Brief communication

Complete nucleotide sequence analysis of *Cymbidium mosaic virus* Indian isolate: further evidence for natural recombination among potexviruses

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The complete nucleotide sequence of an Indian strain of *Cymbidium mosaic virus* (CymMV) was determined and compared with other potexviruses. Phylogenetic analyses on the basis of RNA-dependent RNA polymerase (RdRp), triple gene block protein and coat protein (CP) amino acid sequences revealed that CymMV is closely related to the *Narcissus mosaic virus* (NMV), *Scallion virus X* (SVX), *Pepino mosaic virus* (PepMV) and *Potato aucuba mosaic virus* (PAMV). Different sets of primers were used for the amplification of different regions of the genome through RT-PCR and the amplified genes were cloned in a suitable vector. The full genome of the Indian isolate of CymMV from *Phaius tankervilliae* shares 96–97% similarity with isolates reported from other countries. It was found that the CP gene of CymMV shares a high similarity with each other and other potexviruses. One of the Indian isolates seems to be a recombinant formed by the intermolecular recombination of two other CymMV isolates. The phylogenetic analyses, Recombination Detection Program (RDP2) analyses and sequence alignment survey provided evidence for the occurrence of a recombination between an Indian isolate (AM055720) as the major parent, and a Korean type-2 isolate (AF016914) as the minor parent. Recombination was also observed between a Singapore isolate (U62963) as the major parent, and a Taiwan CymMV (AY571289) as the minor parent.

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1. Introduction

The orchid is one of the most important ornamental plants of the world. The magnificent inflorescence, the wide variety of colours and its long vase life have encouraged its cultivation throughout the world. The orchid is a popular pot plant, and important as a commercial cut flower grown under cover.

Orchids have been reported to be infected with more than 50 viruses (Chang *et al* 2005; Zettler *et al* 1990). Of several orchid-infecting viruses, *Cymbidium mosaic virus* (CymMV) and *Odontoglossum ringspot tobamovirus* (ORSV) have been reported to be two of the most prevalent and important viruses which have attained worldwide distribution (Zettler *et al* 1990; Wong *et al* 1994; Sherpa *et al* 2004). The occurrence of these two orchid viruses results in significant

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Abbreviations used: BAMV, *Bamboo mosaic virus*; ClYMV, *Clover yellow mosaic virus;* CP, coat protein; CsCMV, *Cassava common mosaic virus;* CVX, *Cactus virus X*; CymMV; *Cymbidium mosaic virus*; FoMV, *Foxtail mosaic virus*; LVX, *Lily virus X*; NCR, non-coding protein; NMV, *Narcissus mosaic virus*; ORF, open reading frame; ORSV, *Odontoglossum ringspot tobamovirus*; RDP2, Recombination Detection Program; RdRp, RNA-dependent RNA polymerase; PAMV, *Potato aucuba mosaic virus*; PapMV, *Papaya mosaic virus*; PepMV, *Pepino mosaic virus*; PlAMV, *Plantago asiatica mosaic virus*; PVX, *Potato mosaic virus*; SMYEV, *Strawberry mild yellow edge virus*; SVX, *Scallion virus X*; TGB, triple gene block; WClMV, *White clover mosaic virus*.

economic losses to the orchid industry. Mixed infection with both the viruses can cause blossom brown necrotic streak (Eun *et al* 2002). The negative impact of these viruses on cultivated orchids, especially the more common CymMV, has been reported in the orchid growing areas of the world (Zettler *et al* 1990; Francki *et al* 1985). Orchid cultivars infected with CymMV often show smaller flower size, poor quality, necrosis and flower disfigurement as well as foliage symptoms, resulting in great economic losses (Seoh *et al* 1998). The virus is generally restricted to the Orchidaceae, where it is found in high concentrations, and is extremely stable (Jensen 1950). Generally orchid viruses have not been reported from wild collections but Wey *et al* (2001) found that the wild species and hybrid plants of *Phalaenopsis*, *Doritaenopsis* and *Doritis* collected from Taiwan, Japan, the UK and USA were found to be infected with CymMV and/or ORSV with an infection rate (as per country of origin) of 35.8%, 18.2%, 18.2% and 33.3%, respectively. CymMV is an RNA virus belonging to the group of flexuous rod-shaped potexviruses which are approximately 475–490 nm in length (Frowd and Tremaine 1977; Steinhart and Oshiro 1990). CymMV contains about 6–7 kb of positive-sense genomic RNA that is capped and polyadenylated (Francki *et al* 1985; Srifah *et al* 1996). The protein subunits of 257 amino acid residues have a molecular weight of 27,600 daltons, and the base composition (G, A, C and U) of the viral RNA is 21.1, 28.9, 24.4 and 25.6, respectively (Frowd and Tremaine 1977). Like most of the polyadenylated monopartite positive-strand RNA viruses, the open reading frame (ORF) coding for the viral coat protein (CP) is located at the 3′ end (Chia *et al* 1992). The putative polyadenylation signal, the AATAAA motif, is found in the terminus of the 3′-UTR (Ryu *et al* 1995).

