



Resistance to wheat streak mosaic virus in transgenic wheat engineered with the viral coat protein gene

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Abstract

Wheat (*Triticum aestivum*) plants were stably transformed with the coat protein (CP) gene of wheat streak mosaic virus (WSMV) by the biolistic method. Eleven independently transformed plant lines were obtained and five were analyzed for gene expression and resistance to WSMV. One line showed high resistance to inoculations of two WSMV strains. This line had milder symptoms and lower virus titer than control plants after inoculation. After infection, new growth did not show symptoms. The observed resistance was similar to the ‘recovery’ type resistance described previously using WSMV *Nlb* transgene and in other systems. This line looked morphologically normal but had an unusually high transgene copy number (approximately 90 copies per 2C homozygous genome). Northern hybridization analysis indicated a high level of degraded CP mRNA expression. However, no coat protein expression was detected.

Introduction

Wheat streak mosaic (WSM) is a serious disease affecting wheat (*Triticum aestivum* L.) in the Great Plains region of the United States, Canada and many other wheat producing countries (Brakke, 1971). WSM disease causes an estimated 2% annual loss in crop yield in US (Appel et al., 1991; Christian & Willis, 1993) and localized yield losses of up to 100% are common (McNeil et al., 1996). This disease is caused by wheat streak mosaic virus (WSMV) of the family *Potyviridae*. WSMV is characterized by flexuous rod-shaped particles 700 nm long × 15 nm diameter (Brakke, 1971). The virus is naturally trans-

mitted by the eriophyid mite vector *Aceria tosichella* Keifer (Slykhuis, 1955). The complete nucleotide sequence of WSMV has been determined (Stenger et al., 1998). The genome is a single stranded plus-sense RNA of 9,384 nucleotides, encoding a polyprotein of 3,035 amino acid residues. While previously assigned to the genus *Rymovirus* (Stenger et al., 1998), WSMV was recently re-assigned to the genus *Tritimovirus* (Fauquet & Mayo, 1999).

In the early 1980s it was suggested that expression of viral gene sequences in transgenic plants might provide a means of disrupting the viral life cycle, while leaving host functions unaffected (Hamilton, 1980; Sanford & Johnston, 1985; Carr & Zaitlin, 1993). Virus resistance derived from the expression of viral genes in transgenic plants (often referred to as pathogen-derived resistance, PDR; Sanford &

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Johnston, 1985) has been widely used to develop virus-resistant plants with various degrees of success. Since the first reports of this strategy to protect tobacco and tomato plants against tobacco mosaic virus (TMV) infection by engineering the viral coat protein gene in plants (Powell-Abel et al., 1986; Nelson et al., 1988), there have been many reports of transgenic plants that have been made resistant to viral diseases (reviewed by Lomonosoff, 1995; Beachy, 1997). In most of these reports, resistance has been observed against infecting virus particles as well as viral RNA. In a similar approach in order to obtain resistance against WSMV, we have isolated the WSMV-CP gene and introduced it into wheat. In this report we present data on resistance in transgenic wheat lines transformed with the WSMV-CP sequence under greenhouse conditions.

Materials and methods

Cloning the WSMV CP gene in transformation vectors

A plasmid pSOG27, which contains the full-length WSMV-CP gene (from isolate 'H81', Niblett et al., 1991) and *nos* gene terminator (Rogers et al., 1987), was obtained from Dr. L. Crossland (Novartis). The original promoter was replaced by the maize *ubi1* gene promoter excised from pAHC17 (Christensen & Quail, 1996) to construct plasmid pRQ103. A herbicide bialaphos-resistant *bar* gene construct (a 2.09 kb *HindIII* fragment) from plasmid pRQ101 (Sivamani et al., 2000b), was inserted into pRQ103 to give rise to pRQ105 (Figure 1). The *bar* gene (De Block et al., 1987) was under the control of a CaMV 35S promoter, containing the maize *adh1* intron 1 in the 5' untranslated region and the *nos* terminator (Fromm et al., 1990). This construct was used as a non-translated one (Smith et al., 1994) since no translation initiation codon was introduced to the WSMV-CP gene as revealed by sequencing analysis (data not shown). In addition, two translatable gene constructs were made as follows. The WSMV-CP coding sequence was synthesized by polymerase chain reaction (PCR) from a cDNA clone of a Montana isolate 'Conrad, MT' of WSMV (Carroll et al., 1982), which covers the full length *NiB* gene and the CP gene (Sivamani & Qu, 1997). The downstream and upstream primers used for amplification of the full length CP gene (1256 bp) were: 5'CGGGATCCAACAATGA-

