INTRODUCTION

Chrysanthemum virus B (CVB) belongs to the genus Carlavirus of the family Flexiviridae, characterized by monopartite, positive-sense, single-stranded RNA genomes encapsidated into flexuous filamentous virions (Adams et al., 2004; Martelli et al., 2007). An examination of the partial sequence of the CVB genome has revealed a gene arrangement typical of other carlaviruses (Levay & Zavriev, 1991). The 5'-proximal open reading frame (ORF) encodes the viral replicase and is followed by a triple gene block encoding movement proteins, an ORF for the capsid protein (CP) and a gene for a small cysteine-rich protein (CRP) of unknown function (Adams et al., 2004). Proteins related to the carlavirus CRP are found only in three other genera of the family Flexiviridae, namely Allexivirus, Vitivirus and Mandarivirus. In all of these genera, the CRP ORF is located in the 3'-proximal region of the viral genomic RNA.

All carlavirus CRPs contain a basic motif and a zinc finger-like motif positioned in the central part of the protein (Gramstat et al., 1990; Koonin et al., 1991). Moreover, these CRPs have been predicted to possess moderate sequence similarity to some cellular nucleic acid-binding proteins, implying that carlavirus CRP may bind nucleic acids (Koonin et al., 1991). Indeed, the CRP encoded by the carlavirus potato virus M has been shown to bind RNA and DNA in vitro (Gramstat et al., 1990). However, the nucleic acid-binding properties of the potato virus M CRP were not characterized in detail.
Previously, we demonstrated that the CVB 12 kDa CRP (p12 protein), when expressed from a potato virus X (PVX) vector in Nicotiana tabacum cv. Samsun (both with and without the N gene) or Nicotiana benthamiana plants, induced a hypersensitive response (HR) (Lukhovitskaya et al., 2005b). However, p12 alone was not able to cause an HR upon agroinfiltration (Lukhovitskaya et al., 2005b). Most known viral suppressors of RNA silencing exert dramatic effects on PVX by enhancing the severity of the symptoms, which often become necrotic (Brigneti et al., 1998; Pfeffer et al., 2002; Pruss et al., 1997; Yelina et al., 2002). However, p12 was not able to suppress post-transcriptional gene silencing initiated locally in patch agroinfiltration assays by either weak (ssRNA) or strong (dsRNA) inducers (Lukhovitskaya et al., 2005b). Interestingly, the effect of p12 on the PVX infection phenotype in tobacco depends on the plant genotype. Systemic spread of p12-expressing PVX was promoted in Nicotiana benthamiana leaves, one of three anchored primers and one of 30 different 13 mer arbitrary primers were used. PCR (30 s at 94 °C, 2 min at 40 °C and 30 s at 72 °C) was carried out for 40 cycles followed by 5 min elongation at 72 °C. Differentially expressed sequences were detected by separating PCR products in a 6 % polyacrylamide gel containing 8 M urea, with paired sets of reactions run side by side, followed by gel autoradiography. Selected fragments were recovered from the gel. In parallel, the corresponding region from the adjacent lane where the paired sample was resolved was also taken for DNA recovery. For SSCP analysis (Mathieu-Daudé et al., 1996), pairs of samples were reamplified with the primers that were used initially. To avoid normalization, we reduced the number of PCR cycles to five. Reamplified products were denatured in formamide and separated in a native Hydrolyn MDE gel (FMC BioProducts). After gel autoradiography, all confirmed fragments were ligated into pGEM-T (Promega). The inserts were reamplified and used in two parallel dot-blot hybridization reactions carried out with 32P-labelled cDNA probes synthesized from RNA samples initially taken for differential display analysis.

