Involvement of Residues Within Putative α Helix Motifs in the Behavior of the Alfalfa and Tobacco Mosaic Virus Movement Proteins

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ABSTRACT

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The movement proteins (MPs) of both alfalfa mosaic virus (AMV) and tobacco mosaic virus (TMV) have a 15-amino acid putative α helix motif that is part of a larger domain involved in the cell wall (CW) localization of these proteins. These α helices have common features since they are amphipathic and contain two clusters of acidic amino acids, EE and DE. Mutations were introduced into these helices to investigate their role in CW localization and in the activity of the MPs. Results showed that both these motifs are involved in the CW localization of the MPs, although through different mechanisms: whereas the helical structure and the EE cluster of the AMV-MP were required for optimum CW lo-

calization, the DE cluster of the TMV-MP, but not the helical structure, was involved in this process. Chimeric proteins resulting from an exchange of the α helices between MPs showed that these sequences did not function in the complementary background. Moreover, the region around aa 61 of the TMV-MP is necessary for protein stability. Transgenic tobacco plants expressing a mutated AMV-MP in which the α helix was deleted or had its structure destroyed exhibited an abnormal development and a modified morphology. In parallel, a similar mutation of the TMV-MP yielded a nonfunctional protein that still accumulated in the CW but that did not compete with the viral MP during infection.

Additional keyword: cell wall targeting.

A plant is successfully infected by a virus provided that four steps are entirely completed: entry of the virus into one plant cell, replication of the viral genome, transport of viral components from the initially infected cell to neighboring ones (short distance movement), and transport of the infectious particles from the site of infection to noninfected organs via the conducting vessels (long distance movement). Short distance movement is partially controlled by viral nonstructural movement proteins (MPs), which therefore represent a main component in determining severity of disease.

MPs have been tentatively identified for many viruses (14,24, 29). Movement of viral components from cell to cell is thought to take place via plasmodesmata. It is still uncertain whether virions or nucleic acids are transported from cell to cell. In any case, the diameter of plasmodesmata is too small to allow their passage, suggesting that one of the functions of the MPs would consist in enlarging the channels.

One possible mechanism for enlarging channels is exemplified by viruses that produce tubular structures. Cauliflower mosaic virus (CaMV), cowpea mosaic virus (CPMV), red clover mottle virus (RCMV), and tomato ringspot virus (TomRSV) induce tubular structures that associate with plasmodesmata or provide channels through the cell wall (CW). These tubules contain virus

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particles and are associated with MPs (27,42,46,49). The purified MPs of tobacco mosaic virus (TMV) (6), CaMV (7), alfalfa mosaic virus (AMV) (40), and red clover necrotic mosaic virus (RCNMV) (21,33) exhibit a cooperative but nonspecific nucleic acid binding function in vitro. Citovsky et al. (9) postulated that the MP of TMV might unwind the viral RNA so that the linear protein–nucleic acid structures can enter plasmodesmata. However this hypothesis is not supported by results obtained with the RCNMV MP, which does not unfold RNA (19).

Another mechanism for enlarging channels is the modification of plasmodesmatal size exclusion limit by MPs. Such a modification was demonstrated by microinjection experiments: injections of fluorescent dyes into transgenic plants expressing the MPs of TMV (47,50) and AMV (35), or coinjection of dyes with tobacco rattle virus (TRV) (17). Other experiments demonstrated that some MPs move rapidly from cell to cell when microinjected into one plant cell: the RCNMV MP (19) and the BL1 protein from bean dwarf mosaic geminivirus (31). These studies show that the size exclusion limit is sufficiently increased by the putative MPs to allow the passage of ribonucleoproteins but not of intact virions.

The subcellular localization of most MPs is in accordance with their role in plasmodesmatal modifications. Electron microscopic observations have showed that the TMV MP is present inside plasmodesmata (2,45), as are the MPs of RCMV (42), CPMV (46), and CaMV (7). The AMV MP was observed in the middle lamella of infected cells (43). Other MPs were detected in CW fractions (defined below): RCNMV (32), tomato black ring virus (13), and grapevine chrome mosaic virus (22). However, there are exceptions to this scheme: MP of cucumber mosaic virus was

detected in the nucleolus (28), whereas potato virus X MP was detected in the cytoplasmic inclusion bodies (12).

AMV and TMV are single-stranded RNA viruses, members of the genera *Ilarvirus* and *Tobamovirus*, respectively. The AMV-MP (32 kDa) is translated from the first cistron of RNA3 (30) whereas the TMV-MP (30 kDa) is expressed via a subgenomic RNA (3). Transgenic tobacco plants expressing the TMV-MP are able to complement the transport of the thermosensitive Ls1 TMV strain at the nonpermissive temperature (15). The TMV-MP produced by the plant accumulates in the CWs. Two domains involved in CW localization have been identified: stretches of 19 amino-acids (aa 195 to 213) (4), and 3 amino-acids (aa 3 to 5) (26). Deletion of these domains yields nonfunctional proteins (20) whose CW accumulation rate is lower than the wild-type protein. The AMV-MP accumulates in the CW of transgenic tobacco plants expressing this protein. Deletion of the N-terminal 77 amino-acids yields a protein that mainly localizes in the cytoplasm (18).

Sequence comparisons of the stretch of 19 amino-acids of the TMV-MP with the N-terminal domain of the AMV-MP, which are both involved in the CW localization of these proteins, show that they contain, respectively, part of and a complete 15 amino-acid putative α helix. Moreover, these α helices share several similar features: they are amphipathic and contain two clusters of acidic amino-acids EE and DE. In order to determine whether these α helices are involved in the CW localization of the MPs, deletions, site-specific mutations, and chimeric proteins were engineered. The subcellular localization of these proteins was assessed in transgenic tobacco plants. Some of these transgenic plants were also used to test the effect of the constitutively expressed mutated MPs on virus spread.

