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Sequence characteristics of potato virus Y recombinants

Xiaojun Hu,[1,2] Alexander V. Karasev,[1,3] Celeste J. Brown[2,3] and Jim H. Lorenzen[2,4]

Correspondence
Alexander V. Karasev
akarasev@uidaho.edu

[1]Department of Plant, Soil, and Entomological Sciences, University of Idaho, Moscow, ID 83844, USA

[2]Bioinformatics and Computational Biology Program, University of Idaho, Moscow, ID 83844, USA

[3]Department of Biological Sciences, University of Idaho, Moscow, ID 83844, USA

[4]International Institute of Tropical Agriculture, Kampala, Uganda

Potato virus Y (PVY) is one of the most economically important plant pathogens. The PVY genome has a high degree of genetic variability and is also subject to recombination. New recombinants have been reported in many countries since the 1980s, but the origin of these recombinant strains and the physical and evolutionary mechanisms driving their emergence are not clear at the moment. The replicase-mediated template-switching model is considered the most likely mechanism for forming new RNA virus recombinants. Two factors, RNA secondary structure (especially stem–loop structures) and AU-rich regions, have been reported to affect recombination in this model. In this study, we investigated the influence of these two factors on PVY recombination from two perspectives: their distribution along the whole genome and differences between regions flanking the recombination junctions (RJs). Based on their distributions, only a few identified RJs in PVY genomes were located in lower negative FORS-D, i.e. having greater secondary-structure potential and higher AU-content regions, but most RJs had more negative FORS-D values upstream and/or higher AU content downstream. Our whole-genome analyses showed that RNA secondary structures and/or AU-rich regions at some sites may have affected PVY recombination, but in general they were not the main forces driving PVY recombination.

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INTRODUCTION

RNA recombination is a major evolutionary factor in RNA viruses (Roossinck, 1997, 2003; Strauss & Strauss, 1988). The phenomenon has been observed for many plant viruses (reviewed by Simon & Bujarski, 1994), e.g. bromoviruses (Allison *et al.*, 1989), luteoviruses (Gibbs, 1995), nepoviruses (Le Gall *et al.*, 1995a, b) and cucumoviruses (Fraile *et al.*, 1997). It is especially prevalent in potato virus Y (PVY) (Lorenzen *et al.*, 2006, 2008; Revers *et al.*, 1996).

The replicase-mediated template-switching (copy choice) model is considered the most likely mechanism for forming new RNA virus recombinants (Nagy & Simon, 1997). In this model, the viral replicase switches template from the donor to the acceptor RNA during RNA synthesis and uses the nascent RNA as a primer to generate a recombinant RNA (Nagy & Simon, 1997). AU-rich regions could be recombination-promoting signals in viral RNAs (Shapka & Nagy, 2004). They have been found frequently near recombination sites in many viruses, e.g. picornaviruses (Pilipenko *et al.*, 1995), brome mosaic virus (BMV) (Nagy & Bujarski, 1996, 1997; Shapka & Nagy, 2004), turnip crinkle virus (TCV) and cucumber necrosis virus (Cheng & Nagy, 2003), citrus tristeza virus (CTV) (Vives *et al.*, 2005), bean pod mottle virus (Zhang *et al.*, 2007), turnip mosaic virus (TuMV) (Ohshima *et al.*, 2007), grapevine fanleaf virus and *Arabis* mosaic virus (Vigne *et al.*, 2008). On the other hand, RNA secondary structures, especially stem–loop structures (hairpins), can promote replicase pausing or act as signals for replicase pausing on the template or nascent RNA (Nagy & Simon, 1997). They are also RNA replication enhancers that may promote RNA recombination directly by binding the replicase-aborted nascent-strand complex during the crossover event (Nagy & Simon, 1997; Nagy *et al.*, 1998). Stem–loop structures were also observed in recombination sites in TCV (Cascone *et al.*, 1993; Nagy *et al.*, 1999b), tombusviruses (White & Morris, 1995) and norovirus (Nayak *et al.*, 2008). However, a comprehensive study of how these two factors affect plant RNA virus recombination based on whole-genome analyses has not yet been conducted.

A supplementary figure showing a multiple sequence alignment of breakpoint 502 is available with the online version of this paper.

One method for predicting thermodynamic RNA secondary structure over a viral genome is FORS-D analysis (Forsdyke, 1995; Zhang *et al.*, 2008). A nucleotide segment with a more negative FORS-D value has high potential to develop a stem–loop structure. In human immunodeficiency virus, most recombination breakpoints occurred in more negative FORS-D regions (Zhang *et al.*, 2005). Equivalent analyses have yet to be reported for plant RNA viruses.