Till date, the complete sequence of six isolates of the CymMV is available (accession numbers: AF016914, U62963, AB197937, AY571289, AM055720 and NC_001812). The complete nucleotide sequence of the genome of CymMV was reported to be 6227 nucleotides in length (Wong *et al* 1997), except AB197937 from Japan which has 6226 nucleotides, excluding the poly (A) tail at the 3′- terminus. Similar to other potexviruses, its genome organization comprises five major ORFs (1–5), encoding an M_r 160 kDa putative RNA-dependent RNA polymerase (RdRp); an M_r 26 kDa/13 kDa/10 kDa triple gene block (TGB) and an M_r 24 kDa CP. The CymMV-encoded proteins share a high degree of similarity with the corresponding proteins of other members of potexvirus group. The 5′-end of the genome of the Indian isolate of CymMV starts with GGAAAA, which is in contrast to GAAAA at the 5′-end of most other potexviruses, including the Japanese isolate (accession number AB197937) and Singapore isolate (accession number NC_001812). The nucleotide sequence of the 5′ non-coding region (NCR) of CymMV and all potexviruses starts with GAAAA. CymMV and *Lily virus X* (LVX) possess the shortest 5′ NCR among all the potexviruses. Based on phylogenetic comparisons of RdRp and CP, CymMV shares a close relationship with *Potato aucuba mosaic virus* (PAMV), *Narcissus mosaic virus* (NMV), *White clover mosaic virus* (WClMV), and *Strawberry mild yellow edge virus* (SMYEV) (Wong *et al* 1997).

2. Materials and methods

2.1 *Sample collection and virus identification*

2.1.1 *ELISA:* Different orchid samples were collected and tested for the presence of CymMV by DAS-ELISA according to the manufacturer's (Agdia, USA) instructions.

2.1.2 *Electron microscopy: Phaius tankervilliae* suspected to be infected with virus was tested for the presence of CymMV using electron microscopy (EM) as published earlier (Sherpa *et al* 2003).

2.1.3 *RT-PCR:* Total RNA was isolated using an RNA isolation kit according to the instructions of the manufacturer (Qiagen Rneasy methods) and RNA was used for the first strand complementary DNA (cDNA) synthesis. RdRp was amplified using a pair of primers (accession numbers AM157360 and AM157361) using long PCR according to the instructions of the manufacturer (Fermentas, USA). Various primer combinations used for the amplification of different regions of RdRp (1–923, 856–1766, 1720-–2663, 2598–3522, 3459–4354) were as follows: CymR1U (5′-GGAAAACCAAACCTCACGTCTA-3′) with CymR1D (5′-GGAATATTTGTGGTAGTAGAACA-3′); CymR2U (5′-GGATCTTCGCACACCCAAACT-3′) with CymR2D (5′-CTCTTTCC TTTAAAGCCATGTT-3′); CymR3U (5′-GGATCTTTGGATTCCCCTACT-3′) with CymR3D (5′-TTTGGATTTTAGGGGCAGTGAGA-3′); CymR4U (5′-CTGATGT CGGCAACCGTTGCATG-3′) with CymR4D (5′-GGAGTCTTTTCACAATTGAT AA-3′); CymR5U (5′-ACCTCCGTAAGATGCGGCAAAG-3′) with CymR5D (5′ A ACTAAGTACGCTAGCTCCATCA-3′). TGB proteins (TGB1, TGB2 and TGB3) and the CP gene were amplified using the primer sets given in table 1. The 3′ end of the NCR was amplified using primer accession number AJ566130 and oligo dT (18). Other genes were amplified using the pair of primers shown in table 1. The cloning and sequencing strategies of different cDNA is shown in figure 1.