MATERIALS AND METHODS

Mutagenesis of the movement protein genes. Mutagenesis was carried out with the Altered Sites Mutagenesis System (Promega, Madison, Wis.). The *Hin*dIII site from the pAlter-1 phagemid was eliminated by *Hin*dIII digestion, filling-in, and recircularization, resulting in the pAlter-H vector that was used for mutagenesis. Mutagenesis of the AMV-MP was started with a cDNA encoding a 12-amino acid N-terminally deleted MP, P3 Δ(1-12), (18). The *Xho*I/*Eco*RI fragment from M13mp7.P3, encoding P3 Δ(1-12), has previously been subcloned into pMON316 between the *Xho*I and *Eco*RI sites. The recombinant plasmid was digested with *Bgl*II, filled-in, and subsequently digested with

*Eco*RI. The resulting insert was introduced into pAlter-H between *Sma*I and *Eco*RI, yielding the plasmid named pA. Mutagenesis of the TMV-MP was started with a cDNA encoding a 9-amino acid C-terminally deleted MP, MP1 (4). The cDNA was digested with *Bam*HI and *Eco*RI and subcloned into pAlter-H between the same restriction sites, yielding the plasmid named pT. To simplify the designation of the mutated proteins described in this article, P3 Δ(1-12) and MP1 were renamed mpA and mpT, respectively.

Mutations of the conserved amino-acids in the helical motifs were produced in pA and pT plasmids using oligonucleotides (Fig. 1A), which replaced the (EE) cluster of the mpA protein and the (DE) cluster of the mpT protein with (AA). The mutated proteins are referred to as mpA-E and mpT-D. Introduction of a (GG) cluster inside the helical motifs was done on pA and pT with the oligonucleotides described in Figure 1B. The resulting plasmids encode the mpA-G and mpT-G proteins, respectively.

Destruction of the *Hin*dIII sites (encompassing exactly two codons corresponding to KL residues) originally present in the mpA and mpT open reading frames was done with the pA and pT plasmids using the oligonucleotides described in Figure 1C, resulting in the plasmids named pA-H and pT-H, respectively. In the case of pT-H, the encoded mpT shows a conservative replacement of the K residue by an R residue. pA-H and pT-H were used for subsequent mutagenesis, consisting of the introduction of *Hin*dIII sites bordering the sequence corresponding to the helical motifs of mpA (amino acids 21 to 35) and mpT (amino acids 187 to 201). Creation of these *Hin*dIII sites was done with the oligonucleotides described in Figure 1D and E, respectively. The resulting double-mutated plasmids, pAnt and pTnt, were digested with *Hin*dIII and religated to remove the sequences corresponding to the helical motifs. In the encoded proteins, referred to as mpA-Δα and mpT-Δα, the deletion of the α helical motifs resulted in the addition of a KL cluster to the sequence. The pA plasmid was digested with *Cla*I, filled-in, and cut with *Eco*RI. The insert, encoding a 77-amino acid N-terminally deleted AMV-MP previously described as P3 Δ(1-77) (18), was introduced into pAlter-H between *Bam*HI (filled-in) and *Eco*RI, yielding the pAc plasmid. The cDNA encoding a 73-amino acid C-terminally deleted TMV-MP, MP4 (4) was subcloned into pAlter-H between the *Bam*HI and *Eco*RI sites, yielding the pTc plasmid. P3 Δ(1-77) and MP4 were renamed mpAc and mpTc, respectively. The *Hin*dIII-bordered-sequences encoding the α helices were obtained from *Hin*dIII digestion of pAnt and pTnt. Chimeric cDNAs were engineered by insertion of these α helices encoding sequences into the *Hin*dIII sites naturally present in the cDNAs encoding the mpA, mpT, mpAc, and mpTc proteins. This cloning procedure allowed the recovery of cDNAs (Fig. 2A and B) containing the sequence encoding an MP to which was added the cDNA encoding (i) the homologous α helix (mpAc-αA, mpTc-αT), (ii) the heterologous α helix (mpA-αT, mpAc-αT, mpT-αA, mpTc-αA), (iii) two or four copies of αA (mpAc-2αA, mpTc-4αA), and (iv) the inverse of the sequence of αT (inv-αT). Due to the introduction of *Hin*dIII sites, the chimeric proteins contained one or two additional KL residue to their sequence.

The GUS gene was excised from the plant shuttle vector pBI121.1 (Clontech) as follows: after digestions with *Sac*I (filled-in), followed by *Sma*I, the plasmid was religated and referred to as pBIΔGUS. All the cDNAs were excised from the plasmids with *Xba*I and *Eco*RI (filled-in), and inserted into the pBIΔGUS between *Xba*I and *Bam*HI (filled-in).

DNA sequencing. All mutagenized plasmids were verified by sequencing using the T7 DNA Polymerase Sequencing System from Promega.

Plant transformation and preparation of subcellular extracts. The pBIΔGUS containing the different cDNAs were introduced into *Nicotiana tabacum* cv. Xanthi NN using *Agrobacterium tumefaciens* (strain LBA 4404) mediated plant transformation (23). Subcellular fractionations were carried out using

A mpA 5'CTTCATCAGCTAGTAACATTG<u>CCG</u>CAGCATAAGACACTGAAA3'
   mpT 5'GGGACATCTTCCATGAAC<u>GCA</u>G<u>C</u>AACGACTTCTTCTGTAAG3'

B mpA 5'CTTCATCAGCTAGTAACAT<u>CCCCCC</u>TTCCTCAGCATAAGACAC3'
   mpT 5'GGGACATCTTCCATGAA<u>CCCCCC</u>CTCATCAACGACTTCTTC3'

C mpA 5'GCCTCATTAAAAAG<u>T</u>TTCAAGGTGATGC3'
   mpT 5'CCACTATCAATAAGC<u>CG</u>AACTCCTTTAAGAAGG3'

D mpA 5'TTCCTCAGCATAAGACAC<u>AAGCTT</u>TGAAAAGCTAGAGGAAGA3'
   mpT 5'CTTCTTCTGTAAGTTCCAT<u>AAGCTT</u>GGGCCCTCCGTCTCTCACG3'

