Virology **295,** 307–319 (2002)
doi:10.1006/viro.2002.1332, available online at http://www.idealibrary.com on IDEAL®

Silencing of a Gene Encoding a Protein Component of the Oxygen-Evolving Complex of Photosystem II Enhances Virus Replication in Plants

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Received October 29, 2001; returned to author for revision January 2, 2002; accepted January 2, 2002

It has been suggested that, in addition to viral proteins, host proteins are involved in RNA virus replication. In this study the RNA helicase domain of the *Tobacco mosaic virus* (TMV) replicase proteins was used as bait in the yeast two-hybrid system to identify tobacco proteins with a putative role in TMV replication. Two host proteins were characterized. One protein (designated #3) belongs to a protein family of ATPases associated with various activities (AAA), while the second host protein (designated #13) is the 33K subunit of the oxygen-evolving complex of photosystem II. Using *Tobacco rattle virus* vectors, genes #3 and #13 were silenced in *Nicotiana benthamiana*, after which the plants were challenged by TMV infection. Silencing of gene #13 resulted in a 10-fold increase of TMV accumulation, whereas silencing of gene #3 caused a twofold reduction of TMV accumulation. Additionally, silencing of genes #3 and #13 decreased and increased, respectively, the accumulation of two other viruses. Similar to silencing of gene #13, inhibition of photosystem II by application of an herbicide increased TMV accumulation several fold. Infection of *N. benthamiana* with TMV resulted in a decrease of #13 mRNA levels. Silencing of gene #13 may reflect a novel strategy of TMV to suppress basal host defense mechanisms. The two-hybrid screenings did not identify tobacco proteins involved in helicase domain-induced *N*-mediated resistance.

Key Words: plant-virus interaction; helicase; hypersensitive response; virus-induced gene silencing; VIGS; plant virus replication; chloroplast; two-hybrid system; oxygen-evolving complex; AAA-protein.

INTRODUCTION

RNA viruses depend on RNA replicase enzymes for the replication of their genomes in the host. Plus-strand RNA viruses, including most plant viruses, encode catalytic subunits of the RNA-dependent RNA polymerase (RdRp) necessary for replication in their genomes. There is accumulating evidence that host factors are also involved in the viral RNA replication process. Usually, the replication complexes are associated with membranes, and also host proteins, often factors normally involved in cellular translation, have been identified in purified or enriched RdRp preparations (Lai, 1998). Results of studies on the plus-strand plant RNA virus *Brome mosaic virus* indicated that for replication in yeast several yeast proteins are necessary, and recently, a putative mRNA decapping yeast protein was shown to be required for template selection (Strauss and Strauss, 1999; Diez *et al.,* 2000).

Genetic and biochemical evidence has shown that the *Tobacco mosaic virus* (TMV) 126K and 183K proteins are subunits of the viral replicase. The 126K protein contains domains with similarity to methylases involved in capping of mRNAs, and RNA helicases involved in unwinding of double-stranded regions. The 183K protein arises after read-through of the 126K stop codon and in its unique C-terminal part harbors conserved motifs found in all RNA-dependent RNA polymerases. The region is involved in the formation of the viral replication complex, as it mediates an interaction between the TMV 126K and 183K proteins. Moreover, recently it was found that a chimeric TMV containing a helicase domain from another TMV strain could not spread through the plant, but was capable of infecting single cells or protoplasts (Hirashima and Watanabe, 2001). This suggests that the 126K helicase region is also involved in cell-to-cell movement of the virus.

In addition to its functions in the viral life cycle, the 126K protein of most tobamoviruses has a role in gene-for-gene resistance in plants containing the TMV resistance gene *N*. Gene-for-gene resistance during a plant–pathogen interaction is dependent on the presence of both a resistance gene of the host and a corresponding avirulence gene of the pathogen (Flor, 1971). The resistance reaction usually involves a hypersensitive response (HR), characterized by necrotic lesions at the infection site, and prevents systemic infection. In addition, the plants develop systemic acquired resistance

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0042-6822/02 $35.00

TABLE 1

Interaction of Identified Tobacco Clones with HEL:U1 and MOREHEL:U1 Baits in Yeast

Prey	Bait			
Positive clones	pBCP	HEL:U1	MOREHEL:U1	MOREHEL:Ob
#3—AAA ATPase	−	++/−[a]	+++[b]	−
#13—33K subunit O_2-evolving complex	−	−	+++[b]	−
H#1—14-3-3	nd[c]	+	−	nd
H#1.3, 1.6, 23, 28, 33—Rieske FeS	−	+/−[a]	−	nd
H#13, 17—P-protein	−	+	−	nd
H#14—phenylalanine ammonia lyase	−	+	−	nd
H#16—pyruvate kinase	−	+	−	nd
H#19—no homology	−	+/−	−	nd
H#20, 30—elicitor responsive protein	−	+	−	nd
H#21—threonine synthase	−	+	−	nd

[a] The interaction is dependent on the yeast strain; PJ69-4A gives good growth on quadruple drop-out medium, while the β-galactosidase assay using Y153 is negative.

[b] +++ indicates that the interaction resulted in an intense blue staining that appeared 1 h postincubation; ++ and +, reduced blue staining of colonies; −, no blue staining.

[c] nd, not determined.

(SAR) effective against a broad spectrum of related and unrelated pathogens (reviewed in Sticher *et al.,* 1997). Tobamoviruses elicit HR and SAR responses in *NN* tobacco plants (Holmes, 1929, 1934). Therefore, the TMV–*N* interaction is studied as a model system for gene-for-gene resistance. The HR elicitor of TMV-U1 was recently shown to reside in the RNA helicase domain of the TMV replicase proteins (Padgett *et al.,* 1997; Abbink *et al.,* 1998; Erickson *et al.,* 1999). We showed that the elicitor function resides in the 311 carboxyl-terminal amino acids of the TMV 126K replicase protein, a domain we designated HEL:U1. Its elicitor capacity was however greatly enhanced when the domain was extended at the amino-terminus to 436 amino acids, which was therefore named MOREHEL:U1.

In this study, the yeast two-hybrid system was employed to identify tobacco proteins interacting with the helicase domain of the TMV replicase proteins. Based on the role of the TMV replicase proteins in the two processes, the resulting helicase-interacting host proteins could participate in either virus replication, in gene-for-gene resistance, or both.

RESULTS

Library screenings with the TMV HEL:U1 and MOREHEL:U1 baits

To identify tobacco host proteins that interact with the TMV replicase helicase domain, we used the yeast two-hybrid system originally described by Fields and Song (1989). This assay has proven to be a powerful tool to detect protein–protein interactions. Both HEL:U1 and MOREHEL:U1 domains were fused in-frame with the GAL4 DNA-binding domain. The constructs did not transactivate the ADE and HIS reporter genes in the PJ69-4A strain by themselves. Therefore, we were able to screen a Samsun NN cDNA library that was fused to the GAL4 activation domain. Initial screenings were performed at 30°C. However, the induction of *N*-mediated HR by HEL:U1 and MOREHEL:U1 is temperature sensitive (ts) and does not occur at temperatures above 28°C (Samuel, 1931). In addition, recent findings suggest that this ts defect resides in the HR elicitor activity (Padgett *et al.*, 1997). Therefore, a second screening was performed with the MOREHEL:U1 bait at 25°C. However, none of the identified interactors in this study gave a ts interaction with either HEL:U1 or MOREHEL:U1.

The Samsun NN cDNAs that were positive in these screenings were isolated and retransformed to yeast strains PJ69-4A and Y153 to confirm that the interactions were reproducible, independent of the yeast strain used and specific for the HEL:U1 or MOREHEL:U1 bait. Specificity was tested with pBCP, a bait vector that expresses the *Alfalfa mosaic virus* (AMV) coat protein (CP) fused to the GAL4 DNA-binding domain (Tenllado and Bol, 2000). None of the positive clones was able to interact with the AMV CP or transactivate the ADE, HIS, or β-galactosidase genes in the absence of the HEL:U1 or MOREHEL:U1 bait (Table 1). Screening with the HEL:U1 bait yielded 14 true-positive clones. Most of the p-BLAST searches revealed homology to protein sequences in the GenBank database. One clone (H#1) contained an insert with high similarity to a *Nicotiana tabacum* cDNA encoding the 14-3-3 isoform b T14-3b protein (gb:U91723). Five independent clones (H#1.3, 1.6, 23, 28, 33) contained partial sequences of the *N. tabacum* Rieske FeS protein (gb:X66009), which is part of the cytochrome *b6f* complex in chloroplasts. These clones appeared to have inserts

fused to the GAL4 activation domain at nucleotide positions 205, 243, 380, 303, and 350 of the Rieske cDNA. Two independent clones (H#13 and H#17) contained partial cDNAs similar to the *Solanum tuberosum* P cDNA (gb:Z99770) with fusions at nt positions 2364 and 1629 of the potato P cDNA. One clone (H#14) corresponded to a partial *N. tabacum* phenylalanine ammonia-lyase cDNA (gb:X78269) fused at nt position 1607. Two clones (H#20 and H#30) were identical and encoded a novel "elicitor responsive" protein from *N. tabacum* (gb:AB040409). Neither the nature of the elicitor nor the function of the protein was listed in the database description. The inserts of the H#20 and H#30 clones contained the entire ORF plus 36 nucleotides of the 5′-noncoding region. In addition, the encoded proteins showed a low homology to a senescence-upregulated mRNA encoding a hypothetical protein (SENU-1; emb:CAA99759.1) from *Lycopersicon esculentum*. Two clones (H#16, H#21) showed a low similarity with an *Arabidopsis thaliana* S-adenosylmethionine-sensitive threonine synthase precursor (gb:AB027151) and a *Ricinus communis* pyruvate kinase cDNA (gb:M64737). These clones however may directly or indirectly influence amino acid metabolism and their interaction with the HEL bait should therefore be interpreted with caution. However, it must be emphasized that these H#16 and H#21 clones did not give a positive interaction with the pBCP clone (Table 1) and did not transactivate the ADE and HIS reporter genes by themselves. One clone (H#19) did not show homology to any sequence in the GenBank database.

