Nucleo-cytoplasmic shuttling of the beet necrotic yellow vein virus RNA-3-encoded p25 protein

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The protein p25 encoded by beet necrotic yellow vein virus (BNYVV) RNA-3 is involved in symptom expression of infected plants. Confocal microscopy analysis of wild-type and mutated p25 fused to GFP and transiently expressed in BY-2 tobacco suspension cells identified a nuclear localization signal (NLS) in the N-terminal part of the protein. Functionality of the NLS was confirmed by pull-down assays using rice and pepper importin-α. Furthermore, it was demonstrated that p25 contains a nuclear export sequence sensitive to leptomycin B. The nuclear export signal (NES) was characterized by mutagenesis. A GFP–p25 fusion protein expressed during a BNYVV infection of Chenopodium quinoa leaves had the same subcellular localization as observed during transient expression in BY-2 cells. The symptom phenotype induced by expression of GFP–p25 during infection was similar to that induced by wild-type virus. Studies with mutated derivatives of GFP–p25 revealed that symptom phenotype was altered when the subcellular localization of GFP–p25 was modified.

INTRODUCTION

Beet necrotic yellow vein virus (BNYVV; family Benyvirus) is a positive-stranded RNA virus with rod-shaped virions and an unusual multi-component genome. BNYVV is transmitted by the soil-borne plasmodiophoromycete fungus Polymyxa betae and is responsible for rhizomania disease of sugar beet, which is characterized by extensive rootlet proliferation from the main taproot and other abnormalities. BNYVV genes required for basic housekeeping functions such as genome replication, cell-to-cell movement, packaging and suppression of post-transcriptional gene silencing reside on RNA-1 (6746 nt) and RNA-2 (4812 nt) (Dunoyer et al., 2002; Tamada, 1999). These genome components are necessary for virus multiplication in Chenopodiaceae hosts and are sufficient for local lesion formation on leaves of a diagnostic species such as Chenopodium quinoa (Quillet et al., 1989; Tamada & Abe, 1989). All field isolates of BNYVV contain two additional RNAs. RNA-4 (1467 nt) carries information necessary for vector transmission of the virus (Tamada & Abe, 1989), while RNA-3 (1775 nt) controls rhizomania symptom expression on natural host (Tamada et al., 1999) and local lesion phenotype on leaves (Jupin et al., 1992; Tamada et al., 1989). Some BNYVV isolates contain a fifth RNA (RNA-5, 1342–1347 nt) that can also influence symptom severity in a synergistic fashion with RNA-3 (Tamada et al., 1996). BNYVV RNA-3 encodes a ~25 kDa protein (p25) and there is extensive evidence that p25 is an important determinant of leaf symptom phenotype (Jupin et al., 1992; Tamada et al., 1989, 1999). It also governs BNYVV invasion of the plant root system and induction of rootlet proliferation in sugar beet (Tamada et al., 1999). p25 contains a highly basic N-terminal domain and an acidic C-terminal domain (Jupin et al., 1992). No significant sequence similarity between p25 and currently known proteins has been detected.

BNYVV, like virtually all plant RNA viruses, replicates in the cytoplasm but immuno-gold electron microscopy observations have shown that p25 is present both in the nuclei and in the cytoplasm of infected leaf cells (Haebeler & Stussi-Garaud, 1995). In this paper, we have studied sequences that control the movement of p25 in and out of the nucleus. We have used a biolistic approach to deliver transient expression vectors encoding p25 fused to the jellyfish green fluorescent protein (GFP) to cultured plant cells. This study was performed in order to characterize the nuclear sequences that govern the movement of BNYVV p25 in and out of the nucleus and to determine the pathways involved in such nuclear translocations.
METHODS

Clones and infectious transcripts. Clones and procedures for production of run-off transcripts of RNA-3 have been described (Lauber et al., 1998; Quillet et al., 1989). Fusions between p25 and GFP (Reichel et al., 1996) were generated by overlap extension mutagenesis (Ho et al., 1989) of cloned cDNA by using PCR. In the replicon containing p25-GFP, the p25 termination codon was replaced by CCC and the GFP initiation codon by GGG. In the replicon containing GFP-p25, the GFP termination codon was replaced by CCC and the p25 initiation codon by GGG. The 3’ minimal viral promoter of replicon GFP-p25 was then replaced with the full-length 3’ minimal viral promoter of replicon GFP–p25 was then replaced by CCC and the GFP initiation codon by GGG. In the replicon containing GFP–p25, the GFP termination codon was replaced by CCC and the p25 initiation codon by GGG. The 3’ minimal viral promoter of replicon GFP-p25 was then replaced with the full-length 3’ sequence of RNA-3 using a BamHI–HindIII restriction fragment to create repGFP–p25. GFP–p25ACt was produced from GFP–p25 by subcloning the sequence between the RNA-3 SmaI and BamHI sites into repGFP (Erhardt et al., 2000). The deletion mutant used to construct GFP–p25ABA was from Jupin et al. (1992). The p25 deletion mutants GFP–p25ANt and –p25ASst were produced by eliminating the sequences between the SmaI and filled-in BamHI sites, SmaI and Stul sites, respectively.

