SYSTEMIC ACQUIRED RESISTANCE IN TOBACCO: USE OF TRANSGENIC EXPRESSION TO STUDY THE FUNCTIONS OF PATHOGENESIS-RELATED PROTEINS.

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ABSTRACT. Systemic Acquired Resistance (SAR) is the resistance to a variety of fungal, bacterial, and viral pathogens induced in many plant species by prior inoculation with a necrotizing pathogen. While the mechanism of resistance is unclear, there is a strong correlation in tobacco between the resistant state and the presence of the so-called "Pathogenesis-Related proteins" (PR-proteins). To study the involvement of the PR-proteins in SAR, we have engaged in a comprehensive program to clone and express constitutively in tobacco the cDNAs for all the PR-proteins. In addition to cloning the well-described PR 1-5 gene families, we have used differential cDNA screening to clone several new gene families which are induced by TMV inoculation. Homozygous transgenic seed lines expressing the various genes (or their anti-sense transcripts) are tested against a battery of pathogens for altered disease phenotypes. We have determined that transgenic plants expressing constitutively tobacco PR1 protein show significant resistance to blue mold (*Peronosopra tabacina*). The resistance, exhibited as a delay in symptom development, is observed in several independent transgenic PR1 lines, as well as in F1 crosses with a PR1 transgenic line as parent. Lines expressing the cDNA of a newly defined induced gene, denoted SAR8.2d, exhibit resistance to another oomycete pathogen, Phytophthora parasitica. As with the PR1 resistance, multiple transgenic events show the resistance, which is observed as much delayed symptom development.

1. Introduction

It has long been known that many plants can develop "immunity" to a variety of bacteria, fungi and viruses following an initial inoculation with a necrotizing pathogen (Chester 1933). This systemic acquired resistance (SAR) phenomenon has been most extensively studied in tobacco varieties which exhibit a hypersensitive response (HR) to tobacco mosaic virus (TMV) (Ross, 1961; Bol, *et al.* 1990; White and Antoniw, 1991). SAR can also be induced by chemicals such as salicylic acid (White, 1979) and 2,6-dichloroisonicotinic acid (Metraux, *et al.* 1991). Salt stress, acid damage, or wounding do not induce SAR.

The mechanism for this broad, inducible resistance is not known, but is clearly different from the well characterized "single gene" resistances, which generally work only against individual pathogen isolates and may exhibit high genetic diversity within a spcies (Keen,

E. W. Nester and D. P. S. Verma (eds.),
Advances in Molecular Genetics of Plant-Microbe Interactions, 527–533.

1990). Much attention has been paid to several multigene families of tobacco, termed the "pathogenesis-related proteins", whose coordinate induction correlates with the onset of SAR (Gianinazzi, et al. 1970; Van Loon, 1975; Linthorst, 1991). While several of these PR-proteins, inculding chitinases, glucanases, and permatins, have been shown to have antifungal activity *in vitro* (Mauch, *et al.* 1988; Vigers, *et al.* 1991; Woloshuk, *et al.* 1991), there is still no direct evidence that they play a causal role in disease resistance *in vivo*. Others, such as PR-1, have no known biochemical function and exhibit no homology to known genes in current databases. However, PR1 protein localization (Antoniw and White, 1986) and its high level of induction suggest an active role in disease resistance.

To evaluate the role of PR-protein genes in the development and maintenance of resistance, we have undertaken a program to express the various PR-cDNA types in transgenic tobacco, in sense and anti-sense orientations, under the control of a strong, constitutive promoter. A description of the research program and some early results are presented here.

2. General Approach

We have engaged in a three part program to find and isolate cDNAs for pathogenesis-related genes (GENE DISCOVERY), to engineer transgenic tobacco to express constitutively these PR-proteins (or their anti-sense transcripts) at high levels (TRANSGENIC EXPRESSION), and to test the resulting lines for altered SAR phenotypes (PHYTOPATHOLOGY TESTING).

2.1 GENE DISCOVERY

The gene discovery phase of the research program entailed two approaches, **a)** cloning cDNAs of the known PR proteins, and **b)** discovering cDNAs of new TMV-induced gene families for which no protein product was yet known. The first approach involved the isolation and sequencing of the PR-proteins of Xanthi nc tobacco, followed by synthesis of oligonucleotide probes for cDNA isolation. Full-length clones, generally representing the most highly expressed isoform of each family, were sequenced and used for subsequent expression engineering.

The second approach used differential screening of induced cDNA populations. A cDNA library was constructed using mRNA from upper, uninfected leaves of Xanthi nc plants which had been inoculated with TMV on the lower leaves 11 days earlier. This upper leaf tissue was chosen to avoid mRNAs involoved in the local necrotic response at the TMV lesions. Candidate cDNAs were evaluated for kinetics of induction using Northern analysis. Again the full-length cDNAs of predominant family members were used for expression engineering.