E mpA 5'CCAGAATAGACATTGAGTT<u>AAGCTT</u>TGAAACTTCATCAGCTAG3'
   mpT 5'GCAAGCCTGATCGACATAGG<u>AAGCTT</u>GACATCTTCCATGAACTC3'

Fig. 1. Oligonucleotides for mutagenesis of the mpA and mpT coding sequences. **A,** Oligonucleotides switching the conserved amino acid clusters to AA. **B,** oligonucleotides used to introduce a GG cluster inside the helical domain. **C,** Oligonucleotides used to destroy the original *Hin*dIII sites. **D,** Oligonucleotides used to introduce a *Hin*dIII site at the N-terminus border of the α helices. **E,** Oligonucleotides used to introduce a *Hin*dIII site at the C-terminus border of the α helices. Introduced or substituted nucleotides are underlined.

the highest producing plant lines. Subcellular extracts were prepared as previously described (4). Briefly, after grinding, the homogenate was filtered through a nylon cloth, yielding the filtrate, referred to as the soluble fraction (SOL), and a crude CW fraction (retained in the filter). The CWs were washed three times in the presence of 2% Triton X-100 to remove cytoplasmic contaminants, boiled 10 min in SB buffer (75 mM Tris-HCl pH 6.8, 9M urea, 4.5% sodium dodecyl sulfate [SDS]) and filtered again. This filtrate is referred to as the CW fraction. In some experiments the proteins from the CW fraction were concentrated by precipitation with acetone. Total leaf extracts were prepared by grinding 20 mg of leaf in 80 µl of SB buffer. Subcellular extracts were obtained from fully expanded leaves of 6- to 8-week-old plants (16). Analyses of the subcellular partitioning of the mutated MPs were done on plants expressing comparable amounts of these proteins, e.g., the amount of the mutated MPs in the SOL fraction did not vary by more than fourfold among all plants tested.

Antisera, immunoblotting procedures, and protein quantification. Immunodetection of the AMV movement proteins was done with an antiserum raised against the 12-amino acid N-terminally deleted AMV-MP, designated anti-P3 (18). Immunodetection of the AMV coat protein was done with an anti-AMV serum (43). TMV movement proteins were detected with an antiserum raised against a 23-amino acid synthetic peptide corresponding to amino acid positions 6 to 28. The peptide was injected subcutaneously into a rabbit. The anti-mpT serum was collected after five injections, each containing 100 µg of peptide.

Proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE) using 12% gels and blotted onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, RFA) with the Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, Richmond, Calif.). Electrotransfer was performed at 3.55 mA/cm² of gel for 45 min. Western blots were incubated overnight at 4°C with antisera (diluted to 1/5,000) in phosphate-buffered saline (PBS) (7.9 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 150 mM NaCl; pH 7.5) containing 1% Tween 20 and 3% bovine serum albumin (BSA). After washing (2 × 20 min) with PBS + 1% Tween 20, blots were incubated 4 h with an alkaline phosphatase goat conjugated anti-rabbit antiserum (Bio-Rad) in PBS + 1% Tween 20 + 5% dehydrated skimmed milk. Colorimetric reaction was performed in 100 mM diethanolamine (pH 9.7), 50 µg/ml Bromo-Chloro-Indolyl-Phosphate, and 100 µg/ml Nitro Blue Tetrazolium. Reactions were stopped after 2 h. Immunoblots were scanned at 590 nm with a Shimadzu Flying Spot Scanner CS9000. Caution was taken to scan protein bands for which colorimetric reactions were of similar intensities and always below the saturation level. In the case of proteins that migrate as a doublet, the two bands were scanned together. The relative percentage of mutated protein that accumulated in the CW was calculated for each experiment as well as the average numbers and the standard deviations.

RNA manipulations. Preparation of total RNA was done by grinding 1 g of leaves with the Polytron apparatus in 2 ml of extraction buffer (200 mM Tris-HCl pH 9, 400 mM KCl, 35 mM MgCl₂, 25 mM EDTA). Immediately after grinding, 4 ml of phenol/chloroform (1:1) was added, and the mixture was stirred for 20 min. Nucleic acids in the aqueous phase were precipitated with EtOH. The pellet was washed with 800 µl (3 M sodium acetate pH 5.8) to solubilize DNA. Pelleted RNAs were washed twice with EtOH 70% and resuspended in 200 µl H₂O. Ten micrograms of total RNAs was loaded onto denaturing formaldehyde gels for Northern blotting analyses (38). RNA blots were probed with a random primed cDNA corresponding to the mpT coding sequence.

Movement complementation assays. Inoculation of tobacco leaves with TMV strains L and Ls1 (15) were done at virion concentrations of 0.2 µg/ml and 0.4 µg/ml, respectively, in 100 mM

