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## Molecular Characterization of a New Begomovirus Infecting Honeysuckle in Kobe, Japan

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A virus (HY12) isolated from honeysuckle (*Lonicera japonica* Thunb.) collected in Kobe, Hyogo prefecture, Japan, showing symptoms of yellow vein mosaic, was characterized. HY12 was transmitted to *Nicotiana benthamiana* by sap inoculation while the other honeysuckle isolates could not. The complete DNA- $\alpha$  and DNA- $\beta$  like molecules of the isolate were determined and found to comprise 2763 and 1262 nucleotides (nt), respectively, with a typical begomovirus genome organization. The DNA- $\beta$  molecule had the conserved ORF ( $\beta$  C1) and satellite conserved region (SCR) typical of the *Geminiviridae* family containing a 5'-TAATATTAC-3' motif in the stem loop and the adenine-(A) rich region. Additionally, its entire sequence was interrupted at intervals with sequences that were not viral and whose origin was unknown. A comparison of the DNA- $\alpha$  sequences revealed that HY12 shared 74% with four other honeysuckle begomoviruses but 88–89% with tobacco begomoviruses found in Japan. The isolate shared up to 92% of the IR sequences with the honeysuckle begomoviruses but only 60–88% with those of tobacco. Phylogenetic analysis of full length DNA- $\alpha$  component as well as its CP clustered HY12 with the tobacco begomoviruses. HY12 is an isolate of *Honeysuckle yellow vein mosaic virus*.

### INTRODUCTION

Geminiviruses are plant viruses characterized by their bisegmented (geminate) shape and size (30×20 nm) of their particles, a capsid protein of about 30 kDa and their propensity to infect phloem cells. Each geminate particle encapsidates a small (2.5–3.0 kb) circular single stranded (ss) DNA genome that replicates in the nuclei of host cells via a double stranded (ds) DNA intermediate (replicative form) through a rolling cycle mechanism (Saunders *et al.*, 1991). Based on the host range, genome organization and vector species, the *Geminiviridae* family is divided into four genera (Rybecki, 1994; Briddon and Markham, 1995; Padidam *et al.*, 1995; Fauquet *et al.*, 2000). *Begomovirus* comprises over 100 species (Fauquet and Stanley, 2003) and is the only genus that is transmitted by the whitefly *Bemisia tabaci* (Harrison and Robinson, 1999), infects only dicotyledonous plants and causes most of the economically important diseases of plant species. Members of this genus are mostly bipartite with two components known as DNA- $\alpha$  and DNA- $\beta$ . Until recently, monopartite begomoviruses were known to have only one DNA component that closely resembles DNA- $\alpha$  of bipartite begomoviruses. Some of the DNA- $\alpha$  like molecules have now been found to associate with yet another molecule referred to as

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DNA  $\beta$ : DNA  $\beta$  depends on the begomovirus for its proliferation and is responsible for accumulation of the helper virus to normal levels in infected plants, suggesting its involvement in either replication, systemic movement or the suppression of host defense mechanism (Sounders *et al.*, 2000).

In Japan, only one begomovirus, *Honeysuckle yellow vein mosaic virus* (HYVMV) has been reported infecting honeysuckle (*Lonicera japonica* Thunb.) while three begomoviruses, *Tobacco leaf curl Kochi virus* (TbLCKV-KK), *Tobacco leaf curl Japan virus* (TbLCJV), TbLCJV-[JP3], TbLCJV-[JP2] and *Tobacco leaf curl Yamaguchi virus* (TBLCYV) infect tomato (Ikegami *et al.*, 1987; Onuki, 2003). However, little information is available in the area of genomic recombination and evolution of the Japanese honeysuckle and tobacco begomoviruses. Moreover, it is still not clear whether begomoviruses infecting tobacco are the same ones infecting honeysuckle. To resolve this impasse, we collected samples in Kobe, Hyogo prefecture, Japan and analyzed them by PCR. Based on the results, we report here characterization of a new distinct begomovirus infecting honeysuckle in Kobe, Japan.

