ELSEVIER

Gene 222 (1998) 155–162

GENE
AN INTERNATIONAL JOURNAL ON GENES AND GENOMES

Host-root exudates increase gene expression of asparagine synthetase in the roots of a hemiparasitic plant *Triphysaria versicolor* (Scrophulariaceae)[1]

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Received 23 May 1998; received in revised form 12 September 1998; accepted 17 September 1998; Received by W. Martin

Abstract

Triphysaria is a facultative root parasite in the Scrophulariaceae family. Similar to other related parasites, the development of the parasitic life cycle is initiated by molecular signals released from appropriate host roots. Using a differential display, we isolated cDNAs preferentially abundant in *T. versicolor* roots exposed to *Trifolium repens* (white clover) root exudates in vitro. Sequence analysis indicated that one of the differentially expressed cDNAs had significant homology to the nitrogen-assimilating enzyme, asparagine synthetase (AS). *T. versicolor* AS cDNA clones were isolated and placed into three distinct classes on the basis of nucleotide sequence variations. All three classes encoded identical AS proteins. AS was expressed in both roots and shoots of in-vitro-cultured *T. versicolor*. Steady-state levels of AS mRNA increased in *T. versicolor* roots several-fold when seedlings were exposed to exudate obtained from hydroponically grown *Arabidopsis thaliana* roots. Therefore, AS transcript levels increased in response to exudates from two different hosts (*Trifolium* and *Arabidopsis*). The *T. versicolor* AS message levels increased to a similar magnitude when seedlings were incubated in the dark. Interestingly, AS levels were unaffected by treatment with the *Striga* haustoria inducer 2,6-dimethoxybenzoquinone. The potential role of AS in root parasitism is discussed. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Induced gene; Parasitic plants; Root signal

1. Introduction

Plant roots respond to a variety of signal molecules that are released into the rhizosphere by different organisms. Plant-signaling molecules in the rhizosphere include nutrients, lipo-oligosaccharides, and phenolics. In response to rhizosphere signals, root growth may be either stimulated or repressed. Some signals trigger distinct developmental changes in the plant roots. Nodule development, for example, is triggered in legumes in response to signals released from nitrogen-fixing *Rhizobia*. Similarly, galls form on plant roots in response to signals released by root knot nematodes. In each of these cases, root developmental programs are altered in response to molecules released from a second organism.

Parasitic plants in the Scrophulariaceae family use rhizospheric signals to trigger developmental pathways critical for its success as a parasite. What is unique about these signals is that they are derived from other plants (Parker and Riches, 1993; Press and Graves, 1995). Seed germination in *Striga* and *Orobanche* depends on stimulatory molecules released from host roots. Haustorium development is also mediated through host signals (Chang and Lynn, 1986). Within hours after treating parasite roots with host-root exudates, epidermal hairs begin to proliferate near the parasite root tips. At the same time, cortical cells localized under the growing hairs begin to expand (Riopel and Timko, 1995). These regions rapidly develop into haustoria with the capacity to attach and

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[1] EMBL Accession Nos: TVAS-B AF014056, TVAS-E AF014055, TVAS-6 AF014054.

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Abbreviations: AS, asparagine synthetase; DMBQ, 2,6- dimethoxybenzoquinone; MS, Murashige and Skoog media; RC4D, RFLP-Coupled Domain Directed Differential Display; UB, ubiquitin.

PII: S0378-1119(98)00502-2

invade into a host, and establish vascular connections through which the parasite robs host resources. Additional host-derived signals are likely required for further development in the parasitic lifestyle (Riopel and Timko, 1995; Estabrook and Yoder, 1998).

Triphysaria are small, facultative parasites common in California grassland stands. Similar to other facultative parasites, *Triphysaria* have a broad host range (Atsatt and Strong, 1970). Field studies showed the association of *Triphysaria* with over 25 species in 18 families of both monocots and dicots (Atsatt and Strong, 1970). In vitro, *Triphysaria* aggressively parasitize both *Arabidopsis* and maize (Yoder, 1997; Estabrook and Yoder, 1998). Haustorium development is induced in *Triphysaria* in response to similar haustorial inducing factors as those characterized for *Striga* and *Agalinis* (Riopel and Timko, 1995; Smith et al., 1996). The only haustorial-inducing factor isolated to date from host roots, 2,6-dimethoxybenzoquinone (Chang and Lynn, 1986), also induces haustoria in *Triphysaria*.

The genus *Triphysaria* (previously *Orthocarpus*) is comprised of five species of parasites in the Scrophulariaceae family (Chuang and Heckard, 1991). All *Triphysaria* are simple diploids, and interspecific hybrids can be readily obtained. The ability to make defined crosses and generate segregating F2 populations makes *Triphysaria* an attractive genus for genetic studies (Yoder, 1998). Unlike its close relatives *Striga* and *Orobanche*, *Triphysaria* has no agronomic significance, so genetic experiments can be conducted without quarantine restrictions.

