Tomato ringspot nepovirus protease: characterization and cleavage site specificity

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We have cloned the region of tomato ringspot nepovirus (TomRSV) RNA-1 coding for the putative TomRSV 3C-related protease (amino acids 1213 to 1508) in a transcription vector and in a transient expression vector. Using cell-free transcription and translation systems and plant protoplasts, we have demonstrated that proteins produced from these clones possess a proteolytic activity in *trans* on the cleavage site between the TomRSV movement and coat proteins. By amino acid homology of the TomRSV 3C-related protease with other nepo- and comovirus proteases, His^{1283}, Glu^{1331} (or Asp^{1354}) and Cys^{1433} have been predicted to constitute the catalytic triad. Site-directed mutagenesis of His^{1283} to Asp abolished the TomRSV protease activity, *in vitro* and *in vivo*. The cleavage site between the TomRSV movement and coat proteins has been determined to be Q/G, by direct protein sequencing. Previously, His^{1451} located in the substrate binding pocket of the TomRSV 3C-related protease has been suggested to be involved in the cleavage site specificity. We show that an inactive TomRSV 3C-related protease is obtained after substitution of His^{1451} with Leu. These results are discussed in light of the possible relation of the TomRSV 3C-related protease to 3C-related proteases of nepo-, como- and potyviruses.

Introduction

Tomato ringspot virus (TomRSV) is a member of the nepovirus group. Its genome is composed of two plus sense RNA molecules of 8214 (RNA-1) and 7273 (RNA-2) nucleotides (nt) in length (Rott *et al.*, 1991, 1995). Each RNA component is linked to a VPg protein at the 5′ end and is polyadenylated at the 3′ end. TomRSV RNA-1 and RNA-2 potentially encode polyproteins of molecular mass 244 kDa (P1) and 207 kDa (P2), respectively. The deduced TomRSV genomic organization is similar to that of the other nepoviruses, with the VPg, protease and polymerase located at the C-terminal region of P1 and the movement and coat proteins located at the C-terminal region of P2 (Rott *et al.*, 1991, 1995; for a review see Sanfaçon, 1994). Mature TomRSV proteins are probably released from P1 and P2 by proteolytic processing as shown for two other nepoviruses: tomato black ring virus (TBRV) and grapevine fanleaf virus (GFLV) (Demangeat *et al.*, 1990; Margis *et al.*, 1991). TomRSV, along with peach rosette mosaic, cherry leaf roll and myrobalan latent ringspot viruses have been classified as subgroup II nepoviruses owing to the length of RNA-2 (> 5·4 kb) (Francki *et al.*, 1985). TomRSV is the only sequenced member of nepovirus subgroup II. In contrast, GFLV, TBRV, arabis mosaic virus and grapevine chrome mosaic virus (GCMV) belong to subgroup I nepoviruses because their RNA-2 component is smaller than 5·4 kb.

Picornaviruses and picorna-like viruses, including the comovirus, potyvirus and nepovirus groups (Goldbach *et al.*, 1990) encode 3C proteases and 3C-related proteases, respectively. These proteases are homologous to the chymotrypsin-like serine proteases because their catalytic triad is constituted by His, Glu (or Asp) and Cys (Bazan & Fletterick, 1988; Gorbalenya *et al.*, 1989). For example, His^{40}, Glu^{71} and Cys^{147} are the catalytic triad of the well characterized poliovirus 3C protease (numbering according to the poliovirus 3C protease) (Hämmerle *et al.*, 1991). In addition, a highly conserved histidine (His^{161}, numbered according to the poliovirus 3C protease), found in all the substrate binding pockets of the 3C proteases and of the poty- and comovirus 3C-related proteases is essential for the recognition of the (Q,E)/(G,E) cleavage site consensus of picorna-, poty- and comovirus polyproteins (Bazan & Fletterick, 1988, 1990; Gorbalenya *et al.*, 1989; Allaire *et al.*, 1994; Matthews *et al.*, 1994). Members of nepovirus subgroup I are an exception to this: they have a Leu in the substrate binding pocket of their 3C-related proteases, instead of the conserved His found in the substrate binding pocket of the picorna-, poty- and comovirus 3C-related proteases (Demangeat *et al.*, 1990; Ritzenthaler

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et al., 1991). This could explain why all the cleavage sites identified on polyproteins of subgroup I nepoviruses differ from the (Q,E)/(G,E) cleavage site consensus of picorna-, poty- and comovirus polyproteins (Brault *et al.*, 1989; Serghini *et al.*, 1990; Pinck *et al.*, 1991; Bertioli *et al.*, 1991; Block *et al.*, 1992; Demangeat *et al.*, 1992; Margis *et al.*, 1993; Buckley *et al.*, 1994). Surprisingly, the TomRSV 3C-related protease, like the picorna-, como- and potyvirus proteases, contains the conserved His in the substrate binding pocket. This suggests that TomRSV polyprotein cleavage sites may be similar to picorna-, poty- and comovirus polyprotein cleavage sites (Rott *et al.*, 1995).

In this paper, we have prepared a cDNA clone corresponding to the region of the TomRSV genome encoding the 3C-related protease. Using an *in vitro* and *in vivo* system, we have shown the proteolytic activity of the protein produced from this clone. We have also characterized the cleavage site specificity of the TomRSV 3C-related protease.

Methods

Plasmid constructions

Clones pMR10 and pMR14, containing the complete sequence of TomRSV RNA-1 and TomRSV RNA-2, respectively, were described previously (Rott *et al.*, 1991, 1995).

Protease clones. Plasmid pT7-PRO was constructed by amplifying a 887 nt fragment from plasmid pMR10 (nt 3714 to 4601 of TomRSV RNA-1 sequence) with *Taq* polymerase (Pharmacia). Oligonucleotide 17 (5′ GCTCTAGACCATGGCTTCGACGATTCCCTCCGG 3′) and oligonucleotide 18 (5′ CGGAATTCCCACTTTAGAAACCCC 3′) were used to generate the 5′ and 3′ ends of the fragment, respectively. To allow translation of the protein, oligonucleotide 17 contains an ATG codon (underlined in the above sequence) in frame with the TomRSV RNA-1 nucleotide sequence. Oligonucleotide 17 and oligonucleotide 18 contain respectively a *Xba*I and an *Eco*RI site that were used to subclone the PCR-amplified fragment into the polylinker of plasmid pKS(+) (Stratagene).

Plasmids pT7-PRO^{H1283D} and pT7-PRO^{H1451L} were obtained by site-directed mutagenesis, after substitution of His^{1283} (numbered according to TomRSV P1) by Asp and His^{1451} by Leu, respectively. The method of Kunkel (1985) was employed using pT7-PRO uridyl- single-strand DNA and mutagenic oligonucleotides 24 (5′ CTTTGGCTTTGACTAAAGATCAGGCCTTAACCATAC 3′) and 25 (5′ CAAAATCATAGGGATGCTTGTGGCGGGATCC 3′) respectively. The underlined nucleotides in the above oligonucleotides correspond to the nucleotides substituted in each clone.