Point mutations in p25 were created by overlap extension mutagenesis. pCKeGFP-derived constructs were produced by PCR amplification of repGFP–p25 using a sense primer corresponding to the 3’ end of the GFP gene and XbaI-containing reverse primers complementary to the 3’ end of the p25 gene except for pCKGFP–p25ACT, which was obtained with a reverse primer providing a stop codon after aa 103, followed by an XbaI restriction site. Fragments were placed between the cauliflower mosaic virus 35S promoter and terminator in pCKeGFP (Gaire et al., 1999; Reichel et al., 1996) by using BglII and XbaI sites. All constructs and PCR fragments were characterized by restriction enzyme digestion and sequenced with an ABI prism 373 DNA sequence analyzer (Applied Biosystems) or a Hitachi 3100 Genetic Analyser (Applied Biosystems) using a BigDye Terminator sequencing kit (Applied Biosystems) and specific primers.

Infection of leaves and analysis of infection products. Virus infection procedures were as described previously (Erhardt et al., 2000; Lauber et al., 1998). Polypeptides containing the sequence of p25 and/or GFP were immuno-detected on Western blots with a p25-specific antiserum (Niesbach-Klosgen et al., 1994) and mouse monoclonal anti-GFP antibody (Roche), respectively.

BY-2 transient expression. GFP-fusion proteins were transiently expressed in BY-2 tobacco suspension cells (Nicotiana tabacum cv. Bright Yellow 2) maintained as described (Banjoko & Trelease, 1995). Cells were subcultured every 7 days and harvested 3 days after medium renewal for biolistic bombardment. The harvested cells were filtered onto Whatman discs and placed on 0.8% agar Murashige-Skoog (MS) media plates supplemented with 0.25 M mannitol for 2–4 h. Particle preparation and biolistic assays were performed using identical settings for single-track mode and excitation/emission wavelengths (488/505–545 nm) for GFP. Image processing was carried out with LSM510 version 2.5 (Zeiss) and Photoshop 5.5 (Adobe system).

In vitro GST pull-down assays. Rice GST-importin-z and -f were provided by A. Baba, E. Herzog and T. Hohn (Friedrich Miescher Institute, Basel, Switzerland). Peper GST-importin-z was provided by U. Bonas and B. Szürek (Institut fur Genetik, Martin-Luther Universität Halle-Wittenberg, Halle, Germany). All constructs were cloned into pGEX (Pharmacia Biotech) and expressed in E. coli BL21 as described (Herzog et al., 2000). A rapid translation system (RTS100; Roche Molecular Biochemicals) was used, as described by the supplier, to produce identical amounts of wild-type or mutated 35S-radiolabelled p25 to perform the test. In vitro GST-pull-down assay conditions were as described (Herzog et al., 2000) using identical amounts of GST-tagged proteins and 1% Tween and 1 mM DTT in PBS buffer to perform the assay. Aliquots of the bound protein were separated on 12% SDS-PAGE and the 35S-labelled proteins were visualized by autoradiography.

RESULTS

GFP–p25 is located in the nucleus and the cytoplasm of transfected cells

Transient expression vectors based on the pCK plasmid (Gaire et al., 1999; Reichel et al., 1996) were designed to drive expression of full-length or partially deleted p25 proteins fused to GFP (Fig. 1a) from a constitutive 35S promoter. Plasmids were introduced into BY-2 tobacco cells by means of biolistic gene transfer and the fluorescent cells were analysed by CLSM 16 h post-bombardment. All the biolistic experiments were performed at least twice in the same conditions. In each case, 30 randomly chosen GFP-expressing BY-2 cells were observed. Only very minor variations (<1 in 30) in cytoplasmic versus nuclear localization were detected for a given experimental treatment. In preliminary experiments, transient expression of p25–GFP and GFP–p25 constructs revealed that the two fusion proteins showed similar localizations (data not shown). The GFP–p25 fusion construct was chosen for further studies so as to retain the same initiation codon sequence context for the different p25 deletion constructs (see below).