The complexity of parasite root responses to host-plant signals suggests the action of several genes. For instance, specific protein induction has been shown in *Striga* root cultures after stimulation with haustorial initiating factors have been identified by two-dimensional polyacrylamide gel electrophoresis (Wolf and Timko, 1992; Stranger et al., 1995). As of yet, no parasite genes differentially expressed after exposure to host-root exudates have been identified.

We used a differential cDNA display to identify genes differentially expressed in *T. versicolor* roots after treatment with host-root exudates. A mRNA transcript encoding the enzyme asparagine synthetase (AS) was found to be up-regulated in *T. versicolor* roots after treatment with either *T. repens* or *Arabidopsis* root exudates. AS transcripts are expressed in both roots and shoots of in-vitro-grown *T. versicolor* plants. In response to host exudates, steady-state levels of AS mRNA in *T. versicolor* roots increased several-fold. AS is encoded by a highly polymorphic, small gene family in *T. versicolor*. An elevated level of AS mRNA expression in parasite roots is potentially significant, based on the importance of host nitrogen resources for successful parasitism (Press and Graves, 1995). This is the first case of a parasitic plant gene being differentially regulated by host-root signals.

2. Materials and methods

2.1. Seeds and germination

Seeds from *T. versicolor* were harvested from a grassland field in Napa Valley, California. Field grown *Triphysaria* are almost always parasitic on neighboring plants, in this case a mix of non-native grasses, clovers, and other grassland annuals. Seeds of *Arabidopsis thaliana* var. Columbia and *Trifolium repens* (white clover) were obtained from Lehle Seeds (Round Rock, TX) and a local nursery, respectively.

Seeds were surface-sterilized by rinsing in 80% ethanol for 5 min, then in 50% bleach, 1% Triton for 30 min, followed by several rinses in sterile water. *T. versicolor* seeds were placed on quarter-strength (0.25 ×) MS medium (0.75 mM $CaCl_2$, 0.3 mM KH_2PO_4, 5 mM KNO_3, 0.2 mM $MgSO_4$, 5 mM NH_4NO_3) supplemented with micronutrients (0.01 μM $CoCl_2$, 0.5 μM $CuSO_4$, 70 μM H_3BO_3, 14 μM $MnCl_2$, 10 μM NaCl, 0.2 μM $NaMoO_4$, and 1 μM $ZnSO_4$), 1% sucrose and 0.6% Phytoagar (Life Technologies, Rockville, MD). After examining several parameters, it was determined that *Triphysaria* germination was optimal at 16°C under a 12-h photoperiod. Under these conditions, about 80% of the seeds had germinated by 10 days. *Triphysaria* seeds do not require host germination stimulants.

2.2. In-vitro culture and treatment with host exudates

Three weeks after germination, *T. versicolor* seedlings were transplanted into Magenta boxes containing 0.25 × Hoagland's solution (1.25 mM $Ca(NO_3)_2$, 1.25 mM KNO_3, 0.25 mM KH_2PO_4, 0.5 mM $MgSO_4$), micronutrients, 1% sucrose and 0.4% Phytoagar. The plants were incubated for an additional 5 weeks at 24°C in a 17-h photoperiod before being treated with host-root exudates.

Exudates of *Arabidopsis thaliana* var. Columbia and *Trifolium repens* roots were obtained from hydroponically grown plants. In both cases, seeds were surface-sterilized and placed into Erlenmeyer flasks containing 0.25 × MS medium and incubating at 24°C for 3 weeks in a 17-h photoperiod with gentle shaking. The medium was then removed from the flasks, filter-sterilized, and stored at −20°C until used.

The *T. versicolor* seedlings were gently removed from the Magenta boxes and placed into Petri dishes with their roots submerged in 5 ml of host exudates. The seedlings were then gently swirled in the exudate at 16°C for 3 days. Control plants were treated identically except that the medium in which the roots were swirled did not contain host exudate. The roots and shoots were then physically separated and frozen in liquid nitrogen.

T. versicolor roots were also treated with 2,6- dimethoxybenzoquinone (DMBQ) (Pfaltz and Bauer, Waterbury, CT). In this case, *T. versicolor* seedlings were placed along one edge of a square Petri dish containing growth medium. The Petri dishes were placed in vertically inclined racks in the growth chamber to allow the *T. versicolor* roots to grow down along the surface of the agar. Two milliliters of 100 μM DMBQ was applied to the exposed roots for various periods of time before the roots were harvested and frozen in liquid nitrogen. The roots of the control plants were treated similarly except in the presence of water instead of DMBQ.

