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Accumulation of barley stripe mosaic virus is significantly reduced in transgenic wheat plants expressing a bacterial ribonuclease

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Abstract

An *rnc*70 gene encoding a mutant bacterial ribonuclease III (RNase III) was introduced into wheat (*Triticum aestivum* cv. Bobwhite) by microprojectile bombardment. T1, T2, and T3 plants regenerated from three transgenic callus lines were challenged with barley stripe mosaic virus. Plants expressing RNase III exhibited a high level of resistance to the virus infection. This resistance was evidenced by the absence of virus symptoms and reduced accumulation of virions in these plants. The result demonstrates that this pathogen-targeted resistance strategy can be effectively employed in conferring resistance to viral diseases of cereal crops.

Introduction

Plant viruses cause significant annual losses to crop plants. Currently, a variety of transgenic approaches has been employed to generate virus resistant plants as promising strategies to control viral diseases (Beachy, 1997; Rimann-Philipp & Haan, 1998). Since the first viral pathogen-derived resistance (PDR) approach was published (Abel et al., 1986), there have been growing numbers of reports on a variety of strategies that are based on PDR. The PDR strategies have a potential limitation of application against a broad spectrum of viruses because PDR is usually effective only against related virus strains or viruses. Recently several pathogen-targeted virus resistance (PTR) strategies have been published (see review by Reimann-Philipp & De Haan, 1998). The majority of the resulting resistance seems to result from post-transcriptional gene silencing (PTGS) (Waterhouse et al., 1999). Expressing RNA-specific ribonacleases in the cytoplasm of transgenic plants is one of the approaches that target RNA viruses and viroids (Watanabe et al., 1995; Ogawa et al., 1996; Langenberg et al., 1997; Sano et al., 1997). However, work on virus resistance in transgenic cereals has been limited due to a variety of difficulties including low efficiency of cereal transformation (McGrath et al., 1997). Rice (Hayakawa et al., 1992), corn (Murry et al.,1993), oats and barley (McGrath et al., 1997), and wheat (Karunaratne et al., 1996) have been transformed with viral coat protein genes. However, there was no data on the analysis of coat portein mediated resistance (CPMR) in the transgenic wheat lines described by Karunaratne et al. (1996). Moreover, PTR strategies have not yet been tested in transgenic cereal crops.

We have transformed wheat (*Triticum aestivum* cv. Bobwhite) with a bacterial *rnc*70 gene by microprojectile bombardment (Zhang et al., 2000). This gene is a mutant of the *rnc* gene encoding RNase III, a double-stranded (ds) RNA-specific ribonuclease from *Escherichia coli.* The wild-type gene product binds and cleaves dsRNA, whereas the mutant *rnc*70 gene with codon 117 changed from glutamic acid to lysine encodes a product that retains the binding capacity but is unable to cleave the substrates (Court, 1993). Our previous study (Langenberg et al., 1997) has shown that the *rnc* and *rnc*70 transformed tobacco plants are resistant to infections by viruses with divided genomes.

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Barley stripe mosaic virus (BSMV), a member of hordeivirus, was used in this study to explore the effectiveness of this PTR strategy in transgenic cereals against its infection. The BSMV is a rod-shaped, single-stranded RNA virus with a divided genome. It has been shown that BSMV forms dsRNA, a replicative form, during replication in plants (Pring, 1972). This replicative form RNA is a potential prime target for RNase III.

