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Complementation and disruption of viral processes in transgenic plants

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SUMMARY

RNAs 1 and 2 of alfalfa mosaic virus (AlMV) encode the replicase genes P1 and P2, respectively, whereas RNA 3 encodes the movement protein and the viral coat protein (CP). To investigate the mechanism of cross-protection, tobacco plants were transformed with wild-type and mutant DNA copies of the AlMV CP gene and the two replicase genes P1 and P2. Expression of wild-type CP at relatively low levels resulted in a resistance against infection with AlMV virus particles whereas at higher expression levels CP protected against infection with either AlMV particles or RNAs. Plants transformed with a mutant AlMV CP gene were not resistant to the wild-type virus but were resistant to AlMV with the same mutation in the CP gene. Transformation of plants with the wild-type P1 gene (P1 plants), P2 gene (P2 plants) or both these genes (P12 plants) did not result in resistance to AlMV. Instead, these plants could be infected with an inoculum lacking the gene(s) that was (were) integrated in the plant genome. Infection of non-transgenic plants, P1 plants or P2 plants with a mixture of AlMV genomic RNAs requires the presence of CP in the inoculum but P12 plants could be infected with RNA3 without any requirement for CP in the inoculum. Infection conditions in which 35*S* promoter/AlMV cDNA fusions were present in the inoculum instead of in the plant genome were used to shed light on the early function of CP. Finally, plants were transformed with P2 genes with mutations in the GDD-motif. A number of these transgenic lines showed a high level of resistance to AlMV.

1. INTRODUCTION

In 1929 McKinney first reported that plants infected with a mild strain of a virus showed a reduced susceptibility to a second infection with a severe strain. This phenomenon, which was called cross-protection, has often been used to demonstrate relationships between viruses. Because of the specificity of the interaction it was believed that gene products produced by the protecting virus were responsible for the resistance to the challenging virus. The development of plant transformation techniques in the 1980s permitted an investigation of this hypothesis by an analysis of the susceptibility to virus infection of transgenic plants expressing a viral gene product. In 1986 it was shown that tobacco plants transformed with the coat protein (CP) gene of tobacco mosaic virus (TMV) showed a resistance to TMV infection (Powell Abel *et al.* 1986). Shortly thereafter we showed that expression of the CP genes of alfalfa mosaic virus (AlMV), tobacco streak virus (TSV) and tobacco rattle virus (TRV) in transgenic tobacco resulted in a similar resistance to the homologous viruses and since then CP-mediated resistance has been demonstrated for an ever growing number of plant RNA viruses (for a review, see Register III & Nelson 1992). The demonstration of CP-mediated resistance, however, does not rule out the possibility that non-structural viral genes do play a role in the classical phenomenon of cross-protection as well. We have addressed this question using AlMV as a model system. Figure 1 shows the structure of the tripartite RNA genome of this virus. RNAs 1 and 2 encode the replicase subunits P1 (126 kDa) and P2 (90 kDa), respectively, whereas RNA 3 encodes protein P3 (32 kDa) with a role in cell-to-cell transport of the virus and CP (24 kDa), which is translated from the subgenomic messenger, RNA 4. We have transformed tobacco with DNA copies of the P1 gene (P1 plants), the P2 gene (P2 plants) or both these genes (P12 plants) (Taschner *et al.* 1991). In these plants transcription of the transgene is driven by the CaMV 35*S* promoter and terminated by the *nos* terminator. The P1 plants supported replication of AlMV RNAs 2 + 3, the P2 plants supported replication of RNAs 1 + 3 and the P12 plants could be infected with RNA 3 only. Apparently, expression of functional replicase genes in these plants does not result in a resistance to infection. The P2 functions of the tobamovirus, TMV, and the tobravirus, pea early-browning virus (PEBV), are