**METHODS**

**Recombinant clones.** The clone PVX-p12 has been described previously (Lukhovitskaya et al., 2005b). The mutants p12ZF and p12BM were obtained with specific primers using standard procedures. The coding sequence of Arabidopsis thaliana SCL6-IV (locus At4g00150) was amplified on a cDNA template with primers 5′-CAGCAGGAAAGAAGAAGAAGAGCTTCAAGAAGAC-3′ and 5′-GCTCTAGAAATCCATCGCACTGAGCAAAATAAAATCA-GAAAC-3′. The resulting product was cloned into pRT101 (Toepfer et al., 1999) as an Xhol–BamHI fragment. SCL6-IV and pre-miR171 expression cassettes from N. I. Lukhovitskaya and others (2001). For construction of the p12 gene, the p12 gene was amplified with primers 5′-CGGGATCCATGATGTTGAGATGATG-3′ and 5′-GGGCGGATCCAGGCGATTTATAAGGAC-3′, and the resulting product was digested with Xhol and NcoI and cloned into the similarly digested pRT-GFP-S65T (Solovyev et al., 2000). To construct the reverse-order GFP–p12 fusion gene, the p12 gene was amplified with primers 5′-CGGGATCCATGATGTTGAGATGATG-3′ and 5′-GGGCGGATCCAGGCGATTTATAAGGAC-3′, and the resulting product was digested with Xhol and NcoI and cloned into the similarly digested pRT-GFP-S65T (Solovyev et al., 2000). To construct the reverse-order GFP–p12 fusion gene, the p12 gene was amplified with primers 5′-CGGGATCCATGATGTTGAGATGATG-3′ and 5′-GGGCGGATCCAGGCGATTTATAAGGAC-3′, and the resulting product was digested with Xhol and NcoI and cloned into the similarly digested pRT-GFP-S65T (Solovyev et al., 2000).

**RESULTS**

**Role of the zinc finger motif and the basic motif found in p12 in HR induction**

Expression of CVB p12 in N. tabacum and N. benthamiana plants from a PVX vector has been shown to induce HR (Lukhovitskaya et al., 2005b). To investigate the role of the
p12 zinc finger motif and the basic motif in HR induction, we engineered two p12 mutants. One, p12ZF, carried two alanine amino acids replacing two of the four cysteine residues that are presumed to form the zinc finger structure (Fig. 1a). The other, p12BM, possessed four alanines replacing the positively charged arginine residues of the basic motif (Fig. 1a). The p12 sequence in the PVX-p12 construct (Lukhovitskaya et al., 2005b) was replaced by sequences for p12ZF and p12BM, and the two new constructs were termed PVX-p12ZF and PVX-p12BM, respectively. To exclude possible side effects of N gene-mediated acceleration of PVX-p12 infection (Lukhovitskaya et al., 2005b), PVX-p12ZF and PVX-p12BM as well as PVX and PVX-p12 were inoculated onto the leaves of N. tabacum cv. Samsun plants of a genotype that does not carry the N gene. Inoculated and upper uninoculated leaves were analysed by Northern and Western blotting 7 days post-inoculation (p.i.).

As shown previously, PVX-p12 induced an HR on inoculated leaves at 5 days p.i. and mosaic symptoms in systemically infected leaves, which became necrotic at 7 days p.i. (Fig. 1b). Later, the further development of an HR led to the death of the plant top (data not shown). This infection phenotype was very similar to that induced by PVX-p12 in N. benthamiana plants as described previously (Lukhovitskaya et al., 2005b). The PVX-p12ZF mutant induced mosaic symptoms typical of PVX infection on the upper uninoculated leaves at 7 days p.i. and no symptoms developed on inoculated leaves (Fig. 1b). No symptoms were observed in plants inoculated with the PVX-p12BM mutant (Fig. 1b).

Northern blotting revealed accumulation of the viral RNAs in both inoculated and systemically infected leaves of plants inoculated with PVX, PVX-p12 and PVX-p12ZF (Fig. 2, lanes 2–7). No virus-specific RNAs were detected in plants inoculated with PVX-p12BM (Fig. 2, lanes 8 and 9). The results of Northern blot analysis were confirmed by Western blotting with a PVX CP-specific antiserum (Fig. 2). The observation that PVX-p12ZF did not induce an HR suggested that the p12 zinc finger motif is required for HR induction.

