenuata*

A cDNA library of 3×10^5 clones made from shoots of *N. attenuata* plants that had been fed upon by *M. sexta* larvae continuously for 24 h was screened for terpene synthase (TPS) cDNAs by filter hybridization. Using *NtEAS* as a probe, 48 positive clones were obtained in a primary hybridization and 30 positive clones in a secondary hybridization. All inserts of in vivo excised plasmids larger than 1500 bps were partially sequenced from both ends, revealing 14 full-length cDNAs with high sequence similarity to *NtEAS*. The 14 full-length cDNAs represented three unique, apparently intact *EAS*-like *TPS* transcripts of *N. attenuata*, *NaEAS12*, *NaEAS34* and *NaEAS37*, and two aberrant clones, *NaEAS25* containing an unprocessed intron and *NaEAS36* containing a frame shift mutation.

The deduced amino acid sequences of the *NaEAS12*, *NaEAS34* and *NaEAS37* ORFs all contained 549 amino acids (1650 bps), and are 94, 96 and 93% identical with NtEAS, respectively, and 76% identical with EAS of *Capsicum annuum* (Back et al., 1998). The amino acid sequences NaEAS12, NaEAS34 and NaEAS37 resemble typical TPSa type sesquiterpene synthases in all respects (Bohlmann et al., 1998a), lacking transit peptides, containing a modified RR(x)8W motif with a proline residue replacing the second arginine residue, and possessing an intact DDxxD motif. In addition, all contain the amino acid residues previously inferred to be catalytically important from the crystal structure of NtEAS (Starks et al., 1997).

2.2. Heterologous expression in E. coli and characterization of recombinant EAS from N. attenuata

Because TPS enzymes with very similar amino acid sequences can have different catalytic activities (Bohlmann et al., 1999), the proteins encoded by *EAS*-like cDNAs from *N. attenuata* were heterologously expressed for functional characterization. The three *NaEAS* cDNAs, *NaEAS12*, *NaEAS34* and *NaEAS37*, were cloned into the pSBETa vector which was previously found to yield improved functional expression of plant *TPS* genes in *E. coli* (Bohlmann et al., 1999). The expression vector employs the T7 RNA polymerase promoter and contains the *argU* gene that encodes t-RNAs which use the arginine codons AGA and AGG, which are rare in *E. coli* but commonly found in plant terpene synthases. Extracts of the induced bacteria *E. coli* BL21(DE3) containing pSBET-*NaEAS12*, pSBET-*NaEAS34* or pSBET-*NaEAS37* were assayed for monoterpene, sesquiterpene and diterpene synthase activity using the corresponding prenyl diphosphate substrates. Enzymatic production of terpene olefins by extracts of 100 ml cutures of these three *E. coli* transformants was only observed using [1-^{3}H]FPP as substrate. Extract prepared from *E. coli* BL21(DE3)/pSBETa served as control for terpene formation independent of

recombinant TPS enzyme. These extracts did not yield detectable amounts of sesquiterpene products. Sesquiterpenes generated by the recombinant enzymes, EAS12, EAS34 and EAS37, were analyzed by gas chromatography on a DB5-MS capillary column and identified by comparison of retention times and mass spectra with those of authentic standards, including an authentic standard of 5-*epi*-aristolochene generated in vitro with recombinant EAS enzyme from *N. tabacum*. The single major sesquiterpene product of each NaEAS enzyme was identified as 5-*epi*-aristolochene synthase (Fig. 2). No oxygenated or phosphorylated sesquiterpenes or other sesquiterpene olefins were found in detectable amounts.

