Replicase-Mediated Resistance to Alfalfa Mosaic Virus

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Tobacco plants transformed with the P1 and P2 replicase genes of alfalfa mosaic virus (AMV) have been shown to produce functional replicase proteins, permitting their infection with AMV inocula lacking the genome segments encoding P1 and P2, respectively. To see whether expression of a mutant P2 protein would interfere with the assembly of a functional replicase complex, tobacco plants were transformed with modified P2 genes. When plants were transformed with a P2 gene encoding an N-terminally truncated protein which mimicked the tobacco mosaic virus 54K protein, no resistance was observed with 10 independent lines of transformants. Similarly, when the GDD motif in the full-length P2 protein was changed into VDD, no resistance was observed in 14 transgenic lines. However, when the GDD motif was changed into GGD (5 lines), GGD (15 lines), or DDD (13 lines), 20 to 30% of the transgenic lines showed a high level of resistance to AMV infection. This resistance was effective to inoculum concentrations of 10 to 25 μg/ml of virus and 100 μg/ml of viral RNA, causing severe necrosis of control plants. For all transgenic lines, the expression of the transgenes was analyzed at the RNA level. With the GGD, GVD, and DDD mutants, resistance was generally observed in plants with a relatively high expression level. This indicates that the resistance is due to the mutant replicase rather than to an RNA-mediated cosuppression phenomenon. © 1995 Academic Press, Inc.

INTRODUCTION

Genetically engineered resistance to virus infection has been obtained by transformation of plants with wild-type (wt) or modified viral genes encoding replicase proteins, transport proteins, or coat proteins (see Beachy et al., 1990; Carr and Zaitlin, 1993; Lapidot et al., 1993). For several viruses like alfalfa mosaic virus (AMV) and tobacco mosaic tobamovirus (TMV), coat protein (CP)-mediated resistance could be attributed to the viral protein accumulating in the transgenic plant rather than to the viral RNA (van Dun et al., 1988a; Powell et al., 1990; Taschner et al., 1994). In these plants there was a direct correlation between the level of CP accumulation and the resistance observed. Moreover, the resistance was abolished by mutations that interfered with CP synthesis without affecting the accumulation of CP mRNA. In potyviruses, however, the resistance of transgenic plants expressing nontranslatable CP mRNA was found to be more effective than the resistance of plants transformed with the wt CP gene (Lindbo and Dougherty, 1992; van der Vlugt et al., 1992). Evidence has been presented supporting the hypothesis that accumulation of a transgenic viral RNA may activate a host mechanism that specifically degrades the viral RNA, similar to the phenomenon of cosuppression (Lindbo et al., 1993; Smith et al., 1994). Once activated, this mechanism will also degrade the genomic RNA of an infecting virus.

Replicase-mediated resistance has been reported for plants transformed with wt or modified replicase genes of TMV, pea early browning virus, cucumber mosaic virus (CMV), potato virus X (PVX), potato virus Y (PVY), and cymbidium ringspot virus (see Baulcombe, 1994). For several of these viruses an inverse relationship has been found between the level of expression of the viral transgene and the resistance obtained. This phenomenon has been documented in most detail for PVX. Resistance has been obtained by transformation of plants with the wt 166K PVX replicase gene, aminomethyl terminal portions of this gene, and full-length genes with the GDD motif expressed as ADD (Braun and Hemenway, 1992; Longstaff et al., 1993). The GDD motif (Gly-Asp-Asp) is believed to be part of the catalytic center of the polymerase (Koonin, 1991). More recent data showed that a similar resistance could be obtained by transformation of tobacco with PVX replicase genes containing other mutations in the GDD motif or containing a ribosomal frameshift early in the 166K reading frame (E. Mueller, J. Gilbert, G. Brigneti, G. Davenport, and D. C. Baulcombe, personal communication). In this study, resistance was found to be associated with low level accumulation of the transgenic replicase RNA, whereas plants with a relatively high level of this RNA showed no resistance. It has been suggested that PVX replicase-mediated resistance is due to a specific degradation of viral RNA by a gene-

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silencing mechanism (Baulcombe, 1994) similar to the RNA-mediated resistance observed with potyvirus CP-sequences (Smith et al., 1994).