2.2 *Cloning and sequencing*

The PCR amplified products were purified (Qiagen Ltd, Sussex, UK), ligated into pGEM-T-Easy (Promega,

Table 1. Primers used to amplify different open reading frames

Gene	Primer sequence		Product size	Annealing temperature	Accession number
RdRp	U	5′-TCC**CCCGGG**GGAATGGCTCGAGTACGTGACAC -3′ (32 mer)	4.2 kb (74–4327)	58°C	AM157360
	D	5′-TCC**CCCGGG**GGATCAAAATTAACCCCGTTTTA-3′ (32 mer)			AM157361
CymR1	U	5′- GGAAAACCAAACCTCACGTCTA -3’ (22 mer)	923 bp (1–923)	50°C	AM157362
	D	5′-GGAATATTTGTGGTAGTAGAACA-3’ (23 mer)			AM157363
CymR2	U	5′- GGATCTTCGCACACCCAAACT-3’ (21 mer)	912 bp (856–1766)	50°C	AM157665
	D	5′- CTCTTTCCTTTAAAGCCATGTT-3’ (22 mer)			AM157666
CymR3	U	5′- GGATCTTTGGATTCCCCTACT-3’ (21 mer)	944 bp (1720– 2663)	50°C	AM157667
	D	5′-TTTGGATTTTAGGGGCAGTGAGA -3’ (23 mer)			AM157668
CymR4	U	5′-CTGATGTCGGCAACCGTTGCATG -3’ (23 mer)	924 bp (2598-3522)	48°C	AM157669
	D	5′- GGAGTCTTTTCACAATTGATAA-3’ (22 mer)			AM157670
CymR5	U	5′- ACCTCCGTAAGATGCGGCAAAG -3’ (22 mer)	896 bp (3459–4354)	50°C	AM157671
	D	5′-AACTAAGTACGCTAGCTCCATCA-3’ (23 mer)			AM157672
CymMV MP1	U	5′- TCC**CCCGGG**GGAATGGAGCTAGCGTACTTAGT-3′ (32 mer)	690 bp (4333–5022)	50°C	AM157673
	D	5′-TCC**CCCGGG**GGATCAGGTGGAGAAAACGAA-3′ (30 mer)			AM157674
CymMV MP2	U	5′-TCC**CCCGGG**GGAATGCCAGGTTTAGTTCCA-3′ (30 mer)	339bp (5010–5348)	50°C	AM157675
	D	5′-TCC**CCCGGG**GGATCAGGTCCAAATCAAGGT-3′ (30 mer)			AM157676
CymMV MP3	U	5′-TCC**CCCGGG**GGAATGCTAGGTACCCGAAAT-3′ (30 mer)	276 bp (5203–5478)	50°C	AM157677
	D	5′-TCC**CCCGGG**GGATCAAATTATTGTGGTAA-3′ (29 mer)			AM157678
CymMV CP	U	5′- CG**GGATCC**ATGGGAGAGTCCACTCCA -3’ (26 mer)	672 bp (5481–6152)	50°C	AJ566130
	D	5′- G**GAATTC**TCAGTAGGGGGTCCAGGC -3′ (25 mer)			AJ566131

WI, USA), and the ligated vector was transformed into *Escherichia coli*. The recombinant plasmid DNA for sequencing was isolated by the boiling method (Holmes and Quingley 1981) and purified by using the WIZARD DNA clean-up system (Promega, USA). The nucleotide sequence of the clones was determined by the dideoxy chain termination method (Sanger *et al* 1977) using an automated DNA sequencer (ABI PRISM® 310 Genetic Analyzer).

The viruses used in sequence alignments and phylogenetic analyses were as follows: CymMV (accession number: AM055720), *Bamboo mosaic virus* (BaMV; accession number: AF018156), *Cassava common mosaic virus* (CsCMV; accession number: U23414), *Foxtail mosaic virus* (FoMV; accession number: AY121833), *Papaya mosaic virus* (PapMV; accession number: D13957), *Plantago asiatica mosaic virus* (PlAMV; accession number: NC_003849), *Potato mosaic virus* (PVX; accession number: AF172259), SMYEV (accession number: NC_003794), WClMV (accession number: NC_003820), *Cactus virus X* (CVX; accession number: AF308158), *Clover yellow mosaic virus* (ClYMV; accession number: NC_001753), NMV (accession number: D13747), *Pepino mosaic virus* (PepMV; accession number: AF484251), PAMV (accession number: NC_003632), *Scallion virus X* (SVX; accession number: AJ316085), *Tulip virus X* (TuVX; accession number: NC_004322), LVX (accession number: NC_007192) and *Botrytis virus X* (BVX; accession number: AY055762). All the viruses used in phylogenetic analyses belonged to the potexvirus genus apart from *Garlic latent carlavirus* (GLV: NC_003557), *Indian citrus ringspot mandarivirus* (ICRSV: AF406744) and *Apple stem pitting foveavirus* (ASPV: NC_003462), which were used as outgroups.

