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Reciprocal sequence exchange between non-retro viruses and hosts leading to the appearance of new host phenotypes

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Abstract

Divergence among individuals of the same species may be linked to positional retrotransposition into different loci in different individuals. Here we add to recent reports indicating that individual variance occurs due to the integration of non-retroviral (potyviral) RNAs into the host genome via RNA recombination followed by retrotransposition. We report that in bees (*Apis mellifera*), approximately 30% of all tested populations carry a segment of a *dicistrovirus* in their genome and have thus become virus-resistant. Reciprocally, segments of host sequences have been found within defective-interfering-like sequences of a *dicistrovirus*. Similarly, host sequences were found fused to potyviral sequences, previously described integrated into their host genome. A potential, continuous RNA exchange leading to divergence is discussed.

Keywords: RNA recombination; Virus-host exchange; RNA heredity; evolution

Introduction

Bee mortality has recently become a more salient concern in Israeli apiculture, due to the discovery of a virus (tentatively termed Israeli acute paralysis virus: IAPV) which has been isolated, partially sequenced and characterized as a *dicistrovirus* (Mayo, 2002; Liljas et al., 2002). Bees are known to carry latent viruses, and epidemics can be elicited by environmental and artificial changes, causing disease outbreaks (Anderson and Gibbs, 1988). Thus, the parasitic bee mite *Varroa destructor* can engender disease by transmitting a virus or by simply puncturing a bee: its role in disease outbreaks has yet to be determined (Camazine and Liu, 1998; Bowen-Walker et al., 1999; Chen et al., 2004). One surprising phenomenon associated with IAPV syndrome is that only some hives in a cluster are affected, even though they are all exposed to the same environmental conditions and forage in the same fields. Since all individuals in a hive are progeny of the same queen, their genetic makeup (except for about 50% of the haploid males) is very similar (at least heterozygously), but different from that of the other hives. Therefore, genetic diversity could explain the susceptibility/resistance of one hive population relative to the next.

The *Dicistroviridae* are a family of insect viruses belonging to the picorna-like super family (van Regenmortel et al., 1999; Mayo, 2002). They carry positive-strand RNA of ca. 9 kb with two open reading frames (ORFs), each coding for a polyprotein that is processed to several active proteins during infection. The 5′ proximal ORF expresses non-structural proteins related to viral replication, and the ORF at the 3′ end expresses the various structural proteins (Govan et al., 2000; de Miranda et al., 2004). The two ORFs are separated by an intergenic region. Translation is not initiated by met-tRNA but rather from internal ribosomal entry sites (IRES), one located in the 5′ untranslated region and the other in the intergenic region. The structural polyprotein is processed by proteolysis to four or five capsid proteins, but (due to intermediate structures?) the number of viral proteins in infected tissue is larger (Govan et al., 2000; Wilson et al., 2000).

Recently, two independent papers have described integration into the host genome of DNA versions of non-retro RNA virus genome segments: flavivirus genome segments were found in its mosquito host (Crochu et al., 2004), and a segment of a potyviral genome was found in some (but not all) grapevine

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varieties (Tanne and Sela, 2005). In the latter case, a history of retrotransposition was evident, and RNA recombination was suggested as the step initiating integration.

RNA recombination between viral RNA segments and between a viral RNA and its respective transgene has been well documented (for example, Nagy and Simon, 1997; Adams et al., 2003, Oberste et al., 2004). However, little information has been published regarding viral–host RNA recombination (Monroe and Schlesinger, 1983; Greene and Allison, 1994; Charini et al., 1996). Recently, genome segments of non-retro RNA viruses have been found integrated in their respective host genomes (Crochu et al., 2004; Tanne and Sela, 2005), and in one case, an inserted segment of Potato virus Y (PVY) was shown to reside in a grapevine genome within a retrotranposomal element and a history of RNA recombination was implicated (Tanne and Sela, 2005). It has been suggested that retrotransposition is involved in the generation of pseudogenes (Esnault et al., 2000), but no evidence of transposition leading to speciation was found (Kidwell and Lisch, 2001; Mourier, 2005). Recently, it has been shown that long interspersed nuclear element-1 (LINE-1) causes individual diversity by retrotransposition into different gene loci in different neural cells, altering different gene functions (Muorti et al., 2005). Here we report a case showing not only the integration of a viral segment into the genome of its host (honeybee) but also the reciprocal integration of a host–genome segment into a virus-derived sequence. As a result of integration of a viral sequence into the host genome, a new phenotype has emerged: individual bees harboring the integrated viral sequence were found to be virus-resistant. In addition, we show that exchange of sequences between host and virus also occurs in the case of PVY and tobacco.

