Interfamilial recombination between viruses led to acquisition of a novel translation-enhancing RNA element that allows resistance breaking

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Summary

- Many plant viruses depend on functional RNA elements, called 3′-UTR cap-independent translation enhancers (3′-CITEs), for translation of their RNAs. In this manuscript we provide direct proof for the existing hypothesis that 3′-CITEs are modular and transferable by recombination in nature, and that this is associated with an advantage for the created virus.
- By characterizing a newly identified Melon necrotic spot virus (MNSV; Tombusviridae) isolate, which is able to overcome eukaryotic translation initiation factor 4E (eIF4E)-mediated resistance, we found that it contains a 55 nucleotide insertion in its 3′-UTR. We provide strong evidence that this insertion was acquired by interfamilial recombination with the 3′-UTR of an Asiatic Cucurbit aphid-borne yellows virus (CABYV; Luteoviridae).
- By constructing chimeric viruses, we showed that this recombined sequence is responsible for resistance breaking. Analysis of the translational efficiency of reporter constructs showed that this sequence functions as a novel 3′-CITE in both resistant and susceptible plants, being essential for translation control in resistant plants.
- In conclusion, we showed that a recombination event between two clearly identified viruses from different families led to the transfer of exactly the sequence corresponding to a functional RNA element, giving rise to a new isolate with the capacity to infect an otherwise non-susceptible host.

Introduction

Mutation, recombination, reassortment and combinations of these molecular events can produce new viral forms, a minority of which might become competent enough for replication in a new environment (see, for instance, Escriu et al., 2007). While mutation is a universal and important mechanism of genetic variation that affects all viruses, the occurrence of recombination appears to vary greatly among different viruses, and can also be dependent on the host and the environment (Jaag & Nagy, 2010). For plant RNA viruses, recombination seems to be one of the strongest forces shaping their genomes, and the end results are detectable in phylogenetic analysis. It is likely that plant RNA viruses have tested recombination with all types of genetic material, because indications of recombination have been identified between closely related, but also distantly related, virus genomes and even with host genes. While intraspecific recombination was detected in many RNA viruses (Moury et al., 2006; Ohshima et al., 2007; Pagán & Holmes, 2010), intergeneric recombinants are rare (Aus dem Siepen et al., 2005). Moreover, some plant viruses seem to have acquired host genes through recombination (Dolja et al., 2006).

The evolutionary effect of recombination has been the subject of a wealth of theoretical papers. On the one hand, recombination may play a fundamental role in compensating for deleterious mutations caused by low fidelity polymerases (Chao & Matthews, 1992; Chao & Trang, 1997). On the other, recombination may lead to the acquisition of nonself sequences. Thus, recombination may result in dramatic changes in the biological properties of the virus, potentially playing a role in the emergence of new viral pathogens (Fernández-Cuartero et al., 1994; Domingo, 2010), including resistance-breaking and host-switching strains (García-Arenal & McDonald, 2003; Jegouic et al., 2009; Sztuba-Solińska et al., 2011; Bujarski, 2013). The acquisition of the host-switching ability may force a reduction in the virus’ fitness in the original host, because the new host may impose different selective requirements (Elena et al., 2011). While some studies indicate that recombination may assist host switch (Chare & Holmes, 2006; Codoñer & Elena, 2008; Sztuba-Solińska et al., 2011), others do not support an association between recombination and emergence, suggesting instead that it is circumstantial (Holmes, 2008, 2009). The results presented here support the first statement.

In principle, recombination may affect any function in the viral cycle, including translation of viral RNAs. Viral mRNAs have evolved numerous mechanisms to recruit the translational machinery of the host, allowing them to compete with host...
mRNAs and avoid defense mechanisms that act at the translation level. Only c. 20% of known positive-strand RNA viruses have genomic and subgenomic RNAs with the 5′-cap structure and 3′-poly(A) tail typical of eukaryotic mRNAs (van Regenmortel et al., 2000). Thus, most lack one or both of these features, and often use their 5′- and/or 3′-termini in alternative gene expression strategies (Dreher & Miller, 2006; Kneller et al., 2006). Plant viruses of the families Tombusviridae and Luteoviridae lack both the cap and poly(A) tail. Several of the species from these two families have been shown to control their cap-independent translation with a cap-independent translational enhancer element residing within or near their 3′-UTR (3′-CITE; Miller & White, 2006). Different 3′-CITEs with distinct properties have been described, but all have in common the general mechanism of steps involving recruitment of the translation initiation factors at the 3′-CITE and delivery of these near the translation start site through communication with the 5′-UTR (Simon & Miller, 2013). All members of the genus Luteovirus (family Luteoviridae), and all members of the Necrovirus and Dianthovirus genera (family Tombusviridae) carry 3′-CITEs similar to that of Barley yellow dwarf virus (BYDVM Translational Enhancer, BTE; Shen & Miller, 2004; Kneller et al., 2006). Other structurally unrelated 3′-CITEs are found within the 3′-UTRs of members of the Tombusviridae family, such as I-shaped, Y-shaped and 3′-CITEs similar to the one of Panicum mosaic virus (PMV Translational Enhancer, PTE; Miller et al., 2007; Simon & Miller, 2013). BTE- and PTE-like 3′-CITEs have also been identified in umbraviruses (Wang et al., 2010). Several 3′-CITEs have been shown to bind the eukaryotic translation initiation factor (eIF) 4F, but with variations in the directly bound subunits, either eIF4E or eIF4G: for the BTE 3′-CITE of BYDVM (genus Luteovirus) it was shown to be eIF4G (Treder et al., 2008), while for the PTE-like 3′-CITE of PMV (genus Panicoovirus) (Wang et al., 2011), for the Y-shaped 3′-CITE of Carnation italian ringspot virus (CIRV, genus Tombusvirus) (Nicholson et al., 2013) and for the I-shaped 3′-CITE of Maize necrotic streak virus (MNeSV, genus Tombusvirus) it was shown to be eIF4E (Nicholson et al., 2010).