Plasmids p35S-PRO, p35S-PRO^{H1283D} and p35S-PRO^{H1451L} were constructed by first subcloning the *Nco*I–*Eco*RI fragments of pT7-PRO, pT7-PRO^{H1283D} and pT7-PRO^{H1451L} into the polylinker of plasmid pMTL23 (Chambers *et al.*, 1988). This step was performed in order to insert a stop codon at the end of the three proteases. Then the *Xba*I–*Bgl*II fragments were extracted from each intermediate plasmid and subcloned into the polylinker of plasmid pDH51, containing the cauliflower mosaic virus 35S promoter and polyadenylation signal (Pietrzak *et al.*, 1986).

Substrate clones. Plasmid p35S-MPCAT was constructed by amplifying an 802 nt fragment from plasmid pMR14 (nt 3294 to 4096 of TomRSV RNA-2 sequence) with *Taq* polymerase using oligonucleotides 1 (5′ CGACCTGCAGAATGGCGGGGGATGCAAGCTCG 3′) and 2 (5′ CGACGGATCCTTCGCACAGGTAACTTTGCG 3′). To allow translation of the protein an ATG codon (underlined in the above sequence) was included in oligonucleotide 1 in frame with the TomRSV RNA-2 sequence. A *Pst*I site on oligonucleotide 1 and a *Bam*HI site on oligonucleotide 2 were used to subclone this fragment into the polylinker of plasmid pCAMVCN (Pharmacia). Because an additional *Pst*I site is present on plasmid pCAMVCN downstream of the CAT gene, the ligation was performed on three fragments: the *Pst*I–*Bam*HI digest of the PCR-amplified fragment, the 1072 nt *Bam*HI–*Cla*I fragment of pCAMVCN and the 3093 nt *Pst*I–*Cla*I fragment of pCAMVCN. The resulting plasmid contained an N-terminal fusion of the TomRSV RNA-2 in frame with the chloramphenicol acetyltransferase (CAT) gene, under the control of the 35S promoter.

Plasmid pT7-MPCAT was constructed by cloning the *Pst*I–*Pst*I fragment of p35S-MPCAT into the *Pst*I site of the polylinker of plasmid pKS(+).

Plasmid pT7-MPCP was constructed by amplifying an 1870 nt fragment from plasmid pMR14 (nt 3294 and 5164 of the TomRSV RNA-2 sequence) with *Taq* polymerase using oligonucleotides 1 (described above) and 15 (5′ GGAAGGCGAGCCGGCC 3′). The *Pst*I site on oligonucleotide 1 and a *Pst*I site present on the amplified product at position 4993 (numbered according to the TomRSV RNA-2 sequence) was used to obtain a 1699 nt fragment which was further subcloned into the *Pst*I site of the polylinker of plasmid pKS(+).

Plasmids pT7-MPCPΔQG and pT7-MPCPΔEG were obtained after deletion of the sequence corresponding to the dipeptide QG at position 1320 to 1321 (numbered according to TomRSV P2) and after deletion of the sequence corresponding to the dipeptide EG at position 1326–1327, respectively. The method of Kunkel (1985) was employed using pT7-MPCP uridyl single-strand DNA and mutagenic oligonucleotides 22 (5′ GGAATTCTTCTGTTGGGTCCTGGC 3′) and 23 (5′ GGGTCCTGGCAAACTGAAGCCGC 3′), respectively.

Constructs described above were transfected in *E. coli* strain JM109 by electroporation (Pfau & Youderian, 1990) and the structure of recombinant plasmids was verified by restriction enzyme digestion and DNA sequencing (Sambrook *et al.*, 1989).

In vitro transcription and translation. Run-off transcripts were synthesized from linearized plasmids using bacteriophage T7 RNA polymerase (Stratagene) (Sambrook *et al.*, 1989), purified by phenol–chloroform extractions and precipitated with 2 M-LiCl. [^{35}S]Methionine labelled translation products were synthesized in a micrococcal nuclease-treated rabbit reticulocyte lysate system (NT-RRLS) (Promega) as described by Margis *et al.* (1991). Unlabelled translation products were synthesized as described above but [^{35}S]methionine was omitted and replaced by cold methionine as described by the supplier. After 1 h at 30 °C, RNase A at 50 ng/μl was added for 10 min at 30 °C in order to eliminate the RNA template.

Maturation studies were performed at 30 °C by adding 5 μl of unlabelled protease translation products to 1 μl of [^{35}S]methionine labelled substrate translation products. After 3 h of incubation, reactions were stopped by the addition of an equal volume of electrophoresis loading buffer (62·5 mM-Tris–HCl pH 6·8, 10% glycerol, 5% β-mercaptoethanol, 2% SDS, 0·01% bromophenol blue). Samples were boiled for 2 min and fractionated in 12% or 15% SDS–polyacrylamide gels, according to Laemmli (1970).

Protoplast transfection. Protoplasts from *Nicotiana plumbaginifolia* were prepared and transfected as described by Negrutiu *et al.* (1987). Samples of 6×10^5 protoplasts were used for polyethylene glycol transfection using 10 μg of each plasmid. After 24 h of culture at 26 °C in the dark, protoplasts were harvested and soluble extracts of proteins were prepared as described by Fütterer *et al.* (1989). For each batch of