Results

Occurrence of an IAPV sequence in the bee genome and its expression

We PCR-tested the DNA of 100 individual bees from 19 different, randomly selected hives (located 20 to 150 km apart) and found that 31 of them carry a segment of IAPV, residing between bases 1380 and 1808 (GenBank Accession No. AY738529; see Materials and methods) of the structural protein cistron (Fig. 1A). Sequences upstream of this segment were not detected in the bee genome. This was corroborated by Southern blot analysis (Fig. 1B), where four out of eight individuals (lanes 2, 3, 5, 6) reacted positively.

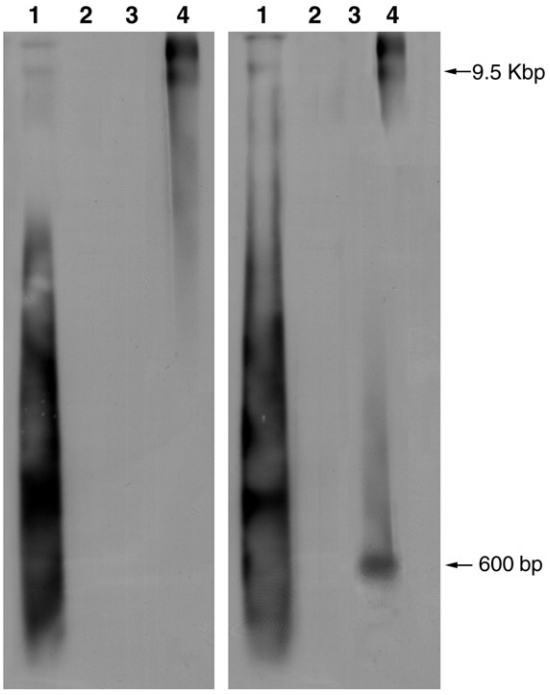

Fig. 2. Northern blot analysis of bee-extracted RNA. Lanes in both panels are identical. Lane 1: RNA was extracted from an IAPV-infected bee. Lane 2: RNA was extracted from a bee that does not carry the viral sequence in its genome. Lane 3: RNA was extracted from a bee carrying a viral sequence in its genome. Lane 4: RNA from a purified virus preparation. The right panel was probed with a viral segment found to be integrated into the bee genome. The left panel was probed with a viral segment that has never been found integrated in the bee genome.

Northern blot analysis indicated that the integrated viral segment is transcribed (Fig. 2), and Western blot analysis indicated the expression of a shorter version of a viral capsid protein only in bees harboring the viral segment in their genome (Fig. 3).

Since bees may carry latent viruses, an RNA-based analysis needs to be able to distinguish between a viral–homologous RNA transcribed from a genome-embedded sequence and that of a potential latent virus. The appearance of a well-defined band only in viral–segment-carrying bees in the northern analysis, and only when probed with the inserted segment (Fig. 2, right frame, lane 3), and the absence of a similar band in the left frame (probed with a viral sequence upstream of the integrated one), is indicative of expression of the integrated sequence and rules out its being a degradation product of latent infection. Nevertheless, we used two additional methods to

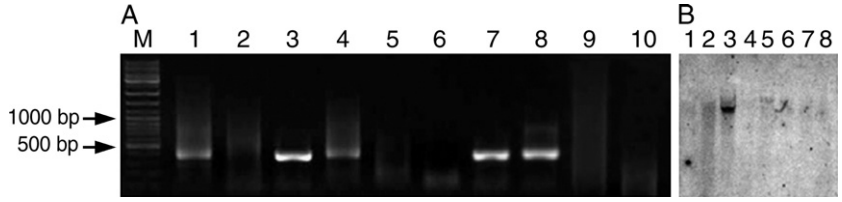

Fig. 1. PCR and Southern blot evidence for the incorporation of a viral sequence into the bee genome. Left panel: PCR was carried out with bee DNA. Lane M: size markers. Lane 7: positive control; PCR was performed with a cloned IAPV. Lane 6: negative control; PCR was conducted without template. In all other lanes, template DNA was extracted from various bees. Right panel: Southern blot analysis of DNA extracted from various individual bees and probed with the cloned structural protein ORF of IAPV. Template DNA in lanes 1, 3, 4, and 8 in panel A correspond (respectively) to lanes 2, 3, 5 and 6 in panel B. Size marker ladder is in 100 bp increments.

