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Toxicity of Tomato Spotted Wilt Virus Glycoprotein Signal Peptide and Promoter Activity of the 5' UTR

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Cloning of the 5' untranslated region (5' UTR) and N-terminus of the glycoprotein precursor (G2G1) open reading frame of tomato spotted wilt virus has been problematic, possibly because of the toxicity of a signal peptide at the beginning of the G2G1 protein precursor. The toxicity of the signal peptide to bacterial growth and the reason for the expression of the peptide gene in *Escherichia coli* were investigated by cloning the 5' UTR and the signal peptide sequence separately. Cells transformed with the plasmid containing both the first 30 amino acids of the glycoprotein and the 5' UTR showed a severe growth inhibition whereas transformants harboring either the plasmid with the signal sequence or the 5' UTR alone did not show any inhibition. An *E. coli* promoter-like sequence was found in the 5' UTR and its promoter activity was confirmed with a promoter-less GUS gene cloned downstream of the 5' UTR. In the cloning of the Tomato spotted wilt virus (TSWV) glycoprotein G2G1 open reading frame all the recovered plasmids contained stop codons in the signal sequence region. However, clones containing no stop codon were recovered when the signal sequence and the 5' UTR were cloned separately.

Keywords : TSWV, signal sequence, growth inhibition, prokaryotic promoter.

High-level expression of foreign proteins in *E. coli* is not always possible because of mRNA instability, poor transcription and/or translation and toxicity of overproduced foreign proteins to their hosts (Das, 1990). One of the main causes for the host toxicity is a short (less than 15 amino acids) stretch of hydrophobic amino acid residues in the signal peptide sequence or transmembrane anchor domain (Steinberg et al., 1995; Brosius, 1984).

The signal peptide sequences of many viral glycoproteins have been implicated as possible causes for host toxicity and low expression level in *E. coli* (Steinberg et al., 1995; Brosius, 1984; Watson et al., 1982). For example, high-level expression of HIV and HTLV-1 glycoproteins could only be archived by deletion of the hydrophobic signal sequences (Sisk et al., 1992). Similarly, host toxicity of overexpressed vesicular stomatitis virus glycoprotein was overcomed by deletion of a hydrophobic signal peptide (Rose and Schafferman, 1981).

Tomato spotted wilt virus (TSWV), which is the type species of the tospovirus genus of the family Bunyaviridae (Francki et al., 1991), causes serious diseases on many crops worldwide. The genome of TSWV consists of two ambisense RNA segments, S (small, 2.9 kb) and M (medium, 5.0 kb) RNA and one negative-sense RNA segment, L (large, 8.9 kb) RNA (Elliot, 1990). The S RNA encodes a nucleocapsid protein (N, 29 kDa) in viral complementary sense and nonstructural protein (NSs, 52.5 kDa) in the viral sense (de Haan et al., 1990). The M RNA encodes two glycoproteins G1 (95 kDa) and G2 (58 kDa) (Adkins et al., 1996) as one open reading frame in viral complementary sense and another nonstructural protein, NSm (33.6 kDa) in viral sense (Law et al., 1992; Kormerink et al., 1992). The L RNA encodes the putative viral polymerase (L) protein of 331 kDa in the viral complementary sense (de Haan et al., 1992). The two glycoproteins are associated with the lipid envelope of virus particle (German et al., 1992). There is evidences indicating that the glycoproteins play an important role in the transmission of TSWV by thrips (Ullman et al., 1989; Bandla et al., 1998). Defective mutants that can not be transmitted by the insect vector have point mutations located in the G2G1 gene of the M RNA (Resende et al., 1991; Verkleij and Perters, 1983).

To investigate the exact function of these proteins, their proteolytic processing and possible roles as ligands for receptors in the insect vector, we cloned the entire G2G1 open reading frame (ORF). In an initial attempt, a PCR product containing the N-terminal half of the G2G1 open reading frame and 5' UTR could not be recovered. However, the PCR product was successfully cloned as three separate pieces (Adkins et al., 1996). In this paper, the presence

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of a toxic sequence in the N-terminus of the G2G1 ORF and the reason for its expression in *E. coli* was elucidated by separate cloning of the N-terminal region and the 5' UTR, and by expressing a transcriptional fusion of a promoter-less GUS gene downstream of the 5' UTR.