We could not exclude the possibility that the inability of PVX-p12BM to infect plants was due to unwanted mutations that may have arisen from the plasmid manipulations and rendered the PVX genome elements essential for virus replication or cell-to-cell movement dysfunctional. Therefore, we constructed a p12BM-stop derivative in which a stop codon was introduced two codons downstream of the p12BM start codon by the substitution of two nucleotides. The p12BM sequence in the PVX-p12BM construct was replaced by that for p12BM-stop, and the new construct was termed PVX-p12BM-stop. As expected, PVX-p12BM-stop induced symptoms similar to those of wild-type (wt) PVX in systemically infected N. tabacum plants (data not shown). These data demonstrated that the expressed protein (p12BM) rather than the p12BM gene sequence itself suppressed PVX infection. A similar effect was observed when the CRP of potato mop-top virus was expressed from barley stripe mosaic virus (Lukhovitskaya et al., 2005a). Additionally, the inability of PVX-p12BM-stop to induce an HR implied that the p12 protein, but not the sequence encoding it, is involved in HR induction in PVX-p12-infected plants.

The finding that PVX-p12BM-stop moved systemically whereas PVX-p12BM did not suggested that the p12BM protein somehow inhibits PVX infection either in cis or in trans. To test this idea, N. tabacum plants were co-inoculated with PVX-p12BM and wt PVX at a 2 : 1 or 10 : 1 ratio with the expectation that PVX would not move systemically if p12BM acts in trans. However, mosaic

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**Fig. 1.** Influence of p12 and its mutants on PVX infection phenotype in N. tabacum. (a) Mutagenesis of CVB p12. Shading indicates the positions of the basic motif (BM) and putative zinc finger motif (ZF). Asterisks show the cysteine residues potentially involved in Zn ion coordination. Numbers in parentheses indicate the length of protein regions not shown here. For the mutants, the positions of the introduced alanine residues are shown. Dashes indicate residues that are identical to the wt protein sequence. (b) Leaves of plants inoculated with the DNA of 35S promoter constructs PVX, PVX-p12, PVX-p12ZF or PVX-p12BM. Leaves were photographed at 7 days p.i.
symptoms typical of PVX infection were observed on the upper leaves in all co-inoculation experiments (data not shown). Analysis of upper uninoculated leaves revealed that the p12 sequence was detected, as expected, in all samples from plants inoculated with PVX-p12, but not in control samples (i.e. mock-, wt PVX- and PVX-p12BM-infected) (Fig. 3). Moreover, the p12 sequence was detected in samples from upper uninoculated leaves of plants co-inoculated with PVX and PVX-p12BM regardless of the co-inoculation ratio. Therefore, these experiments showed that p12BM inhibits PVX infection in cis, but this effect can be alleviated by co-infection with wt PVX.

Differences between PVX-p12, PVX-p12ZF and PVX-p12BM could be due to differences in the stability of the wt protein and its mutants that would result in different accumulation levels of each. To compare the stability of the wt p12, p12ZF and p12BM proteins, we expressed these proteins in N. tabacum leaves by agroinfiltration and analysed their accumulation by Western blotting with a p12-specific antiserum. The wt p12 protein appeared to have a lower-than-expected mobility in the gel, migrating as an 18 kDa protein (Fig. 4). The mutants p12ZF and p12BM were found to accumulate to amounts comparable to those of the wt protein (Fig. 4). These data indicated that differences between PVX-p12, PVX-p12ZF and PVX-p12BM were not due to different stabilities of the p12, p12ZF and p12BM proteins.

Plant genes induced in response to PVX-p12 infection

As p12 somehow alters the plant’s response to PVX infection, we hypothesized that the expression of p12 from...
the PVX genome might modulate host gene expression. mRNA differential display was used in this study as an approach to identify plant genes that were induced or repressed in PVX-p12-infected *N. benthamiana* plants compared with PVX-infected plants. RNA was prepared from leaves systemically infected with PVX and PVX-p12. Samples were collected at 6 days p.i. at the onset of mosaic symptoms for both PVX-p12 and PVX, i.e. 1 day before the pronounced induction of HR. cDNA was amplified using a set of arbitrary PCR primers. The PCR products were resolved by PAGE and visualized by autoradiography.