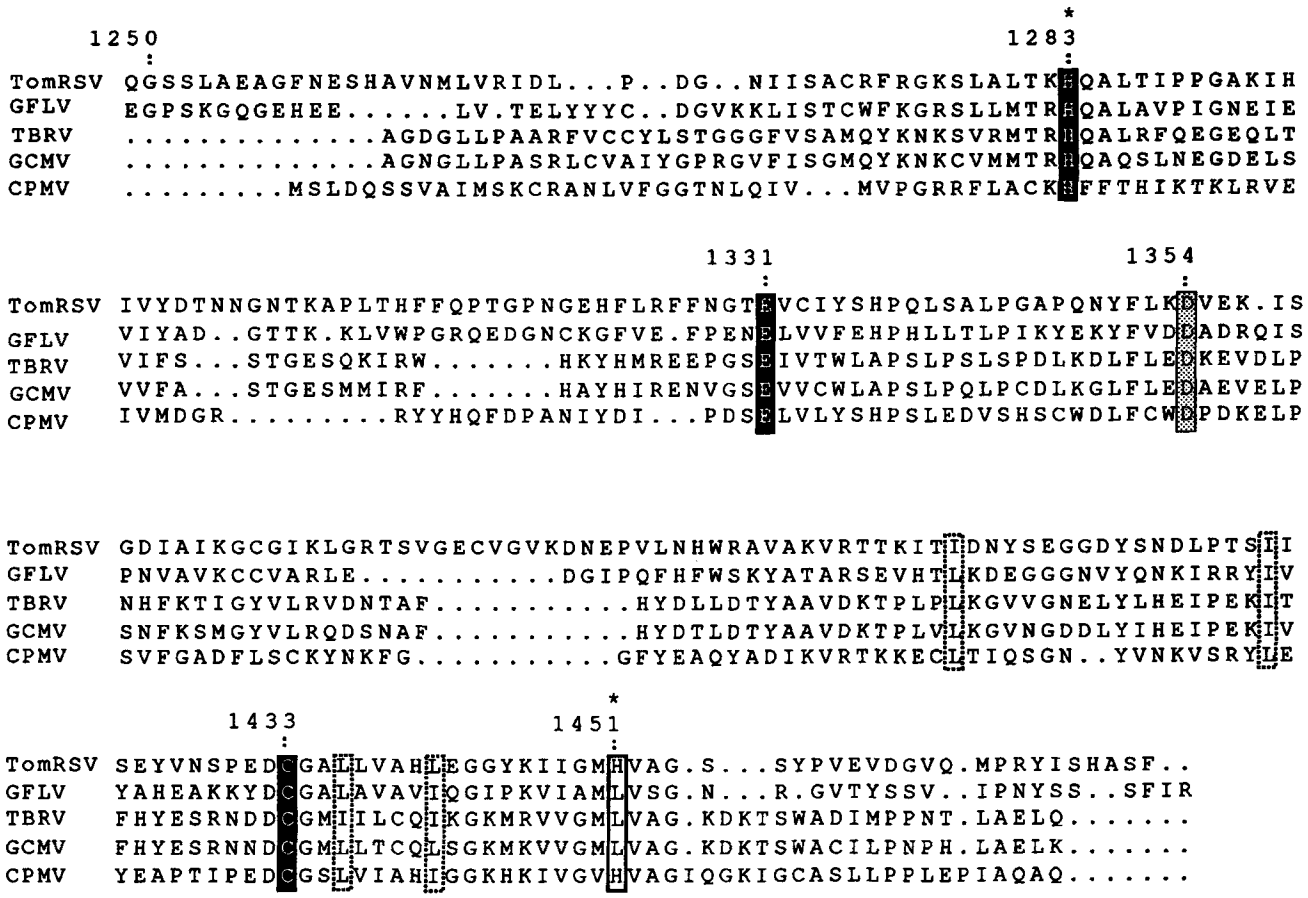

Fig. 1. Comparison of the amino acid sequences from the TomRSV, GFLV, TBRV, GCMV and CPMV 3C-related proteases. The N and C termini of TomRSV, TBRV and GCMV proteases and the C terminus of the GFLV 3C-related protease were arbitrarily chosen since the cleavage sites producing these proteases are unknown. The aligned sequences are numbered according to polyprotein P1 encoded by TomRSV RNA-1. His^{1283}, Glu^{1331} and Cys^{1433}, which in homology to the catalytic triads of CPMV and GFLV (Dessens & Lomonossoff, 1991; Margis & Pinck, 1992) probably constitute the TomRSV catalytic triad, are shown in black boxes. Asp^{1354}, which may be an alternative to Glu^{1331} in the TomRSV catalytic triad (as demonstrated for TEV, Dougherty *et al.*, 1989*a*), is shown in the grey box. His^{1451}, which is also found in the substrate binding pocket of the CPMV protease and the leucines found at the equivalent position in the substrate binding pocket of subgroup I nepovirus proteases, are shown in the white box. Hydrophobic amino acids found in all the reported proteases and potentially belonging to the substrate binding pocket are boxed with dotted lines. Amino acids of TomRSV protease that have been mutated are labelled (*).

protoplasts, CAT assays were performed, as described by Neumann *et al.* (1987), by measuring the rate of conversion of [^{14}C]acetyl-CoA to [^{14}C]acetylated chloramphenicol/million protoplasts.

When labelling of protoplasts was required, 25 μCi (0·92 MBq) [^{35}S]methionine (1000 Ci/mmol; Amersham) was added, 18 h after transfection, to samples of 3×10^5 protoplasts resuspended in 0·5 ml of culture medium, for a period of 6 h.

Immunoprecipitation. Pelleted, ^{35}S-labelled protoplasts or *in vitro* labelled translation products were diluted with 1 vol. of dissociation buffer (125 mM-Tris–HCl pH 6·8, 25% β-mercaptoethanol, 10% SDS), boiled for 3 min and diluted 10-fold with PBS-TDS buffer (150 mM-NaCl, 1 mM-KH_2PO_4, 8 mM-Na_2HPO_4, 2 mM-KCl, 1% Triton X-100, 0·5% sodium deoxycholate, 0·1% SDS). Ascites fluid (1 μl) containing a monoclonal antibody against the movement protein (MP antibody) (Wieczorek & Sanfaçon, 1993) or 5 μl of a polyclonal antibody directed against the CAT protein (CAT antibody) (Boehringer Mannheim) was added. Samples were incubated overnight at 4 °C and immunoprecipitated with 25 μl of 10% Protein A– or protein G–Sepharose CL-4B (Sigma) for 1 h at 4 °C with rotatory shaking. Immunoprecipitates were washed five times in PBS-TDS buffer, resuspended in 25 μl of dissociation buffer containing 0·01% bromophenol blue and analysed on SDS–polyacrylamide gels.

N-Terminal sequence analysis of the viral coat protein. TomRSV was propagated in cucumber plants and virus was extracted from systemically infected leaves 12 days after inoculation. Leaves were ground in 50 mM-borate buffer pH 7 and filtered through cheesecloth. After clarification of the extract at 7000 *g* for 30 min, precipitation of the cellular proteins with 15% $(NH_4)_2SO_4$ and a second clarification, the TomRSV virions were concentrated by ultracentrifugation at 96000 *g* for 2 h. The pellet was resuspended in 50 mM-borate buffer pH 7 and purified through a 4 M-caesium chloride gradient at 14000 *g*, for 16 h. The band of virions was collected and dialysed against 20 mM-borate buffer, pH 7. Coat protein (150 pmol) was loaded onto a 10% SDS–polyacrylamide gel, transferred onto polyvinylidene difluoride protein sequencing membrane (Bio-Rad) as described by the supplier and subjected directly to automated Edman degradation.