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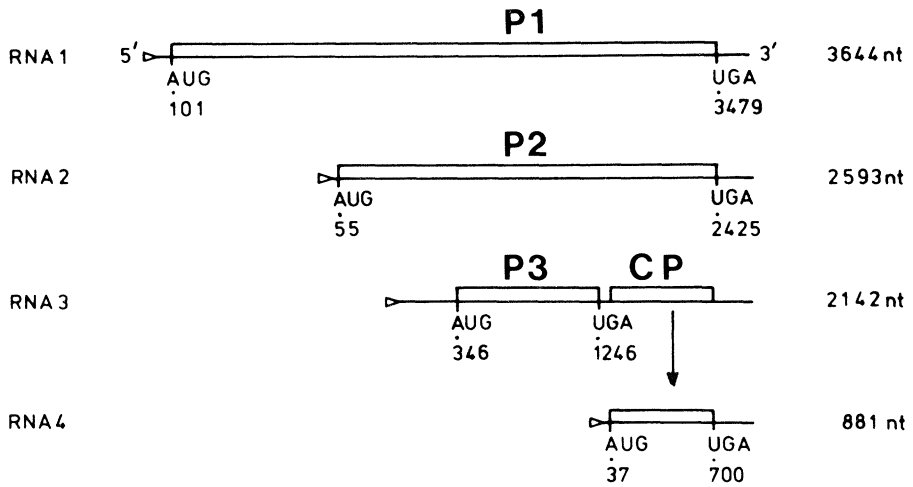

Figure 1. Schematic representation of the tripartite RNA genome of alfalfa mosaic virus. The location of the P1, P2, P3 and coat protein (CP) genes is indicated.

expressed by read-through translation of the corresponding P1 genes. Transformation of tobacco with these 54 kDa read-through sequences of TMV and PEBV resulted in high levels of resistance to the corresponding viruses (for a review, see Wilson 1993). For the potexvirus, potato virus X (PVX), transformation with the wild-type gene encoding the single 165 kDa replicase of this virus resulted in a strong protection of the transgenic plants. In addition, transformation with a mutant 165 kDa gene in which the GDD motif was changed into ADD resulted in plants that were immune to infection with PVX (see Wilson 1993).

A mixture of the genomic RNAs of AlMV is not infectious to plants or protoplasts unless each of the three RNAs has bound a few molecules of CP (for a review, see Jaspars 1985). This indicates that in addition to its structural role and its role in cross-protection, AlMV CP has an early function in the initiation of infection. In the present work we used plants transformed with wild-type and mutant CP genes, and wild-type replicase genes, to study the role of CP in the initiation of infection and in cross-protection. In addition, we analysed the susceptibility to infection of plants transformed with mutant P2 genes.

2. PLANTS TRANSFORMED WITH WILD-TYPE COAT PROTEIN GENES

Among the 27 recognized groups of plant viruses with a single-stranded RNA genome of plus-polarity, two groups with a similar tripartite genome require CP in the inoculum to initiate infection. This phenomenon has been called 'genome activation'. These are the ilarviruses and the monotypic AlMV group. The ilarvirus that has been studied in most detail is tobacco streak virus (TSV). Although there is no obvious sequence similarity between CPs of AlMV and TSV, ALMV CP can activate the TSV genome and vice versa. Moreover, CPs of the two viruses bind specifically to the 3′-termini of the homologous as well as the heterologous RNAs (see Jaspars 1985). We have transformed tobacco with the CP genes of AlMV strain 425 (Van Dun *et al.* 1987) and TSV (Van Dun *et al.* 1988). Table 1 summarizes the susceptibility to infection with various inocula of plants accumulating no viral protein (control plants), plants accumulating AlMV CP and plants accumulating TSV CP. An inoculum of TSV particles infects the control and AlMV CP plants but not the TSV CP plants. Conversely, an inoculum of AlMV particles infects the control and TSV CP plants but not the AlMV CP plants. Apparently, the transgenic plants are only resistant to the homologous virus. A mixture of AlMV RNAs 1–4 infects all three types of plants. The control is infected because RNA 4 can substitute for CP in the inoculum. The observation that AlMV CP plants are resistant to AlMV particles but susceptible to AlMV RNAs 1–4 indicates that CP produced by the plant interferes with uncoating of virus particles. When the plants are inoculated with AlMV RNAs 1, 2 and 3, the control plants are not infected because the inoculum does not contain CP or RNA 4. However, both the AlMV and TSV CP plants are infected by a mixture of AlMV genomic RNAs. Apparently, CP produced by both plants is able to activate the AlMV genome. This demonstrates that AlMV and TSV CP are equivalent in their early functions in genome activation but that they are not equivalent in their function in CP-mediated resistance.

