

Agroinfection of Transgenic Plants Leads to Viable Cauliflower Mosaic Virus by Intermolecular Recombination

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Intermolecular reconstitution of a plant virus has been detected in whole plants in a system using a defective cauliflower mosaic virus genome and transgenic host plants containing the missing viral gene. The information for the gene VI protein of the virus was integrated into the chromosome of host *Brassica napus* plants and leaves of these plants were inoculated with *Agrobacterium tumefaciens* containing the complementing viral sequences. In several cases, upper leaves contained replicating viral DNA which was able to incite CaMV symptoms on turnip plants. The sequence of the resultant recombinant viral molecules suggested that both DNA and RNA recombination events may have been involved in the production of functional virus, one event being gene targeting of the T-DNA. © 1992 Academic Press, Inc.

INTRODUCTION

Traditional methods for combatting virus spread in plants have included spraying pesticides to kill the vector of the virus (reviewed by Matthews, 1991) or pre-treating plants with a nonvirulent strain of the virus to induce cross-protection to related viral strains (reviewed by Fulton 1986). Modern approaches using genetic engineering include the induction of defense responses of the plant prior to pathogen attack. Thus far, the most promising approach has been to use transgenic plants containing viral sequences in their genome. For example, tomato and tobacco plants containing the coat protein gene for tobacco mosaic virus or alfalfa mosaic virus have shown protection against challenge by the respective donor virus and often other related virus strains in greenhouse and field experiments (reviewed by Beachy *et al.*, 1990).

However, the possibility that viral transgenes can be "rescued" by infecting viruses has to be considered, especially since no known plant virus integrates into the plant genome as an obligatory step of its life cycle. As the coat protein-mediated protection approach does not block delivery of viruses to plants, transgenic plants may contain mixtures of nucleic acids from different viruses provided by an external source, e.g., an insect vector. Recombination events between these RNA and/or DNA molecules and the viral gene(s) lo-

cated in the plant nuclear DNA might produce altered infectious material.

To test this, transgenic plants containing one gene of a plant virus in the genome were infected with the complementing part of the virus, and viral replication and spread were analyzed. As test virus, cauliflower mosaic virus (CaMV) was used as it allows analysis of both DNA and RNA recombination events. CaMV infects a variety of *Brassica* species and produces symptoms of chlorotic spots and vein clearing on leaves. The virus, an 8-kb double-stranded DNA virus (for review, see Gronenborn, 1987) is a pararetrovirus which replicates via an RNA intermediate (Bonneville *et al.*, 1988). The virus produces two RNA transcripts, the 19S RNA containing only the information for the gene VI protein, and the 35S RNA containing the complete viral coding information which is also used as the template for reverse transcription. Inoculation of two mutant strains of the virus has been shown to result in viral infection mediated by DNA or RNA extrachromosomal recombination including template switching during reverse transcription (Howell *et al.*, 1981; Lébeurier *et al.*, 1982; Choe *et al.*, 1985; Dixon *et al.*, 1986; Geldreich *et al.*, 1986; Grimsley *et al.*, 1986b; Stratford and Covey, 1989; Vaden and Melcher, 1990).

As delivery vector, *Agrobacterium tumefaciens* was chosen as it provides a convenient and reproducible method for DNA delivery to whole plants. *A. tumefaciens* is a soil-borne plant pathogen whose infection leads to the transfer of a fragment of DNA, the T-DNA, from the bacterium to the plant (for review, see Hohn, 1991). The T-DNA integrates into the plant chromosome where the genes located on this DNA fragment are expressed. *A. tumefaciens* has successfully been

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used to deliver entire viral sequences to a variety of plants (Donson *et al.*, 1988; Elmer *et al.*, 1988; Gardner *et al.*, 1986; Grimsley *et al.*, 1986a; Grimsley *et al.*, 1987; Hayes *et al.*, 1988; Woolston *et al.*, 1988), a procedure termed agroinfection (for review see Grimsley, 1990).

In this work, transgenic *Brassica napus* plants containing the CaMV gene VI were inoculated with *A. tumefaciens* containing the rest of the viral genomic information. These two viral constructions contained two regions of DNA homology, one of 1033 bp, the other of 818 bp to allow recombination between the two viral sequences. Replicating, viable virus was detected in several plants. As there are sequence differences in the viral strains used for the two constructions, analysis of the viral DNAs allowed suggestions of mechanisms for the intermolecular recombination event.

