Expression, localization and effects on virulence of the cysteine-rich 8 kDa protein of *Potato mop-top virus*

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Potato mop-top virus (PMTV) RNA3 contains a triple gene block (TGB) encoding viral movement proteins and an open reading frame for a putative 8 kDa cysteine-rich protein (CRP). In this study, PMTV CRP was shown to be expressed in the course of virus infection, and a PMTV CRP-specific subgenomic RNA was mapped. CRP has previously been shown to be dispensable for infection of PMTV in *Nicotiana benthamiana*. In this study, PMTV CRP was found to increase the severity of disease symptoms when expressed from *Potato virus X* or *Tobacco mosaic virus* in *N. benthamiana* and *Nicotiana tabacum*, suggesting that the protein affects virulence of the virus or might suppress a host defence mechanism. However, PMTV CRP did not show RNA silencing suppression activity in three assays. Host responses to the PMTV CRP expression from different viral genomes ranged from an absence of response to extreme resistance at a single cell level and were dependent on the viral genome. These findings emphasized involvement of viral proteins and/or virus-induced cell components in the plant reaction to CRP. PMTV CRP was predicted to possess a transmembrane segment. CRP fused to the green fluorescent protein was associated with endoplasmic reticulum-derived membranes and induced dramatic rearrangements of the endoplasmic reticulum structure, which might account for protein functions as a virulence factor of the virus.

**INTRODUCTION**

A number of plant viruses with positive-stranded RNA genomes encode small cysteine-rich proteins (CRPs) that contain characteristic sequence motifs (Koonin et al., 1991; Savenkov et al., 1998; Morozov & Solovyev, 1999) that are also found in ‘zinc finger’ proteins and some regulatory proteins, including transcription factors (Berg & Godwin, 1997; Auld, 2001). In general, CRPs encoded by plant viruses can be grouped into several classes based on amino acid sequence similarity. For example, CRPs encoded by *Hordeivirus, Pecluvirus, Furovirus* and *Tobravirus* genera share amino acid sequence similarity (*Hordei*-Peclu-Furo-Tobra class, HBFT) (Savenkov et al., 1998) and are shown to be viral virulence determinants (Gramstat et al., 1990; Donald & Jackson, 1994; Hehn et al., 1995; Dunoyer et al., 2001; Liu et al., 2002; Yelina et al., 2002). Biochemical analysis revealed zinc-binding and RNA-binding properties of *Hordeivirus* CRP (Donald & Jackson, 1996; Bragg et al., 2004). Another common feature reported for CRPs of this class is the ability to self-interact via a characteristic C-terminal coiled-coil domain (Dunoyer et al., 2002; Bragg & Jackson, 2004; Yelina et al., 2005). Additionally, CRPs of *Hordeivirus, Tobravirus* and *Furovirus* genera are functionally interchangeable (Liu et al., 2002; Yelina et al., 2002) further suggesting common functions. Indeed, recent studies have demonstrated that these proteins are effective suppressors of RNA silencing, the host mechanism that defends against invading nucleic acids (Dunoyer et al., 2002; Yelina et al., 2002, 2005; Liu et al., 2002; Bragg & Jackson, 2004).

Another CRP class is found in viruses of genera *Carlavirus* and *Allexivirus*. These proteins have a sequence conservation...
pattern distinct from that found in the HBFT class (Kanyuka et al., 1992). A carlavirus CRP has been shown to contain a zinc finger-like sequence motif and to bind RNA in vitro (Gramstat et al., 1990). However, little is known about the functions of carlav- and alexivirus CRPs. The CRP encoded by Potato mop-top virus (PMTV; the type member of genus Pomovirus) shows no noticeable amino acid sequence similarity to proteins of the two aforementioned CRP classes (Scott et al., 1994; Savenkov et al., 2003).

The tripartite PMTV genome consists of single-stranded RNAs of positive-polarity and contains eight open reading frames (ORFs). RNA1 and RNA2 encode virus replication and encapsidation/transmission functions, respectively (Kashiwazaki et al., 1995; Reavy et al., 1998; Savenkov et al., 1999; Sandgren et al., 2001). RNA3, the smallest RNA in the PMTV genome, encodes a triple gene block (TGB) and an 8 kDa CRP (ORF4) (Scott et al., 1994; Savenkov, 2002). Three TGB-encoded proteins are involved in viral cell-to-cell movement (Morozov & Solovyev, 2003; Zamyatnin et al., 2004), whereas CRP function(s) is yet unknown. Our previous analysis demonstrated that the CRP gene is dispensable for replication, accumulation, movement or virulence of PMTV in Nicotiana benthamiana plants (Savenkov et al., 2003). Furthermore, it is not known whether this protein is expressed in infected plants. Other poxviruses do not encode a CRP similar to the PMTV 8 kDa protein (Koenig et al., 1996, 1998). Analysis of the PMTV sequences from several virus isolates revealed that CRP is highly variable (Pečenková et al., 2004). Additionally, unlike Swedish and Scottish isolates (Tod, in four Danish isolates the AUG start codon of the CRP ORF is replaced by a GUG codon (Pečenková et al., 2004).