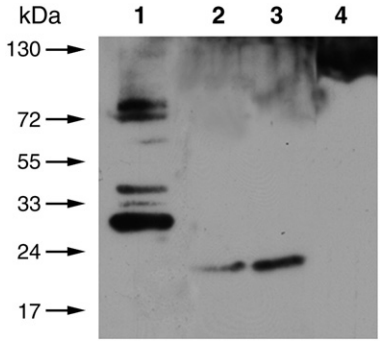

Fig. 3. Expression of an IAPV-derived protein in bees; a western blot of bee proteins. The blot was reacted with antibodies to IAPV. Lane 1: Proteins extracted from purified IAPV virions. Lanes 2 and 3: Proteins extracted from bees carrying an IAPV segment in their genome. Lane 4: Protein extracted from bee not harboring any IAPV segment in their genome. Size markers are indicated on the left.

discern transcription of genome-integrated viral sequences from virus infection: (a) A sequence upstream of the integrated one (bases 1263 to 1400 in the structural protein clone) has never been found in the bee genome, and hence the presence of RNA carrying this sequence is indicative of viral (or latent viral) infection. On the other hand, the occurrence of RNA corresponding to bases 1380 to 1808 and the absence of nucleotides 1263 to 1400 (Figs. 2 and 4) implies transcription from the integrated viral segment in the absence of infection. (b) Since RNA viruses replicate via a double-stranded intermediate, RNA in both sense (viral) and antisense orientations is expected in cells with a replicating virus, whereas RNA transcribed from an integrated sequence should be in either sense or antisense orientation. This was determined by RT–PCR with primers amplifying the two aforementioned viral sequences, as well as with primers for the 1380 to 1808 segments, designed to amplify either sense or antisense RNA.

Genome-walking analyses identified five loci of integration of the viral segment in the bee genome: clone EB29 integrated into the bee chromosome LG3, clone EA19 into chromosome LG5, clone EC5 into chromosome LG6, clone EC42 into chromosome LG12 and clone EC21 into chromosome LG16.

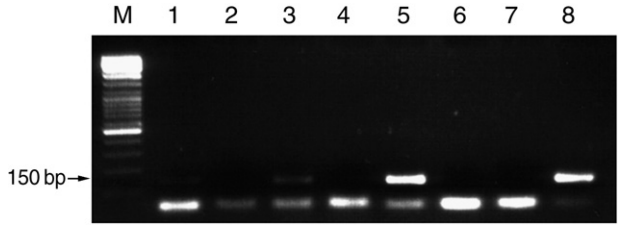

Fig. 4. Discerning viral infection from expression of integrated viral sequences. RT–PCR was performed with primers amplifying a region outside the one found integrated into the bee genome. The various lanes represent RNA samples extracted from various individual bees. The appearance of a 150-bp band is indicative of infection. Arrows indicate sizes in bp. Lane M: size markers. Lane 6: negative control; PCR without a template. Lane 7: negative control, PCR without RT. Lane 8: positive control; the template was viral RNA. Lanes 1–5: RNA was extracted from various bees. Size marker ladder is in 100 bp increments.

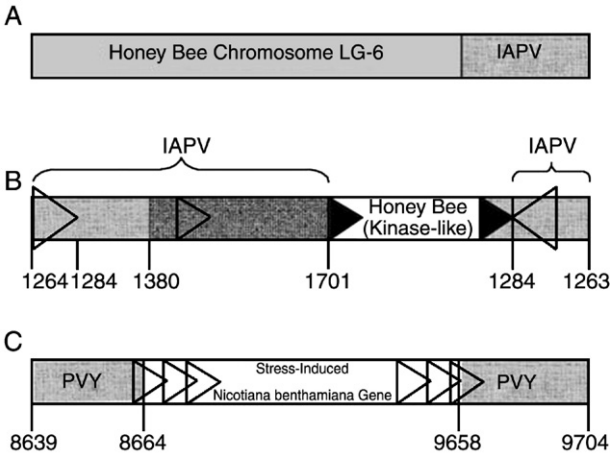

Fig. 5. Schematic illustration (not to scale) of: (A) representative bee locus with an integrated viral segment; (B) the defective viral RNA, extracted from IAPV virions, carrying a bee sequence; (C) the defective viral RNA extracted from PVY virions carrying a sequence of *N. benthamiana*. The triangles represent sequence repeats and their orientation. The numbers indicate the sequence positions on IAPV (Frame B) and PVY (Frame C). Numbering is according to GenBank Accession Nos. AY738529 for IAPV and D00441 for PVY.