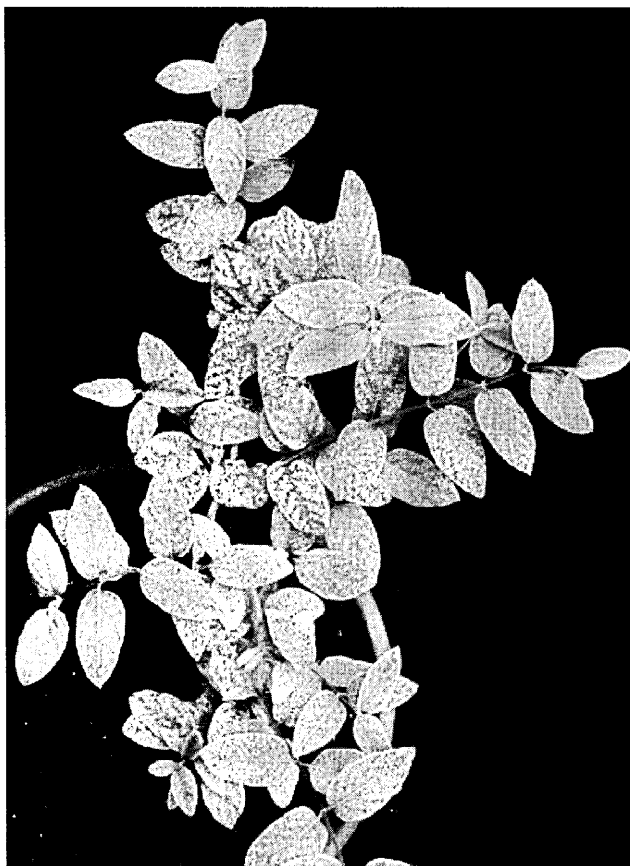
## MATERIALS AND METHODS

### Sample collection and sap inoculation

Honeysuckle samples (stem cuttings) showing symptoms of a viral disease (Fig. 1) were collected in Kobe, Hyogo prefecture, Japan in 2000 and labeled HY12. The cuttings were potted and maintained in an insect-free greenhouse kept at 24–27 °C. To determine infectivity of the virus isolate and symptoms induced in *Nicotiana benthamiana*, and *N. tabacum*, test plant seedlings at the 3- to 5-leaf stage were inoculated by sap obtained by homogenizing infected leaf samples (Table 1) in the standard inoculation buffer [0.05 M K/Na phosphate buffer, pH 7.5 containing 2% (w/v) polyvinylpyrrolidone (PVP), 0.2% (w/v) Na<sub>2</sub>SO<sub>3</sub>]. Leaf homogenates were rubbed on the upper 2–3 abaxial-dusted leaves of the test plants and kept in the greenhouse for symptom development. Virus isolates used for inoculation were HYVMV-HY12, -FK1 (Accession No. in the DNA data bases: AB178945), -OT2 (AB178948) and -SP1 (AB182261).

**Table 1.** Infectivity of Japanese honeysuckle geminivirus isolates on *N. benthamiana* and *N. tabacum* by sap inoculation.

Sample origin	Strain	Plants infected /inoculated	Virus detected in <i>N. benthamiana</i> / <i>tabacum</i>
Fukuoka	FK1	0/10	-/-
Kobe	HY12	6/10	HYVMV/-
Oita	OT2	0/10	-/-
Sapporo	SP1	0/10	-/-



**Fig. 1.** Begomovirus-infected honeysuckle plant collected in Kobe, Japan showing symptoms of yellow vein mosaic and mottle.

### **PCR-mediated amplification and cloning of HY12 DNA-A and DNA $\beta$**

Total DNA was extracted from young virus-infected honeysuckle leaves essentially as described by Dellaporta *et al.* (1983). Amplification of viral sequences was done using degenerate universal geminivirus primers designed by Briddon and Markham (1994). Sequence analysis of the PCR products obtained enabled us to design primers (Table 2) specific for HYVMV and tobacco leaf curl begomoviruses from Japan. For easy amplification and cloning, two primer pairs [HGVU148 // HGVL1560 amplifies 1500 bp (lower DNA-A fragment); REGEU1560 // REGEL148 amplifies 1450 bp (upper DNA-A fragment)] and one primer pair [CHIGu360A // CHIGL360A (amplifies complete DNA-A component)] were used. DNA  $\beta$  was amplified using universal primers (Briddon *et al.*, 2002). PCR amplification of both components proceeded as described previously (Were, 2001). PCR products of half- and full-length DNA-A as well as full length DNA  $\beta$  were purified, cloned into pGEM<sup>T</sup>- vector (promega) essentially as described by Sambrook *et*

