Mapping of functional region conferring nuclear localization and karyopherin α-binding activity of the C2 protein of bhendi yellow vein mosaic virus

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Bhendi yellow vein mosaic disease is caused by a complex consisting of a monopartite begomovirus associated with a β-satellite. The C2 protein of bhendi yellow vein mosaic virus (BYVMV) is a suppressor of post-transcriptional gene silencing and also functions as a transcriptional activator. To explore the molecular mechanisms of its nuclear trafficking and self-interaction, fusion proteins of fluorescent proteins with wild-type or mutated constructs of BYVMV C2 were expressed in tobacco protoplasts. Analyses revealed that the BYVMV C2 nuclear localization signal (NLS) was located in the N terminus of the protein, comprising aa 17–31 of C2. NLSs are recognized by a class of soluble transport receptors termed karyopherins α and β. The BYVMV C2 NLS was found to be necessary for this protein’s interaction with its nuclear import mediator, karyopherin α, ensuring its nuclear localization. Nevertheless, when deleted, C2 was found in both the cytoplasm and the nucleus, suggesting NLS-independent nuclear import of this protein. Homotypic interaction of BYVMV C2 was also found, which correlates with the nuclear localization needed for efficient activation of transcription.

INTRODUCTION

Geminiviruses are a family of small circular ssDNA viruses with a unique geminate particle structure. These viruses replicate via dsDNA intermediates by a rolling-circle mechanism in plant cell nuclei (Gutierrez, 1999; Jeske, 2009; Lazarowitz et al., 2004; Nawaz-ul-Rehman & Fauquet, 2009). Although the majority of begomoviruses are bipartite with the genome comprising two similar-sized DNA components (DNA A and B), some have only a monopartite genome resembling DNA A of the bipartite begomoviruses. The DNA A component encodes a replication-associated protein that is essential for viral DNA replication, a replication enhancer protein, the coat protein and a transcriptional activator protein (TrAP). The DNA B component encodes a nuclear shuttle protein and a movement protein, both of which are essential for systemic infection of plants (Gafni & Epel, 2002; Hanley-Bowdoin et al., 1999).

Bhendi yellow vein mosaic virus (BYVMV) is a monopartite begomovirus whose genome is associated with a β-satellite (previously known as DNA β) (Jose & Usha, 2003).

The β-satellite is essential for inducing symptoms of yellow vein in Ageratum (Saunders et al., 2000, 2004), leaf curl in cotton (Briddon et al., 2001), yellow vein mosaic in bhendi (also known as okra) (Gopal et al., 2003; Kumar et al., 2006), yellow leaf curl in tomato (Zhou et al., 2003) and yellow vein in Vernonia (Packialakshmi & Usha, 2011).

The C2 protein of geminiviruses plays a variety of roles. All positional homologues of this protein in begomoviruses have three functional domains: a basic domain with a bipartite nuclear localization signal (NLS) at the N terminus, a non-classical zinc finger in the middle and an acidic activation domain at the C terminus (Chowdareddy et al., 2009; Jeske, 2009; Sunter & Bisaro, 1997). Tomato golden mosaic virus (TGMV) AL2 and beet curly top virus (BCTV) L2 have been shown to interact with and inactivate adenosine kinase (Wang et al., 2003). Transgenic expression of TGMV AL2 and BCTV L2 causes a genome-wide reduction in cytosine methylation, thus reversing transcriptional gene silencing (Buchmann et al., 2009). African cassava mosaic virus-Kenya AC2 (Voinnet et al., 1999), tomato yellow leaf curl China virus C2 (Dong et al., 2003), mungbean yellow mosaic virus (MYMV) AC2 (Trinks et al., 2005), TGMV AL2 and BCTV L2 (Wang
et al., 2005) function as suppressors of post-transcriptional gene silencing. Tomato yellow leaf curl virus C2 binds to ssDNA in a sequence non-specific manner (Noris et al., 1996). C2 acts as a transcriptional activator and transactivates the transcription of late viral genes (Haley et al., 1992; Sunter & Bisaro, 1992). In the case of the curtoviruses beet severe curly top virus (BSCTV) and spinach curly top virus (SCTV), the transcription activation domain is absent in C2 and hence it does not function as a transcriptional activator (Baliji et al., 2007; Hormuzdi & Bisaro, 1995). BSCTV C2 interacts with S-adenosylmethionine decarboxylase and attenuates its degradation, suppressing DNA methylation-mediated gene silencing (Zhang et al., 2005). Transactivation of the host genes has also been demonstrated in the case of TGMV AL2, where the activity of the cytokinin-responsive promoters is increased (Baliji et al., 2010). MYMV AC2 activates several components of the plant transcriptome (Trinks et al., 2005). Tomato yellow leaf curl Sardinia virus (TYLCSV) C2 interacts with the COP9 signalosome complex and thus interferes with its derubylation activity (Lozano-Durán et al., 2011).