Computer analysis. Sequence alignments between the TomRSV 3C-related protease, the 3C-related proteases of three subgroup I

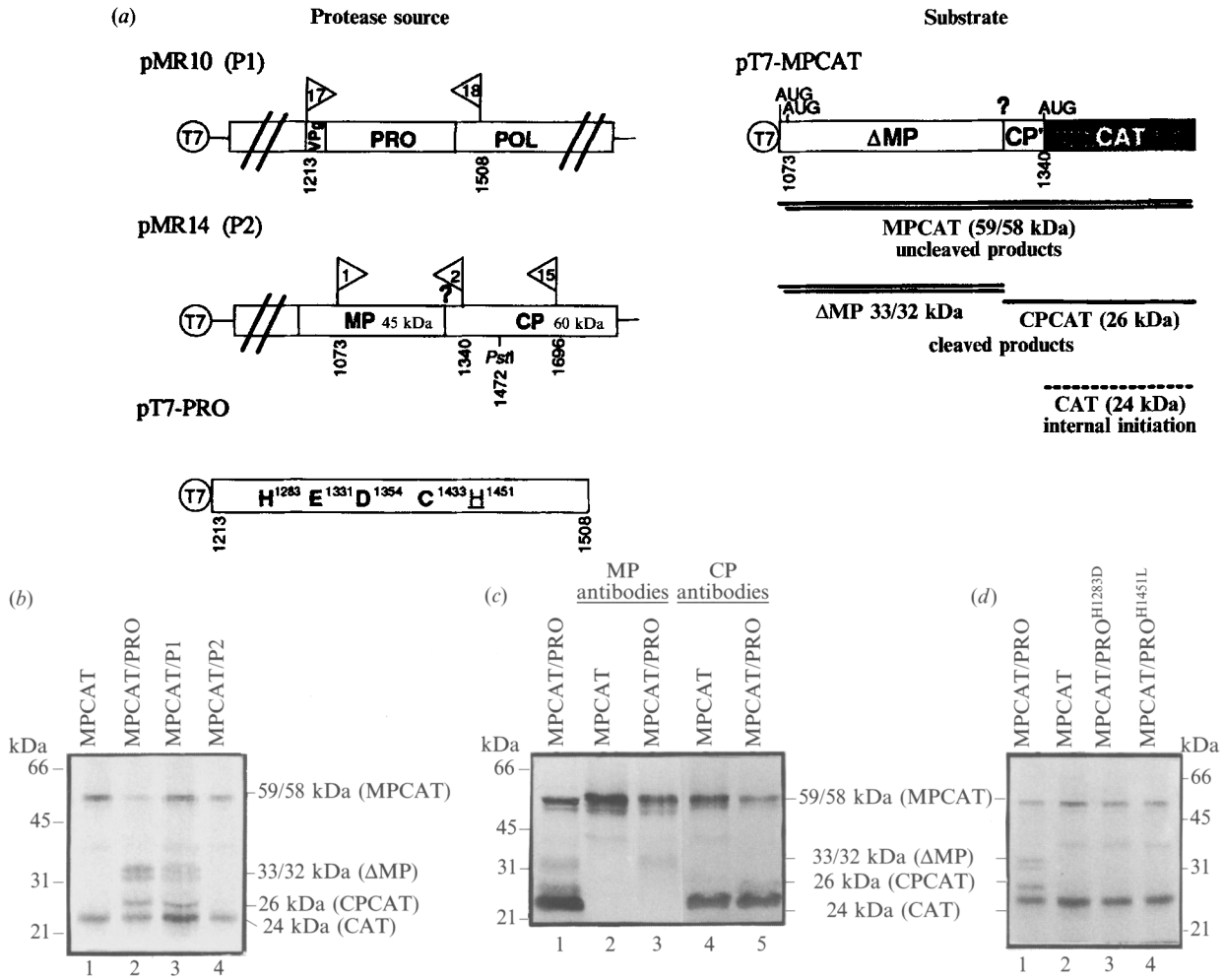

Fig. 2. Proteolytic activity of the 3C-related TomRSV protease in rabbit reticulocyte lysates. (*a*) Schematic representation of the TomRSV cDNA recombinant clones. Boxes and horizontal single lines represent the open reading frame and the non-coding sequences, respectively. The bacteriophage T7 RNA promoter is represented by a circle. In plasmids pMR10 and pMR14 (corresponding to full-length cDNA clones of TomRSV RNA-1 and RNA-2, respectively, and coding for the TomRSV polyproteins P1 and P2, respectively), the approximate location of the VPg, protease (PRO), polymerase (POL), movement protein (MP) and coat protein (CP) are shown. TomRSV polyprotein P1 putative cleavage sites are represented by single vertical lines. The MP–CP cleavage site (?) located on polyprotein P2 is also indicated. Flags above polyproteins P1 and P2 represent the oligonucleotides used to construct plasmids pT7-PRO, pT7-MPCAT and pT7-MPCP. The *PstI* site used to construct clone pT7-MPCP is shown. Numbers below each ORF correspond to amino acids numbered according to P1 and P2. In pT7-PRO, amino acids belonging to the potential catalytic triad of the TomRSV 3C-related protease are indicated. His^{1451}, belonging to the putative substrate binding pocket, is underlined. The MPCAT fusion protein contains the TomRSV RNA-2 region (white box), coding for the C-terminal region of the TomRSV movement protein (ΔMP), the MP–CP cleavage site (?) and the C-terminal region of the coat protein (CP'), in frame with the CAT protein (black box). The MPCAT start and the internal initiator codons, and the CAT start codon are indicated. Tr-MPCAT translation products and their expected molecular mass before (MPCAT) and after (ΔMP and CPCAT) processing are indicated by thick lines. The translation product of tr-MPCAT (and its expected molecular mass) after internal initiation at the CAT start codon is represented by a thick dotted line (CAT). (*b*) Maturations studies using [^{35}S]methionine labelled tr-MPCAT translation products (MPCAT) as substrate and the unlabelled translation products of tr-PRO (PRO) (lane 2), tr-TomRSV RNA-1 (P1) (lane 3) and tr-TomRSV RNA-2 (P2) (lane 4) as a potential source of protease. Lane 1, [^{35}S]Methionine labelled tr-MPCAT translation products. Samples were run on SDS–12% polyacrylamide gels. The positions of molecular mass markers are indicated. Expected positions for the major substrate translation products before (59/58 kDa MPCAT) and after processing (33/32 kDa ΔMP and 26 kDa CPCAT) are also indicated as well as the expected position for the translation product obtained after internal initiation at the CAT start codon (24 kDa CAT). (*c*) Immunoprecipitations of [^{35}S]methionine labelled tr-MPCAT translation products (lanes 2 and 4) and [^{35}S]methionine labelled tr-MPCAT translation products maturated with unlabelled tr-PRO translation products (lanes 3 and 5) using MP antibodies (lanes 2 and 3) and CAT antibodies (lanes 4 and 5). Lane 1, maturation experiments using [^{35}S]methionine labelled tr-MPCAT translation products as substrate and unlabelled tr-PRO translation products as a source of protease. (*d*) Maturations studies using [^{35}S]methionine labelled

nepoviruses (GFLV, TBRV and GCMV) and the cowpea mosaic virus (CPMV) 3C-related protease were performed using the PILEUP program (Devereux *et al.*, 1984).

