Technical Advance

Production of Antimicrobial Defensin in *Nicotiana benthamiana* with a Potato Virus X Vector

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Accepted 29 October 2000.

A recombinant plasmid, pTXS.TH, was constructed to express the gene-encoding wasabi (*Wasabia japonica*) defensin with the potato virus X (PVX) vector. pTXS.TH allows the expression of defensin in the host *Nicotiana benthamiana*, and the defensin protein WT1 can be purified from virus-infected leaves by heat treatment and affinity chromatography. WT1 exhibits strong antifungal activity toward the phytopathogenic fungi *Magnaporthe grisea* (50% inhibitory concentration [IC₅₀] = 5 µg/ml) and *Botrytis cinerea* (IC₅₀ = 20 µg/ml) but is weakly active against the phytopathogenic bacterium *Pseudomonas cichorii*. This virus-mediated expression system is a rapid and efficient method to produce and characterize antimicrobial proteins in plants. It is particularly useful for the study of proteins that are difficult to produce with other expression systems.

Additional keywords: degenerate primer; His₆; immunoblot analysis; PCR; SDS-PAGE.

Overexpression of antimicrobial protein genes by genetic transformation is commonly used to confer enhanced disease resistance to crop plants (Evans and Greenland 1998). Plant transformation, however, is a time-consuming procedure. Therefore, it is crucial that the protein be characterized extensively with a wide variety of microorganisms that cause plant diseases prior to perform plant transformation experiments. Such experiments can be hindered by the difficulty of purifying a large quantity of the antimicrobial protein of interest because, in many cases, this requires large amounts of source material and can be expensive and time consuming.

Recently, it has become possible to express proteins efficiently in bacteria, yeast, or animal cells. The efficiency of expression can vary considerably, and antimicrobial proteins are frequently recalcitrant during expression in bacterial and yeast cells as a result of their toxicity to the host. To address these problems, plant virus vectors are increasingly used and a number of different viruses have been successfully developed into tools for gene delivery and expression of foreign genes in plants (Scholthof et al. 1996).

Total RNA from leaves of wasabi (*Wasabia japonica*) was reverse transcribed into cDNA with an Oligo-dT-M13M4

primer (Takara Biomedicals, Kusatsu, Japan). With the use of this cDNA as template, a cDNA encoding a wasabi defensin gene was amplified by polymerase chain reaction (PCR) with the M13M4 primer (5'-GTTTCCCAGTCACGAC-3') and a defensin-specific degenerate primer (5'-ATGGCTAAGTTGCKTCYATC-3') containing the start codon of the defensin gene designed according to the consensus DNA sequence of defensin genes from radish (Terras et al. 1995) and *Arabidopsis thaliana* (Penninckx et al. 1996). The PCR product (450 bp) was cloned, resulting in pCR2.1WT1, and sequenced. Its high-sequence homology to other known defensin genes suggests that the cDNA originates from a wasabi defensin gene (GenBank accession no. AB012871). To characterize the function of the corresponding protein, we attempted to express the wasabi defensin gene in *Escherichia coli* but failed, presumably a consequence of the toxicity of the protein to the host.

The 243-bp coding region of the wasabi defensin gene (WT1) was amplified from plasmid pCR2.1WT1 by PCR with a primer pair corresponding to the N and C termini of the WT1 protein. The DNA sequence of the N-terminal primer is 5'-dCCGAGCTCGGTACCATGGCTAAGTTTGCTTCTAT-3' (nucleotides underlined and in italics correspond to *SacI* and *KpnI* sites, respectively). The DNA sequence of the C-terminal primer is 5'-dCCGAGCTCTTAGTGGTGATGGTGA TGGTGACATGGGAAGTAACAGATAC-3' (*SacI* site is underlined), which was designed with six histidine codons (italics) so that the cloned and expressed protein carries a C-terminal histidine tag (6× His [His₆]) to facilitate its purification by affinity chromatography. The PCR product encoding WT1-His₆ was digested with *SacI*, blunt-ended with T4 DNA polymerase, and ligated to the *EcoRV* site of the pPC2S PVX expression vector (Baulcombe et al. 1995) to generate pTXS.TH (Fig. 1A).

