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Frequent occurrence of recombinant potyvirus isolates

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We have performed a systematic search for recombination in the region encoding coat protein and the 3′ non-translated region in natural isolates of potyviruses, the largest group of plant RNA viruses. The presence of recombination, and the localization of the cross-over points, were confirmed statistically, by three different methods. Recombination was detected or suspected in 18 out of 109 potyvirus isolates tested, belonging to four out of eight virus species, and was most prevalent in potato virus Y, clear in bean common mosaic virus, and possible in bean yellow mosaic and zucchini yellow mosaic viruses. Recombination was not detected in the four other potyvirus species tested, including plum pox virus, despite the availability of numerous sequences for this last species. Though it was not specifically researched, no evidence for inter-specific recombination was found. For several reasons, including the fact that only a minor portion of the genome was analysed, the above figures certainly represent an underestimate of the extent of recombination among isolates of potyviruses, which might thus be a common phenomenon.

Introduction

There is growing evidence that RNA recombination is a major evolutionary factor in plant RNA viruses (Koonin, 1991; Simon & Bujarski, 1994). Instances of recombination between genomic viral RNAs have been described for natural isolates of tobraviruses (Robinson *et al.*, 1987; Goulden *et al.*, 1991), bromoviruses (Allison *et al.*, 1989), viroids (Rezaian, 1990), nepoviruses (Rott *et al.*, 1991; Le Gall *et al.*, 1995*a*), potyviruses (Cervera *et al.*, 1993) and luteoviruses (Rathjen *et al.*, 1994; Gibbs & Cooper, 1995). The phenomenon has also been observed in laboratory isolates of cucumoviruses (Fernández-Cuartero *et al.*, 1994), nepoviruses (Le Gall *et al.*, 1995*b*), hordeiviruses (Edwards *et al.*, 1992), carmoviruses (Cascone *et al.*, 1990) and bromoviruses (Bujarski & Kaesberg, 1986; Rao & Hall, 1990), and was particularly well studied in the last two cases (Carpenter & Simon, 1994; Nagy & Bujarski, 1995). Recombination between viral and cellular RNAs has also been described, both in natural (Mayo & Jolly, 1991; Sano *et al.*, 1992) and experimental (Greene & Allison, 1994) systems.

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The sequence of the CP region of PVY-LB has been deposited in GenBank, accession number X92078.

To date, however, no efforts have been directed at a systematic search of viral genomes for recombination. The potyvirus group is particularly well suited for such a search for several reasons. Firstly, it is the largest genus of plant viruses (Ward & Shukla, 1991), with many members partially or fully sequenced. Secondly, RNA recombination is known to be possible in this genus: it has been described between field isolates of plum pox virus (Cervera *et al.*, 1993) and may also have occurred between an isolate of turnip mosaic virus and a cellular sequence (Sano *et al.*, 1992). We have attempted to detect recombinant isolates in several potyviruses. Our approach, inspired by that used by Chenault & Melcher (1994) on cauliflower mosaic virus, is based on the search for virus isolates showing different clustering properties in trees constructed using different short regions of their genomes. The results obtained may then be confirmed by various statistical means.

Using this approach, we demonstrate the occurrence of recombination in the region encoding the capsid protein (CP) and in the 3′ non-translated region (3′NTR) of several isolates of potato virus Y (PVY) and bean common mosaic virus (BCMV). Potential recombinants were also observed for bean yellow mosaic virus (BYMV) and zucchini yellow mosaic virus (ZYMV), but apparently not for watermelon virus II (WMV2), turnip mosaic virus (TuMV), plum pox virus (PPV) and papaya ringspot virus (PRSV).

Methods

■ **Viral sequences.** Nucleotide sequences with accession numbers as follows were retrieved from the GenBank database (the designations used in this paper for the different isolates are indicated in parentheses when applicable).

BCMV: L11890 (Mex), L12470 (US1), L15332 (NL1), L19472 (NL2), L19473 (US5), L19474 (US7), L19539 (CH2), L21766 (NL4), S66251/S66275 (NL1b), S66252/S66279 (NY15), S66253/S66280 (BlCMV), U05771 (PStV-Bl2), X63559 (PStV-Bl1), Z15057 (B), Z21700 (PStV-Ib).

BYMV: D00490 (GDD), D00604 (CS), D28819 (MB4), S77515 (S), X53684 (DG), X81124 (MI).

PPV: D13751, M21847, M92280, U27652, S57404, S57405, X16415, X56258, X57975, X57976, X81073, X81074, X81075, X81076, X81077, X81078, X81079, X81080, X81081, X81082, X80183, X81084. A corrected version of sequence X16415 was used (M. Ravelonandro, personal communication).

PRSV: D00594, D00595, D50591, I06996, S89893, U14736, U14737, U14738, U14739, U14740, U14741, U14742, U14743, U14744, X67672, X78557.

PVY: D00441 (Fr), D12539 (O4), D12570 (T), M81435 (US), M95491 (H), U06789 (VN), U09508 (N US), U09509 (O7), U10378 (H VN), U25672 (Ch2), S74810 (36), S74813 (T13), X14136 (O1), X54058 (Ch1), X54611 (Hu), X54636 (N Rus), X68221 (Chil), X68222 (Pot US), X68223 (Eur H), X68224 (NsNr), X68225 (MsNr), X68226 (O'), X79305 (NL2), X92078 (LB). In addition, the sequences of the following isolates, which are not in the databases, were included: PeMV (Dougherty *et al.*, 1985), Is (Rosner & Raccah, 1988), Go16 (Wefels *et al.*, 1989), NL1 (van der Vlugt *et al.*, 1993), Th (Hataya *et al.*, 1990), O2 (Lawson *et al.*, 1990) and M1, partially sequenced by one of us (Le Romancer, 1993).

TuMV: D10601, D10927, L12396, X52804, X65978, X81140, X81141, X83968.

WMV2: D00535, I06999, I06186, L22907.

ZYMV: D00593 (Flo), D00692 (Con), I07016 (Pat15), L29569 (Reun), L31350 (Cal), M35095 (Is), X62662 (Sin).

The three sub-fragments of these nucleotide sequences used in this study were called N-ter, C-ter and 3'NTR. The N-ter region is defined as that encoding the hyper-variable amino-terminal extension of the CP gene (up to, but excluding, the amino acid homologous to the first lysine in the motif KDKDVNAG of PVY-N; see Rybicki & Shukla, 1992). The C-ter region is that encoding the carboxy terminus of the CP [from, and including, the motif RYGLIRN in PVY-N (see Rybicki & Shukla, 1992) and including the termination codon]. The 3'NTR, when available, is the 3' non-translated region, excluding the termination codon.