Materials and Methods

General. Restriction enzymes, AMV reverse transcriptase and Taq polymerase were purchased from Promega (Madison, WI). *E. coli* XL-1 blue strain and plasmid pBluescript KS+ were obtained from Stratagene (La Jolla, CA). Dr. Wagner (University of California, Berkeley) kindly provided the plasmid pJW15 containing the GUS gene.

Virus. TSWV L strain was maintained in Jimsonweed (*Datura stramonium* L.), and purified from the infected leaves RNA was extracted with phenol/chloroform according to Tas et al. (1977).

cDNA synthesis and cloning. cDNA of the 3' end region of the M RNA and N-terminal half of the G2G1 precursor was synthesized by reverse transcription and amplified by polymerase chain reaction (PCR). The primers used for cDNA synthesis and PCR cloning were designed based on the published M RNA sequence of a Brazilian isolate (Kormelink et al., 1992). The primer GPRIME (5'-CGCGGATCCAGAGCAATCAGTGCAAACAA-3') is complementary to the 3' end of the M RNA and has a *Bam*H I restriction site (underlined) and three extra nucleotides for efficient restriction enzyme digestion. The adenine residue at position 10 of GPRIME corresponds to the 3' end of M RNA, which is at position 4821 from the 5' end (Kormelink et al., 1992). The primer MIFOR (5'-CAACTGATGTTAACCCTAAAG-3') is viral sense and located at nucleotide 3024 to 3044, and has a *Hinc* II site in the middle (underlined).

For the first strand cDNA synthesis, 1 μl of viral RNA was mixed with 50 pmole of GPRIME primer in the total volume of 10 μl. After 10 min denaturation at 95°C, the mixture was chilled on ice and reverse transcription was conducted with AMV reverse transcriptase for 1 h at 42°C, followed by 10 min heat inactivation at 70°C. Finally, 2.5 μl of the first strand cDNA synthesis mixture was used for the PCR amplification with *Taq* polymerase without further purification.

The PCR reaction conditions were as follows: 5 min pre-heating at 95°C, a denaturation step at 90°C for 30 sec, an annealing step at 55°C for 45 sec, and a synthesis step at 75°C for 1 min. Forty cycles of PCR reactions were followed by a 3 min extension reaction at 72°C. The PCR product was fragmented into three pieces (113bp, 1179bp, and 508bp) and cloned separately because *Bam*H I/*Hinc* II treated PCR product could not be cloned (Fig. 1). The 508-bp *Hinc* II/*Hind* III fragment was cloned between the *Hinc* II and *Hind* III site of pUC19, producing pUCHH. The 1179-bp *Hind* III-*Spe* I fragment and 113-bp *Bam*H I-*Spe* I fragment were cloned into pBluescript KS+, resulting in pSHL and pSBS, respectively. The sequences of cloned PCR products were determined by the dideoxynucleotide termination method (Sanger et al., 1977) using the Sequenase Version 2.0 (U.S. Biochemicals, Cleveland, OH).

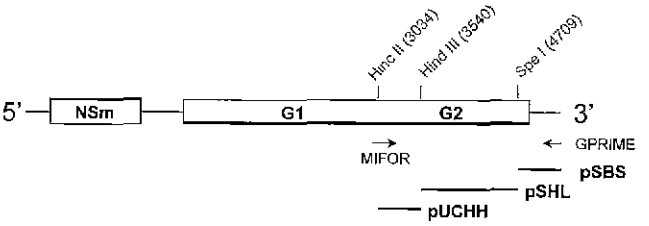

Fig. 1. Structure of TSWV M RNA and cloning of the 5' UTR and G2G1 ORF. The 4821nucleotide ambisense M RNA of the Brazilian isolate of TSWV encodes the NSm protein gene in the positive sense and the G2G1 precursor protein in the negative sense. The thick bars of the ORFs indicate the positive sense strand of transcripts. Primers used for cDNA synthesis and PCR amplification are presented. Three plasmids derived from PCR products are shown with restriction sites used in the cloning, and their location from the 3' end of the genomic RNA is indicated.