Screening with the MOREHEL:U1 bait resulted in 13 true-positive clones which encoded only two different sequences. BLAST analysis of a representative of the first group (which from this point on is designated clone #3) showed the presence of a highly conserved AAA family motif (AAA indicates ATPases associated with various cellular activities). Figure 1 shows the sequence of clone #3 and its encoded protein with the AAA homology domain underlined (GenBank Submission No. AF426837). It should be noted that the Walker A (GxxxxGKS/T, in which x indicates any amino acid) and B (four hydrophobic residues followed by D/E) motifs that are present in AAA motifs are also present in other unrelated ATPases (Walker *et al.,* 1982). However, AAA family members share additional homology in a part of the remaining protein sequence that is approximately 230 amino acids long (Beyer, 1997). This sequence is also known as the AAA module. The second MOREHEL:U1 interacting clone (designated clone #13) proved to be a partial cDNA encoding the 33K subunit of the water-oxidizing, oxygen-evolving complex (OEC) of photosystem II (gb:X64349), which was fused to the GAL4 activation domain at nt position 703 of the database mRNA sequence. This protein is encoded by a nuclear gene (*psbO*) and is transported to the chloroplast, where it resides in the thylakoid membranes.

Since both MOREHEL:U1 and HEL:U1 proteins are able to induce a HR in NN tobacco, we determined if the HEL:U1 interactors were able to interact with the MOREHEL:U1 bait and vice versa. The results are shown in Table 1. To our surprise none of the HEL:U1 interactors was capable of interacting with the MOREHEL:U1 bait, although the HEL:U1 sequence is completely identical to the carboxyl-terminus of MOREHEL:U1. Apparently, the overall structure of the HEL:U1 bait is different from the MOREHEL:U1 bait. The interaction of HEL:U1 with clone #3 could only be detected in the PJ69-4A strain and not in the Y153 strain. Currently, it is unclear why the HEL:U1-#3 interaction differs in the two strains. No interaction between #13 and HEL:U1 was observed.

Since the MOREHEL:U1 elicitor functions more rapidly and severely in establishing *N*-mediated necrosis, we focused on the MOREHEL:U1 interactors. First, the #3 and #13 cDNA inserts were recloned into the bait vector pAS2-1 and were tested in the yeast two-hybrid system with the MOREHEL:U1 sequence in the prey vector pACTII. In this assay only protein #13 was still able to interact with the MOREHEL:U1 sequence, although the level of blue staining in the β-galactosidase assay was less than in the reverse combination (data not shown). The #3 sequence did not interact with MOREHEL:U1 in this set up. On the other hand, the #3 protein was able to form dimers/multimers in the yeast two-hybrid system, a feature that has been described for AAA proteins (Confalonieri and Duguet, 1995).

Next, we tested if the MOREHEL:U1 interactors were able to interact with the equivalent MOREHEL:Ob sequence. The TMV:Ob strain does not induce an HR in *NN* tobacco and the MOREHEL:Ob domain is not able to trigger necrosis in *NN* tobacco plants (Fig. 2). Both MOREHEL sequences are 80% similar (64% identical), suggesting that the MOREHEL:Ob contains mutations that circumvent recognition in the *NN* tobacco host during virus infection. Interestingly, the MOREHEL:U1 interactors #3 and #13 were not able to interact with the MOREHEL:Ob bait (Table 1). Since both MOREHEL domains likely provide the same function in virus replication, i.e., an RNA helicase function, this observation suggests that the interacting proteins do not fulfill a role in this process. Rather, this observation suggests that the protein–protein interactions play a role in the elicitor recognition process required for *N*-mediated HR induction. To further investigate the correlation between the interactions and HR induction, we analyzed the interaction of proteins #3 and #13 with chimeras of U1 and Ob MOREHEL domains, depicted in Fig. 2. These chimeras were recently analyzed for their HR-inducing capacity (Abbink *et al.,* 2001). From these experiments it became clear that interaction with #3 or #13 does not correlate with HR induction (Fig. 2). Chimera 3 (ABCdefgh) is able to interact with both host proteins, yet it does not induce an HR. Chimeras 15, 16, and 17 (abCDEFGH, ABcDEFGH,

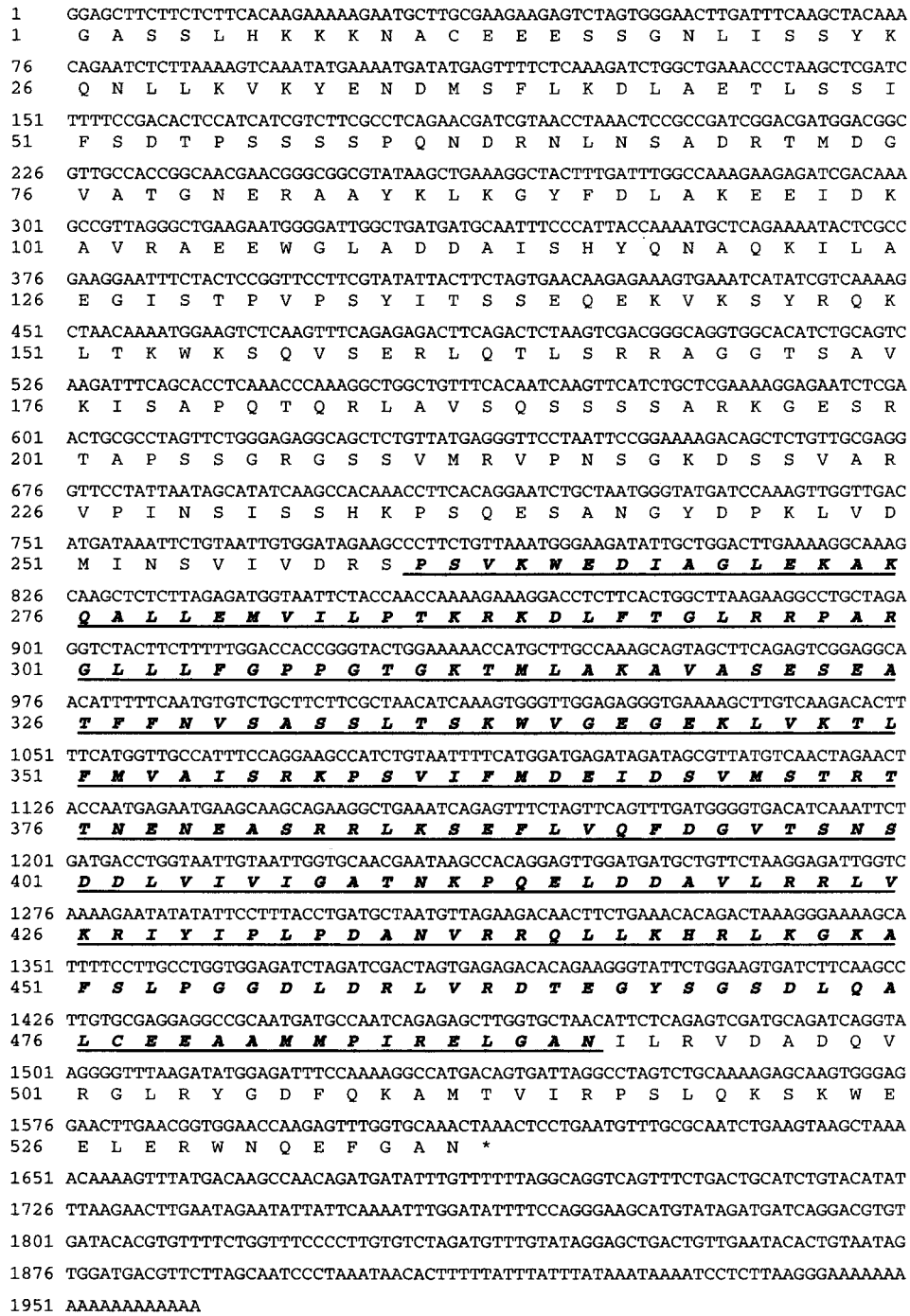


FIG. 1. Nucleotide and amino acid sequences of the insert of the MOREHEL-interacting pACT clone #3 (gb:AF426837). The deduced amino acid sequence of the ORF is depicted underneath the cDNA. Nt 1 indicates the first nucleotide of the #3 cDNA downstream of the linker sequence that was used to construct the pACT-cDNA library. The AAA cassette is underlined and in bold italics. The Walker A and B motifs in the AAA cassette are formed by aa 306 through 312 and 361 through 365, respectively.

and AbcDEFGH, respectively) are not interacting with either host protein and still elicit *N* gene-dependent necrosis.