PVY is the type member of the genus *Potyvirus* (family *Potyviridae*). The PVY genome is a single-stranded, positive-sense RNA of about 9.7 kb, with a virus-encoded protein (VPg) attached covalently to its 5′ end and a 3′ poly(A) tail (Fauquet *et al.*, 2005; Riechmann *et al.*, 1992). The viral RNA encodes a single, large polyprotein, which is processed post-translationally by three virus-encoded proteases (P1, HC-Pro and NIa) into nine gene products (Fig. 1a) (Dougherty & Carrington, 1988). The major strains, defined based on their pathology in tobacco, are the ordinary strain PVY^{O} and the necrotic strain PVY^{N} (de Bokx & Huttinga, 1981). PVY has a high degree of genetic variability (identity range 82–98 %, according to a BLAST search) and is also subject to recombination (Revers *et al.*, 1996). Since the early 1980s, a number of PVY recombinants have been documented, including PVY^{NTN}, PVY^{N}-Wi (in North America termed $PVY^{N:O}$) (Crosslin *et al.*, 2005; Piche *et al.*, 2004; Singh *et al.*, 2003; Thole *et al.*, 1993). A new recombinant that differs from PVY^{NTN} and PVY^{N}-Wi, called NE-11, was recently identified (Lorenzen *et al.*, 2008; Piche *et al.*, 2004).

The PVY^{N}-Wi isolates contain one recombination junction (RJ) between the HC-Pro and P3 region, where the sequence switches from PVY^{N}-like to PVY^{O}-like (see Fig. 1a). Some of these isolates also contain an additional RJ in the P1 N-terminal region (Glais *et al.*, 2002). PVY^{NTN} isolates have an N : O : N : O genomic structure with three or four RJs (see Fig. 1a). The first RJ in the HC-Pro/P3 region is nearly identical to that of the PVY^{N}-Wi RJ; in the 6K2–NIa N-terminal region, the sequence reverts to PVY^{N}-like sequence; and in the CP C-terminal region, there is another switch to a PVY^{O}-type sequence (Boonham *et al.*, 2002; Glais *et al.*, 2002; Lorenzen *et al.*, 2006). Other PVY recombination patterns were also described recently (Ogawa *et al.*, 2008).

Although many PVY recombinants have been reported, the mechanisms of selection of quite a limited number of RJs are poorly understood and the physical factors affecting recombination in PVY are not known. In this study, we strive to understand recombination in PVY. First, we identified all RJs among all 43 PVY whole-genome sequences in GenBank (Table 1) and confirmed statistically that Mont (N) and Oz (O) were the most likely parents of N-Wi and NTN recombinants, using the Monte Carlo simulation tool SeqGen and the likelihood recombination-detection software LARD (Holmes *et al.*, 1999). Second, we predicted thermodynamic RNA secondary structure in the parental isolates, Mont (N) and Oz (O), using FORS-D analysis to investigate the relationship between RNA secondary structures and RJs. Third, we compared the sequences around the breakpoints (or RJs) in the parents with regard to FORS-D values and nucleotide composition. Our whole-genome analyses showed that RNA secondary structures and/or AU-rich regions at some sites affected PVY recombination, but they were not the main forces driving PVY recombination.

METHODS

Recombination analyses. All PVY full-length genome sequences were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/). Retention of only a single sequence from homologues with 100 % sequence identity resulted in 43 sequences for subsequent analyses (Table 1). Sequences were aligned by MUSCLE 3.6 using default parameters (Edgar, 2004). Recombination breakpoints were identified by the RDP2 software (Martin *et al.*, 2005), using the aligned nucleotide sequences as input. We assumed PVY^{O} and PVY^{N} to be the potential ancestral (or parental) strains, and PVY^{NTN} and PVY^{N}-Wi to be potential recombinants. Positions of the identified RJs were verified using several programs implemented in RDP2 with default settings, e.g. RDP (Martin & Rybicki, 2000), MAXCHI (Smith, 1992), GENECONV (Padidam *et al.*, 1999), SiScan (Gibbs *et al.*, 2000) and CHIMAERA (Posada & Crandall, 2001). The criterion for inclusion was a very low empirical *P*-value ($<10^{-15}$) supported by multiple programs. For accurate detection of crossover sites, only parents and recombinants sharing the same crossover sites were chosen. Alignment files for all of the crossovers are provided in Supplementary Fig. S1 (available in JGV Online).

