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Recombination of Engineered Defective RNA Species Produces Infective Potyvirus In Planta†

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Recombination occurred between viral genomes when squash plants were cobombarded with mixtures of engineered disabled constructs of a zucchini yellow mosaic potyvirus. Single and double recombinants were detected in the progeny. Genes involved in the recombination process and the mechanisms of recombination were studied in potyviruses for the first time.

Recombination of RNA viruses permits rapid evolution and adaptation (1, 4, 13, 16, 24). Recombination of plant RNA viruses is thought to involve the RNA-dependent RNA polymerase (12, 19, 20). The recombination process has been studied in several groups of plant RNA viruses (14, 16, 18, 24, 26) and in animal viruses with large RNA genomes, such as coronaviruses (14) and picornaviruses (9). Potyviruses are the largest group of plus strand RNA viruses, many of whose genes are involved in replication (10, 11, 23, 25). However, no experimental research has been performed on recombination of potyviruses, although sequence analysis of potyvirus strains indicates that recombination has occurred naturally (3, 4, 21, 22). Zucchini yellow mosaic virus (ZYMV) is a potyvirus (15), 9,593 nucleotides (nt) in length (2), mainly infecting cucurbitaceous species. Genome rearrangements have been detected in an engineered tobacco etch potyvirus (5) and similarly in an engineered ZYMV (8a).

A method has been developed in our laboratory for infecting cucurbit plants through particle bombardment with a cDNA clone of ZYMV under the control of the 35S promoter, several orders of magnitude more efficient than mechanical inoculation (7). Recombination of ZYMV by particle bombardment with a handheld device (8) was attained with mixtures of two plasmids (0.1 to 0.2 μg of DNA of each plasmid per squash [*Cucurbita pepo* L. cv Ma'ayan] seedling), precipitated together onto tungsten microprojectiles. Each plasmid encoded a different disabled chimeric ZYMV clone.

Constructs were designed specifically for cobombardment. In the first experiment three constructs were used: p35S ZYMV-CP (Fig. 1A), a construct with most of the coat protein deleted (270 of 279 amino acids) (cleaved with *Alw*NI and *Avr*II from the full-length clone p35SZYMV [7], removing nt 8563 to 9397, filled with Klenow fragment), and tagged with a *Sal*I restriction site at the 3′ end of the P1 gene; p35SZYMV-HC (Fig. 1B), where most of the HC-Pro was deleted (cleaved with *Bst*EII and *Bam*HI, removing nt 880 to 2280, filled with Klenow fragment maintaining the frame); and p35SZYMV-P3/CI (Fig. 1C) from which most of the P3 and about two-thirds of the CI genes were removed (ligation after cleavage with *Nde*I, removing nt 2573 to 4801).

None of these constructs alone was infectious by particle bombardment (Fig. 1). However, 30% of the plants cobombarded with constructs A and B and 15% of plants cobombarded with constructs A and C show severe symptoms, suggesting that recombination occurred. To confirm recombination events reverse transcriptase-PCR (RT-PCR) of progeny virions was performed with primers P1 (5′-CATACATATGGCCTCCATCA-3′) at position 133 and P2 (5′-AGGATCCTGGGTAATTC-3′) at position 2286. The existence of the *Sal*I site was confirmed by digestion of the PCR fragment, producing two fragments (Fig. 2, lane 2). The DNA segment produced by RT-PCR of control (untagged) virions cannot be cleaved by *Sal*I (Fig. 2, lane 3). The greater percentage of infected plants following cobombardment with constructs A and B than with constructs A and C is possibly due to the larger deletion in construct C (2.3 kb) than in construct B (1.2 kb). We assume that recombination occurred due to the polymerase switching from one donor strand to another, during the synthesis of the minus strand (Fig. 3A), when the nascent RNA strand released by the first donor strand is complementary base paired to a second donor strand, as suggested previously (14, 26).

To examine the possibility of multiple recombinations following the process of cobombardment, a different approach was attempted, by using disease phenotype markers. Selection with a phenotype marker allows easy determination of differential recombination events in a plant population. Cobombardment was performed with construct A (p35SZYMV-CP, originally produced from a severe isolate) mixed with construct D (p35SZYMV-P3, obtained from a mild strain, where the mild symptom characteristic maps to the 5′ end of the ZYMV genome) (unpublished data) (Fig. 4). In this manner, a population with a mixed phenotype was obtained for the first time: 20% plants with severe symptoms and 8% plants with mild symptoms (Fig. 4). Severe symptoms represent the viral progeny resulting from a single recombination. Double recombination events are less frequent and cause mild symptoms. In the double recombination process, the polymerase switches donor template twice during synthesis of the minus strand, from the first donor to the second donor strand, returning later to the first donor (Fig. 3B). Possibly the experiment reported in Fig. 1 produces progeny carrying double recombination events (without *Sal*I sites), but these were not found in the progeny virions of the small number of infected plants examined from that experiment.