Levels of #13 mRNA in healthy and virus infected *NN* plants

Since the MOREHEL:U1 interaction with proteins #3 and #13 appeared not to correlate with HR induction, we set out to determine a possible role of the protein interactions during TMV infection. First we determined the expression levels of the endogenous #3 and #13 mRNAs in tobacco leaves. The #3 mRNA could only be detected by rt PCR and its expression level did not seem to be affected by TMV-U1 replication in *NN* or *nn* tobacco (data not shown). However, #13 mRNA levels were easily de-

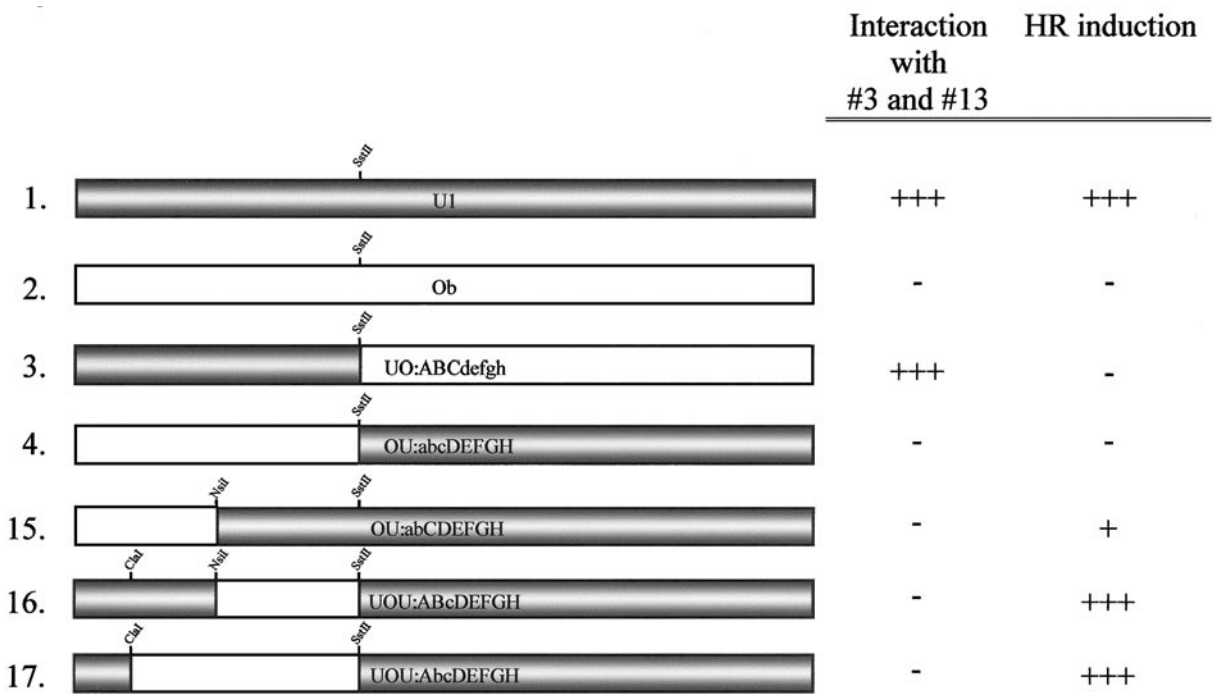


FIG. 2. Interactions of MOREHEL chimeras with host proteins #3 and #13. Interactions of the chimeric MOREHEL proteins with proteins #3 and #13 were analyzed in the yeast two-hybrid system using yeast strains PJ69-4A and Y153. Interaction of MOREHEL:U1 with proteins #3 and #13 was set at "+++"; minus sign: no growth of yeast colonies on selective medium. In the last column the capacity of the chimeras to induce an *N* gene-dependent HR is indicated. MOREHEL:U1 elicitor activity was set at +++. "−" indicates that the leaves did not induce necrosis or leaf yellowing. "+" indicates that a HR was only induced in cells at the underside of the infiltrated leaf tissue.

tected by Northern blot analysis. Since expression of #13 mRNA is under control of a circadian clock, we analyzed total RNA samples that were harvested at the same time point during the day (11:00 AM). It appeared that TMV-U1 infection resulted in a decrease of #13 mRNA in *NN* tobacco, while infection by AMV did not affect #13 mRNA levels (Fig. 3). In addition, the mRNA levels of the nuclear-encoded chloroplast proteins Rieske and the small subunit of Rubisco decreased upon TMV infection in *NN* tobacco (Fig. 3).

Silencing of genes #3 and #13 affects TMV accumulation

To determine roles for proteins #3 and #13 during TMV infection and the *N*-mediated resistance, we silenced the endogenous *#3* and *#13* genes. For this purpose, we used the recently described virus-induced gene silencing (VIGS) method (Ruiz *et al.,* 1998). Virus-induced gene silencing has been shown to be an antiviral response in plants (Ratcliff *et al.,* 1997; Baulcombe, 1999). Upon infection viral genes are silenced by specific breakdown of the viral RNA and consequently, the virus infection is halted. This specific RNA breakdown response can be used to silence endogenous genes by inserting parts of their sequences into the viral genome (Ruiz *et al.,* 1998; Burton *et al.,* 2000; Ratcliff *et al.,* 2001). Thus, partial sequences of the #3 and #13 cDNA were cloned into the cDNA of the *Tobacco rattle virus* (TRV) RNA2. Infection of TRV is known to induce the VIGS response in *Nicotiana benthamiana* plants. As a positive control a part of the *N*

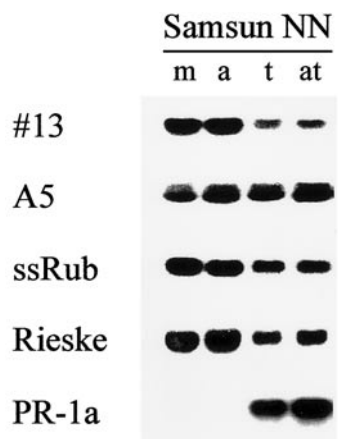


FIG. 3. Effect of virus infection on mRNA levels of chloroplast proteins in Samsun *NN* tobacco plants. Samsun *NN* plants were mock infected (m) or infected with AMV (a), TMV-U1 (t), or coinfected with AMV and TMV (at). Six days after infection total RNA was isolated and subjected to Northern blot analysis. Identical blots were probed for #13, A5, small subunit of rubisco (ssRub), Rieske, or PR-1a mRNA. A5 is a constitutively expressed tobacco gene whose cDNA was identified by yeast two-hybrid screens with the AMV helicase domain (M. van der Heijden and J. F. Bol, unpublished result). A5 is therefore used as control for equal RNA loading of the agarose gel.

gene was inserted into TRV cDNA2 (J. R. Peart and D. C. Baulcombe, unpublished observations), while the empty vector that expressed a noninserted TRV cDNA2 was used as a negative control. *N. benthamiana* plants that are transgenic for the *N* gene were infected with TRV cDNAs 1 and 2 containing the different inserts. It should be pointed out that these *N* transgenic plants do not elicit an HR that confines wild-type TMV-U1 infection to the vicinity of the necrotic lesions. TMV-U1 infection therefore results in a systemic necrosis that follows the systemically spreading virus. To study *N*-mediated resistance, we used a recombinant TMV expressing the green fluorescent protein (GFP). This TMV-GFP showed reduced accumulation when compared to wild-type TMV-U1 and was confined to the site of inoculation in *NN*-transgenic *N. benthamiana*, although necrotic lesions were not commonly formed.

Figure 4 shows the results of the silencing experiments. Silencing of #13 was confirmed by Northern blot analysis (Fig. 4A). Usually, VIGS is not fully effective in all cells of the leaf and frequently tissue areas remain nonsilenced. Indeed, we found #13 mRNA accumulation in silenced leaf reduced, but never absent. However, phenotypically, #13 silencing was evident. Figure 4B shows the symptoms induced by the various TRV vectors in *NN*-transgenic *N. benthamiana*; similar symptoms were observed in nontransgenic *N. benthamiana*. Silencing of *#13* induced significant chlorosis resulting in light green leaves. Silencing of *#3* or *N* did not induce an obvious phenotype when compared to plants infected with the empty vector (TRV-e). Silencing of *N* in *NN*-transgenic *N. benthamiana* increased the number and size of TMV-GFP foci (J. R. Peart *et al.,* unpublished observations) and Northern blot analysis revealed an approximately 10-fold increase in TMV accumulation in the *N*-silenced leaves (Fig. 4D). Eventually, *N*-silenced plants became systemically infected with TMV. Similar to the silencing of *N*, silencing of *#13* in *NN*-transgenic *N. benthamiana* resulted in a significant increase in the number and size of TMV-GFP foci (result not shown). The apparent increase in virus accumulation was confirmed by Northern blot analysis showing TMV RNA levels resembling those after silencing of *N* (Fig. 4D). However, *#13*-silenced plants did not become systemically infected. In contrast to silencing of *N* and #13, silencing of *#3* in *NN*-transgenic *N. benthamiana* resulted in an approximately twofold reduction of TMV accumulation (Fig. 4D).