2.3. Isolation of asparagine synthetase cDNAs

T. versicolor asparagine synthetase (AS) cDNAs were originally identified as being more abundant bands in *T. repens* exudate-induced roots than in non-treated roots by the RFLP-Coupled Domain Directed Differential Display (RC4D) procedure (Fischer et al., 1995). An RC4D reaction using the MADS box primer P038 (5′-GAT CAA G(A/C)G (G/C)AT CGA GAA-3′) (Fischer et al., 1995) was performed using RNA isolated from *T. versicolor* roots induced with *T. repens* exudates and RNA from *T. versicolor* roots similarly treated with medium lacking host-root exudate. Bands of interest were cut out of the gel, boiled for 30 min in 10 mM Tris, pH 7.0, 0.1 mM EDTA and the DNA recovered by ethanol precipitation. The recovered RC4D products were reamplified and cloned using the TA cloning kit from InVitrogen (Carlsbad, CA).

T. versicolor AS clones were obtained from a cDNA library using the RC4D products as hybridization probes. A cDNA library was prepared from 3 μg poly(A)$^+$ RNA isolated from *T. versicolor* root tips induced for 3 days with *T. repens* root exudate. The cDNAs were directionally cloned into the vector, λ-ZapII, and packaged using Gigapack II Gold (Stratagene, San Diego, CA). Approximately 10^5 cDNA clones with an average size of 1 kb were obtained. Plaques were hybridized with the RC4D AS products labeled with ^{32}P by random priming.

DNA sequencing was done using an Applied Biosystems 377 at the Advanced Plant Molecular Genetics Facility (University of California, Davis, CA). DNA sequence homology searches were done using BLAST (Altschul et al., 1990). Amino acid predictions and protein homology alignments were done using the Wisconsin GCG package (Devereux et al., 1984).

2.4. RNA isolation and Northern analyses

Total RNA was isolated by a phenol, LiCl precipitation method (de Vries et al., 1982). Poly(A)$^+$ RNA was isolated using Oligotex (Qiagen, Chatsworth, CA). RNA (4 μg total RNA per lane) was glyxolated and separated by electrophoresis in 1% agarose. RNA was then blotted on to Hybond N (Amersham, Arlington Heights, IL), using 10 × SSC (15 M NaCl, 1.5 M Na_3 citrate, pH 7.6) and fixed on the membrane by baking at 80°C under vacuum. The filters were prehybridized for 1 h at 60°C in 50% formamide, 0.25 M $NaHPO_4$ (pH 7.2), 0.25 M NaCl, 1 mM EDTA, 100 μg/ml of denatured salmon sperm DNA and 7% SDS. Hybridization was conducted overnight under the same conditions using ^{32}P-labeled AS probes. To standardize RNA levels, blots were hybridized at 50°C with an *Arabidopsis* ubiquitin DNA fragment (a kind gift from J. Callis, University of California, Davis, CA). Hybridization intensities were quantified using a STORM phosphoimager (Molecular Dynamics, Sunnyvale, CA).

Random primed probes were made from gel-purified fragments using ^{32}P-dCTP and the Amersham Multiprime DNA labeling system as described by the manufacturer (Amersham, Arlington Heights, IL). Riboprobes were made using the Ambion Maxiscript transcription kit, ^{32}P-UTP and T7 RNA polymerase (Ambion, Austin, TX).

2.5. Southern analysis

Total DNA from aerial portions of *T. versicolor* plants was isolated, cleaved with restriction enzymes, fractionated on 0.7% agarose gels and transferred to Zeta-Probe membrane (Bio-Rad Laboratories, Hercules, CA) using an alkaline transfer method (Reed and Mann, 1985). Filter hybridizations were conducted at 65°C in 4 × SSC, 1 × SPEP (1% SDS, 0.2% sodium pyrophosphate-8 hydrate, 10 mM EDTA, 20 mM sodium phosphate, pH 6.8), 5 × Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% BSA), and 100 μg/ml of denatured herring sperm DNA. The filters were washed in 1 × SSC and 1 × SPEP at 65°C.

3. Results

3.1. Isolation of T. versicolor *AS cDNAs*

RC4D is a modified differential display that utilizes amplification primers specific to conserved sequence domains in conjunction with RFLP analysis to identify differentially expressed genes (Fischer et al., 1995). We used the P038 primer, originally designed to preferentially amplify MADS box genes, in an RC4D reaction to compare *T. versicolor* roots treated with growth medium with those treated with growth medium in which *T. repens* had been grown.

Bands were observed on the RC4D gels that appeared to represent genes up-regulated in exudate-treated *T.*

versicolor roots. Several of these were cloned and sequenced. Many of the cloned fragments contained sequence domains predicted for DNA binding proteins related to the MADS gene family (Wrobel, Delavault, and Yoder, unpublished). In addition, several differentially expressed fragments were isolated that showed a sequence homology to asparagine synthetases (AS) from various organisms.