This study was carried out to determine whether the PMTV CRP is expressed in virus-infected plants and has any functions in vivo. We report immunological detection of PMTV CRP in infected plants, mapping of the subgenomic RNAs (sgRNAs) associated with RNA3, characterization of PMTV CRP-associated effects on the infection phenotypes of heterologous viruses, analysis of the potential of CRP to suppress RNA silencing and localization of GFP-fused CRP in plant cells.

METHODS

Cloning. To obtain pPMTV3-FLAG, PMTV CRP gene and RNA3 3’-UTR were amplified using two pairs of primers: CIS-Nco (5’-AAGCATTGGAAAAAGCATTTATC-3’)/8K-FLAG-Sal-M (5’-GGG-TGACATTTGTGAATCAGCTCCTTGTGATGCATCGCCTAT- TTTATAGTA-3’) and 8K-FLAG-Sal-P (5’-GGGCTGACCTGAG- GATCGTTAATGTTAATG-3’)/RNA3-Spe-Sph (5’-CGCATCT- ACTAGTGGTTCTGATCCCTTCGAC-3’). Restriction enzyme sites are underlined. The resulting amplification products were digested with Avrl/SalI or SalI/SphI, respectively, and ligated into Avrl/SphI digested pPMTV3. To insert the PMTV CRP gene into Barley stripe mosaic virus (BSMV) RNA1, cDNA was cloned in a similar way after amplification of the gene with primers 8K-NhAg-STOP (5’-GCGCTGACTCCTGCAAGCTGCAATAATTATAGTTT- ACTAT-3’) and 8K-Bam-END (5’-GCGAG-TCCCAAAGGCATAATTTATATAGATG-3’). After digestion with Ncol and BamHl, the resulting DNA fragment was ligated with similarly digested BspI (Yelina et al., 2002) to give BSMV[PMTV-CRP]. BSMV[del-PMTV-CRP] was constructed by digestion of BSMV[PMTV-CRP] with Ncol and BamHl, making the ends blunt by Klenow enzyme and religation. To construct BSMV[PMTV-CRP-stop], the CRP gene was amplified with primers 8K-NhAg-BEG and 8K-Xhsa-END (5’-GCGCTGACTCCTGCAAGCTGCAATAATTATAGTTT- ACTAT-3’) (restriction enzyme site underlined) and 8K-Bam-END. After digestion with Ncol and BamHl, the fragment obtained was used to replace the wild-type (wt) sequence in BSMV[PMTV-CRP]. For cloning in Tobacco mosaic virus (TMV; genus Tobamovirus) and Potato virus X (PVX; genus Potexivirus) vectors, the CRP gene was amplified with primers 8K-NhAg-BEG and 8K-Xhsa-END (5’-GCGCTGACTCCTGCAAGCTGCAATAATTATAGTTT-ACTAT-3’) (restriction enzyme site underlined). The resulting product was cloned into PVX vector as Ndel–SalI fragment and in TMV30B as AgeI–Xhol fragment, as described previously for hordeiviral CRPs (Yelina et al., 2002). TMV[PMTV-CRP-stop] was obtained in a similar way after amplification of the CRP gene with primers 8K-NhAg-STOP and 8K-Xhsa-END. To obtain PVX[PMTV-CRP-FLAG], CRP-FLAG gene was amplified with primers CIS-Nco and 8K-FLAG-Sal-M, digested with Avrl/SalI and ligated into Avrl/SalI-digested PVX[PMTV-CRP]. To construct PVX[PMTV-CRP]-GF, the fragment containing 5’-terminal 342 residues of the GFP gene flanked by SalI restriction sites was ligated into the SalI site of PVX[PMTV-CRP], and the orientation of the insert was checked by restriction analysis. The construct PVX-GF was described previously (Yelina et al., 2002), as well as binary vectors containing GFP gene, dsGFP, and PSLV-sGb CRP (Yelina et al., 2005). To obtain a similar construct with PMTV CRP, the gene was cloned under the control of a duplicated Cauliflower mosaic virus 35S promoter and Tobacco etch virus translational enhancer into Ncol/BamHl-digested vector pCK-GFP65ST. Expression cassette flanked by HindIII sites was cloned from the resulting plasmids into a binary vector pLH7000 (Hausmann & Töpfer, 1999) provided by Dr L. Hausmann (Federal Centre for Breeding Research on Cultivated Plants, Germany). To obtain pRT-CRP–GFP, the terminator codon of CRP gene was replaced by GFP (S65T) sequence. pRT-E–YFP (yellow fluorescent protein) construct has been described previously (Zamyatin et al., 2004).