EC5 (as an example) is diagrammatically illustrated in Fig. 5A and the entire sequence is shown in Fig. 6. Chromosomal locations and positions within the chromosomes of all clones are summarized in Table 1.

The clones' sequences were deposited with GenBank (Accession nos. DQ468104–DQ468108). All viral insertions were found in intergenic regions of the various chromosomes.

Bees carrying a viral sequence in their genome are resistant to IAPV infection

Sixty-four randomly selected, healthy-looking bees were tested by DNA–PCR for the presence of integrated viral sequence, and by RT–PCR of the upstream (non-integrated) region for latent viral infection. Nineteen bees (29%) carried the viral segment in their genome, as evidenced by their positive reaction to the DNA–PCR assay. None of them reacted to the RT–PCR of the upstream sequence, and they were thus considered virus-free. The remaining 45 individuals did not carry the viral segment in their genome, but RT–PCR indicated the presence of latent virus in 22 of them. The absence of latent infection in viral–segment-carrying bees raised the question of whether bees harboring a viral segment are resistant/tolerant to viral infection. This was tested directly by inoculating bees with IAPV. The survival rate of bees (not carrying the viral segment) following injection of IAPV (1 μl containing 1 μg of virus per larva or pupa) was tested on over 300 individuals (in groups of 20–100 each) and was found to average less than 2%. We then injected 80 bee pupae with a purified virus preparation. The experiment and its controls are summarized in Table 2. Of the 80 virus-injected bees, 67 individuals died, but 13 emerged as mature, healthy-looking adults, indicating not only survival, but normal development as well. RT–PCR assays with primers amplifying the aforementioned viral sequence (bases 1380 to 1808 of the structural protein clone) in either sense or antisense

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   1  ATCGATTTTC CGCGAGAGAG AAAAAAAGAA AGAAAGAAAG AGACGAAAAG
  51  TGAAAGCCAC CAAAAATCGC CTGTCCCTCG GTTTAACTTT TTCCAGTTTC
 101  GTTCGGGCGT GTCGTTGCGG GTCGATGGAA CGGGAGACAC CGGGAGACAT
 151  ATACAACACC GGATCGGATA GTTGGCAGAC GCGTGTTCAC CACACGGGGC
 201  GAATTTTTCT CTATGTTTCA GCGCGTACAG TTTCCACGTG TCGAGCGAGG
 251  GCCAGATGCA GCCGGTGCCC TTCCCACCGG ATGCGCTCAG CGGACAAGGG
 301  ATACCGCGCC ATGCGCGGCA AACTAATACC CTCAGCCACG GGGAGGTCGT
 351  GTGCGCTGTC ACGATCTCGA ATCCGACCAA ATATGTGTAC ACCGGCGGCA
 401  AGGGATGGGT CAAGGTTTGG GATATAGGGC AAGGTGGCAC GGGCAGCACC
 451  AAGTCCGTGT CCCAGCTCGA CGGCCTCCAA CCTGACAATT ACATCAGGTC
 501  AGTGCTCTTT TATTCTATAC CCTTCCAGCT GCTTTATCTT CAATTAAATT
 551  TTATCATACG ATTTTCTTTT CCACGATGTT CGAATTAAAA AATCGAAAAA
 601  GACACTTTGA ATCTTAGATT AGAATCTGAA AATTTTCTAA AATTTAAACT
 651  TTACACTTTC TCTTTATTAG ATTATTATAG AATTATTTTT TTAATTTTGA
 701  CGATCTATAT CTATATTTAT CAAATAATCT AAACCAGAGT CCATAAACAA
 751  ACGGTGAAAC AAATACTGTC CAAATTGTAT TACTATTTTC TGGAAGGAAA
 801  AAAAGGaAAA AAAAAACAAT TACGATATTC CGCCATCGCG TGGGGGAATA
 851  CAATTAATTT TACCAGAGAG AACACACCTC TGGAAACATT CTTACAATAA
 901  CACGAAGCGA TCGTTGAAAC GATATTATGT AAACCCTTCC CTCCCCCCCT
 951  CCAATAGGAT TTTATTTCAC AAACGGGTCA CCAGCAATCG AGCCGATCGA
1001  ACCGAAACAA AAACCTACCG TTTTGCACCG TTAAAAACGA AAGAGCGAGA
1051  GAAAGGATCC GCCGTGGGGC GAAACGGAGG AGGAGGACGC ATCGTATAAT
1101  GAGATTCAAT GTATTCCATG CACGCTCGAA AAATCGCGGC AATGTGTCGG
1151  TCGTGTAGAA CAAACGGGGG GGGCCGGGCG GGGTGTTCGA CGTTTTCACG
1201  ATACATTTCG CGCTGGATGT GCACTGGGAG ACAGACAAAT CT
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Fig. 6. An example of a bee locus in which a viral sequence has been integrated (clone EC5). Further details are shown in Table 1. Nucleotides 1 to 1190 correspond to an intergenic region on chromosome 6 of the bee genome (99% identity with nucleotides 519505 to 520633 of Accession No. NW622830.1). The integrated viral sequence (bold letters) is 83% identical to IAPV, and is a part of the viral genome that was found integrated by PCR. A diagrammatic representation of this sequence is presented in Fig. 5A.