In the case of the I-shaped 3′-CITE of Melon necrotic spot virus (MNSV, family Tombusviridae, genus Carmovirus) genetic evidence for an interaction with melon eIF4E exists: melon resistance against MNSV was shown to act at the level of translation, being mediated by eIF4E (Nieto et al., 2006; Truniger et al., 2008). The eIF4E allele from resistant melon varieties differs from the susceptibility allele in a single amino acid residue (Nieto et al., 2006). A resistance breaking isolate, MNSV-264, was described (Diaz et al., 2002), but this isolate did not prevail under field conditions (M. A. Aranda, unpublished data). The critical region in this isolate for overcoming the resistance was resolved to a 3′-CITE element. This 3′-CITE was active not only in susceptible melon, as the corresponding 3′-CITEs of avirulent strains, but also in resistant melon (Diaz et al., 2004; Truniger et al., 2008). Thus, it was proposed that an inefficient interaction between the host-specific 3′-CITE of avirulent isolates and eIF4E of resistant melon impedes the correct formation of the translational initiation complex at the viral RNA ends and thereby leads to resistance (Truniger et al., 2008).

In 2011 a new virulent MNSV isolate, that we named MNSV-N, was identified on MNSV-resistant melon plants in southeastern Spain. We set out to determine the resistance-breaking mechanism of MNSV-N. We found that MNSV-N contains a 55 nucleotide (nt) insertion in its 3′-UTR that functions as a virulence determinant. This inserted sequence has the capacity to act as a 3′-CITE in resistant and susceptible melon. The I-shaped 3′-CITE described for avirulent MNSV isolates, only functional in susceptible melon, is also present in this isolate. Both 3′-CITEs required the presence of the 5′-UTR of MNSV in cis for efficient translation to occur. Results show strong evidence that this 55-nt insertion has been acquired by interfamilial recombination with the 3′-UTR of an Asiatic Cucurbit aphid-borne yellow virus (CABYV) isolate. Thus, the sequence acquired by MNSV by recombination is a functional element able to control cap-independent translation of MNSV-N in the otherwise resistant host. To our knowledge this is the first direct proof for the previously proposed modularity and transferability in nature of 3′-CITEs. Additionally, it is one of the first rare recombination events in a plant RNA virus that has been proven to result in resistance breaking. Thus, our results support the hypothesis that recombination in positive sense RNA viruses can widen host range, giving rise to new emergent strains.

Materials and Methods

Plants, viruses and virus inoculations

The susceptible (Nsv/−) Cucumis melo L. cultivars used were the cantaloupe-type accession C-35 (‘La Mayora’ germplasm collection, Málaga, Spain). The resistant C. melo cultivar (nsv/nsv) is the cantaloupe-type accession C-46 (‘La Mayora’ collection). We used MNSV resistance-breaking isolates MNSV-N and MNSV-264 (Diaz et al., 2002) and nonresistance-breaking isolates MNSV-Al (Dutch-type; Genoves et al., 2006) and MNSV-M25 (Diaz et al., 2003). MNSV-N became available through our diagnosis service during the spring of 2011. MNSV-N was biologically cloned by four serial single necrotic lesion passages onto healthy resistant melon C-46 (nsv/nsv), as described in Diaz et al. (2004). MNSV was inoculated mechanically on expanded melon cotyledons as described before (Diaz et al., 2003; Diaz et al., 2004). Plants were grown and maintained after inoculation in a glasshouse with a 16-h photoperiod, day: night temperature of 25 : 18°C, and day: night relative humidity of 70 : 60%.

For the host range study (Table 1) 10 plants from different species from the families Cucurbitaceae (Cucumis melo (C35, C46; ‘La Mayora’ collection), Citrullus lanatus (cv Sugar Baby; Semillas Battle), Cucurbita pepo (cv Pasteleria; Semillas Battle) and Cucumis sativus (cv Markemore; Semillas Arnedo)), Chenopodiaceae (Chenopodium ambrosioides and Chenopodium quinoa (‘La Mayora’ collection)), Amaranthaceae (Gomphrena globosa (‘La Mayora’ collection)) and Solanaceae (Nicotiana benthamiana (‘La Mayora’ collection)) were mechanically inoculated on expanded cotyledons for the cucurbit species, and on young but fully expanded leaves of seedlings for the other species (20 cucurbits for inoculations with MNSV-264, because of very
low systemic infection frequency). Infection was visually evaluated by the appearance of necrotic lesions and by dot-blot hybridization using an MNSV-specific probe at 7 dpi (inoculated leaves) and 14 dpi (evaluation of systemic infection).

Analysis of viral virulence

For this experiment cotyledons of resistant melons were mechanically rub-inoculated with purified virions (Diez et al., 1998) of MNSV-264 or MNSV-N, while cotyledons of susceptible melon were additionally inoculated with MNSV-Al at a concentration of 1 mg ml⁻¹ in 10 mM potassium phosphate buffer. The diameters (mm) of the single necrotic lesions induced by each isolate in at least 10 plants were measured at 3, 5 and 7 dpi. Viral RNA accumulation at 7 dpi was determined by RT-qPCR, as previously described (Gomez et al., 2009). Briefly, total RNA extractions of three biological replicates from a mix of three lesions (6-mm diameter discs) were performed using TRI-Reagent (Sigma-Aldrich, St Louis, MO, USA). RT-qPCR was performed using the Power SYBR® Green RNA-to-CT™ 1-Step Kit (Life Technologies, Carlsbad, CA, USA) following the manufacturer’s protocol. In vitro transcripts were used in serial dilutions to generate standard curves. Primers for qPCR were designed by using Primer Express software (Applied Biosystems International, Foster City, CA, USA) targeting the 3′-UTR region. Primers for MNSV-Al were 5′-ATT TGGTCTCCCATATTCCCTAC-3′ (CE-1291) and 5′-ATACGC GTTAGGTTACGACCAAGAGTGACGCAC-3′ (CE-1292), for MNSV-264 were 5′-GACGGAGTCCTCCAGCAATGCA-3′ (CE-1289) and 5′-GGCC TCCGATAAGACACCCCTCA-3′ (CE-1290), and for MNSV- N were 5′-TTGGGAGATGAGGCTGACT-3′ (CE-1293) and 5′-GAGACCGGGTTGAGTCA-3′ (CE-1294). The virus concentration in each sample (ng of viral RNA per 100 ng of total RNA) was estimated by interpolating the threshold cycle (Ct) in standard curves. Slope values for each standard curve were as follows: MNSV-Al -3.47 and R² = 0.998; MNSV-N -3.60 and R² = 0.997; and MNSV-264 - 3.48 and R² = 0.997. Reaction efficiency was above 90% in all cases.