■ **Computer analysis of the sequences.** Multiple alignments of the selected regions were obtained using the program ClustalV (Higgins *et al.*, 1992). Distance matrices were calculated from these alignments based on the Dayhoff PAM matrix (Dayhoff *et al.*, 1983), using the program DNADIST in version 3.5 of the PHYLIP package (Felsenstein, 1989). Clustering was done from these matrices using the program NEIGHBOR in PHYLIP, implementing the UPGMA (average linkage clustering) method. Unrooted trees could finally be printed using the utility program DRAWTREE in PHYLIP. The statistical significance of the branching order was estimated by performing 100 replications of bootstrap resampling of the original alignment using SEQBOOT (Felsenstein, 1985), and synthesizing the resulting set of trees using CONSENSE.

In addition, the statistical test for recombination developed by Sawyer (1989) was used, as implemented in the program VTDIST (kindly provided by Dr S. Sawyer), to statistically validate the recombination events suspected from the clustering tree topologies. Dr Sawyer also provided us with an unreleased version of this program, VTDIST2, that allows pair-wise comparisons of the input sequences. In this test, the totally conserved positions are omitted ('condensation') from the alignment, and for each sequence pair a set of fragments is defined between successive sites where these two sequences differ. Two working parameters are defined, MCF (maximal size of the condensed fragments), and SSCF (sum of the squares for condensed fragments). Two additional parameters (MUF and SSUF) are defined similarly but omitting the condensation step. Statistical comparison of the parameter values obtained with random permutations of the sequences or with the actual data provides an estimation of the significance of the size distribution of the fragments, and hence of the likelihood that genetic rearrangements have occurred. We found that the results of the VTDIST analysis became very difficult to interpret when sequences that were identical or differed at only one position were present in the dataset. Therefore, in some cases we inserted only one sequence of such pairs into the dataset analysed by VTDIST.

The recombination breakpoints were tentatively localized using the maximum chi-squared approach described by Maynard-Smith (1992), as implemented in the program RecSite (O. Le Gall, unpublished). This statistical method searches along a recombinant sequence for the limit between the blocks, and estimates the probability for the null hypothesis (no recombinant structure). In addition, RecSite determines the percentage identity between the sequences in each block, which helps to understand the structure of the recombinant sequence.

Results

PVY

A databank and literature search allowed identification of the region encoding the CP of 29 PVY isolates, 12 of which also included the 3'NTR. The pepper mottle virus isolate sequenced by Dougherty *et al.* (1985) was included in our analysis since it is now considered to be a strain of PVY (Robaglia *et al.*, 1989). In addition, we sequenced the CP–3'NTR of two more isolates, LB and M1, except for a region in M1 internal to the CP coding region (from positions 8954–9097, numbered according to the complete sequence of isolate Fr, as in Robaglia *et al.*, 1989). Three regions in the PVY sequences were selected and analysed as described above. The N-ter region extends from positions 8573–8659, the C-ter region from positions 9119–9376 and the 3'NTR region from positions 9377–9704 of isolate Fr.

The general topologies of the trees constructed using the N-ter, C-ter and 3'NTR regions are similar (Fig. 1), with two major sub-groups of isolates being delineated beside a less homogeneous set of isolates, as already described by van der Vlugt *et al.* (1993). Sub-groups I and II roughly correspond to the N and O pathogenicity and serology sub-groups of PVY respectively. The rest of the PVY isolates cluster in a third sub-group in the N-ter tree, though with a lower bootstrap value (47%), and are even more widely dispersed in the C-ter tree (Fig. 1*b*). This set of isolates corresponds to the third sub-