Next, we investigated the effect of silencing of *#3* and *#13* in wild-type *N. benthamiana* plants that do not express the N protein. TMV replication was analyzed as before and the results are shown in Figs. 4C and 4E. Clearly, silencing of *#13* greatly enhanced the infection of TMV-GFP as early as 3 days postinoculation. GFP foci were larger in size and number (Fig. 4C) and accumulation of TMV RNA was increased about 10-fold (Fig. 4E). Silencing of *#3* resulted in smaller infection foci (Fig. 4C) and a twofold reduction in the accumulation of TMV RNA, relative to the mock-silenced plant. This twofold reduction was reproducibly observed in three independent experiments. It appeared that the decrease in size rather than in the number of GFP foci is responsible for the decrease in TMV RNA levels. Apparently, #3 and #13 proteins affect TMV accumulation independent of the presence of the *N* gene.

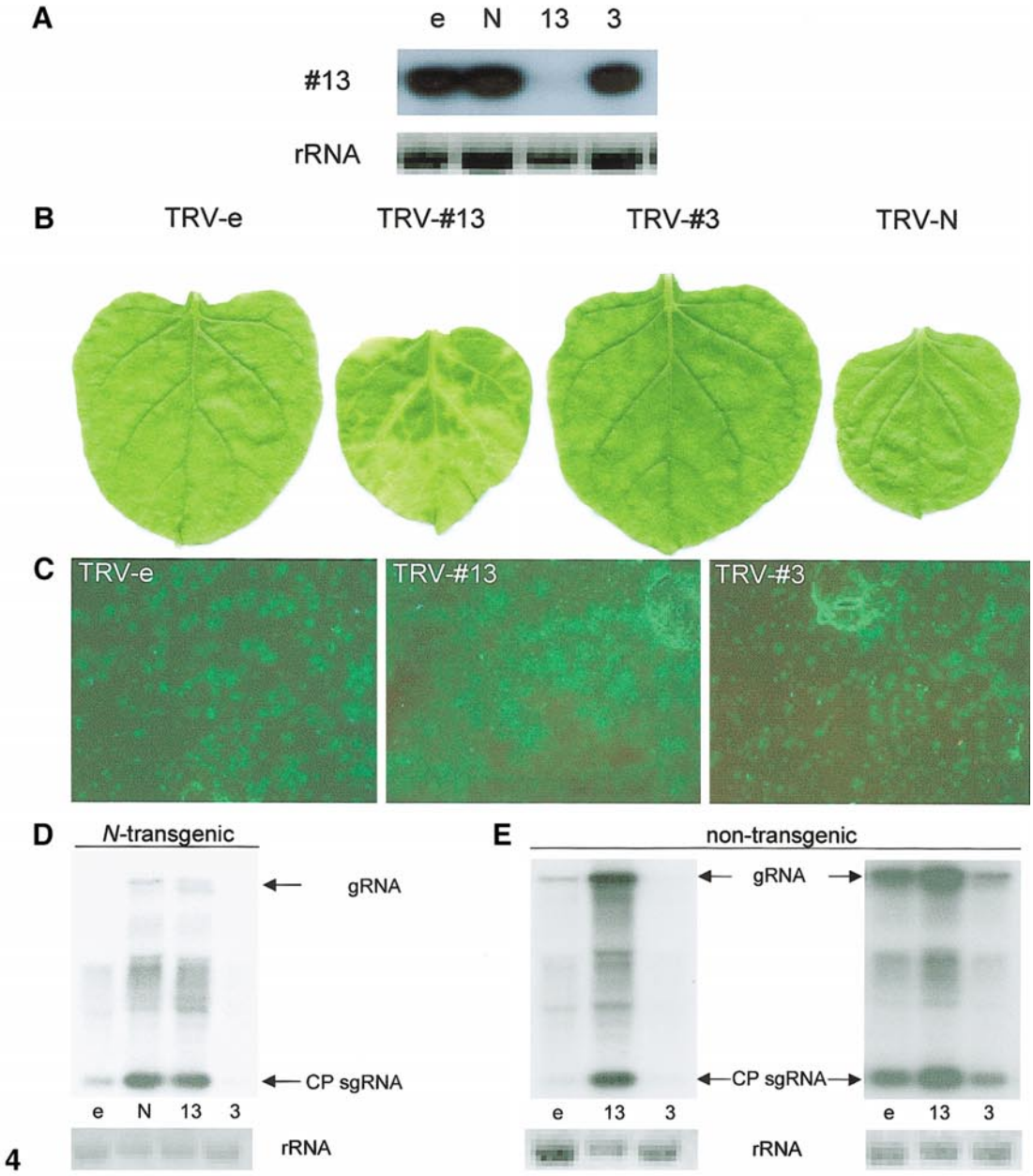
To determine whether these effects are specific for TMV, we analyzed the infection of leaves with AMV and *Potato virus X* (PVX) after silencing of *#3* and *#13*. When inoculation was done with recombinant PVX-GFP, no fluorescent foci were observed on #3-silenced leaves, whereas #13-silenced leaves contained foci that were significantly larger than those on leaves silenced with the empty vector (result not shown). The Northern blot analysis shown in Fig. 5 revealed that silencing of #13 resulted in a several-fold increase in the accumulation of PVX-GFP and AMV RNAs. On the other hand, silencing of #3 resulted in a decrease in the accumulation of the two viruses. Apparently, the #13 and #3 proteins affect the infection cycle of viruses from at least three different genera.

Effect of DCMU treatment on TMV accumulation

Clone #13 encodes the 33K subunit of the OEC of photosystem II. The activity of this complex converts water into protons, electrons, and oxygen and can be inhibited by the herbicide DCMU. To check if the reduced activity of photosystem II resulting from *#13* silencing is the cause for the increase in TMV accumulation in plants, we infiltrated Samsun *NN* and nn plants with DCMU and subsequently infected them with TMV-U1. Treatment of *NN* and nn tobacco plants with this compound resulted in mild chlorosis (Fig. 6B). Upon subsequent TMV-U1 infection, the *N*-mediated HR was triggered in *NN* plants. However, the number of necrotic lesions was increased and the lesions appeared several hours earlier (compare Figs. 6A and 6C). Later in infection, the lesions formed one confluent necrotic patch. Also, in nn plants the accumulation of virus is enhanced, since DCMU-treated plant material contains four to five times more infectious TMV than mock-treated plant material upon TMV infection (Fig. 6D). Eventually, the combination of DCMU treatment and TMV infection results in necrosis in nn plants as well, suggesting that the leaf succumbs under the high viral load or under the combination of different stress factors: inactivation of photosystem II and TMV infection. In summary, these observations support the conclusions from the data obtained with silencing of *#13*.

DISCUSSION

With their limited genome, plus-strand RNA viruses rely on a number of host proteins to assist in processes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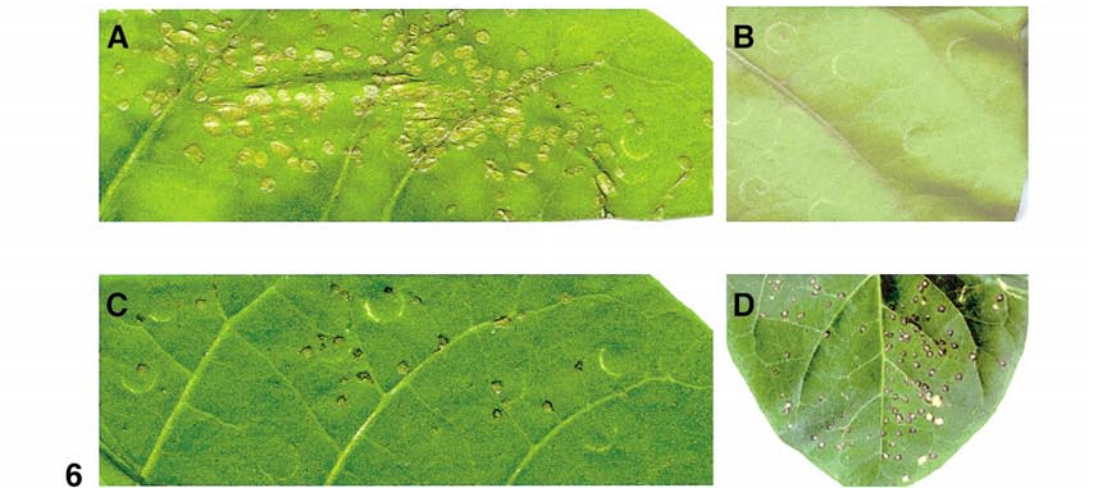
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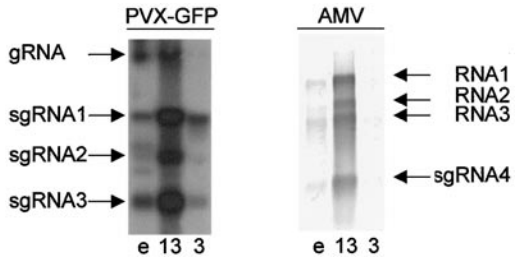

