MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 5, No. 6, pp. 513-515, 1992

Research Notes

A New Multigene Family Inducible by Tobacco Mosaic Virus or Salicylic Acid in Tobacco

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Received 18 February 1992. Revised 21 July 1992. Accepted 30 July 1992.

A previously undescribed cDNA family was isolated from tobacco challenged with tobacco mosaic virus (TMV). A cDNA library was constructed with mRNA from upper leaves of Xanthi nc tobacco plants that had been inoculated with TMV on the lower leaves 11 days previously. The library was screened differentially with radiolabeled cDNA synthesized with mRNA from upper, uninoculated leaves of either TMV-inoculated or mock-inoculated tobacco plants. The new cDNA family, designated SAR8.2, had at least five expressed members, one or more of which were inducible by TMV inoculation and by salicylic acid treatment. The cDNAs encoded small, highly basic proteins containing N-terminal hydrophobic signal peptides and highly conserved cysteine-rich C-terminal domains. One of the SAR8.2 family members contained a direct repeat of the C-terminal domain in tandem. Hybridization of SAR8.2 cDNA to tobacco genomic DNAs indicated a gene family of 10–12 members.

Additional keywords: disease resistance, pathogenesis-related (PR) proteins, systemic acquired resistance.

Resistance to a variety of fungal, bacterial, and viral pathogens is induced in many plant species by prior inoculation with a necrotizing pathogen. This phenomenon, systemic acquired resistance (SAR), has been studied extensively in tobacco varieties such as Samsun NN and Xanthi nc (genotype NN) that exhibit a hypersensitive reaction (HR) to tobacco mosaic virus (TMV) (Ross 1961; Van Loon 1975; Ward *et al.* 1991). The biochemical mechanisms of resistance are unclear. Much attention has been directed to a group of proteins, termed the pathogenesis-related (PR) proteins; PR protein expression is induced throughout the plant in response to pathogen-induced necrosis, and accumulation of PR proteins correlates with the resistant state (Gianinazzi *et al.* 1970; Van Loon and Van Kammen 1970; Van Loon *et al.* 1987). PR proteins in tobacco and other species have been the subject of recent reviews by Bol *et al.* (1990), White and Antoniw (1991), and Linthorst (1991).

Here, we report a newly discovered gene family of Xanthi nc tobacco, identified by differential screening of a cDNA library from uninfected leaf mRNAs of TMV-inoculated plants. Five highly related cDNAs have been isolated and sequenced. Genomic blot analysis suggests that the gene family may contain up to 12 members.

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Nucleotide and/or amino acid sequence data for SAR8.2a–e were submitted to GenBank as accession numbers M97194, M97359, M97360, M97361, and M97362, respectively.

To isolate cDNAs induced systemically by TMV inoculation, we conducted differential screening of a TMV-induced cDNA library by hybridizing library plaque lifts (Benton and Davis 1977) sequentially with two probes (Jakobsen *et al.* 1989). The first probe was ^{32}P-labeled first strand cDNA (Maniatis *et al.* 1982) synthesized with template mRNA from mock-inoculated plants. Filters were not stripped before hybridization of the second probe. The second cDNA probe was made with the same mRNA used for construction of the library. This mRNA was from upper leaves of TMV-inoculated plants (i.e., leaves containing no virus and acropetal to lesion-bearing inoculated leaves) taken 11 days after the initial inoculation. Among the clones isolated was a new class of TMV-induced cDNAs, designated SAR8.2. RNA blot analysis with SAR8.2 labeled a band of approximately 560 bases. SAR8.2 is a trivial name derived from the screening procedure and bears no reference to the properties of the clone or its encoded protein.

Sequence analysis of 24 independent SAR8.2 cDNA clones revealed five different types. For the sake of brevity, the DNA sequences are not shown here. Of the 24 clones, five were type a, eight were type b, four were type c, six were type d, and one was type e. All of the clones showed extensive sequence identity in the coding and untranslated regions.

Figure 1 shows a comparison of the protein sequences encoded by the five cDNAs. SAR8.2e contained a C-terminal repeated domain (discussed below) that is shown on a separate line for alignment comparison. Each of the open reading frames (ORFs) contained an apparent canonical signal peptide sequence at its N terminus (underlined). Assuming that the processing of the signal peptides was in a fashion consistent with the rules of von Heijne (1986), the molecular weights of the resulting mature

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          10        20        30        40        50        60        70        80        90
8.2a  MFSKTNLFLCLSLAILVIVISSQVDAREMSKAPASITQAMNSNIITDQKMGAGITRKIPGWIRKGAKPGGKIIGKACKICSCKYQICSKCPKCHD*
8.2b  .....I....F......M.....A........A.P..............T....I.............-..N.....................*
8.2c  ................L......A...QI...A.P..H.....N..N..T....I................VA.....................*
8.2d  ................L......A........AVP........N..N..T....I................VA.....................*
8.2e  ................L......A....T...T.P...E....NT...--------...---KR-P....N.F.......P...........D.>
                                                                  QN.A..F.......T.........NQN*
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Fig. 1. Amino acid sequence comparison of five SAR8.2 cDNA open reading frames. The putative signal peptides are underlined. Amino acids identical to the SAR8.2a sequence are represented by a dot. Mismatches are shown as capital letters, and gaps are represented by dashes. An asterisk indicates the stop codon; the SAR8.2e sequence is continued on the next line to show its alignment with homologous segments of the other proteins and itself.