We have transformed tobacco with the replicase genes P1 and P2 encoded by AVMV RNAs 1 and 2, respectively (van Dun et al., 1988a; Taschner et al., 1991). The P1 protein contains methylase and helicase motifs; the P2 protein contains the GDD motif. RNA 3 of the tripartite AVMV genome encodes the movement protein P3 and the viral CP which is translated from the subgenomic RNA 4. Plants transformed with the P1 gene (P1 plants) were found to support replication of RNAs 2 and 3, plants transformed with the P2 gene (P2 plants) supported replication of RNAs 1 and 3, and plants transformed with both replicase genes (P12 plants) could be infected with RNA 3 alone. This demonstrates that these plants accumulate functional replicase proteins and that this accumulation does not result in a resistance of the plants to AVMV infection. P1 and P2 have both been found to be subunits of the purified viral replicase (Quadt et al., 1991). To study the effect of the expression of a non-functional P2 protein in transgenic plants on the assembly of a functional replicase by an infecting virus, we transformed tobacco with a 5′-deleted P2 gene and full-length P2 genes with mutations in the GDD motif. Resistance was observed with three GDD mutants. To address the question whether the resistance was RNA- or protein-mediated, the correlation between resistance and expression of the transgene was analyzed.

**MATERIALS AND METHODS**

**Construction of mutant AVMV P2 clones**

Mutations were made in the P2 gene of AVMV strain 425. This gene is present in RNA 2 (2593 nucleotides) between nucleotides 55 and 2424 (Cornelissen et al., 1983). cDNA 2 clone pMAR4, flanked by HindIII sites, was used as starting material. This is the clone that was used by van Dun et al. (1988b) to transform tobacco with the wt P2 gene. To create mutant ΔP2 with a 5′-deleted P2 gene, a 1980-bp SspI–HindIII fragment from clone pMAR4 (nucleotides 613–2587 of RNA 2) was ligated in with Klenow polymerase, ligated to BamHI linkers, and inserted in the sense orientation into the BamHI site of transformation vector pROI1, a pBIN19 derivative (Bever, 1984). Under control of the CaMV 35 S promoter and the nopaline synthase terminator, the resulting clone pPV115 is expected to yield a transcript with an open reading frame starting at Met-191 (nucleotides 625–627). A second in-frame AUG codon is present at nucleotides 655–657 (Met-201). Translation initiation at Met-191 or Met-201 would result in 68K or 67K proteins, respectively, that are coterminal with the C-terminus of P2 (90K).

Site-directed mutagenesis to introduce mutations at the conserved GDD motif of the AVMV 425 P2 gene (nucleotides 1924–1932, amino acids 624–626) was performed using the Altered Site Mutagenesis kit (Promega Co., Madison, WI) as recommended by the supplier. AIVMV cDNA 2 was cloned into mutagenesis vector pSELECT-1 to give pPV65, which was mutagenized with primers PT3 (5′-d-TGCTCCG(C/T)/ATGATGATT-3′) and PT4 (5′-d-TTCCGCTG(C/T/G)/TGATT CAT-3′). The degenerate nucleotides in PT3 and PT4 correspond to nucleotides 1925 (Gly codon) and 1928 (Asp codon) of RNA 2, respectively. The 418-bp Pmfl-BglII fragments (nucleotides 1878–2297) containing mutations were sequenced and exchanged with those of pPV103, a pCI19H derivative containing AIVMV cDNA 2. The 2.6-kb HindIII fragments from the resulting clones (nucleotides 1–2587) were introduced into the HindIII site of transformation vector pPV54, a pBIN19 derivative, between the CaMV 35 S promoter and the nopaline synthase terminator. Plasmids pPV131, pPV133, pPV141, and pPV144 encode AIVMV P2 in which the GDD sequence is replaced by GVD, DDD, GGD, and VDD, respectively. In addition, in all clones a C to T transition at position 2066 was observed compared to the published sequence. This transition does not affect the reading frame and may reflect sequence heterogeneity in the viral RNA.

**Plant transformation**

Transformation vectors were introduced into Agrobacterium tumefaciens strain LBA4404 containing pAL4404 by triparental mating (van Dun et al., 1987). Nicotiana tabacum cv. Samsun NN plants were transformed with A. tumefaciens containing transformation vectors by the leaf disk transformation procedure (Horsch et al., 1985). Seeds from the primary transformants were germinated on MS medium containing kanamycin (100 μg/ml). Some lines were lost because the primary transformants did not produce seeds or produced seeds that did not germinate. It is our experience that this phenomenon is inherent to the transformation procedure and is not specific to a particular gene construct used for transformation.