GCGCTATTATTGCAG3' and 5'CGCGGATCCCTTATCACGCAAGAGCGTTTAC3', respectively. Using the same upstream primer and the following downstream primer, 5'CGGGATCCAACAATGTCAGGATCAGGTTCTG3', a truncated CP gene was amplified to give a product of 923 bp. The downstream primers of the WSMV-CP sequences were designed based on the published sequences of WSMV capsid protein gene (Niblett et al., 1991). Translational initiation and termination codons (italicized) for the WSMV-CP gene and *BamHI* sites (underlined) at both termini of the gene were introduced. The PCR products were digested with *BamHI* and ligated into pAHC17 (Christensen & Quail, 1996). The right orientation of the inserts was confirmed by restriction digestion analysis. Consequently, two plasmids, pESCP45 containing a 1256 bp sequence encoding a full-length 45 kDa WSMV-CP, and pESCP35 containing a 923 bp truncated WSMV-CP gene coding for a 35 kDa truncated coat protein, were obtained. In both plasmids the CP gene coding sequences were driven by the maize *ubi1* promoter and followed by the *nos* terminator (Figure 1). For these two constructs, the plasmid pRQ101 was used as a selectable marker in co-transformation experiments (Sivamani et al., 2000b).

Transformation and regeneration of transgenic wheat plants

Tissue culture procedures and biolistic transformation of immature embryos obtained from greenhouse-grown spring wheat (*Triticum aestivum* L. cv. Hi-Line or cv. Bobwhite) were described as earlier (Sivamani et al., 2000a,b). While Hi-Line is an elite Montana spring cultivar (Lanning et al., 1992), Bobwhite is a breeding line often used for wheat transformation experiments (Weeks et al., 1993).

Transgenic plantlets were transplanted from rooting medium to potting mix (Sunshine Mix #1, Fisons, Canada) in the greenhouse, and were covered with beakers for the first few days to reduce desiccation. Greenhouse day/night temperatures were $25 \pm 2^\circ\text{C}/19^\circ\text{C}$ under a 16 h photoperiod with supplemental lights to provide light intensity of $150 \mu\text{mol m}^{-2} \text{s}^{-1}$.

Leaf paint assay

To examine the *bar* transgene expression in the transgenic plants, a freshly made aqueous solution containing 1 mg l^{-1} herbicide bialaphos (De Block et al., 1987) was used to paint on the mid lamina portion of a second-youngest leaf using a cotton bud. The

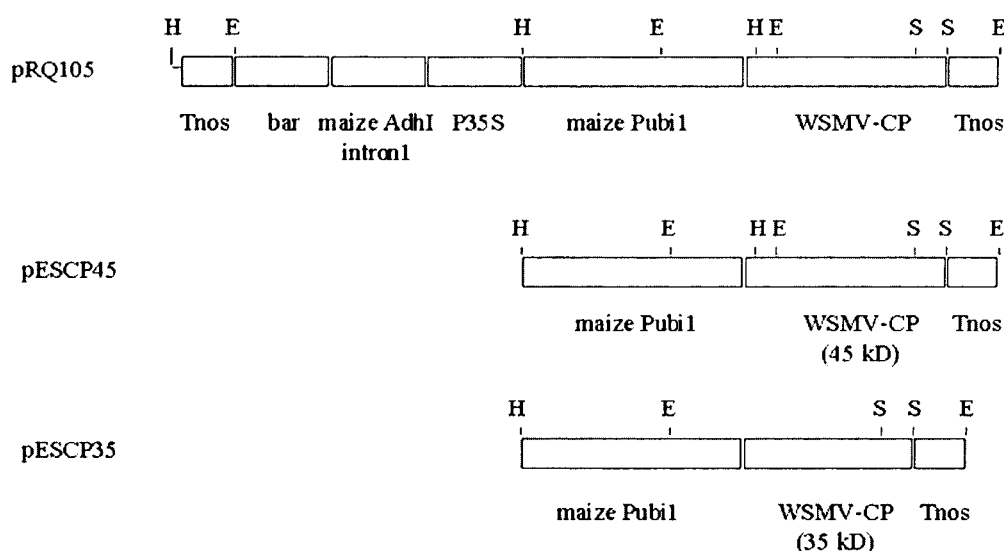


Figure 1. Diagram of three WSMV-CP gene constructs used in wheat transformation. Pubi1: maize ubiquitin 1 gene promoter region including exon 1 and intron 1. P35S: CaMV 35S transcript promoter. Tnos: transcription termination region of *Agrobacterium tumefaciens nos* gene. Relevant restriction sites are labeled above each construct. E: *EcoRI*, H: *HindIII*, S: *SacI*.

painted area (about 2.5 cm long) was marked using a marker pen and observed for damage in the following week.

PCR analysis

PCR was carried out to demonstrate the presence of WSMV-CP sequences in transgenic wheat lines. Genomic DNA extraction and PCR conditions used in the experiments were as previously reported (Sivamani et al., 2000b). The oligonucleotides described above were used as primers for PCR reactions. In some experiments a primer derived from *nos* terminator with the sequence 5'GTAACATAGATGACACCGCG3' was used in combination with the downstream WSMV-CP primer.