As expected from previous studies (Liang & Pardee, 1992), the majority of PCR products were present at identical levels in samples derived from PVX- and PVX-p12-infected plants. However, over 170 differentially displayed PCR products were detected and isolated for further characterization. Reamplified PCR products were subjected to SSCP analysis (Mathieu-Daude et al., 1996), followed by cloning and dot-blot hybridization with probes synthesized from total RNA prepared from PVX- and PVX-p12-infected plants. Ultimately, 12 cDNA clones were identified and subjected to sequencing. Thus, RNA transcripts corresponding to 12 differentially displayed bands were significantly upregulated in PVX-p12-infected plants (Table 1). No differentially downregulated mRNAs were identified. It should be noted that, due to the limited number of arbitrary primers used, our differential display analysis was not intended to be exhaustive.

The results of BLAST sequence analysis of the 12 cDNA clones are summarized in Table 1. Five cDNAs (4AA7, 7AA6, 1AA12, 8AA2 and 3GA24) corresponded to genes induced in plants under oxidative stress conditions, whereas another (4CA22) is needed for tolerance to hydrogen peroxide in yeast (Table 1). Three of the oxidative stress genes (4AA7, 7AA6 and 3GA24) and one other gene (3AA2) are known to be induced upon other types of abiotic stress or treatments with signalling substances (salicylic acid and jasmonic acid) (Table 1). The identification of two ribosomal protein genes (6CA24 and 6GA24; Table 1) was not surprising because ribosomal proteins have been found among proteins induced in plants under stress conditions (Vranova et al., 2002; Zago et al., 2006). Importantly, six of the 12 cDNA clones were associated directly with the plant response to pathogens. Two of these genes are expressed upon induction of systemic acquired resistance (SAR) (4AA7 and 3AA2), whereas four genes are associated with HR induction in plants (7AA6, 1AA12, 8AA2 and 3AA3) (Table 1). One gene of the latter group, 3AA3, is known to be essential for HR development in response to a bacterial elicitor (Lu et al., 2003).

Therefore, our PCR differential display analysis revealed that p12, when expressed from PVX, modulates the host gene expression profile. Moreover, seven out of 12 genes upregulated in response to PVX-p12 infection were associated with plant responses to pathogen attack or abiotic stress.

**p12 does not interfere with the microRNA pathway**

We have shown previously that p12 is unable to suppress RNA silencing in patch agroinfiltration assays (Lukhovitskaya et al., 2005b). In this study, we investigated whether p12 could interfere with microRNA (miRNA) biogenesis or function. To this end, we used the *SCL6-IV* gene, the known target of miR171 (Llave et al., 2002). The *SCL6-IV* gene belongs to the *Scarecrow* family of plantspecific transcription factors involved in a wide range of developmental processes in plants (Di Laurenzio et al., 1996). The *SCL6-VI* gene contains a functional miRNA-responsive element that is complementary to miR171 (Llave et al., 2002). miR171 recruits an effector complex to direct cleavage of the target *SCL6-IV* mRNA. This cleavage event gives rise to an unstable 5’-terminal mRNA part and a stable 3’-terminal mRNA part that can be detected by Northern blotting (Llave et al., 2002).

An *Agrobacterium tumefaciens* infiltration assay was used to test whether p12 could prevent cleavage of the *SCL6-IV* mRNA. In this assay, co-expression of *SCL6-IV* and pre-miR171 resulted in *SCL6-IV* mRNA cleavage (Fig. 5, lane 3). Co-infiltration for expression of *SCL6-IV*, pre-miR171 and the p12 gene also resulted in *SCL6-IV* mRNA cleavage (Fig. 5, lane 5). In contrast, infiltration with potato virus *A* HC-Pro, an RNA silencing suppressor known to interfere with miRNA-mediated cleavage (Kasschau et al., 2003), prevented the cleavage and significant accumulation of the 3’-terminal part of *SCL6-IV* mRNA (Fig. 5, lane 4; compare also lanes 3 and 4). Collectively, these data suggested that p12 does not interfere with miRNA-mediated digestion of cellular mRNA that contains the miRNA-responsive element.