**Inoculation of plants**

Inoculation was done on self-fertilized R1 progeny of the transformants and nontransgenic control plants. Six plants per independently transformed line were inoculated with 20 μl/half-leaf of a solution containing particles or RNAs of AIVMV strain YSMV, using three half-leaves per plant. On tobacco, strain YSMV causes yellow lesions on the inoculated leaves which become necrotic 1 week after inoculation. At this time a systemic yellowing starts to develop in the uninoculated leaves. Local lesions on the inoculated leaves were scored 2 weeks after inoculation.
formed lines with the ΔP2 construct were obtained. In mutants VDD (14 lines), GGD (5 lines), GVD (15 lines), and DDD (13 lines) the GDD motif was mutated into Val–Asp–Asp, Gly–Gly–Asp, Gly–Val–Asp, and Asp–Asp–Asp, respectively.

Resistance analyzed by local lesion assay

Self-fertilized kanamycin-resistant R, progeny plants of the independently transformed lines were inoculated with 10 μg/ml of particles of AIMV strain YSMV. In addition, a number of lines were inoculated with 2.5 μg/ml of AIMV. Six plants per line were inoculated, using three half-leaves per plant. At inoculum concentrations of 2.5 and 10 μg/ml, strain YSMV caused several hundreds to over a thousand yellow lesions per half-leaf, respectively, on nontransgenic control plants. Table 1 shows the results obtained with the inoculum of 10 μg/ml; column "L" gives the line number and column "I" the number of plants showing symptoms per six plants that were inoculated. Plants of the 10 lines transformed with the ΔP2 mutant and the 14 lines transformed with the VDD mutant all showed symptoms at levels comparable to that on control plants. Similar results were obtained with the inoculum of 2.5 μg/ml (results not shown), indicating that none of the ΔP2 or VDD plants showed a significant resistance to infection.

### Table 1

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*Plants were transformed with the mutant AIMV P2 genes shown in Fig. 1.

Table 1. Susceptibility of Transgenic Tobacco Plants to Infection with AIMV Particles

*Plants were transformed with the mutant AIMV P2 genes shown in Fig. 1. L, Line numbers of independently transformed R, progeny plants. L, Six plants of each line were inoculated with particles (10 μg/ml) of AIMV strain YSMV. Symptoms on the inoculated leaves were scored 14 days after inoculation. For each line the number of plants with symptoms is given.
To analyze the susceptibility of the GDD mutant plants, six plants of GVD line 1 and six plants each of DDD lines 4 and 9 were inoculated with RNA of AIMV strain YSMV at a concentration of 100 μg/ml. On six nontransgenic control plants this inoculum caused several hundreds of lesions per half-leaf. However, none of the inoculated GVD or DDD plants showed symptoms on the inoculated or systemic leaves. Thus, the resistance of these lines is effective against inoculation with either AIMV particles or RNAs.

**Viral RNA accumulation in ΔP2 and VDD plants**

Because the lines listed in Table 1 did germinate on medium containing kanamycin, they apparently all expressed the npII gene. However, the absence of resistance in the ΔP2 and VDD plants could be due to a defect in the expression of the mutant P2 gene. Figure 2 shows a Northern blot loaded with polyadenylated RNAs from uninoculated ΔP2 plants probed with cDNA 2. As a control, the blot was loaded with RNA from uninoculated P2 and P12 plants (Fig. 2, lanes 2 and 3). Except for lane 7 (Fig. 2, lane 9) all ΔP2 lines accumulated a truncated RNA molecule of the expected length at levels comparable to those in the P2 and P12 plants. Although the P2 and P12 plants accumulate wt P2 protein at a level sufficient for infection of the plants with RNAs 1 plus 3 and RNA 3, respectively, an antiserum raised to a C-terminal peptide of P2 was unable to detect the protein in homogenates of these transgenic plants (van Dun et al., 1988b). Therefore, we could not analyze the expression of mutant P2 genes at the protein level.

An analysis of the expression level of the transgene in the VDD plants is shown in Fig. 3. Expression was observed in lines 1, 5, 9, 12, 13, 14, and 16, with the highest level of the mutant P2 mRNA accumulating in line 14. In addition to a transcript of the correct size, a truncated transcript accumulated in relatively large quantities in line 18. Northern blot analysis of viral RNA accumulation in the plant lines shown in Figs. 2 and 3 was not performed.
FIG. 4. Northern blot analysis of viral RNA accumulating in uninoculated and infected GGD plants. (A) Polyadenylated RNAs from uninoculated independently transformed plant lines. (B) Total RNA from plant lines inoculated with AIMV particles (10 μg/ml). RNA was extracted from the inoculated leaves. The numbers of the plant lines are given. The blots were probed with labeled cDNA 2 (A) or cDNAs 1, 2, and 3 (B). The positions of AIMV RNAs 1–4 are indicated to the right.

also done after inoculation of the plants with AIMV (10 μg/ml). This showed that all inoculated plants accumulated virus to levels similar to those in inoculated control plants (results not shown). It was concluded that expression of the transgenes in the ΔP2 and VDD plants did not result in a reduced susceptibility of the plants to AIMV infection.