2.3. Expression of EAS in roots and shoots of *N. attenuata* and *N. sylvestris*.

The *NaEAS37* cDNA was employed as a probe to analyze *TPS* transcript accumulation in *N. attenuata* and *N. sylvestris* under constitutive conditions in roots and shoots, and in shoots of *N. attenuata* after feeding for 24 h by larvae of the tobacco hornworm, *Manduca sexta*. Gene expression in roots was analyzed in hydroponically grown plants and in soil grown plants of *N. attenuata*. Strong signals were detected for transcripts hybridizing to the *NaEAS37* probe under constitutive conditions in roots of both *N. attenuata* and *N. sylvestris* (Fig. 3). Because of the similar levels of constitutive expression observed in *N. attenuata* roots harvested from both soil-grown plants and plants grown in hydroponic culture, it is unlikely that this gene expression is due to elicitation by soil microorganisms. In contrast to this constitutive expression in roots, no transcript hybridization was detected with RNA extracted from shoots of *N. attenuata* and only weak hybridization was found with RNA from shoots of *N. sylvestris*. This same pattern of gene expression was observed in each of more than ten plants of each treatment reared and tested individually.

Previously, gene expression of *EAS* has been well characterized in cell suspension cultures and intact plants of *N. tabacum* (Facchini and Chappell, 1992; Yin et al., 1997; Keller et al., 1998). In these systems, elicitors and microbial pathogens induce transcriptional expression of *EAS* leading to accumulation of capsidiol. *EAS* expression was not previously found under constitutive conditions in any organs or tissues of *N. tabacum*. Similarly, the *N. tabacum EAS4* promoter is active only in response to pathogens or elicitation, and does not confer constitutive gene expression on any tissues tested, including leaves, stems and roots (Yin et al., 1997). Thus, the constitutive gene expression of *EAS* detected in roots of *N. attenuata* and *N. sylvestris* in this study (Fig. 3) is a newly described phenomenon in *Nicotiana*. Tan et al. (2000) found that transcripts for δ-cadinene synthase, a sesquiterpene synthase involved in the formation of antimicrobial sesquiterpene aldehydes in species of *Gossypium* (Chen et al., 1995; Davis et al., 1996), are constitutively expressed in seedling roots of *Gossypium arboreum*.

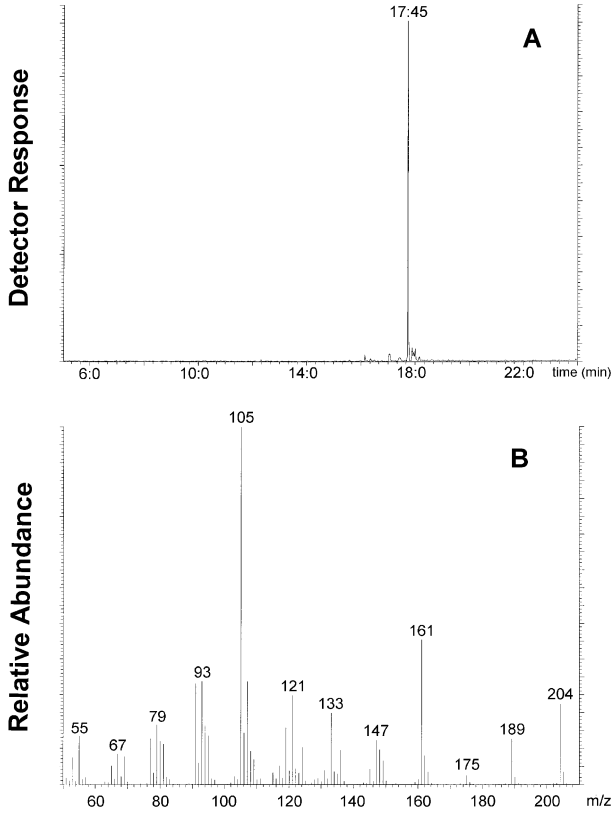

Fig. 2. Identification of the sesquiterpene product derived from farnesyl diphosphate in vitro by recombinant *N. attenuata* NaEAS37 5-*epi*-aristolochene synthase. (A) Total ion chromatogram of reaction products separated on a DB-5MS capillary column. Retention time of the principal product (17:45 min) formed from FPP in vitro by NaEAS37 enzyme corresponds with the retention time of authentic 5-*epi*-aristolochene standard. (B) Mass spectrum of the principal NaEAS37 enzyme product 5-*epi*-aristolochene.