Expression of GFP alone in BY-2 cells (Fig. 1b) resulted in the localization of the fluorescent protein both in the cytoplasm and in the nucleus of the cell, most probably by passive diffusion between the two compartments (Chiu et al., 1996). The 52 kDa fusion protein GFP–p25 also localized to both the nucleus and the cytoplasm (Fig. 1b). Since the diffusion exclusion limit of nuclear pores (for review see Gorlich et al., 1996; Gorlich & Kutay, 1999) has been shown to be around 9 nm, corresponding to a diffusion limit up to ~60 kDa for a globular protein (Bonner, 1978; Mattaj & Englmeier, 1998; Paine et al., 1975),
this observation suggests that GFP–p25 could be actively transported into the nucleus, although we cannot exclude the possibility that some passive diffusion could also occur in parallel, as observed for MAPK (Adachi et al., 1999).

To map a putative NLS within p25, the subcellular localization of different p25 deletion mutants was analysed. The deletion mutants GFP–p25ΔCt (aa 104–219 deleted), GFP–p25ΔBA (aa 104–196 deleted), GFP–p25ΔNt (aa 1–102 deleted) and GFP–p25ΔSSSt (aa 1–70 deleted) were tested as described above. In GFP–p25ΔCt (Fig. 1b) and GFP–p25ΔBA (data not shown) the fusion proteins were localized exclusively in the nuclei of expressing cells, indicating that an NLS is present within the first 102 aa of p25. GFP–p25ΔNt was never observed in the nucleolus of transfected cells. These data also suggest that a sequence in the C-terminal region either acts to retain part of the GFP–p25 in the cytoplasm or promotes export of GFP–p25 from the nucleus to the cytoplasm.

Deletion of the N-terminal domain of p25 (GFP–p25ΔNt) resulted in a mainly cytoplasmic localization of the fusion protein (Fig. 1b), although detection of a faint green fluorescent signal in the nucleus indicated that some passive diffusion of the 39 kDa protein may have taken place. However, even if some passive diffusion of GFP–p25ΔNt occurred the fusion protein no longer accumulated abundantly in the nucleus (compare GFP–p25 and GFP–p25ΔNt, Fig. 1b).

Deletion mutant GFP–p25ΔSSSt (Δ1–70) was also tested in order to map precisely the putative NLS within the first 102 aa of p25. Deletion of the first 70 aa (GFP–p25ΔSSSt) eliminated the NLS-like activity and the protein was only observed in the cytoplasm of the transfected cells (Fig. 1b).

**The sequence KRIRFR is responsible for nuclear targeting of p25 and can partially drive the nuclear localization of a GFP-GUS reporter gene**

p25 contains the sequence motif 57KRIRFR62 (Haeberle & Stussi-Garaud, 1995), which resembles the basic NLS motifs of the simian virus 40-type (Gorlich & Kutay, 1999;
Kalderon et al., 1984). Mutations resulting in short deletions or substitutions of basic residues for alanine were created in this putative NLS (Fig. 2a) in the context of the constitutively nuclear-targeted protein GFP–p25\textsubscript{D\textsubscript{Ct}}. The resulting constructs were tested in transient expression assays as described above. Replacement of all of the basic amino acids within the putative NLS by alanine (57AAIAFA62 in GFP–p25\textsubscript{D\textsubscript{Ct}-m1, Fig. 2a) strongly inhibited nuclear targeting (Fig. 2b). Single alanine substitution of each basic amino acid residue within the NLS motif distinguished three classes of mutants: (i) the mutations K57A in GFP–p25\textsubscript{D\textsubscript{Ct}-m2} and R58A in GFP–p25\textsubscript{D\textsubscript{Ct}-m3} (Fig. 2b) partially blocked nuclear import of the protein, resulting in a distribution of the proteins between the nucleus and the cytoplasm of the cells; (ii) the mutation R60A in GFP–p25\textsubscript{D\textsubscript{Ct}-m4} (Fig. 2b) did not interfere with nuclear targeting, whereas (iii) the mutation R62A in GFP–p25\textsubscript{D\textsubscript{Ct}-m5} completely blocked nuclear import (Fig. 2b), indicating that this particular fusion protein was also unable to diffuse into the nucleus.