cDNA synthesis and sequencing

RNA from MNSV-N infected cotyledons (5 dpi) was extracted using TRI reagent (Sigma-Aldrich). cDNA was obtained with Expand Reverse Transcriptase (Roche) using two different reverse primers: primer A contains the 3′-terminal 10 last nucleotides in the genome that are conserved in all MNSV isolates except MNSV-264 (Fig. 2b; Truniger et al., 2008), and primer B contains the sequence complementary to the 10 nucleotides at the 3′ end of the MNSV-264 genome. PCR reactions for amplification of the 3′-end were performed with the Prime Star HS DNA polymerase (high fidelity; Takara, Shiga, Japan) using either of these two primers together with primer MA245, that lies in a conserved region of the CP-gene. Amplification was obtained only with primer A: this can be explained by the on average higher nucleotide similarity of avirulent isolates with the 3′-end of MNSV-N than with MNSV-264. This PCR fragment was sequenced. The complete MNSV-N sequence was obtained by sequencing in both directions overlapping PCR fragments obtained with primers designed in conserved regions: CE-830: 5′-CACGCAAAATGGTCTTCCACATC-3′; CE-831: 5′-TCTCTATCTGAGGAGGACCAATGGGAGAC-3′; CE-832: 5′-CATGGTAAGGCACTGGAGAC-3′; CE-833: 5′-TCTAATGGGGCGAAAGATAGCC-3′; CE-834: 5′-ACATGCGCTTCCAGGAACGC-3′; CE-835: 5′-CCCGGGGCTATATCTCCTGAC-3′. The whole MNSV genome sequence showed a high similarity to the Dutch-type subgroup of MNSV (95%).

Construction and analysis of chimeric viruses

The amplified 3′ end of MNSV-N was cloned directionally into HpaI/PstI sites of the chimeric clone pBSK+−264/3′-Mz5 (Truniger et al., 2008), resulting in the exchange of its 3′-UTR of MNSV-Mz5 with that of MNSV-N, resulting in pBSK ± 264/3′-N (264/3′-N). Deletion of the 55-nucleotide insertion (see alignment in Fig. 2) in the 3′-UTR of MNSV-N in pBSK ± 264/3′-N was obtained by amplification of the whole plasmid with primers lacking this insertion. Subsequently, DpnI digestion was used to select for the mutant plasmids (in vitro mutagenesis; Sambrook &
Russell, 2001). *In vitro* transcribed RNA (RiboMAX Large Scale RNA production; Promega) from the above constructs, linearized with *Pst*I, was inoculated mechanically onto cotyledons (Diaz *et al.*, 2004) or electroporated into protoplasts from resistant and susceptible melon plants (Truniger *et al.*, 2008). The appearance of necrotic lesions was recorded after visual inspection. The ability of mutants to multiply in melon protoplasts was studied by dot-blot or Northern blot using a cRNA probe complementary to the 3′-UTR of MNSV-Mα5 (Diaz *et al.*, 2004). Each experiment was carried out at least three times. The last 600 nt from the 3′-end of progeny virus genomes was amplified by RT-PCR and sequenced.

**Luc-constructs**

The MNSV-N 5′-UTR was amplified from cDNA (see ‘cDNA synthesis and sequencing’ above) by PCR using a primer that contained restriction sites and the T7 promoter sequence directly in front of the first 10 nt of the 5′-UTR sequence, which is highly conserved in all MNSV isolates. The fragment was directionally cloned into the *KpnI/NcoI* sites of the T7-luc plasmid (modified pGL3, resulting in 5′-N-luc; Truniger *et al.*, 2008). The 3′-UTRs were directionally cloned after PCR amplification with primers containing restriction sites into the *XbaI/HpaI* sites of the T7-luc or 5′-N-luc plasmid (resulting in 3′-N-luc or 5′-N-luc-3′-N, respectively). Deletion of the 55-nt insertion was achieved by *in vitro* mutagenesis (see ‘Construction and analysis of chimeric viruses’ above). The constructs (5′-end-luc-3′-end) were amplified by PCR with the high fidelity Prime Star HS DNA polymerase and transcribed *in vitro* (RiboMAX; Promega). Constructs containing only the first 81, 65 and 37 nts of the 3′-UTR of MNSV-N were obtained by PCR amplification of plasmid 5′-N-luc-3′-N with reverse primers ending at the corresponding position (N81-5′-CCGGGTTTGGAGTACAAGACC; N65-5′-AGACCCAGT GATTGTGACAGGC; N37-5′-ATGCCGGGTG GAGTCAC GCTC; underlined in Fig. 2b), *DpnI* digestion of the input plasmid, followed by *in vitro* transcription (RiboMAX Large Scale RNA production; Promega).

**In vivo translation in melon protoplasts**

*In vivo* translation in melon protoplasts (susceptible or resistant) was performed as described in Truniger *et al*. (2008). Briefly, 10 μg of *in vitro* transcribed RNA was electroporated into 1 × 10⁶ protoplasts (Diaz *et al.*, 2004). To minimize variations between samples, 2 μg of capped *Renilla* luciferase reporter RNA (*pRL-null* vector; Promega) were introduced along with the virus RNA. After 5–6 h incubation in the dark at 25°C, protoplasts were lysed in 1 × PLB (Promega). *Firefly* and *Renilla* luciferase activities were measured with the Dual-Glo™ Luciferase assay system (Promega). These experiments were carried out at least five times for each construct.