Since the differential display procedure yielded relatively small DNA fragments, a *T. versicolor* root cDNA library was screened using the AS clones obtained from RC4D as probes. Eleven clones were analyzed by sequencing. Homology searches clearly indicated that the clones encoded AS. Since the largest cDNA lacked about 40 nt of the 5′ coding terminus, a consensus primer hybridizing to the first 20 nt of plant AS mRNA sequences and *T. versicolor* AS specific primers were used in RT-PCR reactions to obtain the remaining consensus sequence of *T. versicolor* AS (Fig. 1). Sequence analysis revealed that 12 nucleotides at the 3′ end of the MADS box primer were identical to 12 consecutive nucleotides in the *T. versicolor* cDNA sequence.

The deduced *T. versicolor* AS protein was 586 amino acids long and shared between 79 and 90% similarity with other characterized plant AS proteins (Table 1). As previously reported (Chevalier et al., 1996; Waterhouse et al., 1996), conservation among plant AS genes was highest in the N terminal portion of the gene; the similarities between *T. versicolor* and other plant AS genes increased to 86–94% when the last 31 amino acids were excluded from the analysis. The eight invariant amino acid residues of the Cys–His–Asp catalytic triad typical of purF-type glutamine amidotransferases were also observed (Mei and Zalkin, 1989).

Partial sequence and restriction enzyme analyses showed that the 11 AS cDNAs were not identical but could be placed into three distinct groups; TVAS-6,

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   1 ATGTGTGGGATTCTTGCHGTTTTGGGTTGYTCMGATGACTCTCAGGCGAAAAGGGTTCGSGTTCTYGAGCTCTCTCGCAGATTGAAGCAYCGTGGGCCCGACTGGAGTGGCATACATCAT
     M  C  G  I  L  A  V  L  G  C  S  D  D  S  Q  A  K  R  V  R  V  L  E  L  S  R  R  L  K  H  R  G  P  D  W  S  G  I  H  H

 121 CAYGGAGACTGTTATTTRGCWCACCAGCGGCTAGCTATAGTCGATCCCGCCTCCGGMGATCAGCCTCTCTTTAACGAGGACAAAMGGATTGCYGTCACCGTAAATGGAGAAATATACAAC
     H  G  D  C  Y  L  A  H  Q  R  L  A  I  V  D  P  A  S  G  D  Q  P  L  F  N  E  D  K  R  I  A  V  T  V  N  G  E  I  Y  N

 241 CAYGAGGAGCTTCGGGCGCTTTTGCCGAACCAYAAGTTCCGTACCGGAAGCGATTGTGACGTCATAGCGCATCTCTACGAAGAGTAYGGAGAAAACTTTGTCGAAATGCTAGATGGGATG
     H  E  E  L  R  A  L  L  P  N  H  K  F  R  T  G  S  D  C  D  V  I  A  H  L  Y  E  E  Y  G  E  N  F  V  E  M  L  D  G  M

 361 TTCTCGTTTGTGCTKCTCGATTCTCGTGATAACACMTTTATCGCTGCTCGTGATGCWTTCGGRATAACYTCTCTRTATATCGGTTGGGGACTYGACGGTTCTGTTTGGATATCRTCTGAG
     F  S  F  V  L  L  D  S  R  D  N  T  F  I  A  A  R  D  A  F  G  I  T  S  L  Y  I  G  W  G  L  D  G  S  V  W  I  S  S  E

 481 YTAAAAGGGTTACACGATGAATGTGAGAATTTCGAAGTKTTCCCACCKGGACACGTRTACTCGAGCAAAACCGARGGTTTYAGAAGATGGTATAAYCCTCCTTGGTTYTCYGAGGCTATW
     L  K  G  L  H  D  E  C  E  N  F  E  V  F  P  P  G  H  V  Y  S  S  K  T  E  G  F  R  R  W  Y  N  P  P  W  F  S  E  A  I

 601 CCTTCWACTCCTTATGACCCTTTAGTCCTMAGGGGTGCTTTTGAACAAGCTGTGATTAAAAGACTTATGACAGATGTACCTTTCGGAGTTCTTTTATCGGGAGGGCTCGATTCKTCKCTA
     P  S  T  P  Y  D  P  L  V  L  R  G  A  F  E  Q  A  V  I  K  R  L  M  T  D  V  P  F  G  V  L  L  S  G  G  L  D  S  S  L

 721 GTTGCWGCTGTWACKGCTCGWCATTTGGCSGGMACWAAAGCTGCGAAAMGATGGGGATCACAGCTTCAYTCTTTYTGTGTGGGCCTTGAGGGCTCGCCTGATTTGAAAGCYGGAAAGGAA
     V  A  A  V  T  A  R  H  L  A  G  T  K  A  A  K  R  W  G  S  Q  L  H  S  F  C  V  G  L  E  G  S  P  D  L  K  A  G  K  E

