

A Comprehensive Subtractive cDNA Cloning Approach to Identify Nematode-Induced Transcripts in Tomato

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ABSTRACT

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Using a cDNA cloning technique that incorporated polymerase chain reaction amplification of cDNA and subtraction in a single-strand phagemid vector, we constructed a library of transcripts exhibiting up regulation in tomato cells infected with the parasitic nematode *Meloidogyne incognita*. Starting with 51 mg of dissected, nematode-induced giant cells, we constructed a primary cDNA library of 2.2×10^6 recombinants. Subtrac-

tion against uninfected tomato roots gave a 4,860-fold enrichment. Analysis of the library as a whole indicated that contamination by nematode sequences was less than 1%. Transcripts from high copy number genes accounted for 14% of the clones; the remaining 244 recombinants appeared to be derived from distinct, unique genes. Partial DNA sequence analysis of one cDNA revealed homology with the RB7 gene from tobacco, the only gene previously known to be up regulated in giant cells. In addition to permitting a transcriptional analysis of giant cells, our cloning technique should be well suited for isolating genes from other pathogen-infected plant cells.

Root-knot nematodes (*Meloidogyne* spp.) are obligate, sedentary endoparasites of many plant species. After hatching in the soil, larvae invade the root and migrate intercellularly to the developing vascular cylinder where permanent feeding sites are established. Mature feeding sites are characterized by the presence of five to seven multinucleate cells termed *giant cells* (2). These large, avacuolate cells with extensively remodeled cell walls are metabolically active (3) and serve as the obligate nutritive source for the developing nematode. Giant cell formation, coupled with limited proliferation of nearby pericycle and cortical cells, results in the characteristic root-knot gall. Although giant cells share features with other plant cells, notably transfer cells, they are a novel cell type and presumably arise by a pattern of gene expression different from that in other plant cells. It previously has been speculated that gene expression in giant cells might include genes normally expressed at different developmental times or in different cell types (4).

Surprisingly little is known about gene expression in healthy root cells. In a survey of randomly chosen cloned root mRNAs, Evans et al (9) were unable to identify any as being root specific. Using a differential screen, Conkling et al (6) isolated genes encoding four moderately to abundantly expressed root-specific transcripts from tobacco. The expression of one of these, TobRB7, has been reported to be up regulated in giant cells induced in tobacco by *M. incognita* (17). Also using a differential screening approach, Gurr et al (13) identified a gene in potato whose expression is "correlated with events in the immediate vicinity of the pathogen" (the potato cyst nematode, *Globodera rostochiensis*), but the nature of this gene was not revealed.

As a first step in understanding the molecular basis for giant cell formation, we wished to construct a cDNA bank representing genes that are uniquely expressed in giant cells (compared with uninfected root cells) or that have elevated expression levels. Classically, subtractive screening or cDNA cloning approaches have proved effective in identifying transcripts with differential expression profiles, including developmentally regulated mRNAs from *Dictyostelium discoideum* (15) and gastrulation-specific sequences from the African clawed toad, *Xenopus laevis* (21). However, classical subtractive approaches, whether used to con-

struct an enriched library from which individual clones may be characterized or to produce probes for the differential screening of a standard library, have a number of limitations. Because of their requirement for large amounts of starting mRNA (typically tens of micrograms of poly(A)⁺ from the driver source and >1 μ g from the target source), these methods alone are not suited for isolation of giant cell-specific transcripts. Furthermore, the clones that result from either approach represent moderately to abundantly expressed transcripts; rare transcripts tend to be underrepresented. Finally, the differential enrichment that the subtractive steps are designed to achieve also results in enrichment of aberrant clones, and contamination of subtracted cDNA with double-stranded material after hydroxylapatite chromatography can lead to a subtracted library with substantial representation of clones present in both driver and target. Differential screening is then required to confirm subtracted clones.

Recently, a number of techniques that address some of the shortcomings of the subtractive approach have been developed. By exploiting the polymerase chain reaction (PCR) to amplify small amounts of cDNA, researchers have recently produced libraries of approximately 10^6 primary recombinants starting with as little as 10 μ l of J558 myeloma cells (1), 300 mg of mouse brain tissue (7), and 50 mouse ovulated oocytes (24). Analyses of these libraries showed them to be low in contaminating rRNA clones; inserts ranged from 200 bp to >2 kb. PCR amplification also has been used to produce cDNA as driver for subtraction (14).