Segregation analysis

Twenty to 25 seeds from each progeny line were germinated and were first tested by PCR (for the WSMV-CP gene) followed by leaf paint assay and segregation of the transgenes was analyzed.

Southern blot analysis

Genomic DNA was extracted by the procedure of Dellaporta et al. (1983) with minor modifications (Sivamani et al., 2000a,b). Approximately 40 μ g of genomic DNA, undigested or digested with *HindIII* restriction enzyme (Life Technologies, Rockville,

MD, USA), was used for the analysis. After electrophoresis on 0.9% agarose gels, DNA was transferred to a GeneScreenTM nylon membrane (NEN Research Products, Boston, MA, USA) as described (Sambrook et al., 1989). The hybridization was performed according to the manufacturer's recommendations. Blotted DNAs were probed with a 1 kb *HindIII-SacI* fragment of the WSMV-CP coding region labeled with [α -³²P] dCTP (NEN) using the Multiprime DNA labeling system (Amersham, Arlington Heights, IL, USA) according to the manufacturer's protocol. BIOMAXTM MS film (Eastman Kodak, Rochester, NY, USA) was exposed to the blots for an appropriate time period and developed. For copy number determination of line 25, 4 μ g of the genomic DNA was digested with *EcoRI* and compared with a reconstruction lane which contained 'Hi-Line' non-transgenic plant DNA and *EcoRI*-digested plasmid DNA equivalent to 10 copies of the WSMV-CP gene per 1 C wheat genome. The copy number was analyzed on a phosphor-imager (Model STORM840, Molecular Dynamics, Sunnyvale, CA, USA) using the manufacturer's Image Quant software (version 1.0).

RNA analysis

To detect transcription of the WSMV-CP transgene(s), total RNA was isolated from non-inoculated plants (containing the WSMV-CP gene as indicated by PCR

analysis) using FastRNA[®] Kit-Green (Bio 101, Inc. Carlsbad, CA). Approximately 30 µg of total RNA was used in the analysis per plant line. Electrophoresis was performed on a 1.2% agarose gel containing formaldehyde based on Sambrook et al. (1989) and blotted to a GeneScreen[™] nylon membrane (NEN). The hybridization conditions used were as described by Sambrook et al. (1989). The same probes used for Southern hybridization were used for hybridization of the northern blots which were then exposed to BIOMAX[™] MS film. Soybean 18S rRNA gene was used to probe the blot as a molecular marker and as a reference to determine the intactness of the electrophoresed RNA.

Western analysis

To investigate the CP transgene expression, proteins were extracted from transgenic plants, and western blot analysis was performed as described previously (Sivamani et al., 2000a) with WSMV antiserum (Sherwood, 1987).

Inoculation of transformed plants

Segregating individuals of T1 generation of transgenic lines were tested with isolate 'Conrad, MT' of WSMV (Carroll et al., 1982). The presence of the CP transgene in these plants was determined by PCR assays. Ten- to 14-day-old T1 seedlings were mechanically inoculated with the virus as described below.

One hundred microgram of purified WSMV per millilitre of water and/or a homogenate of 1 : 50 (w/v) ratio of WSMV infected leaf tissue in water and 0.03 g carborundum was used as the inoculum. The inoculum was applied by rubbing five times back and forth on each leaf with a cotton bud. Twenty four hours after inoculation, plants were transplanted to individual 10 cm × 10 cm plastic pots and placed in a walk-in growth chamber which was maintained at a photoperiod of 16L/8D, temperature 22–25°C and relative humidity of 10–50%. WSMV symptoms were scored every 5 days using the following scale: 0 = no symptom, 1 = faint green mottling, 2 = light colored green and yellow streaks and a general stunting of the plant, 3 = yellowish green to markedly yellow mottling and striping, 4 = pronounced yellow color over entire leaf surface, 5 = leaf completely dry and necrotic and plant almost dead. Non-transgenic Hi-Line and Bobwhite plants were inoculated as positive controls. The negative control plants were inoculated with distilled water.

To determine whether the transgenic wheat plants were resistant to other WSMV strains, segregating population from T3 or T4 generation of the transgenic lines were inoculated with strain 'Logan' (Stenger et al., 1998) under the same conditions as described above.

ELISA tests

Inoculated transgenic wheat lines were analyzed for the presence of WSMV by antigen-coated plate ELISA. ELISA tests were performed on all inoculated plants at 20 day post inoculation. ELISA tests were also performed at 40 day post inoculation when the strain 'Logan' was used. Total proteins were extracted from the second youngest leaf and ELISA tests performed as previously reported (Sivamani et al., 2000b). The optical density (OD) at A405 was measured using an automated Kinetic Microplate (Molecular Devices Corp., Sunnyvale, CA) ELISA reader. The OD readings were automatically standardized by the ELISA reader by subtracting the reading of the buffer control. The OD readings obtained with non-inoculated, non-transgenic controls were multiplied by two, and values above and below were considered positive and negative, respectively.