 841 GTTGCCGATTATTTGGGTACTGTTCATCAYGAGTTTCTTTTCACWGTGCAGGATGGAATTGATGCGATCGAGGATGTGATTTATCATATCGAGACGTATGAYGTRACTACGATCCGAGCA
     V  A  D  Y  L  G  T  V  H  H  E  F  L  F  T  V  Q  D  G  I  D  A  I  E  D  V  I  Y  H  I  E  T  Y  D  V  T  T  I  R  A

 961 AGCACGCCTATGTTTCTTATGTCACGTAAGATAAAATCGCTCGGGGTTAAAATGGTAATTTCCGGTGAGGGTTCTGATGAGATWTTCGGTGGCTATTTGTACTTYCATAAGGCACCGAAY
     S  T  P  M  F  L  M  S  R  K  I  K  S  L  G  V  K  M  V  I  S  G  E  G  S  D  E  I  F  G  G  Y  L  Y  F  H  K  A  P  N

1081 AAGGAAGAGTTTCATCGTGAAACTTGTCGCAAGATWAAGGCTCTTCATCAATATGATTGTTTGAGAGCRAATAAGGCAACTTCTGCWTGGGGTTTAGAGGCTCGAGTYCCGTTTCTTGAT
     K  E  E  F  H  R  E  T  C  R  K  I  K  A  L  H  Q  Y  D  C  L  R  A  N /K  A  T  S  A  W  G  L  E  A  R  V  P  F  L  D

1201 AAGGAATTTGTGAACTTGGCAATGAGCATCGATCCCGAAGCAAAGATGATAAAACCCGATCAAGGGMGRATCGAGAAGTGGATTTTRAGGAAGGCTTTCGATGAYGAAGAACGMCCWTAT
     K  E  F  V  N  L  A  M  S  I  D  P  E  A  K  M  I  K  P  D  Q  G  R  I  E  K  W  I  L  R  K  A  F  D  D  E  E  R  P  Y

1321 CTTCCCAAGCATATTTTGTATAGGCAGAAAGAACAGTTYAGTGATGGTGTCGGATATAGCTGGATCGACGGGCTTAAAGCCCATGCCGAACAGCATGTAACTGATAAGATGATGCTTAAT
     L  P  K  H  I  L  Y  R  Q  K  E  Q  F  S  D  G  V  G  Y  S  W  I  D  G  L  K  A  H  A  E  Q  H  V  T  D  K  M  M  L  N

1441 GCTGGACATATCTTCCCACATAACACTCCAACMACAAAGGAGGGTTACTACTATAGGATGATATTCGARCGRTTTTTCCCACAGAACTCAGCCAAGCTCACTGTTCCAGGCGGGCCGAGT
     A  G  H  I  F  P  H  N  T  P  T  T  K  E  G  Y  Y  Y  R  M  I  F  E  R  F  F  P  Q  N  S  A  K  L  T  V  P  G  G  P  S

1561 GTGGCTTGYAGCACTGCTACAGCTGTGGCGTGGGATGCWTCGTGGTCGAARAAYYTGGAYCCATCGGGCCGGGCCGCAACTGGCGTGCATGATTTGGCCTATGAGAATCATGTGCCGATC
     V  A  C  S  T  A  T  A  V  A  W  D  A  S  W  S  K  N  L  D  P  S  G  R  A  A  T  G  V  H  D  L  A  Y  E  N  H  V  P  I

1681 GGGAATTTGAAGTCCAAGAAAATGGACTCTGTTTCACTGGGAAATGCAGTGGGCCCTCAAGAGCTCACCATCAGAAGTTAGCGTCGTTTAACGACGTTGATAAATTATCTGTTGTGTGTA
     G  N  L  K  S  K  K  M  D  S  V  S  L  G  N  A  V  G  P  Q  E  L  T  I  R  S  *  ----------

1801 GTTTATTTATGTAGTGTATTATGACAATCAAACTAAAATAAAATGTAAAATTTGGTTGTTTGTAGAGGGATAAGTTTGGTTARGTRCCAGATTTT-CCTAATGYAKT-TKTGTT-TTTGT
                                                                                                        T           A      A
                                                                                                                    @

1921 TTTTGTTTTYCATWTTTCGAGCTTCTTAGTAAAAGAAGCCTTST----CCTTYT-CAGTGTG--AAAAAGTTTGGCATTGTGTATAATGCATKTAGTTTATCTGTATTTCARCTCTTTCT
                                                 TTCA      T       TG

2041 TATATCAAATCTRCATTTTGTATT  2064
                             @
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Fig. 1. Nucleotide and amino acid sequence of *T. versicolor* AS. The nucleotide (top) and amino acid sequence (bottom) using the IUB code (bold) for variable nucleotide positions between the three groups of *T. versicolor* AS cDNA clones are shown. Primer sequences used for RT-PCR are underlined and italicized in the nucleotide sequence. Dashes represent deletions in some of the clones. The symbol '@' denotes the observed poly-A addition sites. The eight invariant amino acid residues of the cysteine–histidine–aspartate catalytic triad are bold and double-underlined.

