Molecular characterization of two evolutionarily distinct endornaviruses co-infecting common bean (Phaseolus vulgaris)

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Two high-molecular-mass dsRNAs of approximately 14 and 15 kbp were isolated from the common bean (Phaseolus vulgaris) cultivar Black Turtle Soup. These dsRNAs did not appear to cause obvious disease symptoms, and were transmitted through seeds at nearly 100% efficiency. Sequence information indicates that they are the genomes of distinct endornavirus species, for which the names Phaseolus vulgaris endornavirus 1 (PvEV-1) and Phaseolus vulgaris endornavirus 2 (PvEV-2) are proposed. The PvEV-1 genome consists of 13 908 bp and potentially encodes a single polyprotein of 4496 aa, while that of PvEV-2 consists of 14 820 bp and potentially encodes a single ORF of 4851 aa. PvEV-1 is more similar to Oryza sativa endornavirus, while PvEV-2 is more similar to bell pepper endornavirus. Both viruses have a site-specific nick near the 5' region of the coding strand, which is a common property of the endornaviruses. Their polyproteins contain domains of RNA helicase, UDP-glycosyltransferase and RNA-dependent RNA polymerase, which are conserved in other endornaviruses. However, a viral methyltransferase domain was found in the N-terminal region of PvEV-2, but was absent in PvEV-1. Results of cell-fractionation studies suggested that their subcellular localizations were different. Most endornavirus-infected bean cultivars tested were co-infected with both viruses.

INTRODUCTION

The family Endornaviridae contains dsRNA viruses that infect plants, fungi and oomycetes and are transmitted vertically via gametes, but not horizontally (Fukuhara & Moriyama, 2008; Okada et al., 2011; Valverde & Gutierrez, 2007). Endornaviruses consist of a non-encapsidated, single, linear dsRNA that ranges in length from 9.8 to 17.6 kbp. These viruses have been reported to infect economically important crops, such as rice (Moriyama et al., 1995), common bean (Wakarchuk & Hamilton, 1990), broad bean (Pfeiffer, 1998), barley (Zabalgogeazcoa & Gildow, 1992), cucurbits (Couuts, 2005), pepper (Okada et al., 2011) and avocado (Villanueva et al., 2012), as well as some plant-pathogenic fungi and the oomycete Phytophthora spp. (Hacker et al., 2005). With the exception of Vicia faba endornavirus (VfEV), which is associated with male sterility, most endornaviruses do not appear to affect the phenotype of the host and are generally found at constant concentrations per cell in every tissue and at every developmental stage (Moriyama et al., 1996).

The full genomic sequences of approved or putative endornavirus species from cultivated rice [Oryza sativa endornavirus (OsEV)] (Moriyama et al., 1995), broad bean (VfEV) (Pfeiffer, 1998), barley (Zabalgogeazcoa & Gildow, 1992), wild rice [Oryza rufipogon endornavirus (OrEV)] (Moriyama et al., 1999a), 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), Tuber aestivum [Tuber aestivum endornavirus (TaEV)] (Stielow et al., 2011), bell pepper [bell pepper endornavirus (BPEV)] (Okada et al., 2011),
Two endornaviruses co-infecting common bean

2011) and avocado [Persea americana endornavirus (PaEV)] (Villanueva et al., 2012) have been reported.

Endornaviruses encode a single polypeptide that is presumed to be processed into several proteins of different functions by virus-encoded proteases. Based on conserved domain database (CDD) comparison, the genomes of all completely sequenced endornaviruses contain conserved motifs of an RNA-dependent RNA polymerase (RdRp, pfam00978) similar to the alpha-like virus superfamily of positive-stranded RNA viruses (Roossinck et al., 2011).

Two dsRNAs of approximately 13 and 15 kbp have been reported in the common bean (Phaseolus vulgaris) cultivar Black Turtle Soup (BTS) (Wakarchuk & Hamilton, 1985, 1990). These dsRNAs were not correlated with cytoplasmic male sterility in common bean and were associated with the mitochondria (Mackenzie et al., 1988). Based on the nucleic acid type, size and partial sequence information for one of the two putative dsRNAs (GenBank accession no. AB185246), Phaseolus vulgaris endornavirus was accepted as a member of the family Endornaviridae (Fukuhara & Gibbs, 2012). However, from which of the two dsRNA the partial genomic sequence was obtained was not determined, and nothing is known about the other dsRNA molecule.