Mont (PVY^{N}) and Oz (PVY^{O}) isolates were identified by RDP2 as the most likely parents of the N-Wi and NTN strains. For statistical confirmation of crossover events between Mont and Oz at identified RJs, the simulation methods were used as described by Holmes *et al.* (1999). Briefly, for each RJ of the parents and one recombinant, 1000 nt segments centred around the RJ were retrieved from the alignment and input into PHYML (Guindon & Gascuel, 2003) under the HKY model (Hasegawa *et al.*, 1985). The tree inferred by this method was used to simulate 500 datasets using SeqGen under the HKY model and no recombination. Then, likelihood estimates of the real and simulated data given the tree with and without recombination were determined by LARD (Holmes *et al.*, 1999). The likelihood ratios for the simulated datasets were used to build the null distribution (H_0, no recombination). If the likelihood ratios for the real data fell outside this distribution, the null hypothesis was rejected in favour of the alternative hypothesis (H_1, recombination), providing statistical support for the recombination event.

FORS-D analysis. To investigate the relationship between local stem–loop potential and natural recombination, parental isolates Mont (PVY^{N}) and Oz (PVY^{O}) were analysed by FORS-D, using a modified Forsdyke method (Forsdyke, 1995). The two full-length sequences were divided into 951 successive 200 nt windows that each overlap the previous window by 190 nt. For each window, 50 randomized sequences were obtained by using the Perl function List : : Util 'shuffle'. The minimum free energy (MFE) values of sequences were calculated by the RNAfold program in the Vienna package (Hofacker *et al.*, 1994). A natural sequence usually has two kinds of information: nucleotide composition and order. By shuffling the order of a sequence, the contribution of the nucleotide order to the stem–loop potential (FORS-D) can be found by subtracting the mean of the MFE values for folding the randomized sequences (FORS-M) from the MFE value for folding the natural sequence (FONS). This provides an estimate of the local base-ordered stem–loop potential.