Ensuring that cDNAs representing transcripts expressed at low abundance are represented requires that enough cDNA remain after subtraction for efficient cloning. By performing the cloning event before the subtraction, the requirement of working with potentially very small quantities of cDNA can be avoided. Several researchers (8,19,22) have developed methods to produce cDNA libraries in phagemid vectors, which may be easily and efficiently transformed following subtractive hybridization.

A major goal of our research is to obtain a general view of the molecular events that lead to the induction and maintenance of giant cells in plant roots by *Meloidogyne* spp. To this end, we wished to clone transcripts from giant cells representing genes with up-regulated expression. We have combined a PCR amplification of cDNA with the efficient and sensitive phagemid subtraction methods to produce a subtracted cDNA bank from a small number of individual tomato giant cells. We have optimized many

of the reactions employed in library construction and introduced a number of novel steps to develop a robust technique that should be applicable to molecular analyses of other plant-microbe interactions. Here we present a description of our bank; detailed analyses of specific clones will be presented elsewhere.

MATERIALS AND METHODS

Culture maintenance. Giant cells and normal root tissue were isolated from tomato (*Lycopersicon esculentum* 'Rutgers Large Red') plants cultured in vitro. Seeds were surface sterilized by vacuum infusion of 0.5% sodium hypochlorite and germinated in Parafilm-sealed petri plates on 2% GelRite (Schweizerhall Inc., South Plainfield, NJ) containing vitamin-supplemented Gamborg's B5 medium (Sigma Chemical Co., St. Louis, MO). Cultures were maintained in the dark at 26 C. When tertiary root branches were evident (after approximately 2 wk), each dish was inoculated with 200 root-knot nematode (*M. incognita*) eggs, which had been surface sterilized by incubation for 10 min in 0.1% streptomycin and 0.01% HgCl₂. Nematode stocks were maintained on greenhouse-grown tomato (cultivar Tropic) plants, and eggs were isolated by hypochlorite extraction (16).

Root galls containing young adult female nematodes were excised from the etiolated plant-nematode cultures at 1–2 mo postinfection. Gall tissue surrounding the giant cell was dissected away, and the exposed, intact nematode was removed. Giant cells were resected and rapidly frozen at –72 C.

RNA isolation. RNA was isolated by a modification of the method of Chomczynski and Sacchi (5). Approximately 50 mg of giant cell tissue was ground under liquid nitrogen in a micro-homogenizer (Biomedix, Middlesex, U.K.) and resuspended in 200 μ l of E-buffer (4 M guanidine isothiocyanate, 25 mM sodium phosphate buffer, pH 7.4, 1% sarkosyl, and 1 mM β -mercaptoethanol) at 40 C. The suspension was incubated at 40 C for 20 min with occasional vortexing. Sodium acetate, pH 4, was added to a final concentration of 50 mM followed by addition of an equal volume of water-saturated phenol, and the sample was placed on ice for 10 min. One-tenth volume of chloroform was added, and the aqueous phase was recovered following centrifugation at 12,000 g for 10 min at 4 C. The organic phase was back extracted with 100 μ l of E-buffer containing 50 mM sodium acetate, pH 4, and the aqueous phases were pooled. RNA was recovered by ethanol precipitation, dried in vacuo, and dissolved in water.

Normal root RNA was extracted from uninfected tissue-cultured seedlings grown in parallel and harvested at the same times as the giant cells. Freshly harvested tissue was frozen in liquid nitrogen and ground with a mortar and pestle. E-buffer was added at 2 ml/g of tissue (fresh weight).

cDNA synthesis. Poly(A)⁺ RNA was captured from giant cell total RNA on oligo(dT)₂₅ coupled paramagnetic beads (DynaL Inc., Great Neck, NY) according to the manufacturer's instructions. Bead-bound RNA was washed twice with 2.5 times the original bead suspension volume of first-strand cDNA synthesis buffer (50 mM Tris HCl, pH 8.0, 50 mM KCl, and 10 mM MgCl₂), and cDNA was synthesized on the bead to produce a solid-phase primary cDNA pool (18). First- and second-strand synthesis and end repair reactions were performed with the Riboclone cDNA synthesis system (Promega, Madison, WI) according to manufacturer's suggestions. Beads were kept in suspension during the synthesis reactions by shaking at 350 rpm.