Viral RNA accumulation in GGD, GVD, and DDD plants

Figure 4A shows the accumulation of polyadenylated viral RNA in the uninoculated GGD plants. Lines 1 and 5 contained relatively high levels of mutant P2 mRNA, a low level was visible in line 8 and little or no accumulation was detectable in lines 6 and 7. Figure 4B shows the average level of virus accumulation in six plants of each line after inoculation with AIMV (10 μg/ml). The plants of line 5 did not show symptoms (Table 1) and no virus was detectable in the plants. Plants of line 6 showed normal symptoms of AIMV infection and accumulated virus (Fig. 4B) to a level similar to that in inoculated control plants (not shown). Lines 1, 7, and 8 showed a partial resistance (Table 1) which is reflected by a reduced level of virus accumulation (Fig. 4B).

FIG. 5. Northern blot analysis of viral RNA accumulating in uninoculated and infected GVD plants. (A) Polyadenylated RNAs from uninoculated independently transformed plant lines. (B) Total RNA from plant lines inoculated with AIMV particles (10 μg/ml). RNA was extracted from the inoculated leaves. The numbers of the plant lines are given. The blots were probed with labeled cDNA 2 (A) or cDNAs 1, 2, and 3 (B). The positions of AIMV RNAs 1–4 are indicated to the right.

Three different phenotypes were observed for tobacco plants transformed with the GGD, GVD, and DDD constructs: (1) lines with a relatively high level of resistance

virus at levels comparable to those in inoculated control plants. Although the data of Table 1 suggested a partial resistance for GVD lines 4, 8, 14, and 17, such a resistance was not detectable when the average level of virus accumulation in six plants of each line was considered. Plants of GVD lines 19 and 20 did not show symptoms after inoculation (Table 1). No virus accumulation was detectable in line 19 but a very low level (less than 1% of the control) could be seen in line 20 (Fig. 5B).

The accumulation of viral RNA in uninoculated and infected DDD plants is shown in Figs. 6A and 6B, respectively. As measured by Northern blot hybridization, lines 9 and 15 showed the highest level of resistance and these lines accumulated relatively high levels of mutant P2 mRNA, comparable to those in wt P2 and P12 plants. Low levels of transcripts were detectable in lines 1 and 4, but only in line 4 was this accompanied by a significant resistance of the plants to AIMV infection. The results of Table 1 suggested a partial resistance of lines 1 and 5 but this was not reflected in the overall virus accumulation in six inoculated plants of these lines.

DISCUSSION

Three different phenotypes were observed for tobacco plants transformed with the GGD, GVD, and DDD constructs: (1) lines with a relatively high level of resistance
in all inoculated plants, (2) “partially resistant” lines with some plants with a relatively high level of resistance and others being fully or partially susceptible to infection, and
(3) nonresistant lines. High resistance was observed with lines GGD-5, GVD-19, GVD-20, DDD-9, and DDD-15 and these lines all showed the highest levels of accumulation of transgenic RNA (Figs. 4, 5, and 6). (Lines GVD-1 and GVD-13 also showed relatively high levels of resistance but the accumulation of viral transcripts in these lines was not analyzed.) In contrast, the nonresistant lines GGD-6, GVD-3, GVD-9, GVD-12, GVD-15, GVD-16, DDD-2, DDD-3, DDD-6, DDD-8, DDD-10, DDD-12, DDD-14, and DDD-16 did not accumulate transgenic RNA or accumulated a truncated transcript of the nuclear P2 gene (e.g., line GVD-12; Fig. 5A). In the partially resistant lines various levels of accumulation of P2 transcripts were observed. Segregation for resistance has been reported for R1 and even R2 progeny of plants transformed with PVY replicase genes (Audy et al., 1994). Possibly, a similar segregation occurred in our partially resistant lines. We have not analyzed individual plants of such a line for a possible correlation between resistance and accumulation of the transcript from the nuclear P2 gene.