In this investigation, we detected two dsRNAs (14 and 15 kbp) in 11 common bean cultivars. The dsRNAs, from BTS, were isolated, characterized, and determined that they consist of the genome of two distinct endornaviruses. We propose the names of Phaseolus vulgaris endornavirus 1 (PvEV-1) and Phaseolus vulgaris endornavirus 2 (PvEV-2) for these viruses with dsRNA genomes of 14 and 15 kbp, respectively. Here, we report their complete nucleotide sequences, genome organization, detection, subcellular localizations, and phylogenetic relationships with other endornaviruses.

RESULTS

BTS contains two distinct dsRNA species

Two dsRNAs of approximately 14 and 15 kbp, which have been reported previously in common bean, were originally detected in BTS and, later in the work, in several other common bean cultivars (Fig. 1, Table 1). These dsRNAs were clearly resolved after agarose gel electrophoresis and ethidium bromide staining (Fig. 1). Experiments of vertical transmission of these dsRNAs using 161 individual BTS plants from accession no. 48724 (National Institute of Agrobiological Resources, Tsukuba, Japan) indicated that they all contained the two dsRNAs (Table S1, available in JGV Online). However, screening 50 BTS plants from a 1984 seed lot provided by R. Provvidenti (Cornell University, Ithaca, NY, USA) resulted in three dsRNA-free plants. Self-pollination of a selected dsRNA-free BTS plant resulted in a dsRNA-free BTS line. Observations of the phenotype of the dsRNA-free and dsRNA-infected BTS lines did not reveal detectable differences. These results indicate that the dsRNAs were transmissible via seed at nearly 100 % efficiency.

As shown in Fig. 1, the two dsRNAs were detected in both developmental stages of BTS. In 10-day-old seedlings, the relative copy number of the 15 kbp dsRNA was lower than that of the 14 kbp dsRNA. However, the concentrations of both dsRNAs were similar when 60-day-old plants were tested. These results were obtained after >30 independent dsRNA extractions.

Nucleotide sequences of PvEV-1 and PvEV-2

The full sequences of the two dsRNAs designated PvEV-1 and PvEV-2 were 13 908 and 14 820 bp respectively (Fig. 2). Both sequences are available from GenBank/DDBJ under accession numbers AB719397 and AB719398, respectively. As the 5’ region of both dsRNAs contained multiple candidate AUG initiation codons, the most favourable initiation codon was determined according to the consensus sequence AA(G)CAUGGGC (Lütcke et al., 1987). In silico analysis showed that a favourable context for translation initiation was found at nt 375 and 231 of PvEV-1 and PvEV-2, respectively; therefore, the estimated 5’ UTRs of PvEV-1 and PvEV-2 were 374 and 230 nt long, respectively. The 3’ UTRs of PvEV-1 and PvEV-2 consisted

Fig. 1. Detection of dsRNAs in seeds and at two developmental stages of the common bean cultivar BTS. dsRNAs were isolated from dry seeds (lanes 1–4), 10-day-old seedlings (lanes 5–8) and mature leaves of 60-day-old plants (lanes 9–12) by the SDS-phenol method followed by DNase I treatment. The dsRNAs derived from 20 mg of each tissue were electrophoresed on 0.5 % agarose gel for 40 h at 30 V, and stained with ethidium bromide. Arrows indicate the positions of the 14 and 15 kbp dsRNAs. Lane M, DNA size markers.
of 46 and 37 nt, respectively. In both cases the 3’ terminus ended in poly(C) sequences of 12 and 11 nt, respectively. No other significant ORFs were found in either the coding or non-coding strand.

**PvEV-1 and PvEV-2 contain a single large ORF with several enzyme motifs**

Both PvEV-1 and PvEV-2 contain a single ORF in the coding strand of 4496 and 4851 codons, respectively.

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**Table 1. Detection of dsRNA in various cultivars of common bean**

+ : Single infection; + + : double infection; – : negative; NT, not tested; CDBN, National Cooperative Bean Nursery, USA; GE, agarose and/or PAGE.