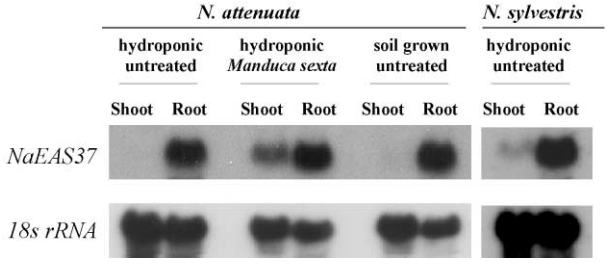

Fig. 3. Transcript accumulation in roots and shoots of *N. attenuata* and *N. sylvestris*. Transcripts hybridizing with a *NaEAS37* probe were detected in constitutive roots of all hydroponic and soil grown plants of *N. attenuata* and in roots of *N. sylvestris*. Transcripts were not detected in shoots of constitutive, rosette stage *N. attenuata*. In some instances, feeding by larvae of the tobacco hornworm, *M. sexta*, induced accumulation of transcripts hybridizing with *NaEAS37* in shoots of *N. attenuata*. Transcript hybridization with 18s rRNA was monitored as a measure for RNA loading.

Differences in *TPS* gene expression among closely related species have been previously reported in other systems, and could also explain our observations of constitutive *EAS* expression in *N. attenuata* and *N. sylvestris* in contrast to strictly inducible *EAS* in *N. tabacum*. For instance, profound differences in expression of a monoterpene synthase, *S*-linalool synthase, were found in floral organs of two closely related *Clarkia* species, *C. concinna* and *C. breweri* (Dudareva et al., 1996). Members of *TPS* gene families can also be differentially expressed in an organ specific manner in the same species. For example, differential expression of members of a *TPS* gene family was shown for the δ-cadinene synthase genes of the *cad*1-C type and *cad*1-A in *Gossypium arboreum* (Tan et al., 2000). Our findings of differential expression of *EAS* genes in shoots and roots of *N. attenuata* and *N. sylvestris* support this concept. In the conifer *Abies grandis*, both inducible and constitutive members of a *TPS* family occur in the genome of the same species, as demonstrated for constitutive and inducible sesquiterpene synthases (Steele et al., 1998; Bohlmann et al., 1998b) and it is likely that inducible and constitutive *TPS* also co-exist in *N. attenuata*.

In *N. attenuata* it has previously been shown that feeding by caterpillars of *M. sexta* causes emission of volatile terpenoids, including monoterpenes (*trans*-β-ocimene, linalool, terpineol) and sesquiterpenes (*cis*-α-bergamotene, *trans*-β-farnesene) from leaves (Halitschke et al., 2000; Kessler and Baldwin, 2001). We therefore tested expression of *TPS* in shoots of *N. attenuata* using the *NaEAS37* probe. Accumulation of transcripts hybridizing with the *EAS* probe was sometimes, but not consistently, observed in foliar tissues after feeding by *M. sexta* (Fig. 3). Some of the induced Northern signal obtained with induced shoots could result from cross-hybridization of the *EAS* probe with other *TPS* genes, such as those potentially involved in insect-induced formation of terpenoid volatiles. This possibility was subsequently tested by sequencing of differentially expressed genes from herbivore-induced leaves of *N. attenuata*. This work confirmed the presence of *EAS* transcripts in induced leaves, however, no other *TPS* genes were found (Baldwin, unpublished results). This result is consistent with our initial cDNA screening of the induced *N. attenuata* cDNA library, described here, in which the *NtEAS* probe hybridized to *NaEAS* cDNAs but failed to identify other *TPS* genes that could be expected in induced foliage. In conclusion, the Northern signals found with *EAS* probe in induced shoot RNA can be accounted for by induction of *EAS* expression. Expression of *EAS* in shoots could be induced by microbial infection of the wound side. 5-*Epi*-aristolochene has not been found as a volatile component of species of tobacco but is transformed into the less volatile capsidiol.