The 143 aa C2 protein of BYVMV plays a vital role in transcription activation of late viral gene promoters, and also acts as a weak suppressor of post-transcriptional gene silencing (Gopal et al., 2007). The activity of C2 protein as a transcriptional activator occurs in the host-plant cell nucleus, where the virus transcribes and replicates its genome. Therefore, a NLS domain in the C2 amino acid sequence is a prerequisite to ensuring its entrance into the nucleus.

All eukaryotic cells transport a variety of molecules into the nucleus, including metabolites and proteins as well as ribosomal subunits, certain RNAs and ribonucleoproteins. The process of signal-mediated nuclear import is now well established for proteins. A typical nuclear protein contains a transferable basic NLS, which is recognized by soluble transport receptors, termed karyopherins or importins α and β (Mosammaparast & Pemberton, 2004; Sorokin et al., 2007). Karyopherin α directly binds the basic NLS sequence and interacts with karyopherin β via its N-terminal region. Small proteins of less than 40–60 kDa can diffuse passively through the nuclear pore complex, whereas larger proteins cannot move freely from the cytoplasm to the nucleus. Although there is no unique consensus sequence for NLSs, most can be classified into two groups: the simian virus 40 (SV40) large T-antigen NLS (PKKKRKV) type (Kalderon et al., 1984) and the bipartite motif type that consists of two basic regions separated by a variable number of amino acid residues, exemplified by the nucleoplasmmin NLS, KR-X<sub>10</sub>-KKKL (Gafni & Epel, 2002).

Whilst it is evident that C2 interacts with a number of cellular proteins to execute its multiple roles in the geminivirus replication cycle, it is unclear how the activities of this viral protein are regulated. Studies of some of these interactions have shown that the C2 homologue AL2 can interact with itself to form dimers and higher-order multimers (Yang et al., 2007). Self-interaction correlates with nuclear localization of AL2 complexes and the ability to activate transcription efficiently. A demonstration of the roles of C2 and an elucidation of the mechanisms involved might enhance our understanding of the propagation of BYVMV and help in the development of a new approach to prevent the disease caused by this virus.

RESULTS

Intracellular localization of BYVMV C2 in tobacco protoplasts

First, we determined the intracellular localization of BYVMV C2 protein in plant cells by fusing its DNA coding sequence in frame to the gene encoding enhanced yellow fluorescent protein (EYFP), an enhanced mutated derivative of GFP, or to another derivative with cyan fluorescence, enhanced cyan fluorescent protein (ECFP). To control the position of the tag relative to BYVMV C2, EYFP was fused to the N terminus (EYFP–C2) and ECFP to the C terminus (C2–ECFP) of C2. Fused chimaeric genes were driven by a cauliflower mosaic virus 35S promoter.

Identification of the NLS motif in BYVMV C2

The N terminus of BYVMV C2 is rich in basic amino acids. Multiple sequence alignment of the C2 proteins of a number of begomoviruses with CLUSTALW (Larkin et al., 2007) showed the presence of a conserved stretch of basic amino acids characteristic of a NLS motif (Dong et al., 2003). The putative BYVMV C2 NLS resembles the SV40 large T-antigen NLS (Kalderon et al., 1984), which is not as common in TrAP proteins of geminiviruses as the bipartite one. To identify the functional NLS sequence of BYVMV C2, the residues in the putative NLS (Fig. 1b) were replaced individually by site-directed mutagenesis. C2 mutants carrying individual substitutions of K17I, K21T, K24T, K25L, R28T, R29G, R30I and R31L were obtained using ECFP-tagged C2 as template and introduced into tobacco protoplasts for expression. The localization pattern of the tagged proteins was studied using confocal microscopy. Analysis of the subcellular localization pattern of these mutants revealed that residues K21, K24, R25 and R30 play a vital role in nuclear transport, as substitution of these amino acids altered the localization pattern such that these mutant C2 proteins localized to both the cytoplasm and the nucleus (Fig. 3a–d). Substitution of K17, R28, R29 and R31 did not have any effect on the nuclear localization. However, aggregation was found in the nucleus in all cases (Fig. 3e–h).
The subcellular localization of ECFP-tagged truncated versions of C2: Tr1 (aa 1–57), Tr2 (aa 1–70), Tr3 (aa 1–90), Tr4 (aa 1–100), Tr5 (aa 31–143) and del-NLS (lacking aa 17–31) (Fig. 1a), were investigated. Tr1, Tr2, Tr3 and Tr4 all localized exclusively to the nucleus, whereas the mutant in which the putative NLS had been deleted (del-NLS) and Tr5 were found in both the nucleus and the cytoplasm (Fig. 4).