<table>
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<th>Cultivar</th>
<th>Market class</th>
<th>Origin/source</th>
<th>GE</th>
<th>RT-PCR</th>
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<td>Heirloom Seed</td>
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<td>+</td>
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<td>Italy</td>
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**Fig. 2.** Schematic representation of the genome organizations of PvEV-1 and PvEV-2, OsEV and BPEV with key nucleotide and amino acid references, including the position of the nick in the coding strand (▼). The box represents the large ORF, whereas lines depict UTRs. MTR, Viral methyltransferase; Hel-1, viral helicase 1; CPS, capsular polysaccharide synthase; UGT, UDP-glycosyltransferase; RdRp, viral RNA-dependent RNA polymerase.
Comparison of amino acid sequences of the polyproteins encoded by these ORFs revealed low levels of mutual identity and similarity (5 and 17%, respectively).

A BLAST search (NCBI database) using deduced amino acid sequences from both ORFs encoded by PvEV-1 and PvEV-2 detected conserved domains of a putative RNA helicase-1 (Hel-1), UDP-glucose glycosyltransferase (UGT) and an RdRp. A putative viral methyltransferase (MTR) domain was only found in PvEV-2, whereas a conserved putative capsular polysaccharide synthase (CPS)-like domain was only present in PvEV-1 (Fig. 2).

A putative Hel-1, a conserved enzyme essential for virus replication, was found in PvEV-1 and PvEV-2 (aa 1368–1618 and 1338–1585, respectively). Important motifs I–VI (Koonin et al., 1993) in the Hel-1-like region were conserved (Fig. S1). Phylogenetic analysis of the Hel-1 domain indicated that PvEV-1 and PvEV-2 were distantly related (Fig. 3a).

As with most endornaviruses, a UGT domain was located between Hel-1 and RdRp regions in both viruses. The UGT-like region consists of four important motifs and a putative steroid-binding domain (Warnecke et al., 1999). The putative UDP-sugar-binding domain (motif IV) was highly conserved in both PvEV-1 and PvEV-2 (Fig. S2). However, the putative steroid-binding domain and motif II exhibited low similarity to the typical UGTs of most endornaviruses. The putative UGT of PvEV-1 was related to the UGT of OsEV, OrEV and PaEV, while that of PvEV-2 was related to that of BPEV (Fig. 3b).

RdRps encoded by PvEV-1 and PvEV-2, which are located in the C-terminal regions of their polyproteins (aa 4013–4476 and 4373–4828, respectively), exhibited high similarity to

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**Fig. 3.** Maximum likelihood-based phylogenetic trees of viral Hel-1 (a), UGT (b), RdRp (c) and MTR (d) of endornaviruses, related taxa and corresponding regions encoded by some representative viruses, fungi and plants inferred by using the method based on the WAG substitution model (Whelan & Goldman, 2001). A discrete gamma distribution was used to model evolutionary rate differences among sites. The rate variation model allowed for some sites to be evolutionarily invariable (G+I) in (a) and (c). Consensus trees obtained after 1000 replicates are presented. The percentage of trees in which taxa clustered together is shown. Branches with 50% bootstrap values are collapsed, as they are considered untrustworthy. GenBank accession numbers of genes are provided in Table S2. Grapevine leafroll-associated virus (GLRaV) is a closterovirus and was used as an outgroup in (a) and (c). AMV, Alfalfa mosaic virus; BSBV, beet soil-borne virus; BMV, brome mosaic virus; CeEV, Chalara elegans endornavirus; CMV, cucumber mosaic virus; ObPV, Obuda pepper virus; PepRSV, pepper ringspot virus; PMMoV, pepper mild mottle virus; TMV, tobacco mosaic virus; TRV, tobacco rattle virus.
the RdRps encoded by known endornaviruses. Multiple sequence alignment of these sequences and RdRp derived from ssRNA viruses showed that motifs A–E (Poch et al., 1989) were conserved in PvEV-1 and PvEV-2 (Fig. S3). A neighbour-joining phylogenetic tree showed that they were related closely to endornaviruses (Fig. 3c). PvEV-1 was related closely to OsEV and OrEV in addition to PaEV, while PvEV-2 was related closely to BPEV. PvEV-1 and PvEV-2 were only distantly related to each other. This phylogeny was similar to that derived from the Hel-1 domain.