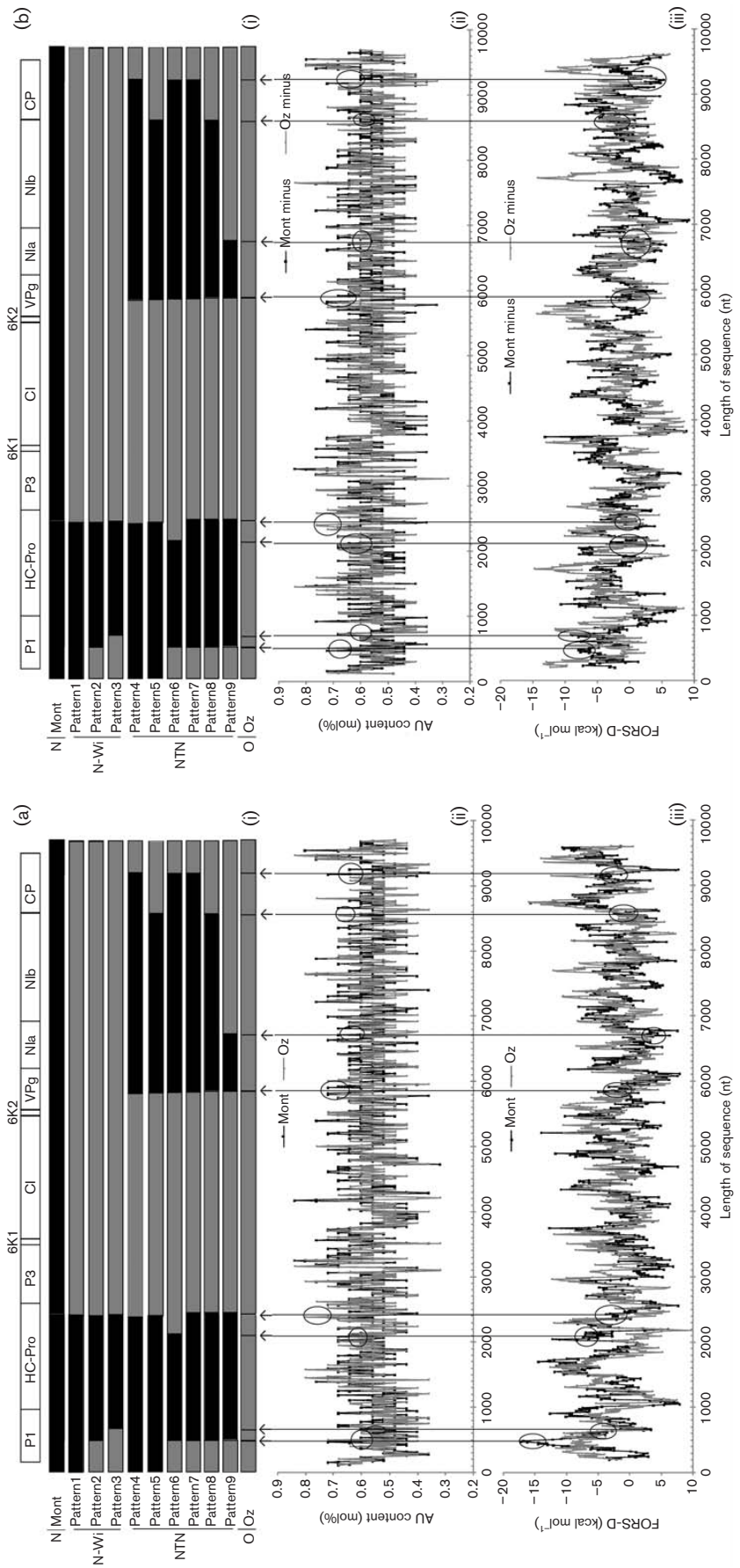

Fig. 1. Relationship between FORS-D values, AU-rich sequences and recombination junctions (RJs) in a positive strand (a) or a negative strand (b) of the PVY genome. (i) PVY genome structure, mosaic patterns and all known RJs. The underlying boxes indicate the various cistrons of the PVY polyprotein. The genome of the Mont isolate is represented in black and that of the Oz in grey; recombinant genomes are represented as combinations of these two colours, with vertical lines indicating known RJs. (ii) AU content of both Mont (PVY^N) and Oz (PVY^O). (iii) FORS-D values of both Mont and Oz. Locations of RJs in the AU, FORS-D distribution are ringed in (ii) and (iii).

Table 1. Full-length PVY sequences used in the analysis

GenBank accession no.	Isolate	Strain	Original host	Reference	Country
AJ585198	SASA-61	N	*Solanum tuberosum*	Barker & McGeachy, unpublished data	UK
AY166866	Tu 660	N		Nie & Singh (2003)	Canada
AY884984	RRA-1	N		Lorenzen *et al.* (2006)	USA
AY166867	N-Jg	N		Nie & Singh (2003)	Canada
AJ890346	Nicola	N	*Nicotiana tabacum*	Schubert *et al.* (2007)	Germany
DQ157180	NE-11	N		Lorenzen *et al.* (2008)	USA
AJ585197	SCRI-N	N	*Solanum tuberosum*	Barker & McGeachy, unpublished data	UK
AY884983	Mont	N		Lorenzen *et al.* (2006)	USA
X97895	N605	N		Jakab *et al.* (1997)	Switzerland
M95491	Hun-NTN	NTN		Thole *et al.* (1993)	Hungary
EF026075	PB312	NTN		Baldauf *et al.* (2006)	USA
AJ890345	Linda	NTN	*Nicotiana tabacum*	Schubert *et al.* (2007)	Germany
AJ585342	NIB-NTN	NTN	*Solanum tuberosum*	Barker *et al.*, unpublished data	Slovenia
AJ890344	Ditta	NTN	*Nicotiana tabacum*	Schubert *et al.* (2007)	Poland
AY884982	423-3	NTN		Lorenzen *et al.* (2006)	USA
AJ890347	Satina	NTN	*Solanum tuberosum*	Schubert *et al.* (2007)	Germany
AJ889867	Wilga156	NTN	*Solanum tuberosum*	Schubert *et al.* (2007)	Germany
AJ890343	Gr99	NTN	*Nicotiana tabacum*	Schubert *et al.* (2007)	Poland
AJ889866	12-94	NTN	*Solanum tuberosum*	Schubert *et al.* (2007)	Poland
AJ890342	34/01	NTN	*Nicotiana tabacum*	Schubert *et al.* (2007)	Poland
AJ889868	Wilga156var	NTN	*Solanum tuberosum*	Schubert *et al.* (2007)	Germany
AB270705	SYR-NB-16	NTN	*Nicotiana tabacum*	Chikh Ali *et al.* (2007)	Japan
DQ008213	PN10A	N-Wi		Lorenzen *et al.* (2006)	USA
AJ890350	Wilga 5	N-Wi	*Solanum tuberosum*	Schubert *et al.* (2007)	Germany
AJ890349	LW	N-Wi	*Solanum tuberosum*	Schubert *et al.* (2007)	Poland
AJ584851	SASA-207	N-Wi	*Solanum tuberosum*	Barker & McGeachy, unpublished data	UK
AY745492	L56	N-Wi		Nie *et al.* (2004)	Canada
AY745491	Mb112	N-Wi		Nie *et al.* (2004)	Canada
EF026076	PB209	N-Wi		Baldauf *et al.* (2006)	USA
DQ157178	ID-1	N-Wi		Lorenzen *et al.* (2008)	USA
AY884985	Alt	N-Wi		Lorenzen *et al.* (2006)	USA
DQ157179	OR-1	N-Wi		Lorenzen *et al.* (2008)	USA
X12456	Fr	O		Robaglia et al. (1989)	France
AJ585195	SASA-110	O	*Solanum tuberosum*	Barker & McGeachy, unpublished data	UK
U09509	O-139	O	*Solanum tuberosum*	Singh & Singh (1996)	Canada
AJ585196	SCRI-O	O	*Solanum tuberosum*	Barker & McGeachy, unpublished data	UK
EF026074	Oz	O		Baldauf *et al.* (2006)	USA
AJ890348	Adgen	C	*Solanum tuberosum*	Schubert *et al.* (2007)	France
AF463399	MN	Non-pot		Fellers *et al.* (2002)	
DQ309028	NC57	Non-pot	*Nicotiana tobacum*	Hari & Kelly, unpublished data	USA
AF237963	NNP	Non-pot	Pepper	Fanigliulo *et al.* (2005)	Italy
AJ439544	SON41	Non-pot	*Solanum nigrum*	Moury *et al.* (2002)	France
AJ439545	LYE84.2	Non-pot	*Lycopersicon esculentum*	Moury *et al.* (2002)	Spain