orientation revealed that (as expected) all dead bees carried RNA in both orientations, whereas all surviving individuals expressed the integrated viral sequence in only the sense orientation, indicating expression only from the integrated segment and that viral replication had not taken place. An example of these results is shown in Fig. 7.

A segment of a bee gene is embedded within an IAPV-associated defective RNA

Viral RNA recombination has hitherto been attributed to template-switching of the viral replicase. Recent reports, however, indicate a non-replicative, non-sequence-specific form of viral RNA recombination attributed to a breakage and joining mechanism (Agol, 1997; Nagy and Simon, 1997; Gallei et al., 2004). The latter is not necessarily dependent on recognition by a replicase, and may theoretically engender recombination between any two RNA species, including viral and host RNAs; a few such cases have been reported (Monroe and Schlesinger, 1983; Charini et al., 1996). While template-switching recombines one RNA species with another, breakage and joining is reminiscent of crossing over in DNA, where a segment of one DNA species may be flanked on both sides by segments of the other DNA species. Since we suggested that in the case of IAPV, the initial step was RNA recombination between a viral sequence and a bee sequence, we postulated that the recombination sites were adjacent to that viral sequence which was found integrated in the bee genome. Therefore, we performed RT–PCR on RNA extracted from CsCl-purified IAPV virions, using primers adjacent to the bee-integrated sequence. This resulted in the appearance of the expected major viral band with a few minor bands alongside it. As demonstrated diagrammatically in Fig. 5B, one of these minor bands (Fig. 8), which is reminiscent of a "defective-interfering" viral RNA (DI-RNA), was found interrupted by 41 nucleotides of a non-viral sequence homologous to *rac1-activated kinase* of several organisms (96% identity with the respective human segment; Fig. 9).

The kinase sequence embedded within the viral sequences is bordered by 10-bp direct repeats of the *kinase* sequences, and the entire virus–host chimeric sequence is flanked on both

Table 1
Characteristics of the loci in which an IAPV viral sequence was integrated into the bee genome

Clone	The bee chromosome into which a viral sequence was integrated	Position on the chromosome	Accession no. of the bee sequence and % identity with that of the clone
EB29	LG3	244882 to 245322	NW_622759; 97%
EA19	LG5	182579 to 182748	NW_622804; 99%
EC5	LG6	519505 to 520633	NW_622830; 99%
EC42	LG12	391790 to 392141	NW_622565; 98%
EC21	LG16	394542 to 394849	NW_622631; 99%

Table 2
Summary of bee analysis following the injection of purified virus to pupae

				Carrying a sense viral sequence	Carrying an antisense viral sequence
H_2O-injected	Died	9	2	+	
			7		
	Emerged as adults	71	9	+	
			62		
Virus-injected	Died	67	67	+	+
	Emerged as adults	13	12	+	
			1	+	+

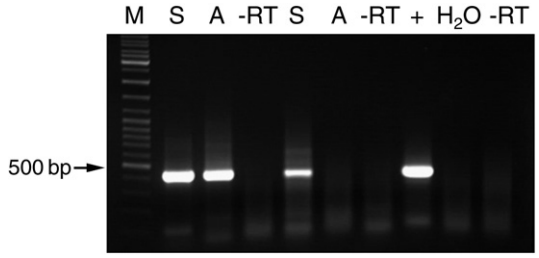

Fig. 7. Example of the PCR analyses carried out as summarized in Table 2. RT–PCR of RNA extracted from bees. M: Size markers. S: Reverse transcription was performed with a primer adhering to sense-oriented RNA. (A) Reverse transcription was performed with a primer adhering to antisense-oriented RNA. −RT: A control reaction to ensure that no DNA is reacting; the reverse–transcription step was performed in the absence of reverse transcriptase. In the second, third and fourth lanes (from left) RNA was extracted from a dead bee. In the fifth, sixth and seventh lanes (from left) RNA was extracted from a surviving bee. +: Positive control with viral RNA; reverse transcription was performed with a sense-adhering primer. Size marker ladder is in 100 bp increments.