Results

Transformation

Three constructs, pESCP45, pESCP35, and pRQ105, were used in the transformation experiments. They represented full-length translatable, truncated translatable, and full-length non-translatable constructs, respectively. All these kinds of CP gene constructs have been shown to confer virus resistance in potyviruses (Lindbo et al., 1993; and reviewed by Wilson, 1993; Smith et al., 1994). From five transformation experiments, 11 independent T0 transgenic wheat lines were obtained: nine from pESCP45 and one each from pRQ105 and pESCP35 (Figure 1). Among the 11 T0 transgenic plants, 10 were derived from cv. Hi-Line and one from cv. Bobwhite. Transformation efficiency ranged from 0.3 to 0.7%. Five plant lines, three from pESCP45 (lines 11B, 15B, and 25), and one from pRQ105 (line 9) and pESCP35 (line 20) each, were chosen for further study on the basis of the presence of WSMV-CP transgene, fertility of the plants, and Mendelian segregation of transgenes in the progeny plants (Table 1). All the primary transgenic lines

Table 1. Transgenic wheat lines tested in the experiments

Transgenic line	Cultivar	Gene Construct	Generation inoculated* with	
			'Conrad, MT'	'Logan'
9	Hi-Line	pRQ105	T1	T3
11B	Hi-Line	pESCP45	T1	T4
15B	Hi-Line	pESCP45	NA	T3
20	Bobwhite	PESCP35	T1	NA
25	Hi-Line	pESCP45	T1	T4

NA – not analysed.

*WSMV virus strains.

and their progenies used in this study looked normal and had no appreciable morphological difference from their parental cultivars.

Molecular characterization of transgenic plant lines

PCR analysis was performed to examine the presence of the WSMV-CP transgene while leaf paint assays were performed to evaluate *bar* gene expression. Leaf paint assays on non-transgenic plants and negative segregants of the transgene showed yellowing in the bialaphos applied area after 2 or 3 days and apparent necrosis after about a week. The segregation of the CP transgene among T1 offspring plants of the lines 9, 11B, 15B, 20 and 25 was in good fit to a 3:1 Mendelian segregation ratio as evidenced by PCR assays for the presence of the WSMV-CP transgene as well as the leaf paint assays for the *bar* gene expression (data not shown), suggesting one locus of each transgene in each transgenic plant line. The CP transgene was co-segregated with bialaphos resistance in each of these lines, implying that the two transgenes were tightly linked in the five lines.

WSMV-CP probes hybridized with undigested genomic DNA at high molecular weight range of all transgenic lines (data not shown) confirming that the transgene was integrated into the plant genome. The various hybridization patterns among the digested DNAs indicated that each line was from an independent transformation event (Figure 2). Among the lines tested, line 25 was quite unusual in Southern analysis by that the lane had much stronger hybridization signals when the exposure was appropriate for other lanes (Figure 2A). Only by reducing the loading amount to one tenth ($4\ \mu\text{g}$ per lane) of the original, can a major band be seen and the CP gene copy number be determined (Figure 2B). It was estimated

that line 25 contained approximately 45 copies of the WSMV-CP gene in a 2C hemizygous wheat genome, or 90 copies in a 2C homozygous genome. The observed Mendelian segregation suggested all the copies were closely linked and inherited as one transgenic locus.

In northern blot hybridization, the CP transgene transcript was only detected in line 25 even though PCR and Southern analysis confirmed the transgene presence in other lines (lines 9, 11B, 15B and 20). The steady state RNA of the WSMV-CP transgene was detected in line 25 as an intensive smear when total RNA was analyzed on northern blot (Figure 3A). Shorter exposure still showed a strong smear of the hybridization signals throughout the lane with four blurred bands, one being at about the expected migration position (1.6kb) and three others in smaller size (Figure 3B). Intactness of the rRNA on the blot was confirmed by the hybridization with soybean 18S rRNA probes (Figure 3B), suggesting that the smear did not result from RNA degradation during extraction and electrophoresis. Attempts to detect WSMV-CP protein in these lines, using WSMV antiserum (Sherwood, 1987) were unsuccessful. The protocol used in western analysis can detect as low as 20ng of virus protein as revealed in a control experiment.

Characterization of resistance in transgenic plants

Preliminary studies confirmed that $100\text{--}150\ \mu\text{g ml}^{-1}$ of WSMV virus solution was sufficient to successfully infect control Hi-Line wheat plants (Sivamani et al., 2000b). Typically, symptoms became evident 10 days post inoculation (dpi). More than 90% of the control plants were ELISA positive at 20 dpi, and exhibited typical visual symptoms of WSMV infection.