A double-point mutation in GFP–p25\textsubscript{D\textsubscript{Ct}-m6} (K57A and R58A) did not significantly diminish nuclear import of the fusion protein, which was located in the nucleus and cytoplasm (Fig. 2b) in relative amounts similar to those observed for the single mutants GFP–p25\textsubscript{D\textsubscript{Ct}-m2} and GFP–p25\textsubscript{D\textsubscript{Ct}-m3}. Replacement of the KIRIRFR sequence by the sequence RS in GFP–p25\textsubscript{D\textsubscript{Ct}-m7} (Fig. 2b) gave similar results as those observed with GFP–p25\textsubscript{D\textsubscript{Ct}-m1}. In both cases the fusion protein was largely confined to the cytoplasm.

All of the aforesaid mutations were then analysed in the context of full-length p25 fused to GFP. Analysis of the subcellular localization following bombardment revealed that, in every case, the nuclear–cytoplasmic partitioning was as observed in the GFP–p25\textsubscript{D\textsubscript{Ct}} context, except that there was an even stronger cytoplasmic localization of the mutant GFP–p25–m1, which exhibited no detectable fluorescent signal in the nuclei (Fig. 2b).

To provide further evidence for active nuclear import of p25, we generated a cDNA construct (GFP–p25\textsubscript{D\textsubscript{Ct}-GUS;
Fig. 2a) in which the GUS sequence was fused to the C terminus of GFP–p25 deleted of its C-terminal domain. This was done in order to create a very large fusion protein (about 106 kDa) that should be totally blocked from entering the nucleus by passive diffusion. As a control, we used the nuclear import-deficient construct GFP–p25ΔCt-m7-GUS (Fig. 2a). The N-terminal region of p25 containing the putative NLS relocated a significant portion of the GFP–p25ΔCt-GUS fusion protein to the nucleus of the transfected cells (Fig. 2b), whereas the GFP–p25ΔCt-m7-GUS fusion protein was exclusively localized in the cytoplasm (Fig. 2b). Western blotting analysis conducted on protein extracted from BY-2 cells expressing GFP–p25ΔCt-GUS and GFP–p25ΔCt-m7-GUS revealed that both proteins were of the expected size (not shown). Thus, these results represent additional evidence that p25 enters the nucleus by an active NLS-dependent process.

**p25 binds importin-α in vitro**

Import of proteins into the nucleus is often mediated by the importin-β-related transport pathway. This pathway involves either a direct interaction of the nuclear-targeted protein with importin-β or -α, which binds to importin-β. To obtain additional information on the 25 nuclear import mechanism, possible interactions between p25 and pepper (Szurek *et al.*, 2001) or rice (Jiang *et al.*, 1998) importin-α and -β (Matsuki *et al.*, 1998) were assayed by *in vitro* pull-down experiments using GST-tagged importins. Radiolabelled wild-type p25 expressed in *E. coli* was able to bind to *E. coli*-expressed importin-α from rice and pepper, whereas p25 carrying the m1 mutation described above was unable to bind importin-α from either species (Fig. 3).

![Fig. 3. In vitro interaction between p25 and rice or pepper GST-importin-α.](image)

Neither protein interacted with the matrix (R), with GST (G) or with rice GST-importin-β (rIβ) (Fig. 3). Similarly, no interaction was observed between identical amounts of p25 carrying the m5 mutation (R62A) and the pepper importin-α (pIα). Co-immunoprecipitation assays using an anti-GST serum gave identical results (data not shown). Thus, these data are consistent with the hypothesis that nuclear entry of p25 is mediated by importin-α and involves the p25 sequence 57KRIRFR62.

**C-terminal domain of p25 mediates active nuclear export**

As noted above, the fact that the NLS sequence in GFP–p25 does not localize the fusion protein exclusively to the nuclear compartment suggests that p25 also contains a sequence which acts either (i) to retain a fraction of the fusion protein in the cytoplasm (for example, by interacting with a cytoplasmic protein or structure) or (ii) to actively promote transport of nuclear GFP–p25 back to the cytoplasm. The observation that C-terminal deletion constructs such as GFP–p25ΔCt and GFP–p25ΔB localize exclusively to the nucleus (see Fig. 1b) suggests that the putative domain, which promotes movement of GFP–p25 to the cytoplasmic compartment and/or its retention there, resides within the C-terminal half of p25. To investigate this hypothesis more, we examined the effect of leptomycin B (LMB) on the subcellular distribution of different GFP–p25 fusion constructs. LMB inhibits the activity of CRM1/Exportin1, a receptor that mediates the nuclear export of proteins containing hydrophobic-rich nuclear export sequences (NES) (Fornerod *et al.*, 1997; Haasen *et al.*, 1999; Kudo *et al.*, 1998). Thus, a protein that shuttles actively between the nucleus and the cytoplasm in the absence of LMB will strongly accumulate in the nucleus upon LMB treatment if its export depends on the CRM1/Exportin1 pathway.