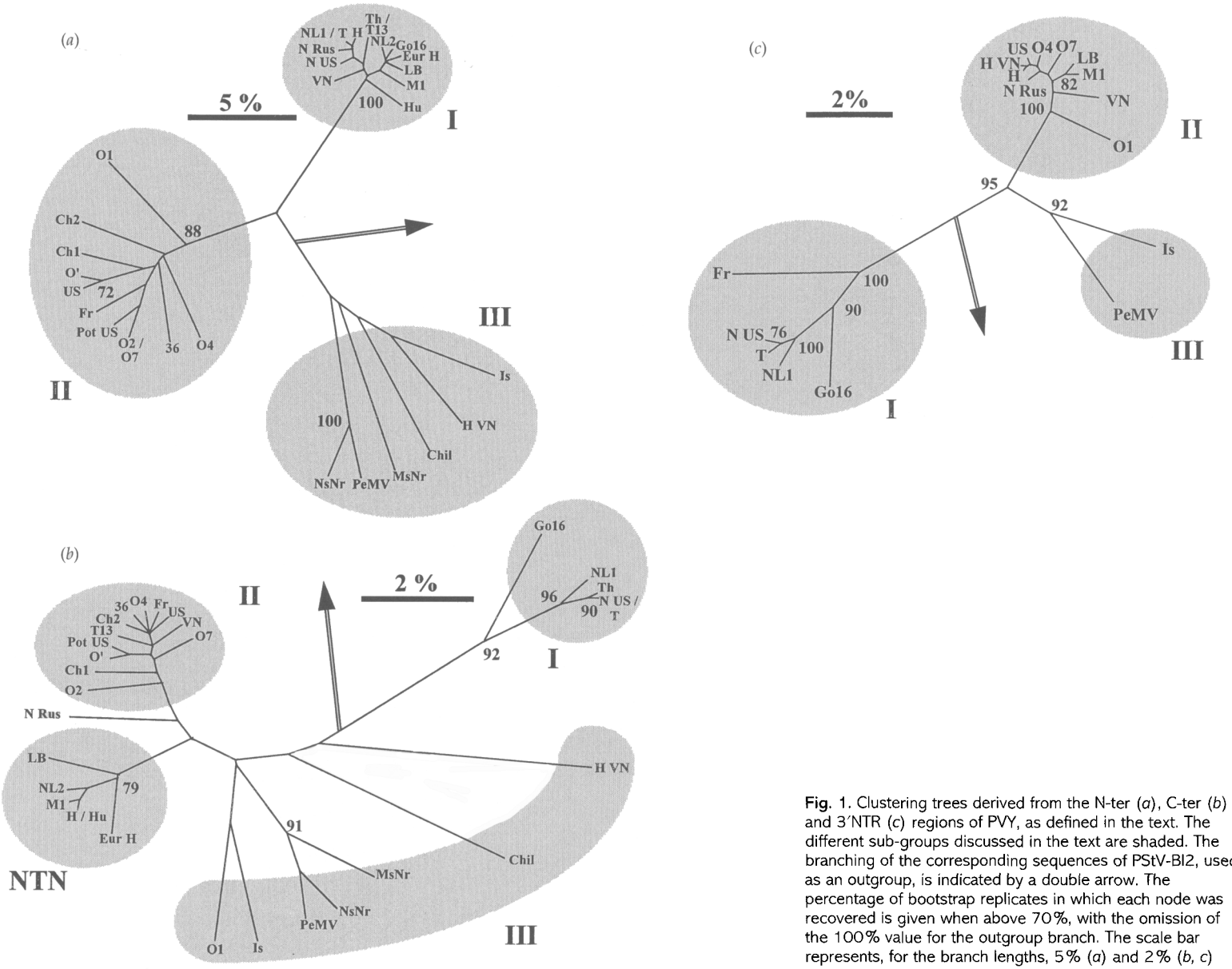

Fig. 1. Clustering trees derived from the N-ter (*a*), C-ter (*b*) and 3′NTR (*c*) regions of PVY, as defined in the text. The different sub-groups discussed in the text are shaded. The branching of the corresponding sequences of PStV-BI2, used as an outgroup, is indicated by a double arrow. The percentage of bootstrap replicates in which each node was recovered is given when above 70%, with the omission of the 100% value for the outgroup branch. The scale bar represents, for the branch lengths, 5% (*a*) and 2% (*b*, *c*) substitutions per site.

Table 1. VTDIST analysis of the potyviruses studied

Virus	Parameter†	Parameter value†	*P*-value‡ (%)	SD above sim. mean§	SD of sims‖
BCMV	SSCF	272142	0·09	4·42	11889·66
	MCF	126	39·57	0·12	26·95
	SSUF	5925582	0·31	3·70	277659·95
	MUF	583	32·88	0·30	122·54
BYMV	SSCF	17736	0·01	5·99	777·48
	MCF	56	0·20	4·74	5·94
	SSUF	605209	< 0·01	8·26	26720·09
	MUF	362	0·04	5·80	33·91
PPV*	SSCF	1621475	3·60	2·05	91193·46
	MCF	195	32·49	0·47	27·06
	SSUF	25187052	42·66	0·11	2369358·76
	MUF	674	62·77	−0·34	82·58
PRSV*	SSCF	171854	10·73	1·26	9584·78
	MCF	168	5·86	1·79	27·17
	SSUF	3845941	17·57	0·88	198327·01
	MUF	703	9·23	1·53	118·65
PVY*	SSCF	1225061	< 0·01	13·51	28537·01
	MCF	230	0·75	2·48	29·76
	SSUF	21199878	< 0·01	12·82	621145·16
	MUF	767	1·16	2·11	93·02
TuMV*	SSCF	6896	21·08	0·71	596·99
	MCF	25	55·58	−0·20	5·79
	SSUF	724803	41·21	0·03	86153·17
	MUF	251	73·70	−0·67	71·27
WMV2	SSCF	4689	57·83	−0·40	1170·72
	MCF	53	57·51	−0·31	13·52
	SSUF	982188	33·42	0·30	185768·77
	MUF	792	39·50	0·37	169·87
ZYMV	SSCF	35126	< 0·01	6·18	2713·347
	MCF	137	0·68	3·03	19·867
	SSUF	1408284	1·35	2·92	131904·01
	MUF	673	13·34	1·17	137·92

* Only the CP coding region was considered in the analysis reproduced here.
† See text for parameter definitions.
‡ Proportion of permuted datasets, out of 10000, yielding higher scores than the actual data.
§ Number of standard deviations above simulation mean.
‖ Standard deviation of the simulations.

group described by van der Vlugt *et al.* (1993), and will thus be named sub-group III in this paper. In the C-ter tree, isolate Eur H and all of the isolates (H, Hu, NL2, LB and M1) belonging to the NTN biotype (necrotic-tuber necrosis: Le Romancer & Kerlan, 1992; Le Romancer *et al.*, 1994; van den Heuvel *et al.*, 1994) are clustered together close to sub-group II.

Several PVY isolates show markedly different affinities depending on the genomic region considered. Such is clearly the case for isolates T13 and VN, which shift from sub-group I in the N-ter tree to sub-group II in the C-ter tree and in the 3′NTR tree for VN (no sequence information is available for T13 in this region). Isolates N Rus and NTN (including Eur H) also have different positions in the different trees, being found in sub-group I of the N-ter tree, and shifting to a position slightly removed from the main sub-group II cluster in the C-ter tree (as already described above for the NTN and Eur H isolates), and well within sub-group II in the 3′NTR tree (3′NTR sequences are not available for some NTN isolates and for Eur H). The bootstrap value of sub-group II is low in the C-ter tree, but the high value of sub-group I in this tree allows us to draw the conclusion that these isolates significantly 'leave' this sub-group in this region, and for the isolates for which the

3′NTR was analysed this is confirmed with a 100% bootstrap value for sub-group II. Isolate Fr clusters in sub-group II in both the N-ter and the C-ter trees, but in sub-group I in the 3′NTR tree, and H VN similarly shifts from sub-group III to sub-group II in the 3′NTR tree. Isolate O1 clusters in sub-group II in the trees derived from N-ter (with a bootstrap value of 88%) and 3′NTR (bootstrap value of 100%) but in sub-group III in the C-ter tree, though with a low bootstrap value (58% for the branching between O1 and Is; data not shown). These 12 isolates are thus good candidates for being the results of RNA recombination. In the case of isolate O1, two successive recombination breakpoints could lead to the double shift observed.

In the second step of our analysis, we proceeded to evaluate the statistical significance of these potential recombination events. Sawyer (1989) devised a statistical test for genetic rearrangement based on the imbalance in size distribution of regions in which pairs of sequences within a dataset are identical. We used a dataset containing the entire CP sequences, rather than the terminal regions used previously, to run the VTDIST program implementing this test. The parameters considered differed significantly from a randomized dataset, with a probability lower than 1·2% of the null hypothesis that no recombination occurred (Table 1). A similar result was obtained when another dataset was used, in which the entire CP-3′NTR sequences were included, but in which the number of isolates was restricted to those for which this information was available (not shown). This analysis provides strong statistical evidence for RNA recombination in PVY, and confirms the previous analysis made on the basis of the topology of the trees; however, it does not tell us which sequences are recombinant.

