Bell pepper endornavirus: molecular and biological properties, and occurrence in the genus Capsicum

Ryo Okada,1 Eri Kiyota,1 Sead Sabanadzovic,2 Hiromitsu Moriyama,1 Toshiyuki Fukuhara,1 Prasenjit Saha,3 Marilyn J. Roossinck,3 Ake Severin4 and Rodrigo A. Valverde5

1Laboratory of Molecular and Cellular Biology, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Tokyo 183-8509, Japan
2Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology, Mississippi State University, Mississippi State, MS 39762, USA
3Plant Biology Division, The Samuel Roberts Noble Foundation, Ardmore, OK 73402, USA
4Laboratoire de Physiologie Vegetale, Universite de Cocody-Abidjan, UFR Biosciences, 22 BP, 582 Abidjan 22, Côte d'Ivoire
5Department of Plant Pathology & Crop Physiology, Louisiana State University Agricultural Center, Baton Rouge, LA 70803, USA

Bell peppers (Capsicum annuum) harbour a large dsRNA virus. The linear genome (14.7 kbp) of two isolates from Japanese and USA bell pepper cultivars were completely sequenced and compared. They shared extensive sequence identity and contained a single, long ORF encoding a 4815 aa protein. This polyprotein contained conserved motifs of putative viral methyltransferase (MTR), helicase 1 (Hel-1), UDP-glycosyltransferase and RNA-dependent RNA polymerase. This unique arrangement of conserved domains has not been reported in any of the known endornaviruses. Hence this virus, for which the name Bell pepper endornavirus (BPEV) is proposed, is a distinct species in the genus Endornavirus (family Endornaviridae). The BPEV-encoded polyprotein contains a cysteine-rich region between the MTR and Hel-1 domains, with conserved CXCC motifs shared among several endornaviruses, suggesting an additional functional domain. In agreement with general endornavirus features, BPEV contains a nick in the positive-strand RNA molecule. The virus was detected in all bell pepper cultivars tested and transmitted through seed but not by graft inoculations. Analysis of dsRNA patterns and RT-PCR using degenerate primers revealed putative variants of BPEV, or closely related species, infecting other C. annuum genotypes and three other Capsicum species (C. baccatum, C. chinense and C. frutescens).

INTRODUCTION

Endornaviruses have a single linear dsRNA genome (9.8–17.6 kbp), infect plants, fungi and oomycetes and are transmitted at a high rate through seeds and spores (Fukuhara et al., 2006; Gibbs et al., 2000). They infect economically important crops, such as rice (Moriyama et al., 1995), French bean (Wakarchuk & Hamilton, 1990), broad bean (Pfeiffer, 1998), barley (Zabalgogeazcoa & Gildow, 1992), cucurbits (Goutts, 2005) and pepper (Valverde & Gutierrez, 2007), as well as some plant-pathogenic fungi and the oomycete Phytophthora (Hacker et al., 2005). The presence of endornaviruses in phenotypically normal plants is a unique feature of particular genotypes of these crops. The full sequence of the genome of approved or putative Endornavirus species from cultivated rice [Oryza sativa endornavirus (OSV)] (Moriyama et al., 1995), wild rice [Oryza nivipogon endornavirus (ORV)] (Moriyama et al., 1999), broad bean [Vicia faba endornavirus (VFV)] (Pfeiffer, 1998), Phytophthora spp. [Phytophthora endornavirus 1 (PEV-1)] (Hacker et al., 2005), Helicobasidium mompa [Helicobasidium mompa endornavirus 1 (HmEV-1)] (Osaki et al., 2006), Gremmeniella abietina [Gremmeniella abietina type B RNA virus XL (GaBRV-XL)] (Tuomivirta et al., 2009) and Tuber aestivum [Tuber aestivum endornavirus (TaEV)] (Stielow et al., 2011) have

The GenBank/EMBL/DDBJ accession numbers for the bell pepper endornavirus sequences reported in this paper are: AB597230, JNO19858, JNO19859, JNO19860, JNO19861, JNO19862 and JNO19863.

Three supplementary figures are available with the online version of this paper.
been reported. Moreover, endornavirus-like dsRNAs have been isolated from bottle gourd (*Lagenaria siceraria*), Malabar spinach (*Basella alba*) and eel grass (*Zostera marina*) (Fukuhara *et al.*, 2006).

Endornaviruses encode a single polypeptide, which is presumed to be processed by virus-encoded proteinases. Genomes of all completely sequenced endornaviruses contain conserved motifs of a viral RNA-dependent RNA polymerase (RdRp, pfam00978), similar to the alpha-like virus superfamily of positive-stranded RNA viruses (Gibbs *et al.*, 2000).

Peppers (*Capsicum* spp.) are perennial plants native to South America and today they are cultivated throughout the world for their nutritional and medicinal value and for use as a cooking condiment (DeWitt & Bosland, 1996). A dsRNA was reported from tissue extracts of apparently healthy bell pepper (*Capsicum annuum*) cultivars (Valverde *et al.*, 1990b). Partial sequence information suggested that the dsRNA constitutes the genome of an endornavirus (Valverde & Gutierrez, 2007).