This relatively simple method can be used to screen viral genes for involvement in recombination (Fig. 5). A construct was designed with the entire ZYMV genome, out of frame (oof) following the *Bam*HI restriction site (200 bp from the 3′ terminus of the HC-Pro) (construct E, p35SZYMV-oof). We

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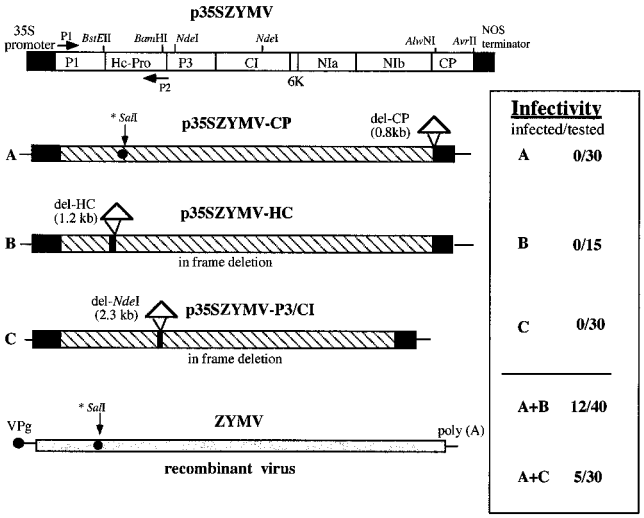

FIG. 1. Schematic representation of truncated infectious clones of ZYMV, showing the identifying features of each clone. The upper panel of the figure (p35SZYMV) represents the full-length infectious clone of ZYMV, under control of the 35S promoter and *Nos* terminator (7). Each ZYMV gene is marked, along with the key restriction enzyme sites and primers used. Panels A to C display the additional characteristics of the truncated clones, marking the identifying features of each. The bottom panel represents the resultant recombinant virus, with the restriction enzyme site used for identification. The panel on the right enumerates the infectivity of each clone (or mixture of clones) following particle bombardment. The numbers given are the number of squash plants with visible symptoms of viral infection/total number of plants test bombarded.

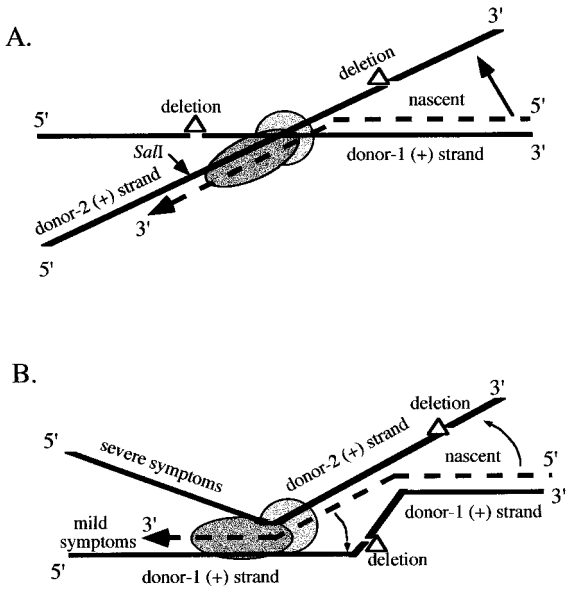

FIG. 3. Diagrammatic representation of homologous recombination by the switching of templates during minus (−) strand RNA synthesis. (A) A single recombination event where the polymerase switches truncated donor strands, to process a recombinant infective RNA. The movement (from one donor strand to the second) of the released 5′ end of the nascent RNA is marked with a solid arrow. The deletions in each donor strand are marked. (B) In the double recombination event the nascent strand switches twice from donor 1 to donor 2 and then returns to donor 1. The movement of the 5′ end of the nascent strand is marked with an arrow.

assume that as only the P1 protein could be processed from this construct, the clone is not infectious by itself (Fig. 5E). Cobombardment of the oof construct with plasmid A (p35S ZYMV-CP) produced an infectivity of 60%. This result indicates that the coat protein itself was not necessary in the recombination process (Fig. 5), while not excluding the possibility that part of the coat protein gene sequence was necessary for replication, as reported (17). However, cobombardment of the oof construct E with construct F (p35SZYMV-P3) does not result in infectivity, implying that the gene product(s) from this region (carboxy terminus of the HC-Pro, P3 and 6K2 genes, and N terminus of the CI) is essential for recovery of infectious RNA.

We wished to prove that recombination actually occurred through viral replicase genes, rather than due to a plant-encoded DNA recombinase. Plants were cobombarded with a mixture of plasmid F (p35SZYMV-P3) and a second plasmid encoding the intact ZYMV genome under the control of the bacterial T7 promoter (infectious through in vitro RNA synthesis) (6). Individually, the constructs are not infective (data not shown). Cobombardment also is ineffective (0/20), supporting our suggestion that recombination occurs through RNA-dependent RNA polymerase not through a host-encoded DNA recombinase.