MATERIALS AND METHODS

Construction of recombination substrates

The vector Ca292 contains the *EcoRI*-*BstEII* fragment of pH79 (Hohn and Collins, 1980), the *BstEII*-*SalI* fragment of CaMV strain S (Franck *et al.*, 1980) including gene VI, the *SalI*-*ClaI* 599-bp fragment from pBR322, and a *ClaI*-*EcoRI* fragment coding for *str*/spec resistance in bacteria. This vector was recombined into *A. tumefaciens* C58 strain 3850 (Zambryski *et al.*, 1983) by standard procedures. Figure 1A shows the gene VI construction, designated gVI, after the T-DNA has integrated into the plant genome. For the preparation of the gl-V construction, pEAP21 (Gal *et al.*, 1991) was digested with *BstEII*, and the fragment containing the genes I-V was cloned into the *BstEII* site of pEAP1 (Gal *et al.*, 1991) to form the intermediate vector pEAPSG1. This vector was mobilized into *Agrobacterium* as above to form the construction gl-V (Fig. 1A). This construction contains 3 different CaMV strains; From the *SalI* to *HindIII* site and from the *EcoRV* to *BstEII* site, strains 4184 (Howarth *et al.*, 1981) and S as described for pEAP1 (Gal *et al.*, 1991), and from the *BstEII* to *SalI* site, strain D/H (Balazs *et al.*, 1982).

Plant growth and transformation

B. napus variety Brutor and *Brassica rapa* variety Just Right (turnip) plants were maintained under greenhouse conditions prior to transformation or inoculation. Transgenic plants were kept in isolated chambers with growth conditions of 16-hr light, 8-hr dark at 24°. Inoculation of *B. napus* or *B. rapa* with transgenic plant ex-

tracts or *A. tumefaciens* was performed as described (Grimsley *et al.*, 1986a).

The method of plant transformation was essentially that of Guerche *et al.* (1987b) using co-inoculation of plant petiole pieces with the *A. tumefaciens* strain containing the CaMV construction described above and a wild-type *Agrobacterium rhizogenes* A4 strain (Pisan, 1990). The resultant hairy roots were cultured and analyzed for the expression of the transgene nopaline synthetase, as described (Petit *et al.*, 1986; Aerts *et al.*, 1979). Transformed roots were regenerated and plants with sufficient root formation were transferred to soil. The plants had a reduced seed set and wrinkled, dark green leaves typical of *A. rhizogenes* transformed *B. napus* plants.

DNA isolation, Southern, and slot blot analysis

Isolation of total plant DNA was performed by two methods (Burr and Burr, 1981; Saghai-Marroof *et al.*, 1984). Southern blots were performed using nitrocellulose membranes, blotted under neutral conditions, and hybridized in the presence of nonfat dried milk as described (Sambrook *et al.*, 1989). Slot blots were performed using the Milliblot-S system (Millipore Corp., Bedford, MA) and Zeta probe nylon membrane (Bio-Rad Laboratories, Richmond, CA). DNA samples, typically 20–400 ng total DNA (in 43 μ l), were denatured in a solution containing 60 mM Tris-HCl, pH 7.5, 0.2 M NaOH, and 7 \times SSC (20 \times SSC: 3 M NaCl, 0.3 M trisodium citrate) by heating at 80° for 10 min and then loaded using a mild vacuum in slots prerinsed with 0.4 M NaOH. The membrane was rinsed with 2 \times SSC and hybridized as for Southern blots. Radioactive probes for slot blots and Southern blots were made using the random primer labeling kit from Boehringer-Mannheim (Mannheim, Germany) and [α -³²P]dATP (Amersham, Little Chalfont, England).