LMB treatment of BY-2 cells expressing GFP did not significantly influence its subcellular distribution between the nuclear and cytoplasmic compartments (Fig. 4). On the other hand, when the cells were bombarded with a construct expressing GFP fused to the well-characterized HIV rev nuclear export sequence (GFP–NESrev+; Haasen *et al.*, 1999), the fluorescent signal was stronger in the cytoplasm than in the nucleus and LMB treatment caused the GFP–NESrev+ to localize predominantly in the nuclear compartment (Fig. 4). A GFP construct fused to the rev NES, which had been mutated to abolish its export activity (GFP–NESrev−; Haasen *et al.*, 1999), displayed similar subcellular distribution in the presence and the absence of LMB. We conclude that LMB inhibits CRM1/Exportin1-mediated nuclear export in BY-2 cells in our experimental conditions.

The effect of LMB on nuclear export of p25 was investigated using the mutant GFP–p25An in this mutant, which lacks the N-terminal p25 NLS sequence (see above), can...
move into the nucleus only by passive diffusion. GFP–p25ΔNt was present in the both the nucleus and the cytoplasm in the absence of LMB, with the cytoplasmic fluorescent signal stronger than that observed in the nucleus (Fig. 4). However, after LMB treatment the relative distribution of fluorescence in the two compartments altered in favour of the nucleus (Fig. 4). We conclude that a CRM1/Exportin1 NES is situated somewhere in the C-terminal part of p25.

Characterization of p25 nuclear export sequence

Nuclear export sequences are rich in hydrophobic residues although there is not a strict consensus motif (for review see Macara, 2001). Analysis of the C-terminal p25 sequence revealed a region rich in hydrophobic residues between aa 164 and 196. To study the effect of this hydrophobic domain (HD, Fig. 5) upon nuclear export, we fused it to the C-terminal part of the GFP sequence, leading to GFP–HD. When expressed in bombarded BY-2 cells, GFP–HD behaved exactly as did GFP–NESrev + (see Fig. 4), i.e. only low levels of GFP–HD were detected in the nucleus in the absence of LMB but the protein accumulated strongly in the nucleus following LMB treatment (Fig. 5). We conclude that the HD domain (aa 164–196) contains an NES.

To map precisely the sequence motif responsible for nuclear export, we examined the effect of LMB treatment on the subcellular localization of GFP fused to three different portions of the HD domain subdomains, HDa, HDb and HDc (Fig. 5). GFP–HDa and GFP–HDc were observed in both the nucleus and the cytoplasm of transfected cells and their subcellular distribution was not affected by LMB treatment (Fig. 5). On the other hand, GFP–HDb was in relatively low abundance in the nucleus in the absence of LMB, whereas LMB treatment strongly induced its accumulation in the nucleus (Fig. 5). We conclude that the NES sequence is localized within the HDb subdomain (residues 169–183, Fig. 5).

Sequence alignment of the p25–HDb domain with several well-characterized NES sequences revealed a NES-like motif consisting of the hydrophobic residues V<sup>169</sup>, V<sup>172</sup>, V<sup>175</sup> and V<sup>178</sup> (Fig. 6a). A set of HDb mutants was produced in which each of the aforesaid hydrophobic residues was replaced by an alanine (Fig. 6b). Alanine substitution mutants targeting three other hydrophobic residues (L<sup>174</sup>, V<sup>181</sup> and L<sup>182</sup>; Fig. 6b) that were not part of the NES-like motif were produced as well. The mutant HDb sequences were fused to GFP and the effect of LMB treatment on the subcellular distribution of fluorescence in bombarded BY-2 cells was studied as before. GFP–NESrev + and GFP–NESrev – provided positive and negative controls, respectively.