phosphate buffer (pH 7). One half of each leaf was inoculated with L and the other half with Ls1. Following inoculation, plants were kept at 33°C for 8 days (40 µE s⁻¹ m⁻², 16 h light per day) and temperature was shifted to 25°C for 2 days to allow necrotic lesions to develop. AMV inoculum was prepared by grinding 1 g of systemically infected leaves in 2 ml of phosphate buffer (pH 7). Leaves were inoculated with 300 µl of inoculum and inoculated plants were kept at 25°C during 10 days.
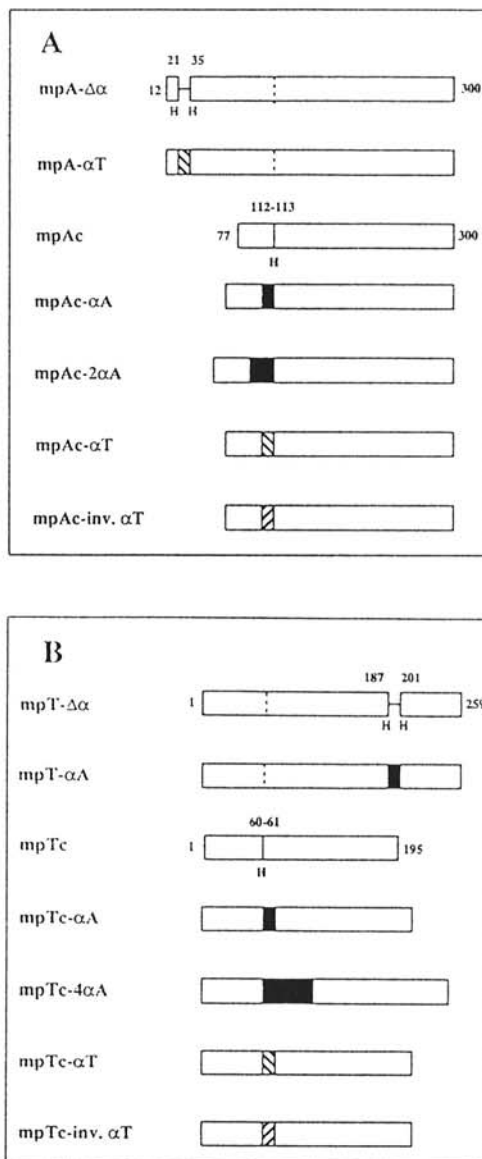

Fig. 2. Characterization of the deleted and chimeric movement proteins. Proteins derived from the AMV-MP (movement protein of AMV deleted from 12 aa at the N-terminus) and from the TMV-MP (movement protein of TMV deleted from 9 aa at the C-terminus) are shown in **A** and **B**, respectively. The original *Hin*dIII sites (H) coding for aa 112 to 113 in the AMP-MP and aa 60 to 61 in the TMV-MP were destroyed (dashed lines) and *Hin*dIII sites (H) were introduced at the borders of the sequences encoding the α helix (dashed box for the AMV-MP and and black box for the TMV-MP). Deletion of the α helices yields mpA-Δα and mpT-Δα. Chimeric proteins mpA-αT and mpT-αA result from the replacement of the natural α helix by the heterologous one. Sequences encoding the α helices were introduced at the natural *Hin*dIII sites of mpAc (movement protein of AMV deleted from 77 aa at the N-terminus) and of mpTc (movement protein of TMV deleted from 73 aa at the C-terminus). One or two AMV α helices (αA) were introduced into mpAc (mpAc-αA, mpAc-2αA), and one or four αA were introduced into mpTc (mpTc-αA, mpTc-4αA). One TMV α helix (αT) was introduced into mpAc (mpAc-αT) and mpTc (mpTc-αT). The sequence encoding the inverted orientation of αT (inv-αT) was introduced into mpAc (mpAc-inv-αT) and mpTc (mpTc-inv-αT).

RESULTS

The aim of this work was to determine whether the putative 15-amino acid α helical motifs of the AMV-MP and TMV-MP are involved in their CW localization.

The initial constructs encoded slightly truncated MPs, which were known to localize in the CW of transgenic tobacco plants (4, 18) in a manner similar to that of the complete MPs. The 12-amino acid N-terminally deleted AMV-MP (mpA) and the 9-amino acid C-terminally deleted TMV-MP (mpT) both contained a 15-amino acid motif (amino acids 21 to 35 and amino acids 187 to 201, respectively) and were predicted to adopt an α helical conformation (5) (Fig. 3A and C). Mutated MPs were engineered by in vitro mutagenesis. In one set of experiments, the sequences encoding the α helices were deleted or the properties of the putative α helices were modified. In another set of experiments, α helices were relocated to another part of the protein and/or exchanged to create chimeric proteins. The cDNAs encoding the mutated MPs described in Figures 2 and 3 were introduced into tobacco plants. For each construct a minimum of six independent transformants were obtained and grown to seed. Plants producing the highest amounts of foreign proteins were selected by immunodetection of MPs in total leaf extracts from the R_1 progeny of at least three of the transformants for each construct. The partitioning of the mutated MPs between the SOL and the CW fractions was assessed in two to four of these selected plants by scanning four to seven immunoblots. A typical immunoblot is presented in Figure 4 and values of the relative CW accumulation of each protein are reported in Table 1. The ability of some mutated MPs to potentiate virus movement was also tested.

Characterization of putative α helices from AMV- and TMV-MPs. The putative α helices of mpA and mpT, designated as αA and αT respectively, share some similarities. They are of the same length and contain two clusters of acidic amino acids, EE and DE, separated by hydrophobic residues. Moreover, helical wheel diagrams (39) revealed the amphipathic nature of these helices (Fig. 3B and D). This is particularly obvious for αT, in which six acidic residues out of seven compose the polar half of the helix. The polar half of αA is not as highly charged as αT (four polar residues out of seven), and the hydrophobic part contains one polar residue. Interestingly, αT is flanked by GGP and P residues located at the N-terminal and C-terminal borders, respectively. These residues are strong helix breakers and are often found to border α helices (25). However, αA is not flanked by such amino acids. In the chimeric proteins mpAc-inv-αT and mpTc-inv-αT (Fig. 2A and B), the inverse of the sequence of αT, inv-αT, was predicted to adopt a strong α helical conformation because of the presence of H residues near the borders of the helix (positions 4 and 15) (1,5). This helix is amphipathic (Fig. 3E) because its polar half contains seven polar residues out of nine but, as opposed to αT, it contains basic and acidic residues.