**Analysis of RNA structure**

The 65-nt sequences of the new 3′-CITC were inserted into the previously described SHAPE cassette (Wang *et al.*, 2010). This plasmid was linearized with *SmaI* and transcribed using MEG-shortsense™ Kit (Ambion). SHAPE experiments were performed essentially as previously reported (Kraft *et al.*, 2013). Briefly, 500 ng of RNA refolded in SHAPE buffer was treated with 60 mM of benzoyl cyanide (BzCN; Sigma-Aldrich) and reverse transcribed by primer extension of a radiolabeled primer. Products were resolved on an 8% denaturing polyacrylamide gel after primer extension. Normalized BzCN reactivity values for each nucleotide position were calculated by SAFA Footprinting Software (Das *et al.*, 2005). The RNA secondary structure was determined by using the MC-Fold computer program (Parisien & Major, 2008), using SHAPE reactivity data. *Trans*-inhibition assays of *in vitro* translation with wheat germ extract (Promega) were performed as described (Kraft *et al.*, 2013).

**Nucleotide sequence accession numbers**

The sequence of the genomic RNA of MNSV-N obtained here has been made available in GenBank (accession number KF060715).

**Results**

MNSV-N breaks down the melon *nsv* resistance and is more virulent than MNSV-264

After its biological cloning, the host range and multiplication efficiency of MNSV-N was studied and compared to that of MNSV-Al (Genoves *et al.*, 2006) and the previously described resistance-breaking MNSV-264 (Diaz *et al.*, 2004). The result of the host range study presented in Table 1 showed that MNSV-N infected the same hosts as MNSV-Al, but additionally, as for MNSV-264, it also infected plants of the resistant melon cultivar (*nsv/nsv* genotype). On directly inoculated leaves or cotyledons virus multiplication was evaluated by the appearance of necrotic lesions and dot-blot analysis, (Table 1). The number of systematically infected plants was detected by appearance of necrotic lesions on upper noninoculated leaves and confirmed by dot-blot (Table 1). The ability of MNSV to systemically infect melon plants depends on the plant genotype, the virus isolate and the environmental conditions. Very often systemic infection occurs in < 10% of the inoculated plants (Mallor *et al.*, 2006; Gosalvez-Bernal *et al.*, 2008). Under our conditions, systemic infection of the resistant melons (C46) was never observed with MNSV-264 (Table 1). However, 30% of the resistant melons (C46) singly inoculated with MNSV-N showed systemic infection. These results substantiated previous observations under field and laboratory conditions, suggesting that this new isolate was more virulent than MNSV-264.

Thus, the multiplication efficiency of MNSV-N was estimated in susceptible and resistant melons and compared to those of MNSV-264 and MNSV-Al. Measuring lesion diameter in a time course experiment showed that MNSV-264 lesions did not increase much with time in either melon genotype. However, the diameter of lesions induced by the other two isolates increased significantly with time, resulting in 3–4-fold bigger lesions (Fig. 1a). Virus RNA accumulation in single
lesions was measured by RT-qPCR at 7 dpi, showing significant variations depending on the melon genotype and isolate inoculated: In susceptible melon, MNSV-Al RNA accumulated to higher concentrations than MNSV-N RNA which, in turn, accumulated more than MNSV-264 RNA. In resistant melon, MNSV-N RNA concentration was again nearly three-fold higher than that of MNSV-264 (Fig. 1b). Additionally, as mentioned above, MNSV-N was the only isolate able to systemically infect resistant melon plants. Therefore, while MNSV-N is not more virulent than MNSV-Al in susceptible melons, its virulence is higher than that of MNSV-264 in resistant melons.

MNSV-N is a natural recombinant between MNSV and CABYV

The nucleotide sequence from the complete genome of MNSV-N was determined and compared with known MNSV sequences. In general terms, the MNSV-N complete genome sequence showed high similarity to the ones of avirulent MNSV isolates (>92%). A sharp decrease in similarity was detected in a small region of the 5′ end of its 3′-UTR (Fig. 2a). However, a general decrease in sequence similarity in the 3′-UTR was found between the genome sequences of MNSV-N and MNSV-264 (Fig. 2a), the region where the virulence determinant of MNSV-264 had been localized before (Diaz-Pendon et al., 2005; Truniger et al., 2008). Conversely, the 3′-UTR of MNSV-N had, on average, a much higher similarity to the 3′-UTRs of avirulent isolates (>76%) than that of MNSV-264 (<50%).

Alignment of the 3′-UTR sequences of MNSV-N and avirulent MNSV isolates clearly showed that a fragment size of 55 nt had been inserted at its 5′-end, after the tenth nucleotide of its 3′-UTR (Fig. 2b). Thus, if this insertion was excluded, the sequence similarity of the 3′-UTR of MNSV-N with those of avirulent isolates increased to 95%. A BLAST search with the first 65 nt of the 3′-UTR of MNSV-N, including the 55-nt sequence insertion, identified a highly similar sequence in the 3′-UTR of an Asiatic isolate of the polerovirus Cucurbit aphid-borne yellows virus (CABYV) (family Luteoviridae). As can be observed in Fig. 2(c), 49 nt of the 55-nt insertion were identical to the 5′-end of the 3′-UTR of the isolate CABYV-Xinjiang. Similarly high conservation was observed with the 3′-UTRs of all other Asiatic CABYV isolates (Supporting Information Fig. S1). Interestingly, CABYV has a world-wide distribution in cucurbits, including melon, and co-infection with MNSV has been reported (Kassem et al., 2007; Juarez et al., 2013). This high sequence identity suggested the occurrence of a recombination event. Thus, we carried out a recombination occurrence analysis using the RDP3 software, which implements several recombination-detecting algorithms (Martin et al., 2010;
Fig. 2d). As hypothesized, a recombination event with very high statistical significance ($P < 0.01$) was detected by all methods implemented in RDP3, strongly suggesting that the 55-nt insertion in MNSV-N was acquired through recombination between MNSV and CABYV, both belonging to different virus families.

**Fig. 2**

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The recombined sequence allows MNSV-N to break nsv-mediated resistance.