In this investigation, we report the full nucleotide sequence, genome organization and some biological properties of the putative endornavirus, tentatively designated Bell pepper endornavirus (BPEV), isolated from Yolo Wonder (YW) bell pepper reported by Valverde & Gutierrez (2007) and an isolate from Kyousuzu (KS) bell pepper from Japan. Furthermore, we analysed a number of additional pepper cultivars for endornavirus-sized dsRNAs and we report its occurrence in other *C. annuum* genotypes and three other *Capsicum* species. Partial sequence analysis of the RdRp(s) from additional pepper isolates suggests that its evolution is congruent with that of its host.

## RESULTS AND DISCUSSION

### Screening *Capsicum* spp. and cultivars for BPEV

BPEV dsRNA was detected in all seven bell pepper cultivars from Japan. Testing for BPEV dsRNA in *Capsicum* genotypes from the USA, including bell peppers and other horticultural types, resulted in 40 additional positive genotypes, shown in Table 1. BPEV was detected in all tested bell peppers and *Capsicum baccatum* lines. Some *C. annuum*, *Capsicum chinense* and *Capsicum frutescens* genotypes were BPEV-positive while others were negative (Tables 1 and 2). A sample of PAGE results is shown in Fig. 1. Several dsRNAs with a lower molecular mass than BPEV were associated with some *Capsicum* genotypes. The majority of these dsRNAs were determined to constitute the genome of plant partitiviruses (Sabanadzovic & Valverde, 2011). BPEV-KS dsRNA was detected in pollen, callus and cultured cells and the relative concentration was similar to that of the dsRNA in leaf tissue (data not shown), unlike OSV and ORV that were reported to be tenfold more concentrated in pollen than in the leaf tissue of rice (Moriyama *et al.*, 1999). BPEV appears to be common among domesticated *Capsicum* species, particularly in cultivated peppers (*C. annuum*, *C. frutescens* and *C. chinense*) (Valverde & Fontenot, 1991) and in *C. baccatum*. These closely related species share a mutual ancestral gene pool (Moscione *et al.*, 2007). One can speculate that the gene pool from which the domesticated peppers originated included pepper genotypes that were infected with BPEV. Nevertheless the origin of BPEV in pepper remains unclear. Two lines of the most primitive *Capsicum* species tested in this study (*Capsicum chacoense*) were free of BPEV. In addition, five accessions of *C. annuum* var. *glabriusculum*, also known as chiltepepin, were negative. This pepper is eaten by indigenous peoples in Mexico, and is thought by some to be an ancestor of many cultivated peppers.

As in previous investigations, all tested bell pepper contained BPEV (Valverde & Fontenot, 1991; Valverde *et al.*, 1990b). Bell pepper cultivars have a narrow gene pool, and, while making crosses to develop new cultivars, breeders may have inadvertently introduced and spread BPEV among bell peppers. Since Japanese bell pepper breeders have used *Capsicum* germ plasm from the Americas to develop Japanese cultivars, it was not surprising to find that bell pepper cultivars from Japan were also infected with BPEV.

### Occurrence of BPEV in bell pepper seedlings

The level of vertical transmission of BPEV is higher through the ovule than through pollen, thus it is very high when both parents are infected (Valverde & Gutierrez, 2007). Screening of seedlings from two bell pepper cultivars, Yolo Wonder (YW) and Marengo (MR), with both parents being BPEV-infected, resulted in 50 of 50 and 136 of 137 positive plants, respectively. These results confirm that high rates of vertical transmission are obtained when both parents are virus carriers. Screening of seedlings from three other bell pepper cultivars, originating from seeds purchased at a local store, resulted in different percentages of BPEV infections. A high proportion of seedling of KS (47 of 49) and California Wonder (CW, 36 of 36) were infected by BPEV, indicating that both parents contained the virus. In contrast, only 8 of 28 seedlings of *Capsicum annuum* L. ‘Kyonami’ (KY) tested BPEV positive, suggesting that the virus was not present in one of the parents.

In agreement with the general properties of endornaviruses, the BPEV-free and BPEV-infected lines of MR pepper were phenotypically undistinguishable, revealing a lack of visible effect on the host. Furthermore, in preliminary experiments, co-infections of BPEV with four acute ssRNA viruses [pepper mild mottle virus, potato virus Y (PVY), cucumber mosaic virus and tomato spotted wilt virus] in MR pepper did not have a visible effect on the symptoms caused by these viruses when compared with single infections of the BPEV-free MR line by any of these viruses (not shown).

http://vir.sgmjournals.org
Attempts to transmit BPEV

None of the BPEV-free MR scions grafted onto BPEV-infected rootstocks tested positive for BPEV. ELISA detection of PVY in the scions confirmed successful grafts. The presence of PVY in the rootstocks and subsequent systemic infection did not aid the movement of BPEV from the rootstocks into the scions. The failure to transmit BPEV by grafting supports the idea that endornaviruses lack systemic movement. Infection of the BPEV-infected