In vitro runoff transcripts were synthesized by T7 RNA polymerase with PVX vectors linearized by restriction endonuclease *SpeI*. The transcripts were inoculated directly to young leaves of *Nicotiana benthamiana* plants by rubbing carborundum-dusted leaves. Each leaf was inoculated with transcript products from 0.5 µg of the plasmid template. Infectious particles generated from pTXS.TH after inoculation are called PVX.TH.

Fourteen days postinoculation (dpi), the plants exhibited systemic infection (Fig. 1B). PVX.TH-infected *N. benthamiana*

plants as well as pPC2S-infected, PVX.GFP (containing jellyfish green fluorescent protein gene)-infected and nonvirus-infected plants were challenged with *Botrytis cinerea*, applying a high concentration of spores to their leaves. No inhibition of fungal growth was observed in PVX.TH-infected plants as compared with the plants infected with PVX.GFP and pPC2S (data not shown). *B. cinerea* growth was pronounced in all PVX (PVX.TH, PVX.GFP, and pPC2S)-infected plants as compared with the nonvirus-infected plants, suggesting that PVX preinfection greatly compromised plant resistance against the fungus. The concentration of WT1 in the PVX.TH infected plants, even if produced, was not high enough to confer an enhanced resistance against *B. cinerea*.

Thus, to isolate WT1 protein from the PVX.TH-infected plants, leaves were harvested, a crude extract was prepared (Kavanagh et al. 1992), and the extract used to inoculate 30 *N. benthamiana* plants. Seven days later, inoculated leaves and noninoculated upper leaves were harvested, and protein extracts were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were blotted to polyvinylidene difluoride membranes, as described (Santa Cruz et al. 1996). Rabbit antiserum raised against the WT1 C terminus and anti-PVX antibody (Roche Molecular Biochemicals, Minato-ku, Japan) were used for immunodetection.

Immunoblot analysis of the protein extracts from the inoculated and upper leaves was carried out with extracts pre-

Fig. 1. A, Organization of the pPC2S and PVX.TH genomes. Boxes represent coding region; horizontal line symbolizes untranslated sequences. pPC2S carries a duplication of the coat protein subgenomic RNA promoter (dashed boxes) and a multiple cloning site. Sizes of virus-encoded proteins are in kilodaltons. CP = coat protein; T = WT1; H = His₆. **B,** Phenotype of pPC2S- (left) and pTXS.TH-infected (right) *Nicotiana benthamiana* plants 14 days postinoculation. pTXS.TH-infected plants exhibited slightly severe mosaic symptoms.

pared at 7 dpi. The results indicate the presence of WT1 antigen and PVX-CP antigen (Fig. 2).

Partial purification of WT1 from PVX.TH-infected *N. benthamiana* leaves was carried out, essentially as described (Terras et al. 1992). After the final centrifugation (15 min at $12,000 \times g$), supernatant was dialyzed extensively against distilled water (Spectra/Por, cutoff 1,000 Da; Spectrum Medical Industries, Terminal Annex, LA, U.S.A.). Dialyzed solution (i.e., the thermostable fraction) was filtered through a 0.45- μ m filter. WT1-His₆ protein (the ProBond affinity-purified fraction) was purified from the filtrate with the Xpress System Protein Purification (Invitrogen, Carlsbad, CA, U.S.A.).