Cloning and sequencing of recombinant viral molecules

Isolation of CaMV particles and viral DNA was performed by the method of Gardner and Shepherd (1980). Viral DNA was digested with *XhoI* (cutting in gene II region of CaMV) and *SalI* (both from Biofinex, Praroman, Switzerland) and the 4840-bp fragment containing the gene VI region was cloned into standard vectors. Sequencing was performed on double-stranded plasmid DNA prepared by an alkaline lysis procedure (Sambrook *et al.*, 1989) using Sequenase (U.S. Biochemicals, Cleveland, OH) and gel purified

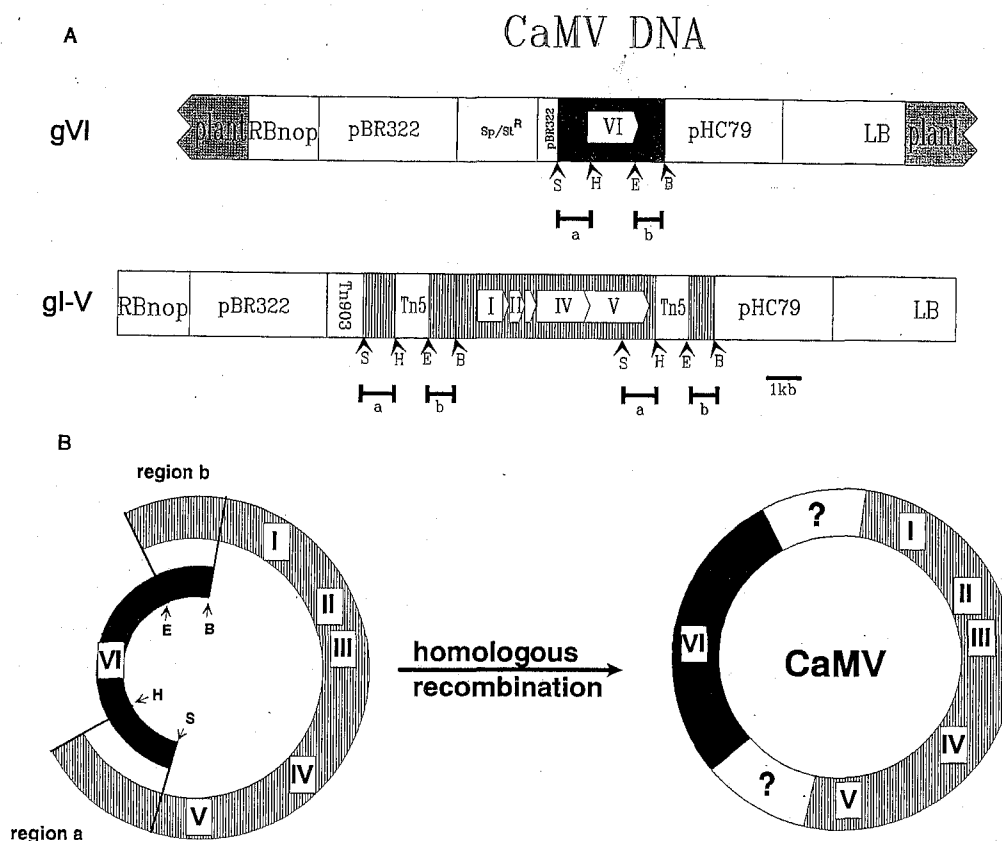


FIG. 1. (A) Constructions used to test intermolecular reconstitution of CaMV DNA. The T-DNA carrying plasmid Ca292 with the gene VI information from CaMV strain S is shown integrated in the plant genome, gVI. Similarly, the plasmid pEAPSG1 carrying the CaMV genes I through V was separately integrated into the T-DNA of *Agrobacterium tumefaciens* and the structure of the T-DNA is shown, gl-V. RB and LB indicate the right and left borders, respectively, of the T-DNA, nop is the gene for nopaline synthetase. The CaMV sequences are indicated by (▨) blocks (D/H and 4184 strains) and (■) blocks (S strain) with the open reading frames for some of the CaMV genes shown as open arrows, the larger ones labeled with Roman numerals. Tn903 confers kanamycin resistance in bacteria and Tn5 confers kanamycin resistance in plants because it is under the control of the CaMV 19S promoter. The str/sp^R expresses resistance in bacteria against streptomycin and spectinomycin, and the small region of pBR322 between this and the gene VI information contains part of the tetracycline resistance gene. The two homologous regions provided are marked as a and b, the a region located between the *SalI* (marked S) and *HindIII* (marked H) sites and the b region located between the *EcoRV* (marked E) and *BstEII* (marked B) sites, respectively, as indicated. These restriction enzyme sites are indicated only within the CaMV part of the construction; the indicated *HindIII* and *EcoRV* sites are not unique. (B) Simplified structure of CaMV sequences to form the recombinant viruses. Only the relevant parts of the CaMV containing sequences of the constructions are shown. Recombination between the provided constructions in the two regions indicated would produce viable virus. As these regions could be derived from either viral partner provided, they are designated by a "?" in the recombinant virus.

viral DNA specific primers as well as the universal and reverse primers complementary to the vector.