C2 protein interacts with itself and with karyopherin α1

The interaction of C2 with itself and with karyopherin α1 was demonstrated directly in planta by fluorescence resonance energy transfer (FRET) analysis using confocal microscopy, which allows the detection of protein interactions within living cells, as well as the subcellular localization of the interacting proteins. EYFP–karyopherin α1 (Kunik et al., 1999; Mizrachy et al., 2004; Yaakov et al., 2011) or EYFP–C2 with either ECFP–C2 or ECFP–C2 del-NLS were transiently co-expressed in tobacco protoplasts and their location within the protoplasts was determined. In all cases except for ECFP–C2 del-NLS, the interacting partners were found to co-localize in the nucleus. ECFP–C2 del-NLS alone was found to localize in both the nucleus and the cytoplasm (Fig. 5). FRET between these proteins was detected by acceptor photobleaching. Fig. 6 indicates the co-localization and energy transfer between EYFP–C2 and ECFP–C2 and between EYFP–karyopherin α1 and ECFP–C2 in the nucleus. However, there was no energy transfer between ECFP–C2 del-NLS and EYFP–karyopherin α1. Quantification of the ECFP signal after photobleaching of EYFP revealed an increase in the intensity of the donor fluorescence with a FRET efficiency (E_F) of 15.97 and 24.5 % for EYFP–karyopherin α1/ECFP–C2 and EYFP–C2/ECFP–C2, respectively (Fig. 6e), which is indicative of FRET. In contrast, ECFP–C2 del-NLS/EYFP–karyopherin α1 revealed a negligible increase in donor fluorescence intensity. Co-expression of EYFP and ECFP did not yield detectable FRET, indicating that there was no interaction between these two proteins and that the cases described earlier, where FRET was detected, were due to interactions between the proteins to which they were fused.

DISCUSSION

We identified and characterized the karyophilic nature of the C2 protein of BYMV and a sequence of amino acids, the NLS, in its N terminus, needed for its nuclear import. This protein is a homologue of related proteins of similar sequence in other geminiviruses that are known to function

Fig. 1. (a) A schematic representation of the different mutants of BYVMV C2 protein used in this study. The positions of the amino acid residues encoded by each construct are indicated above the boxes. Number 1 indicates the first ATG in the C2 protein. All constructs encoded a fusion of ECFP (hatched box) with either a full-length (1) or mutated (2–7) C2 protein: 1, Wild-type C2; 2, Tr1 (aa 1–57); 3, Tr2 (aa 1–70); 4, Tr3 (aa 1–90); 5, Tr4 (aa 1–100); 6, Tr5 (aa 31–143); 7, del-NLS (lacking aa 17–31). The dashed line indicates internal deleted amino acids. (b) The N-terminal 40 residues of BYVMV C2 are shown, with the basic amino acids that were mutated in the substitution mutants indicated in uppercase and underlined.

Localization pattern of truncated versions of C2

The subcellular localization of ECFP-tagged truncated versions of C2: Tr1 (aa 1–57), Tr2 (aa 1–70), Tr3 (aa 1–90), Tr4 (aa 1–100), Tr5 (aa 31–143) and del-NLS (lacking aa 17–31) (Fig. 1a), were investigated. Tr1, Tr2, Tr3 and Tr4 all localized exclusively to the nucleus, whereas the mutant in which the putative NLS had been deleted (del-NLS) and Tr5 were found in both the nucleus and the cytoplasm (Fig. 4).

Fig. 2. Subcellular localization of EYFP–C2 (a) and C2–ECFP (b) in Nicotiana tabacum protoplasts. The ECFP signal is shown in blue and the EYFP signal in yellow. Plastid autofluorescence was filtered out in (a) and (b). All images are projections of several confocal sections.
as transcription activators and are thus expected to be located in the nucleus of the host-plant cell. To analyse the subcellular localization of BYVMV C2 protein in the absence of other viral proteins, plasmids expressing C2 fused to ECFP and EYFP were constructed.