Identification of features related to RJs. The stem–loop potential and compositions of the upstream (5′) and downstream (3′) sequences of the two parents (Mont and Oz) around RJs were compared. The mean of five FORS-D values before and after the breakpoint represents the value for each flanking side. The statistical significance of differences between two sides was compared by Student's *t*-test. Others have shown that a 25 nt AU-rich sequence can promote recombination (Shapka & Nagy, 2004); therefore, the 25 nt block compositions of the upstream and downstream regions around the breakpoints were compared.

For an overview of the AU distribution along the genome, the AU content was calculated, using sliding windows of size 25 and step 10. The AU distributions of Mont and Oz were drawn and the RJs were marked.

RESULTS

Recombination-detection analyses

For detecting recombination breakpoints and viewing mosaic structures, we used several recombination-detection programs included in the RDP2 software. Oz and Mont were chosen as the best parents (around 99 % similarity for

Table 2. Recombination patterns of PVY detected by recombination-detecting programs

Pattern	Group	Recombination breakpoint(s)*	Isolates	*P*-value†
1	N-Wi	2419	SASA-207, L56, Mb112, PB209, ID-1, Alt, OR-1	8.08×10^{-27}
2	N-Wi	502, 2395–2398	LW, Wilga5, PN10A	8.08×10^{-27}
3	N-Wi	652, 2419	261-4	8.08×10^{-27}
4	NTN	2419, 5834, 9193	Linda, Ditta, NIB-NTN, Hun-NTN, PB312, v942490, 423-3	1.48×10^{-18}
5	NTN	2419, 5857, 8607	SYR-NB-16	1.48×10^{-18}
6	NTN	502, 2177, 5834, 9193	Gr99	1.48×10^{-18}
7	NTN	502, 2395–2419, 5834, 9193	PVY-12, 12-94, 34/01	1.48×10^{-18}
8	NTN	502, 2398, 5834, 8585	Wilga156	1.48×10^{-18}
9	NTN	502, 2398, 5834, 6714	Wilga156var	1.48×10^{-18}

*Breakpoints indicate positions in the alignment of all sequences. Ambiguous breakpoints are indicated by a range of values.
†Greatest *P*-value among recombinants identified by the recombination-detecting programs RDP, GENECONV, BootScan, MaxChi, CHIMAERA and SiScan in RDP2.

the respective daughter sequences) for N-Wi and NTN recombinants, as determined by the lowest *P*-values for the programs RDP ($P<4.029 \times 10^{-127}$), GENECONV ($P<2.342 \times 10^{-129}$), BootScan ($P<7.163 \times 10^{-205}$) and MAXCHI ($P<2.54 \times 10^{-43}$). Summarizing these recombinants according to the RJs showed nine recombination patterns with eight RJs [Table 2; Fig. 1, panel (iii)]. As mentioned, this did not include all known PVY recombinants, but only those best represented by the postulated parents: Mont (N strain) and Oz (O strain).