In order to answer this question statistically, we performed two further types of analysis using VTDIST, either by comparing the sequences pair-wise or by comparing each of them with consensus sequences representative of each sub-group, as defined in Fig. 1. First of all, we selected sequences clustering unequivocally in the same manner in all the trees to represent their sub-groups. Sub-group I thus contained isolates Go16, NL1, N US, T and Th, sub-group II contained isolates O2, O4, O7, O′, US, Ch1, Ch2, 36 and Pot US, and sub-group III contained isolates Is, PeMV, Chil, NsNr and MsNr. Consensus sequences were then inferred for each sub-group from a multiple alignment made using ClustalV. In sub-group III, the 3′NTR sequence has been determined only for isolates Is and PeMV, so that a consensual sequence was not possible to determine, and the PeMV sequence was arbitrarily used as the 'consensus' in this region.

All of the PVY sequences were then analysed using VTDIST in a reduced dataset consisting of the three consensus sequences and the isolate to be analysed (Table 2). Depending on their availability for each isolate, either the CP–3′NTR or only the CP coding region was used for these analyses. The 3′NTR of PeMV was excluded from the analysis because of the previous arbitrary choice of this sequence as the consensus for sub-group III. Analysis of isolate M1 was restricted to the region for which its sequence is known. For Fr and H VN, comparisons were made only with isolates for which the sequence of the entire CP–3′NTR is known. The SSCF-associated *P*-values are shown for all the PVY isolates in the first column of Table 2. All of the 12 isolates detected above as possible recombinants, on the basis of their clustering, were confirmed to be so at the 3% level, except for Hu and O1. We also compared all the pairs of PVY sequences using VTDIST2, and for each sequence calculated the mean of the *P*-values obtained when this sequence was compared with isolates representing each subgroup. The results of this pair-wise analysis are given in columns two to four of Table 2. This approach allows us to determine not only which isolates are recombinant, but also between which sub-groups they shift. Again, the results described above were confirmed at the 3% level, except for isolate O1. This time, isolate Hu was confirmed to be a putative recombinant between sub-groups I and II. One of the parents of H VN was predicted to belong to sub-group II, but no prediction could be made as to its second parent using this approach.

Finally, we looked for the location of the recombination breakpoints in the candidate PVY recombinant isolates. For this analysis, we used the approach described by Maynard-Smith (1992), as described above. This approach first determines the structure of a recombinant sequence, and then attributes a significance level to this structure. Here again, each of the PVY sequences was compared with the consensus representing each sub-group. The results of this last analysis are given in columns five and six of Table 2. Using this approach, all the putative recombinants determined above were confirmed at the 3% level, except O1 (but this approach is not optimal for detecting multiple recombinants; Maynard-Smith, 1992). No recombinant structure could be detected that connected with the low *P*-values found for sub-group II isolates O4, O′, O2 and Ch2. Visual inspection of the alignment confirmed that in these four isolates, the significantly high chi-squared value maps to a position close to either end of the alignment, where random mutations in the query sequence weigh more heavily on the statistics than elsewhere (data not shown).

In all cases the structures, as displayed in column five of Table 2, confirm the polarity of recombination predicted from the cluster analysis, except again for isolate O1. At least six different recombination breakpoints were detected in the eleven recombinant isolates (because the recombinant nature of O1 could not be confirmed, this isolate is not included here), four of them in the coding region and two of them in the 3′NTR. As an example, Fig. 2 shows the sequence alignments around the five recombination breakpoints found between sub-groups I and II. In all of these isolates except Fr, the sequences 5′ to the recombination point are of sub-type I and those 3′ to this point are of sub-type II. The location of the recombination

Table 2. Statistical determination of the PVY recombinant isolates and prediction of their recombination breakpoints

PVY isolate	VTDIST*				Maynard-Smith	
	%cons†	%I‡	%II‡	%III‡	Structure/site§	P‖
Go16	7·58	32·60	44·20	21·02	Not applicable	6·1
NL1	48·37	67·46	56·87	45·06	Not applicable	99·8
N US	46·17	58·55	51·10	40·77	Not applicable	9·3
T	22·38	59·92	54·74	45·27	Not applicable	7·2
Th	8·12	64·12	43·07	62·65	Not applicable	64·9
VN	< 0·01	0·03	0·63	47·61	I/8714-8715/II	< 0·1
N Rus	< 0·01	0·21	2·04	59·78	I/8747-8748/II	< 0·1
T13	< 0·01	0·26	0·33	60·14	I/9137-9144/II	< 0·1
LB	< 0·01	0·15	1·24	36·46	I/9170-9183/II	< 0·1
H	< 0·01	0·16	1·46	41·49	I/9170-9183/II	< 0·1
Hu	13·10	2·09	2·92	62·48	I/9170-9183/II	0·3
NL2	2·06	1·83	0·51	58·15	I/9170-9183/II	< 0·1
Eur H	2·75	0·70	0·54	67·54	I/9170-9183/II	< 0·1
M1	< 0·01	NT	NT	NT	I/9170-9183/II	< 0·1
Fr	< 0·01	< 0·01	< 0·01	34·37	II/9455-9466/I	0·1
O4	27·78	68·79	19·79	65·40	None found	0·3
O7	20·75	60·94	22·01	43·95	Not applicable	27·7
US	54·28	75·98	28·95	68·58	Not applicable	20·8
O′	7·87	22·23	15·58	76·35	None found	1·0
O2	77·74	32·76	35·44	67·00	None found	1·1
Ch1	78·62	67·23	73·29	34·58	Not applicable	48·5
Ch2	94·26	66·53	14·74	73·99	None found	3·1
36	11·69	33·81	22·91	81·19	Not applicable	13·4
Pot US	73·31	21·70	34·29	70·17	Not applicable	40·4
O1	78·81	54·97	47·05	43·05	Not applicable	23·0
H VN	< 0·01	22·45	< 0·01	24·92	III/9378-9379/II	2·1
Is	17·00	33·30	73·10	8·11	Not applicable	53·1
PeMV	18·79	37·39	70·35	32·12	Not applicable	98·5
Chil	70·87	25·39	22·48	36·03	Not applicable	26·1
NsNr	13·18	48·16	73·73	40·34	Not applicable	96·8
MsNr	31·96	70·52	83·24	38·13	Not applicable	59·7

* SSCF-associated *P*-values (as percentages) determined using VTDIST. NT, Not tested.
† Comparison of the PVY sequences with the consensus representing the sub-groups defined in Fig. 1.
‡ Mean *P*-value of the null hypothesis, as determined after pair-wise comparisons of each sequence with those of sub-groups I, II or III.
§ Recombination breakpoint as predicted by RecSite; the sequences 5′ to the site indicated are of the type indicated on the left; those 3′ to it are of the type indicated on the right.
‖ Percentage probability of the null hypothesis against this structure, as determined after testing 1000 randomly mutated sequences.

breakpoint within the C-ter region in isolates LB, H, Hu, Eur H, NL2 and M1 explains their clustering slightly outside the rest of sub-group II in the C-ter tree (Fig. 1*b*).