<table>
<thead>
<tr>
<th>Capsicum species</th>
<th>Type</th>
<th>Cultivar or PI line</th>
<th>Origin</th>
<th>GE</th>
<th>RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. annuum</em></td>
<td>Bell</td>
<td>California Wonder</td>
<td>USA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>C. annuum</em></td>
<td>Bell</td>
<td>Yolo Wonder</td>
<td>USA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>C. annuum</em></td>
<td>Bell</td>
<td>Kyousuzu</td>
<td>Japan</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>C. annuum</em></td>
<td>Bell</td>
<td>Kyounami</td>
<td>Japan</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>C. annuum</em></td>
<td>Bell</td>
<td>Kyoumidori</td>
<td>Japan</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>C. annuum</em></td>
<td>Bell</td>
<td>Ace</td>
<td>Japan</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>C. annuum</em></td>
<td>Bell</td>
<td>Suigyokunigou</td>
<td>Japan</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>C. annuum</em></td>
<td>Bell</td>
<td>High Green</td>
<td>Japan</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>C. annuum</em></td>
<td>Bell</td>
<td>Jumbo Colour</td>
<td>Japan</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>C. annuum</em></td>
<td>Bell</td>
<td>Marengo</td>
<td>USA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>C. annuum</em></td>
<td>Bell</td>
<td>Avelar</td>
<td>USA</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td><em>C. annuum</em></td>
<td>Bell</td>
<td>Casca Dura</td>
<td>Brazil</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td><em>C. annuum</em></td>
<td>Bell</td>
<td>King Arthur</td>
<td>USA</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td><em>C. annuum</em></td>
<td>Bell</td>
<td>VR-4</td>
<td>USA</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td><em>C. annuum</em></td>
<td>Bell</td>
<td>Magda</td>
<td>USA</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td><em>C. annuum</em></td>
<td>Bell</td>
<td>Bonnie’s Green Bell,</td>
<td>USA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>C. annuum</em></td>
<td>Bell</td>
<td>Red Bell</td>
<td>USA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>C. annuum</em></td>
<td>Bell</td>
<td>Chocolate Beauty Sweet</td>
<td>USA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>C. annuum</em></td>
<td>Bell</td>
<td>Pimento Sweet</td>
<td>USA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>C. annuum</em></td>
<td>Cayenne</td>
<td>Cayenne Long Red Thick</td>
<td>USA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>C. annuum</em></td>
<td>Cayenne</td>
<td>Super Cayenne</td>
<td>USA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>C. frutescens</em></td>
<td>Tabasco</td>
<td>Greenleaf</td>
<td>USA</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td><em>C. frutescens</em></td>
<td>Tabasco</td>
<td>LSU</td>
<td>USA</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td><em>C. frutescens</em></td>
<td>Tabasco</td>
<td>PI 159239</td>
<td>USA</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td><em>C. frutescens</em></td>
<td>Tabasco</td>
<td>PI 193470</td>
<td>Ethiopia</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td><em>C. baccatum</em></td>
<td>Aji</td>
<td>Monk’s Hat</td>
<td>USA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>C. baccatum</em></td>
<td>Aji</td>
<td>PI 238061</td>
<td>Bolivia</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td><em>C. baccatum</em></td>
<td>Aji</td>
<td>PI 633752</td>
<td>Paraguay</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td><em>C. baccatum</em></td>
<td>Aji</td>
<td>PI 260549</td>
<td>Peru</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td><em>C. baccatum</em></td>
<td>Aji</td>
<td>PI 215699</td>
<td>Peru</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td><em>C. baccatum</em></td>
<td>Aji</td>
<td>PI 260590</td>
<td>Peru</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td><em>C. baccatum</em></td>
<td>Aji</td>
<td>PI 260543</td>
<td>Brazil</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td><em>C. baccatum</em></td>
<td>Aji</td>
<td>PI 441589</td>
<td>Brazil</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td><em>C. baccatum</em></td>
<td>Aji</td>
<td>PI 257135</td>
<td>Ecuador</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td><em>C. baccatum</em></td>
<td>Aji</td>
<td>PI 337524</td>
<td>USA</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td><em>C. baccatum</em></td>
<td>Aji</td>
<td>PI 337522</td>
<td>Argentina</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td><em>C. baccatum</em></td>
<td>Aji</td>
<td>C00754 (AVRDC)</td>
<td>Argentina</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td><em>C. baccatum</em></td>
<td>Aji</td>
<td>C01218 (AVRDC)</td>
<td>Egypt</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td><em>C. baccatum</em></td>
<td>Aji</td>
<td>C01527 (AVRDC)</td>
<td>USA</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td><em>C. baccatum</em></td>
<td>Aji</td>
<td>C01300 (AVRDC)</td>
<td>Germany</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td><em>C. chinense</em></td>
<td>Habanero</td>
<td>PI 159236</td>
<td>USA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>C. chinense</em></td>
<td>Habanero</td>
<td>PI 315008</td>
<td>Peru</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td><em>C. chinense</em></td>
<td>Habanero</td>
<td>PI 315023</td>
<td>Peru</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>C. chinense</em></td>
<td>Habanero</td>
<td>PI 315024</td>
<td>Peru</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td><em>C. chinense</em></td>
<td>Habanero</td>
<td>PI 273426</td>
<td>USA</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td><em>C. chinense</em></td>
<td>Habanero</td>
<td>C00943 (AVRDC)</td>
<td>Peru</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>C. chinense</em></td>
<td>Habanero</td>
<td>C00949 (AVRDC)</td>
<td>England</td>
<td>+</td>
<td>NT</td>
</tr>
</tbody>
</table>

Table 1. *Capsicum* spp. and cultivars that tested positive for bell pepper endornavirus by RT-PCR and/or agarose gel electrophoresis (GE) analyses

+, Positive; NT, not tested; LSU, Louisiana State University; AVRDC, Asian Vegetable Research and Development Center.
rootstock by PVY did not aid transmission. Similar results were reported for a partitivirus infecting Jalapeño M pepper (Valverde & Gutierrez, 2008).