We identified the virulence determinant of MNSV-N by construction of chimeric mutants. For these experiments we decided to use the background of the MNSV-264 genome, because the sequence similarity of the genome without the 3′-UTR of MNSV-N is higher with this isolate (95%, the same % for MNSV-Al and other dutch-type isolates) than with MNSV-Ma5 (91%). Thus, we exchanged the 3′-UTRs of MNSV-N and the 3′-UTR of MNSV-N contains a sequence of 55 nt that is highly similar to the 3′-UTR of Cucurbit aphid-borne yellows virus (CABYV). (a) Nucleotide sequence similarity plot (performed with the AlignX program from the Vector NTI software package (Invitrogen)), comparing nucleotide sequence of avirulent Melon necrotic spot virus (MNSV) isolates vs MNSV-N and MNSV264 vs MNSV-N. The x-axis represents the nucleotides of the virus sequence, with a window size of 5. The y-axis represents the nucleotide similarity, being 1.0 for identical, 0.5 for similar nucleotides and lower depending on the number of different nucleotides and the number of sequences compared. (b) Alignment (CLUSTALX) of the 3′-UTRs of all Dutch-type MNSV isolates (avirulent) and MNSV-N showing that MNSV-N contains an insertion of 55 nt after the tenth nucleotide of its 3′-UTR. High similarity between the sequences of all these MNSV isolates can be observed after this insertion (95%). The previously identified 3′-CITE sequence, highly conserved in avirulent isolates (four variations in the 45 nt), but also in MNSV-N (only one nucleotide different from other isolates), is highlighted in a gray box. The last 10 nt at the 3′ end, invariant in all MNSV isolates, are framed. On the MNSV-N sequence 3′ end of constructs N37, N65 and N81 are marked with arrows. GenBank accession numbers of MNSV sequences included in the alignment are Ma5-AY122286, HM-GU480022.1, Chiba-AB250684, Yamaguchi-AB250687, Yamaguchi CP gene (YS)-AB189944, Nagasaki-AB250686, AI-DQ339157, Dutch-NC001504, ISR (Israel)-DQ922807, Kochi-AB250685, Kouchi CP gene (KS)-AB189943, Mx24-EU589616, Mx71-EU589619, Mx3-EU589618, Mx68-EU589622, Pm58-EU589620, Pm57-EU589621, Pm54-EU589617. (c) Alignment of the first 65 nt of the 3′-UTR of MNSV-N (including the first 10 nt conserved in avirulent isolates (indicated by vertical line) plus the 55-nnt insertion) with the complete 3′-UTR sequence of Cucurbit aphid-borne yellows virus isolate CABYV-Xinjiang (GenBank accession: EU636992). Identical nucleotides are marked below with an asterisk. (d) Recombination hypothesis generated by the RDP3 software, a computer program for characterizing recombination events in sequence alignments using several different recombination analysis methods and tests for recombination hot-spots. The sequences included in this analysis are the 3′-UTRs of MNSV (see part (b) of this figure) and CABYV isolates (GenBank accession numbers are CABYV-Xinjiang: EU636992; CABYV-Beijing: EU000535; CABYV-FJ: GQ221223; CABYV-JAN: GQ221224; CABYV-R_TW82: JQ700306; CABYV-C_TW20: JQ700305.). RDP3 colors similar sequences with similar colors. The predicted recombination in MNSV-N starts at nucleotide position 4 and ends at nucleotide 63. The statistical significance is very high, with a P-value < 0.01.
infectious chimeric virus 264/3′-Mα5 (genome of MNSV-264 with its 3′-UTR exchanged with the one of MNSV-Mα5, thus able to infect susceptible but not resistant melon; Truniger et al., 2008). This new chimeric virus (264/3′N) was able to infect not only susceptible, but also resistant melon, similar to MNSV-264, as shown by mechanical inoculation of susceptible and resistant melons with in vitro transcribed RNA from this clone (264/3′-N; Fig. 3a). This result was confirmed by inoculating melon protoplasts with this RNA (Fig. 3b). Thus, although the 3′-UTR sequences of the two resistance-breaking isolates, MNSV-N and -264, showed low similarity (see Fig. 2a), the virulence determinants of both were located in their 3′-UTRs (Truniger et al., 2008).

In order to determine if the recombinated sequence itself is required for breaking resistance, the multiplication capacity of mutants that have this sequence deleted was analysed in susceptible and resistant melon plants and protoplasts (Fig. 3a,b). While the chimeric virus containing the 3′-UTR of MNSV-N (264/3′-N) was able to multiply in both melon varieties, the deletion mutant (264/3′-A55-N), although viable in susceptible melon, lost this capacity in resistant melon. From this result it can be concluded that the recombinated sequence is necessary for resistance breaking. As expected, because the 3′-UTR sequence is expected to contain a diversity of functional elements (e.g. involved in replication (Zhang & Simon, 2003; Wu et al., 2009)), a chimeric virus with only the recombinated sequence at the 3′-UTR (264/3′-N65) was viable neither in melon plants (Fig. 3a) nor in protoplasts of any of the two varieties (not shown).

The recombinated sequence is a 3′-cap-independent translational enhancer (3′-CITE)

In a previous study, a 44-nt fragment of the 3′-UTR sequence of avirulent isolates had been shown to be essential for viral multiplication, functioning as a 3′-CITE in susceptible melon (Truniger et al., 2008). This 3′-CITE sequence (nucleotides 142–186) was found to be highly conserved in this new isolate, with only one nucleotide difference (Fig. 2b, gray box). Thus, MNSV-N contained the avirulent 3′-CITE, that was not functional in resistant melon, and not, as might be expected from localization of the virulence determinant in the 3′-UTR, the different 3′-CITE of MNSV-264 (functional in both susceptible and resistant melon; Truniger et al., 2008). These and the above data suggested that the 3′-UTR of MNSV-N could possibly contain a second new 3′-CITE, functional in resistant melons, possibly located in the recombinated region.