As shown in Fig. 6(b), alanine substitution of any of the four valine residues in the putative NES motif (mutants GFP–HDbA1, –HDbA2, –HDbA4 and –HDbA5) abolished the LMB-mediated redistribution of the fluorescence from the cytoplasm to the nuclear compartment, that is the fluorescence associated with compared to the situation observed in the absence of LMB treatment. Alanine substitution of the three other residues mentioned above (mutants GFP–HDbA3, –HDbA6 and –HDbA7), on the other hand, did not exhibit LMB-mediated redistribution of the fluorescence (Fig. 6b). We conclude that V<sup>169</sup>, V<sup>172</sup>, V<sup>175</sup> and V<sup>178</sup> form part of the NES signal and that each is necessary for efficient CRM1/Exportin1-mediated export of p25.
Altered subcellular localization of p25 is associated with changes in virus symptoms

The foregoing experiments were all carried out by transient expression of p25-based constructs in single cells and in the absence of other viral proteins. To assess the subcellular distribution of p25 in the context of a BNYVV infection of whole plants, we produced a transcription vector in which the p25 gene of BNYVV RNA-3 was replaced by the sequence encoding the GFP–p25 fusion protein. Previous experiments have shown that BNYVV RNA-3 transcripts encoding GFP fusion proteins can multiply in leaves when co-inoculated with BNYVV RNA-1 and -2 (Stras12) and that the fusion protein is expressed (Erhardt et al., 2000). Inoculation of C. quinoa leaves with Stras12 plus an RNA-3 transcript encoding GFP fused to the N terminus of p25 (repGFP–p25) produced numerous fluorescent rings (2–3 mm diameter) on the inoculated leaves by 4–5 days post-inoculation (d.p.i.) (data not shown). The rings appeared at positions where local lesions subsequently became visible in natural light.

CLSM observations of epidermal cells in the fluorescent rings produced by inoculation with Stras12 plus repGFP–p25 revealed that fluorescence was present in both the nuclear and cytoplasmic compartments (Fig. 7c, lower panel), as observed when GFP–p25 was expressed independently in BY-2 cells (Table 1). Thus, these experiments illustrate that neither the method of introduction of the fusion protein into plant cells (biolistics of BY-2 cells versus mechanical inoculation of leaves) nor the expression vector used (DNA plasmid versus a virus replication-dependent RNA replicon) influences the subcellular distribution of p25. We can also conclude that p25’s subcellular localization is not altered by the presence of other BNYVV proteins during its expression.

Similar infection experiments with Stras12 plus repGFP–p25 containing various mutations in p25 moiety also resulted in the appearance of fluorescent rings at lesion sites. Northern blot hybridization of total leaf RNA with riboprobes specific for BNYVV RNA and GFP (Erhardt et al., 2000) revealed that all the RNA-3 transcripts containing GFP-mutant p25 constructs replicated to levels comparable to those observed with wild-type GFP–p25 (data not shown). Similarly, Western blot analysis of total extracted proteins using GFP- or p25-specific antisera detected an immunoreactive protein of the expected size in every case (data not shown). CLSM observations of epidermal cells in the fluorescent rings revealed that, for each construct, the subcellular localization of the virally

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**Fig. 5.** Effect of LMB upon the location of GFP fused to different p25 C-terminal hydrophobic domains. (a) The p25 hydrophobic sequences (HD, aa 164–196; HDa, aa 164–175; HDb, aa 169–183; HDc, aa 177–196) containing putative nuclear export signal were fused to the C terminus of GFP. (b) CLSM analysis of typical transfected BY-2 cells in the absence (left panel) or presence (right panel) of 40 nM LMB. C, NC and N>C refer to location of the GFP marker in the cytoplasm, in both nuclear and cytoplasmic compartments or a predominant nuclear location of the GFP marker in the nucleus, respectively. Bar, 10 μm.
expressed GFP-mutant p25 fusion protein was identical to that observed when the corresponding fusion was expressed out of viral context by bombardment of BY-2 cells (Table 1 and Fig. 7).

As noted above, RNA-3 is an important determinant of symptoms on BNYVV-infected hosts. Thus, on leaves of *C. quinoa* an inoculum containing only BNYVV RNA-1 and -2 produces mild chlorotic local lesions or CS symptoms similar to those presented in Fig. 7a (Quillet et al., 1989; Tamada & Abe, 1989). The YS phenotype is dependent on expression of p25 (Jupin et al., 1992; Tamada & Abe, 1989). Thus, CS symptoms were observed following inoculation with Stras12 plus RNA-3 in which the p25 gene had been deleted (rep0) or replaced with the GFP gene (repGFP; Fig. 7a, Table 1). Importantly, inoculation of leaves with Stras12 plus repGFP–p25 produced YS local lesions (Fig. 7c) similar to those provoked by wild-type RNA-3, illustrating that the presence of the N-terminal GFP moiety does not interfere with the effect of p25 on leaf symptoms (compare Fig. 7b and c).