Primer extension. To map the 5’ ends of sgRNAs, primer extension was performed using 5 μg total RNA extract isolated from PMTV-infected N. benthamiana as templates. Template RNAs were annealed with 50 pM primer EXT-p2 (complementary to PMTV RNA3 nt 2032–2055) or EXT-CIS (complementary to PMTV RNA3 nt 2510–2532). [α-32P]ATP-labelled reverse transcription was performed using Moloney murine leukemia virus (Mo-MuLV) reverse transcriptase (MBI Fermentas) and templates according to manufacturer’s protocol.

Plant inoculations and analyses. Inoculation of N. benthamiana plants with recombinant viruses, Western and Northern blot analyses were carried out as described by Yelina et al. (2002) and Savenkov et al. (2003), respectively. Agroinfiltration assay and fluorescent microscopy were done according to Yelina et al. (2005).

Sequence analysis. For prediction of transmembrane protein segments, programs TopPred II (Claroś & von Heijne, 1994; http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html), TMpred (Hofmann & Stoffel, 1993; http://www.ch.embnet.org/software/TMPRED_form.html), MEMSAT (Jones et al., 1994; http://saier.ucdavis.edu/memsat.html) and PRED-TMR (Pasquier et al., 1999; http://biophysics.biol.uoa.gr/PRED-TMR/) were used.
RESULTS

Detection of the PMTV 8 kDa CRP in infected leaves

In order to test whether the 8 kDa CRP is expressed in PMTV-infected plants, the CRP cistron was fused to the sequence encoding the FLAG epitope and used to replace the CRP gene in pPMTV3, the full-length cDNA clone of PMTV RNA3 (Savenkov et al., 2003). The resulting clone pPMTV3-FLAG (Fig. 1) was transcribed in vitro and inoculated onto N. benthamiana plants, along with RNA1 and RNA2 transcripts produced on pPMTV1 and pPMTV2 templates (Savenkov et al., 2003). The plants exhibited yellow mosaic symptoms typical of PMTV infection 1 week post-inoculation (p.i.). At 14 days p.i. samples were taken for immunoblotting with anti-FLAG M2 monoclonal antibodies (mAbs). A specific band corresponding to ca. 8 kDa was detected in PMTV-inoculated leaves (Fig. 2a), which indicated accumulation of the FLAG-epitope-tagged CRP during viral infection. The presence of a specific band of approximately 17 kDa (Fig. 2a) suggested dimerization of the protein, which is in agreement with previous reports showing oligomerization of plant virus-encoded CRPs (Dunoyer et al., 2002; Bragg & Jackson, 2004; Yelina et al., 2005).

Mapping of RNA3-specific sgRNAs

In many positive-stranded RNA viruses expression of 5′-distal genes in viral polycistronic RNA occurs via sgRNAs (Miller & Koev, 2000). Accordingly, it was likely that the expression of the PMTV CRP gene requires an sgRNA, since the CRP ORF encompasses a 3′-terminal position on RNA3. Moreover, the current model of TGB expression (Morozyov & Solovyev, 2003) predicts that the 5′-proximal TGBp1 gene is expressed directly from RNA3, whereas two other TGB proteins, TGBp2 and TGBp3, are probably translated from a single sgRNA by a leaky scanning mechanism. However, we cannot exclude the possibility that PMTV TGBp3 might be expressed via its own sgRNA. Thus, the experiment was set up to map the 5′-ends of three putative RNA3-specific sgRNAs: sgRNA-p2/p3, sgRNA-p3 and sgRNA-CRP, respectively.