RESULTS

To test for intermolecular interactions of CaMV sequences, two deletion mutants of CaMV were constructed such that neither one alone would produce infectious virus (Fig. 1A). The first, gVI, contained the *SalI*-*BstEII* fragment from CaMV strain S which included the C-terminal half of gene V and the complete coding information for the gene VI protein, the inclusion body protein of CaMV. This protein has also been

shown in plant protoplasts to be a trans-activator for translation of the 35S polycistronic message (Bonneville *et al.*, 1989). The gVI construction contains the promoter for the 19S transcript of the virus and poly(A) addition and processing sites of this transcript. The 19S promoter has been shown to be active in several plant species including *B. napus* protoplasts in chimeric constructs (Guerche *et al.*, 1987a). The second construction, gl-V, contains the coding information for genes I through V. The gene VI information in this construction has been replaced by the kanamycin resistance coding region of Tn5. Thus this virus would be defective in inclusion body formation and trans-activat-

TABLE 1

VIRAL SYMPTOMS ON *B. RAPA* PLANTS FOLLOWING INOCULATION WITH *AGROBACTERIUM* CONTAINING TWO DEFECTIVE PARTS OF CaMV

Inoculum	Number of plants inoc.	Number of plants with CaMV symptoms
At.gVI	expt 1 2	0
	expt 2 4	0
At.gI-V	expt 1 2	0
	expt 2 4	0
At.gVI + At.gI-V	expt 1 6	6
	expt 2 4	4

Note. Three to 6-week-old *Brassica rapa* plants were inoculated on two leaves with 25 μ l each of an overnight culture of *Agrobacterium tumefaciens* (At) as described (Grimsley *et al.*, 1986a). Symptoms were scored after 2–4 weeks.

ing function, and therefore not be viable. Both constructions were mobilized into *A. tumefaciens* and tested by inoculation of turnip plants. Inoculation of *B. rapa* test plants with either construction alone did not produce viral symptoms, but when the two constructions were inoculated together, 100% of the plants showed CaMV symptoms (Table 1). This demonstrates that the combination of the strains contained all genetic information necessary for full virus infectivity.

To test for interactions between these two viral constructions in transgenic plants, the gVI construction was integrated into the chromosome of *B. napus* plants (see Materials and Methods). The resultant transgenic plants did not show a dramatic alteration in leaf phenotype due to the gene VI protein as has been shown for *Nicotiana tabacum* (Baughman *et al.*, 1988; Takahashi *et al.*, 1989) and *Nicotiana edwardsonii* and *Datura innoxia* (Goldberg *et al.*, 1991). However, as the transformation procedure using *A. rhizogenes* alone produced plants with dark green, wrinkled leaves, visualization of a possible alteration was difficult. The inoculated gVI plants were siblings of one original transgenic plant. From segregation data of the screenable marker nopaline synthetase, it was concluded that the transgene is integrated at a single identical locus in the plants tested. Hybridization to T-DNA left and right border probes showed that the actual locus contained between 5 and 10 copies of the integrated T-DNA (data not shown). Western blot analysis using gene VI protein antibodies revealed less than 1% of cross-reacting material as compared to transgenic plants containing replicating CaMV (Pisan, 1990). This is not surprising given the fact that there is a much lower copy number of gene VI in transgenic plants compared to plants with actively replicating CaMV. This may also explain why

these plants did not exhibit an altered phenotype due to the gene VI protein.