**Table 2.** Primers used for amplification of honeysuckle geminivirus DNA–A fragments.

Primer	Sequence (5'–3')	Strand	Target
HGVU148	CGTCTGAACCTTCGACGGCCCGTATGTC	Virion	CP
HGVL1560	GCAACACCAAGTACGGGAAACCA	Complementary	AC1
REGEU1560	TGGTTTCCCGTACTTGGTGTT	Virion	AC1
REGEL148	GCTTGTGCCTTGGGCAATGGG	Complementary	CP
CHIGU360A	ACTTCGACRGGCCGTATGYC	Virion	CP
CHIGL360A	TCAGACGGCGMCGTRCCYTG	Complementary	CP

*al.* (1989). The cloned DNA fragments were sequenced by a commercial company (GreenGene BioTech. Seoul, Korea).

### Sequence determination and analysis

HY12 DNA–A sequence was phylogenetically compared to those of other honeysuckle, tobacco, *Eupatorium* and tomato using the multiple sequence alignment application of DNAMAN version 4.0 (Lynnon Biosoft, Quebec, Canada) full optimal sequence alignments and neighbor-joining method options of Saitou and Nei (1987) with 1000 bootstrap replications. Percent DNA–A nucleotide (nt) and ORF amino acid (aa) sequence identities between virus isolates were calculated using the distance between all pairs of sequences in the multiple alignments. The begomovirus sequences used for comparison and their database accession numbers were as follows: *Honeysuckle yellow vein mosaic virus* (HYVMV, AB020781; HYVMV–FK1, AB17845; HYVMV–OT1, AB178947; HYVMV–OT2, AB178948), *Tobacco leaf curl Yamaguchi virus* (TbLCYV, AB079765), *Tobacco leaf curl Japan virus* (TbLCJV, AB028604; TbLCJV–JP2, AB055008; TbLCJV–JP3 AB079689), *Eupatorium yellow vein virus* (EpYVV, AB007990; EpYVV–Yamaguchi, AB079766; EpYVV–MNS2, AJ438936), *Tomato leaf curl Taiwan virus* (ToLCTV, U88692), *Ageratum yellow vein China virus*–[Hn2] (AYVCNV–Hn2, AJ495813), *Ageratum yellow vein Sri Lanka virus* (AYVSLV, AF314144), *Malvastrum yellow vein virus*–[Y47] (MYVV–Y47, AJ457824), *Tomato yellow leaf curl China virus*–Tb[Y5] (TYLCCNV–Tb[Y5] AJ319674), *Tobacco leaf curl Yunnan virus*–[Y3] (TbLCYNV–Y3, AF240674), *Tobacco leaf curl Zimbabwe virus* (TbLCZV, AF350330), *Tomato yellow leaf curl Thailand virus* (TYLCTHV, AY514630) and *East African cassava mosaic Zanzibar virus* (EACMZV, AF422174). Recombination analysis was done using a recombination detection software, RDP version 2.0.

## RESULTS

### Symptoms and sap inoculation

Symptoms of a begomovirus-infected honeysuckle plant collected in Kobe, Hyogo prefecture, are shown in (Fig. 1). They consist of yellowing of veins, at times mosaic and/or yellow mottle on the leaves resulting in honeysuckle yellow vein mosaic disease. Results of sap inoculation are presented in Table 1. HY12 was found to be mechanically transmissible to *N. benthamiana* while FK1, OT2 and SP1 were not. *N. benthamiana* infected with the HY12 developed mild symptoms (data not shown), yet were positive for

systemic viral infection on analysis by PCR. Attempts to transmit the viruses to *N. tabacum* by sap inoculation were not successful.

### DNA–A sequence determination and analyses

The complete DNA–A nucleotide sequence of HY12 was determined. It comprised 2763 nucleotides (AB178948) and a genome organization similar to that of other monopartite begomoviruses with two open reading frames ORFs [AV1 (CP) and AV2] in the virion sense strand and four in the complementary sense strand (AC1, AC2, AC3 and AC4). The intergenic region (IR) separated the virion and complementary sense genes and contained a putative stem loop structure with the conserved nonanucleotide sequence 5'–TAATATTAC–3' that is characteristic of the *Geminiviridae* in the loop. The TATA boxes were identified in the IR sequence at nucleotides, 259–262.