Cloning of the signal sequence. To clone the signal sequence downstream of an inducible lac promoter, the N-terminal half of the G2G1 ORF was first subcloned from the clone pRNTR (Adkins et al., 1996) into pET11a (Novagene), and then the sequence corresponding to amino acid residues from 18 to 568 was removed. In the resulting clone pETGS, the G2G1 ORF starts as MARILK instead of MRILK and contains 18 amino acid residues instead of 17. The clone pETNSM containing the entire ORF of TSWV NSm gene, which does not have the signal sequence at the N-terminus and has no toxic effect on *E. coli*, was used as negative control. *E. coli* strain BL21(DE) was transformed with these clones for toxicity test.

Toxicity of the signal peptide. Toxicity of the induced signal peptide was tested as follows: 50 ml DYT media containing 100 μg/ml ampicillin was inoculated with an overnight culture of *E. coli* transformed with pETGS and pNSM. After 2.5 h incubation at 37°C, 10 ml of each culture was mixed with 25 ml fresh DYT media. The OD_{600} of the mixture was 0.3. Two flasks were prepared for each clone and IPTG was added to one of the pair at a final concentration of 1 mM. The other flask was used as negative control. Aliquots (1 ml) were taken from each flask every 30 min for 5 h and the OD_{600} was measured.

Promoter activity test of the 5' UTR. The GUS gene was cloned downstream the 5' UTR of the TSWV glycoprotein gene to test promoter activity of the UTR. A *Bam*H I-*Sac* I fragment containing the GUS gene isolated from the clone pJW15 was cloned into pBKSΔSE digested with *Bam*H I and *Sac* I, resulting in the clone pGUSΔSMA. The plasmid pBKSΔSE had been constructed by removing the sequence between the *Eco*R V and *Sma* I site of pBluescript KS+. A *Bam*H I-*Dra* I fragment containing the 5' UTR and DNA sequence for the first 4 amino acids of G1G2 protein was isolated from the clone pSBS17 and cloned into pGUSΔSMA digested with *Bam*H I and *Sma* I. In the resulting clone pGLGUS, the GUS gene was fused to DNA fragment encoding the four N-terminal amino acid residues, which was under the control of a cryptic prokaryotic promoter present in the 5' UTR. GUS enzyme activity in *E. coli* transformed with pGUSΔSMA or pGLGUS was examined as follows: cells were grown overnight in DYT media containing 100 μg/ml ampicillin, from

which 1.5 ml culture was taken for pellet preparation. After resuspension in 1.5 ml GUS extraction buffer (50 mM NaHPO4, pH7.0; 10 mM BME; 10 mM EDTA, pH7.0; 0.1% Triton X-100), the cells were sonicated for 10 sec 3 times. The total protein concentration of the cell lysates were determined by Braford assay (Braford, 1976). For the GUS assay, 5 μg total protein was added to 1495 μl GUS assay buffer (GUS extraction buffer plus 0.5 mM 4-methylumbelliferyl-D-glucuronide) and the mixture was incubated at 37°C. Aliquots (150 μl) were transferred to 1350 μl stop buffer (0.2M Na_2CO_3) after 2, 5, 10 and 20 min. Two μl of the stopped reactions was mixed with 4 ml dilution buffer (1:10, extraction buffer: stop buffer) and the fluorescence was measured with a TK100 fluorometer (Pharmacia Biotech, Sweden).