Run-off reverse transcription reactions were performed with total RNA extracted from PMTV-infected N. benthamiana plants with primers EXT-p2, EXT-p3 and EXT-CIS annealing to positive-sense RNA3-specific RNAs downstream of start codons of TGBp2, TGBp3 and CRP genes, respectively. The primer extension reaction with EXT-p2 was terminated at positions corresponding to residues 1705 and 1706 of the genomic RNA3 (Fig. 2b). Presumably, the 5′ terminus of the sgRNA-p2/p3 corresponds to an adenine residue at position 1706, whereas the 1 base longer run-off product may arise from incorporation of a residue complementary to the cap structure, as suggested by White & Mackie (1990). Similarly, two major products corresponding to positions 2372 and 2373 were identified in the extension reaction with the primer EXT-CIS (Fig. 2c). These data indicate that sequences of sgRNA-p2/p3 and sgRNA-CRP start at 42 and 89 bases upstream of the translation initiation codons of TGBp2 and CRP, respectively. As expected, the primer extension reaction with primer EXT-p3 did not result in any extension product (data not shown), confirming the hypothesis that PMTV TGBp3 lacks its own sgRNA.
PMTV CRP influence on BSMV, TMV and PVX infection

BSMV (genus Hordeivirus) encodes a CRP previously known as a virulence determinant for viral infection (Donald & Jackson, 1994) and which was recently demonstrated to be a suppressor of RNA silencing (Yelina et al., 2002; Bragg & Jackson, 2004). To determine whether BSMV CRP could be functionally replaced by PMTV CRP, a BSMV (the ND18 strain) chimera was constructed with the PMTV CRP gene replacing that of BSMV (BSMV[PMTV-CRP]; Fig. 3a) under the control of the γb subgenomic promoter. As reported previously, control inoculations with BSMV resulted in mild mosaic symptoms in the systemically infected N. benthamiana leaves at 10–14 days p.i. and local lesions on inoculated Chenopodium amaranticolor leaves at 4–6 days p.i. (Petty et al., 1994; Solovyev et al., 1999). In contrast to BSMV, BSMV[PMTV-CRP] produced no visible symptoms in N. benthamiana and C. amaranticolor (data not shown). In these plants, the BSMV coat protein (CP) was undetectable by Western blotting up to 30 days p.i. (the latest time point observed) (Fig. 4a and data not shown). By contrast, BSMV from which the CRP gene was deleted remained infectious (Yelina et al., 2002).

To analyse whether the observed effect was mediated by the PMTV CRP gene sequence or the encoded CRP protein, a stop codon was introduced into BSMV[PMTV-CRP] 6 nt downstream of the PMTV CRP start codon or the PMTV CRP gene was deleted from this clone. The resulting viruses, BSMV[PMTV-CRP-stop] and BSMV[del-CRP] (Fig. 3a), regained the ability to infect both N. benthamiana and C.
amaranticolor plants, and the BSMV CP was readily detected in the inoculated leaves of both species as demonstrated by Western blot analysis (Fig. 4a). In N. benthamiana, BSMV[PMTV-CRP-stop], as well as BSMV[del-CRP], were restricted to the inoculated leaves, which was consistent with the previously reported finding that BSMV devoid of its CRP is unable to spread systemically in N. benthamiana (Yelina et al., 2002). These data indicate that PMTV CRP, when expressed in the BSMV genome context, exerts an inhibitory non-host-specific effect on BSMV infection.

Protoplasts of Nicotiana tabacum were inoculated to distinguish between the two possible mechanisms, PMTV CRP-mediated suppression of virus accumulation in initially infected cells and an inhibited BSMV cell-to-cell movement. BSMV CP could be detected in BSMV-, BSMV[PMTV-CRP-stop]- and BSMV[del-CRP]-inoculated protoplasts, in contrast to those inoculated with BSMV[PMTV-CRP] at 48 h p.i. (Fig. 4b). These results indicated that PMTV CRP could suppress BSMV accumulation at the single cell level.

The CRP gene of PMTV was inserted into engineered cDNAs of TMV and PVX to determine whether it has any inhibitory effects on infection with viruses from genera other than Hordeivirus. A TMV-based expression vector TMV30B (Shivprasad et al., 1999) was modified to carry the PMTV CRP gene under the control of an sgRNA promoter (TMV[PMTV-CRP]; Fig. 3b). A control construct TMV[PMTV-CRP-stop] contained the CRP gene with a stop codon introduced 6 nt downstream of the PMTV CRP start codon (Fig. 3b). The recombinants were inoculated onto N. benthamiana and N. tabacum cv. Samsun (nn) plants. In both hosts, the parental construct TMV30B caused no visible symptoms on the inoculated leaves and displayed mild mosaic symptoms in the systemically infected leaves, as described previously (Li et al., 1999; Shivprasad et al., 1999). Similar symptoms were induced by TMV[PMTV-CRP-stop] (data not shown), whereas TMV[PMTV-CRP] showed a different phenotype. In N. benthamiana, localized necrotic lesions about 2 mm in diameter first appeared on TMV[PMTV-CRP]-inoculated leaves at 4 days p.i. and expanded later to cover a substantial part of the leaf (Fig. 5a), which resulted in a fast death of the leaf. In N. tabacum, leaves inoculated with TMV[PMTV-CRP] displayed small necrotic lesions about 1–2 mm in diameter at 3 days p.i. (Fig. 5b). Thus, TMV[PMTV-CRP] induced a necrotic reaction in inoculated leaves in both N. benthamiana and N. tabacum. In systemically infected leaves of N.