Alignment of the retrieved complete DNA–A nt sequences with those from the DNA data bases revealed a high degree of variability ranging from 42% to 89% depending on geographical location and or different hosts/diseases. HY12 showed a higher degree of sequence similarity with other begomoviruses found in Japan and rest of Asia than those found in Africa. For instance it shared 74% with HYVMV, 73% with HYVMV–OT1 and HYVMV–FK1, 89% with 74–89 with tobacco leaf curl begomoviruses from Japan, 46% with AYVCNV–[Hn2] but 42 and 44% with TbLCZV and EACMZV, respectively (Table 3). Phylogenetic analysis of the complete DNA–A components divided the Japanese begomoviruses into two major groups, the Japanese honeysuckle infecting begomoviruses (JHBVs) group and the Japanese *Eupatorium* infecting begomoviruses (JEpBVs). The

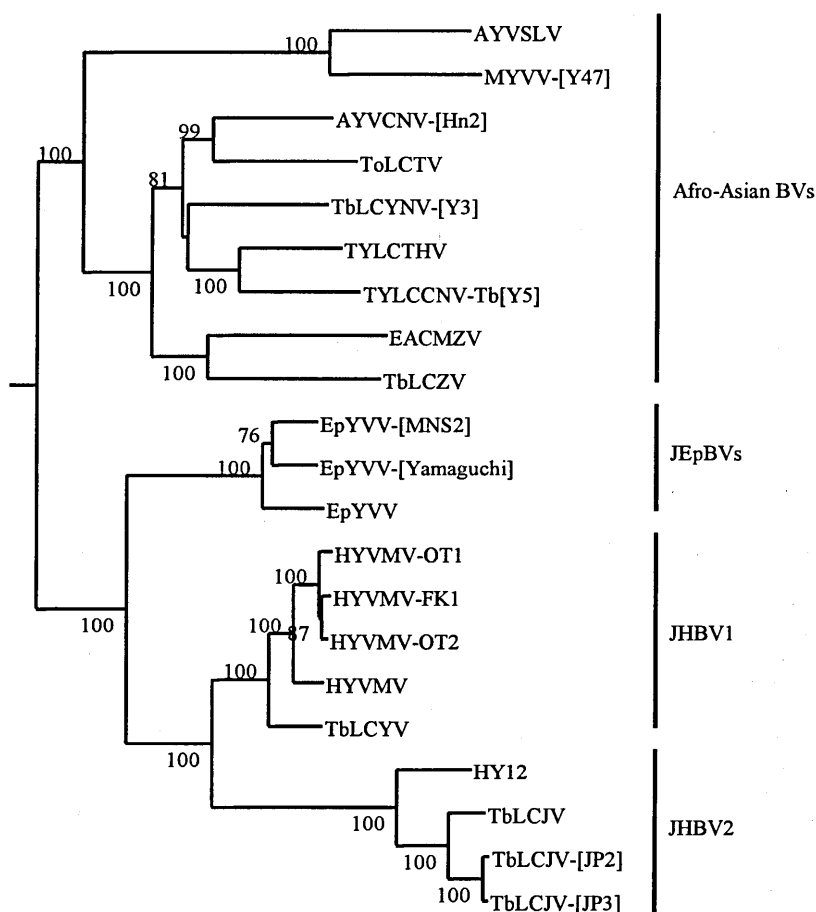
**Table 3.** Percentage nucleotide or amino acid sequence identities between HY12 and 20 other begomoviruses from Asia and Africa.