To further analyse statistically whether Mont and Oz can form a recombinant at a specific RJ, we used all eight RJs in simulation with Mont, Oz and recombinants. For each RJ, the simulated 500 datasets and the natural data were analysed by LARD, which determined the breakpoint for each dataset by the maximum-likelihood estimate. The null distribution (hypothesis H_0, no recombination) was obtained from simulated sequences' log-likelihood ratios as given by LARD. Its range was 1.3–16.2 for all eight RJs. However, the values for the natural RJs were 54–149 (Table 3), well outside the null distribution, which statistically supported the alternative hypothesis of recombination (H_1). Therefore, crossover events between Mont and Oz might truly happen in nature. We can use them to analyse the features that affect recombination.

FORS-D and AU distribution along the PVY genome

Previous studies on small RNA viruses have shown that RNA secondary structure and/or AU-rich sequence may promote RNA virus recombination in the template-switching model (Nagy & Simon, 1997). A stem–loop structure could form a heteroduplex and bring two recombination RNA substrates into close proximity (Nagy & Bujarski, 1993). Thus, both positive-strand and negative-strand RNAs at this region could be the templates for RNA replicase to produce new recombinants. Because FORS-D reflects the local base-ordered stem–loop potential, it can be used to estimate RNA secondary structure in the virus genome. To investigate roles of RNA secondary structure and AU-rich regions in PVY recombination, we

Table 3. Comparison of the log-likelihood ratios (LLRs) for PVY natural recombinant sequences to their respective simulated data at different breakpoints

Breakpoint*	Gene	Recombination patterns	LLRs for natural recombinants†	Range of LLRs for simulated datasets
502	P1	2	149.7 ± 4.3	1.6–13.0
652	P1	3	162.5	1.6–12.2
2177	HC-Pro	6	72.5	1.6–14.0
2395–2419	HC-Pro	1–5, 7	77.2 ± 5.4	1.5–16.2
5834–5857	VPg	4–9	89.1 ± 2.8	1.3–14.1
6714	NIa	9	99.8	1.6–13.3
8585–8607	NIb	5, 8	69.3 ± 4.1	1.6–12.3
9193	CP	4, 6, 7	54.4 ± 4.1	1.2–14.8

*Breakpoints indicate positions in the alignment of all sequences. Ambiguous breakpoints are indicated by a range of values.
†Mean ± SD; for breakpoints 652, 2177 and 6714, only one recombinant was identified, so no SD is given.

used the sliding-windows method to compute AU content and FORS-D values along the Mont and Oz genomes and drew their distributions [Fig. 1, panels (ii, iii)].

The AU content along the PVY genome fluctuated [Fig. 1, panel (ii)]. The mean was 56 mol% for both Mont and Oz and the range was 28–84 mol%. AU-rich ($\geqslant$60 mol% AU) regions in Mont and Oz isolates represented 42 and 37 % of the entire genome length, respectively. The most prominent RJs, located at positions 2395–2419 and 5834–5857, were in AU-rich regions (see alignments provided in Supplementary Fig. S1). However, there were no RJs in the PVY genome regions with the highest AU content, e.g. the regions around positions 1445, 3155, 4175, 7645 and 9465, which had AU contents >80 mol%.

The distribution of FORS-D values is shown in Fig. 1, panels (iii), in which the eight identified RJs are marked. The overall mean FORS-D value of Mont and Oz isolates of PVY was -1.78 and -2.34 kcal mol^{-1} from 951 points, respectively; 63 and 69 % of Mont and Oz points were negative along the genome, respectively. The mean for the FORS-D values around RJs of Mont and Oz was -3.56 kcal mol^{-1} in both cases. These values were lower than the means [P=0.04 (statistically significant) for Mont, P=0.11 for Oz]. In a positive-strand FORS-D distribution, RJs 502 and 2177 were located in the more negative FORS-D values for Mont (N parent). At the same time, in a negative-strand FORS-D distribution, RJs 2395–2419 and 5834–5857 were located in the more negative FORS-D values for Oz (O parent). It needs to be pointed out that most of the regions with the lowest FORS-D values had no RJs.

Identification of features related to recombination breakpoints

After analysing the whole genome, we focused attention on the small regions around the RJs to identify sequence features distinguishing identified recombinant breakpoints in the PVY genome. We calculated FORS-D values for the sequences flanking the corresponding RJs of the parents (Mont and Oz) (Table 4). In the positive strand, we found that, in most cases, upstream (5′) FORS-D values were lower than downstream (3′) values: six of eight in Oz and eight of eight in Mont. This difference was highly significant for four RJs (502, 652, 2177 and 9193) (P<0.01; Table 4) in Mont, and highly significant for one RJ (652) and significant for three RJs (2395–2419, 5834–5857, 9193) (0.01<P<0.05; Table 4) in Oz. We also found a highly significant difference in the FORS-D value for negative-strand RJs, e.g. Mont negative-strand RJ 5834–5857. So, we concluded that the stem–loop structures might enhance RNA recombination at these RJs.