To test for intermolecular interactions between CaMV sequences in the genome and an extrachromosomal DNA element (T-DNA), transgenic *B. napus* plants containing gene VI sequences were inoculated with *Agrobacterium* containing the gI-V construction. Inoculations were performed on lower leaves, and 4 weeks later samples of upper leaves were harvested for viral analysis. The presence of viral DNA was tested by probing slot blots with a CaMV specific probe, and the presence of infectious viral material was tested by inoculation of turnip plants. The combined results of two inoculation experiments are shown in Table 2. Of a total of 12 inoculated transgenic nopaline synthetase positive gVI plants, four plants were shown by slot blots to produce viral DNA in leaves above the inoculated leaves. Neither noninoculated transgenic gVI plants nor inoculated wild type, nor transgenic plants containing the Tn5 coding information (pEAP1 from Gal *et al.*, 1991) inoculated with the same *Agrobacterium* produced viral DNA (Table 2). Leaf extracts from all four CaMV DNA positive plants incited CaMV symptom formation on turnip plants. This result indicates that the transgenic plants produced infectious virus and shows that recombination must have occurred in all cases. The viral DNA in the transgenic plants did not contain Tn5 sequences and appeared to be full length as judged by Southern analysis (data not shown).

In order to produce viable virus, recombination between the two viral sequences must have occurred in the two homologous regions (marked a and b in Figs. 1A and 1B), one between the *SaI*I and *Hind*III sites (a) and the other between the *Eco*RV and *Bst*EI sites (b). The a region, 1033 bp in length, contains the 19S pro-

TABLE 2

DETECTION OF CaMV DNA IN *B. NAPUS* IN PLANTS INOCULATED WITH *AGROBACTERIUM TUMEFACIENS*

Plant	Inoculum	Number of plants with CaMV DNA/number of plants inoculated
Nontransgenic	At.gI-V	0/1
Tn5 transgenic	At.gI-V	0/1
gVI transgenic	mock	0/1
gVI transgenic	At.gI-V	4/12

Note. Four to 8-week-old plants at the 4–6 leaf stage were inoculated on two leaves with the indicated *Agrobacterium* strain. Upper leaf samples were taken 4 weeks later from the youngest fully developed leaf and DNA analyzed on slot blots for the presence of CaMV DNA. Samples that produced hybridization signals of 10-fold or greater intensity than controls (mock inoculated) were considered to contain viral DNA.

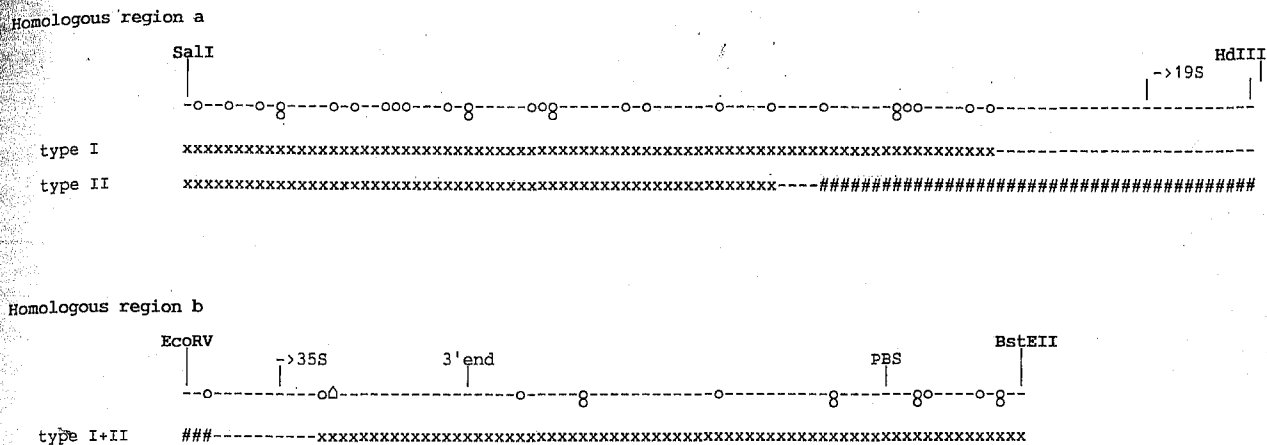


FIG. 2. Nucleotide sequences of recombinant CaMV from inoculated transgenic plants. Sequences as symbols for the homologous region a (top) and region b (bottom), with the upper line of each showing a comparison of the homologous sequences. Each character represents 10 bp, (-) represents sequence identity between the two CaMV strains, (O) stands for one base difference in 10 bp, and (\ominus) in region b for a 6-bp deletion in the CaMV S strain. Transcription start sites of the 19S and 35S RNAs, the 3' ends of both RNAs and the primer binding site (PBS) used for the first DNA strand synthesis are marked. The lower lines represent the sequences of the nine clones of the recombinant viral DNAs from four different gVI transgenic plants inoculated with *A. tumefaciens* containing gl-V. The (x) indicates 4184 CaMV strain-specific sequence; (#) S CaMV strain, and (-) indicates crossover regions between the strains. As described in the text, there were two types of CaMV sequences detected marked as type I and type II in the figure. The two sequence types were different in the homologous region a, but identical (Type I + II) in sequence in the homologous region b.