proteins of SAR8.2a–d would be approximately 7.5–7.7 kDa. However, the fifth cDNA, SAR8.2e, contained a direct repeat of a C-terminal peptide. This additional domain as well as other changes in the ORF increased the molecular weight of the putative mature protein to 9.6 kDa. Amino acid identity ranged from 88 to 96% for SAR8.2a–d. All were greater than 93% homologous when conservative changes were considered. SAR8.2e was only 72% identical to the other four, mainly because of a deletion in the center portion of the ORF.

The C-terminal domains of the four shorter cDNAs, SAR8.2a–d, were cysteine-rich and identical in the final 22 amino acids (aa) (6 cys/22 aa). There were no other cysteine residues in any of the mature protein segments. This segment was conserved and repeated in tandem in SAR8.2e. In this case, each domain had a single amino acid change in the 17-aa segment bounded by the first and last cysteines.

Data base searches with the full-length SAR8.2 cDNA or protein sequences yielded no significant matches (GenBank 69, Swiss-Prot 19, PIR 29). However, when the query sequence was narrowed to the 25-aa cysteine-rich peptide, the cysteine motif showed homology to many peptides known to be involved in divalent cation binding, especially those of metallothionins (Aitken 1990). This, with its conservation and duplication in SAR8.2e, may indicate a functional importance. However, we presently have no evidence of any specific function for these gene products. Chiang and Hadwiger (1991) recently reported *Fusarium*-induced cDNAs of pea that encode cysteine-rich proteins with some homology to proteinase inhibitors. We found no significant homology between these proteins and the SAR8.2 proteins.

The size of this gene family was estimated by hybridization of the SAR8.2 cDNA to tobacco genomic DNA (Bernatzky and Tanksley 1986). Approximately 10–12 hybridizing restriction fragments of various intensities were visible in *Nicotiana tabacum* L. DNA digested with either *Bam*HI, *Sst*I, or *Xba*I (Fig. 2). None of these restriction sites occurred in any of the five SAR8.2 cDNAs. The additional hybridizing bands may represent as yet undetected active genes, or they may be pseudogenes.

Many questions remain regarding SAR8.2 gene regulation. We do not presently know how many of the genes respond to TMV. However, the low level of mRNA in the mock-inoculated plants and the roughly equal distribution of at least four gene products in the TMV-induced cDNA population suggest several genes may be induced. Ward *et al.* (1991) recently reported induction kinetics of

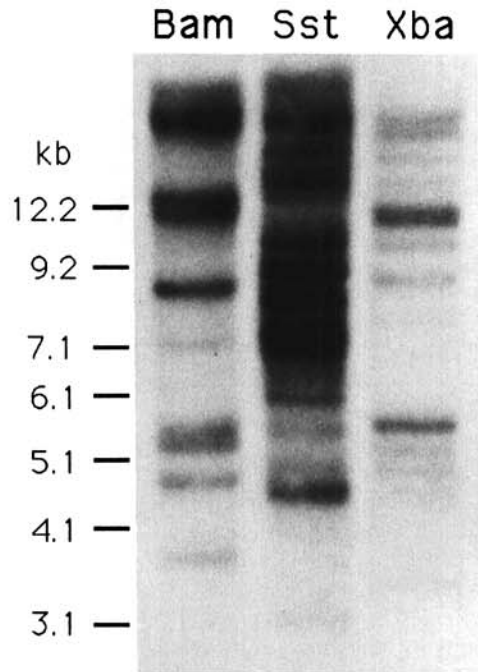

Fig. 2. Southern blot analysis of *Nicotiana tabacum* genomic DNAs. The probe was a nick-translated SAR8.2c cDNA insert. Approximately 10 μg of each genomic DNA was digested with *Bam*HI, *Sst*I, or *Xba*I. Fragments were separated on a nondenaturing 0.7% agarose gel and transferred to nitrocellulose. The blot was washed at 42° C in 0.1× SSC (1× = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0), 0.1% sodium dodecyl sulfate.

10 SAR-related mRNA families, including SAR8.2, induced by TMV, salicylic acid, or isonicotinic acid derivatives. In that study, levels of SAR8.2 mRNAs in control plants were easily detectable. However, in many similar experiments, the SAR8.2 mRNAs were often practically undetectable in untreated plants. Clearly, TMV and salicylate always induced the genes to the same high level, but there seemed to be other variables that affected mRNA accumulation in untreated plants. It is possible that small variations in growth conditions, or exposure to some undetected biotic or abiotic agent, may cause significant expression of at least some of these genes.

ACKNOWLEDGMENT

We thank Bill Schreckengost for performing the DNA sequencing.

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