Fig. 5. PMTV CRP influence on TMV and PVX symptom expression. (a) Necrotization of N. benthamiana leaves inoculated with TMV[PMTV-CRP] imaged at 5 days p.i. The arrow points to the initial local lesion that appeared at 4 days p.i., which is surrounded by an area of secondary necrotization. For imaging, the leaf with the single initial lesion (which appeared after treatment with a diluted inoculum) was taken, since leaves exhibiting many initial lesions died very quickly due to extensive secondary necrotization. (b) Local lesions on N. tabacum cv. Samsun (nn) induced by TMV[PMTV-CRP]. Picture was taken at 7 days p.i. (c) Accumulation of viral RNA in inoculated and systemic leaves of N. tabacum plants infected with TMV30B and TMV[PMTV-CRP]. Northern blotting was carried out with a TMV-specific and a PMTV CRP-specific probes as indicated on the left. Mock, control plants inoculated with water. Positions of viral genomic RNA (gRNA), an sgRNA for viral movement protein (sgRNA1) and an sgRNA for PMTV CRP (sgRNA2) are indicated on the right. (d) Loading control for (c). (e, f) Necrosis of the upper leaves of N. benthamiana plants systemically infected with PVX[PMTV-CRP] (e) and PVX[PMTV-CRP-FLAG] (f) imaged at 10 days p.i. (g) Detection of PMTV CRP with anti-FLAG M2 mAbs in protein samples taken at 14 days p.i. from plants infected with PVX[PMTV-CRP] (lane 2), PVX[PMTV-CRP-FLAG] (lanes 3 and 4) and mock-inoculated leaves (lane 1). Positions of the protein monomer (‘<’) and dimer (‘↔’) are indicated on the right.
**N. benthamiana** TMV[PMTV-CRP] caused mild mosaictype symptoms closely resembling those caused by TMV30B at 7–9 days p.i. (data not shown). The upper non-inoculated leaves in the TMV[PMTV-CRP]-inoculated *N. tabacum* plants remained symptomless up to 30 days p.i.

RNA samples from inoculated and the upper non-inoculated leaves were analysed by Northern blotting with a TMV-specific probe at 16 days p.i. (Fig. 5c). The upper symptomless leaves of TMV[PMTV-CRP]-infected and TMV30B-infected *N. tabacum* plants contained considerable amounts of virus-specific RNA (Fig. 5c). Thus, the PMTV CRP gene altered TMV symptoms in both inoculated and systemically infected leaves of *N. tabacum*. Northern blotting with a CRP-specific probe confirmed the presence of PMTV CRP gene in the symptomless leaves systemically infected with TMV[PMTV-CRP] (Fig. 5c).

The PMTV CRP gene was inserted into an engineered PVX cDNA to yield a PVX[PMTV-CRP] chimera (Fig. 3c). This construct was inoculated onto *N. benthamiana* plants, along with wt PVX used as a control. Systemic infection with wt PVX caused mild mosaic symptoms in the upper non-inoculated leaves at 7 days p.i., while the inoculated leaves remained symptomless (Yelina et al., 2002; data not shown). In contrast, PVX[PMTV-CRP] caused localized necrosis on the inoculated *N. benthamiana* leaves at 5 days p.i., followed by the development of severe necrosis and wilting of the whole upper non-inoculated leaves 9–10 days p.i. (Fig. 5e). These plants died 2–3 days later. To confirm further that the necrotic phenotype induced by PVX[PMTV-CRP] is attributable to the PMTV CRP expression from the PVX vector, we engineered a PVX[PMTV-CRP-FLAG] chimera and inoculated it onto *N. benthamiana*. PVX[PMTV-CRP-FLAG] induced disease phenotype similar to that of PVX[PMTV-CRP] chimera (necrosis of the upper non-inoculated leaves). Expression of the PMTV CRP-FLAG was assayed by Western blotting with anti-FLAG antibodies (Fig. 5g) as before. Overall, this finding suggests that PMTV CRP expressed in the PVX genome dramatically enhances symptom severity of PVX. Moreover, in all experiments, Danish type of PMTV CRP (Fig. 7b), when expressed from three viral vectors, induced disease phenotypes and had an effect on viral vector expression similar to that observed for the original Swedish type of PMTV CRP (data not shown).