It is striking to note that all the isolates (H, Hu, NL2, LB and M1) belonging to the biological type NTN are recombinants between sub-groups I and II, with recombination breakpoints within the same short region. Isolate Eur H, whose biological type is not as clearly defined (Sudarsono *et al.*, 1993), is characterized by the same recombination breakpoint. Whether or not there is a link between this recombination event and the NTN phenotype cannot be ascertained at the moment. These isolates could either have a common (recombinant) ancestor that had the NTN phenotype independently of its recombinant status, or the NTN phenotype could be a direct result of the recombination event, which could then have happened several times independently. The first hypothesis is strengthened by the presence, at several positions within the CP–3′NTR, of nucleotide variations unique to these isolates (not shown).

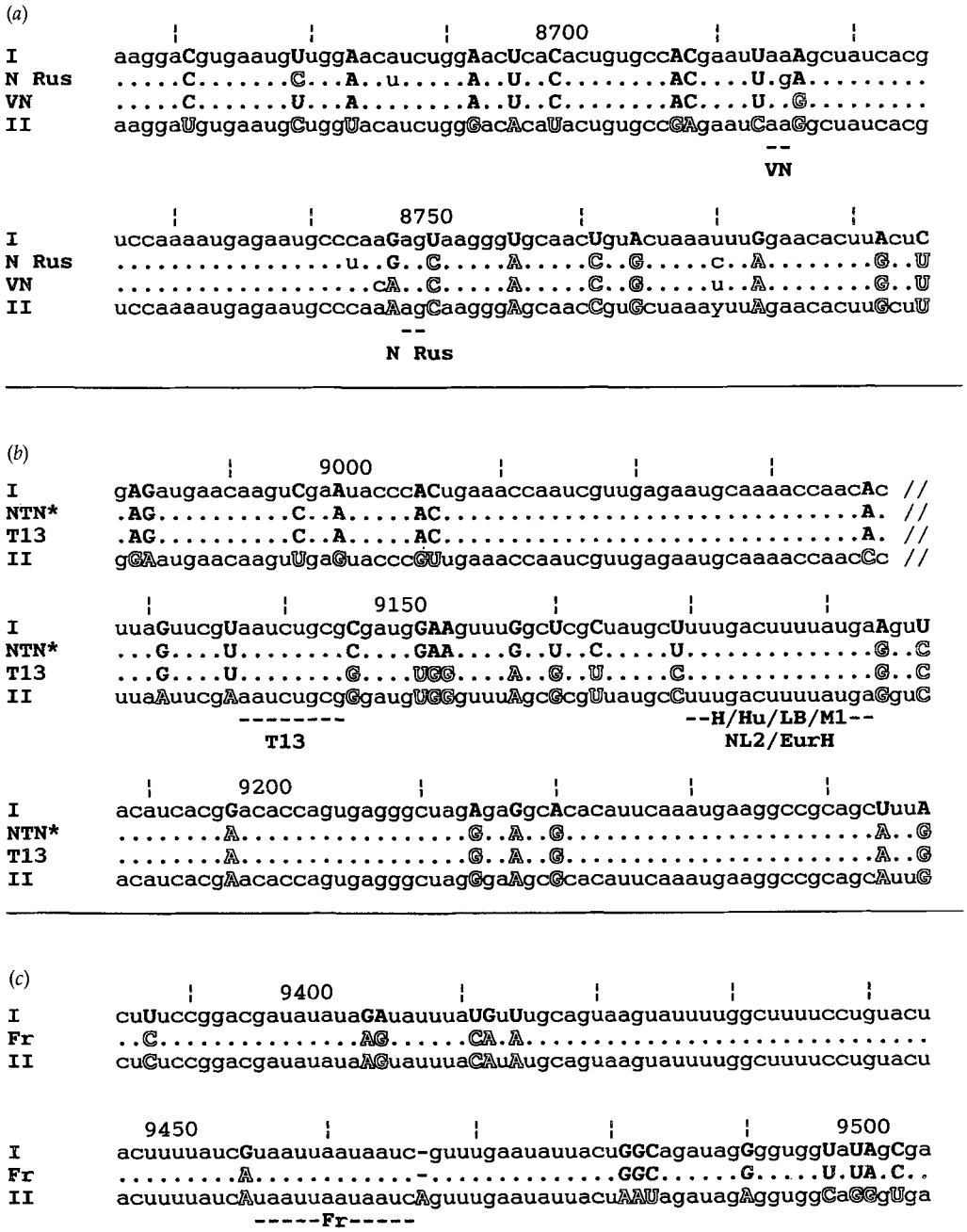

Fig. 2. Alignments of the putative recombinant PVY sequences shown with the consensus sequence of sub-types I (line I) and II (line II). Only the regions around the recombination breakpoints are shown [(*a*) recombination breakpoints in isolates VN and N Rus, (*b*) in isolates T13, LB, H, Hu, NL2, Eur H and M1, (*c*) in isolate Fr]. At the polymorphic sites, the nucleotides are shown in capitals, in bold for those of sub-type I, and in outline for those of sub-type II. The locations of the putative recombination breakpoints are indicated by dashes below the alignments. NTN* denotes the consensus sequence for isolates H, Hu, NL2, LB and M1, all of them of the NTN biotype, and Eur H, of uncharacterized biotype. The numbering is that of isolate Fr (Robaglia *et al.*, 1989), the last digit of each figure indicating the corresponding position. Y denotes C/U when these nucleotides were equally represented at a given position in a sub-group. Dots indicate nucleotides identical to the consensus; // indicates a deletion, in the part of the alignment displayed in (*b*), of a region with no polymorphic sites between sub-types I and II (positions 9039–9127).

