An RGG sequence in the replication-associated protein (Rep) of *Tomato yellow leaf curl Sardinia virus* is involved in transcriptional repression and severely impacts resistance in Rep-expressing plants

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Truncated versions of the replication-associated protein (Rep) of *Tomato yellow leaf curl Sardinia virus* (TYLCSV) can interfere with various viral functions and the N-terminal 130 aa are sufficient for strongly inhibiting C1-gene transcription and virus replication and confer resistance in transgenic plants. In this work, we analysed the relevance of an RGG sequence at aa 124–126, highly conserved in begomoviruses, in these inhibitory functions as well as in the subcellular localization of Rep. Although no role of this RGG sequence was detected by cell fractionation and immunogold labelling in Rep localization, this sequence appears relevant for the transcriptional control of the C1-gene and for the inhibition of viral replication and dramatically impacts resistance in transgenic plants. These results are discussed in the context of the model of Rep-mediated resistance against TYLCSV.
kinase-specific phosphorylation sites (NetPhosK at http://www.cbs.dtu.dk/services) (Blom et al., 2004), the 0.476 score obtained was below the default limit of 0.5. At amino acid position 124–126 a highly conserved RGG triplet was noted, which was present in most sequences retrieved except for some New World species. Proteins with RGG-box motifs are involved in transcriptional repression and in binding and processing of RNA; these motifs are also typical of proteins localized in the nucleus/nucleolus (Burd & Dreyfuss, 1994; Godin & Varani, 2007; Corley & Gready, 2008). Since transcriptional repression, a process expected to take place in the nucleus, is fundamental for the resistance conferred by Rep-210 and Rep-130, we focused on this RGG sequence and tested its role in the subcellular localization in the inhibition of the C1-gene transcription and TYLCSV replication by using truncated Rep mutants with the RGG sequence substituted by SAA (Rep-210SAA and Rep-130SAA). We also analysed the impact of this mutation on TYLCSV resistance by using transgenic plants. To obtain truncated Rep with a mutation in the RGG sequence, plasmids pTOM-100 and pJTR-130 encoding Rep-210 and Rep-130, respectively (Noris et al., 1996; Lucioli et al., 2003), were mutated to pTOM-100SAA and pJTR-130SAA with the Quik Change Site-directed mutagenesis kit (Stratagene) and with primers TY-SARMUT-9 to TY-LARMUT-2263 (5’-GTTGGCTGTCTGTTGTGCTGCACTAGC-AGATCGTCCGTC-3’ and TY-SARMUT-2263(−) (5’-G-ACGGACGATCTGCTAGTGCAGCACAACAGACAGCCA-AC-3’). The expression cassettes from pJTR-120 (Lucioli et al., 2003), pTOM-100SAA and pJTR-130SAA were cloned into pBin19 to be used in the transformation of N. benthamiana, transgenic plants accumulating high level of Reps were self-pollinated.

To analyse the subcellular compartmentalization of the different Rep versions, nuclear and cytoplasmic fractions were prepared from transgenic lines expressing Rep-210 (line 102.22; Noris et al., 1996), Rep-130 (line 308; Lucioli et al., 2003), Rep-210SAA (lines #1, #2 and #3), Rep-130SAA (line #4) and Rep-120 (line #7) using the CellLytic Plant Nuclei Isolation/Extraction kit (semi-pure protocol; Sigma). Four hundred nanograms of total protein quantified using the Experion Pro260 Analysis kit (Bio-Rad) was separated by SDS-PAGE and probed with Rep-antibodies, and also with antibodies against fibrillarin (kindly provided by M. Taliansky, Dundee, UK), used as nuclear control marker. Rep-210, Rep-210SAA, Rep-130 and Rep-130SAA mostly accumulated in nuclear extracts (Fig. 2a), where a polypeptide specifically recognized by the anti-fibrillarin antibodies was also detected (Fig. 2a). However, relatively higher proportions of Rep-130 and -130SAA were found in the cytoplasm compared with Rep-210 and Rep-210SAA; Rep-120 appeared similarly distributed in nuclear and cytoplasmic fractions (Fig. 2a). To support and extend these results, Rep-transgenic plants were also subjected to immunogold electron microscopy (IGEM). Sections (50–60 nm) from tissue embedded into LR-Gold resin (TAAB) were probed with Rep antibodies, followed by goat antirabbit IgGs linked to 15 nm gold particles. Specific labelling in nuclei and nucleoli was found in plants expressing Rep-210, Rep-210SAA and Rep-130 (Fig. 2b); in addition, Rep-210 was detected in nuclei/nucleoli of transgenic tomato plants (line 47 x wild-type; Brunetti et al., 1997) (not shown). However, Rep-130SAA and Rep-120 could not be detected by IGEM, either in transgenic tissue or in agroinfiltrated leaves (not shown), possibly because of poor availability of N-terminal epitopes due to sample preparation or because of an intrinsic paucity of immunogenic epitopes in the N terminus of Rep, this latter hypothesis being substantiated by the inability of the Rep-antibodies to detect truncated Reps shorter than Rep-120 even by Western blot (see Supplementary Fig. S1, available in JGV Online). The karyophilic property of Rep of begomoviruses is consistent with its role during virus replication. Our results are in accordance with previous immunocytochemical staining of the Tomato golden mosaic virus (TGMV) Rep in infected plants (Nagar et al., 1995) and nuclear targeting of African cassava mosaic virus Rep–GFP fusion proteins (Hong et al., 2003). Our work further shows that a large C-terminal deletion (aa 131–359) in TYLCSV Rep does not prevent nuclear accumulation and suggests that the RGG sequence is not involved in this process. Although globular proteins up to 70 kDa can enter the nucleus by simple diffusion (Görlich & Kutay, 1999), active nuclear import processes are generally required even for small proteins such as histones (Breeuwer & Goldfarb, 1990; Jäkel et al., 1999).