Involvement of the putative α helical structure and sequence in the behavior of the MPs. The importance of the putative helical structure in the localization of mpA and mpT was investigated by introducing a GG cluster into the helices. These residues introduced flexibility in the molecule and destroyed the helical structure (5,36). The GG clusters were inserted into the αA and αT helix as described in Figure 3A and C, respectively. An immunoblot of the resulting mutated proteins (mpA-G, mpT-G) in the SOL and CW fractions is presented in Figure 4A and B. Comparison of the migration pattern of mpA-G versus mpA and mpT-G versus mpT indicates that these proteins did not migrate according to their predicted molecular weight. Whether these differences were due to structural alterations or post-translational modifications was not determined. MpA and mpT localized mainly in the CW fraction (Table 1) as do the wild-type proteins (16,18). Interestingly, the relative amount of mpA-G in the CW fraction was reduced by about threefold compared with that of
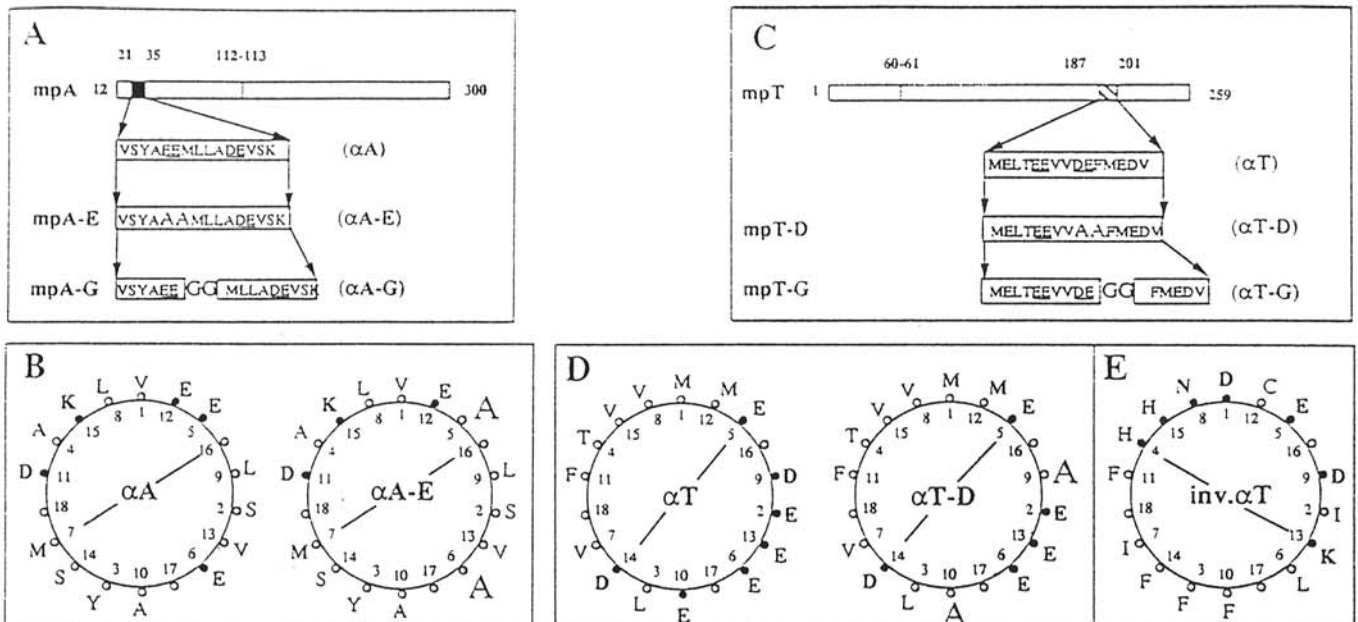

Fig. 3. Characterization of the mutated movement proteins. **A,** The cDNA encoding the 12-amino acid N-terminally deleted AMV movement protein, mpA, contains the putative α helical motif αA (aa 21 to 35). The amino acid sequence of this domain is shown in the αA box. The acidic amino acid clusters are underlined and mutated or introduced residues are in larger characters. Replacement of the EE cluster of αA by AA yields αA-E and the corresponding mutated protein is referred to as mpA-E. Introduction of a GG cluster inside αA yields the αA-G motif and the corresponding protein is referred to as mpA-G. **B,** Helical wheel diagram of the αA and αA-E putative helical motifs. Helical wheels are separated into a polar and a nonpolar face. Acidic residues are in bold and mutated residues are in larger characters. **C,** The cDNA encoding the 9-amino acid C-terminally deleted TMV movement protein, mpT, contains the putative α helical motif αT (aa 187 to 201). The amino acid sequence of this domain is described in the αT box. The acidic amino acid clusters are underlined and mutated or introduced residues are in larger characters. Replacement of the DE cluster of αT by AA yields αT-D and the corresponding protein is referred to as mpT-D. Introduction of a GG cluster after the DE cluster yields the αT-G motif and the corresponding protein is referred to as mpT-G. **D,** Helical wheel diagram of the αT and αT-D putative helical motifs. Helical wheels are separated into a polar and a nonpolar face. Acidic residues are in bold and mutated residues are in larger characters. **E,** Helical wheel diagram of the peptide encoded by the inverted orientation of the αT encoding cDNA (inv-αT).

mpA. In contrast, the relative amount of mpT-G in the CW was similar to that of mp-T.

The role of the complete sequences encoding the α helices for mpA and mpT behavior was investigated by removing the entire 15-amino acid α helical motifs from the proteins. The deletion of the α helical motif of mpA lowered the relative CW amount of mpA-Δα (Fig. 2A) by about threefold compared with mpA (Table 1). The deletion of αT in mpT was done together with a conservative mutation at amino acid 60 (K changed to R). The resulting deleted protein (mpT-Δα, Fig. 2B) was never detected in plants although it was verified by Northern analysis that the corresponding RNA was expressed at the same level as in mpT expressing plants (data not shown). The more likely explanation is that mpT-Δα is unstable, assuming that the conservative amino acid substitution cannot by itself destabilize the protein.