**Subcellular localization of p12 and its mutants**

As the p12 zinc finger motif is required for HR induction and the basic motif seems to interfere with infectivity of the virus, we next investigated the subcellular localization of p12 and its two mutants. The GFP coding sequence was fused to the p12 gene to give either GFP–p12 or p12–GFP translational fusions. *N. benthamiana* leaves were infiltrated with *A. tumefaciens* containing the binary vector with the 35S::GFP–p12 or 35S::p12–GFP cassette. Epidermal cells of the infiltrated leaves were examined at 2 days p.i. by CLSM. p12–GFP was observed mostly in the nucleus (Fig. 6a), but a minor amount of the protein localized to small granules dispersed in the cytoplasm (Fig. 6d). Interestingly, the distribution of p12–GFP in the nucleus was not uniform, showing a granular pattern (Fig. 6a). GFP–p12 gave a much lower fluorescent signal than p12–GFP and showed a localization pattern similar to that of p12–GFP (data not shown).

To investigate the role of the zinc finger motif and the basic motif in protein localization, the coding sequences of the two p12 mutants were inserted into the binary vector to
Table 1. Sequence analysis of identified cDNA clones corresponding to mRNAs upregulated in PVX-p12-infected leaves in comparison with PVX-infected plants

<table>
<thead>
<tr>
<th>cDNA clone</th>
<th>Similar to:</th>
<th>GenBank accession no.</th>
<th>Sequence similarity</th>
<th>Encoded protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>4AA7</td>
<td>N. tabacum SAR8.2b</td>
<td>AAP23856</td>
<td>Expect 0.0</td>
<td>Associated with systemic acquired resistance*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Identities 99 %</td>
<td></td>
</tr>
<tr>
<td>7AA6</td>
<td>N. tabacum PDR1</td>
<td>AB109389</td>
<td>Expect 7e–77</td>
<td>ABC transporter†</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Identities 85 %</td>
<td></td>
</tr>
<tr>
<td>1AA12</td>
<td>N. tabacum cDNA clone</td>
<td>AJ344591</td>
<td>Expect 5e–21</td>
<td>Induced upon oxidative stress‡</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Identities 95 %</td>
<td></td>
</tr>
<tr>
<td>4CA22</td>
<td>N. tabacum transketolase 1</td>
<td>DQ198165</td>
<td>Expect 5e–67</td>
<td>Transketolase 1§</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Identities 90 %</td>
<td></td>
</tr>
<tr>
<td>6CA24</td>
<td>N. tabacum cDNA clone</td>
<td>DW002693</td>
<td>Expect 1e–103</td>
<td>Similar to acidic ribosomal protein P1all</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Identities 97 %</td>
<td></td>
</tr>
<tr>
<td>3AA2</td>
<td>N. tabacum PR-Q</td>
<td>M29868</td>
<td>Expect 3e–99</td>
<td>PR-Q§</td>
</tr>
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<td></td>
<td></td>
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<td>Induced by AvrPto and hydrogen peroxide treatment#</td>
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<td></td>
<td></td>
<td></td>
<td>Identities 91 %</td>
<td></td>
</tr>
<tr>
<td>3AA3</td>
<td>N. benthamiana cDNA clone</td>
<td>AY310790</td>
<td>Expect 6e–19</td>
<td>Necessary for HR development**</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Identities 100 %</td>
<td></td>
</tr>
<tr>
<td>1GA29</td>
<td>N. tabacum cDNA clone</td>
<td>EB450178</td>
<td>Expect 9e–65</td>
<td>Membrane-anchored ubiquitin-fold protein 2††</td>
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<td></td>
<td></td>
<td></td>
<td>Identities 99 %</td>
<td></td>
</tr>
<tr>
<td>1CA25</td>
<td>N. tabacum cDNA clone</td>
<td>EB435045</td>
<td>Expect 0.0</td>
<td>Similar to pectin methylesterase inhibitor‡‡</td>
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<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
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<td>Expect 6e–72</td>
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<td>AM780783</td>
<td>Expect 5e–48</td>
<td>Similar to ribosomal protein L14e¶</td>
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<td></td>
<td></td>
<td></td>
<td>Identities 95 %</td>
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</table>

*Proteins of the SAR8.2 family, associated with SAR, are locally and systemically induced in response to TMV infection and salicylic acid treatment (Alexander et al., 1992), as well as after different biotic and abiotic stresses (Lee & Hwang, 2003). SAR8.2 is induced in a MEK2-dependent manner (Kim & Zhang, 2004).