Therefore, we studied the role of the 3′-UTR of MNSV-N in cap-independent translation initiation. For this, we flanked the firefly luciferase gene (luc) with the 5′- and/or 3′-UTRs of this new isolate and studied the in vivo translation efficiency in susceptible and resistant melon protoplasts. In susceptible as well as in resistant melon, the 5′-UTR of MNSV-N alone, like the 3′-UTR, was not able to enhance cap-independent translation (Fig. 4; first and eight bar above (susceptible) and below (resistant), respectively), while the presence of both UTRs of MNSV-N resulted in a > 40-fold increase of the translation efficiency (third bar). Thus, the 3′-UTR of MNSV-N was able to enhance cap-independent translation to levels similar to those of the 3′-UTRs of MNSV-Mα5 in susceptible melon and MNSV-264 in resistant melon.

In order to determine if the 55-nt insertion was involved in translational control, this sequence was deleted from the 3′-UTR of MNSV-N. The shortened 3′-UTR (Δ55-N) still enhanced cap-independent translation in susceptible melon, even increasing its activity, but was unable to facilitate translation in resistant melon (fourth bar). These results suggested that the shortened 3′-UTR of MNSV-N still contained a 3′-CITE that is functional in susceptible, but not in resistant melon. Thus, the recombinated sequence was necessary for cap-independent translation.

In order to determine if the extra sequence in the MNSV-N 3′-UTR is sufficient for cap-independent translation in resistant melon, three different constructs were tested containing the first 81, 65 or 37 nt of the 3′-UTR of MNSV-N together with its 5′-UTR flanking the luc. The two longer fragments, N65 and N81, enhanced translation in both susceptible and resistant melon (bars 5 and 6, respectively), while the shorter one, N37, lost this capacity (bar 7). These results suggested that the first 65 nt of the 3′-UTR of MNSV-N, that are highly similar to the first 65 nt of the 3′-UTR of Cabyv-Xinjiang, were sufficient to function as a 3′-CITE that is active in susceptible and in resistant melon. The activity of this 3′-CITE was dependent on the presence of the 5′-UTR in cis; no translation activity controlled by the 3′-UTR of MNSV-N (3′N) or only its first 65 nt (N65) was found in the absence of the 5′-UTR (bars 8/9).

The newly identified 3′-CITE belongs to a new structural class of 3′-CITEs and functions in the absence of eIF4E

The prediction of the secondary structure of the first 65 nt of the 3′-UTR of MNSV-N obtained by Mfold was different to the previously described 3′-CITEs, suggesting that this was a new class of translation enhancer element formed by two stem-loops (Fig. 5b). Thus, the secondary structure of this new 3′-CITE in solution was studied by Selective 2′-Hydroxyl Acylation analyzed by Primer Extension (SHAPE; Wilkinson et al., 2006) using benzoyl cyanide (BzCN; Mortimer & Weeks, 2008). This chemical quickly modifies flexible and therefore possibly single-stranded nucleotides in a sequence-independent manner, forming 2′-O-adducts that block reverse transcriptase. The RNA segment used in this structure probing assay was inserted into the SHAPE cassette described in Wang et al. (2010) and the functionality of the 3′-CITE in this context was confirmed by trans-inhibition assay (Fig. S2), using as reporter mRNA the luciferase construct with the 5′- and 3′-UTR of MNSV-Mα5 flanking the luc gene (Truniger et al., 2008). Both the MNSV-N 3′-CITE alone and in the context of the cassette used for SHAPE analysis inhibited translation of reporter mRNA when added in 200-fold excess indicating structural and functional integrity of the 3′ CITE in the SHAPE cassette.

 Primer extension after modification with BzCN revealed two highly modifiable regions forming the loops of two stem-loop structures (Fig. 5), consistent with the structure predicted by
Mfold. The first stem-loop (SL1) consists of 34 nucleotides (3990–4023), including two highly reacting bases, U4003 and G4004, and one, G4006, with weak activity in the loop. The second stem-loop (SL2) is formed by 25 nucleotides with four strongly modified nucleotides located in the loop (C4033, G4036, U4037 and C4038) and three nucleotides with weak activity (A4040, A4041, U4043). Magnesium titration experiments showed that the folding of this structure was independent of this divalent cation. Thus, the 3'-CITE folded into two helices protruding from a central hub in magnesium independent manner. This structure was supported by the nucleotide variations found in the 3'-UTR sequences of the known Asiatic-type CABYV isolates (light blue arrows Fig. 5b), because most of them were located in the unpaired loop regions L1 or L2. Additionally, two of the five variations that appeared in base-paired regions did not disrupt base pairing (orange arrows). The other three possibly had little effect on the structure as they were located in the base of the stems (gray arrows; see alignment Fig. S1).

Data on resistance breaking suggested that the new 3'-CITE could function either with the different eIF4E variants expressed by susceptible or resistant melon, or independently of eIF4E. To learn if this new 3'-CITE was eIF4E-dependent, its activity in melon protoplasts of a previously described eIF4E knock-down line was studied (Rodríguez-Hernández et al., 2012). Through expression of a hairpin construct targeting and thus silencing melon eIF4E, its expression had been shown to be reduced more than six-fold. As can be seen in Fig. 6(a), low cap-independent translation activity was obtained for the construct with 5'- and 3'-URS of MNSV-MΔ5 flanking the luc gene, in agreement with the eIF4E-dependence of its 3'-CITE (Truniger et al., 2008; Rodríguez-Hernández et al., 2012). On the other hand, the construct with both wild-type MNSV-N UTRs and even with only the first 65 nt of the 3'-UTR (N65) resulted in 8–10-fold higher translation activity. This result shows that translation controlled by the new 3'-CITE in melon can occur in the absence of Cm-eIF4E. Consequently, MNSV-N was able to infect eIF4E-silenced melon plants (Fig. 6b).