BCMV

The nucleotide sequences of 15 BCMV isolates are available in GenBank, and all contain the 3′NTR. The sequences of isolates of peanut stripe (PStV) and black-eye cowpea mosaic (BlCMV) viruses were also included in this analysis since they have been shown to represent isolates of BCMV (McKern *et*

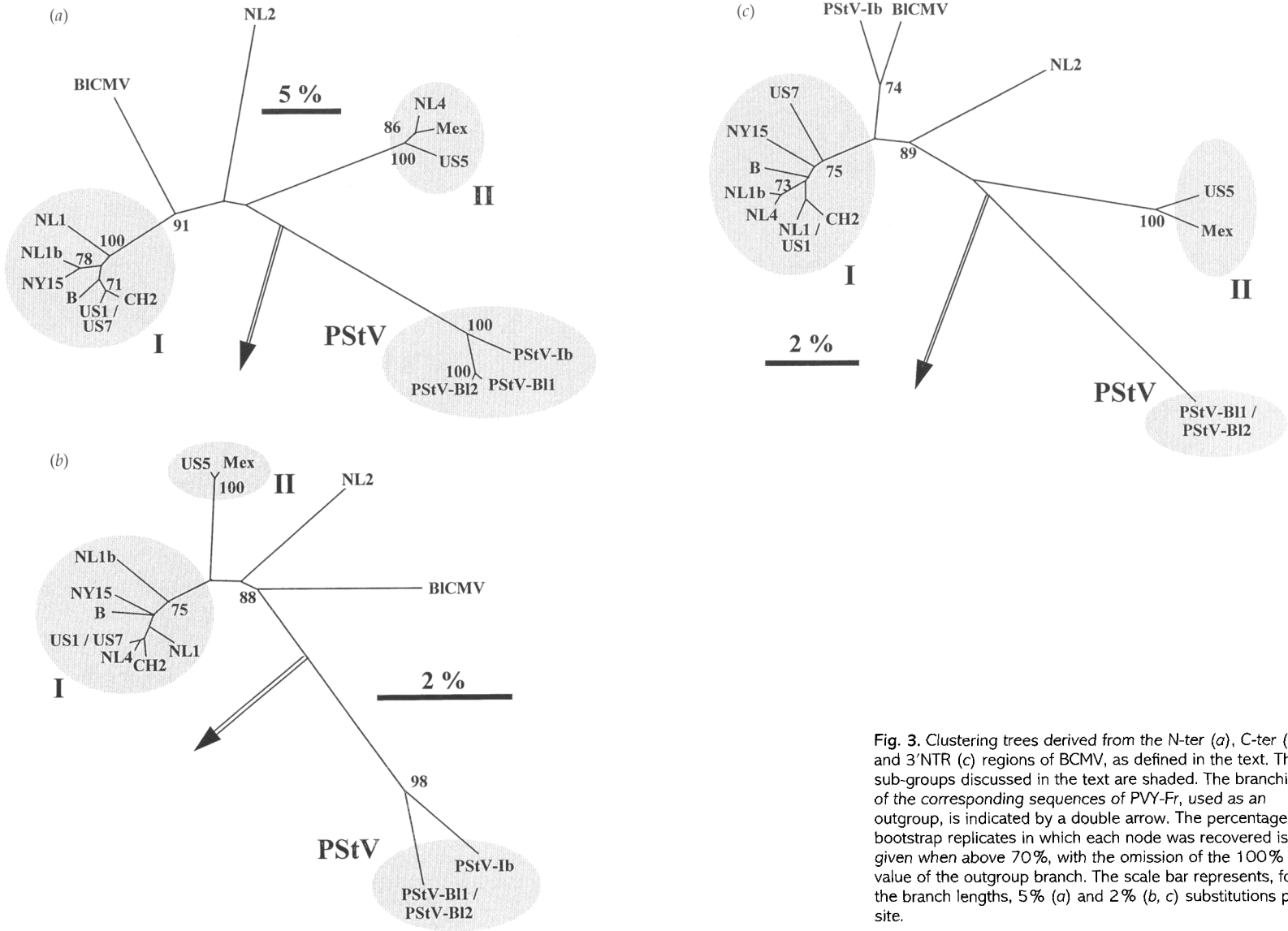

Fig. 3. Clustering trees derived from the N-ter (*a*), C-ter (*b*) and 3′NTR (*c*) regions of BCMV, as defined in the text. The sub-groups discussed in the text are shaded. The branching of the corresponding sequences of PVY-Fr, used as an outgroup, is indicated by a double arrow. The percentage of bootstrap replicates in which each node was recovered is given when above 70%, with the omission of the 100% value of the outgroup branch. The scale bar represents, for the branch lengths, 5% (*a*) and 2% (*b*, *c*) substitutions per site.

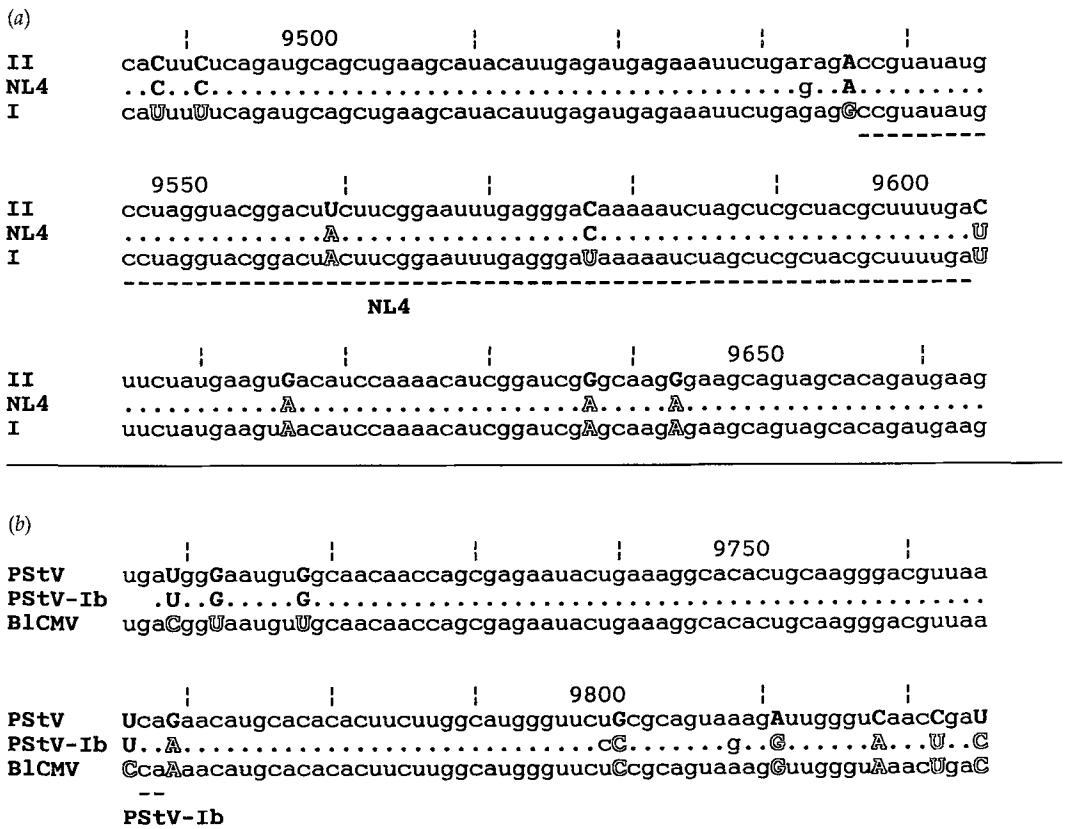

Fig. 4. Alignments of the putative recombinant BCMV sequences with the consensus sequence of various sub-types (I, II, PStV or BlCMV). Only the regions around the recombination breakpoints are shown [(*a*) recombination breakpoint between sub-types I and II in isolate NL4, (*b*) between PStV and BlCMV in isolate PStV-Ib). At the polymorphic sites, the nucleotides are shown in capitals, in bold for those of sub-type II or PStV, and in outline for those of sub-type I or BlCMV. The locations of the putative recombination breakpoints are indicated by dashes below the alignments. The numbering is that of PStV-Bl2 (Gunashinge *et al.*, 1994), the last digit of each figure indicating the corresponding position. R denotes G/A when these nucleotides were equally represented at a given position in a sub-group. Dots indicate nucleotides identical to the consensus.