Virus	DNA <sup>a</sup>	IR <sup>a</sup>	V1 <sup>a</sup>	V2 <sup>a</sup>	C1 <sup>a</sup>	C2 <sup>a</sup>	C3 <sup>a</sup>	C4 <sup>a</sup>
EpYVV	60	48	87	88	85	68	77	80
EpYVV–[MNS2]	59	52	87	85	86	70	76	79
EpYVV–[Yamaguchi]	60	51	87	84	86	70	78	77
HYVMV–OT1	73	92	88	91	92	82	82	86
HYVMV–FK1	73	92	86	91	93	83	83	84
HYVMV–OT2	74	92	88	91	93	82	84	84
HYVMV	74	62	88	90	93	84	84	85
TbLCYV	74	64	88	91	92	91	90	78
TbLCJV–[JP2]	89	87	91	85	94	81	81	89
TbLCJV–[JP3]	89	88	92	84	95	81	81	91
TbLCJV	88	64	91	91	95	81	81	–
AYVCNV–[Hn2]	46	45	47	57	58	60	69	69
ToLCTV	47	48	44	75	58	58	68	74
TLCYNV–[Y3]	45	49	47	60	51	57	66	59
TYLCTHV	45	47	45	64	58	55	66	70
TYLCCNV–Tb[Y5]	44	47	42	59	55	58	69	73
AYVSLV	41	43	42	63	58	53	64	73
MYVV–[Y47]	41	48	44	55	55	55	64	69
EACMZV	44	45	41	53	50	53	66	–
TbLCZV	42	53	41	65	51	52	66	–

<sup>a</sup> Nucleotide sequence identity. <sup>a</sup> Amino acid sequence identity.

former was further subdivided into two subgroups namely Japanese honeysuckle infecting begomoviruses subgroup I (JHBV1) and the Japanese tomato infecting tobacco begomoviruses, herein referred to as Japanese honeysuckle infecting begomoviruses subgroup II (JHBV2). The naming of the subgroups was based on the fact that all tobacco and tomato infecting begomoviruses in Japan pass the winter in honeysuckle. The JHBV1 comprise HYVMV-OT1, HYVMV-FK1, HYVMV-OT2 and TbLCYV while JHBV2 comprised HY12, TbLCJV-[JP2], TbLCJV-[JP3] and TbLCJV (Fig. 2). The begomoviruses from China, Taiwan and Thailand were clustered together and so were those from Africa. Hosts also played a big role in the separation of the viruses, *Eupatorium* geminiviruses were clearly separated from those of honey suckle and tobacco (Fig. 2).

A detailed analysis of the aligned CP nt as well as the deduced amino acid (aa)

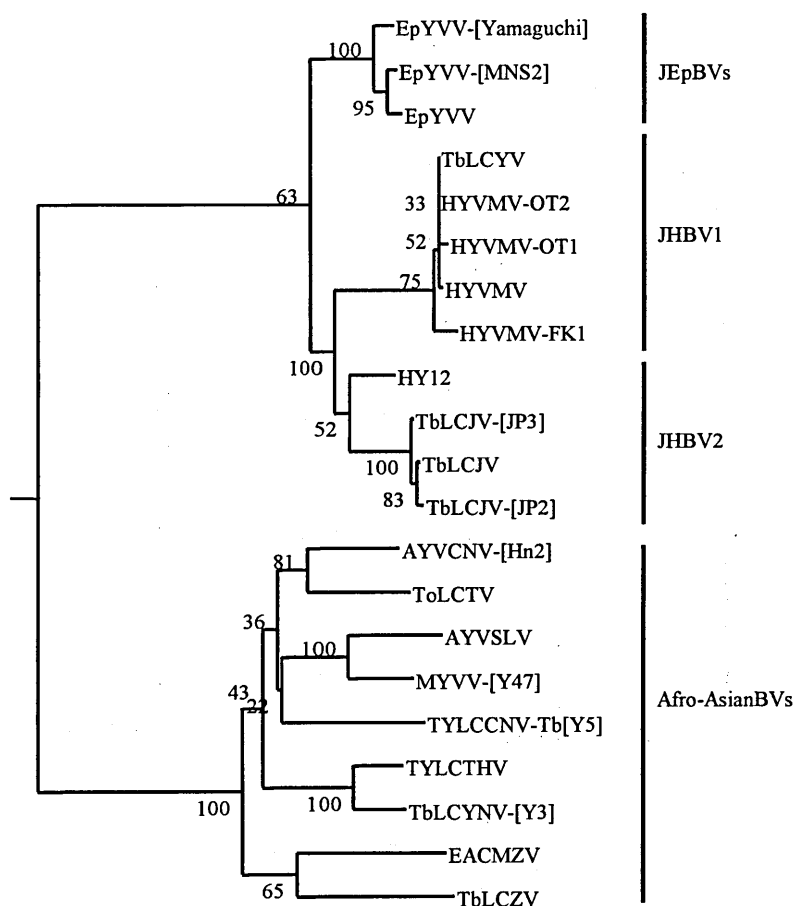


**Fig. 2.** Phylogenetic tree generated from alignment of full-length DNA-A nucleotide sequences of HY12 and 20 other honeysuckle, tobacco, *Eupatorium* and other Asian, and African begomoviruses.