Fig. 1. Sequence conservation among begomoviruses of a region of 30 aa encompassing the 121–130 aa sequence of TYLCSV Rep, as a result of a CLUSTAL_X and WebLogo analysis.
The differences in the relative partitioning between nucleus and cytoplasm of Rep-210, Rep-130 and particularly Rep-120 indicate that residues 121–210 contribute to a certain extent to nuclear targeting, due to yet unidentified nuclear localization signals or to domains interacting with nuclear import factors. In spite of the lower level of Rep-120 expression in the transgenic lines compared with those expressing the other Rep variants, the observed similar distribution of Rep-120 between nucleus and cytoplasm (Fig. 2a) could result from a free nuclear transport or the preservation of an inefficient nuclear localization signal.

To test the impact of the RGG sequence on the C1-gene transcriptional repression mediated by truncated Reps, N. benthamiana protoplasts were co-transfected with the GUS reporter construct plntS/GUS, together with each of the Rep-expressing plasmids pTOM-100 (Rep-210), pTOM-100SAA (Rep-210SAA), pJTR-130 (Rep-130), pJTR-130SAA (Rep-130SAA) and pJTR-120 (Rep-120) or pGEM-P as control, as described previously (Lucioli et al., 2003). Rep-130 and Rep-210 strongly inhibited GUS expression (GUS residual activity 3.5 and 1.9, respectively, Fig. 3a) as reported previously (Lucioli et al., 2003), while residual activities of 18.1 for Rep-130SAA (5.2-fold higher than Rep-130) and of 3.4 for Rep-210SAA (1.8-fold higher than Rep-210) (Fig. 3a) were recorded. Control of Rep expression in protoplast experiments using Western blot (see Supplementary Fig. S2, available in JGV Online) allows us to exclude that these results are due to a lower accumulation of the RepSAA polypeptides or their degradation.

As Rep-130SAA could not be immunolocalized by IGEM, protein fractionations (Fig. 2a) indicate that the reduced transcriptional repression of RepSAA mutants compared with their non-mutated counterparts is not even a consequence of defects in nuclear localization. The relevance of the RGG sequence in the transcriptional repression activity, particularly evident in the Rep-130 context, is congruent with the role exerted by proteins containing RGG motifs (Alex & Lee, 2005; Corley & Gready, 2008). Interestingly, a lower repression of the AL1 gene transcription in vitro was reported for an RS-R125 mutant of TGMV Rep (where R125 of TGMV Rep corresponds to R124 of TYLCSV Rep) (Orozco et al., 2000).

It has been shown that C1-gene transcriptional repression is linked to the inhibition of virus replication in protoplasts. We then tested Reps mutated in the RGG sequence for their inhibitory activity on TYLCSV replication, according to Lucioli et al. (2003). Rep-210SAA was strongly inhibitory, similar to Rep-210 and Rep-130 (Brunetti et al., 2001; Lucioli et al., 2003), while Rep-130SAA had only a limited effect (Fig. 3b), indicating that, as in the transcriptional inhibition assays, the RGG to SAA mutation had a different impact on Rep-130 than on Rep-210.