al., 1992; Vetten *et al.*, 1992; Khan *et al.*, 1993; Saiz *et al.*, 1994). On the other hand, an additional sequence referenced as BCMV, that of strain NL3 (accession number U20818), was not included in our analysis since Khan *et al.* (1993) showed that this isolate should be considered as the member of a distinct virus species. As in the PVY analysis, three regions were selected and analysed. With nucleotide numbering according to the complete sequence of isolate PStV-Bl2 (Gunashinge *et al.*, 1994), the region N-ter extends from positions 8945–9118, C-ter from 9548–9808 and 3′NTR from 9809–10061.

Fig. 3 shows the trees constructed using the N-ter, C-ter and 3′NTR regions. There are three main groups in each tree. A large sub-group (sub-group I) contains most of the isolates, and two smaller sub-groups (sub-groups II and PStV) contain two sequences each, Mex/US5 in sub-group II and PStV-Bl1/PStV-Bl2 in sub-group PStV. In addition, the sequences of BlCMV and BCMV-NL2 diverge in two other directions and could thus be the only currently sequenced representatives of two additional sub-groups. However, two isolates, NL4 and PStV-Ib, cluster differently in the trees displayed in Fig. 3. NL4 belongs to sub-group II in the N-ter tree, but to sub-group I in the C-ter and 3′NTR trees, and PStV-Ib belongs to sub-group PStV in the N-ter and in the C-ter trees, but is closer to BlCMV in the 3′NTR tree. These observations, supported by high bootstrap values, indicate that these two isolates are possible recombinants.

When the statistical relevance of this possible recombination was estimated using VTDIST, a high probability for recombination within the 15 BCMV sequences was found according to the SS parameters, but not to the M parameters (Table 1). Such a difference in the prediction made with the two types of parameter has been described in some cases by Sawyer (1989). Again, a 'consensus' sequence was determined for the five sub-groups shown in Fig. 3. Mex was arbitrarily chosen to represent sub-group II, and similarly PStV-Bl2 was chosen for the PStV subgroups. The consensus sequence for sub-group I was determined and found to be identical with that of isolate US1. BlCMV and NL2 were considered to be the only representatives of their respective sub-groups. When each of the BCMV sequences was compared, using VTDIST, to these five consensus sequences, only isolates NL4 and PStV-Ib

gave SSCF-associated *P*-values lower than 3% (data not shown). Isolates US7 and NL1 also gave relatively low *P*-values (4·93% and 8·90% respectively), and none of the other isolates had a *P*-value lower than 32% (data not shown). This result confirms the hypothesis that isolates NL4 and PStV-Ib are recombinants. The pair-wise comparisons, performed as for PVY in Table 1, again confirmed this result at the 1% level, and indicated that NL4 was probably a recombinant between sub-groups I and II, and that PStV-Ib was probably a recombinant between the PStV and the BlCMV sub-groups (data not shown), as predicted above from the cluster analyses.

The Maynard-Smith approach was then used to determine the fine structure of these two recombinant isolates (Fig. 4). The recombination breakpoint was mapped between positions 9537 and 9603 in isolate NL4, with sub-type II sequences 5′ to this region and sub-type I sequences 3′ to it. In PStV-Ib, the breakpoint was located between positions 9767 and 9768, with PStV-type sequences upstream and BlCMV-type sequences downstream.

BYMV

The nucleotide sequences of six BYMV isolates, all of them including their 3′NTR, were available in GenBank. As above, three regions were selected and clustering trees derived thereof. No major differences were observed in the topology of these trees, except that the isolates MB4 and MI converged to DG in the 3′NTR and that GDD diverged from DG in this same region (data not shown). The program VTDIST found evidence at the 0·01% level for genetic rearrangement in BYMV when the SS parameters were considered, and at the 0·2% level with the M parameters (Table 1), and VTDIST2 confirmed, at the 2% level, the implication of MB4 and MI in this process (data not shown). The closer relationship of MB4 with MI and DG, as well as the divergence of GDD, in the 3′NTR were confirmed by visual inspection of the entire alignment (data not shown) but, in the rest of the CP–3′NTR region, no other available sequence could be related to these isolates. The Maynard-Smith approach did not detect any significant mosaic structure in isolates MB4, MI or GDD (not shown). In conclusion, it cannot be excluded that recombination has occurred in BYMV, between isolates belonging to a sub-group represented by DG, and another one not represented in the available dataset. Thus, the case of BYMV, no firm conclusion about the presence or absence of recombination is possible, essentially because of the relatively restricted dataset.

ZYMV

The nucleotide sequences of seven ZYMV isolates, all including their 3′NTR, were available in GenBank. Regions were defined and clustering trees derived as above. In all the trees, two sub-groups could be defined, one containing Sin and Reun, and another one containing four other sequences (data not shown). Isolate Is lies between these two sub-groups. Within the major sub-group, isolates Con and Cal cluster together and Pat15 is slightly aside. Isolate Flo clusters with Pat15 except in the C-ter tree where it is closer to Cal and Con (data not shown), and could thus be a double recombinant, detected despite the overall close relationships between the isolates of this sub-group. In the dataset provided (the isolate Reun, very divergent in the 3′NTR, was excluded), VTDIST detected possible recombination at significant levels except with parameter MUF (Table 1). The Maynard-Smith approach showed that, at the 1% confidence level, the segment of Flo extending to position 9169 was of the Pat15 type, then of the Con/Cal type up to position 9407, and then shifted back to Pat15 (the numbering refers to Cal, as in Wisler *et al.*, 1995) (data not shown). Flo could thus be a double recombinant between related ZYMV isolates; however, these observations rely on only 13 polymorphic sites in the intermediate region.