Discussion

In this study we provide the first direct proof for the hypothesis that 3'-CITEs are in nature modular transferrable RNA elements and show that this phenomenon can be associated with an advantage for the recombinant virus created. We have characterized a new 3'-CITE in MNSV, the third characterized in this virus. This new 3'-CITE gives the natural recombinant virus the capacity to infect resistant melon cultivars (nsv/nsv genotype). It was most probably acquired through a specific and unique recombination event, leading to the acquisition of only the translational enhancer element; the parental viruses can be clearly identified among still existing viruses, an Asiatic isolate of CABYV (CABYV-Xinjiang) and MNSV. Thus, we call this new class of 3' CITE, the CABYV-Xinjiang-like translation element, short CXTE. The first two 3'-CITEs characterized in MNSV were the one present in all avirulent MNSV isolates (and highly conserved), unable to infect the resistant melon, and that of MNSV-264, able to overcome this resistance (Truniger et al., 2008). The 3'-CITE of MNSV-264 was also proposed to have been acquired through recombination, but in this isolate the complete 3'-UTR was accepted from an unknown heterologous source (Nieto et al., 2011).

The modularity and transferability of 3'-CITEs have been proposed before, because different types of 3'-CITEs can be found in a single genus and the same type of 3'-CITE appears in different virus genera (Nicholson & White, 2011). The first direct, but artificial, evidence came from the viability of engineered chimeric viruses: the Y-shaped 3'-CITE from CIRV was exchanged with the I-shaped one from MNeSV or the PTE-like 3'-CITE of Cucumber leaf spot virus (CLSV). The results also supported the modularity of 3'-CITEs (Nicholson et al., 2013). In these

experiments, the 3′-CITEs exchanged belonged to viruses from the same family as CIRV, the *Tobuviridae*. Additionally, all three 3′-CITEs are known to depend on eIF4E/eIF4G for proper functioning (Nicholson et al., 2010, 2013; Wang et al., 2011). However, in the case of the natural resistance-breaking MNSV isolates, MNSV-264 and MNSV-N, the possible recombination event occurred between viruses of different families: *Tobuviridae* and *Luteoviridae* for MNSV-N. Additionally, the 3′-CITEs of these two isolates have been shown to function in the absence of eIF4E, in contrast to the 3′-CITEs of avirulent MNSVs (Fig. 6 and Rodríguez-Hernández et al., 2012). These results suggest that 3′-CITEs, when interchanged, can be active in very similar (belonging to the same family) and very heterologous (belonging to different families) viral genomes, even if they recruit different translation initiation factors for their activity.

Our results also confirm that eIF4E-mediated resistance breaking by MNSV is controlled by the host-specificity of its 3′-CITE. While the 3′-CITEs of the avirulent isolates are not functional in resistant melon, the 3′-CITEs of MNSV-N and -264 are active in this melon genotype. Remarkably, the previously identified MNSV 3′-CITEs (from avirulent isolates and MNSV-264) have both been predicted to be I-shaped (Truniger et al., 2008; Nicholson et al., 2010). In contrast, the new 3′-CITE described here has a double stem-loop structure that is different from all 3′-CITEs described to date (Miller et al., 2007; Nicholson & White, 2011). The *in vivo* translation experiments and 3′-UTR sequence alignment analyses lead to the conclusion that the 3′-UTR of MNSV-N contains two 3′-CITEs: one is functional only in susceptible melons and the other functional in both susceptible and resistant types. To our knowledge the existence of more than one 3′-CITE with varying mechanisms and different host-specificity in the same viral genome has not been observed before. The 3′-UTR of PEMV RNA2 has also been reported to contain two 3′-CITEs, a PTE-like CITE and a T-shaped CITE like that of *Turnip crinkle virus*, but the effect, if any, of these two CITEs on host-specificity is unknown (Gao et al., 2012).

How would cap-independent translation occur in the presence of both the CITE and the I-shaped 3′-CITE? In susceptible melon both 3′-CITEs would be active, while in resistant melon and in the absence of eIF4E only the CXTE would be functional. We have shown that both 3′-CITEs depend on the presence of the 5′-UTR of MNSV-N in cis for their activity (Fig. 4; Truniger et al., 2008). Long-distance RNA–RNA interactions have been shown to be essential for translation, transcription and replication of viral RNAs (Wu et al., 2009). Thus, RNA circularization in the case of MNSV-N would occur through the I-shaped 3′-CITE/5′-UTR sequence complementarity (V. Truniger, unpublished data). In the chimeric virus 264/3′-N the same interaction of this 3′-CITE with the 5′-UTR of MNSV-264 would occur, because the 5′-UTR sequences of these two isolates are highly conserved (the first 24 nt, containing the complementary sequence stretch, are identical; Fig. S3). But interestingly, the CXTE of MNSV-N alone also showed 5′-UTR dependent *in vivo* cap-independent translation-enhancing capacity in melon protoplasts (N65; 100%; second bar – upper graph), or of MNSV-264 in resistant melon protoplasts (≈100%; second bar – lower graph). All constructs, with exception of these two positive controls and the two constructs in the last two bars (that contain a plasmid sequence at the 5′-end (5′pl.)), contained the 5′-UTR of MNSV-N and the different 3′-UTRs indicated below. 3′-UTRs: 3′-pl. = plasmid sequence; MΔ5 or 264 = 3′-UTR of MNSV-MΔ5 or 264; MNSV-N or 3′N = 3′-UTR of MNSV-N; Δ55N = 3′-UTR of MNSV-N with the 55 nt of insertion deleted; N65/81/37 = first 65/81/37 nt of the 3′-UTR of MNSV-N, respectively. Error bars are ± SD.