Oligonucleotides P35 (GTAAGCGGCCGAGCGTCAG-TAACTC) and P36 (TACTGACGCTGCGGCCGCTTAC) were annealed under standard conditions (25). This formed a double-stranded adapter molecule with an internal *NotI* site. The molecule was blunt at one end and had a noncomplementary, four-base overhang at the opposite end. P36 was end labeled with polynucleotide kinase prior to annealing. Annealed adapter was ligated onto the free end of the bead-bound double-stranded (ds) cDNA at 15 C for 16 h with shaking at 350 rpm. Unligated adapter was removed from the cDNA by washing with sterile water until free counts were no longer detected in the wash.

Second-strand cDNA was eluted from bead-bound first-strand cDNA by heating to 94 C for 3 min. The beads were then rapidly chilled on ice, and the aqueous phase was removed from the beads. The cDNA yield was estimated by liquid scintillation counting of an aliquot of the eluate. An additional batch of second-strand cDNA was synthesized on the beads by primer extension with P36. Following elution, this material was pooled with the previously synthesized second-strand cDNA. Pooled cDNA was converted to double-stranded form by extension from the poly(A) tract at the 3' end with P39 primer (ACTCTTGGGCCGAG-TTGCC[T]₁₅). The ds-cDNA was purified from excess P39 by spin column chromatography over Sephadex G-50 equilibrated with 200 mM NaCl.

PCR amplification. One-fourth of the cDNA was amplified by 15 cycles of PCR (94 C, 2 min; 58 C, 1 min; 72 C, 5 min) with primers P36 and P40 (ACTCTTGGGCCGAGTTG-GCC[T]₄). Amplification products were fractionated on 1% low melting point agarose, and size ranges of DNA (400–700 bp, 700 bp to 1.3 kb, and 1.3–5 kb) were resected and eluted by Gelase digestion (Epicentre Technologies, Madison, WI). One-fifth of each size class was reamplified by 10 cycles of PCR under the same conditions as the original amplification. Each reaction was diluted fivefold into fresh reaction mix and amplified a further five cycles. The 400- to 700-bp fraction and the 700-bp to 1.3-kb fraction were faithfully reamplified and then were pooled.

cDNA cloning. Single-stranded amplification products were removed by mung bean nuclease digestion, and the cDNA was restricted with *NotI* and *SfiI*. Fifty nanograms of digested cDNA insert was ligated into 300 ng of dephosphorylated pGem 11zf(+) vector (Promega) digested with *NotI* and *SfiI*. The ligation reaction was phenol extracted, ethanol precipitated, resuspended in 4 μ l of water, and electroporated at 200 Ω , 25 μ F, 18.8 kV/cm into 200 μ l of electrocompetent *Escherichia coli* DH12s cells (Bethesda Research Laboratories, Gaithersburg, MD). Cells were incubated for 40 min in 100 ml of SOC medium at 37 C with shaking (350 rpm), and an aliquot was removed to determine the number of primary transformants. Ampicillin was added to the bulk of the culture to 50 μ g/ml, and the culture was grown to an A_{600} of 0.1. Two milliliters of the culture was removed, grown overnight at 37 C, and stored as a glycerol stock. M13K07 phage was added to the main culture at a multiplicity of infection of 10, and the culture was grown for an additional 2 h. Phage-infected cells were selected by addition of kanamycin (70 μ g/ml), and the culture was incubated with vigorous shaking at 37 C for 15 h.

Recombinant M13 virions were harvested by precipitation from 4% polyethylene glycol 8,000 and 500 mM NaCl, and single-stranded (ss) DNA was prepared and resuspended in 200 μ l of water. The sample was digested with *HindIII*, and ssDNA was purified by fractionation on low melting point agarose.