When T1 progenies of lines 9, 11B, 20 and 25 were challenged with WSMV isolate 'Conrad, MT', line 25 displayed a 'recovery' type resistance (Lindbo et al., 1993) while the plants of other lines did not show obvious resistance (data not shown). The CP gene-positive plants (as shown by PCR analysis) of line 25 initially developed milder WSM symptoms, that is, light greenish-yellow streaks/spots on the inoculated leaves, about 10 days after inoculation. The newly grown leaves of these plants showed stronger resistance ranging from a delay of symptom appearance to no symptom through the plant life cycle. Meanwhile, offspring plants of line 25 that tested negative for the WSMV-CP transgene by PCR (CP-) showed

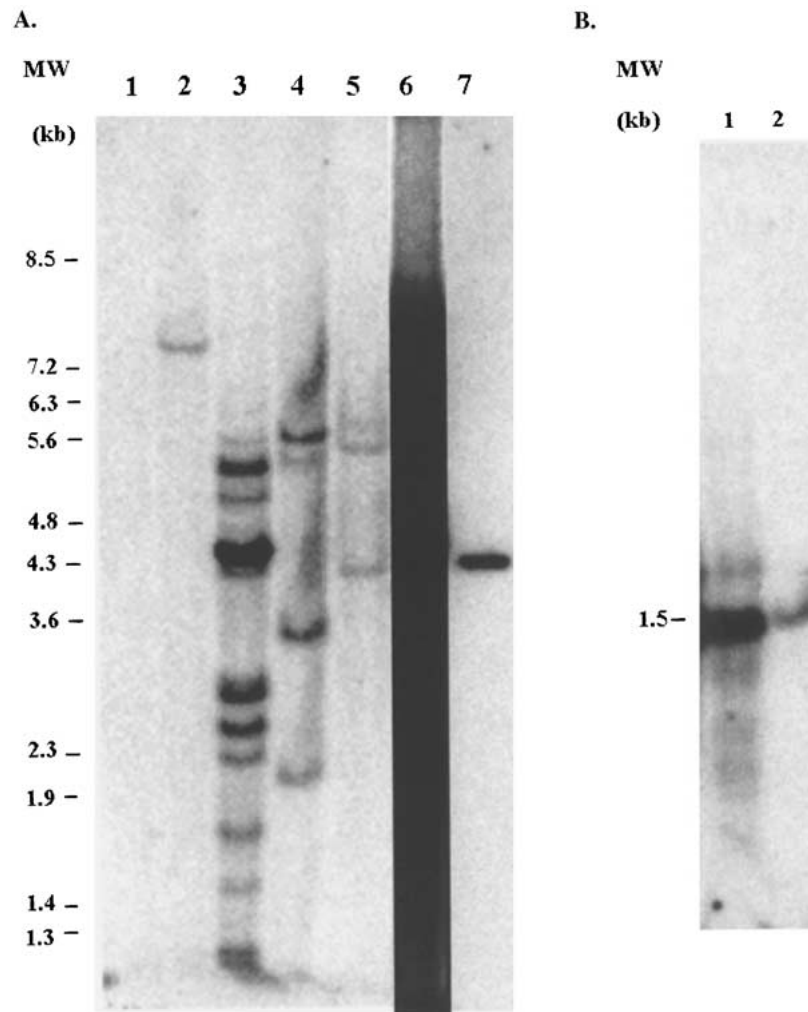


Figure 2. Southern hybridization of T2 generation transgenic wheat plants. (A) Forty micrograms of genomic DNA were digested with *Hind*III and fractionated by 0.9% agarose gel electrophoresis. The blot was hybridized with a 1 kb *Hind*III-*Sac*I fragment of the WSMV-CP gene (Figure 1). Lanes 1–6 are: Hi-Line (negative control), lines 9, 11B, 15B, 20 and 25. Lane 7 is the positive control (*Hind*III digested pRQ103 plasmid and carrier DNA with an expected fragment size of 4.3 kb equivalent to 1 copy per 1C wheat genome). The migration positions of the molecular weight markers were indicated on the left. (B) Determination of the transgene copy number of line 25 by Southern hybridization. Lane 1: Four micrograms of genomic DNA of a hemizygous plant from line 25 was digested with *Eco*RI. Lane 2: pRQ103, equivalent to 10 copies of the transgene in wheat genome of lane 1, together with carrier DNA, was digested with *Eco*RI. The expected size of the digested fragment is indicated on the left.

typical symptoms of WSMV comparable with the control plants. ELISA tests showed substantially lower OD ($OD_{405} = 0.108 \pm 0.129$) in the CP+ resistant plants indicating a lower virus titer than in the CP-plants ($OD_{405} = 0.387 \pm 0.166$) or the inoculated non-transgenic plants ($OD_{405} = 0.300 \pm 0.282$) (Figure 4).