2.2 TRANSGENIC EXPRESSION

Full-length cDNAs were cloned between the enhanced CaMV promoter (double-35S) (Comai, *et al.* 1990) and the 3' terminator region of the Ti-plasmid *tml* gene. Both "sense" and "anti-sense" versions were created for most cDNAs. The reulting expression cassettes were then cloned into a binary vector (McBride and Summerfelt, 1990) containing a kanamycin (NPT II) plant selectable marker gene. The kan gene was adjacent to the left T-DNA border, and was expressed from a Ti-plasmid *mas* gene promoter. Transcription was toward the right T-DNA border. The expression cassettes were inserted adjacent to the right T-DNA border in either possible orientation.

Early experiments compared PR1 expression (as measured by ELISA) in transgenic plants transcribing the PR1a cassette either toward the right border in parallel with the kan gene, or toward the left border, in opposition to the kan gene. Measurements of approximately 25 plants in each group showed no significant difference in constitutive PR1 expression, whether compared on an average-plant or best-plant basis.

Typically, 25-30 Xanthi nc transgenic plants were generated from each chimeric gene construction. We designated primary transgenic plants obtained directly from tissue culture as T1 plants. T1 plants in soil were grown to approximately 30 cm tall, and leaf tissue was harvested for ELISA or Western blot analysis. Those "sense" orientation plants showing the highest constitutive protein expression were allowed to self-pollinate and set T2 seed. T2 seed were tested in a germination assay in the presence of kanamycin (150 mg/l), and those seed lots exhibiting 3:1 (r/s) segregation of the kan^r trait were advanced. Ten T2 plants were allowed to self-pollinate and set T3 seed. The resulting T3 seed was tested for kan segregation as above. Homozygous seed lots (*i.e.* those exhibiting 100% kan^r seed) were advanced to the phytopathology screening program. In almost all cases lines were identified which constitutively expressed the engineered protein at equal or higher levels compared to TMV-inoculated plants.

"Anti-sense" lines, and "sense" lines expressing genes for which antibodies to the encoded proteins were not yet available were selected only on the basis of kan segregation. All lines showing 3:1 (r/s) kan segregation were advanced to the homozygous T3 stage.

2.3 PHYTOPATHOLOGY TESTING

T3 lines showing highest expression of the engineered protein were used in initial diesease tests against a broad variety of pathogens. Any indications of altered disease phenotypes were followed up by larger tests against those pathogens showing the effect. Pathogens tested included TMV, PVY, *Pseudomonas tabaci*, *Pseudomonas syringae*, *Cercospora nicotianae*, *Phytophthora parasitica*, *Peronospora tabacina*, *Heliothis virescens*, and *Melodigyne incognita*.

Six to ten plants of each seed line, in six-inch clay pots, were treated in a random design. Disease ratings and data tabulations were performed in a double blind fashion. The two pathogens for which results are given here are *Phythpthora parasitica* (black shank) and *Peronospora tabicina* (blue mold). *P. parsitica* zoospores were applied to soil as a spore suspension in water. Disease was rated as degree of wilting on a scale of 1-5, with 5 representing total collapse. *P. tabacina* spore suspensions were sprayed on leaves in a dew chamber, and disease was rated as a percentage of leaf area infected.

3. RESULTS

3.1 OVERALL SCREENING RESULTS

Transgenic lines that have been tested to date include the "sense" orientations of PR1a, PR2c (acidic glucanase), PR3 (acidic chitinase), class I (basic) glucanase, class I (basic) chitinase, cucumber class III chitinase, PR-Q' (an acidic glucanase), SAR8.2d, class I glucanse less the vacuolar targeting peptide (-vtp), and class I chitinase (-vtp). Several other constructions representing new or related gene families are still advancing through the program.

To date we have identified two pathogenesis-related cDNAs which conferred significant disease tolerance to tobacco when expressed in a constitutive manner. They are the SAR8.2d transgenic lines, which showed resistance to *Phytophthora parasitica*, and the PR1a lines, which were resistant to *Peronospora tabacina*. These will be discussed in more detail below. Also, lines expressing PR3, class I (basic) chitinase, or cucumber chitinase gave clear indications of resistance in a damping-off assay against *Rhizoctonia solani*, which is in agreement with the results of Broglie, *et al.* (1991).

3.2 SAR8.2

The SAR8.2 cDNA family was discovered by differential screening of a cDNA library representing TMV-inoculated plants. The mRNA used for the library construction and for screening was taken from upper leaves of plants inoculated 11 days previously on the lower leaves with TMV. Upper leaves of mock-inouclated plants furnished mRNA for the differential comparison.

Northern analysis using SAR8.2 probes showed that the mRNAs were approximatly 550-600 nucleotides. They were strongly induced by TMV-inoculation, salcylic acid treatment, or 2,6-dichloroisonicotinic acid (INA) (Ward, *et al.* 1991). We have seen considerable variation in SAR8.2 levels in untreated plants, indicating that there may be other unknown factors affecting their regulation. However, even when the levels are relatively high in the untreated plants, TMV or the chemical inducers always cause additional induction.

We sequenced 25 cDNAs representing the SAR8.2 family, and identified five distinct cDNAs. The open reading frames (ORFs) of the cDNAs encoded small highly basic proteins with N-terminal signal peptides. Assuming processing of the signal peptides, four of the proteins were approximately 7.6 kDa, and the fifth was about 9.6 kDa.