Driver preparation. A solid-phase cDNA pool was prepared from 75 μ g of total RNA from uninfected roots, and 10 ng of the resultant ds-cDNA was subjected to 15 cycles of PCR, exactly as described for the giant cell library, except that the dTTP concentration was reduced to 75 μ M and biotin-16-dUTP (Boehringer Mannheim, Indianapolis, IN) was added to 25 μ M (14). The reaction was diluted fivefold, and PCR amplification continued for five cycles. The final yield of biotinylated cDNA was 5 μ g.

Subtraction and transformation. Biotinylated root cDNA (4 μ g) was coprecipitated with 2 μ g of M13 DNA with giant cell cDNA inserts, giving a 10-fold molar excess of driver to insert. The DNA was resuspended in 20 μ l of water, and 0.5 μ g of oligo(dT)₁₅ was added. One microliter of 2 \times hybridization solution (400 mM NaCl, 100 mM Tris HCl, pH 7.5, and 4 mM EDTA) was added, and the mixture was concentrated in vacuo to 2 μ l. The solution was covered with a drop of mineral oil, heated to 94 C for 5 min, and incubated at 65 C for 25 h.

The annealing reaction was diluted to 50 μ l with 0.5 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and then incubated at room temperature for 30 min with 600 μ l of streptavidin-coated paramagnetic beads (Promega), previously

equilibrated to 0.5× SSC. The beads were separated from the solution on a magnetic stand, and the binding step was repeated. Oligo(dT)₁₅ was removed from unbound ssDNA by fractionation on Sephadex G-50 equilibrated with 200 mM NaCl, and salts were removed by fractionation on water-equilibrated Sephadex G-50. ssDNA was converted to double-stranded form by Klenow extension from P46 primer (GGCCAAGTTGGCC[T]₅), desalted on water-saturated Sephadex G-50, concentrated to 3 μl in vacuo, and electroporated into *E. coli* DH12s cells as described. Cells were incubated at 37 C with shaking (250 rpm) for 1 h in SOC medium and plated in the presence of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside). White colonies were picked into individual wells of 96-place microtiter plates containing SOC and grown overnight at 37 C, and glycerol stocks were prepared.

Library analysis. Colonies were replica stamped from the microtiter plate glycerol stocks onto nitrocellulose membranes (12) and probed with nick-translated genomic tomato and *M. incognita* DNA and cDNA inserts under standard conditions (23).

ssDNA was prepared from recombinants by rescue with the helper phage M13K07 and subjected to sequencing with the dideoxy chain-termination method (20). Hybridization probes for Southern and RNA dot blot analyses were prepared by primer extension of single-stranded templates with universal sequencing primer. Tomato and nematode genomic DNA was prepared by grinding tissue in liquid nitrogen followed by proteinase K digestion and phenol-chloroform extraction (11).

RESULTS AND DISCUSSION

cDNA synthesis. Fifty-one milligrams of essentially pure giant cells was accumulated by dissection. On the basis of comparative ethidium bromide fluorescence, the yield of total RNA from this sample was estimated to be 11 μg. This represents a yield per gram of tissue (fresh weight) approximately four times higher than that which we obtained from cultured whole roots and possibly is a reflection of increased transcriptional activity in giant cells.

Performing the cDNA synthesis on paramagnetic beads provided a number of advantages over liquid synthesis, including the ability to readily recover poly(A)⁺ RNA and cDNA into essentially zero volume. Because the bead effectively blocked one end, addition of a *NotI* adapter after cDNA synthesis was restricted to the 5' end (with respect to the message). Additionally, the use of solid-phase cDNA synthesis provided a reusable pool of first-strand cDNA.

On the basis of incorporation of ³²P-labeled P36 (half of the adapter), conversion of poly(A)⁺ RNA into eluted second-strand cDNA was 24%. Subsequent resynthesis of second-strand cDNA corresponded to a 15% conversion of poly(A)⁺ RNA. It is likely that this reduced efficiency resulted from inefficient priming. Conversion of pooled, eluted second-strand cDNA to double-stranded form by priming with an oligo(dT)-anchored *SfiI* primer (P39) yielded 43 ng of dsDNA. Analysis of an aliquot by electrophoresis on alkaline agarose (data not shown) revealed an average size of 800 bases for this cDNA pool, a portion of which extended longer.