†PDR1 is a member of the superfamily of ABC (ATP-binding cassette) transporters involved in the active ATP-dependent transport of many chemically and structurally unrelated compounds across membrane bilayers (Higgins, 1992). In different plant species, PDR1 expression is induced in response to hydrogen peroxide (Vandenabeele et al., 2003), elicitors of bacterial and fungal nature, and also jasmonic acid, but not salicylic acid (Sasabe et al., 2002). PDR1 is involved in the secretion of anti-pathogen terpenoids (Jasinski et al., 2001).

§Identified as one of the mRNAs induced under conditions of oxidative stress (Vranova et al., 2002). Similar to the β-subunit of ATP citrate lyase, the enzyme is involved in synthesis of acetyl-CoA in the cytoplasm. Its mRNA is upregulated in the early stages of the HR in pepper (Suh et al., 2001).

||Transketolase 1 is one of the enzymes of the pentose phosphate pathway. Although no role in defence responses in plants has yet been documented for this enzyme, enzymes of the pentose phosphate pathway are necessary for yeast tolerance to hydrogen peroxide-induced oxidative stress (Juhnke et al., 1996).

||Encodes a protein similar to a Solanum tuberosum acidic ribosomal protein P1a-like protein.

¶PR-Q is a pathogenesis-related protein with the properties of an acidic chitinase, thought to be involved in plant defence against phytopathogenic fungi (Stintzi et al., 1993). As with other PR proteins, PR-Q is expressed in response to stress or pathogen attack (Payne et al., 1990).

#Identified as one of the mRNAs induced by the bacterial elicitor AvrPto. Expression of this gene is also induced in tobacco following hydrogen peroxide treatment and cell-death induction (Vandenabeele et al., 2003; Zago et al., 2006). The functions of the encoded protein in tobacco and other plant species are unknown.

**Identified as one of 79 genes necessary for the development of the HR (Lu et al., 2003). The encoded product is similar to a photosystem II 10 kDa polypeptide, the function of which in photosynthesis is unknown.

††The cDNA encodes a protein similar to A. thaliana membrane-anchored ubiquitin-fold protein 2 (AtMUB2). AtMUB2 is localized to the plasma membrane; the functions of this protein are unknown (Downes et al., 2006).

‡‡Encodes a protein similar to pectin methylesterase inhibitor protein (Giovane et al., 2004), which is associated with ripening.

§§Encodes a protein exhibiting a high degree of similarity to Solanum peruvianum small heat-shock protein Hsp20.1, which belongs to a family of small stress-induced proteins containing an α-crystallin domain. In addition to expression induction of proteins of this family under heat-shock conditions, they are induced by abiotic stresses of varying kinds (Sun et al., 2002).

¶¶¶Encodes a protein similar to Solanum tuberosum ribosomal protein L14e.
SCL6-IV vector without an expression cassette. RNA was analysed with an -specific probe. Staining of rRNA carried out to verify equal gel loading.

**Fig. 5.** Northern blot analysis of the potential of p12 to influence miRNA functions. *N. benthamiana* leaves were infiltrated with *Agrobacterium* cultures mediating expression of the genes indicated above the lanes. C, Control infiltration with a binary vector without an expression cassette. RNA was analysed with an SCL6-IV-specific probe. Staining of rRNA carried out to verify equal gel loading.

give 35S::p12BM–GFP and 35S::p12ZF–GFP constructs. After agroinfiltration with these constructs, epidermal cells were examined at 2 days p.i. p12BM–GFP was almost completely excluded from the nucleus and localized to cytoplasmic inclusions of different sizes, with most of the protein concentrated in a few inclusions (Fig. 6e, g). In contrast, p12ZF–GFP basically retained the localization pattern of p12–GFP, residing predominantly in the nucleus and also in the cytoplasm, where it was associated with irregularly shaped small structures (Fig. 6h–j). The proportion of the protein localized to the cytoplasm was considerably higher for p12ZF–GFP compared with p12–GFP (Fig. 6d, h). These data indicated that the p12 basic motif, but not the putative zinc finger motif, is needed for localization of the protein to the nucleus.