sequences revealed that the 5' terminal and the core region was the most conserved area of the CP in all begomoviruses compared. Furthermore, the HY12 shared a high degree of sequence identity (87–92%) with the Japanese begomoviruses and less than 50% with those found in rest of Asia and Africa. Phylogenetic analysis of CP aa sequences generated a tree structure in which HY12 was clustered alone but more related to JHBV2 than to JHBV1 (Fig. 3).

When the IR nt sequences were compared, HY12 shared a high percentage (92%) with HYVMV-OT1, HYVMV-OT2 but HYVMV-FK1 isolates and 64–87% with JHBV2. Other begomoviruses from rest of Asia and Africa compared poorly with HY12. The IR had the grossest variation of the whole DNA-A component.

A comparison of the C1 sequences revealed that HY12 shared a higher degree (92–95%) of sequence identity with JHBV1 and JHBV2 than with those found in the rest

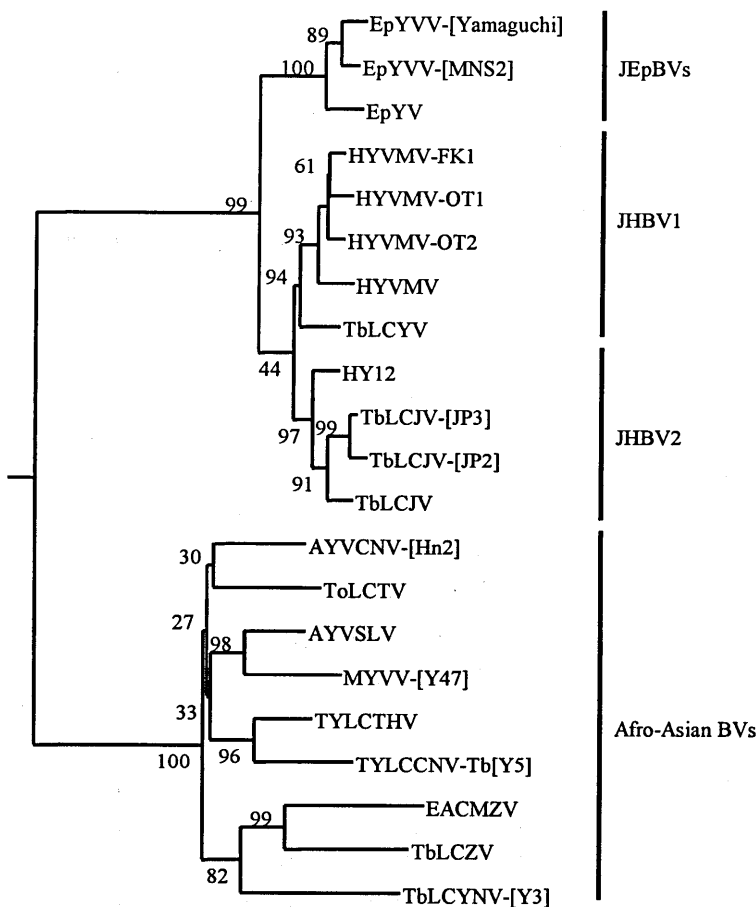


**Fig. 3.** Phylogenetic tree generated from CP amino acid sequence alignments of HY12 and 20 other honeysuckle, tobacco, *Eupatorium* and other Asian and African begomoviruses.



of Asia and Africa. Generally, the C1 sequences of Japanese begomoviruses were more related compared to those from the rest of Asia and/or Africa. Phylogenetic analysis of the C1 aa sequences revealed a tree structure in which HY12 was clustered with JHBV2 (Fig. 4).

When individual encoded proteins were compared, the highest amino acid sequence similarity was between HY12 and TbLCJV with 91% CP, 91% V2, 95% C1, 81% C2 and C3. Generally, among the Japanese begomoviruses the CP was the most conserved sequence of the genome followed by C1 and C4 in that order. It is interesting to note that the CP of HY12 was most related to JHBV2 than to JHBV1 (Table 3). The contrast is true for the IR, which was in addition found to be most diverse part of the begomovirus genome.