FIG. 5. PVX and AMV replication in VIG-silenced *N. benthamiana*. Nontransgenic *N. benthamiana* plants were silenced for *#3* or *#13* or were mock-silenced (e) by recombinant TRV vectors. Silenced leaves were inoculated with recombinant PVX-GFP or AMV. At 3 d.p.i., accumulation of PVX-GFP and AMV RNA was analyzed by Northern blot hybridization. Genomic and subgenomic RNAs are indicated.

involved in their multiplication. With the current experiments we have attempted to characterize host proteins with affinity for the replicase proteins of TMV. We have aimed at the helicase domain of the 126K protein since this region not only plays a role in viral RNA replication, but is also involved in cell-to-cell transport of the virus in the infected plant (Hirashima and Watanabe, 2001) and functions as avirulence factor (Avr) triggering the resistance response in plants harboring the TMV resistance gene *N* (Abbink *et al.,* 1998, 2001; Erickson *et al.,* 1999).

Apart from a few cases in which interactions between resistance and avirulence gene products were indeed detected (Jia *et al.,* 2000; Scofield *et al.,* 1996; Tang *et al.,* 1996), many research groups have been unsuccessful in establishing such direct interactions. Also, our attempts to find a positive interaction between the tobacco N protein and the TMV MOREHEL in the yeast two-hybrid system failed (data not shown). Although there may be technical difficulties due to the size of the R proteins, there are also indications that additional host proteins are involved in the R–Avr protein interaction (Kooman-Gersmann *et al.,* 1996; Leister and Katagiri, 2000).

The yeast two-hybrid system is widely used for identification of protein–protein interactions and we used this system to screen an *NN* tobacco cDNA library with a smaller and larger part of the 126K protein of TMV strain U1 containing the helicase region (HEL:U1 and MOREHEL:U1, respectively) as bait. Although both screenings provided interesting proteins to study in more detail, we focused on the MOREHEL:U1 interactors since this elicitor induces the strongest *N*-dependent HR. Two positive clones were identified that interacted specifically and strongly with MOREHEL:U1 in the yeast two-hybrid system and the possible role of these proteins in *N*-mediated resistance and TMV replication was investigated.

The first MOREHEL:U1 interactor, clone #3, was shown to contain an ATPase motif characteristic for AAA proteins. These ATPases are involved in very divergent cellular processes ranging from peroxisome biogenesis to ^{26}S-proteasome functioning and cell-cycle regulation (reviewed in Confalonieri and Duguet, 1995; Beyer, 1997). The AAA motif is approximately 230 amino acids long and can be divided into 10 subdomains, of which the amino-terminal subdomain A is least conserved among different AAA family members (Beyer, 1997). Subdomains D and F contain the Walker A and B motifs that are also typical for other ATPases (Walker *et al.,* 1982). Biochemical data on AAA ATPase activity show that the specific activity is generally low when compared to other ATPases. Affinity values for ATP vary largely among different AAA proteins (Confalonieri and Duguet, 1995). AAA proteins have been shown to form oligomeric ring structures and indeed #3 proteins interact and form dimers or multimers in the yeast two-hybrid system. Protein #3 has a strong homology in subdomains B through K with AAA proteins that belong to subfamily 7 (SF7), which was recently extended by the human and mouse spastin proteins (Beyer, 1997; Hazan *et al.,* 1999). While other subfamilies contain AAA members with similar or identical functions, SF7 members do not have a common function. However, the SF7 AAA protein Sap1p is a yeast protein located in the nucleus and the mouse and human spastin proteins contain a putative nuclear targeting signal (Liberzon *et al.,* 1996; Hazan *et al.,* 1999). Interestingly, protein #3 contains a stretch of positively charged amino acids (HKKK at positions 6 through 9), which may be part of a nuclear targeting signal. Outside the AAA cassette, the homology with other proteins is

FIG. 4. TMV replication in VIG-silenced *N. benthamiana*. *NN*-transgenic or nontransgenic *N. benthamiana* plants were silenced for *#13*, *#3*, or *N* or were mock-silenced (e). (A) Northern blot showing #13 mRNA accumulation in VIG-silenced leaves of *NN*-transgenic *N. benthamiana* (the TRV silencing vectors are indicated). (B) Phenotype of the silenced leaves. (C) Silenced leaves of nontransgenic *N. benthamiana* were infected with recombinant TMV-GFP and GFP foci were visualized by fluorescence microscopy. (D) Northern blot analysis of TMV infection in the inoculated leaf of silenced *NN*-transgenic *N. benthamiana* 6 days after inoculation (d.p.i.) with TMV-GFP (the position of genomic RNA and subgenomic CP mRNA is indicated). (E) Similar Northern blots showing TMV RNAs at 3 (left) and 5 (right) d.p.i. in nontransgenic, silenced *N. benthamiana*. The panels below the autoradiographs of A, D, and E show the ethidium bromide stained ^{28}S rRNA band after electrophoresis.

FIG. 6. Effect of DCMU treatment on TMV infection. *N. tabacum* cv. Samsun *NN* plants were infiltrated with 10 μM DCMU in 0.96% ethanol (A, B). Infiltration with 0.96% ethanol was performed as a negative control (C). Three days after infiltration leaves in A and C were inoculated with TMV-U1 and photographed at 4 d.p.i. The mock-inoculated, DCMU-treated leaf in B was photographed 7 days after infiltration (B). DCMU-treated and control-treated nn tobacco plants were infected with TMV-U1. Two days postinoculation equal amounts of infected plant material were homogenized and used to inoculate opposing leaf halves of *NN* plants. Lesions were counted 3 d.p.i. (D). The left leaf half shows the lesions induced by inoculum from the control-treated leaf material, while the right leaf half shows the lesions induced by inoculum from the DCMU-treated leaf material.

very low. The highest similarity is found with two hypothetical AAA proteins from *A. thaliana* and *Oryza sativa* (gb:T00863 and BAA88371, respectively). A common function for all AAA members has been suggested: ATP-dependent anchoring of protein (or other macromolecular) structures (Confalonieri and Duguet, 1995; Beyer, 1997). However, these observations do not allow us to propose a function for clone #3 in tobacco plants.

The second MOREHEL:U1 interactor, clone #13, proved to be identical to a partial cDNA of the tobacco 33K subunit of the OEC of photosystem II. This complex is located in chloroplasts and catalyzes part of the so-called "light reaction." In the presence of light, the complex forms oxygen out of water and produces protons and high-energy electrons that are used to establish a proton gradient across the chloroplast thylakoid membrane. This proton gradient is subsequently used by the ATP-synthase to synthesize ATP. The OEC consists of 33K, 23K, and 16K protein subunits. While the 23K and 16K subunits are thought to regulate the OEC activity, the 33K subunit is absolutely required for OEC activity by stabilizing the tetranuclear manganese center (Jain *et al.,* 1998 and references therein). Although replication of some plant viruses is associated with chloroplasts, the replication of TMV RNA is believed to occur in structures derived from the endoplasmic reticulum (Reichel and Beachy, 1998). However, #13 and TMV replicase proteins are both synthesized in the cytoplasm where they could interact.

The significance of protein–protein interactions observed in the yeast two-hybrid system needs confirmation by other experimental approaches. To study interactions *in vitro*, we have expressed GST fusions of HEL, MOREHEL, #3, and #13 proteins in *Escherichia coli* and by translation in the rabbit reticulocyte lysate. Due to insolubility of the proteins produced in *E. coli* and aspecific precipitation of *in vitro* translation products by glutathione beads, GST pull-down assays were not feasible. However, the observation that #3 and #13 did not interact in yeast with the MOREHEL domain of the tobamovirus Ob prompted a further characterization of these proteins. MOREHEL:Ob does not induce the *N*-mediated HR in tobacco (Abbink *et al.,* 2001), suggesting that the elicitor function of the TMV-U1 protein is correlated with its binding to these two host proteins. A possible role of this binding in the *N*-mediated resistance response was analyzed by two approaches. First, genes #3 and #13 were silenced in *NN*-transgenic *N. benthamiana*. Silencing of the *N* transgene or gene #13 both resulted in a 10-fold increase in TMV accumulation. However, the observation that silencing of #13 in nontransgenic *N. benthamiana* resulted in a similar increase in TMV accumulation indicates that #13 is not a component of the *N*-mediated resistance response pathway. The second approach was to study a possible correlation between the binding of #3 and #13 to U1/Ob MOREHEL chimeras and the ability of these chimeras to induce the *N*-mediated HR. Again, no evidence supporting a role of #3 and #13 in the resistance response was obtained. However, the results with chimeras 3 and 4 in Fig. 2 demonstrate that sequences in the N-terminal 182 amino acids of MOREHEL are involved in the interaction with #3 and #13. Moreover, the results with chimeras 15, 16, and 17 indicate that similar sequences in this N-terminus interact with both #3 and #13.