### Nucleotide sequence analysis of BPEV-YW and BPEV-KS

The complete sequences of BPEV-KS (14,727 nt) and BPEV-YW (14,728 nt) were determined. The two genomes were very similar, except for the presence of a guanine at the 5' end in the case of BPEV-YW. They shared 88.2% identical nucleotide sequences in the whole genome, with highly conserved 5' and 3' UTRs, and only single nucleotide differences in each of the UTRs, which underscores their functional importance in virus replication.

A single ORF was found on the plus strand of both BPEV-KS and BPEV-YW, starting at nucleotide 225 in the case of BPEV-KS (nt 226 in the case of BPEV-YW) and ending at nucleotide 14,670, which could encode a polyprotein of 4815 aa with an estimated molecular mass of 545 kDa (Fig. 2a). The genome size and protein-encoding strategy are typical of endornaviruses. The putative protein products of BPEV-KS and BPEV-YW shared 92% identical amino acid residues (96% similarity). Nevertheless,

### Table 2. Capsicum spp. and cultivars that tested negative for bell pepper endornavirus by PAGE analyses

<table>
<thead>
<tr>
<th>Capsicum species</th>
<th>Type</th>
<th>Cultivar or PI line</th>
<th>Country of origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. annuum</td>
<td>Cayenne</td>
<td>Thai Cluster</td>
<td>Thailand</td>
</tr>
<tr>
<td>C. annuum</td>
<td>Unknown</td>
<td>Pico de Pájaro</td>
<td>Honduras</td>
</tr>
<tr>
<td>C. annuum</td>
<td>Unknown</td>
<td>MI-2</td>
<td>Sri Lanka</td>
</tr>
<tr>
<td>C. annuum</td>
<td>Serrano</td>
<td>Criollo de Morelos</td>
<td>Mexico</td>
</tr>
<tr>
<td>C. annuum</td>
<td>Jalapeno</td>
<td>Jaloro</td>
<td>USA</td>
</tr>
<tr>
<td>C. annuum</td>
<td>Unknown</td>
<td>PBC-535</td>
<td>Taiwan</td>
</tr>
<tr>
<td>C. annuum</td>
<td>Yellow wax</td>
<td>Sweet Banana</td>
<td>USA</td>
</tr>
<tr>
<td>C. annuum</td>
<td>Yellow wax</td>
<td>Rio Grande Gold</td>
<td>USA</td>
</tr>
<tr>
<td>C. annuum</td>
<td>Serrano</td>
<td>Hidalgo</td>
<td>USA</td>
</tr>
<tr>
<td>C. annuum</td>
<td>Cayenne</td>
<td>Thai Cluster</td>
<td>Thailand</td>
</tr>
<tr>
<td>C. annuum var. glabriusculum</td>
<td>Chiltepin</td>
<td>PI 224405</td>
<td>Mexico</td>
</tr>
<tr>
<td>C. annuum var. glabriusculum</td>
<td>Chiltepin</td>
<td>PI 310488</td>
<td>Mexico</td>
</tr>
<tr>
<td>C. annuum var. glabriusculum</td>
<td>Chiltepin</td>
<td>PI 511887</td>
<td>Mexico</td>
</tr>
<tr>
<td>C. annuum var. glabriusculum</td>
<td>Chiltepin</td>
<td>PI 631135</td>
<td>Guatemala</td>
</tr>
<tr>
<td>C. annuum var. glabriusculum</td>
<td>Chiltepin</td>
<td>PI 632932</td>
<td>Guatemala</td>
</tr>
<tr>
<td>C. chacoense</td>
<td>Unknown</td>
<td>PI 260429</td>
<td>Argentina</td>
</tr>
<tr>
<td>C. chacoense</td>
<td>Unknown</td>
<td>PI 260435</td>
<td>Bolivia</td>
</tr>
<tr>
<td>C. chinense</td>
<td>Habanero</td>
<td>PI 152225</td>
<td>USA</td>
</tr>
<tr>
<td>C. frutescens</td>
<td>Tabasco</td>
<td>PI 368084</td>
<td>Malaysia</td>
</tr>
<tr>
<td>C. frutescens</td>
<td>Tabasco</td>
<td>PI 193470</td>
<td>Ethiopia</td>
</tr>
<tr>
<td>C. frutescens</td>
<td>Tabasco</td>
<td>PI 439502</td>
<td>Costa Rica</td>
</tr>
<tr>
<td>C. frutescens</td>
<td>Tabasco</td>
<td>PI 441650</td>
<td>Brazil</td>
</tr>
<tr>
<td>C. frutescens</td>
<td>Tabasco</td>
<td>PI 593924</td>
<td>Ecuador</td>
</tr>
<tr>
<td>C. pubescens</td>
<td>Rocoto</td>
<td>C00640</td>
<td>Guatemala</td>
</tr>
<tr>
<td>C. pubescens</td>
<td>Rocoto</td>
<td>C00778</td>
<td>Guatemala</td>
</tr>
<tr>
<td>C. pubescens</td>
<td>Rocoto</td>
<td>PI 593639</td>
<td>Guatemala</td>
</tr>
<tr>
<td>C. pubescens</td>
<td>Rocoto</td>
<td>PI 593630</td>
<td>Ecuador</td>
</tr>
<tr>
<td>C. pubescens</td>
<td>Rocoto</td>
<td>Grif 1614</td>
<td>Mexico</td>
</tr>
</tbody>
</table>