Results

Genomic localization and cDNA cloning of the TomRSV 3C-related protease

Based on amino acid sequence alignments between TomRSV polyproteins and polyproteins encoded by subgroup I nepoviruses (GFLV, TBRV and GCMV), a comovirus (CPMV), a potyvirus (tobacco etch virus, TEV) and a picornavirus (poliovirus), Rott *et al.* (1995) located the potential TomRSV 3C-related protease on TomRSV polyprotein P1. Using the models of Bazan & Fletterick (1988) and Gorbalenya *et al.* (1989), amino acid sequence alignments published by Rott *et al.* (1995) and our own amino acid sequence alignments (Fig. 1), we predicted that amino acids His^{1283}, Glu^{1331} (or Asp^{1354}) and Cys^{1443} (numbering according to the TomRSV polyprotein P1) potentially constitute the catalytic triad of the TomRSV 3C-related protease. Furthermore, Rott *et al.* (1995) predicted potential cleavage sites on the TomRSV polyprotein P1: dipeptide Q^{1212}/S^{1213} between the NTP binding protein and the VPg, dipeptides Q^{1236}/S^{1237} or Q^{1239}/G^{1240} between the VPg and the protease, and dipeptide Q^{1465}/M^{1466} between the TomRSV protease and the polymerase.

Based on the above information, the clone pT7-PRO, encoding the potential TomRSV protease, was constructed using the method of Margis *et al.* (1991). A PCR product, encompassing the potential TomRSV protease region (amino acids 1213 to 1508) was obtained using plasmid pMR10 as template (Rott *et al.*, 1995) and primers 17 and 18 (see Methods and Fig. 2*a*). The unique *Xba*I and *Eco*RI restriction sites present on primers 17 and 18, respectively, were used to place the PCR-amplified fragment under the control of the T7 promoter. As primer 17 also contains a *Nco*I site, an ATG start codon was introduced in frame with the amplified TomRSV RNA-1 sequence. This would allow the translation of a 32 kDa protein corresponding to the putative TomRSV 3C-related protease, probably flanked by the entire putative VPg and by 42 amino acids downstream from the potential Q/M cleavage site between the protease and the polymerase (Fig. 2*a*). *In vitro* transcription of *Eco*RI-linearized pT7-PRO gave transcript tr-PRO. *In vitro* translation of tr-PRO in a micrococcal nuclease-treated rabbit reticulocyte lysate system (NT-RRLS) for 1 h, in the presence of [^{35}S]methionine, resulted in one major labelled protein of 32 kDa (data not shown).

In trans cleavage by the TomRSV protease in vitro

Experiments were designed to determine whether the TomRSV protease was able to recognize and to process in *trans* cleavage sites on the TomRSV polyprotein P2. NT-RRLS was chosen rather than wheat germ extract because proteolytic processing can be inhibited in wheat germ extract (Shih *et al.*, 1987; Margis *et al.*, 1991). *In vitro* translation of the entire P2 coding region resulted in a very complex banding pattern (data not shown), presumably due to internal initiation or premature termination event, or to degradation of the very long polyprotein P2 (207 kDa). We decided to test the proteolytic activity of the TomRSV 3C-related protease on an isolated P2 cleavage site. We chose the cleavage site between the movement and coat protein (MP–CP cleavage site), because a monoclonal antibody against the movement protein (MP antibody) was available (Wieczorek & Sanfaçon, 1993). The chimeric pT7-MPCAT clone was constructed as follows: a PCR product, corresponding to the TomRSV RNA-2 region from amino acids 1073 to 1340 was obtained, using plasmid pMR14 as template (Rott *et al.*, 1991) and primers 1 and 2 (see Methods and Fig. 2*a*). According to the estimated molecular masses of the TomRSV movement and coat proteins (Wieczorek & Sanfaçon, 1993; Allen & Dias, 1977), the PCR-amplified fragment contains approximately 30 kDa of the coding region of the C-terminal extremity of the TomRSV movement protein (ΔMP) and 2 kDa of the coding region of the N-terminal extremity of the coat protein (CP′). The PCR product was further cloned in frame with the CAT reporter gene. The entire insert was placed under the control of the T7 promoter. An ATG codon, present on primer 1, was introduced in frame with the insert in order to allow the translation of the MPCAT fusion protein with a predicted molecular mass of 56 kDa. Because the MPCAT fusion protein should contain the MP–CP cleavage site, we predicted that it would be processed by the TomRSV 3C-related protease to give the ΔMP protein and the CPCAT protein with a predicted molecular mass of 26 kDa, containing approximately 2 kDa of the N-terminal extremity of the CP followed by 24 kDa of the CAT protein (Fig. 2*a*).

Digestion of plasmid pT7-MPCAT by *Xho*I followed by transcription with bacteriophage T7 RNA polymerase produced transcript tr-MPCAT. Translocation of tr-

tr-MPCAT translation products as substrate and unlabelled translation products of tr-PRO (Lane 1), tr-PRO^{H1283D} (PRO^{H1283D}) (lane 3) and tr-PRO^{H1451L} (PRO^{H1451L}) (lane 4) as a potential source of protease. Lane 2, [^{35}S]methionine labelled tr-MPCAT translation products.

MPCAT in NT-RRLS for 1 h, in the presence of [^{35}S]methionine, produced three major labelled proteins of apparent molecular masses 59 kDa, 58 kDa and 24 kDa. The same pattern was obtained after 4 h of incubation, in the presence of a fivefold excess of micrococcal nuclease-treated rabbit reticulocyte lysate, proving that the NT-RRLS did not contain endogenous proteolytic activity (Fig. 2*b*, lane 1). To further characterize these three labelled proteins, immunoprecipitations using various antibodies were performed. The 59 kDa protein was immunoprecipitated by the MP antibodies and also by CAT antibodies (Fig. 2*c*, lanes 2 and 4), demonstrating that this protein corresponded to the MPCAT fusion protein. The difference between the calculated and apparent molecular mass of the MPCAT fusion protein could be explained by abnormal migration of the translation products in SDS–polyacrylamide gels. The less abundant 58 kDa protein also reacted with both antibodies (Fig. 2*c*, lanes 2 and 4), suggesting that this protein was also an MPCAT fusion protein. This protein may be produced by an internal initiation at the methionine located 19 amino acids downstream from the initiator codon (Fig. 2*a*). The 24 kDa protein was immunoprecipitated only by CAT antibodies (Fig. 2*c*, lanes 2 and 4). Thus, we conclude that the 24 kDa protein is the CAT protein synthesized by internal initiation at the initiator AUG codon of the CAT gene.