Resistance mediated by activation of a host mechanism that specifically degrades viral RNA was proposed to explain genetically engineered resistance to tobacco etch and potato virus Y potyvirus and PVX potexvirus. This phenomenon is characterized by an inverse relationship between accumulation of RNA and protein expressed from the transgene and the resistance observed (Lindbo et al., 1993; Smith et al., 1994; Baulcombe, 1994). Our observation that resistance was obtained only in plants with a relatively high expression level of the P2 gene indicates that this resistance is not due to a cosuppression phenomenon but is caused by a mutant P2 protein accumulating in the plants. Although the titer of the available antiserum was too low to detect the P2 protein in unfractionated homogenates of transgenic plants, this serum detected P2 in a purified replicase preparation isolated from uninoculated P12 plants (M. de Graaff, M. R. Man in't Veld, and E. M. J. Jaspers, submitted for publication). The P2 gene constructs in the P12 plants and the GGD, GVD, or DDD plants differ only in a point mutation in the GDD motif. Thus, the viral transcripts produced in wt and mutant P2 plants are expected to be translated with similar efficiencies but we cannot rule out the possibility that the mutations affect the stability or turnover of the P2 protein. On the other hand, a single point mutation in the GDD motif probably does not convert the wt P2 transcript into a mediator of resistance. The resistance is more likely to be due to the mutant P2 protein translated from the modified P2 transcript.

Mutations at the region encoding the GDD motif destroyed the infectivity of the genome of QB phage (Inokuchi and Hirashima, 1987), the L-A double-stranded RNA virus of yeast (Ribas and Wickner, 1992), and PVX (Longstaff et al., 1993) and greatly reduced the activity of the poliovirus replicase in vitro (Jablonski et al., 1991). Expression of the mutant QB replicase component in transformed Escherichia coli affected susceptibility to infection by the phage (Inokuchi and Hirashima, 1987). Possibly, the mutant P2 accumulating in our GGD, GVD, and DDD plants associates with P1 expressed from inoculum RNA 1, thereby preventing the association of P1 with wt P2 translated from inoculum RNA 2. The formation of a nonfunctional replicase complex would block the viral replication cycle. Accumulation in transgenic plants of high levels of PVX 165K replicase with mutations in the GDD motif did not affect the susceptibility of the plants to infection with PVX (Baulcombe, 1994). Also, plants transformed with the PVY Nb replicase deleted for the GDD motif were not resistant (Audy et al., 1994). This may reflect a difference in the composition of the replicase complexes involved in replication of the tripartite AIMV genome and the monopartite PVX and PVY genomes.

Transformation of plants with replicase sequences of a number of viruses resulted in a resistance that was more specific than CP-mediated resistance (Carr and Zaitlin, 1993). However, transformation with the TMV 183K gene containing an IS10-like insertion resulted in a broad resistance to tobamoviruses (Donson et al., 1993). Our plants transformed with mutant P2 genes of AIMV strain 425 showed resistance to a challenge inoculation with AIMV strain YSMV. Available sequence data
indicate a 95% overall sequence similarity between the genomes of strains 425 and YSMV and also other AIMV strains (Langereis et al., 1986; Neeleman et al., 1991; L. Neeleman, unpublished data). Exchange of RNAs 1 or 2 between strains 425 and YSMV results in viable pseudorecombinants, indicating that P2 of strain 425 can interact with P1 of strain YSMV (Dingjan-Verssteeg et al., 1972). After infection of the mutant P2 plants with a high inoculum dose, P1 may be translated from RNA 1 in excess over the mutant P2, permitting the association of P1 with P2 translated from inoculum RNA 2. At such a high inoculum dose only a few local lesions were observed without systemic symptoms, indicating that the virus remained localized at the infection site.

AIMV and bromo mosaic bromovirus (BMV) have a similar genome structure and AIMV P1 and P2 correspond to BMV 1a and 2a proteins, respectively. The N-terminal 115-residue segment preceding the polymerase-like domain in the BMV 2a protein was found to contain the sequence required for its binding to the 1a protein (Kao and Anhquist, 1992). This binding may be functionally equivalent to the covalent linkage of the “126K” and “54K” sequences in the TMV 183K readthrough protein. Probably, the N-terminal deletion in the AIMV ΔP2 mutant will interfere with the possibility of this protein interacting with P1 but the deletion does not extend into the sequence of P2 corresponding to the TMV 54K protein. Transformation of plants with the truncated P2 gene did not result in resistance to AIMV infection. However, we have no proof that the viral transcript in these plants is translated into the ΔP2 protein. The AIMV P2 gene is homologous to the CMV 2a gene. Resistance has been obtained by transformation of plants with a 3'-truncated 2a protein from which an 84-base segment including the GDD-motif was deleted (Anderson et al., 1992).

The viral transcripts accumulating in the VDD plants are presumed to be translated with an efficiency similar to that of transcripts in the wt P2 plants but no resistance was observed with any of the VDD lines. Currently, we are investigating whether the glycine to valine substitution in the GDD motif of this mutant affects the polymerase activity of the P2 protein.

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