Although proteins #3 and #13 may not be involved in the *N*-mediated resistance response, both host proteins clearly affect the accumulation of TMV and two unrelated viruses. Silencing of #3 with a TRV vector resulted in a consistent reduction of the accumulation of TMV, PVX, and AMV in the silenced leaves. This indicates that the AAA-type protein encoded by gene #3 has a positive role in the replication of a wide spectrum of plant RNA viruses. Conversely, silencing of #13 resulted in an up to 10-fold increase in the accumulation of TMV, PVX, and AMV in infected leaves. Recently, it was found that the TMV replicase proteins 126K and 183K interact via the helicase domain region (Goregaoker *et al.,* 2001). Although the inhibitory effect of #13 on TMV replication may be due to its interfering with this interaction, the effects of #13 silencing on AMV and PVX RNA accumulation suggest a more general role, in which the 33K subunit of the OEC is required for constituting a basal defense mechanism against viral infections. It cannot be excluded that in the absence of a functioning OEC a general effect on plant metabolism results in an enhanced susceptibility to viral infections. However, experiments with the herbicide DCMU showed that inhibition of the oxygen generation via photosystem II did not compromise HR induction and therefore, gene-for-gene resistance appears unaffected by inhibiting electron transport and oxygen generation via photosystem II. The HR was even accelerated, possibly because of the increased accumulation of TMV. The experiment with DCMU corroborates the conclusion that silencing of #13 results in an increase in TMV accumulation by (partially) blocking the OEC. Possibly, the OEC is involved in generation of active oxygen species that activate basal defense mechanisms after infection of the plant by viruses or other pathogens.

Because #3 mRNA is not detectable by Northern blot hybridization, the effect of TMV infection on levels of this mRNA was not analyzed in detail. However, TMV-U1 infection of *NN* tobacco resulted in a decrease in the levels of several chloroplast proteins including #13, the Rieske protein, and the small subunit of Rubisco (Fig. 3). This is not a general property of plant viruses, as AMV infection did not affect the level of these messengers. Interestingly, infection with TMV-Ob did not affect the mRNA levels of #13, Rieske, and Rubisco (result not shown). Protein #13 does not interact in yeast with the MOREHEL domain of Ob (Table 1) or the helicase do-

main of AMV (our unpublished result). Possibly, the interaction between the helicase domain of TMV-U1 with protein #13 (i.e., the OEC 33K subunit) plays a role in triggering the coordinate decrease in mRNA levels of a number of chloroplast proteins. Recently, it was reported that protein levels of the 23K and 16K subunits of the OEC were decreased upon infection with a tobamovirus (Rahoutei *et al.,* 2000). It has been suggested that the inhibition accounts for symptom development during viral infections. Indeed the TMV replicase proteins have been implicated in mosaic symptom formation (Bao *et al.,* 1996; Shintaku *et al.,* 1996). It has been proposed that inhibition of photosystem II is caused by the presence of the TMV CP in chloroplasts, although a correlation of chloroplast-localized CP levels with symptom development has not been found. TMV mutants that fail to express CP still induced the typical mosaic symptoms (Culver and Dawson, 1989). In addition, photosystem II appeared to be inhibited in inoculated leaves, which do not display mosaic symptoms. Therefore, the mechanism for the inhibition of photosystem II and symptom development has not been firmly established. One could propose a model in which the TMV-U1 replicase proteins sequester the 33K subunit of the OEC, resulting in the inhibition of the complex and photosystem II. In this way, the virus could optimize the infection conditions by suppressing basal defense mechanisms of the plant. Inhibition of photosystem II also promotes accumulation of other viruses such as AMV, but this virus is apparently unable to trigger a decrease of #13 mRNA levels itself. The mechanism that coordinates the reduction of #13 mRNA with mRNAs of other chloroplast proteins remains to be solved.

In summary, we have identified two tobacco host proteins that interact with the helicase region of the 126K TMV-U1 replicase protein. Although neither protein appears to be involved in HR induction, both proteins affect the infection cycle of TMV and several other viruses. Recently, avirulence proteins from bacterial pathogens were shown to stimulate pathogen growth on susceptible plants (Chang *et al.,* 2000; Shan *et al.*, 2000; White *et al.,* 2000). It has been suggested that resistance gene products recognize pathogen proteins that affect pathogenicity proteins in the plant (Van der Biezen and Jones, 1998). In this respect, the TMV replicase could be considered as a pathogenicity protein. To our knowledge this paper reports the first observation that inhibition of photosystem II enhances the levels of virus accumulation.

MATERIALS AND METHODS

Constructs

The HEL:U1 and MOREHEL:U1 cDNAs in pUC-HEL and pUC-MOREHEL (Abbink *et al.,* 1998) were cloned downstream of the GAL4 DNA-binding domain of the pAS2-1 vector (Clontech) as *Nco*I-*Sal*I and *Nco*I-*Pst*I fragments resulting in the pAS-HEL:U1 and pAS-MOREHEL:U1 constructs. The sequence of the fusions between the GAL4 DNA-binding domain and the helicase inserts was confirmed by DNA sequencing (Amersham Pharmacia Biotech). The HEL:U1 and MOREHEL:U1 cDNAs were also cloned into the *Nco*I and *Xho*I sites of pACTII (Li *et al.,* 1994). The resulting constructs were designated as pACT-HEL:U1 and pACT-MOREHEL:U1. The *Nco*I-*Xho*I inserts of pACT-#3 and pACT-#13, two clones that were identified in this study as MOREHEL:U1 interactors, were cloned into pAS2-1 to study the interaction with pACT-MOREHEL:U1.

A full-length open reading frame (ORF) of the 33K subunit of the oxygen-evolving complex of photosystem II was obtained by a standard reverse transcriptase (rt) PCR. Total RNA isolated from Samsun *NN* leaf was reverse transcribed by AMV reverse transcriptase using primer CCACCCCGTCCCTTTGGG (5′ → 3′). The resulting cDNA was amplified by PCR using the same primer and primer GGAACCATGGCTGCCTCTCTACAAGC (5′ → 3′). The PCR product was subsequently cloned into pACT-#13 as an *Nco*I-*Eco*RI fragment to create a full-length #13 cDNA fused to the GAL4 activation domain-encoding DNA region (pACT-#13fl).

The MOREHEL sequence of the non-HR-inducing TMV strain Ob was amplified in a standard PCR reaction with the full-length clone of TMV-Ob, pOb as template (Padgett *et al.*, 1997; kindly provided by Dr. Beachy, St. Louis, MO) using primers (5′ → 3′) GGGCCATGGTCGCAGCTGAATCAGTTATTAGTAACAAGATGGC and CCCCTGCAGTTACTATGCACTCACACTATCTACC. The MOREHEL:Ob sequence was subsequently sequenced and cloned into pAS2-1 downstream of the GAL4 DNA-binding domain. Chimeric MOREHEL ORFs were cloned into the bait vector pAS2-1 downstream of the GAL4 DNA-binding domain using the *Nco*I and *Pst*I restriction sites. These bait proteins were analyzed in the yeast two-hybrid system for their ability to interact with host proteins #3 and #13 expressed from the pACT-#3 and pACT-#13 plasmids. The MOREHEL chimeras have been cloned into binary vectors for analysis of their HR-inducing capacity upon expression by agroinfiltration (Abbink *et al.,* 2001).

All plasmids were propagated in *E. coli* DH5α. In addition, the binary constructs were introduced into *Agrobacterium tumefaciens* LB4404 for agroinfiltration studies.

The nucleotide sequence of clone #13 has been deposited with GenBank and assigned Accession No. AF426837.