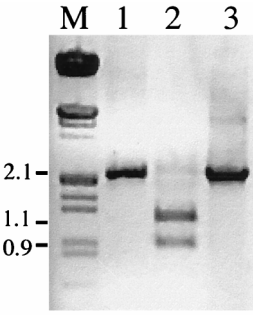

FIG. 2. Analysis of tagged recombinant progeny virus by digestion of RT-PCR products. Gel-purified RT-PCR fragments were separated by electrophoresis on a 1% agarose gel. Numbers indicate the expected size of digested and undigested fragments (in kilobase pairs). Lane M, Lambda phage cut with *Hin*dIII/*Eco*RI molecular weight markers; lane 1, the undigested RT-PCR fragment from the tagged progeny virus; lane 2, the products of the tagged virus cleaved by *Sal*I; lane 3, the nontagged progeny RT-PCR products, after *Sal*I digestion.

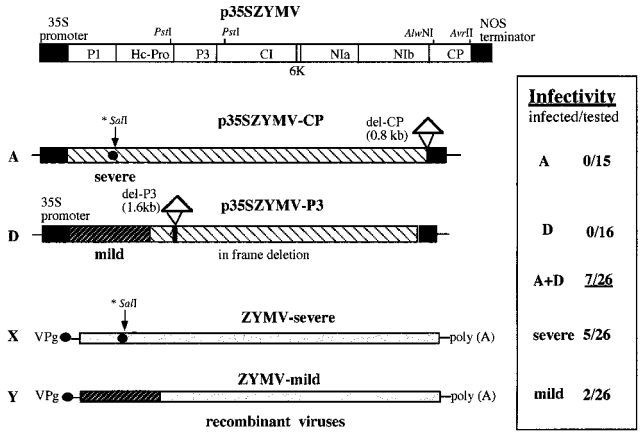

FIG. 4. Schematic representation of truncated infectious clones of ZYMV used for demonstration of the double recombination process. The top panel is the standard map of ZYMV. Panels A and D are truncated clones, D bearing the weak symptom sequence. X and Y are the product viruses, respectively bearing one and two recombination events. The right panel shows the infectivity of the clone(s) after bombardment.

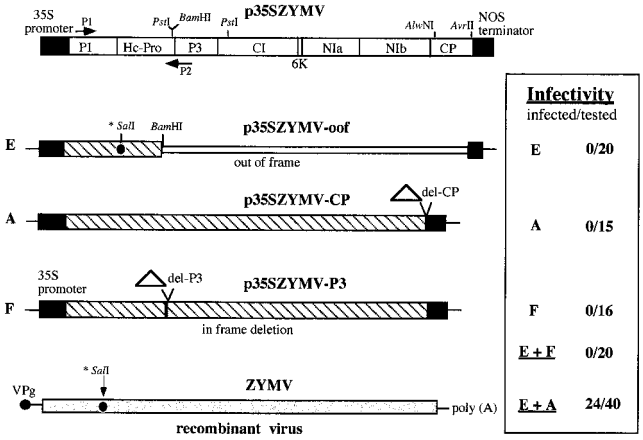

FIG. 5. Mapping of the genes involved with the recombination process. The top panel shows the map of the ZYMV genome. E is a construct of the intact ZYMV genome that is largely untranslatable, and A and F are truncated constructs with deletion of the CP and P3 genes, respectively. The lowest panel is the progeny recombinant virus. The panel on the right shows the infectivity of the bombardments of the clones and their mixtures.

We assume that the great efficiency of infectivity by particle bombardment is because cDNA is delivered directly into intact host cells (7). Precipitation of premixed cDNAs ensures that a high percentage of tungsten particles are coated with both types of cDNA, ensuring delivery of both cDNA species to the same host cell. Recombination was never obtained through mechanical inoculation of cDNA or in vitro synthesized transcript. Nonreplicating constructs introduced to the same cell were translated and assembled to produce an active viral replicase. We assume that during the synthesis of the minus strand the replicase switches donor (plus) strand templates, as described previously (26). During potyvirus replication a high ratio of plus-to-minus-strand RNA is produced (4), and therefore there is a higher probability that the plus strand is the donor. However, the possibility that the recombination event could be due to switching minus strand templates during plus strand synthesis cannot be ruled out. Computer analysis of the 3′ nontranslated region of many strains of potyviruses statistically confirmed recombination between natural strains of potyviruses, including ZYMV (21).

This publication is the first to demonstrate direct evidence that recombination occurs in plant cells infected with truncated cDNAs of a potyvirus by particle bombardment. This system is now being used for the investigation of intra- and interspecies recombination in potyviruses. This methodology will allow the construction of new infective clones from parts of large RNA viruses.

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