The proteolytic activity of the complete TomRSV P1, encoded by plasmid pMR10 (Rott *et al.*, 1995) and of the potential TomRSV protease, encoded by plasmid pT7-PRO, was assayed in *trans* on the MP–CP cleavage site present in the MPCAT fusion protein. Plasmids pMR10 and pT7-PRO were linearized by *Xba*I and *Eco*RI, respectively, and transcriptions were carried out with T7 RNA polymerase to give transcripts tr-TomRSV RNA-1 and tr-PRO. Unlabelled translations of tr-TomRSV RNA-1 and tr-PRO and [^{35}S]methionine labelled translations of tr-MPCAT were done separately in NT-RRLS for 1 h. Maturation experiments were performed by incubating [^{35}S]methionine labelled tr-MPCAT translation products with either unlabelled tr-TomRSV RNA-1 translation products or unlabelled tr-PRO translation products, for 3 h. The pattern of [^{35}S]methionine labelled tr-MPCAT translation products obtained after maturation experiments revealed the presence of three additional proteins of 33 kDa, 32 kDa and 26 kDa (Fig. 2*b*, lanes 2 and 3). The 33 kDa and 32 kDa proteins were immunoprecipitated only by MP antibodies (Fig. 2*c*, lanes 3 and 5) and therefore correspond to ΔMP, released after processing of the MPCAT fusion protein at the MP–CP cleavage site. Two bands were probably detected because of an internal initiation arising at the second methionine present on the MPCAT chimeric construct. The 26 kDa protein reacted only with CAT antibodies (Fig. 2*c*, lanes 3 and 5) demonstrating that it corresponds to the CPCAT protein also expected after processing of the MPCAT fusion protein.

The potential proteolytic activity of the TomRSV P2 was also tested on the MP–CP cleavage site. Plasmid pMR14 (Rott *et al.*, 1991), encoding the entire P2, was linearized by *Xba*I, transcriptions were carried out with T7 RNA polymerase to give transcripts tr-TomRSV RNA-2 and unlabelled translations of these transcripts were done in NT-RRLS for 1 h. After incubation of [^{35}S]methionine labelled tr-MPCAT translation products with unlabelled tr-TomRSV RNA-2 translation products, only bands corresponding to the unprocessed MPCAT translation products were observed (Fig. 2*b*, lane 4).

These results show that polyprotein P1 but not polyprotein P2 possesses a proteolytic activity able to process in *trans* the MP–CP cleavage site present on the MPCAT fusion protein. Our results indicate that this proteolytic activity also exists on the protein encoded by pT7-PRO.

His^{1283} probably belongs to the catalytic triad

From the predictions of Bazan & Fletterick (1988) and of Gorbalenya *et al.* (1989) and also by homology with the previously mutagenized GFLV 3C-related protease (Margis *et al.*, 1992), His^{1283} was predicted to be the base component of the catalytic triad of the TomRSV 3C-related protease (Fig. 1). To verify this hypothesis and also to possibly generate an inactive protease as a control for the precursor processing studies, plasmid pT7-PRO^{H1283D}, in which His^{1283} was converted to Asp, was obtained by site-directed mutagenesis. The proteolytic activity of the mutated protease encoded by pT7-PRO^{H1283D} plasmid was assayed in *trans* on the MP–CP cleavage site. Full-length transcripts tr-PRO^{H1283D} were synthesized and unlabelled *in vitro* tr-PRO^{H1283D} translation products, produced in NT-RRLS, were mixed with [^{35}S]methionine labelled tr-MPCAT translation products, for 3 h. Analyses of the cell-free translation products revealed that only bands corresponding to the unprocessed MPCAT translation products were observed (Fig. 2*d*, lane 3). Thus, the mutated TomRSV protease is unable to process the tr-MPCAT translation products, indicating that His^{1283} is required for the proteolytic activity of the TomRSV protease, *in vitro*.

Proteolytic activity of the TomRSV 3C-related protease in vivo

To test whether we could detect a TomRSV proteolytic activity *in vivo*, i.e. in plant protoplasts, we used the strategy developed by Torruella *et al.* (1989), dem-

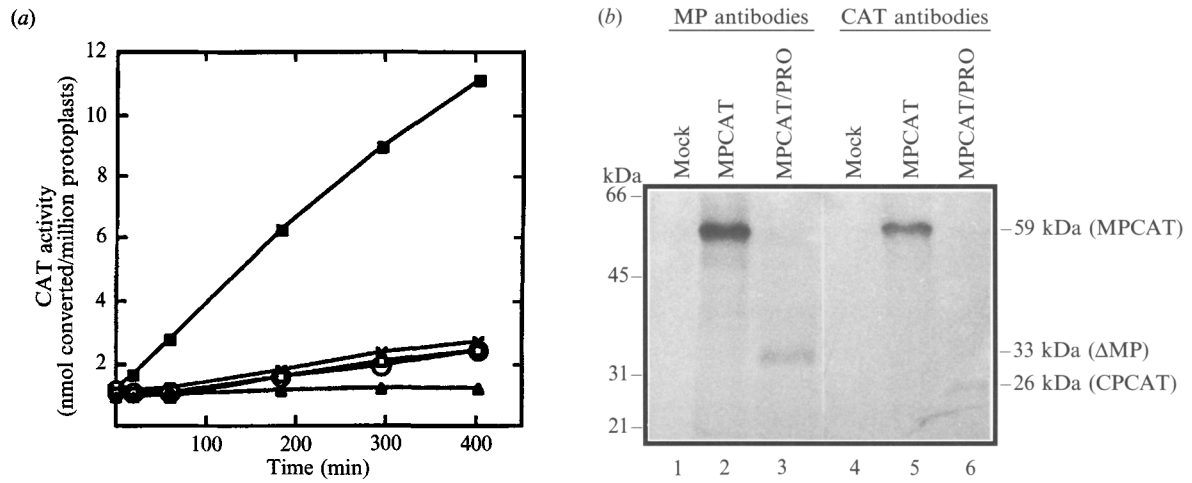

Fig. 3. Proteolytic activity of the 3C-related TomRSV protease in protoplasts. (*a*) CAT activity detected in protoplasts is shown as nmol [^{14}C]acetyl-CoA converted to [^{14}C]acetylated chloramphenicol/million protoplasts. CAT activity was detected in non-infected protoplasts (▲), protoplasts transfected with p35S-MPCAT (□), with p35S-MPCAT and p35S-PRO (■), with p35S-MPCAT and p35S-PRO^{H1283D} (○), and with p35S-MPCAT and p35S-PRO^{H1451L} (X). Each experiment was done at least three times and a representative experiment is shown. (*b*) Immunological analysis of the processing of MPCAT protein in protoplasts. Lanes 1 and 4, un-infected protoplasts; lanes 2 and 5, protoplasts transfected with p35S-MPCAT; lanes 3 and 6, protoplasts transfected with the p35S-MPCAT and p35S-PRO. Immunoprecipitation was done on extracts from 3×10^5 protoplasts using MP antibodies (lanes 1, 2 and 3) and CAT antibodies (lanes 4, 5 and 6). Immunoprecipitates were separated on an SDS–12% polyacrylamide gel. The positions of molecular mass markers are indicated on the left and expected positions for the substrate products before (59 kDa MPCAT) and after processing (33 kDa ΔMP and 26 kDa CPCAT) are indicated on the right.

onstrating the proteolytic activity of the cauliflower mosaic virus aspartic proteinase. The inserts of plasmid pT7-PRO coding for the 32 kDa TomRSV 3C-related protease and of plasmid pT7-MPCAT coding for the 56 kDa fusion protein were placed under the control of the 35S promoter, in order to direct protein synthesis in plant protoplasts (plasmids p35S-PRO and p35S-MPCAT).