**Fig. 1.** Polyacrylamide gel electrophoresis (5% acrylamide) of bell pepper endornavirus dsRNA from *C. annuum* YW (lane 1) and related endornavirus dsRNAs infecting various *Capsicum* species; lane 2, *C. frutescens* LSU Tabasco; lane 3, *C. chinense* PI 315023; lane 4, *C. chinense* PI 159236; lane 5, *C. chinense* PI 273426; lane 6, *C. chinense* PI 315008; lane 7, *C. chinense* PI C00943; lane 8; *C. baccatum* PI 260590; lane 9, dsRNAs extracted from *Nicotiana benthamiana* infected with grapevine leafroll-associated virus 2 (GLRaV-2); lane 10, dsRNA-free *C. annuum*. Arrow indicates endorna-like dsRNAs. Sizes of visible GLRaV-2 dsRNAs are indicated.
detailed analyses revealed three variable regions in the two polyproteins (Fig. 2b). Interestingly, this pattern roughly corresponds to regions of low similarity between ORV and OSV (Moriyama et al., 1999). Considering that the genomes of these two viral isolates shared all their major characteristics, further discussion is based on the BPEV-KS genome data (except when otherwise stated).

The estimated 5′ UTR of BPEV-KS was 225 nt long (226 nt in the case of BPEV-YW). Although two AUG codons were found at nucleotides 18–21 and 121–123, in silico analyses showed that these two codons were probably not utilized owing to their unfavourable initiation context. Compared with the consensus sequence for plants AA(G)CA AUG-GC (Lütcke et al., 1987), the third AUG codon (AGUGAUGGC) of BPEV is in the most favourable context for translation initiation and is assumed to be the starting codon for the translation of a long ORF. The 3′ end of BPEV consists of a 57 nt-long UTR, ending in the unique sequence GGGGAGGGAGGGCCCCCCCCC and lacking a poly(A) tail.

A BLAST search using the BPEV sequence detected conserved domains of a putative viral methyltransferase (MTR), RNA helicase 1 (Hel-1), UDP-glucose–glycosyltransferase (UGT) and an RdRp (Fig. 2a). The BPEV genome organization is unique since it is the only endornavirus to contain these four domains (Roossinck, et al., 2011).

The search in the protein family (Pfam) database (Finn et al., 2010) revealed the presence of a highly conserved MTR (pfam01660) domain between amino acids 288 and 482 with an expect (E) value of 1.1 × 10^{-10}. Similar results were obtained in analyses performed with the conserved domain database (CDD) database (Marchler-Bauer et al., 2007). Multiple-sequence alignment of the putative MTR region of BPEV and several ssRNA viruses belonging to the...
alpha-like superfamily showed the conserved motifs I–IV of the ‘Sindbis-like’ supergroup (Rozanov et al., 1992). The nearest relative of this motif is the MTR domain from a tobramovirus, *Sunn hemp mosaic virus*; other relatives are related to tymoviruses. The invariant amino acid residues for MTR activity, a histidine in motif I, the DXXRArg signature in motif II and a tyrosine in motif IV, are found in BPEV.

A putative Hel-1 domain was found between amino acids 1342 and 1584 and contained conserved motifs from I to VI (Koonin et al., 1993). A BLAST search of this region against the Pfam database produced significant matches with the consensus sequences of the viral superfamily I helicase (pfam01443; E = 1.7 × 10⁻¹⁰). This type of helicase is found in many ssRNA viruses (Roossinck, et al., 2011).

The presence of a region with significant similarity (E values from 9 × 10⁻⁴ to 5 × 10⁻¹⁶) to cellular UDP-glycosyltransferases is located between amino acids 3049 and 3362, and is in similar location to glycosyltransferase domains found in other endornaviruses (Fig. 3a). The UGT-like region in the BPEV genome showed greatest similarity to motifs A and B, which were reported by Hacker et al. (2005) for PEV-1 and are present in some other endornaviruses, hypoviruses and cellular UGTs. While no direct effect of endornaviruses on plants has been reported (except for VFV being associated with the cytoplasmic male sterility; Pfeiffer, 1998), the fungal endornavirus HmEV-1 reduces the virulence of the host fungus, *H. mompa* (Ikeda et al., 2003). Osaki et al. (2006) suggested that the hypovirulence caused by HmEV-1 might be due to the UGT, as the cellular sterol UGT gene of the host fungus, which is essential for pathogenesis in plant-pathogenic fungi, might be repressed by gene silencing.

The RdRp_2 domain encoded by BPEV is located in the carboxy-terminal region of the ORF and contained conserved motifs I–IV (Poch et al., 1989). It shares similarity with the RdRp domains encoded by other endornaviruses (E values ranging from 8 × 10⁻²¹ to 5 × 10⁻⁷¹) and was slightly more related to PEV-1 (43 % identity, 63 % similarity) than to endornaviruses reported from plants (40–42 % identity, 55–57 % similarity) (Supplementary Fig. S1, available in JGV Online). The nearest non-endornavirus relatives are members of genera *Ampelovirus* and *Closterovirus* in the family *Closteroviridae*.