moter. This region has been used previously in studies of intramolecular genomic homologous recombination, and cross-over events all along this sequence were observed in the recombinant viral DNAs (Gal *et al.*, 1991). The 818-bp long b region includes the viral 35S transcript 5' end and is the site of the natural virus template switch during reverse transcription of the CaMV RNA (Bonneville *et al.*, 1988). Since these two homologous regions were derived from the two viral strains 4184 and S, their sequences are slightly different (28 differences in region a, 23 differences in region b). Sequence analysis therefore was expected to allow deductions as to the mechanism of homologous recombination in this system.

CaMV particles were purified from the transgenic *B. napus* plants, the viral DNA was digested with *Xho*I (cutting in gene II) and *Sal*I, and the fragment containing the gene VI region was cloned into a *Sal*I cut vector (see Materials and Methods). In this cloning strategy, the two homologous regions from one virus were maintained on the same clone. One clone each was isolated from two of the plants, two from a third plant, and five from the fourth plant. All nine clones were completely sequenced in both homologous regions. The sequences in the homologous region a were of two types (Fig. 2). Eight of the nine clones consisted entirely of CaMV strain 4184 sequences in the region a (type I) while one clone was partially derived from CaMV S strain sequences (type II in the upper part of Fig. 2). All clones showed the same sequence in the

region b: the first 30 bp were derived from the S sequence while the rest was CaMV strain 4184 specific.

DISCUSSION

This work demonstrates the production of infectious virus following inoculation of a defective virus onto a transgenic host plant which contains the complementing gene in its genome. This is the first demonstration of a viral recombination event of this type in plants. Whereas complementation of defective viruses on plants transgenic for the appropriate complementing gene information was demonstrated (van Dun *et al.*, 1988; Hanley-Bowdoin *et al.*, 1990; Osbourn *et al.*, 1990; Holt and Beachy, 1991), recombination or reconstitution of infectious virus has not been reported. Comparisons of these reports with the present work are at best difficult since, different gene functions, different virus types, and different plant hosts have been used. An analysis of the viral DNA sequences suggested both RNA and DNA recombination events were involved in the intermolecular event (see below) which suggests that the observation might be generalized to include other virus types.

Mechanism of recombination event

Production of viable virus using the two viral sequences in the described system must involve two recombination events in the overlapping regions pro-

vided (Fig. 1B). The two events could in principle both be at the RNA level, one at the DNA and the other at the RNA level, or only at the DNA level. The recombinant viral DNAs analyzed in this system provided evidence for the first two types of recombination events.

The majority of the viral DNAs (eight of nine clones) had an identical sequence in the two regions a and b (labeled type I in Fig. 2). These DNAs are CaMV 4184 specific in the entire region a and in region b downstream of the 35S start site, but contain a small region of CaMV S strain sequence in the region b upstream of the 35S transcript start site. This sequence is consistent with two "RNA recombination" events caused by two steps of template switching during reverse transcription, as shown in Fig. 3A: an illegitimate switch combining the 5' end of the "35S" transcript to the 3' end of the 19S transcript of the gene VI coding transgene, and a second illegitimate switch back to the original "35S" transcript. (The term "35S" transcript is used for this RNA since it is not wild-type 35S RNA as it contains Tn5 coding sequences in place of the gene VI information.)