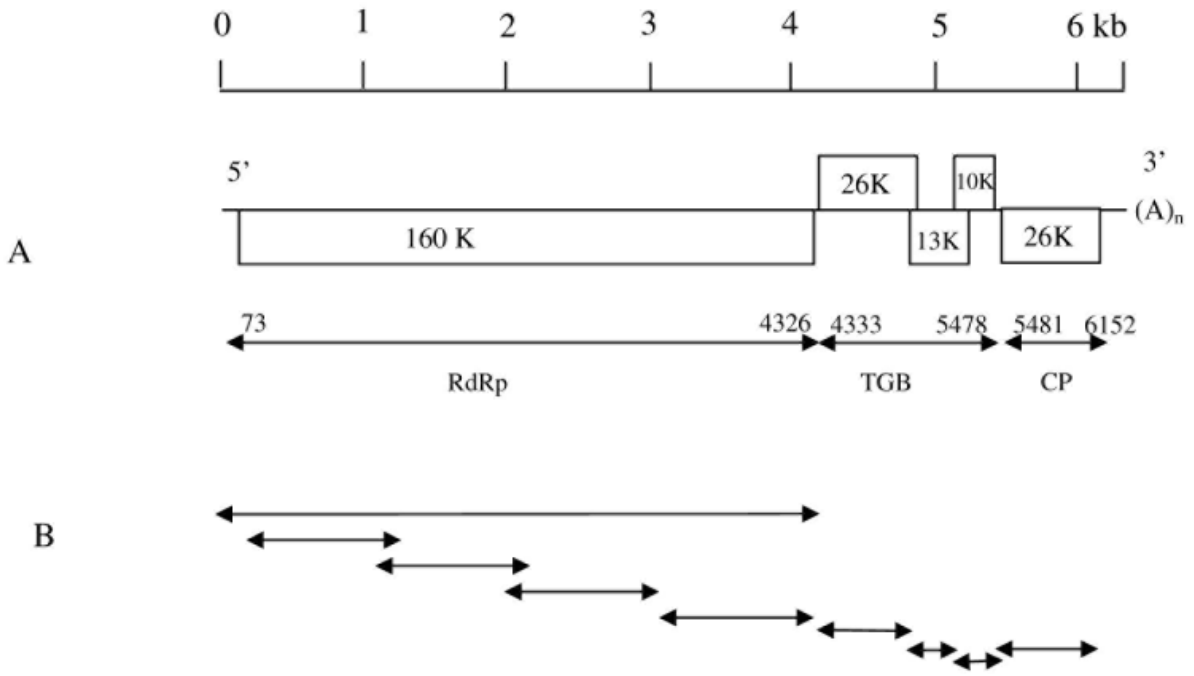

Figure 1. Schematic representation of the CymMV genome. (**A**) Genome organization with scale, boxes represent the coding regions for RdRp (4254 nt), TGB1 (690 nt), TGB2 (339 nt), TGB3 (276 nt) and CP (672 nt). (**B**) Sequencing strategy. Double arrowed line shows amplified fragments used for cloning and sequencing.

2.3 *Recombination detection*

Detection of potential recombinant sequences, identification of likely parental sequences and localization of possible recombination breakpoints were carried out using the RDP2 and GENECONV (Padidam *et al* 1999), MAXIMUM CHI SQUARE (Maynard Smith 1992), CHIMAERA (Posada and Crandall 2001) and SISTER SCAN (Gibbs *et al* 2000) methods implemented in RDP2 (Martin *et al* 2005) with the following settings: window size=10, highest acceptable probability=0·001, internal reference sequences.