**CVB p12 binds nucleic acids**

The nucleic acid-binding activity of purified recombinant p12, p12ZF and p12BM was examined *in vitro* using a GMSA. In our experiments, increasing amounts of p12 were incubated with radioactively labelled run-off transcripts representing the 5′ 340 nt of the GFP mRNA (5′GF) or the corresponding DNA fragment.

In the presence of Mg$^{2+}$, as the amount of p12 was increased, the 5′GF DNA was progressively retarded (Fig. 7a, left upper panel). However, formation of the complexes was rather inefficient because only a small proportion of DNA was found in the retarded complexes at a protein : DNA ratio of 20 : 1 (Fig. 7a, left upper panel). In the presence of Zn$^{2+}$, the formation of fully retarded complexes was stimulated and, additionally, intermediate-sized complexes were observed (Fig. 7a, right upper panel). Similar results were obtained in DNA-binding experiments with two other DNA probes (data not shown).

Next, we investigated DNA-binding activity of the two p12 mutants. p12ZF formed the fully retarded complexes in the presence of both Mg$^{2+}$ and Zn$^{2+}$ (Fig. 7a, middle panels). No intermediate-sized complexes were seen (Fig. 7a, middle panels). Conversely, p12BM did not form the fully retarded complexes, but intermediate-sized complexes were observed in the presence of Zn$^{2+}$ (Fig. 7a, lower panels). This analysis demonstrated that both motifs found in the p12 sequence contribute to DNA binding: the basic motif is needed for formation of the fully retarded complexes, whilst the putative zinc finger motif is required for Zn$^{2+}$-dependent formation of the intermediate-sized complexes.

GMSAs for p12 binding to RNA demonstrated that both single-stranded 5′GF RNA and double-stranded 5′GF RNA were shifted to the fully retarded complexes in the presence of both Mg$^{2+}$ and Zn$^{2+}$ (Fig. 7b, upper panels, and Fig. 7c). p12ZF formed complexes with ssRNA (Fig. 7b, middle panel), whereas p12BM did not bind ssRNA (Fig. 7b, lower panels). Therefore, we concluded that binding of p12 to RNA is determined by the basic motif, which is therefore able to determine interactions with ssRNA, dsRNA or dsDNA, whereas Zn$^{2+}$-dependent binding determined by the p12 zinc finger motif is specific to DNA.

To compare the affinity of p12 for DNA and RNA, we used a competition assay. Radiolabelled 5′GF RNA was mixed with unlabelled competitor DNA before the addition of p12. Conversely, radiolabelled 5′GF DNA was mixed with unlabelled competitor RNA. The nucleic acid–protein complexes were then analysed using a GMSA (Fig. 7d and e). In this assay, the competitor RNA efficiently competed with DNA in formation of the fully retarded complexes in the presence of Mg$^{2+}$ (Fig. 7d). In a reciprocal competition assay, the competitor DNA efficiently competed with RNA in the presence of both Mg$^{2+}$ and Zn$^{2+}$ (Fig. 7e). These data are consistent with our conclusion that the basic motif exhibits no preference for the type of nucleic acid. On the other hand, in DNA-binding experiments carried out in the presence of Zn$^{2+}$ and the competitor RNA, a considerable amount of the intermediate-sized complexes was seen, even when competitor RNA was added at a 10:1 molar ratio (Fig. 7d). This observation suggests that, in the presence of Zn$^{2+}$, p12 exhibits a strong preference for DNA.
DISCUSSION

We analysed the role of two motifs conserved in carlavirus CRPs, namely the basic motif and the zinc finger motif (Gramstat et al., 1990; Koonin et al., 1991), in subcellular localization of CVB p12, its binding to nucleic acids and HR induction upon expression from a PVX vector. The subcellular localization of p12, which was mainly in the nucleus, was determined by the basic motif, which could therefore represent part of the nuclear location signal, resembling numerous known highly basic nuclear location signal sequences (Pemberton & Paschal, 2005). The HR induced upon p12 expression in PVX-infected plant tissue (Lukhovitskaya et al., 2005b) was found to be associated with the zinc finger motif. Interaction of p12 with nucleic acids in vitro involved both the basic motif and the zinc finger motif, although they were found to play distinct roles. Whilst the putative zinc finger determined Zn²⁺-dependent DNA-specific binding activity, the p12 basic motif determined a rather weak Zn²⁺-independent interaction with both RNA and DNA with no preference for the type of nucleic acid. Collectively, our analyses of the p12 mutants showed that the zinc finger motif is involved in both DNA binding and HR induction, whereas the basic motif is required for localization of the protein to the nucleus.