T3 or T4 offspring plants from lines 9, 11B, 15B, and 25 were challenged with Logan strain of WSMV. Again, the offspring plants of line 25 that harbored the

WSMV-CP transgene, initially showed milder symptom after inoculation and a recovery type resistance in the new growth as evidenced by ELISA tests and symptom evaluation. At 20 dpi, the six bialaphos resistant plants in line 25 showed a mean symptom score of 0.5 compared to the 10 negative control plants (cv. Hi-Line) which had a mean symptom score of 1.8. The offspring plants that contained the WSMV-CP gene also had lower OD readings in ELISA (Figure 5). The progeny of lines 9, 11B and 15B did not show

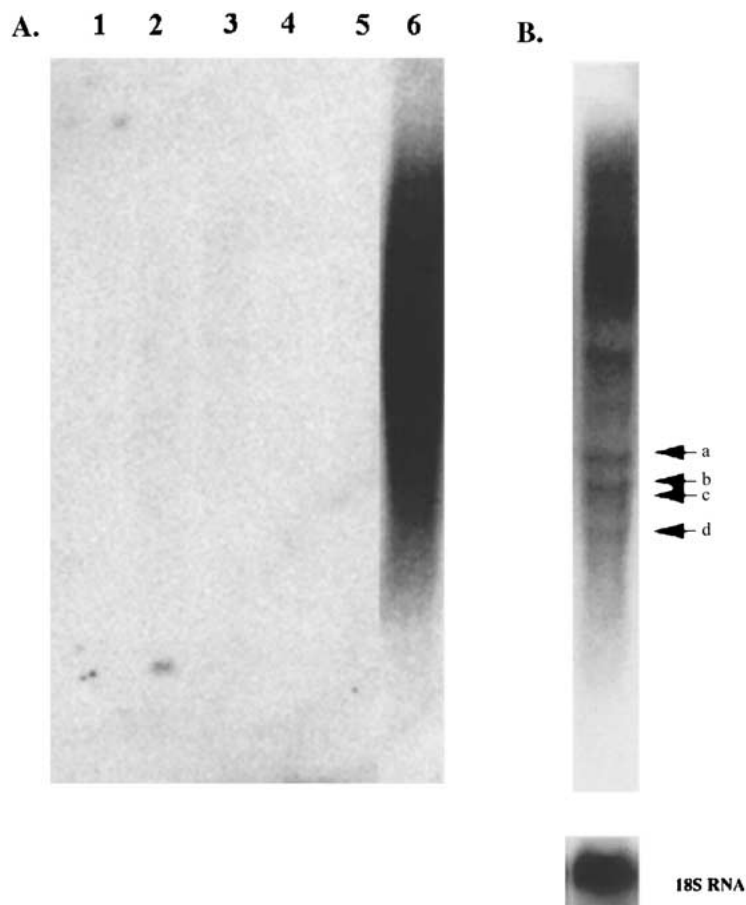


Figure 3. Northern analysis of the T2 generation of the transgenic wheat lines. (A) Lanes 1–6: Thirty micrograms of total RNA from Hi-Line (negative control), lines 9, 11B, 15B, 20 and 25, were fractionated by 1.2% agarose gel electrophoresis and the blot was probed with a 1 kb *HindIII-SacI* fragment of the WSMV-CP gene (Figure 1). (B) A shorter exposure of a line 25 plant sample still showed a strong smear of the hybridization signals throughout the lane with four blurred bands, one being at about the expected migration position (1.6 kb, band a) and three others in smaller size (bands b–d), as pointed by the arrows.

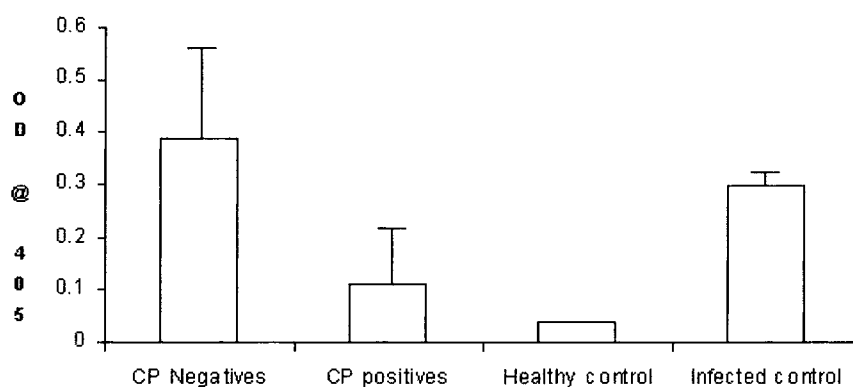


Figure 4. ELISA results of T1 offspring plants from line 25 and non-transgenic Hi-Line plants after inoculation with WSMV isolate ‘Conrad, MT’. A total of 32 plants from a segregating T1 offspring of line 25 was used in which 23 plants showed the presence of WSMV-CP transgene by PCR (marked as ‘CP positives’ in the chart) and nine plants tested negative in PCR (marked as ‘CP negatives’ in the chart). The OD₄₀₅ readings from two each non-inoculated and inoculated Hi-Line plants were used as negative and positive controls, respectively. Each column in the chart represents the mean value of the OD₄₀₅ readings while bars indicate standard deviation observed within each group.