Tobacco plants have also been transformed with the CP gene of the S strain of AlMV (Tumer *et al.* 1987). These plants were resistant to infection with both AlMV particles and RNAs. A comparison showed that in plants transformed with CP of the S strain the expression level of the transgene was about four times higher than that in plants transformed with the CP gene of strain 425 (P. E. M. Taschner *et al.*, unpublished results). This indicates that at a relatively low level CP interferes with uncoating of virus particles whereas at a higher level it also inhibits a later step in the infection cycle. The sequence similarity between CPs of AlMV strains 425 and S is about 97% and the S-CP plants were resistant to particles and RNAs of strain 425 (P. E. M. Taschner *et al.*, unpublished results).

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Table 1. *Susceptibility to infection of plants transformed with the coat protein genes of AlMV or TSV*

(The AlMV CP plants used were those made by Van Dun *et al.* (1987). Susceptibility to infection is expressed as: +, susceptible to infection; −, resistant to infection.)

inoculum	control plants	AlMV CP plants	TSV CP plants
TSV particles	+	+	−
AlMV particles	+	−	+
AlMV RNAs 1–4	+	+	+
AlMV RNAs 1–3	−	+	+

3. PLANTS TRANSFORMED WITH WILD-TYPE REPLICASE GENES

The ability of P12 plants and P12 protoplasts to support replication of AlMV RNA 3 has been extensively used to map *cis*- and *trans*-acting functions involved in the replication of RNA 3 (Van der Kuyl *et al.* 1991*a–c*; Neeleman *et al.* 1991; Van der Vossen *et al.* 1993). A surprising phenomenon was the observation that CP is required in an inoculum of AlMV RNAs for the infection of non-transgenic control plants, P1 plants and P2 plants but not for the infection of P12 plants (Taschner *et al.* 1991). In non-transgenic plants P1 and P2 are expressed from the inoculum RNAs 1 and 2 whereas in P12 plants these proteins are expressed from integrated nuclear cDNAs. It could be that CP is required for the former type of expression but not for the latter. On the other hand, in P12 plants P1 and P2 are present at fixed amounts at the time of inoculation whereas in non-transgenic plants they have to be synthesized after inoculation. It could be that this difference in timing of P1 and P2 synthesis affects the requirement for CP in the inoculum. To investigate these two possibilities we have fused full-length DNA copies of the AlMV RNAs with the 35*S* promoter and *nos* terminator in such a way that the cDNAs were infectious when inoculated to plants. This permitted a study of the requirement for CP in the inoculum under conditions where P1 and P2 were expressed from cDNAs either integrated in the plant genome or present in the inoculum. When non-transgenic plants are inoculated with a mixture of cDNAs 1 and 2 and RNA 3, P1 and P2 are expressed from cDNAs as in the P12 plants but the replicase proteins are synthesized after infection as in the non-transgenic plants. It turned out that under these conditions CP was required in the inoculum to initiate infection (Neeleman *et al.* 1993). Inoculation of non-transgenic plants with a mixture of cDNAs 1, 2 and 3 resulted in virus replication but the infection level was increased several fold by addition of CP to the inoculum. Similarly, P1 plants could be infected with cDNAs 2 + 3 and P2 plants could be infected with cDNAs 1 + 3. However, when one cDNA in these inocula was replaced by its corresponding RNA, infection depended on the presence of CP in the inoculum. From these results it was concluded that the role of CP in the inoculum is to protect the inoculum RNAs from degradation until they can enter the replication complex (Neeleman *et al.* 1993). In P12 plants this replication complex, which contains both P1 and P2 (Quadt *et al.* 1991), is already available at the time of inoculation and no protective role of CP is required. If we inoculate non-transgenic plants with cDNAs 1, 2 and 3, the nuclear transcripts that serve as messengers for P1 and P2 may have been degraded by the time that a functional replication complex has been assembled. However, cDNAs 1, 2 and 3 will serve as templates for multiple rounds of transcription by polymerase II, resulting in a transient supply of corresponding transcripts that are able to program replication complexes. This may explain the observation that a mixture of these cDNAs is able to initiate infection relatively independent of CP in the inoculum (Neeleman *et al.* 1993).