An additional putative domain of approximately 300 aa was identified by *in silico* analyses of BPEV-encoded polyproteins. This domain, located downstream of the MTR, had significant matches with corresponding regions in other endornaviruses (E values ranging between 5 × 10⁻⁴ in the case of OSV to 1 × 10⁻¹⁵ in the case of HmEV-1). The carboxy-terminal part of this putative product was identified as a ‘cysteine-rich region’ (CRR), and is a candidate for a protease domain in PEV-1 (Hacker et al., 2005) and in GaBRV-XL (Tuomivirta et al., 2009). In particular, the region between amino acids 700 and 777 was rich in cysteine residues (22 %) and characterized by the presence of four CXCC motifs. Two of these CXCC motifs (motifs 1 and 3), along with two histidine residues, were
highly conserved in BPEV and several other recognized and putative species of the genus *Endornavirus*, while ORV and OSV contained only conserved motifs 1 and 2 (Fig. 3b and not shown). Although the importance of this region, shared among endornaviruses, is not yet clear, its conservation suggests an important role in the life cycle of endornaviruses. The large endornavirus-encoded polyprotein has been assumed to be processed by viral proteinase(s). This region could be a candidate region for a protease function. However, the importance and function of the CRR region is yet to be experimentally demonstrated.

**RT-PCR**

RT-PCR products (381 bp) were generated from the following *Capsicum* species: *C. annuum* YW, KS, Cayenne Long Red Thick and Super Cayenne; *C. chinense* lines C00943, PI 159236 and PI 315023; and *C. frutescens* Tabasco-LSU and Greenleaf Tabasco. Sequence analysis showed variation among them. Sequences of multiple clones generated from the same specimen were uniform (99–100% amino acid identity), whereas amino acid variation of up to 5% was found among genotypes of the same *Capsicum* species (e.g., maximum level of differences between clones generated from YW and from Super Cayenne pepper). The same level of amino acid diversity was observed among clones generated from *C. chinense* and *C. frutescens*. However, the most significant differences in amino acid variation (up to 16%) were observed between YW and *C. chinense*. The level of identity between BPEV and orthologous sequences of the endornavirus amplified from *C. chinense* and *C. frutescens* (84–85%) was lower than identities between ORV and OSV (94%) in the same region. This suggests that the endornaviruses infecting these two species could be distinct, BPEV-related species.

Sequence differences among RT-PCR products generated from different *Capsicum* species mirrors the taxonomic relationships of the hosts. Phylogenetic analysis using the RdRp region of the various endornavirus sequences (Fig. 4) shows a tree that is congruent with what is known about relationships in the *Capsicum* group, based on isozyme data (McLeod et al., 1982). These closely related species share a mutual ancestral gene pool (Moscone et al., 2007). In the case of *C. frutescens* and *C. chinense*, some have argued that they should be combined into one species owing to their minimal differences and easy interbreeding (Pickersgill, 1966, 1971). This supports our suggestion of host–virus relationship and that BPEV was present in the common ancestor of these three *Capsicum* species and co-evolved with its hosts. Nevertheless, the origin of BPEV in pepper remains unknown and could be elucidated by testing more domesticated genotypes and wild *Capsicum* species. Not all lines of *C. frutescens* and *C. chinense* showed evidence of an endornavirus, and many *C. annuum* cultivars also lack the virus (Table 2). Assuming that the common ancestor of these *Capsicum* species was infected with BPEV, these results suggest a rather inefficient vertical transmission of the virus in certain genotypes. Curiously, two lines of the most primitive *Capsicum* species (*C. chacoense*), five of the non-cultivated *C. annuum* var. *glabriusculum* (Chilepepper) and five of *C. pubescens* (Rocoto) were free of BPEV. The nucleotide-sequence diversity found among various isolates of BPEV from different *Capsicum* species may reflect how long this virus has been associated with these hosts.

### Detection and characterization of the nick in the sequence of BPEV-KS

Purified BPEV-KS was subjected to electrophoresis on a 0.8% denaturing agarose gel, blotted onto a membrane and hybridized using three cDNA probes located between nucleotides 805 and 1135 (Probe 1, Cads577), nucleotides 906 and 1528 (Probe 2, Cads532) and nucleotides 1251 and 1704 (Probe 3, Cads759-5′) from the 5′-ends of the plus strand of the dsRNA. These probes detected both (plus and minus) strands, which were separated by denaturing agarose gel electrophoresis. Approximately 14 and 15 kb RNA fragments were detected with all probes (Fig. 5). A fragment of approximately 1 kb was detected with probe 1 but not with probe 3. With probe 2, the 1 kb fragment was faintly detected. These results indicate that BPEV-KS has a nick in the plus strand between nucleotides 800 and 1100. Eighteen clones were obtained (by using 5′ RACE) to determine the position of the nick on the plus strand (Supplementary Fig. S2, available in JGV Online). Three independently obtained clones indicated a nick in the plus strand at nucleotide 880. Of the remaining clones, nicks were detected at nucleotides 931, 937, 940, 941, 946, 954 or 962, 1183, 1185 and 1187 or 1199. These results suggest that BPEV-KS has a nick in the plus strand at nucleotide 880, but it is likely that this virus has several nicks in the

![Fig. 4.](https://example.com)
plus strand, as reported for PEV-1 and OSV (Hacker et al., 2005; Fukuhara et al., 1995).