Pilot experiments indicated that, under our conditions, the rate of amplification began to diminish between 10 and 15 rounds of PCR while, in *pari passu*, the amount of single-stranded product increased. Consequently, the giant cell cDNA was subjected to only 15 cycles of PCR amplification prior to size fractionation. The size classes were reamplified, and single-stranded amplification products were removed by mung bean nuclease digestion (10) prior to cloning. Comparison of cDNA before and after nuclease treatment indicated that up to two-thirds of the amplified cDNA, primarily the larger material, consisted of single-strand product (data not shown). The size of the final cDNA population averaged about 300 bp and ranged from 200 to 500 bp. Preferential extension of smaller cDNAs over larger ones as PCR progresses has been observed previously (1).

Cloning. To maximize the efficiency of the cloning step and

to eliminate the possibility of cloning uninfected-root driver cDNA, amplified giant cell cDNA was ligated into a phagemid vector. A test plating of the library at this stage indicated that it contained 1.4×10^6 transformants. After conversion to single-stranded form by rescue with M13K07, the cloned giant cell cDNAs were annealed with normal-root cDNA driver to a C_0t value of 500. At the K value for a typical mammalian cell (15,000 unique transcripts), the fraction of cDNAs common to both uninfected roots and giant cells that annealed at C_0t 500 is 96.5%.

After the annealed cDNA had been removed, the single-stranded recombinants were converted to double-stranded form. Transformation by electroporation of double-stranded plasmid DNA has been shown to be several hundred times more efficient than that of single-stranded plasmid DNA (19). We utilized a primer that spans the *SfiI* cloning site and the poly(A) tail of the insert. This primer is not complementary to insert-minus clones nor to incorrectly oriented insert-containing clones. Production of double-stranded plasmid DNA prior to transformation with this primer allowed for more efficient cloning of genuine subtracted clones over insert-minus or aberrant clones that may have escaped the subtraction procedure.

Following transformation, 287 insert-containing clones, as determined by blue-white colony screening on X-Gal-IPTG, were recovered. This corresponded to an overall 4,860-fold enrichment of giant cell sequences over normal root sequences present in the cDNA library. This figure is substantially higher than is typically obtained by subtraction. Duguid et al (8), for example, achieved an approximate 100-fold enrichment of scrapie-modulated RNAs after subtraction.

Library analysis. A variety of criteria was used to assess the overall quality of the subtracted library. Insert size was determined by restriction analysis. Inserts resected from 10 randomly selected clones were 200–500 bp (data not shown). Seven of these probes were also hybridized back to the entire library. One clone detected two recombinants; the remaining six probes detected only the clones from which they were constructed (data not shown). This result suggests that the complexity of the subtracted library might be high.

The total number of highly repeated genomic sequences represented in the library was determined by hybridization of replica filters with nick-translated tomato total DNA (Fig. 1). Forty-two of the 287 clones gave a high signal. Four of these

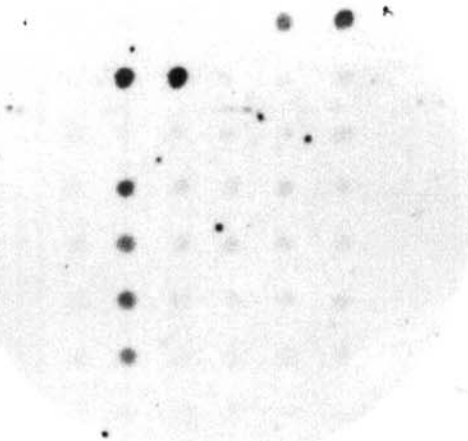


Fig. 1. Grunstein analysis (12) of *Escherichia coli* DH12s colonies containing recombinant cDNA clones derived from *Meloidogyne incognita*-induced tomato giant cells. Filters were hybridized with total tomato DNA that had been nick translated to a specific activity of 10^8 dpm/μg, and positive clones were identified by autoradiography for 23 h at -80 C with an intensifying screen. A signal was detected for eight of the 48 colonies on this filter, indicating that they harbored recombinant cDNAs derived from high copy number tomato genes.

clones were sequenced; three were derived from 25S rRNA and one from 16S rRNA. To further confirm that clones not detected in this assay were indeed derived from low copy number genes, genomic Southern blots were performed with 36 randomly selected clones as probes. Hybridization banding patterns identified one apparent multicopy gene (data not shown). However, most appear to be low copy genes (e.g., DB#210; Fig. 2) or unique genes (e.g., DB#212; Fig. 2). Partial sequences analysis of DB#212 (data not shown) confirmed the presence of a *Bam*HI site implicated by the Southern blot result (Fig. 2). These results also confirm that the clones are of plant origin. To determine the number of contaminating nematode sequences in the library, the replica filters were probed with nick-translated *M. incognita* total DNA. One colony gave a signal detectable above background (data not shown). A second clone encoding a nematode transcript was subsequently identified by genomic blotting (data not shown).