2.4. Identification and quantitative analysis of capsidiol in shoots and roots of *N. attenuata* and *N. sylvestris*

Constitutive expression of *EAS* has not been described in *N. tabacum* or *C. annuum*, the two species from which *EAS* genes have been cloned and characterized before. Thus, after finding constitutive expression of EAS in roots of both *N. attenuata* and *N. sylvestris,* we determined the levels of capsidiol, the antimicrobial sesquiterpenoid formed from 5-*epi*-aristolochene (Fig. 1), present in roots and shoots by quantitative GC–MS. Using authentic capsidiol in a standard addition analysis protocol, capsidiol could not be detected in the shoots of either species with a detection limit of 0.5 $ng \times g^{-1}$ fresh weight consistent with gene expression patterns. However, low constitutive levels of capsidiol were found in the roots of *N. attenuata* (10 $ng \times g^{-1}$ fresh weight) and *N. sylvestris* (4.5 $ng \times g^{-1}$ fr. wt.) (Fig. 4).

The differences in constitutive expression of *EAS* between roots and shoots of *N. attenuata* and *N. sylvestris* are consistent with the formation of capsidiol in roots of both species and the lack of detectable levels of capsidiol in constitutive shoots (Fig. 4). The constitutive formation of capsidiol in roots of *N. attenuata* and *N. sylvestris* could provide protection against pathogens in the rhizosphere where pathogen ingress is more likely due to abrasion of root epidermal cells and lack of other physical protection such as a cuticula. The amounts of capsidiol detected per gram fresh weight were very low based on total organ weight, nearly three orders of magnitude less than the amount accumulated in induced tobacco cell suspension cultures (Vögeli and Chappell, 1988). However, similar to localized induced expression of the *EAS4* promoter in specific cell types of the periderm and vascular tissue in roots of *N. tabacum* (Yin et al., 1997), constitutive EAS expression and formation of capsidiol in *N. attenuata* and *N. sylvestris* could also be spatially restricted, leading to higher localized capsidiol concentrations. In future work, the ecological significance of *EAS* expression and formation of capsidiol in roots of *N. attenuata* will be addressed in plants that have been altered for *EAS* expression.

3. Experimental

3.1. Plant materials and insects

Plants of *Nicotiana attenuata* (inbred line DI-92) and *Nicotiana sylvestris* were either hydroponically grown (Baldwin et al., 1998) or grown in soil (Baldwin and Ohnmeiss, 1994). Roots and shoots were harvested four weeks after germination. Shoot material included leaves and stem tissue of rosette plants. Tissues were stored at −20 °C until used for extraction of capsidiol. Tissues for RNA isolation were frozen in liquid nitrogen and stored at