The biological properties, unique genomic organization and phylogeny indicate that the dsRNAs isolated from the bell pepper cultivars KS and YW represent two closely related isolates of a distinct and novel species of the genus Endornavirus (family Endornaviridae), for which the name Bell pepper endornavirus (BPEV) is proposed.

**METHODS**

**Plant materials.** Bell pepper plants Kyousuzu (KS), Kyounami (KN), Kyoumideri and Ace were from TAKII Seed Company; Suigyokunigou from Sakata Seed; High Green from Tohoku Seed; and Jumbo Colour Bell Pepper from Kaneko Seeds. Seed of KS, KN and bell pepper California Wonder (CW) were purchased from commercial stores in Japan. Bell peppers Red Bell, Bonnie’s Green Bell, Pimiento Sweet and Chocolate Beauty Sweet were purchased as young plants from commercial garden stores in Ardmore, Oklahoma, USA, and Marengo (MR) seeds were from Asgrow Seed. The source of Yolo Wonder (YW) and all other *C. annuum* genotypes and *Capsicum* species used in this investigation were reported in previous publications (Valverde et al., 1990b; Valverde & Fontenot, 1991) or were obtained from L. L. Black (Louisiana State University, Louisiana, USA). Plant introductions (PI) and accessions that included selections from different geographical locations of *C. annuum*, *C. baccatum*, *C. chacoense*, *C. chinense*, *C. frutescens* and *C. pubescens* were provided by R. L. Jarret (USDA-ARS, Griffin, Georgia, USA) and L. M. Engle (AVRDC, Taiwan). Seed used in some experiments was increased by self-pollination. All plants and seedlings were grown in a greenhouse with day/night temperatures of about 25/18°C, respectively, and 16 h days.

**Extraction, purification and analysis of dsRNA from *Capsicum* spp.** Leaf tissues were pulverized in a mortar and pestle after being frozen in liquid nitrogen, and total nucleic acids were extracted by using 2× STE buffer (200 mM NaCl, 20 mM Tris/HCl and 2 mM EDTA, pH 8.0). Total nucleic acids were further purified with phenol and subjected to agarose gel electrophoresis. Alternatively, dsRNA was fractionated from total nucleic acid extracts by column chromatography on CF-11 cellulose (Whatman), as described by Morris & Dodds (1979) or as modified by Valverde et al. (1990a), Márquez et al. (2007) and Roossinck et al. (2010). The dsRNAs from all other *C. annuum* genotypes and *Capsicum* species were extracted from 3.5 g of leaf tissue and analysed on 5% polyacrylamide gels. For each *Capsicum* genotype and *Capsicum* species, follicar samples from at least three plants were tested. In the case of KS pepper, dsRNA was also extracted from pollen, callus and cultured cells. The nature of the purified nucleic acids was ascertainment by digestion with RNase-free DNase I (Promega), followed by selective treatments with DNase-free RNase A in high (2× SSC) and low (0.2× SSC) salt conditions (20× SSC is 3 M sodium chloride, 0.3 M sodium citrate). Preparations were further treated with Proteinase K (Sigma) followed by phenol/chloroform purification and ethanol precipitation.

**Occurrence of BPEV in bell pepper seedlings.** Seeds from KS, KN, CW, YW and MR bell peppers were planted and 8-week-old plants screened for BPEV by RT-PCR or by electrophoretic analyses of dsRNA. A total of 49, 28, 36, 30 and 137 plants of KS, KN, CW, YW and MR, respectively, were screened. A single MR plant was found free of BPEV and was self-pollinated in order to generate a virus-free line for further use in graft-transmission experiments. Seed of CW, YW and MR originated from self-pollinated BPEV-infected plants while seed of KS and KN were purchased from commercial stores, and thus with no knowledge of the parental status in regard to BPEV infections.

**Attempts to transmit BPEV.** BPEV-infected MR was used as rootstock and the BPEV-free line as the scion in graft inoculation experiments. A total of eight rootstocks were used. Four were infected with PVY, which was mechanically inoculated to the plants when they were 6 weeks old. One month after the PVY inoculation, the stems of all plants were cut and scions from the BPEV-free line graft were inoculated (Supplementary Fig. S3, available in JGV Online). Six weeks after grafting, foliar samples were taken and tested for BPEV by electrophoresis and RT-PCR. Scions grafted on PVY inoculated plants were tested for PVY by ELISA.

**Cloning, sequencing and sequence analyses.** BPEV dsRNA from KS (BPEV-KS) and YW (BPEV-YW) were gel purified, heat denatured and reverse transcribed with random hexadeoxynucleotide primers (TaKaRa). Generated cDNA fragments were cloned into pUC109 (TaKaRa) or pGEM-T Easy plasmid (Promega) and the resulting recombinant plasmids were transferred into *Escherichia coli* DH5α competent cells. DNAs from selected colonies were sequenced by automated sequence analysis using a capillary sequencer, 3130xl Genetic Analyzer (Applied Biosystems). After computer-assisted analysis of the initial sequence data, specific primers were designed in order to fill the gaps between adjacent clones by RT-PCR. Each nucleotide in both genomes (BPEV-KS and -YW) was sequenced from multiple clones from independent experiments in order to ensure at least fivefold coverage.