Monitoring the intracellular localization of the fluorescently tagged BYVMV C2 protein did indeed reveal its clear nuclear localization (Fig. 2). To identify the amino acid domain responsible for this karyophilic activity, known as the NLS, we carried out sequence alignment of C2 proteins of a number of begomoviruses (Chowda-Reddy et al., 2009; Dong et al., 2003) using CLUSTALW software, revealing a conserved putative NLS motif (17KVQHKEAKRVHRRR31) rich in basic amino acids and located within the first 31 N-terminal residues of the protein. This NLS resembles monopartite NLS sequences comprising only one basic amino acid-rich motif. The specific amino acid residues required for nuclear import of the C2 protein were determined by mutating the basic amino acids in the cluster at aa 17–31. None of these mutations completely abolished the nuclear import of this protein. Some caused the protein to be located in both the nucleus and the cytoplasm, whilst others did not affect its nuclear localization at all (Fig. 3). This type of distribution differs from that described previously for the C2 homologue (AC2) in the bipartite geminivirus TGMV, where the wild-type protein was found in both the nucleus and the cytoplasm (Wang et al., 2003). C2, known as a pathogenicity factor, is a multifunctional protein, which might indicate that not all roles suggested for this protein in one group of geminiviruses are shared with related proteins in others. Recently, the C2 proteins of TYLCSV and BCTV were shown to interact with the COP9 signalosome protein complex and disrupt its activity over CULLIN1, interfering with the function of the CULLIN1-based SCF ubiquitin E3 ligase (Lozano-Durán & Bejarano, 2011; Lozano-Durán et al., 2011). This might explain why, in some geminiviruses, not all of the C2 protein is needed exclusively in the nucleus as suggested for BYVMV C2. The fact that deleting the entire NLS did not completely abolish the nuclear localization of the C2 protein may be explained by the small size of this protein, which might not have a visible effect on the intracellular localization of ECFP alone (Fig. 5) distributed between the cytoplasm and the nucleus. Nevertheless, due to the importance of the C2 protein in the virus life cycle, we suggest that there might be another, as yet unidentified, nuclear localization domain that can redirect a small portion of the C2 protein into the nucleus in the absence of the main NLS sequence. BYVMV C2 localizes predominantly to the nucleus of the

Fig. 3. Subcellular localization of the BYVMV C2 mutants in tobacco protoplasts. Results are shown for the ECFP–C2 mutants R30I (a), K21T (b), K24T (c), R25L (d), K17I (e), R28T (f), R29G (g) and R31L (h). The ECFP signal is blue and plastid autofluorescence is red.
Fig. 4. Subcellular localization of C2 truncation mutants in tobacco protoplasts. Results are shown for the ECFP–C2 mutants Tr1 (a), Tr2 (b), Tr3 (c), Tr4 (d), Tr5 (e) and del-NLS (f). The ECFP signal is blue and plastid autofluorescence is red.

Fig. 5. Subcellular co-localization of C2 with itself and with karyopherin α1. (a, b) ECFP–C2 co-expressed with EYFP–C2 or EYFP–karyopherin α1, respectively, in N. tabacum protoplasts. (c) EYFP–karyopherin α1 co-expressed with ECFP–C2 del-NLS in N. tabacum protoplasts. (d) EYFP alone co-expressed with ECFP del-NLS in N. tabacum protoplasts. The ECFP signal is blue, the EYFP signal is yellow and the signal produced by comparable levels of the co-localizing proteins is white; plastid autofluorescence is red. All images are projections of several confocal sections.
cells, consistent with its proposed nuclear role in transcriptional regulation.