**Fig. 4** The recombined sequence is a 3′-CITE that is functional in resistant melon. *In vivo* cap-independent translation assayed in melon protoplasts. Each bar represents the relative luciferase activity (corresponding to the translation efficiency) obtained with a construct (as indicated below) either in susceptible (upper part) or resistant (lower part) melon protoplasts. The reference values are the activity obtained with the construct of the luc gene flank by both 5′- and 3′-UTRs either of MNSV-MΔ5 in susceptible (≈100%; second bar – upper graph), or of MNSV-264 in resistant melon protoplasts (≈100%; second bar – lower graph). All constructs, with exception of these two positive controls and the two constructs in the last two bars (that contain a plasmid sequence at the 5′-end (5′pl.)), contained the 5′-UTR of MNSV-N and the different 3′-UTRs indicated below. 3′-UTRs: 3′-pl. = plasmid sequence; MΔ5 or 264 = 3′-UTR of MNSV-MΔ5 or 264; MNSV-N or 3′N = 3′-UTR of MNSV-N with the 55 nt of insertion deleted; N65/81/37 = first 65/81/37 nt of the 3′-UTR of MNSV-N, respectively. Error bars are ± SD.
Fig. 5 Secondary structure probing of the new 3′-CITE. (a) Structure probing by SHAPE of the first 65 nt of the 3′-UTR of Melon necrotic spot virus isolate MNSV-N, including the new 3′-CITE. Primer extension products separated on denaturing PAGE of RNA treated (fourth to sixth lane (concentrations of Mg^{2+} (mM) added are indicated above, (0/1/3)) or untreated (third lane, (−)) with benzoyl cyanide (BzCN). The sequencing ladder was generated by reverse transcription of unmodified RNA in the presence of deoxyCTP (ddCTP; lane C) or ddATP (lane A). The positions of A4000, A4027 and A4045 are indicated on the left. The positions in the PAGE corresponding to the stems (S1/S2) and loops (L1/L2) of stem-loop structures, SL1 and SL2, are marked at the right. (b) Secondary structure of new 3′-CITE probed in panel (a). SHAPE reactivity of nucleotides superimposed on secondary structure predicted by Mfold. Color-coded bases indicate the levels of BzCN modification, with warmer colors indicating greater modification (inset). The nucleotide variations found with respect to the 3′-UTRs of the Asiatic-type Cucurbit aphid-borne yellows virus (CABYV) isolates are indicated by color-coded arrows: orange, nucleotide variations that maintain the secondary structure in double-stranded regions because they do not disrupt base-pairing; light blue, nucleotide variations located in single-stranded regions, gray, three sequence variations in double-stranded regions predicted to disrupt base-pairing.

Our results strongly suggest the occurrence of a recombination event between a polerovirus and a carmovirus, viruses belonging to different families. This is supported by the very high nucleotide sequence identity of the fragment inserted at the 5′-end of the 3′-UTR of MNSV-N with the first 60 nt of the 3′-UTR of CABYV and supported by recombination-detecting algorithms. The presence of MNSV and CABYV co-infecting field-grown cucurbit plants makes this recombination event plausible (Kassem et al., 2007). In this case, recombination has resulted in a broadened host range, supporting the studies that indicate that recombination may assist host switching (García-Arenal & McDonald, 2003; Chare & Holmes, 2006; Codoñer & Elena, 2008; Sztaba-Solińska et al., 2011). The analysis by García-Arenal & McDonald (2003) on the durability of different resistance genes against different viruses showed that pathosystems for which no resistance-breaking strains had been reported were those in which recombinants or reassortants were relatively rare. This result suggested that viruses that undergo genetic exchange were more prone to generating resistance-breaking strains than those that do not. The new MNSV isolate studied here is one example. In this case, the recombination event is the direct reason for resistance breaking. This is one of the first rare recombination events described (Stupina et al., 2008, 2011; Iwakawa et al., 2012).

Imprints of RNA recombination can be found within the genomes of natural populations of plant viruses. RNA recombination seems to be particularly frequent among members of the family Potyrividae, the largest family of plant RNA viruses, but also members of the family Luteoviridae seem to be very recombination prone, and they have been proposed as having emerged from intergeneric recombination events. Thus, several recombinants between poleroviruses and luteoviruses have been described (e.g. Moonan et al., 2000; Domier et al., 2002). These two genera have been proposed to have a common ancestor from which they diverged into different genera by recombination with a sobemovirus and a tombusvirus, respectively (Miller et al., 2002; Pagán & Holmes, 2010). Also stable intergeneric recombinants between potex- and sobemovirus yielded to the new virus species Poinsettia latent virus (PnLV, potexovirus; Aus dem Siepen et al., 2005). Some luteoviruses have even been suggested to have acquired host-chloroplast sequences (Mayo & Jolly, 1991).
events in a plant RNA virus that have been proven to result in resistance breaking or host switching. The adaptation of the multipartite virus CMV to the host alstroemeria through recombination between its RNAs has been described earlier (Chen et al., 2002), but in this case the recombinant virus does not infect an otherwise nonsusceptible host.

The acquisition of the host-switching ability by a virus may impose a reduction in its fitness in the original host, because the new host may impose different selective requirements (Elena et al., 2011). While this was the case for MNSV-264, the first nsv resistance-breaking isolate described, no reduced virulence with the original host has been observed for MNSV-N. Under field conditions, MNSV-264 was not able to become prevalent in MNSV populations, as concluded from the inability to identify it again (M. A. Aranda, unpublished data; Diaz et al., 2004). Our analyses indicate that MNSV-N is slightly less virulent than MNSV-Al in susceptible melons, but its virulence is definitely higher than that of MNSV-264 in resistant melons. These results suggest that MNSV-N may be a more important threat for melon cultivation than MNSV-264 was, especially as the use of resistant melon cultivars is increasing (M. A. Aranda, unpublished data).

Interestingly, the MNSV-264 3′-UTR has <50% nucleotide sequence identity to its counterpart in the other avirulent MNSV strains sequenced to date, whereas the coding regions of all strains show >85% identity (Diaz et al., 2004). In this case, the different 3′-UTR of MNSV-264, gave this isolate the capacity to overcome the melon resistance (Diaz et al., 2004), but this reduced virulence in melon. On the other hand, the precise insertion into the MNSV-N genome of only the 3′-CITE sequence that is functional in resistant melon, did not affect the virulence of this isolate.

In conclusion, we have provided the first direct proof that 3′-CITEs consist of modular elements that can be transferred among viral species most probably through RNA recombination, interacting differentially with host elements to confer host specificity and giving the virus the possibility to infect new hosts.

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**Supporting Information**

Additional supporting information may be found in the online version of this article.

**Fig. S1** Sequence alignment of the 3′-UTR of Asiatic CABYV isolates with the recombined sequence of MNSV-N.

**Fig. S2** Trans-inhibition assays testing the functionality of MNSV-N 3′-CITE in the SHAPE cassette.

**Fig. S3** Sequence alignment of the 5′-UTRs of isolates MNSV-264 and MNSV-N.

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