Previously, we have shown that deletions in the P3 gene do not affect replication of RNA 3 in P12 protoplasts but that deletions or frameshifts in the CP gene result in a 100-fold reduction of the accumulation of plus-strand RNA 3 and a five- to ten-fold increase in the accumulation of minus-strand RNA 3 (Van der Kuyl *et al.* 1991*a*; Van der Kuyl *et al.* 1991*c*). This indicates that CP has at least three functions in the AlMV replication cycle: (i) protection of the inoculum RNAs from 3′-terminal degradation by plant ribonucleases until the replication complex has been assembled; (ii) induction of a switch from the synthesis of symmetric amounts of viral plus- and minus-strand RNAs to asymmetric plus-strand AlMV RNA synthesis; and (iii) encapsidation of the progeny RNAs into virions.

4. PLANTS TRANSFORMED WITH MUTANT COAT PROTEIN GENES

The N-terminal sequence of native AlMV CP starts with three serine residues of which the first residue is acetylated. Tobacco plants have been transformed with a mutant CP gene of AlMV strain S encoding a protein of which the first serine residue was changed into a glycine residue. These plants showed no resistance to infection with AlMV particles or RNAs but could be infected with a mixture of AlMV RNAs 1, 2 and 3 (Tumer *et al.* 1991). This indicated that the mutant CP had lost its role in cross-protection but was still able to activate the genome. When the same mutation was engineered in an infectious clone of AlMV RNA 3 and the transcript of this clone was combined with wild-type RNAs 1 and 2, a viable virus was obtained although the symptoms produced by this mutant on tobacco were slightly necrotic in contrast to the chlorotic symptoms of the wild-type. We have analysed the resistance of wild-type (wt)-CP plants and mutant-CP plants against wt and mutant virus and corresponding RNAs (P. E. M. Taschner *et al.*, unpublished results). The results are summarized in table 2. The resistance of wt-CP plants produced in Leiden (Van Dun *et al.* 1987) and at Monsanto Co., St. Louis (Tumer *et al.* 1987), against infection with wt-virus and wt-RNA has been discussed in a previous paragraph. However, the Monsanto wt-CP plants were also completely resistant to the mutant virus. Moreover, the Monsanto mutant-CP plants were

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Table 2. *Resistance of transgenic plants to infection with wild-type and mutant viruses*

(wt-CP plants (Leiden) and (MON): plants transformed with the wild-type AlMV coat protein gene at Leiden University and Monsanto Co., respectively. The N-terminal amino acid of coat protein produced by mutant-virus and mutant-CP plants is changed from serine into glycine. P12 plants were preinoculated with RNA 3 seven days before a challenge inoculation with virus or RNA. Resistance to infection is expressed as: +, resistance to infection; −, no resistance to infection; N.D., not determined.)