We also examined the AU composition of the sequences on either side of the RJs in Mont and Oz (Table 5). All RJs had at least one AU-rich region, which we defined as having $\geqslant$60 mol% AU. RJs 652, 2395–2419 and 5834 had a high AU content for both parents. When we checked regions

Table 4. FORS-D values (kcal mol^{-1}) around breakpoints on both strands of the parent isolates Mont and Oz

Significantly different FORS-D values (and their corresponding P-values) are shown in bold.

Breakpoint*	Mont plus			Oz plus			Mont minus			Oz minus		
	Left (5′)	Right (3′)	P-value†	Left (5′)	Right (3′)	P-value†	Left (5′)	Right (3′)	P-value†	Left (5′)	Right (3′)	P-value†
502	**−15.3 ± 2.1**	**−8.2 ± 2.0**	**0.0006**[a]	−7.8 ± 1.0	−7.9 ± 3.2	0.94	**−6.8 ± 0.8**	**−3.1 ± 2.2**	**0.008**[a]	−1.2 ± 2.0	−5.7 ± 4.2	0.06
652	**−5.1 ± 2.6**	**2.1 ± 2.0**	**0.001**[a]	**−6.9 ± 2.7**	**1.4 ± 1.3**	**0.0002**[a]	**−3.5 ± 1.2**	**−6.9 ± 1.8**	**0.009**[a]	−2.1 ± 2.6	−3.0 ± 2.6	0.63
2177	**−5.4 ± 1.8**	**1.2 ± 3.5**	**0.005**[a]	2.9 ± 2.1	3.3 ± 5.3	0.87	0.49 ± 0.6	−0.9 ± 2.6	0.28	**2.9 ± 0.8**	**−5.2 ± 3.7**	**0.001**[a]
2395—2419‡	−1.7 ± 1.1	−1.7 ± 2.0	0.96	**−3.1 ± 4.5**	**2.7 ± 2.0**	**0.03**[b]	1.4 ± 2.0	−1.6 ± 2.4	0.08	−1.5 ± 3.7	−1.1 ± 3.1	0.76
5834–5857‡	−1.9 ± 2.6	−0.8 ± 1.4	0.43	**−2.7 ± 1.2**	**1.2 ± 2.8**	**0.02**[b]	**−6.1 ± 2.7**	**−0.9 ± 1.8**	**0.008**[a]	−0.6 ± 2.0	2.1 ± 2.2	0.07
6714	3.1 ± 2.7	3.2 ± 0.8	0.90	2.0 ± 1.5	0.2 ± 2.0	0.14	3.8 ± 1.4	1.5 ± 2.1	0.08	1.8 ± 1.9	2.9 ± 1.7	0.35
8585—8607‡	−0.4 ± 1.9	0.9 ± 4.5	0.57	−1.1 ± 1.2	−3.3 ± 3.1	0.17	−1.5 ± 1.8	−1.0 ± 3.3	0.76	−3.6 ± 1.5	−1.8 ± 2.4	0.19
9193	**−2.9 ± 2.0**	**3.5 ± 3.7**	**0.009**[a]	**−6.8 ± 3.6**	**−0.5 ± 2.6**	**0.01**[b]	3.9 ± 1.0	3.6 ± 2.0	0.73	−0.7 ± 3.5	0.09 ± 3.1	0.72

*Breakpoints indicate positions in the alignment of all sequences. Ambiguous breakpoints are indicated by a range of values. The true positions for Mont and Oz were used to calculate FORS-D means.

†P-values were calculated by Student's t-test with flanking sites' FORS-D values. [a]P<0.01; [b]P<0.05.

‡The values flanking these regions were used to calculate the mean FORS-D value.