The MPCAT fusion protein synthesized *in vivo* from p35S-MPCAT would allow us to monitor the proteolytic activity of the TomRSV protease synthesized from the p35S-PRO construct. Indeed, the unprocessed 56 kDa MPCAT fusion protein (Fig. 2*a*) was designed such that the long (30 kDa) N-terminal extension, corresponding to the TomRSV RNA-2 region from amino acids 1073 to 1340, would inhibit the CAT activity. If the MPCAT fusion protein were processed at the MP-CP cleavage site, the ΔMP and CPCAT proteins with predicted molecular masses of 30 kDa and 26 kDa, respectively, would be released (Fig. 2*a*). Because the CPCAT protein contains a very short 2 kDa N-terminal extension (corresponding to the N-terminal extremity of the TomRSV coat protein), the CPCAT protein would recover the CAT activity, at least partly.

Upon transfection of *N. plumbaginifolia* protoplasts with p35S-MPCAT alone, only low residual CAT activity was detected (compare with mock transfected protoplasts, Fig. 3*a*). After co-transfection of *N. plumbaginifolia* protoplasts with both p35S-PRO and p35S-MPCAT plasmids, a substantial increase of CAT activity was observed (Fig. 3*a*), suggesting that the MPCAT protein is cleaved *in vivo* by the TomRSV 3C-related protease. After transfection of protoplasts with p35S-PRO^{H1283D} and p35S-MPCAT, the CAT activity measured was similar to that of protoplasts transfected with p35S-MPCAT alone (Fig. 3*a*), indicating that His^{1283} also appears to play an essential role in the proteolytic activity of the TomRSV 3C-related protease *in vivo*.

To further establish that the CAT activity detected in protoplasts transfected with p35S-MPCAT and p35S-PRO is correlated with the processing of the MPCAT protein by the TomRSV protease, immunoprecipitations were performed on protoplasts labelled with [^{35}S]methionine. No protein was immunoprecipitated from non-infected protoplasts with either MP antibodies or CAT antibodies (Fig. 3*b*, lanes 1 and 4). After transfection of protoplasts with p35S-MPCAT, a protein of 59 kDa was immunoprecipitated with both MP and CAT antibodies (Fig. 3*b*, lanes 2 and 5), leading to the conclusion that this protein is the MPCAT fusion protein. This is in agreement with our results obtained with NT-RRLS (see Fig. 2*b*). No other proteins were immunoprecipitated with either antibody, demonstrating that internal initiation events did not occur in protoplasts. After transfection of protoplasts with p35S-MPCAT and p35S-PRO, a 33 kDa and 26 kDa protein were immuno-

precipitated with MP antibodies and with CAT antibodies, respectively (Fig. 3*b*, lanes 3 and 6). The 33 kDa protein corresponds to ΔMP and the 26 kDa protein to the CPCAT protein, as already shown using NT-RRLS (Fig. 2*b*).

Identification of the cleavage site between the TomRSV movement and coat proteins

To identify the nature of the MP–CP cleavage site, we determined the amino acid sequence of the N-terminal region of the TomRSV coat protein by automated Edman degradation, using virus particles isolated from cucumber plants. This amino acid sequence was found to be GGSWQEG, which is identical to the amino acid sequence of the polyprotein P2 located between residues 1321 to 1327 (numbered according to P2) (Rott *et al.*, 1991). Thus, the MP–CP cleavage site is Q^{1320}/G^{1321}. This is in agreement with the estimated location of the MP–CP cleavage site deduced from our *in vitro* experiments (Fig. 2*a*).

To confirm that the Q^{1320}/G^{1321} dipeptide is the MP–CP cleavage site recognized *in vitro* by the TomRSV protease, site-directed mutagenesis were performed on plasmid pT7-MPCP, which contains the MP–CP cleavage site (see Methods and Fig. 2*a*). After deletion of dipeptide Q^{1320}/G^{1321}, plasmid pT7MPCPΔQG was obtained. As a control, dipeptide E^{1326}/G^{1327}, another potential cleavage site, was also deleted to give plasmid pT7MPCPΔEG. As expected, maturation experiments revealed that *in vitro* processing was completely abolished by deletion of Q^{1320}/G^{1321}, while it remained unaffected by deletion of E^{1326}/G^{1327} (results not shown).

Substitution of His^{1451} by Leu leads to an inactive TomRSV protease

The TomRSV MP–CP cleavage site, Q/G, is similar to the como-, poty- and picornavirus polyprotein cleavage sites but differs from the cleavage sites of the subgroup I nepovirus polyproteins identified so far. According to Bazan & Fletterick (1988, 1990) and Gorbalenya *et al.* (1989), His^{1451} located in the putative substrate binding pocket of the TomRSV protease (Fig. 1) is predicted to be involved in the cleavage site specificity.

To test whether His^{1451} is required for the proteolytic activity of the TomRSV 3C-related protease at the RomRSV MP–CP cleavage site, plasmid pT7-PRO^{H1451L}, in which His^{1451} is replaced with Leu, was obtained by site-directed mutagenesis. This would partially reconstitute the substrate binding pocket of subgroup I nepovirus proteases. Transcripts tr-PRO^{H1451L} were synthesized from *Eco*RI-linearized pT7-PRO^{H1451L}. Maturation experiments were conducted by mixing [^{35}S]methionine labelled tr-MPCAT translation products with unlabelled tr-PRO^{H1451L} translation products. The pattern of MPCAT translation products obtained revealed that tr-PRO^{H1451L} translation products were unable to cleave tr-MPCAT translation products (Fig. 2*d*, lane 4). The construct encoding the PRO^{H1451L} TomRSV protease was also placed under the control of the 35S promoter (see Methods), to give plasmid p35S-PRO^{H1451L}. After co-transfection of plant protoplasts with p35S-MPCAT and p35S-PRO^{H1451L}, the CAT activity measured was similar to that of protopolasts transfected with p35S-MPCAT alone (Fig. 3*a*). We concluded that this mutated protease was inactive both *in vitro* and *in vivo*. Thus, our results provide evidence that His^{1451} plays an essential role in the proteolytic activity of the TomRSV 3C-related protease.