Table 1
Amino acid similarities among plant asparagine synthetases

Plant	Accession No.	Overall	N terminal[a]
Medicago sativa	L40327	90.26	93.15
Asparagus officinalis	X67958	87.95	94.2
Arabidopsis thaliana	P49078	86.80	94.05
Lotus japonicus	X89409	86.63	93.87
Brassica oleracea	X84448	86.63	93.51
Pisum sativum (ASN2)	P19252	85.97	92.97
Pisum sativum (ASN1)	S11444	85.64	92.61
Oryza sativa	U55873	85.64	90.09
Glycine max	U77679	82.23	93.15
Zea mays	P49094	79.04	85.77

[a] Excludes last 31 amino acids.

TVAS-B and TVAS-E. Sequence comparisons between the three groups identified 75 variable nucleotides in the codons, of which 70 were in the wobble position and 5 were in the first position. None of the nucleotide changes resulted in any amino acid changes; therefore, the cDNAs all encoded identical proteins. Thirty-one changes were in the non-coding regions; 21 of these were small deletions or insertions.

Group TVAS-6 showed an alternative poly-A addition site that resulted in the 3′ end of the cDNA being 157 nucleotides shorter than the other two classes. Group TVAS-B had a different stop codon due to a 10-nucleotide deletion involving the wobble position of the stop codon.

3.2. Genomic organization

The genetic complexity of *T. versicolor* AS was investigated by gel-blot analysis of total genomic DNA. Fig. 2 shows the hybridization pattern obtained by using a probe synthesized from a 1.4-kb fragment of cDNA TVAS-B. This cDNA clone had two *Xho*I sites and no *Bam*HI, *Eco*RI, or *Hin*dIII sites. Two or three bands per lane were observed, with one band being more intense than the other two. The Southern results are consistent with AS being encoded by a small gene family.

3.3. Expression of AS in T. versicolor

The tissue specificity of AS expression in *T. versicolor* was examined by Northern analyses of RNA isolated from roots and shoots of 5-week-old, in-vitro-grown *T. versicolor* seedlings. The AS probe pTVAS-B hybridized to a 2.3-kb RNA isolated from both *T. versicolor* roots and shoots (Fig. 3). Quantification of the hybridizing bands indicated that the level of AS in the shoots was 1.6 times that in the roots.

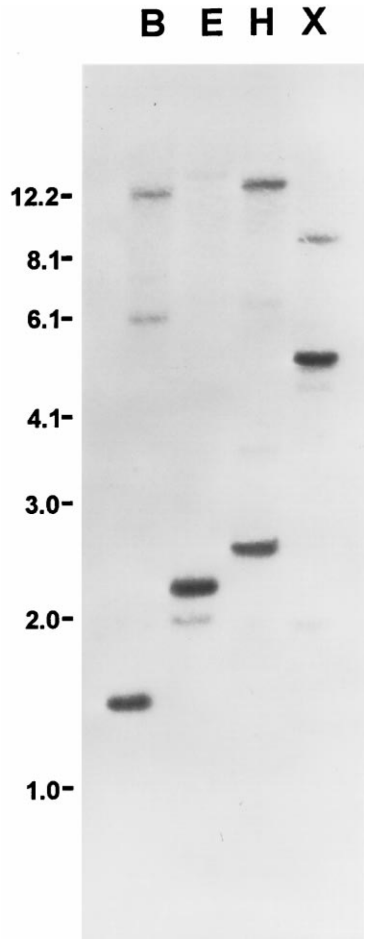

Fig. 2. Southern analysis of the AS gene family in *T. versicolor*. Total genomic DNA (~10 μg) from *T. versicolor* was digested with *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), or *Xho*I (X) as noted above lanes, electrophoretically fractionated on a 0.7% agarose gel, transferred to Zeta-Probe membrane and probed with the 1.4-kb insert of pTVAS-B cDNA clone. The migration positions of the molecular-weight standards are indicated on the left (kb).

The AS clones were originally isolated on the basis of being differentially abundant after treating *T. versicolor* with *T. repens* exudates. To determine whether the response was specific for *T. repens*, a similar experiment was carried out, using root exudates from hydroponically grown *Arabidopsis*. Fig. 4 shows that there is an approximately threefold increase in the steady-state AS levels following treatment with *Arabidopsis* root exudate. Therefore, the AS message increases following exposure to two different host-root exudates. In contrast, there was no increase in AS message levels when the *T. versicolor* roots were treated with 2,6-DMBQ (Fig. 4). When *T. versicolor* roots were treated with either *Arabidopsis* root exudates or 2,6-DMBQ, haustoria formed in over 50% of the roots tips by 24 h (data not shown).