Involvement of the conserved acidic amino acids of the helices in the behavior of the MPs. In order to gain information on the role of the conserved acidic amino acids, one of the clusters was replaced with AA in both mpA and mpT, changing the net charge of the helices. These mutations did not modify the helical structure of the helices (5). In mpA, switching of the EE cluster (aa 25 to 26) to AA (mpA-E, Fig. 3A) resulted in an α helix (αA-E) showing a diminution of the charge in the polar half. However the amphipathic character was even more pronounced than in the wild-type helix because the hydrophobic part of the mutated helix lost its only polar residue (Fig. 3B). In αT, switching of the DE cluster (aa 195 to 196) to AA (mpT-D, Fig. 3C) resulted in an α helix conserving its amphipathic character even though the net charge of the polar half was reduced (Fig. 3D). Immunodetection of these proteins in total extracts from R_1 transgenic plants revealed that they were expressed at less than 10% of the level of mpA and mpT, respectively, in no more than 20% of the R_1 plants, whereas production of the corresponding RNAs was similar to the mpA and mpT expressing plants (data not shown). Some R_2 plants expressing mpA-E and mpT-D to levels comparable to those of MPs were subjected to subcellular fractionation. Immunoblots revealed that mpA-E did not migrate at a rate consistent with its predicted molecular weight (Fig. 4A), probably because of charge modifications. The migration of mpT-D did not seem affected by such a change (Fig. 4C). These mutations reduced the relative accumulation in the CW by about threefold for mpA-E compared with mpA, and by about 11-fold for mpT-D compared with mpT (Table 1).

Are the AMV and TMV MPs α helices equivalent in subcellular partitioning? In order to determine whether αA and αT could be exchanged without modifying the subcellular localization of the proteins, chimeric proteins were engineered by switching the natural α helix with the one from the heterologous protein. Immunodetection of mpA-αT (Fig. 2A) is presented in Figure 4. This protein always migrates as a doublet. In contrast, mpT-αA (Fig. 2B) was never detected in plants expressing the corresponding RNA at a normal level. Table 1 shows that the replacement of αA by αT in mpA did lower by about threefold the relative CW accumulation of the chimeric protein compared with mpA, meaning that the TMV-MP α helix is not sufficient by itself to promote the AMV-MP CW localization.

Complementary experiments were undertaken to determine whether the α helices themselves have the ability to translocate proteins to the CW. In this perspective, the putative α helical motifs, αA and αT (Fig. 3A and C), were introduced into the mpAc and mpTc proteins (Fig. 2A and B), known to localize mainly in the cytoplasm and no longer containing the α helical motif (4, 18), to determine whether they would change their localization. In previous studies, neither mpAc nor mpTc could be detected in the CW (4,18) but optimization of the experimental procedure allowed the detection of mpAc and mpTc in the CW fraction. Figure 4A shows that mpAc always migrated as a doublet and that all the mpAc derived proteins were detectable. In contrast, the mpTc

derived proteins were never detected although the corresponding RNAs were produced at a normal level (data not shown). This suggests that a domain of the TMV-MP located around aa 60 to 61 that is important for stability was disrupted in the chimeric proteins. Analyses of the subcellular partitioning of the chimeric mpAc derived proteins gave some unexpected results (Table 1). Actually, introduction of αA into mpAc (between aa 112 and
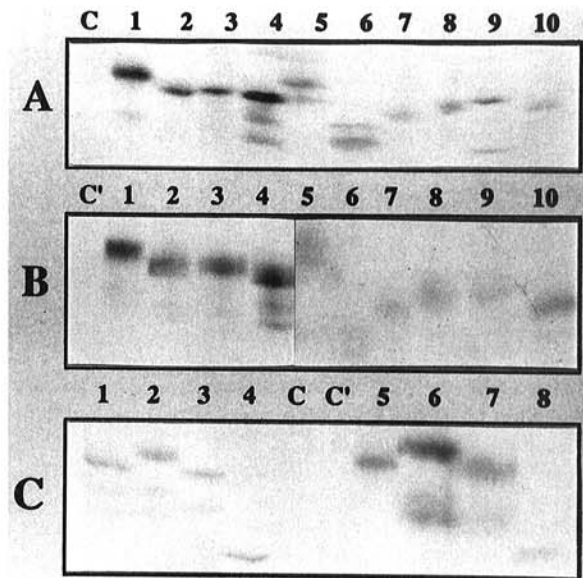

Fig. 4. Immunodetection of the movement proteins in transgenic plants. Plant extracts were separated on 12% polyacrylamide gels. The amount of fresh leaf (mg) corresponding to the material loaded on each lane is indicated in parentheses. The AMV-MP (P3) derived proteins were immunodetected in the soluble (SOL) (A) and cell wall (CW) fractions (B) with an anti-P3 serum. A, Lanes 1: mpA (5.7); 2: mpA-G (8.6); 3: mpA-E (5.7); 4: mpA-Δα (4.3); 5: mpA-αT (34); 6: mpAc (34); 7: mpAc-αA (5.7); 8: mpAc-2αA (8.6); 9: mpAc-αT (26); 10: mpAc-inv-αT (8.6). B, Lanes 1: mpA (4); 2: mpA-G (30); 3: mpA-E (60); 4: mpA-Δα (20); 5: mpA-αT (240); 6: mpAc (240); 7: mpAc-αA (120); 8: mpAc-2αA (240); 9: mpAc-αT (480); 10: mpAc-inv-αT (240). C, Immunodetection of the TMV-MP derived proteins in the SOL (lanes 1 to 4) and in the CW (lanes 5 to 8) fractions with an anti-mpT serum. Lanes 1: mpT (8.6); 2: mpT-G (8.6); 3: mpT-D (17); 4: mpTc (17). Lanes 5: mpT (40); 6: mpT-G (40); 7: mpT-D (240); 8: mpTc (240). Lanes C and C′ correspond respectively to 8.6 mg of SOL and 120 mg of CW extracts from non-transformed plants. Electrophoretic mobilities of the complete AMV-MP (A) and TMV-MP (B and C) are indicated by arrows in the margin.