	resistance to			
plants	wt-virus	wt-RNA	mutant-virus	mutant-RNA
wt-CP plant (Leiden)	+	−	N.D.	N.D.
wt-CP plant (MON)	+	+	+	N.D.
mutant-CP plant (MON)	−	−	+	−
P12 plant + wt-RNA3	+	+	N.D.	N.D.
P12 plant + mutant-RNA3	+	N.D.	N.D.	N.D.

susceptible to wt virus but resistant to mutant virus. Apparently, the susceptibility of the mutant-CP plants to wt virus is an exception and there is no reason to fear that one amino acid substitution in the CP of a challenging virus would be sufficient to overcome CP-mediated resistance of the plant. The expression level of CP in mutant-CP plants was found to be similar to that in wt-CP plants made in Leiden (P. E. M. Taschner *et al.*, unpublished results). This may explain the observation that the mutant-CP plants are susceptible to infection by mutant RNA.

P12 plants infected with wt-RNA 3 of AlMV strain 425 do not show symptoms but produce CP at levels comparable to those in non-transgenic plants infected with strain 425. These infected P12 plants are highly resistant to a challenge infection with the necrotic AlMV strain YSMV. P12 plants infected with mutant RNA 3 produced levels of CP that were ten- to 100-fold higher than those in healthy mutant-CP plants and these infected P12 plants were resistant to a challenge inoculation with AlMV strain YSMV (P. E. M. Taschner *et al.*, unpublished results). Apparently, at this relatively high level of accumulation the mutant CP is able to protect against wt virus.

5. PLANTS TRANSFORMED WITH MUTANT REPLICASE GENES

The protein encoded by the P2 gene of AlMV contains the GDD motif that has been found in most RNA-dependent RNA-polymerases and is believed to be part of the catalytic centre of the viral replicase (Kamer & Argos 1984). We have transformed tobacco with P2 genes in which the encoded GDD motif was mutated to DDD, GGD, GVD and VDD and the transgenic lines were challenged by inoculation with the necrotic AlMV strain YSMV (F. Th. Brederode *et al.*, unpublished results). The following numbers of lines with a high level of resistance to AlMV infection were obtained: DDD-construct, three out of 13 tested; GGD-construct, one out of five tested; GVD, two out of five tested; VDD-construct, zero out of 13 tested. A preliminary analysis of the expression levels of the transgenes by Northern blot hybridization indicated that at least for the DDD-construct there was a correlation between expression level and resistance obtained. Possibly, the mutant P2 protein produced by the plant associates with the P1 protein synthesized by the challenge virus into a non-functional replication complex and thereby prevents replication of the genome of the invading virus.

6. CONCLUSIONS

In addition to its early function, its role in the regulation of the balance between viral plus- and minus-strand RNA synthesis and its structural role, AlMV CP has a function in symptom formation in tobacco (Neeleman *et al.* 1991) and in aphid-transmission of the virus. Its role in cross-protection may be related to one or more of these functions or may represent another property of this protein. A detailed mutation analysis could delineate the domains of CP involved in its various functions. CP produced in transgenic plants at relatively low levels seems to interfere predominantly with uncoating of an invading virus. It could be that the function of CP in regulating the balance between plus- and minus-strand RNA synthesis is dose-dependent and is disturbed only at higher levels of CP such as are obtained in the Monsanto CP plants. The Leiden CP plants could be infected with AlMV RNAs 1, 2 and 3 but did not complement infection when the CP gene in RNA 3 in this inoculum was defective (Dore *et al.* 1991). Apparently, CP produced by these plants is able to carry out the early function of CP in genome activation but is not sufficient to perform one of the later functions of this proteins. On the other hand, the amount of the replicase subunits produced in P1, P2 and P12 plants was sufficient to permit wild-type levels of AlMV replication. Apparently, the time-course and stoichiometry of the synthesis of P1 and P2 during the replication cycle is not very critical. However, mutant replicase subunits produced in transgenic plants are probably useful tools to block virus multiplication.

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