Use of the 19S RNA as template has been proposed in a similar manner to explain the recombinational hot-spot in genomic recombination substrates (Gal *et al.*, 1991) and extrachromosomal recombination in inoculation and agroinfection experiments (Stratford and Covey, 1989; Vaden and Melcher, 1990; Grimsley *et al.*, 1986b). This mechanism requires the presence of both RNA species in the same cell and cellular location in the plant. The 19S promoter as used for the gVI construction has been shown to be active in *B. napus* protoplasts (Guerche *et al.* 1987a). In CaMV-infected *B. napus* plants, this transcript dominates over the 35S transcript (Covey *et al.*, 1990). The "35S" RNA is driven by a truncated 35S promoter which includes 90 bp upstream of the transcript start site. This promoter has been shown to be active in transgenic *N. tabacum* plants and to be root specific (Benfey *et al.*, 1989), although the tissue specific expression has not been characterized in *B. napus*.

The sequence of one virus isolate suggests another mechanism for the intermolecular recombination event. Type II virus contained a long stretch of CaMV S strain sequence in the region upstream of the 19S RNA start site (Fig. 2) precluding a template switch between the 19S and "35S" transcripts at the 5' end of the former RNA. The sequence of this virus suggests that the first recombination step may have involved homologous DNA integration as depicted in Fig. 3B. Transcription of the recombination product results in 35S RNA which can be used as a template for reverse transcriptase to produce viable viral DNA. This virus was

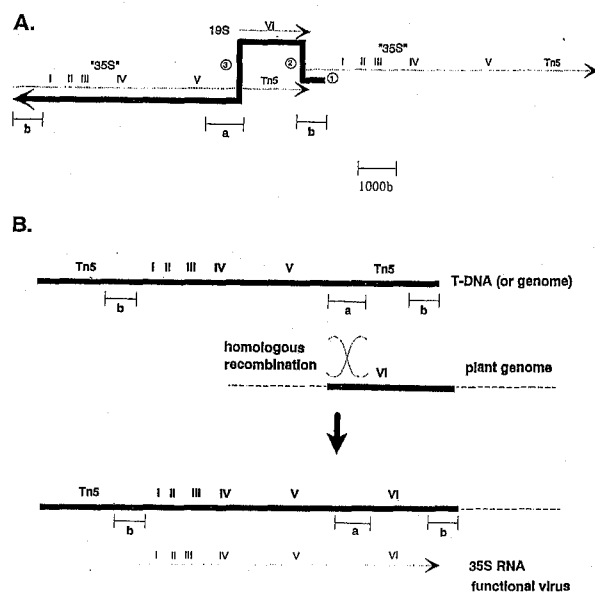


FIG. 3. Possible mechanisms of intermolecular viral recombination. Two proposed mechanisms for the formation of CaMV by intermolecular recombination. DNA is indicated as bold lines and RNA as dotted lines with arrows. The gene products are indicated as Roman numerals above the nucleic acids and the two homologous regions, a and b, are marked. (A) Mechanism involving only RNA template switching: Reverse transcriptase initiates at the natural site, the primer binding site (PBS, marked 1) on the "35S" transcript and continues to the 5' end of this RNA. At this point the reverse transcriptase switches to the 3' end of the 19S transcript (labeled 2) using the region identical to that of the 35S transcript which is used during natural virus replication. This RNA is then used as template for reverse transcription up to its 5' end at which point the enzyme switches back to the "35S" transcript (labeled 3) (either the one used before or another similar transcript) and completes the synthesis of the first strand of infectious viral DNA. (B) Mechanism involving DNA gene targeting: Homologous recombination within the region a between the genomic gVI construction and a T-DNA intermediate containing the gI-V (or a genomic copy thereof following integration of this T-DNA into the plant chromosome) produces the DNA shown below containing a linear arrangement of the viral genes. RNA synthesis from this moiety (driven by the -90 35S promoter) can produce complete 35S RNA and thus functional virus. It is worth noting that the first RNA produced from this proposed DNA structure would have two different sequences in the duplicated region of the RNA between the 35S start and its 3' end, since these regions are coded for by two different parts of the DNA molecule. But in the synthesis of the first DNA strand from this RNA by reverse transcriptase, this difference would be lost as the PBS to 35S start region is used only in the region upstream of the gene I and as such would only be derived from the CaMV 4184 strain.

derived from a plant which also contained a virus of type I suggesting that two events of intermolecular recombination have occurred in one plant.

The viruses of type I could be derived as well from a DNA targeting event in the region of the 19S transcript start. However, since there are no sequence differences to allow a precise mapping of the cross-over

point (Fig. 2), a distinction between these two possible mechanisms cannot be made.