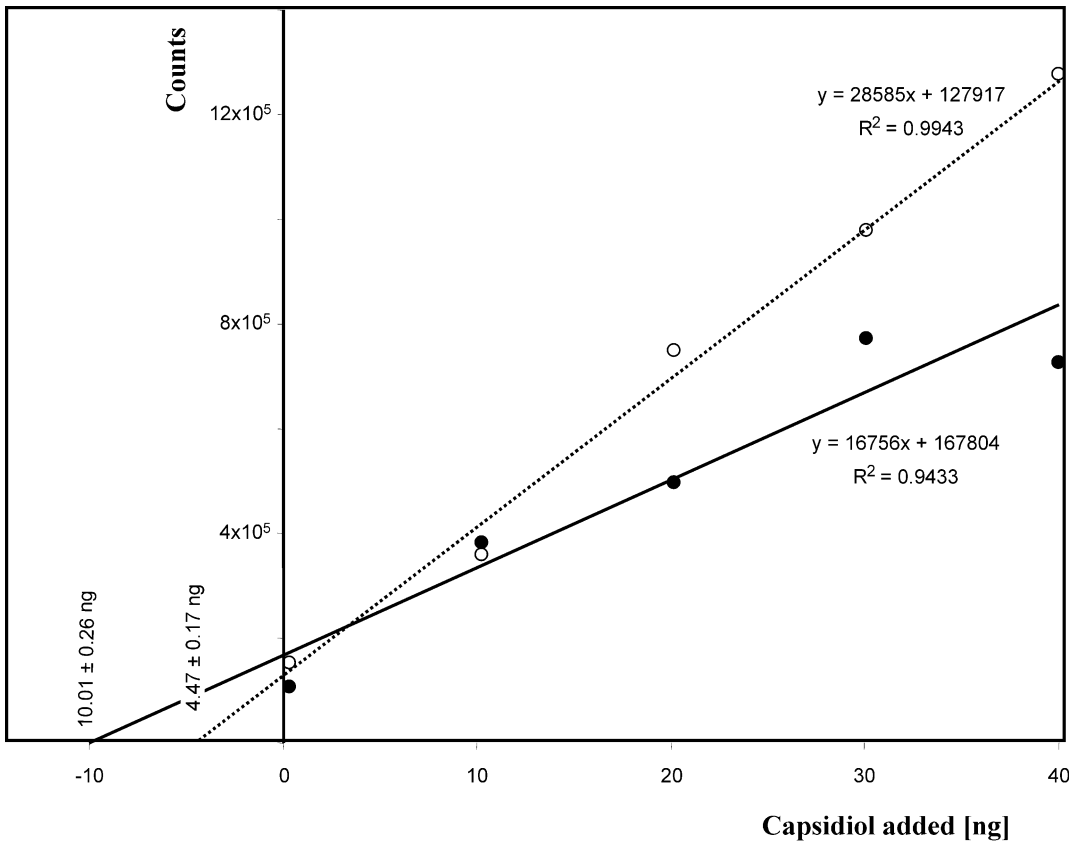

Fig. 4. Quantitative analysis of constitutive levels of capsidiol in roots of *N. attenuata* and *N. sylvestris*. Standard addition quantitative analysis of constitutive levels of capsidiol in roots of *N. attenuata* (●, solid line) and *N. sylvestris* (○, dashed line). Capsidiol was identified by GC–MS.

−80 °C. Larvae of the tobacco hornworm (*Manduca sexta* L., Lepidoptera, Sphingidae) were reared and allowed to feed on plants as described (Hermsmeier et al., 2001).

3.2. Substrates, reagents and cDNA library

[1-^{3}H]Farnesyl diphosphate (125 Ci/mol) was a gift of Dr. Rodney Croteau, Pullman WA, USA. The authentic capsidiol standard was kindly provided by M.-L. Milat, Dijon, France. A tritium labeled 5-*epi*-aritsolochene standard was generated from [1-^{3}H]farnesyl diphosphate using recombinant *N. tabacum* EAS enzyme (NtEAS) expressed in *E. coli* from a pSBET vector construct with an *NtEAS* insert sequence supplied by Dr. Joseph Chappell (Lexington, KY, USA). Expression of NtEAS, enzymatic formation and purification of 5-*epi*-aristsolochene are detailed below. All other chemicals and reagents were purchased from Sigma Chemical Co. or Aldrich Chemical Co., unless otherwise noted. The *Nicotiana attenuata* Uni-ZAP XR cDNA library was described before (Hermsmeier et al., 2001).