sides by inverted repeats of the adjacent viral sequences, suggesting a history of retrotransposition followed by further translocations by DNA transposition (note that the inverted viral repeats are the immediate upstream sequences of the segment of the IAPV genome that was found integrated into the bee genome).

A segment of a Nicotiana gene is embedded within a PVY-associated defective RNA

Since PVY sequences were also found integrated in a plant genome (Tanne and Sela, 2005), a similar search with postulated PVY-DI-like sequences was also carried out. A PVY-DI carrying a segment of a stress-induced gene of a tobacco species (*Nicotiana benthamiana*; 99 bp, 86% homology) was found embedded in the PVY-DI sequence (Figs. 5C and 10). This stress-inducible gene segment carries several direct repeats (homology among the various repeats was 83–95%). Thus, the reciprocal exchange of genomic segments between virus and host at the RNA level was experimentally demonstrated.

Discussion

Evidence for reciprocal exchange of genetic material between virus and host

Previous papers have reported that DNA versions of RNA viral segments can be found in the respective host genome (Crochu et al., 2004; Tanne and Sela, 2005). This paper reports not only the integration of a DNA version of an RNA segment from a bee-affecting *dicistrovirus* into the genome of the honeybee, but also the reciprocal integration of an RNA version of a segment of the host DNA into the virus. This indicates RNA–DNA mobility in both directions in a non-retroviral system. Based on structural features of the integrated viral sequences, we postulated an initial step of recombination between viral RNA and host mRNA, or a host RNA sequence carried on a retrotransposable element (Tanne and Sela, 2005). This is further corroborated in the present paper by the direct and inverted repeats in the RNA sequences as well as by the fact that the chimeric structure represented in Fig. 5B carries a viral sequence which has never been found integrated into the bee genome, implying that the recombination was at the RNA level. The five clones of the bee genome found to harbor IAPV sequences are positioned within intergenic regions of various bee chromosomes. This suggests that their origin may have been a transposable element, now deteriorated and non-autonomous. However, IAPV-derived RNA and protein are expressed, indicating that some IAPV-harboring loci remained as autonomous, expressible, transposable elements, or were integrated, in-frame, into the coding sequences of a host gene, or downstream of a host promoter.

Postulated mechanisms for RNA-based exchange of genomic information

Once a viral sequence has been mobilized into the host genome, it may serve, due to its inherent repeats, as a hot spot for further DNA transpositions within the DNA genome, resulting in gradual deterioration of the inserted sequence (for example, Vitte and Panaud, 2005). The grapevine locus into which a viral segment has been integrated (Tanne and Sela, 2005) and the aforementioned DI-like RNA sequences carry footprints of both transposition and retrotransposition. Recombined RNA, suggestive of a mechanism resembling crossing over (such as that of the DI-RNAs in Figs. 8 and 9), indicates the potential for recombination of any two RNAs (probably at a very low rate, depending on their proximity and abundance). Retrotransposition of heterologous sequences has been reported; however, it was assumed that the mechanism of fusion of two sequences had been DNA recombination, or that a transcript of a retroelement extended into a neighboring gene was spliced, joining together retro and host gene sequences (Derr, 1998; Mourier, 2005). Currently, we are developing a model system which may confirm the mobilization of a non-retro RNA into the genome, the frequency of these events, and the frequency of integration events leading to expression.

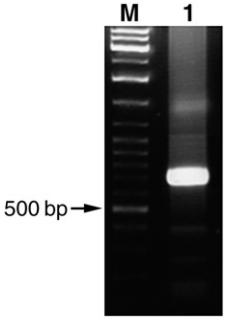

Fig. 8. Electrophoretic pattern of the products of RT–PCR performed on RNA extracted from purified IAPV virions. PCR was conducted with primers corresponding to IAPV sequences immediately adjacent to the viral-integrated sequence. The sequence presented in Fig. 9 is of the minor band indicated by the arrow. Size marker ladder is in 100 bp increments.