In the present study, we demonstrated that BYVMV C2 requires an intact NLS for nuclear localization and that disruption of the NLS abolishes the exclusive nuclear localization, leading to both cytoplasmic and nuclear localization. This is consistent with the function of C2 as a transcription effector, as has been found for other begomovirus C2 proteins (Yang et al., 2007). Such localization requires the involvement of the cytoplasmic-soluble receptor, karyopherin α (Mosammaparast & Pemberton, 2004). Therefore, the effect of omitting the BYVMV C2 NLS on its interaction with karyopherin α1 was investigated. FRET analyses showed a positive interaction between karyopherin α1 and BYVMV C2 protein, indicating that karyopherin α1 mediates C2 nuclear import; in contrast, mutated C2, in which the NLS domain was deleted, appeared to be both cytoplasmic and nuclear (Fig. 5c), and the interaction between it and karyopherin α1 was not detectable (Fig. 6c). The fact that a BYVMV C2 NLS domain that lacks a proline residue is capable of interaction with the karyopherin α receptor contrasts with the suggested role of a proline residue in interactions with karyopherin α (Chowda-Reddy et al., 2009). Thus, the presence of a proline residue adjacent to the basic amino acids of an NLS motif is not always needed for interaction with karyopherin α and further studies are needed to resolve this issue. We also demonstrated that BYVMV C2 is capable of self-interaction, a feature that has been described for other geminiviruses (Yang et al., 2007), and this is suggested to be a prerequisite for its role in transcriptional activation.

METHODS

Plasmid construction. For subcellular localization studies, BYVMV C2 and the truncated versions Tr1, Tr2, Tr3, Tr4 and Tr5 were tagged at the N terminus with ECFP by cloning the PCR-amplified ORF of C2 or its truncated versions into the Xhol/EcoRI sites of pSAT6-ECFP-C1 (Tzfira et al., 2005), resulting in pSAT6-ECFP-C1-C2, and pSAT6-ECFP-C1-C2-Tr1 to pSAT6-ECFP-C1-C2-Tr5 (Fig. 1a). Similarly, BYVMV C2 protein was tagged at its N terminus with EYFP by cloning the PCR-amplified C2 ORF into the Xhol/EcoRI sites of pSAT6-EYFP-C1, resulting in pSAT6-EYFP-C1-C2 (Fig. 1a). The C-terminal fusion of ECFP with BYVMV C2 was formed by cloning...
the PCR-amplified C2 ORF into XhoI/EcoRI sites of pSAT6-ECFP-N1 to create pSAT6-ECFP-N1-C2 (Fig. 1a).

The substitution mutations K17I, K21T, K24T, R25L, R28T, R29G, R30I and R31L (Fig. 1b) were obtained using a Quick Change Site-directed Mutagenesis kit (Stratagene), with pSAT6-ECFP-C1-C2 as template. NLS deletion (del-NLS) was performed according to Imai et al. (1991), using pSAT6-ECFP-C1-C2 as the template. All PCRs were performed with high-fidelity Pfu polymerase and their products were verified by sequencing. The primers used for generating the constructs are listed in Table S1 (available in JGV Online).

**Electroporation of plant protoplasts.** Mesophyll protoplasts were isolated from tobacco (*N. tabacum* cv. Samsun NN) plants grown under sterile conditions (Draper et al., 1988). Electroporation of ~5 x 10⁵ protoplasts per construct was performed in pre-chilled electroporation medium (Fromm et al., 1985) using 1–5 μg plasmid DNA. After electroporation, the protoplasts were transferred to growth medium, incubated in the dark at 25 °C for 48 h and analysed by confocal microscopy.

**Fluorescence imaging.** For confocal imaging, an Olympus IX 81 inverted laser-scanning confocal microscope (Fluoview 500) equipped with an argon ion laser and a 60 x 1.0 numerical aperture. A PlanApo water-immersion objective was used. ECFP and EYFP were excited at 458 and 515 nm and imaged with BA 480–495 and BA 535–565 nm emission filters, respectively. For chlorophyll autofluorescence, a BA 660F emission filter was used. Transmitted light images were obtained using Nomarski differential interference contrast.

**Detection of protein–protein interactions by FRET microscopy.** The FRET procedure was performed by the acceptor photobleaching method (Kenworthy, 2001). ECFP (donor) and EYFP (acceptor) were excited at 70 and 3 % laser power, respectively. All other conditions were as described for confocal imaging. The microscope was configured with a 458–515 nm dichroic mirror for dual excitation and a 515 nm beam splitter to separate ECFP and EYFP fluorescence. The acceptor was bleached by scanning a region of interest (ROI) at 100 % laser power, resulting in photobleaching of at least 90 % of the original fluorescence. The pre- and post-bleach images were collected, and the fluorescence intensity of the region of interest was measured using Fluoview 500 software. Each measurement was conducted on a set of ten different cells. The percentage of FRET efficiency was calculated as $E_F = (I_{0} - I_{1})/I_{0} \times 100$, where $I_{0}$ and $I_{1}$ are the CFP intensities at the time points between which the bleaching occurred (Karpova et al., 2003).

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