3. Results and discussion

The complete nucleotide sequence of the Indian strain of the CymMV was found to be 6227 nucleotides excluding the poly(A) tail at the 3′ terminus and contains five ORFs encoding an M_r 160 kDa putative RdRp; an M_r 26 kDa/13/10 kDa TGB and an M_r 24 kDa CP as other potexvirurses. Phylogenetic analyses carried out on RdRp, TGB proteins and CP amino acid sequences revealed that CymMV is closely related to the NMV, SVX, PepMV, and PAMV. The RdRp of the Indian isolate of CymMV shared 40–54% similarity with other potexvirus at the amino acid level. Likewise, TGB1, TGB2, TGB3 and the CP gene of the Indian isolate of CymMV was found to share 11–34%, 25–35%, 16–25% and 20–53% similarity, respectively, with other potexviruses at the amino acid level. The full genome of the Indian isolate of CymMV from *Phaius tankervilliae* shares 96–97% similarity with CymMV isolates reported from other countries. The RdRp of the Indian CymMV showed 97–98% similarity with other published sequences both at the nucleotide level and the amino acid level, indicating that the RdRp in CymMV isolates is conserved. On sequence alignment, it was also found that there is variability in the TGB1gene among the different CymMV isolates under study. The C-terminal of AB197937 and AF016914 contains the 'TFIALTRAKGNLTIFDFNARFSSTT' amino acid domain and the other three isolates (NC-001812, U62963, and AY571289) contain the conserved amino acid domain ('SLHRSHPRQGQPHHLRFQCQV') at the 3′ terminal. At the C-terminal of TGB1, the Indian isolate contains the amino acid domain 'SLHGSHPRQGQPHHFRFLCQV' which is similar to NC-001812 (Singapore), U62963 (Singapore), and AY571289 (Taiwan); therefore, the Indian isolate falls under this group. Other genes of CymMV show good similarity both at the nucleotide level and the amino acid level.

It was found that the CymMV CP and RdRp share a high sequence similarity with each other and with other potexviruses. On sequence analyses of potexviruses on the

basis of RdRp, TGB proteins and the CP gene, the Indian isolate of CymMV (accession number: AM055720) was found to share good similarity with PAMV, NMV, SVX, PepMV and WClMV. On phylogenetic analyses based on different genes of potexviruses, two major clusters were formed and potexviruses can thus be divided into two groups. CymMV falls into the cluster that comprises NMV, PepMV, PAMV, WClMV, PVX, SVX, LVX and SMYEV. Another cluster comprises ClYMV, CsCMV, *Tulip mosaic virus* (TuMV), PlAMV, FoMV, BaMV and CVX. Most of the viruses of the potex group fall into the cluster that contains CymMV; the significance of these two major clusters is not clear.

On RDP2 analysis, two isolates seem to be recombinant due to intermolecular recombination of two other CymMV isolates. The phylogenetic analyses, RDP analyses, and