Yeast two-hybrid assay

The two-hybrid assay in *Saccharomyces cerevisiae* (Fields and Song, 1989; Chien *et al.,* 1991) was used to

identify tobacco host proteins that interact with the HR elicitors HEL:U1 and MOREHEL:U1. A pACTII-based Samsun *NN* cDNA library was constructed in our laboratory by M. van der Heijden and was used for both screenings. The titer of the library was 0.6×10^6 independent clones. The PJ69-4A strain was first transformed with pAS-HEL:U1 or pAS-MOREHEL:U1 and subsequently with the cDNA library (James *et al.,* 1996; Agatep *et al.,* 1999). Screened with the HEL:U1 bait were 0.6×10^6 yeast transformants and 0.85×10^6 yeast transformants were screened with the MOREHEL:U1 bait. Initial screenings were performed at 30°C. With the MOREHEL:U1 bait an extra screening of 1.1×10^6 yeast transformants was performed at 25°C. Yeast transformants were plated on medium lacking leucine, tryptophan, adenine, and histidine. Positive clones were restreaked on the quadruple drop-out medium containing 20 mM 3AT to inhibit the endogenous PJ69-4A HIS3 protein. The library plasmids were isolated and electroporated to *E. coli* DH5α. Plasmids were tested again in PJ69-4A for their capacity to give the Ade+/His+ phenotype when transformed with the HEL:U1 or MOREHEL:U1 bait. In addition, the interaction was analyzed in Y153 and CG1945 strains using a β-galactosidase assay. For this assay, yeast cells were plated on Whatman filters, lysed by immersion in liquid nitrogen, and incubated on a second filter moistened with 5-bromo-4-chloro-3-indolyl-2-deoxy-β-D-glucosamide (X-gal) at 30°C for 8 h. Inserts of the positive clones were partially sequenced at their 5′ ends to confirm that the insert's ORF was in frame with the yeast GAL4 activation domain. The sequences were subsequently used in an advanced BLAST search of the GenBank database of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST).

Northern blot analysis

Total RNA was isolated and quantified as described (Brederode *et al.,* 1991). The quality of the RNA preparations was assessed by electrophoresis in agarose gel. Equal amounts were applied to formaldehyde-agarose gels. After blotting the gels to Hybond-N paper (Amersham Pharmacia Biotech), blots were hybridized with various probes as described (Memelink *et al.,* 1994). Probes were labeled with [^{32}P]dCTP using the NEBlot kit (New England Biolabs) according to the manufacturer's protocol. cDNA fragments were derived from pACT clones containing inserts corresponding to #13, A5, the small subunit of Rubisco, and Rieske. The PR-1a probe has been described (Brederode *et al.,* 1991). To determine the levels of TMV infection, the *Kpn*I-*Hin*dIII cDNA fragment of TMV-U1 containing the 3′-terminal sequence of the TMV genome was labeled. AMV RNA levels were determined as described (Neeleman and Bol, 1999). PVX-GFP specific probes were created by labeling the *Apa*I-*Xho*I cDNA fragment of the infectious PVX-GFP cDNA clone.

Virus-induced gene silencing of #*3* and #*13*

The *Sal*I-*Bgl*II fragment of pACT-#3 (nt 497-1368) was cloned into the pTV00 cDNA2 clone of tobacco rattle virus. The resulting construct was designated pTRV-#3. Also, the *Eco*RI-*Bgl*II fragment of pACT-#13 (nt 704-1123) was inserted into pTV00, resulting in pTRV-#13. The pTV00 clone contains the minimal sequences required for coreplication with TRV RNA1 and does not contain subgenomic promoter sequences. Therefore, the TRV coat protein is the only protein expressed from RNA2, while the #3 and #13 sequences are not translated from this silencing vector. As a positive control, we used a pTV00 derivative that contains a partial *N* gene sequence and was hence designated pTRV-N. As a negative control empty vector pTV00 was used, which was indicated by pTRV-e. *A. tumefaciens* strain GV was transformed with the independent pTV00 derivatives. Transformed cultures were grown and coinfiltrated with a culture of *A. tumefaciens* transformed with an infectious clone of TRV RNA1 in a ratio of 10 to 1. The infiltrated *N. benthamiana* plants were either wild-type or transgenic for the *N* gene. Infiltration was done with four-week-old plants that contained four true leaves. Three weeks postinfiltration the fifth, sixth, and seventh leaves had become systemically infected with the recombinant TRVs and had been silenced for the endogenous *#3*, *#13*, or *N* genes. These so-called silenced leaves were infected with AMV, recombinant TMV, or PVX expressing GFP from an additional subgenomic promoter. The level of virus accumulation was measured in at least three independent experiments by visualization of GFP and Northern blot analysis at several time points. Northern blots showing the levels of viral RNAs in one representative experiment have been depicted in the figures. Silencing of *#13* was determined by Northern blot analysis of #13 mRNA levels, while silencing of *#3* was determined by rt-PCR. TMV-GFP was inoculated by two methods. For visual assessments of TMV-GFP accumulation, manual inoculation of TMV-GFP sap using a light dusting of Carborundum was performed and accumulation monitored under UV light. For RNA gel blot analysis TMV-GFP was infiltrated at a 50-fold dilution from an $OD_{600} = 1$ (J. R. Peart *et al.,* unpublished observations). After spread of the TMV-GFP virus, systemically infected leaves were ground in a blender and infectious leaf sap was stored at −80°C as virus stock. PVX-GFP infectious leaf material was obtained and stored similarly.

DCMU treatment

(3-(3,4-Dichlorophenyl)-1,1-dimethylurea) (DCMU) is a specific inhibitor of electron transport and photosystem II resulting in inhibition of oxygen generation by the OEC.

DCMU was infiltrated into leaf halves of *N. tabacum* cv. Samsun *NN* or nn plants. The concentrations used were 10 and 100 μM. Both concentrations resulted in leaf chlorosis that was not observed in mock-infiltrated leaf halves. One or three days after infiltration plants were inoculated with TMV-U1 as previously described (Knoester *et al.,* 1998). TMV replication was analyzed by the appearance of necrotic lesions in *NN* plants. In nn plants TMV levels were determined by grinding eight leaf discs into 1 ml of phosphate buffer. Fifty microliters of this leaf sap was used to infect leaf halves of *NN* tobacco plants and necrotic lesions were counted to determine the level of TMV replication in the original nn plants.

ACKNOWLEDGMENTS

We thank M. van der Heijden for use of the Samsun *NN* cDNA library and the A5 cDNA construct. In addition, we are indebted to Dr. J. Kijne for fruitful discussions. Drs. R. Beachy and B. Baker are acknowledged for supplying the pOb and N genomic DNA clones. Work at Leiden University was supported by the Earth and Life Sciences Foundation (A.L.W.), which is subsidized by the Netherlands Organization for Scientific Research (N.W.O.). Work in the Sainsbury Laboratory is supported by the Gatsby Charitable Foundation. Use of genetically modified viruses in the Sainsbury Laboratory is authorized by the United Kingdom Ministry of Agriculture Fisheries and Food (license PHL24B/3654).

REFERENCES

Abbink, T. E. M., De Vogel, J., Bol, J. F., and Linthorst, H. J. M. (2001). Induction of a hyersensitive response by chimeric helicase sequences of tobamovirus U1 and Ob in *N*-carrying tobacco. *Mol. Plant Microbe Interact.* **14,** 1086–1095.

Abbink, T. E. M., Tjernberg, P. A., Bol, J. F., and Linthorst, H. J. M. (1998). Tobacco mosaic virus helicase domain induces necrosis in N gene-carrying tobacco in the absence of virus replication. *Mol. Plant Microbe Interact.* **11,** 1248–1246.

Agatep, R., Kirkpatrick, R. D., Parchaliuk, D. L., Woods, R. A., and Gietz, R. D. (1999). Transformation of *Saccharomyces cerevisiae* by the lithium acetate/single-stranded carrier DNA/polyethylene glycol (LiAc/ss-DNA/PEG) protocol. *Technical Tips Online* http://tto.trends.com.

Bao, Y., Carter, S. A., and Nelson, R. S. (1996). The 126- and 183-kilodalton proteins of tobacco mosaic virus, and not their common nucleotide sequence, control mosaic symptom formation in tobacco. *J. Virol.* **70,** 6378–6383.

Baulcombe, D. C. (1999). Fast forward genetics based on virus-induced gene silencing. *Curr. Opin. Plant Biol.* **2,** 109–113.

Beyer A. (1997). Sequence analysis of the AAA protein family. *Protein Sci.* **6,** 2043–2058.

Brederode, F. Th., Linthorst, H. J. M., and Bol, J. F. (1991). Differential induction of acquired resistance and PR gene expression in tobacco by virus infection, ethephon treatment, UV light and wounding. *Plant Mol. Biol.* **17,** 1117–1125.

Burton, R. A., Gibeaut, D. M., Bacic, A., Findlay, K., Roberts, K., Hamilton, A., Baulcombe, D. C., and Fincher, G. B. (2000). Virus-induced silencing of a plant cellulose synthase gene. *Plant Cell* **12,** 691–706.

Chang, J. H., Rathjen, J. P., Bernal, A. J., Staskawicz, B. J., and Michelmore, R. W. (2000). AvrPto enhances growth and necrosis caused by *Pseudomonas syringae* pv. tomato in tomato lines lacking either Pto or Prf. *Mol. Plant Microbe Interact.* **13,** 568–571.

Chien, C., Bartel, P. L., Sternglanz, R., and Fields, S. (1991). The two-hybrid system: A method to identify and clone genes for proteins that interact with a protein of interest. *Proc. Natl. Acad. Sci. USA* **88,** 9578–9582.

Confalonieri, F., and Duguet, M. (1995). A 200-amino acid ATPase module in search of a basic function. *Bioessays* **17,** 639–650.

Culver, J. N., and Dawson, W. O. (1989). Tobacco mosaic virus coat protein: An elicitor of the hypersensitive reaction but not required for the development of mosaic virus symptoms in *Nicotiana sylvestris. Virology* **173,** 755–758.