TABLE 1. Relative cell wall (CW) accumulation of mutated and chimeric movement proteins (MP) expressed in transgenic tobacco plants

Protein[a]	Percent CW[b]	Protein[c]	Percent CW[b]
mpA	80 (10.2)[d]	mpT	55 (5.2)
mpA-G	24 (6.9)	mpT-G	55 (10.5)
mpA-Δα	24 (7.2)	–	
mpA-E	28 (11.2)	mpT-D	5 (3.8)
mpA-αT	17 (6.3)	–	
mpAc	4 (2.3)	mpTc	9 (4)
mpAc-αA	13 (5)		
mpAc-2αA	5 (0.8)		
mpAc-αT	12 (11.5)		
mpAc-inv-αT	10 (2.4)		

[a] Alfalfa mosaic virus (AMV)–MP derived.

[b] Soluble (SOL) and CW fractions were prepared from plants expressing modified MPs and immunoblotted. The bands corresponding to the modified proteins were scanned and the relative percentage of CW accumulation of each protein was calculated. At least 4 immunoblots were done for each protein and the average values are presented as 100 × CW accumulation / (CW + SOL) accumulation.

[c] Tobacco mosaic virus (TMV)–MP derived.

[d] The standard deviation is indicated in parentheses.

113) resulted in a threefold increase of the CW accumulation of mpAc-αA versus mpAc. However, no increase was gained when two αA or αT were introduced in mpAc (mpAc-2αA, mpAc-αT). Introduction of inv-αT did result in a slight increase of CW accumulation of mpAc-inv-αT versus mpAc.

Phenotypic modifications and virus movement in some transgenic plant. Important phenotypic modifications were observed for plants expressing mpA-Δα and mpA-G. The growth rate of these plants was reduced proportionally to the amount of foreign protein produced. Leaves were rounded, curved, and thick, with a pale green color resulting from a fine yellow mosaic. Dimorphism of the two leaf halves was often observed, one half being atrophied. At similar expression levels, plants expressing mpA-Δα exhibited slightly less pronounced phenotypic modifications than mpA-G producing plants. Such modifications were also observed on plants expressing mpA-αT.

Infection of plants expressing mpT-G, mpT-D, or mpTc with L and Ls1 viruses at 33°C showed lesion size and numbers similar to those observed on nontransformed control plants. This implies that these mutated proteins are not able to complement the Ls1 movement. Moreover, even if the nonfunctional mpT-G accumulated normally in the CW it was not able to protect the plant against TMV.

Infection of plants expressing mpA-Δα and mpA-G with TMV resulted in local lesions of normal size but their number was about 10-fold lower than that on nontransformed control plants. Infection of mpA-G expressing plants with AMV resulted in a reduction of coat protein production in inoculated leaves of about fivefold compared with control plants. Moreover, it seemed that long distance transport was reduced; at 14 days postinoculation, the upper leaves were not infected, whereas systemic infection was detected in more than 70% of nontransformed control plants.

DISCUSSION

This study deals with the role of putative α helical motifs, present in the AMV-MP and TMV-MP in their subcellular localization. The respective roles of the helical structure and of the primary sequence were investigated by deletions, point mutations, and engineering of chimeric proteins. These modifications were made on slightly truncated proteins that were previously shown to accumulate in the CW in a manner similar to the intact ones: an AMV-MP and a TMV-MP deleted by 12 and 9 amino acids, respectively, at the N-terminal end (mpA) or C-terminal end (mpT). Moreover, mpT had already been shown to retain its transport function (4). In addition, truncated proteins, mpAc (77 aa N-terminally deleted AMV-MP) and mpTc (73 aa C-terminally deleted TMV-MP), which exhibit a highly reduced accumulation in the CW (4,18), were also used.

In order to determine whether the putative α helix motif is involved in the CW accumulation of the MPs, these motifs were deleted from mpA and mpT to yield mpA-Δα and mpT-Δα. Analysis of the localization of the deleted proteins showed that the CW accumulation of mpA-Δα is reduced by about threefold compared with mpA. Assuming that switching the M residue (aa 36) to an L one is not sufficient to affect the behavior of mpA-Δα, this means that the α helix is probably involved in optimizing the CW localization of mpA. However, such a deletion seems to destabilize mpT-Δα so no conclusion can be drawn for this construct.

The role of the helical structure per se in the CW accumulation process was investigated by engineering mutated proteins containing a GG cluster inside the helical domain. Such a mutation will result in the breakage of the helical structures of the mutated proteins mpA-G and mpT-G (5). As a consequence of these mutations, the CW accumulation of mpA-G was reduced by about threefold compared with mpA, whereas mpT-G accumulated in the CW with the same efficiency as mpT. This indicates that the

helical structure of the 15-amino acid motif is necessary for the CW accumulation of mpA but is dispensable for the CW localization of mpT. Another explanation, which seems less likely, is that the tertiary structure of the mutated proteins was changed by the deletion of the 15-amino acid sequence or the introduction of a GG cluster. Such a change might modify the subcellular partitioning of these proteins.

The role of the primary sequence of the α helices in the CW localization was also examined. Actually, the α helices of the AMV-MP and the TMV-MP both exhibit an amphipathic character and contain two similar acidic amino acid clusters, EE and DE, flanked by hydrophobic residues. The EE cluster of mpA (aa 25 to 26) and the DE cluster of mpT (aa 195 to 196) were switched to AA since replacement of charged amino acids with alanine residues is supposed to avoid the disruption of the tertiary structure (10). These clusters do play a role in the CW accumulation of mpA and mpT because their replacement with AA residues lowered the relative CW accumulation of the mutated mpA and mpT by threefold and 11-fold, respectively. Charged residues found in clusters in the primary sequence are generally located at the surface of the folded protein and can be involved in protein-protein interactions (10,48).