In positive-stranded RNA plant viruses, the functional significance of nuclear localization has been uncovered for only a few viral proteins. One such example is provided by the groundnut rosette virus (GRV; genus Umbravirus) ORF3 protein, which is able to deliver nucleolus protein fibrillarin to the cytoplasm, where it participates in the formation of protein complexes with viral genomic RNA destined for transport through the plant vascular system (Kim et al., 2007a, b; Ryabov et al., 1998). Other examples include two viral silencing suppressors, namely the 2b protein of cucumber mosaic virus (CMV; genus...

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**Fig. 6.** Subcellular localization of GFP-fused p12 and its mutants, imaged by CLSM 2 days after agroinfiltration of *N. benthamiana* leaves. (a–d) Localization of p12–GFP (a) and mRFP expressed to visualize the cell shape and nucleus (b), and superimposition of the two images (c). In (d), the cell imaged in (a) is shown with a higher gain setting, showing the presence of a minor amount of p12–GFP in the cytoplasm. (e–g) Localization of p12BM–GFP (e) and mRFP (f), and superimposition of the two images (g). (h–j) Localization of p12ZF–GFP (h) and mRFP (i), and superimposition of the two images (j). All images were reconstructed from a series of optical sections. Bars, 20 μm.
Cucumovirus) and polerovirus P0 protein, which inhibit the activity of the nuclear protein AGO1, a small RNA-guided endonuclease that belongs to the Argonaute protein family, playing one of the key roles in RNA silencing pathways (Baumberger et al., 2007; Bortolamiol et al., 2007; Zhang et al., 2006). We have recently shown that CVB p12 is unable to suppress silencing induced locally by either dsRNA or ssRNA (Lukhovitskaya et al., 2005b). In this paper, we demonstrated that p12 does not interfere with processing of the miRNA precursor resulting in production of functional miRNA or with miRNA interaction with AGO1 and the catalytic activity of their complex. Therefore, p12 appears to be functionally dissimilar to 2b and P0, although we cannot rule out the possibility that, similar to the GRV ORF3 protein, p12 is involved in the long-distance movement of viral genomic RNA in plants.

On the other hand, the data reported in this paper suggest another p12 function. We found that p12, when expressed from a PVX vector, was able to alter the host plant...
expression profile, which preceded HR development. Importantly, seven out of 12 mRNA species, identified in our limited differential display survey as being upregulated in response to PVX-p12 infection, encoded pathogenesis-related and/or stress-related proteins. Two of these proteins are known to be expressed upon SAR induction, indicating that SAR initiated by viral infection of inoculated leaves (Durrant & Dong, 2004), although not leading by itself to an HR, can contribute to changes in the transcription profile in upper leaves. To date, signalling pathways activated in plants in response to compatible and incompatible pathogens, phytohormones and environmental stress (heat shock, oxidative stress, drought, salinity, etc.) are known largely to overlap (Bostock, 2005; Fujita et al., 2006). Thus, activation of such pathways by p12 may cause HR development, as has been described for other systems (Eulgem, 2005).

Thus, this study established a link between p12 nuclear localization, its DNA-binding activity and its ability to induce an HR. It should be noted, however, that p12 is unlikely to be able to act as a transcription factor that directly activates promoters of particular plant defence genes, as our data showed that p12 binds DNA sequences non-specifically. We propose that p12-dependent upregulation of defence-related genes and subsequent HR responses are mediated by other factors. This hypothesis is supported by our earlier observations that a p12-induced HR requires PVX infection (Lukhovitskaya et al., 2005b). As an example of a potential mechanism of p12 action, it is possible that the plant response to virus infection might normally be strictly limited by the host cell to prevent an excessive defence reaction leading to plant tissue death, and that p12 is able to hinder the mechanism of such a control resulting in an unlimited response including an extensive HR in PVX-p12-infected plants (Lukhovitskaya et al., 2005b).

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References