Discussion

In this paper, we have cloned the region of TomRSV RNA-1, corresponding to the putative 3C-related protease. We have studied the proteolytic activity of the TomRSV 3C-related protease in *trans*, on the TomRSV MP–CP cleavage site, using an *in vitro* cell-free expression system, and an *in vivo* transient expression system. The fusion of the TomRSV MP–CP cleavage site to the CAT protein allowed us to follow the processing both *in vitro* and *in vivo* by immunoprecipitation with CAT antibodies and by direct measure of the CAT activity. Because of its simplicity, the *in vivo* plant protoplast system coupled with the measure of the CAT activity will be used to further characterize the processing of the TomRSV polyproteins and the regulation of expression of viral genes products.

We showed that both the entire polyprotein P1 and the protein encoded by the pT7-PRO plasmid (which corresponds to the putative TomRSV 3C-related protease, the entire putative VPg, and a small region of the polymerase), are able to process in *trans* the TomRSV MP–CP cleavage site. Further studies are underway that will aim at comparing the proteolytic activities of the entire TomRSV P1, of the mature TomRSV 3C-related protease and of different precursors of the protease. Indeed, differential proteolytic activities of precursor and mature forms of the GFLV and CPMV 3C-related proteases have been shown on different GFLV and CPMV cleavage sites, respectively (Margis *et al.*, 1994; Dessens & Lomonossoff, 1992). In addition, the proteolytic activity of the CPMV 3C-related protease is also regulated by a co-factor of this protease, encoded by the CPMV B-RNA (Peters *et al.*, 1992).

Although we demonstrated proteolytic activity of the TomRSV protease encoded by the pT7-PRO in *trans* on

a P2 cleavage site, attempts to demonstrate an autocatalytic processing of this protease were unsuccessful. Time course experiments on production of [^{35}S]methionine labelled tr-PRO translation products in NT-RRLS did not result in processing of the 32 kDa TomRSV protease precursor (data not shown). However, based on the predictions of Rott *et al.* (1995), the 32 kDa protein encoded by the pT7-PRO construct should contain the cleavage site between the VPg and protease and that between the protease and polymerase. One possibility is that processing at these cleavage sites is relatively inefficient as demonstrated for the GFLV cleavage site between the VPg and protease (Margis *et al.*, 1994) and for the TBRV cleavage site between the protease and polymerase (Demangeat *et al.*, 1990). It is also possible that the cleavage sites recognized by the TomRSV protease are different from those predicted by Rott *et al.* (1995). For instance, the dipeptide Q^{1495}/E^{1496} could be an alternative candidate to the predicted dipeptide Q^{1465}/M^{1466} for the cleavage site between the protease and the polymerase. If this site is recognized by the products of the pT7-PRO construct, autocatalytic processing would probably not be detected, because the Q/E dipeptide is only separated by 13 amino acids from the C-terminal extremity of the protein.

The mutated TomRSV protease encoded by pT7-PRO^{H1283D}, in which the putative His of the catalytic triad (Fig. 1) is replaced by Asp, lost its proteolytic activity. Inactive poliovirus 3C-protease, and CPMV and TEV 3C-related proteases were also obtained after mutation of the His potentially belonging to the catalytic triad (Hämmerle *et al.*, 1991; Dessens & Lomonossoff, 1991; Dougherty *et al.*, 1989*a*). Margis & Pinck (1992) also predicted the His of the GFLV 3C-related protease as the base component of the GFLV protease catalytic triad (see Fig. 1). Thus, our results are fully consistent with the suggestion that His^{1283} constitutes the base component of the putative catalytic triad of the TomRSV 3C-related protease.

The amino acid sequence of the TomRSV 3C-related protease shows a greater degree of overall similarity to that of the GFLV 3C-related protease than to those of the picorna-, como-, poty- and other subgroup I nepovirus proteases (Rott *et al.*, 1995). However, the TomRSV 3C-related protease possesses a His at position 1451, in its putative substrate binding pocket (as found in the substrate binding pocket of the picorna-, como- and potyvirus proteases), instead of the typical Leu found at this position in the substrate binding pocket of the subgroup I nepovirus 3C-related proteases (Fig. 1). Our results show that an inactive protease is obtained after substitution of His^{1451} by Leu. According to the model of Bazan & Fletterick (1988) and Gorbalenya *et al.* (1989), we propose that His^{1451} is involved in the recognition of the TomRSV MP–CP cleavage site. Substitutions of the corresponding His of the poliovirus 3C protease (His^{161}, numbered according to the poliovirus 3C protease) by Gly or Arg also resulted in inactive proteases (Ivanoff *et al.*, 1986; Andino *et al.*, 1993). Interestingly, a mutated GFLV protease, in which the typical Leu belonging to the substrate binding pocket has been replaced by a His also lost its proteolytic activity (Margis & Pinck, 1992).

Recent studies of X-ray crystal structures of the hepatitis A virus and of human rhinovirus 3C proteases have revealed that, in addition to the typical His of the substrate binding pocket, other amino acids, predominantly hydrophobic, are part of the substrate binding pocket (Allaire *et al.*, 1994; Matthews *et al.*, 1994). Similar hydrophobic residues flank His^{1451} of the TomRSV 3C-related protease and are also conserved in the proteases of the other nepoviruses of subgroup I, as well as in the CPMV protease (Fig. 1). They may therefore be part of the substrate binding pocket of these various proteases.

The determination of the first TomRSV polyprotein cleavage site, Q/G, led to the suggestion that TomRSV polyprotein cleavage sites more closely resemble the picorna-, como- and potyvirus polyprotein cleavage sites than the cleavage sites of polyproteins from subgroup I nepoviruses. Moreover, conserved amino acids present around the cleavage sites have been demonstrated to regulate the processing activity (Dougherty *et al.*, 1989*b*; Bazan & Fletterick, 1990). It is noteworthy that a serine exists at the −4 position in the MP–CP cleavage site (see Rott *et al.*, 1991, for the sequence of TomRSV RNA-2). Serines at the −4 or at the −5 position are also present in all the GFLV cleavage sites (Ritzenthaler *et al.*, 1991). Identification of other TomRSV cleavage sites is now required, to confirm the presence of this Ser at this specific position and also to study its potential role in the regulation of cleavage efficiency.

Our results suggest that TomRSV 3C-related protease and TomRSV MP–CP cleavage site share characteristics of the 3C-related proteases and cleavage sites of subgroup I nepoviruses, and of poty- and comoviruses. Because TomRSV is the only sequenced member of subgroup II nepoviruses, it would be interesting to determine whether other proteases of subgroup II nepoviruses also have characteristics similar to the TomRSV 3C-related protease.

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