Results

Cloning of N-terminal half of TSWV Glycoprotein gene. A DNA fragment of 1.82 kb was obtained from the PCR with primers GPRIME and MIFOR. The purified PCR product digested with *Bam*H I and *Hinc* II could not be cloned into *Bam*H I and *Hinc* II-digested pBluescript KS+. Despite several trials, no colony was recovered from the transformation. However, the *Spe* I-*Hind* III fragment (1179bp) and the *Hind* III-*Hinc* II fragment (508bp) were cloned, resulting in the clone pSHL and pUCHH, respectively. The *Bam*H I-*Spe* I fragment (113bp), which was ligated into pBluescript KS+ digested with *Bam*H I and *Spe* I producing pSBS, could not be recovered.

Instead of obtaining pSBS, we recovered four independent mutant clones from the *E. coli* transformed with the pSBS, which were named as pSBS2, 14, 17, 23. All these clones have the same sequence in the 5' UTR of 84 nucleotides (Fig. 2). However, their nucleotide sequences in the open reading frame following the 5 UTR were different from each other (Fig. 3). One interesting feature of the sequences is the introduction of stop codons in the short ORF by either deletion of one nucleotide (pSBS14 and 17) or change of the nucleotides (pSBS2 and 23).

Toxicity of the signal peptide. To test the toxicity of the hydrophobic signal peptide to *E. coli*, an ORF containing the signal sequence gene was cloned next to an inducible promoter, producing the clone pETGS. When *E. coli* transformed with the plasmid pETGS was induced with 1 mM IPTG, the cell did not grow for first 2 h and then started to grow slowly (Fig. 4A). However, the cell growth inhibition was much less in *E. coli* transformed with the plasmid pET-NSM containing TSWV NSm gene (Fig. 4B). The difference shown in Fig. 4C depicts the cell growth ratios between uninduced and induced *E. coli* containing each plasmid.

Promoter Activity of the 5' UTR. Because of the toxicity of the signal peptide and cloning problem with the N-terminal half of the G2G1 ORF with the 5' UTR, the sequence of the 5' UTR of G2G1 protein was examined for the presence of prokaryotic promoters. Although five independent plasmids derived from the plasmid pSBS showed sequence variations after the AUG initiation codon, their 5' UTR sequences were the same. A putative prokaryotic promoter sequence was found in the 5' UTR of these plasmids (Fig. 5). There was 100% nucleotide identity with the Pribnow box (TATAAT) and 5 out of 6 nucleotides (TTGAAA) were identical in the -35 sequence (TTGACA). These two regions were separated by 16 nucleotides. A GUS gene was used to test the promoter activity of the 5' UTR by cloning it downstream and measuring the enzymatic activity of cell lysates. As shown in Fig. 6, lysates of *E. coli* transformed with the plasmid pGLGUS, which containing the 5' UTR-GUS gene fusion, showed over 10 times higher enzymatic activity than that of cells transformed with the plasmid pGUSΔSMA, a plasmid containing a promoter-less GUS gene.

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B  5' AGAGCAATCAGTGCAAACAAAAACCTTAATCCAGACATCTTGA
H  5' AGAGCAATCAGTGCAAACAAAAACCTTAATCCAGACATCTTGA

B  AATTAATCACACAACCATTGTAATCTGGGTAGACATCTAAG
H  AATTAATCACATAACCATTATAATCTGAGTAGACGTGTAAG
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Fig. 2. The 5 UTR sequences of the M RNA of Brazilian and Hawaiian isolates of TSWV. The first 84 nucleotides of the M RNA of Brazilian isolate (B) and Hawaiian strain (H) of TSWV used in this study are shown in the viral complementary sense. Nucleotides differing in two strains are underlined.

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A  Brazilian Isolate  ATGAGAATTCTAAAACTACTAGAACTAGT
       pSBS2          ATGAGAATTTTAAAACTACTATAACTAGT
       pSBS14         ATGAGAATT★TAAAACTACTAGAACTAGT
       pSBS17         ATGAGAATTTTAAAACTACTAGA★CTAGT
       pSBS23         ATGAGAATTTAAAAACTACTAGAACTAGT

B  Brazilian Isolate  M R I L K L L E V
       pSBS2          M R I L K L L ★ V
       pSBS14         M R I ★ N Y ★ N ★
       pSBS17         M R I L K L L D ★
       pSBS23         M R I ★ K L L E L
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Fig. 3. Mutations in the signal peptide sequences derived from one clone. Four plasmids recovered from the clone pSBS which contain the 5' UTR and the DNA sequence encoding the first 8 amino acids were and compared to each other and with that of the Brazilian isolate. (**A**). The nucleotide sequence encoding the signal peptide. The asterisks indicate missing nucleotides and the underlined nucleotides indicate stop codons introduced by mutation. (**B**) The deduced amino acid sequences. The asterisks indicate the introduced stop codons.