To define to what extent the mutation of RGG altered the Rep-mediated resistance, transgenic plants expressing Rep-210SAA, Rep-130SAA, Rep-210 and Rep-130 were challenged with TYLCSV. Since high levels of Rep accumulation are required for conferring resistance (Brunetti et al., 1997), only plants expressing similar amounts of Rep were tested and systemic infection was scored in newly emerged leaves by tissue print from 2 weeks post-inoculation (p.i.)
onwards. As can be seen in Fig. 3(c), the SAA mutation compromised TYLCSV resistance, more dramatically in the case of Rep-130. The resistance conferred by Rep-130 is determined by its tight control of C1-gene transcription and the susceptibility of Rep-130SAA plants could be a direct consequence of the reduced C1-gene repression, which in turn dramatically affects its ability to inhibit TYLSCV replication (see Fig. 3a, b). Similarly, the lower resistance of the three Rep-210SAA lines compared with Rep-210 plants can be reconciled with this mechanism, but it remains to be explained why Rep-210 and -210SAA induced lower resistance than Rep-130, albeit they inhibited transcription and viral replication to a similar extent in protoplasts. We have previously postulated that in planta Rep-mediated resistance is a two-step process (Lucioli et al., 2008) wherein virus accumulation is initially dictated by the interfering activity of the truncated Rep and then is regulated by a fine balance between this interference and the ability of the virus to silence transgene expression. According to this model, the lower resistance induced by Rep-210 and -210SAA compared with Rep-130 could result from a different susceptibility of their mRNAs to TYLCSV-induced gene silencing. Coherently, analysing the C1-derived small interfering RNA population of TYLCSV-infected tomato plants, we observed that the 5' region of the C1-gene was under-represented compared with the downstream sequence (Tavazza et al., 2003). In addition, Rep-210 differs from Rep-130 by a C-terminal extension of 80 aa that...
contains the highly conserved begomovirus Rep-interacting domain mediating Rep self-oligomerization (Lucioli et al., 2003), helicase activity (Choudhury et al., 2006) and interaction with host factors, such as plant-pRBR homologues (Kong et al., 2000). In this scenario, Rep-210 could perform antagonistic functions, inhibiting virus infection through CI-transcriptional repression, while favouring virus replication through its interacting domain. The interaction between TYLCSV Rep and pRBR, though not directly tested, could modulate host gene expression. In fact, Rep-210, but not Rep-130 transgenic plants, showed an altered phenotype (Brunetti et al., 1997) that became strongly attenuated following the mutation of L147 (D. Sallustio & M. Tavazza, unpublished data), a residue expected to be critical for pRBR-interaction similarly to L148 in TGMV Rep (Arguello-Astorga et al., 2004). Besides, several host genes showed similar expression levels in wild-type plants infected by TYLCSV and in healthy plants expressing Rep-210, but not Rep-130 (A. Lucioli & M. Tavazza, unpublished data).

Although TYLCSV Rep cannot be strictly considered a canonical RGG-box protein, we demonstrate in this work that a single RGG triplet highly conserved among begomoviruses is important for the transcriptional repression activity of the truncated Rep-210 and Rep-130, in accordance with the function exerted by this kind of polypeptide. This mutation has a consequent drastic impact on virus resistance in plants, but does not appear to influence the nuclear/nucleolar localization of truncated Rep, at least for Rep variants comprising aa 1–130. In the case of Rep-120 only, the extremely low transcriptional repression could be further attributed to a modified partitioning between nucleus and cytoplasm.

In addition, we unambiguously showed by using IEGM the presence of truncated TYLCSV Reps in the nucleolus, an organelle involved in the synthesis and packaging of rRNAs, in stress response, cell cycle regulation and gene silencing (Pontes & Pikaard, 2008; Boisvert et al., 2007; Brown & Shaw, 2008). Many DNA viruses, retroviruses and even RNA viruses interact with the nucleolus (reviewed by Hiscox, 2007). The functional role of nucleolar localization of Rep awaits further elucidation and its contribution in the regulation of host gene expression or in virus replication in concert with the interaction with plant cell nucleolar components could be envisaged.

Acknowledgements

We thank A. M. Vaira for help in producing transgenic plants and M. Vecchiati for plant care. A special thanks to our late friend R. G. Milne for helpful discussions. L. S. was recipient of a fellowship from Fondazione CRT – Progetto Lagrange (Torino, Italy).

References