Sixty-three of the clones have been partially sequenced. All inserts are flanked by the oligonucleotide pairs (P35/36 and P39/40) used for library construction and are cloned in the correct orientation. With the exception of the four rRNA clones, all inserts begin with a homopolymeric A tract, corresponding to the poly(A) tail of the transcripts and ranging from 4 to 75 A residues. The average length is 11.6. A database search with a partial sequence from the cDNA clone DB#249 as a query revealed that its inferred product shares homology with that of the RB7-5A gene from tobacco (26) (Fig. 3). Significantly, this gene is strongly up regulated in *M. incognita*-induced tobacco giant cells. It will be

interesting to characterize the promoter of the DB#249 gene to see whether it too has a nematode-inducible element (17).

Taken together, these data show that the subtracted library contains authentic cDNA inserts with a low level of contamination by high copy or nematode sequences as well as a high degree of complexity. This bank will permit the first comprehensive analysis of transcriptional events associated with nematode-induced plant giant cells.

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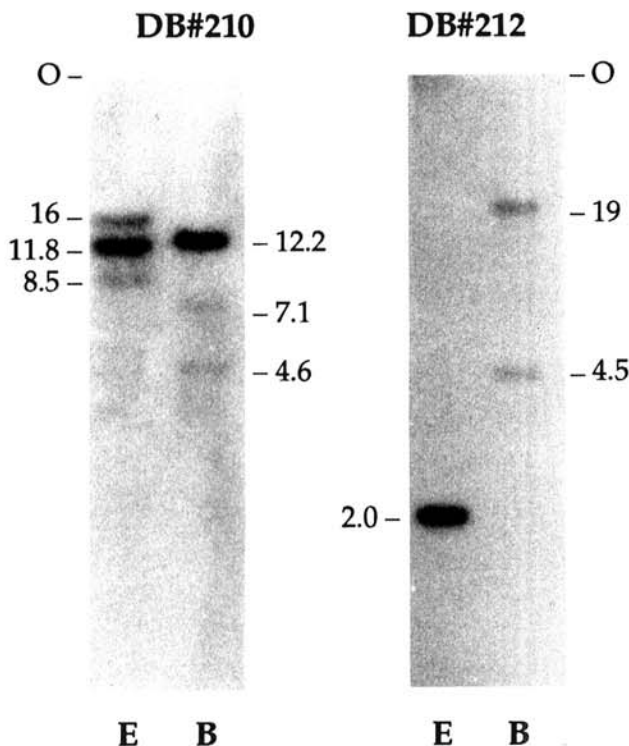


Fig. 2. Tomato genomic DNA, digested with either *Eco*RI (E) or *Bam*HI (B), was Southern blotted and hybridized with cDNA clone DB#210 or DB#212. Sizes of bands are indicated in kilobase pairs. The DB#210 gene appears to be represented several times in the genome, whereas the DB#212 gene appears to be unique.

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DB#249:  AAGPFSGGSMNPARSSGPAMVSGNSEGFWIYWSGPLYGGSLAGLIYTNVMTQEHAPLSNEF
          *****+*****+*****+*****+*****+*****+*****+*****
TobRB7-5A: AAGPFSGGSMNPARSFGPAVAVGDFSQNWYIYWSGPLYGGSLAGLIYTNVMTQEHAPLSNEF
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Fig. 3. Alignment of the carboxyl portions of the deduced proteins from the DB#249 cDNA sequence and the TobRB7-5A gene from tobacco (26). * = Identical amino acids; + = similar residues. A single gap has been introduced into the TobRB7-5A sequence to maximize alignment.

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