Materials and methods

Isolation and cloning of rnc*70 gene and vector construction*

The *rnc*70 gene was obtained from the binary plasmid pAM1551 (Langenberg et al., 1997) by polymerase chain reaction (PCR) with the forward primer (5′-CCCGGGTACCATGAACCCCATCGTAATT-3′) and the reverse primer (5′-GCGGCCGCGGTCATTCCA-GCTCCAGTTT-3′) and subcloned into a plant expression vector, pLZ03, resulting a construct pLZ12 (Figure 1). The vector, pLZ03 was constructed by insertion of the maize ubiquitin promoter and first intron, and *NOS* terminator from plasmid pAHC25 (Christensen & Quail, 1996) into pUC118. A modified pAHC25 plasmid (pAHC25M) was used as a selectable marker. The *gusA* gene, NOS terminator, ubiquitin promoter and intron in front of the *bar* gene were removed from plasmid, pAHC25. The resulting construct, pAHC25M, contains the ubiquitin promoter and intron originally in front of the *gusA* gene, the selection marker gene encoding phosphinothricin acetyl transferase (PAT) and the NOS terminator.

Plant transformation and regeneration

Immature embryos of wheat variety Bobwhite were precultured on a callus induction medium (CI) for 5 days before osmotic treatment and microprojectile bombardment. The callus induction medium was MS medium (Murashige & Skoog, 1962) supplemented with 100 mg/l each of Vitamin B1 (Gibco-BRL, Grand Island, NY, USA), myo-inositol

Figure 1. Map of pLZ12, containing the maize ubiquitin promoter, first intron and exon, *rnc*70 and NOS terminator.

(Sigma, St Louis, MO, USA), and casein hydrolysate (Sigma), 30 g/l sucrose (Sigma) and 0.25% Phytogel (Sigma) as a solidifying agent, plus 2,4-D (2,4-dichlorophenoxyacetic acid, Sigma) at 2.0 mg/l. The embryos were biolistically bombarded with pLZ12 DNA together with pAHC25M DNA at 1:1 molar ratio, followed by selection on the CI medium containing 5 mg/l of bialaphos, the selective agent for the *bar* gene. T0 plants were self-pollinated and grown to maturity. T1 seeds were harvested. T2 and T3 seeds were collected from self-pollinated T1 and T2 plants, respectively.

Nomenclature of transformants and their progeny plants

Each bialaphos resistant callus was numbered, for example, C1, C2, etc and plants regenerated from each callus (T0 plants) were given a suffix number, for example, C1-1, C1-2, etc. T1 plants germinated from these T0 seeds were numbered similarly, for example, C1-1-1, and so on.

PCR and western blot analysis

Genomic DNA and total proteins were isolated from T0, T1, T2, and T3 plants using Puregene DNA isolation kit (Centra Systems, Inc., Minneapolis, Minnesota, USA) and TriPure^{TM} Isolation Reagent (Boehringer Mannheim, Indianapolis, Illinois, USA). PCR was performed according to the procedure described by Zhang et al. (1993) except for the annealing temperatures of 70°C for the *rnc*70 gene. For western blots, extracts containing 5 μg of total soluble proteins were separated on 4–20% acrylamide gradient gels along with 100 ng purified bacterial RNase III or PAT proteins or purified BSMV as positive controls. The purified recombinant RNase III protein and the rabbit polyclonal antibodies against RNase III were gift from Dr. D. L. Court, NCI-Frederick Cancer Research and Development Center, Frederick, MD, USA. The purified PAT protein and polyclonal antibodies were courtesy of Drs. R. Shillito and L. Huang at AgrEvo (Wilmington, DE, USA), respectively. The BSMV was purified from infected barley plants according to the procedure described by Brakke et al. (1988). Rabbit raised BSMV polyclonal antibody reagent was a gift from Dr. E. Ball, University of Nebraska. The primary antibodies were used at 1:1000 dilution. The Amplified Alkaline Phosphatase Goat Anti-Rabbit Immuno-Blot Assay Kit (Bio-Rad,