Protein fractions from the leaves of infected plants were analyzed by electrophoresis and immunoblotting. Figure 3 shows the results of an immunoblot for total leaf protein (1 μ g; lane 3), the thermostable fraction (1 μ g; lane 4), and the ProBond affinity-purified fraction (100 ng; lane 5) with an antiserum against the WT1 C-terminus. Each purification step enriches for and concentrates the WT1 protein. Figure 4 shows SDS-PAGE of the affinity-purified fraction (500 ng) stained with Coomassie blue. The major band in this fraction has an apparent molecular mass of 5.5 kDa. The mature WT1 protein (51 amino acids) includes a 29 amino acid signal peptide (Terras et al. 1995; Von Heijne 1986). The predicted molecular mass of the mature WT1-His₆ corresponds with the size of the fragment detected by SDS-PAGE. Thus, a mature form of WT1-His₆ was successfully purified from PVX.TH-infected *N. benthamiana* plants. The yield of purified WT1-His₆ was 40 μ g from 100 g of PVX.TH-infected *N. benthamiana* leaves.

Purified WT1-His₆ was evaluated for antimicrobial activity through testing its effect on the growth of two phytopathogenic fungi, *M. grisea* and *B. cinerea*, and a phytopathogenic bacterium, *P. cichorii*, with a microspectrophotometer (Broekaert et al. 1990), under the conditions specified previously (Cammue et al. 1992).

The growth of *M. grisea*, *B. cinerea*, and *P. cichorii* was measured in the absence or presence of WT1-His₆ at various concentrations (Fig. 5A). The 50% inhibitory concentration was calculated from the dose response curve to be 5 μ g of *M. grisea* and 20 μ g *B. cinerea* per ml. Figure 5B shows that mycelial growth of *M. grisea* and *B. cinerea* was inhibited effectively by WT1-His₆ at a concentration of 10 μ g per ml. The purified WT1-His₆, however, did not have significant antibacterial activity toward *P. cichorii* (minimal growth inhibition was observed only at 20 μ g of WT1-His₆ per ml). This result is consistent with the report that plant defensins show considerable antifungal activity but little antibacterial activity

Fig. 3. Immunoblot of 1 μ g of total leaf protein (lanes 1, 2, and 3) from a mock-inoculated control plant and pPC2S- and PVX.TH-infected leaves challenged with anti-WT1 C-terminus antiserum. Lanes 4 and 5, thermostable protein fraction (1 μ g) and ProBond resin-bound fraction (100 ng), respectively. Molecular masses are in kilodaltons.

Fig. 2. Immunoblot analysis of total leaf protein (5 μ g) from *Nicotiana benthamiana* leaves probed with either **A**, anti-PVX antibody or **B**, anti-WT1 C-terminus antiserum. Lane 1, protein from a mock-inoculated control plant. Lanes 2 and 3, proteins from pPC2S inoculated leaves and noninoculated upper leaves, respectively. Lanes 4 and 5, proteins from PVX.TH inoculated leaves and noninoculated upper leaves, respectively. Molecular masses are in kilodaltons.

Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of 500 ng of Probond resin-bound fraction. Molecular masses in kilodaltons.

(Broekaert et al. 1995). The antifungal activity of purified WT1-His₆ was quantitatively similar to that of other plant defensins reported (Osborn et al. 1995; Terras et al. 1992; Terras et al. 1993).

This work describes the production of a functional antimicrobial protein in *N. benthamiana* leaves with the PVX vector. An attraction of this method over classical bacterial (Piers et al. 1993) or yeast (Vilas Alves et al. 1994) expression-purification systems is that protein folding and maturation should occur normally. Thus, further development of plant virus vectors should facilitate the production and characterization of proteins encoded by foreign genes in plants.

ACKNOWLEDGMENTS

We thank D. C. Baulcombe (Sainsbury Laboratory, Norwich, U.K.) for the gift of plasmid pPC2S and G. Kahl (University of Frankfurt, Germany) for improvements to this manuscript.

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Fig. 5. A, Inhibitory effects of WT1-His₆ on the growth of *Magnaporthe grisea*, *Botrytis cinerea*, and *Pseudomonas cichorii*. Growth was monitored by measuring light absorbance at 595 nm and expressed as a percentage of the growth in the absence of the WT1-His₆. Standard error ranges from three independent experiments are shown. **B,** Microscopic views of *M. grisea* and *B. cinerea* growth 24 h after the start of culture in 10 µg of buffer per ml with and without WT1-His₆. Bar = approximately 100 µm.

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