**Fig. 4.** Phylogenetic tree generated from C1 amino acid sequence alignments of HY12 and 20 other honeysuckle, tobacco, *Eupatorium* and other Asian and African begomoviruses.

### DNA $\beta$ sequence determination and analysis

The complete satellite DNA of HY12 was determined and found to be a recombinant/deletion mutant with 1262 nucleotides (AB182263). The molecule had a satellite conserved region (SCR), typical of the *Geminiviridae* family containing a 5'-TAATATTAC-3' motif in the stem loop, the C1 gene and adenine- (A) rich region. The molecule was closely related (80–83%) to that of honeysuckle yellow vein mosaic disease (HYVMDA, AJ316140) except for the following additions, deletions and recombinations with unknown donors which were certainly not viral in origin:

HY12  $\beta$  : 1–89. . . 152–216. . . 254–271. . . 612–721. . . 824–867. . . 987–1078. . .  
1201–1262.  
HYVMDA : 1–90. . . 156–200. . . 239–555. . . 596–706. . . 810–853. . . 97–1066. . .  
1283–1344.

The numbers (range) show areas where HY12  $\beta$  has high sequence homology with HYVMDA while dotted lines represent areas where HY12  $\beta$  has unrelated sequences from unknown source.

### Recombination in the DNA-A of HY12 sequence

HY12 had significant as well as several insignificant recombination events with a major one (762–1853 nt) starting from the core region of the CP spanning the entire C3, C2 and part of C1 (C-terminal). In this event HY12 sequence formed part of two sequences, TbLCJV-[JP3] and TbLCYV appearing as major and minor parents, respectively. Two events, one (1871–2316 nt) within the C1 but covering half of C4 and the other, (2505–14) starting at the N-terminal of C1 and covering almost the entire IR were also identified. Both events involved TbLCJV as a minor donor while the major parent was unknown.

## DISCUSSION

The results of this investigation in which HY12 was sequenced and compared with other begomoviruses from the databases demonstrate that the isolate is a new distinct previously unrecognized begomovirus. The sap inoculation experiment revealed that HY12 could be transmitted to *N. benthamiana* and that it was not possible to transmit the isolate to tobacco using this means. This coupled with the fact that HY12 induced different symptoms not similar to those induced by HYVMV-FK1, HYVMV-OT1 or HYVMV-OT2 in honeysuckle, implies that HY12 is somehow, somewhere different from the other three isolates. Apparently, this could be due to DNA  $\beta$ , which was not retrieved from the other three isolates despite repeated attempts to do so. DNA  $\beta$  has been shown to modulate symptoms with a conserved coding region referred to as  $\beta$  C1. Whether or not this is true remains to be seen.

Comparison of the nucleotide sequences of HY12 with those from the databases, explored their relationship in great detail. It was shown that HY12 shares a high degree of DNA-A nt as well as CP aa sequence homology with JHBV2 but only a small percentage with JHBV1. In contrast, HY12 shared a high degree of IR sequence homology (92%) with JHBV1 but only 64–88% with JHBV2. This can be attributed to a recombination event

detected in and covering the CP, C3, C2 and part of C1 and involving TbLCJV-[JP3] and TbLCYV as major and minor parents, respectively. However, HY12 CP varied significantly from those found outside Japan. When a complete DNA-A of HY12 was carefully examined different blocks of the sequence had different affinities indicating that genetic recombination had occurred. For instance, the CP of TbLCJV-[JP2] and TbLVJV-[JP3] shared >90% but the percentage of their IR sequences shared was very low. It has been shown that IR sequences of begomoviruses give the most sensitive indication of the extent of viral similarity and difference (Padidam *et al.*, 1995) because it contains the presumed Rep binding site (Harrison and Robinson, 1999).

Padidam *et al.* (1995), observed that strains of anyone virus should share >90% of their DNA-A sequence, later, Mayo and Pringle (1998) stated that, they should share >90% of their CP sequences in addition. However, the multiplicity of variants due to recombination and other factors associated thereto, increasingly complicate and consequently harden the decision on whether two virus isolates should be considered strains or distinct viruses (Zhou *et al.*, 2001). In conclusion, sequence analyses results have clearly demonstrated that HY12 is a new previously unrecognized begomovirus, a strain of HYVMV.

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