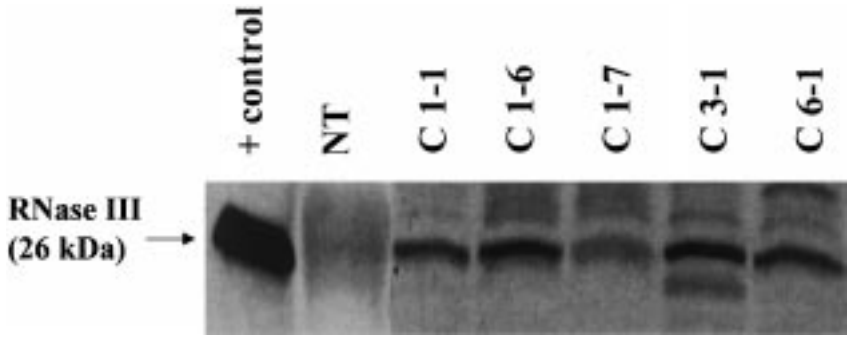

Figure 2. Western blot analysis for presence of RNase III in total proteins extracted from transgenic T0 plants, C1-1, C1-6, C1-7, C3-1 and C6-1. NT, non-transformed plant; +: a positive control of 100 ng of purified RNase III protein (26 kDa). The blot was probed with RNase III antibodies.

Hercules, California, USA) was used for western blot analysis.

Virus inoculation

The BSMV (strain ND-18) inoculum was prepared in 50 mM phosphate buffer (pH 7.4) at a concentration of 0.5 μg/ml. Five transgenic lines originated from three callus lines, C1, C3, C6 and regenerated non-transformed plants were challenged with the virus. Each plant at the 2–4-leaf stage was mechanically inoculated with 0.5 ml of the inoculum. After inoculation, the plants were rinsed with water and subsequently maintained in a greenhouse. The plants were observed during a period of 33 days.

Results and discussion

Transformation and expression of mutant RNase III in wheat

The *E. coli rnc* gene codes for a ds-specific endoribonuclease, RNase III. The wild-type gene product binds and cleaves dsRNA, whereas the product of a mutant gene, *rnc*70, binds dsRNA, but does not cleave the substrates (Court, 1993). In our previous study (Langenberg et al., 1997), tobacco plants transformed with both wild-type and mutant genes were resistant to infection by RNA plant viruses with divided genomes. However, some tobacco plants transformed with wild-type *rnc* exhibited stunted growth and failed to develop fully. The mutant gene (*rnc*70)-transformed tobacco plants, however, were normal in growth and development. Therefore, we transformed wheat plants only with the mutant gene, *rnc*70.

Table 1. Symptomatic responses of T1, T2, and T3 plants to BSMV Infection

Lines	Reaction of parental lines to BSMV	No. of symptomatic plants/No. of total plants tested
NT	N/D	34/35
T1 generation		
C1-6	N/D	7/8
C1-7	N/D	8/8
C3-1	N/D	4/8
C6-1	N/D	1/5
T2 generation		
C1-1-8	N/D	18/34
C3-1-1	Resistant	1/15
C6-1-2	Resistant	0/4
C6-1-5	Susceptible	6/6
T3 generation		
C1-1-8-9	Resistant	0/3
C1-1-8-11	Resistant	0/5

N/D, not determined.

The mutant gene, *rnc*70, was placed under the transcriptional control of the maize ubiquitin promoter and first intron (Figure 1). A total of 195 immature embryos was co-bombarded with two plasmids, pAHC25M and pLZ12. Five transgenic fertile plants (T0), containing both the selection marker gene (*bar*) and the *rnc*70 gene as determined by Southern blot analysis (data not shown), expressed RNase III protein in the cytoplasm at variable levels (Figure 2). Additional bands detected in plant extracts from the non-transformed plant and transgenic wheat in Figure 2 were probably due to cross-reaction of the

Figure 3. Growth comparison of two transgenic lines, C1-1-4 (right) and C1-1-8 (left), with or without BSMV symptoms, respectively, at 28 d.p.i.

polyclonal antibody reagent to endogenous RNase III-like proteins or other plant proteins. These nonspecific bands were detected in all plants tested and are distinct from the authentic RNaseIII band. Expression of the mutant *rnc*70 gene had no apparent effect on normal growth and development of the transgenic wheat plants.