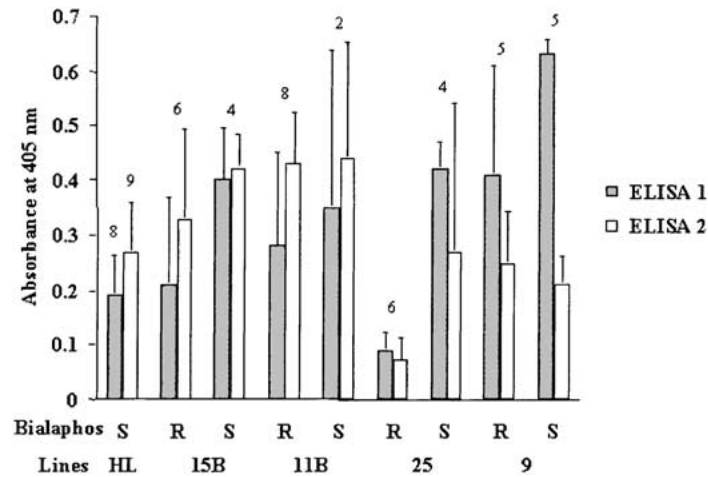


Figure 5. ELISA results of T3 or T4 offspring plants of transgenic lines 9, 11B, 15B and 25 and the non-transgenic control Hi-Line plants (HL) after inoculation with WSMV strain 'Logan'. All the transgenic lines were segregating and the plants in each line were categorized into two groups: bialaphos resistant (R) and susceptible (S). Number of the plants in each group was marked above the columns. Each column represents the mean value of the OD₄₀₅ readings while bars indicate standard deviation observed within each group. ELISA 1 and 2 tests were taken 20 and 40 days after inoculation, respectively.

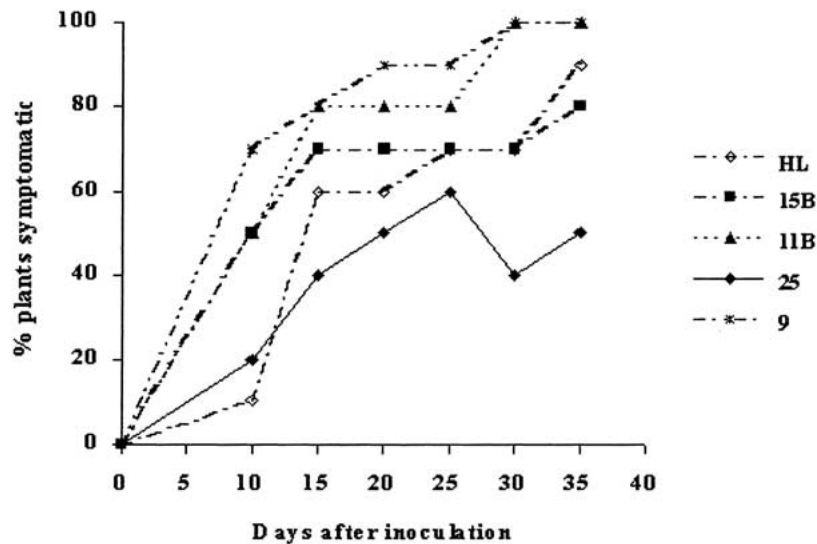


Figure 6. Time course of symptom development on offspring plants of T3 or T4 generation from transgenic lines 9, 11B, 15B and 25 and the non-transgenic control Hi-Line plants (HL) after inoculation with WSMV strain 'Logan'. Ten plants were examined in each of the lines at every time point and the mean score of each line at each time point is presented.

appreciable resistance. In these plants, the typical WSM symptoms were observed in inoculated leaves at about 10 dpi. The symptoms developed in the fresh growth without any delay. More than 50% of the plants of lines 9, 11B and 15B and non-transgenic Hi-Line plants showed typical symptoms of WSM after about 15 days post inoculation. While the offspring plants of line 25 showed a significant retardation in symptom development (Figure 6).