### Analysis of PMTV CRP silencing suppression potential

Virus-encoded suppressors of RNA silencing are known to dramatically enhance symptom severity of viruses such as PVX and TMV, which often results in necrotization of infected tissues or plant death (Pruess et al., 1997; Brigneti et al., 1998; Li et al., 1999; Yelina et al., 2002). On the other hand, a necrotic reaction of plant hosts can result from the expression of a virus-encoded Avr gene, provided that plants carry the respective resistance gene (R-gene) (Keen, 1990). *Agrobacterium*-mediated expression of Avr genes is known to induce necrosis of the agroinfected areas (Tang et al., 1996). Thus, as a first step *N. benthamiana* and *N. tabacum* leaves were infiltrated with an *Agrobacterium tumefaciens* culture harbouring a binary vector plasmid, in which the PMTV CRP gene was placed under the control of a duplicated 3S promoter and a translational enhancer. However, the PMTV CRP was unable to induce necrotization of infiltrated leaves (data not shown), suggesting that this protein per se could not induce necrotic reaction in either plant host.

The ability of PMTV CRP to induce necrotic reaction in both *N. benthamiana* and *N. tabacum* when expressed from a heterologous virus prompted us to test CRP for anti-silencing properties. To this end, we employed a previously described assay that involves *Agrobacterium*-mediated co-expression of a reporter gene GFP3 with dsGF (a double-stranded inducer of GFP3-targeted silencing) and a candidate silencing suppressor (Yelina et al., 2005). Infiltration of *N. benthamiana* leaves with a GFP3-containing culture resulted in bright GFP fluorescence visible under long-wave UV at 3 days p.i., whereas a co-infiltration with two *Agrobacterium* cultures carrying GFP3 and dsGF gave no visible fluorescence under the same conditions due to efficient silencing of the GFP gene, as demonstrated by Yelina et al. (2005). When *A. tumefaciens* carrying PMTV CRP gene was mixed with cultures carrying GFP3 and dsGF prior to inoculation, the infiltrated area showed no visible fluorescence, whereas a culture carrying the CRP gene of *Poa semiannulata virus* (PSLV; genus *Hordeivirus*), a known silencing suppressor (Yelina et al., 2005), resulted in restored GFP fluorescence in the infiltrated area (data not shown). To confirm visual observations, the infiltrated areas were examined by Western blotting with a GFP-specific antiserum and Northern blotting with a GFP-specific probe. Three repeated experiments gave similar results. The representative data of blot analyses are shown in Fig. 6(a). The levels of GFP and its mRNA were considerably reduced in the presence of dsGF, whereas co-expression with PSLV CRP resulted in slightly elevated levels of GFP and mRNA accumulation as compared with areas expressing GFP only (Fig. 6a). The levels of GFP and its mRNA accumulation observed in areas co-infiltreted with PMTV CRP were consistently similar to those expressing GFP and dsGF (Fig. 6a). Moreover, both PMTV CRP and PSLV CRP (used as a control) had no effect on the levels of GFP-specific small interfering RNA (siRNA) accumulation in leaf areas expressing GFP and dsGF (Fig. 6a). Thus, PMTV CRP failed to suppress RNA silencing in the agroinfiltration assay. In parallel experiments carried out in *N. tabacum* plants, PMTV CRP also failed to exhibit silencing suppression activity (data not shown).

Two other types of assay were also performed to determine whether PMTV CRP could suppress RNA silencing. Viral silencing suppressors are known to enhance transient expression of reporter genes (Voinnet et al., 2003; Canto et al., 2004) due to their ability to affect silencing induced by single-stranded RNA known to be a weak silencing inducer.
Fig. 6. Analysis of PMTV CRP potential to suppress RNA silencing in agroinfiltration assays. (a) Western blot detection of GFP accumulation and Northern blot detection of GFP mRNA and GFP-specific siRNA in experiment with GFP-targeted silencing induced by a double-stranded RNA construct dsGF. Samples were taken 3 days p.i. from areas of \textit{N. benthamiana} leaves infiltrated with agrobacterial cultures harbouring the genes indicated above the panel. pH. A control agroinfiltration with a culture containing binary vector without inserted gene. (b) PMTV CRP influence on transient expression of reporter GFP gene in \textit{N. benthamiana} leaves infiltrated with agrobacterial cultures harbouring a binary vector with \textit{GFP} gene. Western blot with \textit{GFP}-specific antiserum and Northern blot detection of \textit{GFP} mRNA and \textit{GFP}-specific siRNA with a \textit{GFP} mRNA-specific probe are shown. In the siRNA blots, positions of oligoribonucleotide markers of 21 and 25 bases are indicated. Ethidium bromide staining of rRNA and 5S RNA is shown below mRNA and siRNA Northern blots as loading controls.