For determination of the sequence of the 3’ end of BPEV-KS, first-strand cDNAs were synthesized by using 5’-end-phosphorylated RT primer and single-stranded cDNAs were circularized or concatamers were formed at 16 h and at 15°C by using RNA Ligase (TaKaRa).
Circular cDNAs or concatamers were amplified by PCR. The 3′ viral end of BPEV-YW was determined by the method described by Lambden et al. (1992), involving the ligation of a 5′-phosphorylated/3′-amino-blocked oligonucleotide (primer 1) to the target dsRNAs, followed by cDNA synthesis primed by a complementary oligonucleotide (primer 2) and amplification of the 3′ end with a virus-specific primer and primer 2. For determination of the sequence of the 5′ end of BPEV-KS, cDNAs were amplified by PCR after adding a homopolymeric tail at the 3′-end of the single-stranded cDNAs by terminal deoxynucleotidyltransferase (TaKaRa). Amplification products were cloned and sequenced by using M13 RV-N (5′-TGTGGAATTGTGAGCGG-3′) and M13 reverse primers (TaKaRa). For MR pepper samples, dsRNA was isolated and converted to cDNA, followed by multiplexing and sequence analysis on a 454/GS FLX system (Roche), as described previously (Roossinck et al., 2010).

Amino acid sequences were assembled and analysed using GENETYX system (Roche), as described previously (Roossinck et al., 2010). Multiple sequence alignments were conducted by using CLUSTAL W (Thompson et al., 1994), and alignments were edited manually using MESQUITE (version 2.74) (Maddison & Maddison, 2010). Phylogenetic analyses were done using MBBAYES (Huelsenbeck & Ronquist, 2001), implemented via a plug-in for GENIOUS. VFY (GenBank accession # CAA04392) was used as an out-group. Rate matrix was set to a Poisson distribution with a gamma rate variation. Burn-in was 100,000 and total chain length was 1,100,000. Branch lengths were unconstrained. PhyML (Guindon & Gascuel, 2003) and PAUP* version 4.0 beta 4b10 (Swofford, 2002) were used to confirm the tree topology (not shown).

Molecular hybridization. For Northern blot analysis, 100 ng of BPEV-KS dsRNA was separated on a 0.8% agarose MOPS gel with 6% formaldehyde and transferred to a nylon Zeta-Probe membrane (Bio-Rad) by capillary blotting (Moriyama et al., 1999). After UV cross-linking and prehybridization in hybrid solution (250 mM phosphate buffer, pH 7.2, 1 mM EDTA, 7% SDS, 1% BSA), blots were hybridized for 16 h at 65 °C in the same solution with 32P-labelled DNA-probes specific for BPEV-clones Cads77, Cads532 and Cads795-S. Probes were synthesized with a random prime labelling system, BoaBEST Labeling kit (TaKaRa). Membranes were washed twice at 65 °C with 40 mM phosphate buffer containing 5% SDS for 1 h and twice at 65 °C with 40 mM phosphate buffer, pH 7.2, containing 1% SDS for 1 h. Hybridization signals were detected with a BAS 1500 system (FujiFilm). Images taken by the BAS 1500 system were prepared with IMAGE GAUGE (FujiFilm).

RT-PCR. To confirm the presence of BPEV in C. annuum genotypes and to investigate its occurrence in three other Capsicum species, selected genotypes and species that yielded BPEV-like dsRNAs by gel electrophoresis were subjected to RT-PCR using a pair of degenerate primers: Endor-F (5′-AACAGAATWATHGTGCGA-3′) and Endor-R (5′-CTAGWCGKTBGTAGCTTGWC-3′), which were designed to amplify a 381 nt fragment of the RdRp of plant endornaviruses.

Alliquots of dsRNAs were heat denatured at 95 °C for 5 min, cooled rapidly on ice and used for RT-PCR detection. RT-PCR was performed using an Access one-tube, two-enzyme system RT-PCR System (Promega), following the manufacturer instructions. The following PCR conditions were applied for BPEV detection: (i) initial denaturation at 94 °C for 2 min, (ii) denaturation for 1 min at 94 °C, annealing for 45 s at 50 °C, extension for 45 s at 70 °C (40 cycles) and (iii) final extension for 5 min at 70 °C. PCR products were cloned by using a PGEM-T Easy Vector System. For each dsRNA sample, three recombinant plasmids were sequenced and the amino acid sequences derived were compared with sequences in the GenBank database by using BLAST.

ACKNOWLEDGEMENTS

We wish to thank R. Jarret (USDA/ARS, Griffin, Georgia, USA) for providing seeds of Capsicum species; Guoan Shen (Noble Foundation) and Dina Gutierrez (Louisiana State University, USA) for helping with some experiments; Dr Tomohide Natsuaki (Utsunomiya University) for helpful comments; Estación Experimental ‘La Mayora’, CSIC, Spain for providing facilities for some experiments. Approved for publication as journal article no. 12047 of the Mississippi Agricultural and Forestry Experiment Station, MSU.

REFERENCES


An endornavirus from bell pepper