Discussion

Pathogen derived resistance offers a promising approach for creating virus-resistant varieties of agriculturally important crop plants via biotechnology. This strategy has been successfully used to protect transgenic plants against several genera of viruses in various plant species (Beachy, 1997). We have observed that transgenic wheat plants engineered with

WSMV *Nib* gene were resistant to the virus isolate 'Conrad, MT' from which the gene was isolated, likely through a post-transcriptional gene silencing (PTGS) mechanism (Sivamani et al., 2000b), but not to the strain Logan (unpublished data). In this communication, we demonstrated that expression of the CP gene of the isolate 'Conrad, MT' in transgenic wheat conferred resistance to both these strains. The resistance of the transgenic wheat plant line 25 to WSMV was significant as the fresh growth of a majority of the plants was free from disease symptoms.

The observed resistance in line 25 was similar to the recovery type resistance of PTGS as reported in wheat plants transformed with the WSMV-*Nib* gene (Sivamani et al., 2000b) as well as in the plants transformed with CP genes of other potyviruses (Lindbo et al., 1993; Smith et al., 1994). Although the plants from line 25 exhibited a resistance phenotype similar to one of the wheat plant lines transformed with the WSMV-*Nib* gene (Sivamani et al., 2000b), the molecular characteristics of line 25 were quite different from the *Nib* line. In the WSMV-*Nib* transgene case, the recovery type was associated with no detectable transgene mRNA. It was proposed that the plants had a pre-established resistance state as indicated by milder symptoms after inoculation and by the presumably active degradation of the transgene/viral RNA. The only WSMV-*Nib* line (line 4.8) that had a detectable transgene RNA (intact transgene mRNA) did not show strong resistance (Sivamani et al., 2000b). In this study, the offspring plants of line 25 which exhibited intensive, smeared RNA signals, showed highest resistance to the WSMV inoculations, while the offspring plants from lines 9, 11B, 15B and 20 which did not have detectable transgene mRNA expression (Figure 3) were susceptible to WSMV infection. The CP transgene mRNA smear in line 25 appears to indicate that the transgene RNA degradation was initiated even before inoculation with the virus (as the RNA samples examined were collected from non-inoculated plants). RNA degradation of CP transgene was observed in the post-transcriptionally silenced leaf tissue in transgenic tobacco plants resistant to another potyvirus, tobacco etch virus (TEV) (Tanzer et al., 1997). The degraded RNA may act as aberrant RNA to activate suppression mechanism as proposed in models trying to explain the PTGS phenomenon (Baulcombe, 1996; Metzloff et al., 1997; Wassenegger & Pelissier, 1998; Waterhouse et al., 1998). However, the case of line 25 was complicated by the high number of closely-linked transgene copies and the strong hybridization signals

at positions higher than the expected RNA transcript, which could be resulted from the rearrangement of the linked transgene copies. However, we have not analyzed the linkage of these transgene copies in details. It is not clear whether these high molecular weight RNAs also played a role in the observed resistance.

It is noticeable that among the two viral transgenes, cloned from the isolate 'Conrad, MT' of WSMV, the WSMV-CP transgene conferred resistance to a different WSMV strain ('Logan') but not the WSMV-*Nib* gene. It is actually in accordance with majority cases observed when engineering these two genes of potyviruses and other plant viruses in plants for resistance to the viral diseases (Beachy, 1997). Although exceptions exist, in general, the CP gene mediated resistance had broader protection than the replicase gene mediated resistance (Namba et al., 1991; Maiti et al., 1993; Beachy, 1997; Jones et al., 1998).

Two other CP gene constructs, the non-translatable pRQ105 and the truncated pESCP35, were also used in the experiments. The plants transformed with the constructs did not show resistance to WSMV. Transgenic plants expressing untranslatable RNA (Smith et al., 1994) and transgenic plants with truncated CP genes (Lindbo et al., 1993) have been shown to confer viral resistance. However, no conclusion can be drawn from the current results since only one plant line from each construct was analyzed.

The resistance in line 25 appears to be associated with the high expression of the transgene RNA, which was likely due to the expression of an unusually high copy number (90 copies per 2 C homozygous genome) of the transgene. Although it is often suggested that high copy number of transgene tends to induce transcriptional gene silencing (reviewed by Flavell, 1994), it was not the case for line 25. Up to the T4 generation so far analyzed, the transgene RNA expression was still intense and the resistance held. The results were in accordance with some reported observations. Goodwin et al. (1996) showed a relationship between copy number of transgenes and virus resistance. Transforming tobacco with the CP gene of tobacco etch virus (TEV), they showed that lines with three or more insertions were highly resistant to TEV whereas lines with one or two insertions showed the ability of inducible resistance. Zhong et al. (1999) also reported high level aprotinin expression in transgenic corn with at least 20 transgene copies. Thus, it appears that high transgene copy number does not necessarily induce transcriptional gene silencing, and sometimes even enhances transgene expression.

The data described in this paper suggest that it is possible to have a broad protection against various WSMV strains in wheat through CP gene mediated resistance.

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