Despite the fact that the acidic clusters are involved in CW localization of the MPs, other residues contained in the α helices are also important. The replacement of the α helix of the AMV-MP with that of the TMV-MP produced a chimeric protein (mpA-αT) that accumulated in the CW only at a level similar to mpA-Δα. This suggests (i) that the two putative helical sequences are not equivalent in the CW accumulation process and (ii) that, although the helical structure and the presence of the acidic clusters are important, they are not sufficient to allow CW accumulation of mpA. Moreover, the C-terminally deleted mpTc protein, which stops just after the DE cluster (aa 196), and which does not contain the entire α helix, did not accumulate readily in the CW. Given the fact that the breakage of the α helix by the introduction of GG residues following the DE cluster did not reduce the CW accumulation of the mutated protein (mpT-G), it is likely that the residues that follow the DE cluster, which are not present in mpTc, are involved in the CW accumulation process. Interestingly, among these residues is an ED cluster (aa 199 to 201) that could be important for CW localization, as is the DE cluster.

In addition to the requirement of residues other than EE for CW localization of mpA, some results strongly suggest that sequences around the α helix are also required in this process. Removal of the entire α helix motif lowers the CW localization by about threefold, but the CW accumulation level of mpAc, which no longer contains a large region around the α helix, is reduced by about 20-fold compared with mpA. Interestingly, a highly hydrophobic domain (aa 46 to 67) located in this region could be necessary for attachment to membranes, thereby mediating the translocation to or the accumulation of mpA in the CW. Moreover, the introduction of one mpA α helix (αA) into mpAc, between aa 112 and 113, results in a threefold increase of the CW accumulation of the resulting chimeric protein (mpAc-αA) versus mpAc. Other hydrophobic domains are also present in mpAc, between amino acids 86 and 100, and between amino acids 157 and 176. Because mpAc is detectable in the CW, these domains could also be involved in the CW localization of mpA. These domains might help αA target mpAc-αA to the CW. However, doubling the number of αA does not increase the CW accumulation compared with mpAc. The same negative results are found for chimeric proteins corresponding to the introduction of αT into mpAc. However, introduction of a strong α helical structure (inv-αA) into mpAc slightly increases the CW accumulation of the chimeric protein compared with mpAc. These experiments suggest that when the mpA α helix is located at position 112 it might be able to promote by itself the CW accumulation of the recombinant protein (mpAc-αA), with, however, far less efficiency than

it does when located at its natural position (aa 21). Because inv-αT does and αT does not increase the CW localization of the chimeric proteins, this reinforces the idea that helical sequences are not equivalent for CW localization.

Mutated and chimeric proteins also provided information about the role of the α helices in protein stability. The introduction of an α helix between amino acids 60 and 61 of mpT or mpTc yields unstable chimeric proteins. Citovsky et al. (9) reported that this region (aa 65 to 86) is involved in correct folding of the TMV-MP. The deletion of the entire αT helix (aa 187 to 201) of mpT probably destabilizes the deleted protein, because it is unlikely that instability results only from the conservative change of the K residue (aa 60) to an R one, or to the replacement of αT by a KL cluster. The 15-amino acid helical structure is not required for the stability of mpA and mpT because mpA-G and mpT-G are stable. Reduction of the production of mpA-E and mpT-D versus nonmutated MPs is in accordance with the fact that acidic amino acids stabilize proteins (11).

The AMV-MP and the TMV-MP share several properties. Both are associated with membranous fractions, are single-stranded RNA binding proteins, and bind RNA cooperatively (6,40). Reports on viral proteins containing an N-terminal amphipathic α helix show that these proteins generally interact with nucleic acids or membranes (34,37) and can mediate protein dimerization (44). It is tempting to speculate that the putative α helices of the AMV-MP and the TMV-MP are involved in at least one of these activities. Because these α helices are acidic they are not good candidates for interaction with nucleic acids. The AMV-MP nucleic acid binding site has been localized between aa 35 and 81 and the α helical domain is not required for cooperative binding (41). On its own, the TMV-MP contains two nucleic acid binding domains: domain A extends from aa 112 to 185 and domain B extends from aa 185 to 268. Domain B, which contains phosphorylation sites (8) and the α helix (187 to 201), was suspected by Citovsky et al. (9) of being involved in protein-protein interactions. These authors showed that the deletion of domain B does not affect cooperative binding, suggesting that the α helix is probably not involved in the dimerization of the TMV-MP. Results presented here show that the helical structure is necessary for optimal CW localization of mpA and that the α helical motifs of mpA and mpT are indeed involved in CW localization via acidic residues.

In conclusion, the α helices of the TMV-MP and the AMV-MP probably interact with membranes or cellular proteins, thereby promoting the translocation or the fixation of the MPs to the CW. For the putative movement protein of the potato leafroll luteovirus such interactions arise via the hydrophobic portion of the helix (44). However, as acidic amino acid clusters are involved in CW localization of the MPs, interactions with cellular proteins might arise via the hydrophilic half of the helix.

Interestingly, the expression of mpA-Δα and mpA-G in transgenic tobacco plants produced phenotypic modifications of these plants. The reduction of the number of infection sites observed after infection of these plants with TMV could be due to the specific phenotype of these plants rather than to a reduction of virus movement. The deleterious effect induced by the mutated MPs probably results from modifications of interactions with cellular proteins rather than from the modification of the subcellular repartition of these MPs. This reinforces the hypothesis that the α helical motif is involved in protein-protein interactions.

Some information was also gained about the involvement of the TMV putative α helix in movement function. Plants expressing a mutated protein obtained by replacement of the DE cluster by AA residues are unable to complement the movement-defective TMV strain Ls1. Because the CW accumulation of this protein is reduced, compared with the wild-type protein, and because the CW is the presumed site of MP activity, it was not possible to conclude that the mutation destroyed the movement activity. On the contrary, introduction of GG residues into mpT (mpT-G) did not lower the CW accumulation of the mutated protein but the movement of Ls1 was not complemented, indicating that mpT-G is a nonfunctional protein. Unfortunately, plants expressing mpT-G are not protected against infection. It could be that conformational changes of the protein weaken the binding of mpT-G to the site of action, so that the mutated MP will be easily displaced by the MP from the infecting TMV.

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