Discussion

Signal peptides usually consist of 16-26 amino acids with polar, basic termini and central non-polar amino acids. Although the signal peptide plays an important role in co-

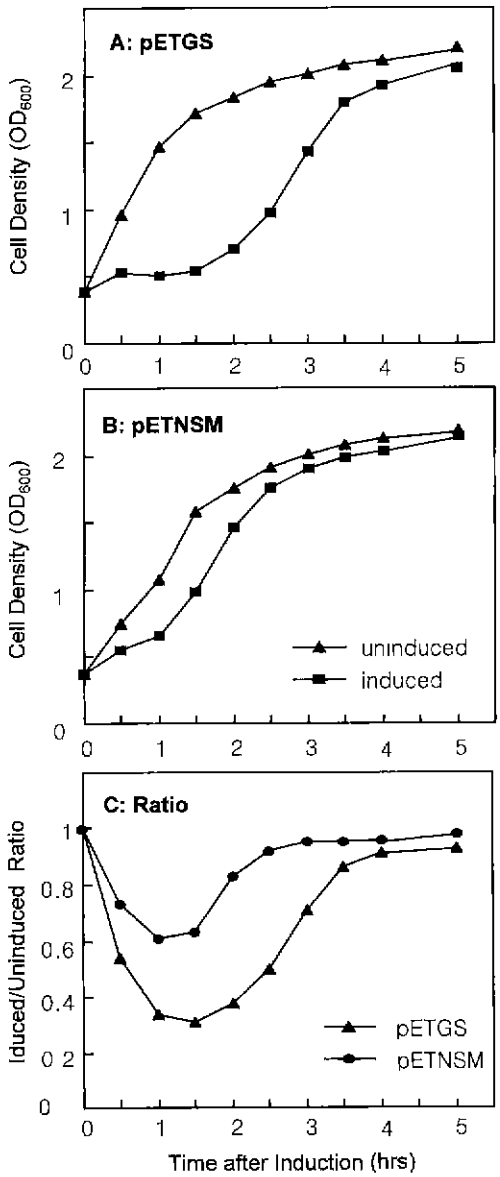

Fig. 4. Growth inhibition of *E. coli* by induced signal peptide. *E. coli* transformed with indicated plasmids were induced with 1 mM IPTG and their growth was compared with uninduced cells by measuring the OD_{600} every 30 min 5 h. The plasmid pETGS contains the signal peptide of first 17 amino acids (A), and the clone pETNSM contains the NSm protein gene of TSWV (B). The growth ratios of induced and uninduced are presented in (C). Results are mean values of three replications.

translational insertion of glycoproteins in the rough endoplasmic reticulum, it also causes problems in overexpression of cloned glycoproteins in *E. coli*. Despite several trials, no colony was recovered from PCR products containing the 5' UTR and N-terminal half of the TSWV glycoprotein gene. However, the PCR product could be cloned into three separate pieces.

Four mutant clones were recovered from *E. coli* trans-

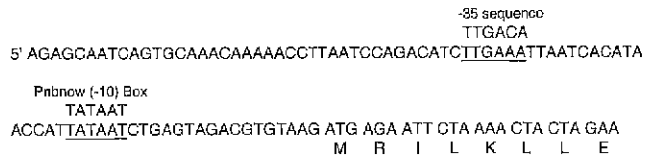

Fig. 5. Putative promoter sequence in the 5' UTR. The 5' UTR and part of ORF of the Hawaiian isolate is shown. The conserved prokaryotic promoter regions are presented and corresponding to -10 and -35 nucleotides are underlined.