Gene targeting

The link in the second model between CaMV gene VI in the plant genome and an extrachromosomal element containing CaMV genes I through V (Fig. 3B) reflects targeted integration of T-DNA. DNA targeting of genes using *A. tumefaciens* has been described in two systems using tissue culture to select for the rare homologous targeting event (Lee *et al.*, 1990; Offringa *et al.*, 1990). This is the first description of such an event taking place in a whole plant. Comparison of the efficiency of the systems is not feasible as in the present case it is not possible to determine the number of cells inoculated. The frequency of intermolecular recombination using co-inoculated *Agrobacterium* strains cannot be estimated for the same reason, but it appears higher than in the case in which one of the recombinant partners is derived from the plant genome (compare Table 1 and Table 2).

Despite the fact that T-DNA integration generally involves the T-DNA ends (Gheysen *et al.*, 1991; Mayerhofer *et al.*, 1991), it is likely that the targeting moiety is free DNA (and not an integrated copy thereof) since double- and single-strand ends of DNA are highly recombinogenic (Baur *et al.*, 1990; Puchta and Hohn, 1991). Theoretically the CaMV part of the T-DNA element could replicate by reverse transcription as it contains all the sequences and coding elements necessary for replication.

Using the symptom appearance and sensitivity of a plant virus as a marker for the intermolecular homologous recombination event provides a simple method to score for the successful recombination event. Since this system provided only one apparent event of DNA targeting, improvements in the efficiency of the event should be devised. As the two target regions provided only 1033 or 818 bp of homology, longer regions could be used. The low targeting efficiency in this system may also be due to the large number of mismatches in the homologous regions. Such mismatches have been shown to reduce recombination efficiency (Shen and Huang, 1986; Waldman and Liskay, 1988). The CaMV homologous regions could be replaced by perfect homology in the incoming DNA. As the ends of the T-DNA are the recombinogenic sites for integration into the plant genome (Gheysen *et al.*, 1991; Mayerhofer *et al.*, 1991), the distance between the T-DNA borders and the site for recombination could be reduced.

Complementation of CaMV functions

As the partners in the recombination experiment code for separate RNAs, complementation of viral

functions could have been expected. This cannot as yet be ruled out, but in all cases where replicating virus was detected, infectious virus was found as well. Detection of such a phenomenon may require analysis of early events and thus probing of the inoculated leaf. It is likely that complementation occurs prior to the recombination event to provide reverse transcriptase protein and perhaps other gene products required. In the future, a complementation-based CaMV vector approach should avoid any sequence overlaps to eliminate the possibility of recombination, as was demonstrated by Gronenborn (1987).

Cross-protection

Cross-protection has been seen in transgenic plants containing viral coat protein genes when they are challenged with the same or a related virus (Beachy *et al.*, 1990). The gene VI containing *B. napus* plants described in this study appear not to be protected against CaMV super-infection as virus replication and spread following inoculation and recombination demonstrates. These plants produced replicating virus as well when inoculated with *A. tumefaciens* carrying the complete coding region for CaMV (Pisan 1990), but virus inoculation of these plants has not been performed. This apparent lack of cross-protection may mean that this protein does not act in this capacity which is not surprising as it is not the viral coat protein. Alternatively, the level of the gene VI protein in the transgenic plants may not be high enough. Indeed, the protein was not detected on Western blots (Pisan, 1990) and the plants did not exhibit an altered phenotype associated with the gene VI protein observed in other plant species (Baughman *et al.*, 1988; Takahashi *et al.*, 1989; Goldberg *et al.*, 1991). The low expression of the gene VI may be due to cosuppression of the multiple copies of the transgene in the genome as has been observed in other systems (Linn *et al.*, 1990; Mittelsten Scheid *et al.*, 1991). Cross-protection with CaMV has been observed in plants previously infected with a nonvirulent strain of the virus (Tomlinson and Shepherd, 1978; Zhang and Melcher, 1989) and in transgenic plants with replicating virus when challenged with a super-infecting virus (Pisan, 1990).

Biological safety of transgenic plants carrying viral genes

This work provides important evidence that molecular recombination events can occur between extrachromosomal (T-DNA) and chromosomally located viral genes. This information may be relevant in considering the use of transgenic plants containing viral genes.

Other viruses could be tested in a similar manner to determine the generality of this observation.

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