PPV, PRSV, TuMV and WMV2

Multiple alignments of the regions designated as described in Methods were obtained for PPV, PRSV, TuMV and WMV2, and used to infer clustering trees. For each of these viruses, the topologies of the trees derived from the different regions were not significantly different (data not shown). Thus, we have not detected any evidence for recombination between the sequenced isolates of these viruses in the CP–3′NTR region. As above, we then analysed these sequence sets using VTDIST (Table 1). As described in Methods, PRSV accessions I06996 and U14740, and TuMV accessions D10927 and X81141 were not considered in this VTDIST analysis because they are identical to, or differ at only one position from another sequence. The VTDIST analysis failed to detect significant probability of recombination either when all the available sequences for a given virus were considered in only their CP coding region (PPV, PRSV and TuMV; see Table 1) or when only those for which the 3′NTR information is available were considered in their CP–3′NTR region (PPV, PRSV, TuMV, data not shown; WMV2, see Table 1). The lowest percentage found for the null hypothesis was 3·6% for the PPV SSCF parameter, but this was not confirmed by any other PPV parameter, and close examination of the data showed that this low value was due to a pattern of conserved and very divergent regions in the El-Amar sequence (X56258), so that, even for PPV, recombination was ruled out.

Discussion

So far, only two possible or probable instances of recombination in the genus *Potyvirus* have been described (Sano *et al.*, 1992; Cervera *et al.*, 1993). In this work we have systematically searched for the presence of RNA recombination in potyvirus sequences present in the databases, or published. We focused on the coat protein region because it is the one that is most often targeted in sequencing projects, leading to a larger available dataset than for any other region of the genome. We first focused on PVY, since this virus is of

considerable economic importance which has prompted more studies than on other potyviruses, resulting in a particularly large (and indeed continuously increasing) dataset. The search was then extended to other potyviruses for which the CP gene sequences of at least four different isolates were available in the databases.

We first used a simple approach for rapid identification of putative recombinant isolates, similar to the one that allowed Chenault & Melcher (1994) to demonstrate the occurrence of recombination in a plant DNA virus, cauliflower mosaic virus. The two terminal domains of the region encoding the CP were analysed separately, and the trees obtained were compared. In the absence of recombination, the general topologies of the trees should be similar, whereas if recombination had occurred in some isolates, the corresponding branches would be expected to differ in their location in the two trees, being more closely related to a different parental cluster in each tree. An advantage of this approach is that the parents themselves do not have to be present in the dataset, as long as other members of the cluster to which they belong are present. A statistical analysis was then performed on the entire CP-encoding region, so as to confirm the occurrence of recombination suspected from the tree topology. Using this approach, we identified a number of recombinant isolates in the case of PVY and of BCMV. Recombination was also proposed in BYMV and ZYMV, but no recombination was detected in PPV, PRSV, TuMV and WMV2. In total, recombination in the CP–3′NTR region of the genome was demonstrated in 13 isolates (with at least nine independent recombination breakpoints), and suspected in five more, out of a total of 109 isolates tested. The recombinant isolates, found in 50% (4/8) of the virus species tested, thus represent between 8% (9/109) and 17% (18/109) of the total population examined.

The reasons for the difference in prevalence of recombination between different virus species are not clear. For some viruses (WMV2, TuMV), the size of the sample (four sequences for WMV2, eight for TuMV) may simply have been too small. Such an explanation cannot, however, apply to PPV and PRSV, for which large datasets were available. In the case of PPV, a possible explanation could be the different geographical distributions of the two major groups of isolates (Candresse *et al.*, 1995), which would clearly preclude mixed infections, a prerequisite for recombination to take place. Indeed, the only PPV recombinant isolate detected so far (Cervera *et al.*, 1993) was isolated in former Yugoslavia, one of the few countries where both types of PPV isolates occur together. Another explanation, which cannot be ruled out, is that individual potyviruses could differ significantly in their recombination rates due to differences in the intrinsic properties of their replication machineries.

The approach that we have used has, however, several limitations that actually lead to underestimating the extent of recombination. First of all, only viable recombination products can be detected, so that in this work 'recombinant' means 'viable recombinant'. Secondly, it is very important to have a dataset large enough to establish a clear clustering of the different isolates (the uncertainty about recombination in BYMV is an illustration of this point), and also to increase the chance that it contains a recombinant isolate. Thirdly, we would have failed to identify recombination between closely related isolates since no visible shift in the clustering of such a recombinant isolate would have resulted. This is illustrated by ZYMV-Flo, a possible recombinant between two closely related isolates, which shows a barely detectable shift between the different trees. Fourthly, multiple recombination events might escape detection since these types of isolate will cluster similarly in regions situated on the two sides of the two recombination breakpoints. Finally, the non-detection of recombination in the CP–3′NTR region does not preclude the possibility of recombination in other parts of the genome. The region we have chosen is, in potyviruses, about one-tenth the entire size of the genome; thus, if recombination breakpoints are evenly distributed along the entire genome, our approach would only have detected about one-tenth of the recombination events between the isolates represented in the dataset. Indeed, in the case of PPV, one of the viruses for which we did not detect any recombination event in this study, RNA recombination with a cross-over point outside the region analysed in this work, in the putative viral polymerase coding domain, has been described (Cervera *et al.*, 1993). This last limitation can be removed only when more complete virus sequences are known.

Although we made no specific efforts to detect interspecific recombination, such events would have been characterized by the presence of highly divergent isolates in some of the trees (comparable to that of the outgroup sequences used in Figs 1 and 3), something we never observed in our dataset. In addition, interspecific recombination would also have been detected by the VTDIST program. We therefore conclude that, if it exists, interspecific recombination has to have a frequency much lower than intraspecific recombination in the genus *Potyvirus* (less than 1 isolate in 109). There may be several reasons for the paucity of interspecific recombination. First of all, the products of such an event could be less likely to be viable than those of intraspecific rearrangement. Alternatively, recombination might not occur at all because lower levels of sequence identity may not allow the template switch by the viral polymerase. Finally, recombination between different virus species might be hindered by spatial separation of the virus replication sites (different host species or sub-cellular, cellular, tissue, or geographical localization, etc.).

The genetic shuffling caused by recombination may lead to the appearance of new virus isolates in the field, some of which may thus acquire new biological properties (as may have happened with the NTN biotype of PVY, as discussed above). Recombination could also happen, as described for a bromovirus (Greene & Allison, 1994), between a replicating virus and the product of a transgene introduced into the genome of a

crop plant for protection purposes. In this respect, it is worthwhile to note that we did not detect interspecific recombination. However, spatial separation, suggested above to be a possible inhibitor of interspecific recombination, might differ in the cases of transgenic versus infected plants.

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