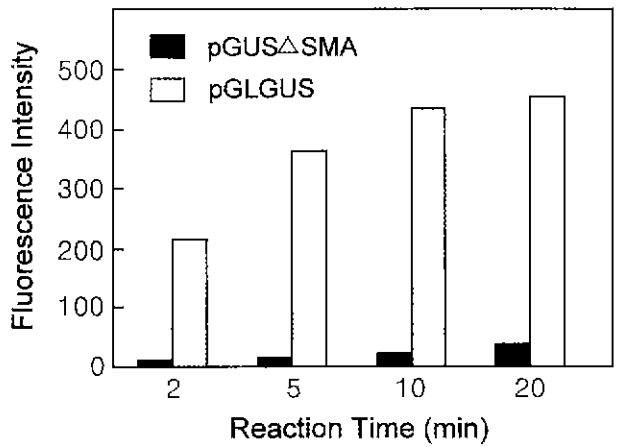

Fig. 6. Promoter activity of the 5' UTR. Lysates of *E. coli* transformed with pGUSΔSMA or pGLGUS were tested for enzyme activity by measuring fluorescence for up 20 min. Results are mean values of three replications.

formed with the plasmid pSBS which contains the 84 nt- 5' UTR and the following N-terminal 9 amino acid residues. All of them have the same 5' UTR sequences, which differs from that of the Brazilian isolates at 5 positions (Fig. 2). However, all these plasmids were different from each other and contained stop codons at different location after the first AUG initiation codon (Fig. 3A). The mutations resulted in peptides consisting of 3 to 8 amino acids instead of cloned 9 amino acid residues (Fig. 3B).

Lethal effects of the hydrophobic signal peptide on *E. coli* have been reported from many viral glycoproteins (Steinberg et al., 1995; Rose and Schafferman, 1981; de Haan et al., 1990). The toxicity of the signal peptide of TSWV glycoprotein was examined by placing it downstream of an inducible promoter. As shown in Fig. 4C, the growth of E. coli cells containing the signal peptide showed more growth inhibition for a longer time and did not make up the growth until 5 h after induction. The recovery of growth of induced cells containing the plasmid pETGS after 2 h may be due to the signal peptide peptidase produced which degrade the signal peptide for recycling of the amino acids (Hussain et al., 1982).

The 5' UTR were examined for the presence of a prokaryotic promoter. Interestingly, there were putative -35 and -10

conserved promoter sequences upstream of the AUG initiation codon (Fig. 5). The -10 conserved sequence (TATAAT) is located between -23 and -18 upstream of the initiation codon and the putative -35 conserved sequence (TTGAAA) is located between -45 and -40. Considering the location, the sequence and the distance between the two conserved sequences, the 5' UTR is a possible promoter. This was confirmed by fusing a GUS reporter gene downstream of the 5' UTR. As shown in Fig. 6, over 10 times enzymatic activity was detected from the 5' UTR-GUS fusion than the GUS gene without the promoter. This strongly suggests the promoter activity of the 5' UTR.

The results presented in this paper suggest that the 5' UTR of the G2G1 precursor gene acts as a prokaryotic promoter and makes the gene expressed in the transformed *E. coli*, and the signal peptide in the expressed precursor exhibits the lethal effects on the host bacteria. Although the presence of the promoter in the 5' UTR of TSWV glycoprotein gene is by chance, it provides an important precedent for the cloning of signal peptides, especially from glycoprotein genes.

Many small peptides have been known to have antibacterial activity and studied to solve drug resistance problem of bacterial pathogens (Gabay, 1994; Travis, 1994). Many glycoproteins could be used as possible antibacterial substances if they can be modified in a stable and easily absorbable form. The signal peptide containing the first 22 amino acids of the TSWV glycoprotein was synthesized in D and L-Form to test their toxicity on microorganisms. However, both oligopeptides had very low solubility in water probably because of its hydropobicity. Further studies are in progress to increase their solubility.

Acknowledgments

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