(Johansen & Carrington, 2001). Therefore, we tested the ability of PMTV CRP to suppress silencing induced by and targeted to GFP mRNA expressed in \textit{Agrobacterium}-infiltrated leaves. Western and Northern blot analyses revealed that PSLV CRP, used as a control, increased the levels of GFP and its mRNA in leaf areas co-infiltrated with GFP and PSLV CRP-bearing \textit{Agrobacterium} strains as compared with areas infiltrated only with a GFP-expressing culture (Fig. 6b). On the other hand, PMTV CRP had little effect on accumulation of GFP, its mRNA and siRNAs (Fig. 6b), indicating that in the \textit{Agrobacterium}-mediated assay PMTV CRP was unable to suppress RNA silencing induced by single-stranded RNA.

To analyse whether PMTV CRP could influence virus-induced RNA silencing in the course of infection, we used a previously described cross-protection assay (Yelina et al., 2002). Co-inoculation of \textit{N. benthamiana} plants with TMV–GFP (GFP-tagged TMV; Fig. 3b) and PVX-GF, a PVX derivative carrying a portion of the \textit{GFP} gene (Fig. 3c), resulted in blocked systemic movement of TMV–GFP due to RNA silencing triggered by two replicating chimeras carrying the homologous GFP sequence (Yelina et al., 2002). When a derivative of PVX-GF expressing PSLV CRP (PVX-PS/b-GF; Yelina et al., 2002) was inoculated first, followed by inoculation of the same leaves with TMV–GFP 3 days later, extensive apical necrosis induced by CRP expression from PVX-GF-CRP was observed in the systemically infected leaves. This was preceded by appearance of GFP fluorescence in systemically infected leaves, which indicated systemic infection with TMV–GFP allowed by the suppression of RNA silencing by PSLV CRP in the co-inoculated leaves (Yelina et al., 2002; data not shown). In contrast, when PVX-GF was modified to express PMTV CRP (PVX[PMTV-CRP].GF; Fig. 3c) and used for inoculation followed by inoculation of the same leaves with TMV–GFP, no GFP fluorescence was observed in the upper leaves before their necrosis and death induced by PMTV CRP (data not shown). Thus, PMTV CRP was unable to suppress virus-induced RNA silencing in the cross-protection assay.

**Subcellular localization of GFP-fused PMTV CRP**

Analysis of PMTV CRP amino acid sequence revealed that the central region of the protein is highly hydrophobic (Fig. 7a), suggesting that PMTV CRP could interact with cell membranes. CRP sequences of six PMTV isolates were analysed using four different prediction algorithms (see Methods). The central CRP region was always predicted, with a high probability, to represent a transmembrane segment (Fig. 7b). As a result of amino acid substitutions, the length of the predicted CRP transmembrane segment in four Danish isolates was 5 aa longer than in the Sw and Todd isolates (Fig. 7b). However, its hydrophobic properties were preserved (Fig. 7b). Taken together, these data suggest that PMTV CRP is an integral membrane protein.

To analyse the subcellular localization of PMTV CRP, we fused the CRP gene with the \textit{GFP} gene and cloned the resulting construct under the control of the 35S promoter (plasmid pRT-CRP–GFP). Microprojectile bombardment was used to introduce the construct to leaves of \textit{N. benthamiana}. Confocal laser scanning microscopy of the leaves bombarded with pRT-CRP–GFP revealed that the GFP-fused CRP accumulated in amorphous structures distributed throughout the cell (Fig. 7c). As PMTV CRP was predicted to interact with cell membranes, CRP–GFP was co-expressed with ER–YFP, a marker of endoplasmic reticulum (ER) (Zamyatnin et al., 2004). Surprisingly, co-expression with CRP–GFP drastically changed the localization of ER–YFP, which was found in CRP–GFP-containing amorphous bodies (Fig. 7c). Similarly, ER–YFP-containing bodies were observed in cells in which ER–YFP was co-expressed with non-fused PMTV CRP (data not shown).
Therefore, these structures originated from the ER membranes. Moreover, CRP–GFP-induced rearrangements of the native ER structure, showing a destructive effect on the cell endomembrane system.