3.3. cDNA isolation

A mixed hybridization probe of two fragments (660 bps and 1590 bps) of the *NtEAS* cDNA was generated by PCR. For PCR, the oligonucleotide primer teas1 (5′-ATG GCC TCA GCA GCA GTT GC-3′) was combined with primer teas2 (5′-TTG ATG GAG TCC ACA AGT AGG-3′) or with primer teas4 (5′-GAG CAA GTT GAA ATC CAA TTT GGC-3′). Two rounds of PCR were performed each in a total volume of 50 μl containing 20 mM Tris/HCl (pH 8.4), 50 mM KCl, 5 mM MgCl2, 200 μM of each dNTP, 0.1 μM of each primer, and one unit of *Taq* polymerase (BRL). Template in the first round of PCR was 5 ng of pbluescript-*NtEAS*. Five microlitres of the completed primary reaction served as template in a secondary amplification. The temperature program for PCR was: denaturing at 95 °C for 2 min, 35 cycles: 1 min at 95 °C, 1 min at 45 °C, 1 min at 72 °C using a Gradient 96 Robocycler (Stratagene). Fifty nanograms of each PCR product were purified by agarose gel electrophoresis and extraction using the Qiagen (Hilden, Germany) gel extraction kit, and randomly labeled with [α-^{32}P]dCTP using Readiprime II random prime labeling system from Amersham Pharmacia (Braunschweig, Germany). The unincorporated nucleotides were removed from the labeling reaction using Probe Quant G-50 Micro-columns from Amersham Pharmacia. Replica filters of 3 × 10^5 plaques of the *N. attenuata* cDNA library were prehybridized for three hours at 45 °C in 3 × SSPE, 0.1%

SDS, 200 mg/l PVP, 200 mg/ml Ficoll and 50 μg/ml blocking DNA. Hybridization was performed for 18 h at 55 °C in 3 × SSPE, 0.1% SDS, 200 mg/l PVP and 200 mg/mL Ficoll using the two NtEAS fragments as a probe. Filters were washed two times for 15 min at 55 °C in 3 × SSPE with 0.1% SDS, and for a third time for 15 min at 55 °C in 1 × SSPE with 0.1% SDS. Filters were exposed for 17 h to Kodak BioMax-MS film at −80 °C. Thirty clones yielding positive signals were purified in a second round of filter hybridization and phagemids excised by in-vivo excision and the plasmids isolated for DNA sequencing.

3.4. Sequence analysis

Insert ends of size selected, recombinant plasmids were sequenced using T3 and T7 vector primers. All full-length cDNAs and inserts of expression constructs were completely sequenced on both strands via primer walking using the DyeDeoxy Terminator Cycle Sequencing method (Applied Biosystems). Sequence analysis was done using programs from the Wisconsin Package Version 9.1, Genetics Computer Group (GCG) Madison, WI