Table 5. Sequence composition (mol%) in 25 nt of the detected recombination breakpoints in parent isolates Mont and Oz

Breakpoint*	Mont				Oz			
	Left (5′)		Right (3′)		Left (5′)		Right (3′)	
	A/U	G/C	A/U	G/C	A/U	G/C	A/U	G/C
502	48	52	48	52	52	48	60	40
652	56	44	64	36	60	40	56	44
2177	44	56	60	40	44	56	56	44
2395	64	36	56	44	80	20	60	40
2398	60	40	56	44	80	20	64	36
2419	64	36	36	64	68	32	48	52
5834	72	28	52	48	64	36	56	44
5857	56	44	48	52	60	40	52	48
6714	48	52	64	36	48	52	68	32
8585	56	44	56	44	68	32	56	44
8607	60	40	48	52	60	40	48	52
9193	52	48	60	40	52	48	56	44

*Breakpoints indicate positions in the alignment of all sequences. The true positions for Mont and Oz were used to calculate sequence composition.

more distant from RJs, AU contents were usually around 50 mol%. Hence, we concluded that the AU-rich sequences might play the same role in PVY recombination as in other RNA viruses.

Did both factors affect PVY recombination simultaneously at the RJ? After checking every RJ with two values (FORS-D and AU content), we found that some sites, such as 502, 652, 2177, 2395–2419, 5834–5857 and 9193, had significantly more negative upstream FORS-D values combined with an AU-rich region downstream; some sites, such as 6714 and 8585–8607, only had an AU-rich region. Taken together, we concluded that secondary structure and AU-rich regions may affect PVY recombination in some regions.

DISCUSSION

The frequency of viral RNA recombination is affected by several factors, including host genes, the viral replication proteins and various features of the viral RNA templates involved (Nagy, 2008). In this study, we investigated two features of the template: stem–loop structure and AU-rich regions. By analysing the distribution of stem–loop structures and AU-rich regions along the PVY genome, we found that 63 and 69 % of the regions of Mont and Oz had stem–loop potential (FORS-D value <0), respectively, whilst 42 and 37 % of the regions of Mont and Oz were AU-rich ($\geqslant$60 mol% AU), respectively. Theoretically, if template features determine locations of crossover sites, many more RJs should be found. However, why are there just a limited number of recombination patterns reported for PVY? There are two possible explanations for this discrepancy. One explanation is that there is a strong selective pressure against successful survival of new PVY recombinants. In PVY, different genes were reported to be under different selective constraints, e.g. P1 (Ogawa *et al.*, 2008), CP and 6K2 (Moury *et al.*, 2002) were found to evolve by positive selection. Most recombinants formed by the template-switching mechanism thus may not survive. One of the reasons is that imprecise recombination in an AU-rich region may lead to nucleotide additions or deletions. Thus, resulting frameshifts in the PVY genome would result in non-viable recombinants. Another explanation is that other factors, such as host genes, may affect PVY recombination.

When we compared FORS-D value and AU content around RJs, we found that most RJs had the following features: lower negative FORS-D value upstream and/or high AU content downstream, especially in the most common sites (502, 2395–2419, 5834 and 9193), which are usually used for identifying N-Wi and NTN isolates. Many other RNA viruses have AU-rich regions downstream of their recombination sites, but this is the first report of a lower FORS-D value around RJs in a plant virus. Here, we first noticed that upstream FORS-D values are significantly lower than downstream ones at some RJs, such as 502, 652, 2177 and 9193 in Mont and 502, 652 and 9193 in Oz. This means that upstream stem–loop potentials are higher than downstream ones at these RJs. We think that RNA secondary structure might pause or slow down the viral RNA replicase before RJs and promote template switching. However, further biological and biochemical experiments are needed to test this conclusion.

GC-rich ($\geqslant$60 mol% GC content) and AU-rich ($\geqslant$60 mol% AU) regions were termed 'homologous-recombination activators', which can influence homologous recombination (Nagy *et al.*, 1999a). The upstream GC-rich region and downstream AU-rich region were observed

in RNA recombinants in CTV (Vives *et al.*, 2005), noroviruses (Rohayem *et al.*, 2005) and TuMV (Ohshima *et al.*, 2007). However, in our study, we did not observe higher GC contents upstream of RJs, except for RJ 2419 (data not shown). Thus, GC-rich regions do not seem to play a role in PVY recombination, in contrast to another potyvirus, TuMV (Ohshima *et al.*, 2007). Whether GC-rich regions may have a recombination-silencer function, as in BMV recombination (Nagy & Bujarski, 1998), needs to be determined.

Based on the distribution of FORS-D values and AU-content variation along the whole PVY genome, we concluded that, whilst RNA secondary structures and AU-rich regions may be enabling forces, they cannot fully explain the recombination observed in PVY. These two factors may play roles in some recombination events; however, other, as-yet-unidentified factors, perhaps host and ecological selection constraints, may drive PVY recombination. One other possible factor affecting PVY recombination is as-yet-unidentified *cis*-acting enhancers of replication (Nagy & Simon, 1997; Nagy *et al.*, 1998), which may also contribute to RJ selection.

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