**DISCUSSION**

Despite the importance of PMTV as a potato pathogen (Sandgren et al., 2002), little is known about virulence variation between isolates and virulence determinants of this virus. In this study, we have shown that CRP is a candidate virulence factor of PMTV.

As a starting point, CRP was immunologically detected in PMTV-infected leaves, and a CRP-specific sgRNA was mapped, showing that CRP is a functional gene.

In a previous study, we demonstrated that PMTV CRP is not required for systemic movement of the virus and symptom induction in *N. benthamiana* (Savenkov et al., 2003). Here, we report that PMTV CRP expression from PVX or TMV vectors enhances the virulence of both viruses and results in systemic necrosis in PVX[PMTV-CRP]-infected *N. benthamiana* and localized necrotic reaction in *N. benthamiana* and *N. tabacum* leaves inoculated with TMV[PMTV-CRP] (Fig. 5a and b). Many viral suppressors of RNA silencing have similar effects when expressed in the PVX or TMV genome background (e.g. Pruss et al., 1997; Brigneti et al., 1998; Li et al., 1999; Yelina et al., 2002). However, PMTV CRP failed to suppress gene silencing in three different assays carried out in *N. benthamiana* plants. Thus, PMTV CRP resembles the 4·8 kDa protein of TMV shown to enhance virulence of PVX and TMV, even though it is not an RNA silencing suppressor (Canto et al., 2004). Conversely, it has been shown that some of the mutations in the *Cucumber mosaic virus* 2b protein, which prevented elicitation of necrosis by the protein, did not prevent its silencing suppression activity (Lucy et al., 2000). Our data show that the PMTV CRP functions as virulence determinant are manifested depending on the viral genetic background from which the CRP is expressed. Indeed, in *N. benthamiana*, the host plant in which the PMTV infection phenotype does not depend on the presence of the CRP gene (Savenkov et al., 2003), expression of CRP induces necrotic plant responses to PVX and TMV infections. Similarly, *N. tabacum* cv. Samsun plants infected with *Tomato aspermy virus* (*TAV*; genus *Cucumovirus*) exhibit mild systemic symptoms, whereas expression of *TAV* 2b protein from the TMV or PVX genomes triggered the hypersensitive response in this host (Li et al., 1999). It is plausible to assume that PMTV CRP, when expressed in the native viral genetic background, may form complexes with other PMTV-encoded products, thereby preventing strong response in *N. benthamiana* plants. Alternatively, TMV and PVX might provide functions which, when combined with PMTV CRP action, result in induction of a strong plant response. We cannot exclude the possibility that PMTV CRP acts in a dose-dependent manner: the observed strong plant response to CRP accumulation could be attributed to its overexpression from PVX and TMV vectors (Shivprasad et al., 1999).

When PMTV CRP was expressed from a BSMV genome in which the BSMV CRP ORF was replaced with that of PMTV, no detectable accumulation of BSMV was observed. This finding was unexpected, since the BSMV CRP is dispensable for virus replication and cell-to-cell movement in *N. benthamiana*, *C. amaranticolor* and barley (*Hordeum vulgare*). A mutant virus in which the gene encoding the BSMV CRP is deleted is able to infect barley plants...
systemically, but is restricted to the inoculated leaves of *N. benthamiana* (Petty et al., 1990; Yelina et al., 2002). The presence of translatable PMTV CRP gene in BSMV genome led to suppression of virus accumulation at a single cell level that represents an example of extreme virus resistance (Valkonen, 2002). This might imply that the PMTV CRP-induced response in *N. benthamiana* is greatly enhanced by BSMV infection. Interestingly, a similar extreme resistance to PMTV CRP-expressing BSMV was also found in *C. amaranticolor*, the local lesion indicator host of the virus. These data suggest that the PMTV CRP-induced plant response may be common for different host species. PMTV CRP and behaves as the virulence determinant in some properties of a cysteine-rich suppressor of gene silencing protein expressed in the plant cells. Importantly, expression of GFP-fused CRP in plant cells can be favourable (or neutral) for PMTV, TMV and PVX replication, but deleterious for BSMV replication.

In this study, while reporting the ability of PMTV CRP to dramatically enhance viral symptoms and disease severity of heterologous viruses, does not resolve the putative CRP function(s) in PMTV infection. The putative TMV ORF6-encoded protein shows properties similar to those of PMTV CRP and behaves as the virulence determinant in *N. benthamiana* but not in other hosts such as *N. tabacum* and tomato (*Lycopersicon esculentum*) (Canto et al., 2004). Therefore, assuming the possibility of a similar host-specific PMTV CRP action, further studies involving a variety of other PMTV host species are required to determine the role of this protein in the virus life cycle.

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