The effect of light on AS expression was examined by growing the plants in the light and dark for various amounts of time. Light-grown plants had AS RNA levels about 2.5 times lower than plants incubated in the dark (Fig. 5).

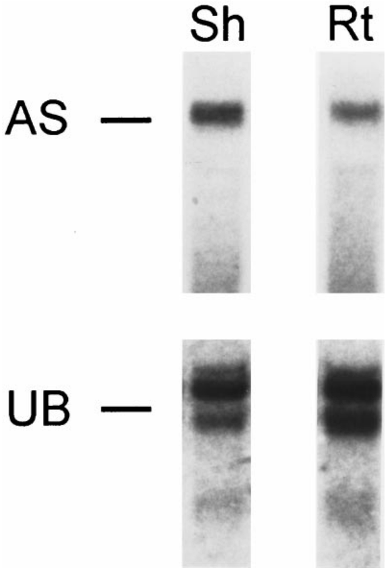

Fig. 3. *T. versicolor* AS is expressed in roots and shoots. Ten days after transplanting *T. versicolor* to 0.25 × Hoagland's medium, roots and shoots were collected separately. Total RNA (4 μg) isolated from *T. versicolor* roots and shoots was separated by electrophoresis, blotted on to Hybond N and hybridized to the random primed cDNA inserts of pTVAS-B (AS) and p2324 (UB).

4. Discussion

The induction of AS mRNA in the roots of a parasitic plant in response to host-root signals is intriguing. Parasitic plants rely on host mineral resources to varying degrees. Mistletoes and other parasites that invade above-ground parts are completely dependent on host nitrogen. In contrast, root parasites such as *Triphysaria* have access to nitrogen both from their host and from the soil (Press and Graves, 1995). Host nitrogen is likely an important resource for parasitic plants. Neither ammonium nor nitrate had any growth effects on *Rhinanthus* minor roots grown in pot cultures in the absence of host plants (Press et al., 1993). Similarly, inorganic nitrogen supplied to unattached *Bartsia trixago* did not evoke the growth or photosynthetic rates of those parasitizing host plants (Press et al., 1993). The importance of host nitrogen to parasitic plants is consistent with the preference of many broad host-root parasites for leguminous hosts (Atsatt and Strong, 1970).

If a parasite is to derive organic nitrogen from a host, it must be able to move the nitrogen efficiently from the site of host attachment to other locations in the parasite. Asparagine, with its higher N:C ratio compared to other amino acids, is the preferred nitrogen transporter amino acid for many plants, particularly under high nitrogen or low carbohydrate conditions. For example, asparagine is the principle N transport compound in many temperate legumes (Scott et al., 1976). Nitrogen-fixing root nodules are enriched in both AS enzyme and its respective mRNA (Ta et al., 1988; Kuester et al., 1997). AS is also enhanced when plants are carbohydrate stressed or grown in the dark (Tsai and Coruzzi, 1990, 1991; Chevalier et al., 1996). Asparagine is the predominant amino acid in xylem sap of *Striga hermonthica*

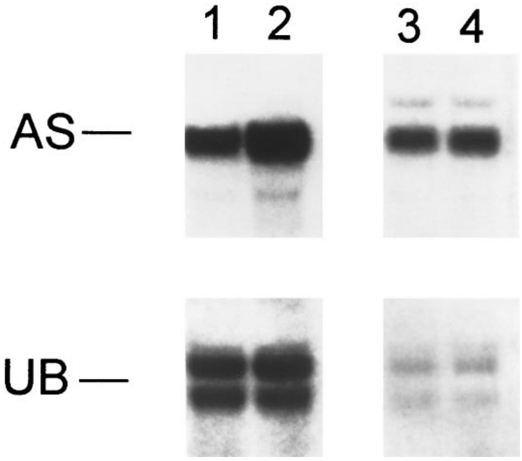

Fig. 4. AS expression following treatment with DMBQ or *Arabidopsis* root exudate. *T. versicolor* plants were treated for 72 h in growth medium (lane 1), in *Arabidopsis* root exudate (lane 2), in 100 μM DMBQ (lane 3), or water (lane 4). The filters were subsequently probed with AS and then ubiquitin (UB) as a loading control. Reduced hybridization of UB in lanes 3 and 4 is a result of low radioactivity incorporation in this experiment.