Table 1 summarizes the lesion types provoked by inoculation of *C. quinoa* leaves with Stras12 plus repGFP–p25 containing various p25 mutant forms. It can be seen (Table 1) that all of the mutant constructs in which the GFP–p25 was localized principally in the cytoplasm produced CS symptoms. Three of the constructs tested, repGFP–p25ΔCt, repGFP–p25ABA and repGFP–p25nes– (GFP–p25 containing the mutated NES sequence YMYACLVNTV from visna maedi virus [VMV] rev proteins (Meyer et al., 1996)), localized strongly to the nuclear compartment when inoculated along with RNA-1 and -2 to leaves (see Table 1 and Fig. 7d, e). RepGFP–p25nes–induced CS local lesions, whereas repGFP–p25ΔCt induced necrotic local lesions (Nec; Table 1). The reason why repGFP–p25ΔCt and repGFP–p25ABA but not repGFP–p25nes– provoked necrotic lesions is not known, but one

Table 1. Lesion types provoked by inoculation of *C. quinoa* leaves with Stras12 plus repGFP–p25 containing various p25 mutant forms.

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Fig. 6. (a) Sequence alignment of BNYVV p25 putative NES with other characterized NESs derived from lipoma preferred partner (LPP; Petit et al., 2000), Ras-related nuclear binding protein 1 (Ran-BP1; Richards et al., 1996), cyclin B1 (Toyoshima et al., 1998), mitogen-activated protein kinase kinase (MAPKK; Fukuda et al., 1996), human immunodeficiency virus (Fischer et al., 1995) and visna maedi virus (VMV) rev proteins (Meyer et al., 1996). Conserved residues are shaded in grey. (b) Alanine scanning mutagenesis of p25 HDb domain fused to GFP sequence. Hydrophobic residues (highlighted in grey) were replaced by alanine and the localization of the resulting fusion proteins was monitored in BY-2 transfected cells in the presence or absence of LMB. C, NC and N>C refer to location of the GFP marker in the cytoplasm, in both nuclear and cytoplasmic compartments or a predominantly nuclear localization of GFP marker in the nucleus, respectively. ND, Not determined.

Fig. 7. Effect of p25 on leaf symptoms in the context of a BNYVV infection of *C. quinoa* 6 d.p.i. *C. quinoa* leaves were inoculated with Stras12 supplemented with repGFP (a), full-length RNA-3 (b), RNA-3 expressing wild-type GFP–p25 (c) or the mutated forms GFP–p25nes– (d), GFP–p25ΔCt (e) and GFP–p25ΔCt-m1 (f). CLSM images showing the subcellular localization of the GFP in cells within the local lesions from each leaf are shown below. GFP marker appeared green whereas chloroplasts appeared red. No GFP fluorescence was observed when wild-type RNA-3 construct was used (not shown).
possibility is that non-specific binding to nuclear DNA of repGFP–p25<sup>ΔCt</sup> and repGFP–p25<sup>BA</sup>, which (unlike repGFP–p25<sup>nes-</sup>) have a high pI, causes cell death. However, whatever the cause of the toxicity of GFP–p25, our findings show that alteration of the wild-type distribution of GFP–p25 between the nuclear and cytoplasmic compartments is accompanied by alterations in p25-related symptoms during virus infection.

**DISCUSSION**

Comparison of the subcellular localization patterns of various truncated GFP–p25 constructs revealed that the N-terminal residues 1–103 are required for nuclear translocation of p25. Alanine-scanning mutagenesis identified the sequence<sup>57</sup>KRIRFR<sup>62</sup> as the p25 NLS motif, with amino acids K<sub>57</sub>, R<sub>58</sub> and R<sub>62</sub> being of particular importance. This monopartite NLS was able to drive nuclear import of a 106 kDa GFP–p25<sup>ΔCt-GUS</sup> protein. Using GST-importin-a<sub>a</sub> fusion proteins, we have demonstrated efficient binding of wild-type p25 to importin-a<sub>a</sub>, an interaction that depends on the presence of the above-described NLS. No interaction was observed when the NLS was mutated, indicating that no secondary NLS is present in p25.