Significant inhibition of BSMV accumulation in transgenic wheat plants expressing RNase III

The progenies of the five transgenic T0 plants were tested for virus resistance (Table 1). They included four T1 lines from their parental T0 plants (C1-6, C1-7, C3-1 and C6-1), four T2 lines (C1-1-8, C3-1-1, C6-1-2 and C6-1-5), and two T3 lines (C1-1-8-9 and C1-1-8-11). The parental T0 lines were not tested for BSMV resistance, as the lines were selected based on expression of RNase III and PAT and were used to produce T1 seeds. Regenerated non-transformed Bobwhite plants were used as controls (NT). In addition, a few segregating transgenic plants containing only the selectable marker gene were also used as controls (Presting et al., 1995). The plants were challenged with BSMV. Yellow chlorotic striping or mosaic symptoms appeared one week after inoculation. Table 1 shows that at 28 d.p.i 34/35 control plants (NT) and some transgenic plants exhibited typical symptoms (Figure 3), whereas, out of a total 96 transgenic plants 51 plants were symptom-free (Table 1 and Figure 3). Marker only transgenic plants were also uniformly infected by BSMV. The responses of T1 lines to BSMV infection were mixed. The T1 plants of transgenic lines C3-1 and C6-1 had better virus resistance than those of lines C1-6 and C1-7. Overall, T2 and T3 progenies exhibited better virus resistance because only the seeds from the resistant T1 plants, except C6-1-5, were used. The C6-1-5 plants showed disease symptoms in the T1 generation test (Table 1) and all progeny plants of C6-1-5 also developed symptoms after inoculation with BSMV. The BSMV ND-18 strain is highly seed transmissible in barley (Murray

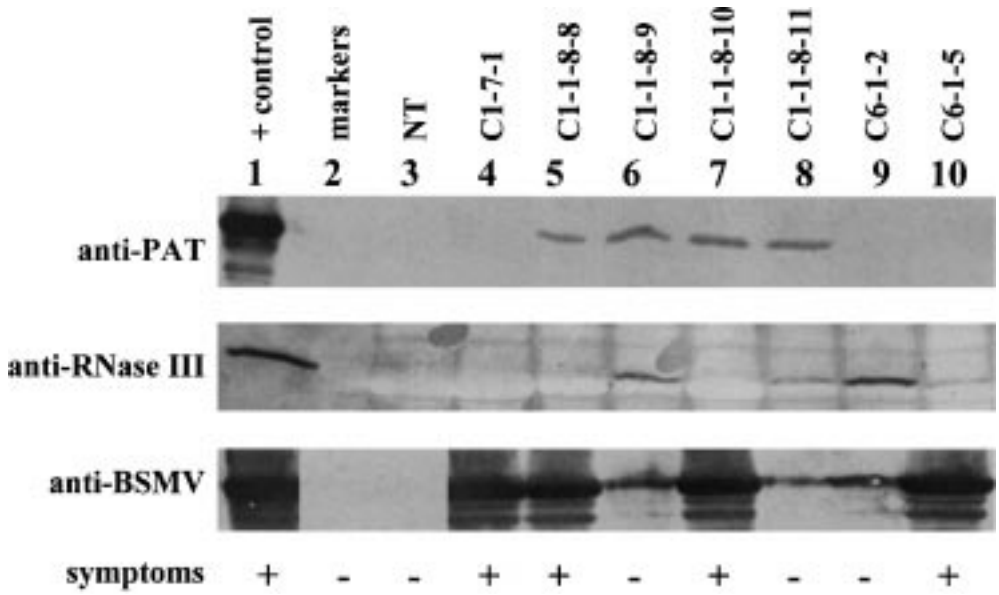

Figure 4. Western blot analysis of the expression of two transgenes, *rnc*70 and *bar*, and the accumulation of BSMV in control and transgenic wheat plants at 21 d.p.i. The blots were individually probed with PAT, or RNase III, or BSMV antibodies as indicated. C1-1-8-8, C1-1-8-9, C1-1-8-10, and C1-1-8-11 are T2 plants. C1-7-1, C6-1-1 and C6-1-5 are T1 plants. NT is a non-transformed non-inoculated control plant. Lanes 4 to 10, transgenic lines as labeled; lanes 1 and 3, positive and negative controls, respectively; and lane 2, protein size markers. A plus (+) or minus (−) sign indicates the presence or absence of visual symptoms. Immunoblots showed a single protein band of about 21 (PAT), 26 (RNaseIII) or 22 (WSMVCP) kDa in extracts from some transgenic plants.