3.5. cDNA Expression in E. coli and enzyme assays

Full-length cDNAs *NaEAS12*, *NaEAS34*, *NaEAS37* and *NtEAS* were subcloned in frame into the pSBETa expression vector (Schenk et al., 1995). Restriction sites *Nde*I and *Bam*HI for subcloning were introduced by sticky-end PCR (Zeng, 1998). Sticky end cDNA inserts were amplified using four primers for each cDNA in two PCR reactions combining primer St1 with primer St3, and primer St2 with primer St4. The following primers were used: NaEAS12-St1 (5′-TAT GGC CTC AGC AGC AGT TGG CAA CTA TGA AGA AG-3′), NaEAS12-St2 (5′-TGG CC CAG CAG CAG TTG GCA ACT ATG AAG AAG-3′), NaEAS12-St3 (5′-CTC AAA TTT CGA TGG AGT CCA CAA GTA G-3′), NaEAS12-St4 (5′-GAT CCT CAA ATT TCG ATG GAG TCC ACA AGT AG-3′), NaEAS34-St1 (5′-TAT GGC CTC AGC AGC AGT TGG CAA C-3′), NaEAS34-St2 (5′-TGG CCT CAG CAG CAG TTG GCA AC-3′), NaEAS34-St3 (5′-CTC AAA TTT TGA TGG AGT CCA CAA-3′), NaEAS34-St4 (5′-GAT CCT CAA ATT TTG ATG GAG TCC ACA AG-3′), NaEAS37-St1 (5′-TTAT GGC CTC AGC AGC AGT AGC CAA-3′), NaEAS37-St2 (5′-TGG CCT CAG CAG CAG TAG CCA AC-3′), NaEAS37-St3 (5′-CTC AAA TTT GAA TGG ATT CCA CAA GTA G-3′), NaEAS37-St4 (5′-GAT CCT CAA ATT TGA ATG GAT TCC ACA AG-3′), NtEAS-St1 (5′-TAT GGC CTC AGC AGC AGT TGC AAA-3′), NtEAS-St2 (5′-TGG CCT CAG CAG TTG CAA AC-3′), NtEAS-St3 (5′-CTC AAA TTT TGA TGG AGT CCA CAA G-3′), NtEAS-St4 (5′-GAT CCT CAA ATT TTG ATG GAG TCC ACA AG-3′). PCR amplifications, purification of PCR products, denaturing, re-annealing and ligation into pSBETa were performed as described before (Bohlmann et al., 2000), yielding plasmid pSBET-*NaEAS12,* pSBET-*NaEAS34*, pSBET-*NaEAS37*, and pSBET-*NtEAS*. Plasmids were then transformed into *E. coli* BL21(DE3). For functional expression, transformed bacteria were grown to $A_{600} = 0.5$ at 37 °C in 5 ml of LB medium supplemented with 30 μg kanamycin/ml. Cultures were then induced by addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside and grown for another 12 h at 20 °C. Cells were harvested by centrifugation and disrupted by sonication using a sonicator model Bandeln HD2070, MS72 (Berlin, Germany) at 20% power for 15 s, and the resulting soluble enzyme preparations were assayed for sesquiterpene synthase activity using [1-^{3}H]farnesyl diphosphate (125 Ci/mol) as described previously (Bohlmann et al., 1997). The assay mixture (1 ml) was overlaid with 1 ml of pentane to trap volatile products. After incubation at 30 °C for 1 h, the reaction mixture was extracted with pentane (3 × 1 ml) and the combined extract was passed through a 1.5 ml column of anhydrous $MgSO_4$ and silica gel (Sigma, 60 Å) to provide the terpene hydrocarbon fraction free of oxygenated metabolites. To collect any oxygenated products, assay mixtures were subsequently extracted with (3 × 1 ml) Et_2O and columns were rinsed with Et_2O (3 × 1 ml) of diethyl ether. To test for the formation of phosphorylated products (Wise et al., 1998), the aqueous phase was then treated with excess potato grade III apyrase, adjusted to pH 8.0–8.5 and treated with excess calf intestinal alkaline phosphatase to hydrolyze diphosphate esters. The incubation mixtures were extracted with Et_2O (3 × 1 ml) and water removed from combined extracts using anhydrous $MgSO_4$. Aliquots of each fraction were taken for liquid scintillation counting to determine conversion rate. To obtain sufficient product for analysis by GC–MS, the enzyme assay was scaled up by a factor of 20 and assays were incubated for 5 h. Controls for product formation independent of expressed cDNA were performed using extracts of *E. coli* BL21(DE3) transformed with plasmid pSBETa.

3.6. Product analysis by GC/MS

Gas chromatography/mass spectrometry (GC/MS) of the product fraction extracted from enzyme assays was performed on a Micromass MasSpec (Micromass, Manchester, UK) double-focusing magnetic sector mass spectrometer (geometry EBE) connected to a Hewlett Packard HP6890 II gas chromatograph, equipped with an DB-5MS (J&W Scientific) capillary column (30 m × 0.25 mm × 0.25 μm). The GC injection port was operated at 220 °C and the transfer line at 280 °C. The oven

temperature programmed from 30 to 280 °C at 10 °C min^{-1}, with initial and final isothermal times of 5 min. Helium was used as a carrier gas at 1 ml min^{-1} and the sample was injected in the splitless mode. Mass spectra were measured in electron impact (EI) mode at 70 eV, with a source temperature of 200 °C, an acceleration voltage of 8 kV, and a resolution of 800. The instrument was scanned between m/z 35 and m/z 300 at 1 scan s^{-1}. Perfluorokerosene (PFK, Aldrich, Deisenhofen, Germany) was used as a calibration gas.