We also showed that nuclear export of GFP–p25<sup>ΔNt</sup> involves a domain containing hydrophobic residues (164–196) and is sensitive to the export inhibitor LMB. Thus, sensitivity to LMB indicates that the CRM1 pathway actively exports p25. The subcellular distribution of wild-type GFP was not affected by the export inhibitor (Fig. 4).

Although wild-type GFP localized almost equally to both the nucleus and cytoplasm due to the diffusion of the protein, GFP fused to the C-terminal domain of p25 (GFP–p25<sup>ΔNt</sup>) or to the HIV rev NES (GFP–NESrev + ), which is recognized by the plant CRM1/Exportin1 receptor (Haasen et al., 1999), was localized mainly in the cytoplasm and only weakly in the nucleus (Fig. 4). These results suggest that the low molecular masses of GFP–p25<sup>ΔNt</sup> (40 kDa) and GFP–NESrev + (30 kDa) permit their diffusion into the nucleus (Bonner, 1978; Gorlich & Kutay, 1999) but, once inside the nucleus, most of the protein is exported back into the cytoplasm by the CRM1/Exportin1 pathway. Our findings indicate that the CRM1/Exportin1-dependent export sequence is localized in the C-terminal part of p25 and encompassed within the sequence<sup>169</sup>VYMVCLVN<sup>TV</sup>. When this latter was replaced by the sequence<sup>169</sup>AYM<sup>ACLVNTV</sup><sup>178</sup>, virally expressed GFP–p25<sup>nes-</sup> protein accumulated abundantly in the nucleus (Fig. 7d).

Transport of bacterial proteins into the nucleus of eukaryotes (i.e. nuclear targeting of pathogen-encoded proteins) has been demonstrated in several instances. Thus, the AvrB<sub>3</sub> protein of Xanthomonas<sup>campestris</sup>, which localizes to the host cell nucleus after infection (Szurk et al., 2002), carries two NLS that interact with the nuclear receptor machinery (Szurk et al., 2001) and a transcription activation domain required for mesophyll cell hypertrophy in susceptible plants (Marois et al., 2002); the nuclear-targeted Agrobacterium<sup>tumefaciens</sup> 6b protein induces phytokinin-dependent division of cells and alteration of leaf morphology by interacting with the putative host

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Symptoms</th>
<th>p25 cellular location</th>
<th>Viral expression</th>
<th>Transient expression</th>
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<tr>
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<td>N</td>
<td>NT</td>
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</tr>
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</table>

*rep0, Bleykasten-Grosshans et al. (1997).
†Also see Haeberle & Stussi-Garaud (1995).

Table 1. Effect of symptoms on C. quinoa leaves of virally expressed GFP–p25 fusion proteins; comparison of cellular locations for the fusion proteins following viral or transient expression

N, Nuclear; C, cytoplasmic; N/C, nuclear and cytoplasmic; CS, chlorotic spot; YS, yellow spot; Nec, necrotic spot; NT, not tested; NA, not applicable.
transcription activator NtSIP1 (Kitakura et al., 2002). Plant virus proteins such as CMV 2b (Lucy et al., 2000) and TEV NLb (Li et al., 1997) have also been shown to be addressed actively to the nucleus of infected cells. However, to our knowledge, together with GRV ORF3 protein (Ryabov et al., 2004), p25 is one of the first proteins encoded by a plant RNA virus, which has been shown to shuttle between the nucleus and the cytoplasm. Furthermore, our findings suggest that p25 belongs to the family of proteins whose activity is regulated by its cellular localization.

When p25 is able to access both the cytoplasm and the nuclear compartment, increase of symptom severity on leaves is observed. Production of the necrotic symptom phenotype (Nec) is poorly understood but may reflect interference with normal nuclear processes such as those described for AvrBs3 (Marois et al., 2002) or generalized toxicity of the highly basic fusion protein in the nucleus of C. quinoa cells as no necrosis was observed on Tetragonia expansa (unpublished results). However, whatever the mechanism, the nec symptoms must be because of the nuclear localization of the fusion protein, as no such symptoms were observed when the NLS sequence was mutated (compare Fig. 7e and f) or when the export sequence was present (compare Fig. 7e and c). Future studies of the p25 nucleo-cytoplasmic shuttling upon the BNYVV infection will aim at understanding the functions of such a protein in the rhizomania process.

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REFERENCES