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  1  GACACCAATC ACGGACCTCA CAAACACCAC AGATGCTCAG GGTCGAGACT
 51  ATATGTCTTA CCTGTCCTAT TTATACCGAT TTTATCGAGG AGGCCGGCGT
101  TATAAATTCT TTAATACCAC CCCTCTCAAA CAATCTCAAA CATGCTATAT
151  AAGAAGCTTT CTTATACCAC GTAATTACTC AGCTGATGAA ATTAACGTAG
201  ACGGACCTTC ACATATAACA TACCCCGTAA TCAATCCTGT GCATGAAGTA
251  GAAGTTCCAT TCTATTCTCA GTATAGGAAA ATACCTATCG CTTCAACATC
301  GGATAAAGGT TATGATTCCT CTCTAATGTA TTTTTCAAAT ACAGCAGCAA
351  CTCAAATTGT TGCCAGAGCA GGAAACGATG ACTTTACCTT TGGTTGGATG
401  ATAGGTCCAC CCCAGCTACA AGGCGAATCA CGCTCCGTTT GCTCTGGGGT
451  TCCTACCATG GCGCTCCGTT TGCTCTGGTG AGGTCCGTGA TTGGTGTCT
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Fig. 9. The sequence of the minor band shown in Fig. 8. IAPV sequences are in bold letters. The segment of the non-viral sequence (*rac1-activated kinase*; Accession No. BC109299) is underlined. A schematic representation of the sequence is shown in Fig. 5B.

Possible impact of RNA recombination and reciprocal mobilization on genome dynamics and evolution

The potential impact of viral RNA recombination on viral as well as host evolution, disease severity and avoidance of the host immune system has been discussed at length (Bujarski and Kaesberg, 1986; Strauss and Strauss, 1988; Jarvis and Kirkegaard, 1992; Lai, 1992; Gallei et al., 2004). A case showing transposition-mediated divergence among individuals has also been demonstrated (Muorti et al., 2005). The present report experimentally supports a hypothesis of perpetual emergence of variance within a population leading to the appearance of new phenotypes within the same species or sub-species (strains, races, varieties, etc.). Thus, another factor may be a presumed cause of variation in both viruses and hosts. Moreover, we suggest that events leading to genome mosaicism and perpetual genome dynamics also take place at the RNA level. Stress, including viral infections, increases the rate of retrotransposition (Wessler, 1996) and the tobacco Tnt1 retrotransposon shares transcriptional activation regions with stress-inducible genes (Mhiri et al., 1997). The abundance of viral RNA and transcripts of some host genes, and the template-switching capacity of both reverse transcriptases and the viral replicases may enable RNA recombination and insertion into the host as well as the viral genomes.

Bi-directional RNA mobilization may lead to dual co-evolution of host and virus. Expression of viral sequences from the host genome is probably an infrequent event prone to deterioration. However, when expressible, it may lead to the appearance of a new host phenotype. The engendering of resistance might be a result of an RNAi mechanism now directed towards the viral sequence, or due to the removal of the pertinent host gene segment (Esnault et al., 2000). However, the reciprocal mobilization of a host sequence into the virus leads to a more stable, possibly replicative, virus-derived form, affecting virus divergence and virus–host interactions. This is due to the postulated ability of the native virus to support replication of the chimeric virus–host entities. Furthermore, if the viral part of the chimera carries signals for assembly, the newly formed RNA will be encapsidated with the viral coat protein(s), as demonstrated in the present report. This may be a basis for viral divergence and may explain, for example, the origin of a host-homologous heat-shock protein in all closteroviruses (Karasev, 2000). The resultant virus–host chimeras, stabilized, and possibly replicative as "parasites" of the native virus, or even encapsidated within viral particles, can now be transmitted horizontally to other host individuals, to other host species, or even acquire new host range. Viruses are transmission-competent entities, and may be better suited than transposons for horizontal transmission of genetic material.

Transgenic resistance and environmental risks

The aforementioned integration of a viral segment into the bee genome results in "naturally transgenic" bees with a pronounced new phenotype: resistance to subsequent viral infection. Man has now successfully created transgenic organisms: in plants, transformation with viral sequences has brought about (in many cases) viral resistance (Sela, 1996). In some cases, this resistance is RNA-mediated and does not require expression of a protein from the transgene (Silva-Rosales et al., 1994; Smith et al., 1994). Apparently, man has unknowingly been mimicking a natural process.