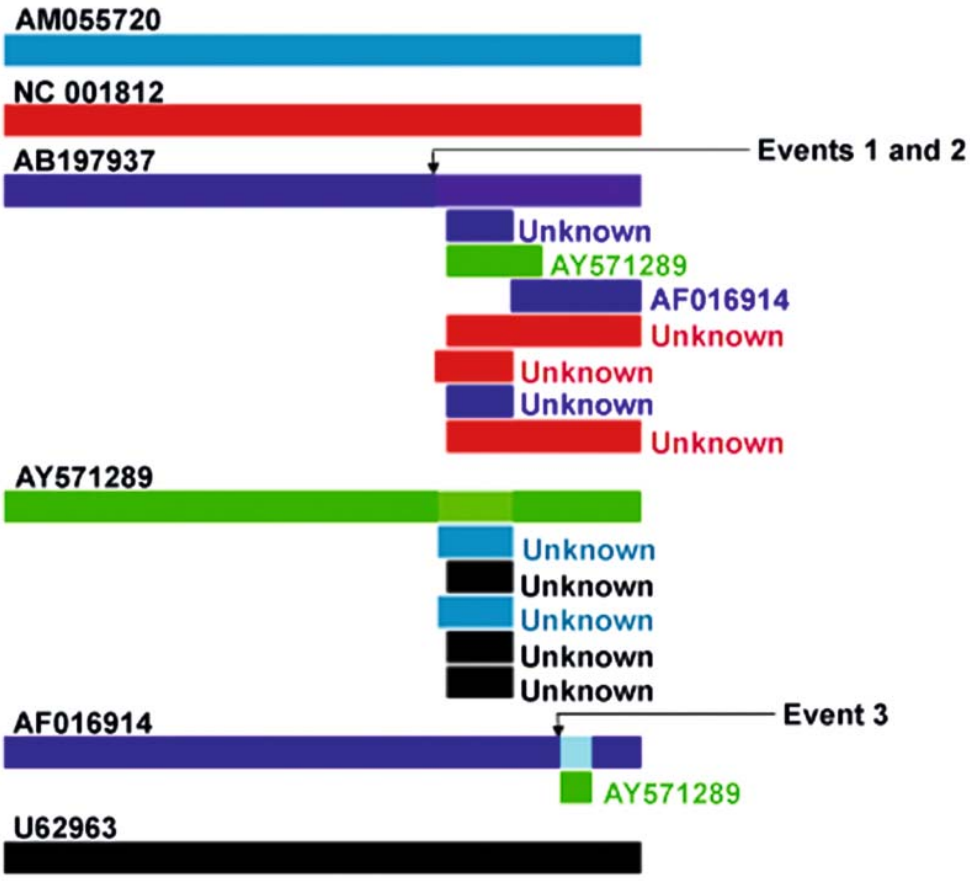

Figure 2. Description of recombination events. As can be seen in the figure, the RDP2 program identifies three recombination events with a *P* value of 4.188×10^{-24}. These events were detected by three programs, viz. GENECONV, MAXIMUM CHI SQUARE and CHIMAERA. The three recombination events can be described as follows:

Event 1: Recombination between an Indian CymMV isolate (AM055720) as the major parent, and a Korean type 2-CymMV isolate (AF016914) as the minor parent. In this recombination event, a region (4973–6226 nt) of the AM055720 genome was replaced with the corresponding genome sequences of AF016914 (RDP *P* value=4.188×10^{-24}), which included the N-terminal of TGB and C-terminal of coat protein.

Event 2: Recombination was detected with the same *P* value as for event 1, between the Singapore isolate (U62963) as the major parent, and the Taiwan isolate (AY571289) as the minor parent. In this recombination event, a region (4337–5262 nt) of the Singapore isolate (U62963) was replaced with the corresponding genome sequence of the Taiwan isolate (AY571289), which included the N-terminals of TGB1 and TGB3.

Event 3: A recombination event was also detected between AB197937 as the major parent and the Taiwan isolate (AY571289) as the minor parent. In this case a region (5468–5753 nt) of the Japanese isolate (AB197937) was replaced with the corresponding genome sequence of AY571289.

sequence alignment survey provided evidence for the occurrence of recombination between an Indian CymMV (AM055720) as the major parent, and a Korean type 2-CymMV (AF016914) as the minor parent. In this recombination event, a region (4973–6226 nt) of the AM055720 genome was replaced with the corresponding genome sequences of AF016914 (RDP *P* value=4.188 x 10^{-24}), which included the N-terminal of TGB and C-terminal of CP as can be seen in event 1 (figure 2). Occurrence of recombination was also observed between the Singapore CymMV (U62963) as the major parent, and the Taiwan CymMV (AY571289) as the minor parent. In this recombination event, a region (4337–5262 nt) of the Singapore CymMV (U62963), was replaced with the corresponding genome sequence of the Taiwan CymMV (AY571289), which included the N terminal of MP1 and N terminal of MP3 as can be seen in event 2 (figure 2). A recombination event was also detected between AB197937 as the major parent and the Taiwan CymMV (AY571289) as the minor parent. In this case, a region (5468–5753 nt) of the Japanese CymMV (AB197937) was replaced with the corresponding genome sequence of AY571289 as can be seen in event 3 (figure 2). All the above recombination events were supported by three separate recombination detection programs, viz. GENECONV, MAXIMUM CHI SQUARE and CHIMAERA.

Genome analysis of the Indian CymMV isolate reveals that various isolates of CymMV share good similarity among themselves and this information may be used to develop transgenic virus resistance plants and design molecular probes for the detection of other potexviruses. In recent years, many potexviruses have been identified and their genomes sequenced. This decade is likely to focus on identifying unique features of their genomes that may provide new insights into methods of control. In this era of functional genomics, studies may focus more on identifying host factors involved in susceptibility and resistance. As we learn more about the molecular basis for pathogenecity, perhaps alternative control methods will be developed. Since there are societal pressures to reduce the use of agrochemicals, more attention must be focused on developing novel control methods that farmers can use to reduce the effects of viral diseases and maintain their crop yields.

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