3.7. RNA isolation and northern hybridization

Total RNA was isolated from 2 g of roots or shoots of 4–6 plants for each treatment according to Hermsmeier et al. (2001). Gel electrophoresis of 20 μg RNA per sample, blotting and northern hybridization was performed exactly as described in Hermsmeier et al. (2001). Radio-labeled *N. attenuata* EAS probe was generated from the *NaEAS37* cDNA with [α-^{32}P]dCTP using the Readiprime II random prime labeling system from Amersham Pharmacia (Braunschweig, Germany). The unincorporated nucleotides were removed from the labeling reaction using Probe Quant G-50 Microcolumns. Hybridization was done for 16 h at 42 °C, followed by washing membranes three times at 45 °C for 5 min in 2 × SSC and 1% SDS, 20 min in 2 × SSC and 1%SDS and 20 min in 1 × SSC and 1% SDS. Membranes were exposed for 4 h to Kodak BioMax-MS film at −80 °C.

3.8. Extraction of capsidiol

Fresh plant tissue (5 g) were ground in liquid nitrogen and weighed into 5 aliquots. Each aliquot was transferred into an extraction vessel and the capsidiol standard and 75 ml cyclohexane/ethyl acetate (1:1, v/v; Merck, Darmstadt, Germany) were added. Plant tissue was extracted by microwave assisted extraction (Soxwave 3.6, Prolabo, Briare, France) with the following microwave radiation program: 2 min at 30 W, 3 min at 45 W, and 10 min at 75 W. The sample was filtered through No. 2 filter paper (Whatman, Maidstone, UK) into a round bottom flask and the residue was extracted again under the same conditions. The combined extracts were reduced to approximately 0.5 ml in a rotary evaporator (Büchi, Konstanz, Germany) at 230 mbar and 40 °C water bath temperature. The sample was taken up in cyclohexane (2 ml) and transferred to a 3-ml silica SPE cartridge (Supelco, Deisenhofen, Germany), which was previously conditioned with cyclohexane (10 ml). The cartridge was washed with cyclohexane/ethyl acetate (75:25, v/v) (20 ml) and capsidiol was eluted with cyclohexane/ethyl acetate (60:40, v/v) 12 ml. The solvent of the capsidiol fraction was evaporated at 50 °C in a gentle nitrogen stream. The sample was transferred with two aliquots of 100 μl iso-octane into 2 ml vials with 250 μl inserts.

3.9. GC/MS determination of capsidiol

One microlitre of each sample was injected into a Saturn 2000 GC/MS system (8200 autosampler, 1079 injector, 3800 GC, and 2000 Saturn ion trap, Varian, Darmstadt, Germany). GC conditions were as follows: helium at 1 ml min^{-1}, splitless injection, 250 °C injector temperature, column temperature program: 60 °C for 1 min, from 60 to 150 °C at 10 °C min^{-1}, to 200 °C at 20 °C min^{-1}, to 230 °C at 3 °C min^{-1}, and to 270 °C at 40 °C min^{-1}, hold at 270 °C for 1.5 min (total run time: 25 min). Chemical ionization was performed with isobutane as reactant gas and masses from 60 to 250 m/z were recorded. Capsidiol was quantified by m/z 201.

3.10. Capsidiol standard addition for quantitative GC/MS analysis

A 50 ng/μl capsidiol standard solution was used for the standard addition to the 5 plant tissue aliquots of each sample. To the first aliquot no standard was added while 50, 100, 150, and 200 μl of standard were added, respectively, to the other aliquots. The quantity of endogenous capsidiol was determined by regression analysis of these samples.

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