Man has been concerned with the possible hazards of virus–host RNA recombination. The authors tend to agree with Falk and Bruening (1994) that man-engendered RNA recombination is statistically insignificant relative to the seemingly frequent natural process.

Materials and methods

Virus isolation

The homogenate of a single dead bee was injected into healthy bees and the virus was isolated from them. Bee homo-

```
  1  CAGCCAAACC CGAACAAAGG GAAGCAGTTT AAGTTTGCAA TTCAAGTTTT
 51  AAGGAAAAGC AATGCAAGTG TTCAGCAATT AGGAGCCCAC TTGGAGAACA
101  AAGGGAAAGT AGCGAGGTTC AAAGGAGTTC AACAATCAAT AGCCCACCTG
151  AAGAACAAAG GGAAGCAGTT CAAGTATTGA GGAAAGGCAT CGCAAGTGTT
201  GTGATTCCGT CATAACAGTG ACTGTAAACT TCAATCAGGA GACCCGGGTT
251  AGA
```

Fig. 10. A sequence of a PVY-derived DI. PVY-homologous sequences are in bold. The segment of the stress-induced gene of *N. benthamiana* (Accession No. CK282886) is underlined. A schematic representation of the sequence is shown in Fig. 5C.

Table 3
List of the employed primers

Primer number	Primer sequence (5′→3′)	Use
936 (forward)	CCACCCCTCTCAAACAATCTCAAACA	Amplifying IAPV segment from the bee genome and amplifying IAPV-DI. Primer # 935 was also used for genome walking
935 (reverse to IAPV, forward in nested genome walking)	AGATTTGTCTGTCTCCCAGTGCACAT	
937 (reverse to IAPV, forward in genome walking)	TATATCCAGTTCAAGTGTCGGTTTTC	Used for nested genome walking
708 (forward)	AGA CAC CAA TCA CGG ACC TCA C	Determining viral infection in bees
780 (reverse)	GAG ATT GTT TGA GAG GGG TGG	
Ap1 provided by Clontech's kit	GTAATACGACTCACTATAGGGC	Genome walking Adaptor
Ap2 provided by Clontech's kit	ACTATAGGGCACGCGTGGT	Genome walking Adaptor (nested)
383 (forward)	CAG CCA AAC CCG AAC AAA G	PVY DI Primers
107 (reverse)	CTA ACC CGG GTC TCC TGA TTG AAG TTT ACA GTC	PVY DI Primers

genates (in 0.01 M phosphate buffer, pH 7.6) contained a considerable amount of bacteria which were lysed with 0.3% sodium deoxycholate and 3Brij 58 (Sigma) followed by centrifugation at 10,000×*g* for 10 min. The pellet was resuspended in the aforementioned buffer and subjected to two cycles of centrifugation (10,000×*g* alternating with 100,000×*g*). The final pellet was resuspended in a solution of CsCl (1.33 g/ml) and centrifuged at 100,000×*g* for 24 h. The resultant single band was collected, diluted with buffer and re-centrifuged at 100,000×*g* for 1 h.

Molecular procedures

Nucleic-acid extraction, PCR, Southern, Northern and Western blot analyses, DNA sequencing and other molecular procedures were performed according to established protocol (Sambrook and Russell, 2001). Genome-walking was carried out according to the protocol of the kit provider (Clontech).

To pinpoint the ends of the viral sequence integrated into the bee genome, we first performed PCR with randomly selected primers to various parts of the viral non-structural and structural protein ORFs. Only one set of primers (of the structural proteins' ORF) reacted positively. We then conducted PCR assays, changing one primer at a time (in increments of 10–20 bp) upstream and downstream of the reacting region, until the PCR no longer reacted. The position of the insert's end was further specified when the junction of the bee genome/IAPV sequences was determined by genome walking. The primers used for the various (reacting) PCR assays are specified in Table 3.

Genome walking

Genome walking from the IAPV sequence into the flanking bee sequences was carried out using Clontech's Universal Genome Walker Kit according to the manufacturer's protocol. The DNA for the genome-walking experiments was RNase-treated prior to cleavage with restriction enzymes. All genome-walking primers were with T_m values of about 70 °C. Primers used for genome walking are listed in Table 3. Primers #937 and AP1 were used to amplify IAPV-harboring genomic fragments of the bee. Primers #935 and AP2 were used for the nested PCR stage of this amplification. The resultant genome-walking PCR products were cloned into the vector pDrive (Qiagen) and sequenced therefrom.

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