Diez, J., Ishikawa, M., Kaido, M., and Ahlquist, P. (2000). Identification and characterization of a host protein required for efficient template selection in viral RNA replication. *Proc. Natl. Acad. Sci. USA* **97,** 3913–3918.

Erickson, L. F., Holzberg, S., Calderon-Urrea, A., Handley, V., Axtell, M., Corr, C., and Baker, B. (1999). The helicase domain of the TMV replicase proteins induces the *N*-mediated defense response in tobacco. *Plant J.* **18,** 67–75.

Fields, F., and Song, O. (1989). A novel genetic system to detect protein-protein interactions. *Nature* **340,** 245–346.

Flor, H. H. (1971). Current status of gene-for-gene concept. *Annu. Rev. Phytopathol.* **9,** 275–296.

Goregaoker, S. P., Lewandowski, D. J., and Culver, J. N. (2001). Identification and functional analysis of an interaction between domains of the 126/183-kDa replicase-associated proteins of *Tobacco mosaic virus. Virology* **282,** 320–328.

Hazan, J., Fonknechten, N., Mavel, D., Paternott, C., Samson, D., Artiguenave, F., Davoine, C.-S., Cruaud, C., Dürr, A., Wincker, P., Brottier, P., Cattolico, L., Barbe, V., Burgunder, J.-M., Prud'homme, J.-F., Brice, A., Fontaine, B., Heilig, R., and Weissenbach, J. (1999). Spastin, a new AAA protein, is altered in the most frequent form of autosomal dominant spastic paraplegia. *Nature* **23,** 296–303.

Hirashima, K., and Watanabe, Y. (2001). Tobamovirus replicase coding region is involved in cell-to-cell movement. *J. Virol.* **75,** 8831–8836.

Holmes, F. O. (1929). Local lesions in tobacco mosaic virus. *Bot. Gaz.* **87,** 39–55.

Holmes, F. O. (1934). Inheritance of ability to localize tobacco mosaic virus. *Phytopathology* **24,** 984–1002.

Jain, P. K., Kochhar, A., Khurana, J. P., and Tyagi, A. K. (1998). The *psb*O gene for 33-kDa precursor polypeptide of the oxygen-evolving complex in *Arabidopsis thaliana* — Nucleotide sequence and control of its expression. *DNA Res.* **5,** 221–228.

James, P., Halladay, J., and Craig, E. A. (1996). Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. *Genetics* **144,** 1425–1436.

Jia, Y., McAdams, S. A., Bryan, G. T., Hershey, H. P., and Valent, B. (2000). Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. *EMBO J.* **19,** 4004–4014.

Jones, L., Hamilton, A. J., Voinnet, O., Thomas, C. L., Maule, A. J., and Baulcombe, D. C. (1999). RNA–DNA interactions and DNA methylation in post-transcriptional gene silencing. *Plant Cell* **11,** 2291–2302.

Knoester, M., Van Loon, L. C., Van Den Heuvel, J., Hennig, J., Bol, J. F., and Linthorst, H. J. M. (1998). Ethylene-insensitive tobacco lacks nonhost resistance against soil-borne fungi. *Proc. Natl. Acad. Sci. USA* **95,** 1933–1937.

Kooman-Gersmann, M., Honee, G., Bonnema, G., and De Wit, P. J. G. M. (1996). A high-affinity binding site for the AVR9 peptide elicitor of *Cladosporium fulvum* is present on plasma membranes of tomato and other solanaceous plants. *Plant Cell* **8,** 929–938.

Lai, M. M. C. (1998). Cellular factors in the transcription and replication of viral genomes: A parallel to DNA-dependent RNA transcription. *Virology* **244,** 1–12.

Leister, R. T., and Katagiri, F. (2000). A resistance gene product of the nucleotide binding site — Leucine rich repeats class can form a complex with bacterial avirulence proteins in vivo. *Plant J.* **22,** 345–354.

Li, L., Elledge, S. J., Peterson, C. A., Bales, E. S., and Legerski, R. J. (1994). Specific association between the human DNA repair proteins XPA and ERCC1. *Proc. Natl. Acad. Sci. USA* **91,** 5012–5016.

Liberzon, A., Shpungin, S., Bangio, H., Yona, E., and Katcoff, D. J. (1996). Association of yeast SAP1, a novel member of the "AAA" ATPase family of proteins, with the chromatin protein SIN1. *FEBS Lett.* **388,** 5–10.

Memelink, J., Swords, K. M. M., Staehlin, L. A., and Hoge, J. H. C. (1994). Southern, Northern, and Western blot analysis. *In* "Plant Molecular Manual" (S. B. Gelvin, R. A. Schilperoort, and D. P. S. Verma, Eds.), Kluwer Academic Publishers, Dordrecht, The Netherlands.

Neeleman, L., and Bol, J. F. (1999). Cis-acting functions of alfalfa mosaic virus proteins involved in replication and encapsidation of viral RNA. *Virology* **254,** 324–333.

Padgett, H. S., Watanabe, Y., and Beachy, R. (1997). Identification of the TMV replicase sequence that activates the N gene-mediated hypersensitive response. *Mol. Plant Microbe Interact.* **10,** 709–715.

Rahoutei, J., García-Luque, I., and Barón, M. (2000). Inhibition of photosynthesis by viral infection: Effect on PSII structure and function. *Physiol. Plant.* **110,** 286–292.

Ratcliff, F., Harrison, B. D., and Baulcombe, D. C. (1997). A similarity between viral defense and gene silencing in plants. *Science* **276,** 1558–1560.

Ratcliff, F., Martin-Hernandez, A. M., and Baulcombe, D. C. (2001). Tobacco rattle virus as a vector for analysis of gene function by silencing. *Plant J.* **25,** 237–245.

Reichel, C., and Beachy, R. N. (1998). Tobacco mosaic virus infection induces severe morphological changes of the endoplasmatic reticulum. *Proc. Natl. Acad. Sci. USA* **95,** 11169–11174.

Ruiz, M. T., Voinnet, O., and Baulcombe, D. C. (1998). Initiation and maintenance of virus-induced gene silencing. *Plant Cell* **10,** 937–946.

Samuel, G. (1931). Some experiments on inoculating methods with plant viruses, and on local lesions. *Ann. Appl. Biol.* **18,** 494–507.

Scofield, S. R., Tobias, C. M., Rathjen, J. P., Chang, J. H., Lavelle, D. T., Michelmore, R. W., and Staskawicz, B. J. (1996). Molecular basis of gene-for-gene specificity in bacterial speck disease of tomato. *Science* **274,** 2063–2065.

Seo, S., Okamoto, M., Iwai, T., Iwano, M., Fukui, K., Isogai, A., Nakajima, N., and Ohashi, Y. (2000). Reduced levels of chloroplast FtsH protein in tobacco mosaic virus-infected tobacco leaves accelerate the hypersensitive reaction. *Plant Cell* **12,** 917–932.

Shan, L., He, P., Zhou, J. M., and Tang, X. (2000). A cluster of mutations disrupt the avirulence but not the virulence function of AvrPto. *Mol. Plant Microbe Interact.* **13,** 592–598.

Shintaku, M. H., Carter, S. A., Bao, Y., and Nelson, R. S. (1996). Mapping nucleotides in the 126-kDa protein gene that control the differential symptoms induced by two strains of tobacco mosaic virus. *Virology* **221,** 218–225.

Sticher, L., Mauch-Mani, B., and Métraux, J. P. (1997). Systemic acquired resistance. *Annu. Rev. Phytopathol.* **35,** 235–270.

Strauss, J. H., and Strauss, E. G. (1999). With a little help from the host. *Science* **283,** 802–804.

Tang, X., Frederick, R. D., Zhou, J., Halterman, D. A., Jia, Y., and Martin, G. B. (1996). Initiation of plant disease resistance by physical interaction of AvrPto and Pto kinase. *Science* **274,** 2060–2063.

Tenllado, F., and Bol, J. F. (2000). Genetic dissection of the multiple functions of alfalfa mosaic virus coat protein in viral RNA replication, encapsidation and movement. *Virology* **268,** 29–40.

Van der Biezen, E. A., and Jones, J. D. G. (1998). Plant disease-resistance proteins and the gene-for-gene concept. *Trends Biochem. Sci.* **23,** 454–456.

Walker J. E., Saraste, M., Runswick, M. J., and Gay, N. J. (1982). Distantly related sequences in the alpha- and β-subunits of ATP synthase, myosin, kinases, and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J.* **1,** 945–951.

White, F. F., Yang, B., and Johnson, L. B. (2000). Prospects for understanding avirulence gene function. *Curr. Opin. Plant Biol.* **3,** 291–298.

Whitham, S., Dinesh-Kumar, S. P., Choi, D., Hehl, R., Corr, C., and Baker, B. (1994). The product of the tobacco mosaic virus resistance gene N: Similarity to toll and the interleukin-1 receptor. *Cell* **78,** 1101–1115.