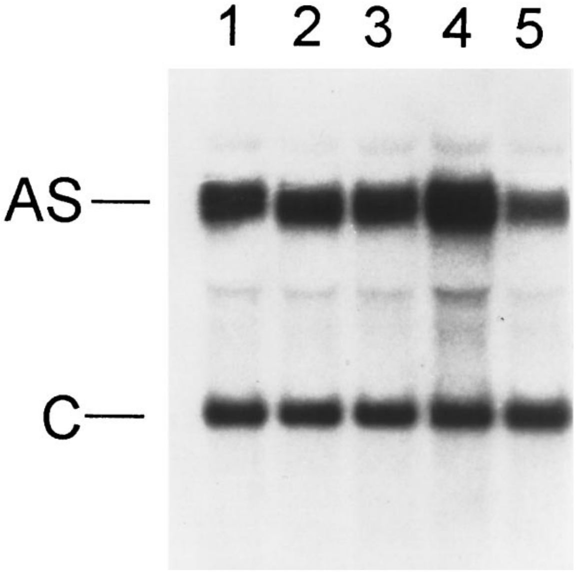

Fig. 5. AS levels in roots are reduced by light. Total RNA (4 μg) isolated from *T. versicolor* roots grown for different periods of time in the light was separated by electrophoresis, blotted on to Hybond N, and hybridized with a riboprobe of pTVAS-E (AS). RNA loading was checked by the control RNA (C). Plants were grown in a 17-h photoperiod for 1 week after transplanting on to 0.25 × Hoagland's medium. RNA was isolated from roots collected after 8, 12 and 16 h in the light (lanes 1, 2, and 3). Some plants were then incubated in the dark for 7 h, and RNA was isolated from collected roots (lane 4). RNA was then extracted from roots isolated after an additional 8 h in the light (lane 5).

(Shah et al., 1984). These authors suggest that the nitrogen assimilation pathway in *Striga* changes before and after it infects a host. The increase in AS mRNA transcript levels in *T. versicolor* in response to host signals might be diagnostic of a shift in the source–sink relationship to favor the movement of nitrogen into the parasite. One test of this hypothesis will be to determine whether AS is similarly increased in response to organic N sources such as aspartate or glutamine.

We have shown in this study that *T. versicolor* AS can be regulated by molecules released by roots of two species of host plants, *Trifolium* and *Arabidopsis*. We do not yet know which host molecules are responsible for this response. It is interesting that whereas DMBQ induces haustoria in *T. versicolor* (Albrecht, Yoder, Phillips unpublished), it does not stimulate AS mRNA. This suggests that multiple signals might be released by host roots and recognized by the parasite, some initiate haustoria development while others signal different processes.

AS transcripts are expressed in *T. versicolor* roots and shoots in our culture conditions even without host exudate treatment. Constitutive expression of AS has been seen in other experimental systems as well (Tsai and Coruzzi, 1991; Chevalier et al., 1996). AS mRNA transcript levels are elevated for a considerable length of time (3 days) after exposure to host exudates. In this regard, it is interesting that AS expression in alfalfa root nodules is maximally expressed 17 days after inoculation with *Rhizobium meliloti* (Ta et al., 1988). In both the parasite and nodule systems, the increased expression of AS continues for extended periods.

We isolated three classes of AS cDNAs from a *T. versicolor* root cDNA library. Two to three bands were observed on Southern hybridizations when AS was used as a probe. We find it unlikely that the different cDNA classes are expressed from different genes. Based on the high sequence similarities between the different AS cDNA classes, we favor the hypothesis that these sequence variants represent alternative alleles of a single AS locus. The different *T. versicolor* cDNAs were 96% identical at the nucleotide level and did not have a single amino acid difference. They were insufficiently variant at the sequence level to allow us to make class-specific probes. In contrast, the two AS genes in *Pisum sativum* and *Lotus japonicus* encode proteins with only 85% similarity (Tsai and Coruzzi, 1990; Waterhouse et al., 1996). Our root cDNA library was prepared from *T. versicolor* seedlings grown from field-collected seeds derived from hundreds of different parents. *T. versicolor* is a self-incompatible, obligate outcrossing annual that, as judged from frequent morphological variations, is highly polymorphic (Yoder, 1998). We suggest that the high polymorphism rate is reflected in the AS cDNA sequence variations.

Three different actin genes have been isolated from *Striga*, and the expression of these was shown to be tissue-dependent. However, no differences in transcript levels were detected before or after DMBQ induction (Wolf and Timko, 1994). Two cDNAs, one encoding cytochrome B(5) and a second a hybrid proline-rich protein, have been isolated from cytokinin induced haustoria from the unrelated stem parasite *Cuscuta reflexa* (Subramaniam et al., 1994). It was not reported whether or not these genes were differentially abundant following induction. Thus, the AS gene of the parasitic angiosperm *Triphysaria* represents a novel case of a plant gene being responsive to host-root signals.

Acknowledgement

We wish to gratefully acknowledge the technical assistance of Denneal Jamison and advise and suggestions of Professor P. Thalouarn and Professor A. Fer. The work was funded by NSF grant #94-07737 and a Conseil Général de Loire-Atlantique, France post-doctoral award to P. Delavault.

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