et al., 1998). However, transgenic wheat plants used in this study did not show noticeable seed transmission, presumably because most seeds were harvested from resistant plants. Western blots were performed on 14 of the plants, with or without symptoms, for analysis of the transgene expression and BSMV's accumulation at 21 d.p.i. Figure 4 depicts western blots, probed with antibodies to the PAT, RNase III, and BSMV proteins, respectively, along with symptom assessments of the plants. Control plant (NT) extracts did not react with any of the antisera. Figure 4 demonstrates a correlation between visible symptoms and a high level of virus accumulation (lanes 4, 5, 7, and 10), as well as a correlation between the expression of RNase III and a considerable reduction of virus accumulation (lanes 6, 8, and 9). These plants exhibited no visual symptoms and they contained reduced levels of the viral coat protein as evidenced from western blots (Figure 4). Lack of symptoms in these plants is probably due to low level of virus accumulation. Accumulation of RNase III in some plants (lanes 4, 5, 7 and 10) was not detected by western blot analysis even though PCR revealed that the *rnc*70 gene sequence was present in the genomes of these plants (data not shown). This could be due to either very low expression levels or silencing of the transgene. Gene silencing or segregation of RNase III in the progeny of a resistant line could account for the susceptible phenotype in some progeny plants. The anti-PAT panel shows that expression of the marker *bar* gene was not affected by BSMV accumulation (lanes 5 and 7).

Brakke et al. (1988) reported that the maximum concentration of BSMV virions in young acutely infected barley leaves varies between 0.5 and 1 μg per mg of leaf tissue. In general, the infectivity of a virus increases at higher inoculum concentrations. To asses the resistance against BSMV at elevated levels of inoculum, the transgenic plants were challenged with a high concentration (0.5 μg/ml) of BSMV, which was about a 10-fold dilution of the maximum concentration of the virus in infected-leaf tissue. All control plants had typical BSMV symptoms 5 days after inoculation indicating viability of inoculum and success of virus infection. This was further supported by low level accumulation of BSMV in the resistant transgenic plants (Figure 4).

Only 1 out of 35 regenerated control plants failed to develop BSMV symptoms, compared to 51 plants without symptoms out of a total 96 transgenic plants. Therefore, somaclonal variation, which occurs in tis-

sue culture, is not responsible for virus resistance in the transgenic wheat plants. On the other hand, a correlation between expression of RNase III and a significant reduction of BSMV accumulation in the same transgenic wheat plants indicates that resistance was due to the *rnc*70 gene product, RNase III. The resistance is probably due to interference with normal RNA virus replication because the mutant RNase III only binds but does not cleave dsRNA, a replicative form of RNA viruses. Binding of RNaseIII to replicating BSMV RNA can conceivably prevent completion of replication process and prevent subsequent translation.

The methodology for wheat plant transformation has been available since 1993 (Weeks et al., 1993). However, a PTR strategy has not been reported in wheat and other cereals, although similar strategies have been reported in several other dicotyledeous plant species (Watanabe et al., 1995; Ed Feyter et al., 1996; Ogawa et al., 1996; Langenberg et al., 1997; Sano et al., 1997; Abad et al., 1997; Kwon et al., 1997; Moon et al., 1997; Yang et al., 1997). Both PDR and PTR strategies have been successfully employed in transgenic dicotyledeous plants. This study demonstrates that this PTR strategy can also be successfully employed in cereal crops.

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