The most interesting feature of the putative proteins is the presence of a cysteine-rich domain at the C-terminus. The four smaller proteins have this domain perfectly conserved. The fifth protein (SAR8.2e) is larger because it duplicates this domain in tandem at the C-terminus. The two cysteine-rich domains of SAR8.2e differ from the other four proteins by only one amino acid in each domain. Database searches using the full-length DNA or protein seuences turned up no significant matches. However, searches with only the cysteine-rich domain revealed many matches, based mainly on the conservation of cysteine spacing. All these proteins, mostly metallothioneins, Bowman-Birk proteinase inhibitors, or zinc-finger proteins, bind divalent cations. While the conservation and duplication of this domain in the gene family may indicate some functionl importance, we have no evidence for a specific biological role for SAR8.2.

Southern analysis showed the SAR8.2 gene family to be quite large, with 10-12 hybridizing bands generated from genomic DNA by enzymes which do not cut the cDNAs. Thus the five cDNAs may indicate only the minimum number of functional genes. Some or all of the additional bands may represent pseudogenes.

When the first SAR8.2d transgenic T3 line was tested in our phytopathology screen, most pathogen infections were indistinguishable from control lines, which included non-transformed Xanthi nc, Xanthi nc transformed with an empty expression cassette, and the SAR8.2 anti-sense line. However, *Phytophthora parasitica* infection was dramatically delayed in the "sense" expression plants. After eight days all the controls showed near total collapse, while the SAR8.2 plants were beginning to exhibit mild to moderate wilting. Two repeats of this expriment with the same line gave a similar result. Expanded experiments with additional lines, representing independent transgenic events, confirmed that the presence of the SAR8.2 chimeric gene was responsible for the observed resistance,

although none of the other lines gave such spectacular results as the initial one. We investigated the possibility that exression of SAR8.2 might cause induction of some or all of the other SAR genes, leading to the resistant phenotype. Western blot analysis showed that none of the known PR proteins were detectable in the untreated SAR8.2 transgenic line. We do not know if the observed resistance is the result of decreased pathogen growth, or only a delay of symptom development.

3.3 PR1a

Early tests with PR1a "sense" transgenic lines gave indications of resistance to *Peronospoa tabacina*. To test the validity of this observation an expanded experiment was designed, incorporating several indpendent transgenic homozygous lines expressing PR1a, and F1 crosses of a PR1a line with lines expressing other transgenic PR-proteins (PR1a heterozygotes). Controls included all the other homozygous transgenic PR-protein lines (non-PR1), untrasformed and untreated Xanthi nc, and untransformed Xanthi nc treated with an immunizing chemical (positive control).

All PR1a-expressing lines except one, whether homozygous or heterozygous, showed significant resistance seven days after inoculation. ELISA tests later revealed that the one non-resistant PR1a line was indeed not expressing PR1a. All the other PR1a lines were expressing high levels of the protein. Using pair-wise t-test comparisons, the group of PR1a homozygous plants were found to differ from the non-PR1 plant group with >99% confidence at day 7. The heterozygous plant group differed with a confidence of 91%. After 9 days the homozygous group retained the >99% confidence level, while the heterozygous group confidence had fallen to 82%. Northern analysis of transgenic PR1a plants revealed that they do not express significant levels of other PR-protein mRNAs.

It is interesting that in none of the transgenic plants do we see resistance as strong as in the chemically immunized plants, even though the PR1 levels of the transgenic plants often equals or exceeds levels in induced plants. This suggests that PR1a may be only one part of a more complex mechanism aimed at this pathogen by the SAR response.

Previous reports of transgenic expression of PR1 proteins in tobacco (Cutt, *et al.* 1989; Linthorst, *et al.* 1989) have concluded that PR1 is not involved in virus resistance. We have extended these observations to PVY, with similar results. However, none of the earlier studies addressed fungal pathogens, and our results are the first indication of a function for PR1 proteins.

4. CONCLUSION

Our goal is to elucidate the functions of the plant genes involved in the SAR response. We have shown evidence for the involvement of two pathogenesis-related protein genes in resistance to two important oomycete pathogens. The nature of the resistance is unknown, but this work provides the materials to ask important genetic and biochemical questions.

Our working hypothesis is that the systemic resistance against any particular pathogen is likely to be multigenic in nature, and that different sets of overlapping genes will be involved in resistance to various pathogens. One way to test this hypothesis is the use of "anti-sense" expression lines, an approach which we have not yet exploited fully, but for which we have a considerable amount of transgenic germplasm in hand. We feel that blocking one necessary gene in a complex function is more likely to lead to an altered

disease phenotype than the constitutive expression of that gene, which by itself may not be sufficient to affect disease.

We are also engaged in a program to introduce two or more of the chimeric PR-cDNAs into tobacco, both by genetic crosses and by combinations of double or triple gene constructs and crosses. Plants expressing multiple "sense" or "anti-sense" genes should be useful in elucidating PR-protein function.

5. LITERATURE CITED

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