A viral MTR domain was found only in PvEV-2, but not in PvEV-1. The MTR domain is also found in the N terminus of two recently reported endornaviruses, GaBRV-XL and BPEV (Tuomivirta et al., 2009; Okada et al., 2011). The PvEV-2 viral MTR domain was found in the N-terminal region (aa 250–608). The MTR is involved in 5′-capping to mRNAs to enhance their stability, and this process occurs in the nucleus. Therefore, many viruses that replicate in the cytoplasm encode their own MTR enzymes. Because the MTR is commonly found in ssRNA viruses of the alphavirus superfamly, the MTR-like region of PvEV-2 was aligned with the corresponding region of selected ssRNA viruses of the families Virgaviiridae and Bromoviridae. A multiple sequence alignment revealed conserved motifs I–IV of the ‘Sindbis-like’ supergroup (Rozanov et al., 1992) in PvEV-2. The invariant amino acid residues for MTR activity, a histidine in motif I, the DXXR signature in motif II and a tyrosine in motif IV, were conserved (Fig. S4). The closest relative of this domain was that from the plant endornavirus, BPEV (65% identity, 79% similarity). Phylogenetic analysis showed that the putative MTR in endornaviruses formed an independent clade in the tree (Fig. 3d).

A CPS domain was found in PvEV-1, but not in PvEV-2. It was located upstream of the UGT domain (aa 2623–2904). According to a BLASTP search, the CPS domain of PaEV had the highest similarity to that of PvEV-1 (35% identity and 55% similarity), whilst the mannosyltransferase of Shigella boydii, which is a Gram-negative bacteria that causes dysentery in humans, also has the second highest similarity (31% identity and 48% similarity), followed by those of OsEV (28% identity and 43% similarity) and OrEV (27% identity and 48% similarity). All of the rest were bacterial mannosyltransferase and CPS. This CPS domain was previously identified in some, but not all, endornaviruses.

A cysteine-rich domain (CRR), identified in other endornaviruses and suggested as a candidate for a protease (Hacker et al. 2005; Tuomivirta et al., 2009), was found in both PvEV-1 and PvEV-2. Multiple sequence alignment showed that most endornaviruses, including PvEV-2, had four CXCC signatures, although cysteine at the third character in signatures 2 and 4 was often substituted (Fig. S5). In contrast, rice endornaviruses (OsEV and OrEV), PaEV and PvEV-1 lacked signatures 1 and 2, and contained two conserved CXCC signatures (Fig. S5).

Detection and determination of the position of the nicks

The 5′ region of the coding strand of most endornaviruses contains a site-specific nick, while the non-coding (minus) strand does not (Fukuhara & Moriyama, 2008). To identify the presence of a nick in PvEV-1, denatured dsRNA blots were hybridized with PvEV-1-derived cDNA probes located between nt 34 and 633 (YpbT44) and nt 8390 and 9374 (YpbT82) of the coding strand. A band of approximately 13 kb was detected by both probes, while a fragment of approximately 1 kb was detected only with probe YpbT44 (Fig. 4a). The presence of the 1 kb fragment implied that a discontinuity was located approximately 1000 nt from the 5′ end of the coding strand. These probes could detect both (coding and non-coding) strands separated by denaturing agarose gel electrophoresis, but the 13 kb coding strand and intact non-coding strand (14 kb) were not separated by electrophoresis due to their similar molecular mass. Therefore, signals of both molecules appeared as one band [dark grey and grey arrows in Fig. 4(b)]. The same experiments were carried out to determine the presence of a nick in PvEV-2 (Fig. 4). A fragment of approximately 1 kb was detected when probed with YpbT104 (nt 258–692) for the 5′ region, indicating that the nick was located at the 5′ region of PvEV-2, as with PvEV-1, which was similar to other endornaviruses.

However, further 5′ RACE experiments were carried out in order to determine the exact sites of the nicks in both PvEV-1 and PvEV-2. Sequence analyses of multiple RACE-generated clones indicated that the majority of cDNAs showed a nick in the coding strand at nt 1111 for PvEV-1 and nt 881 for PvEV-2 (Fig. 4a).

Subcellular localizations of PvEV-1 and PvEV-2

Subcellular fractionation was performed to determine the localization of PvEV-1 and PvEV-2 in host cells. PvEV-1 was associated mainly with the microsomal fraction (Fig. 5, lanes 9–10), while the subcellular localization of PvEV-2 was not clearly defined; PvEV-2 was associated mainly with the crude chloroplast and mitochondrial fractions (Fig. 5, lanes 5–8). DNA was purified from the chloroplast fraction and digested with BamHI, EcoRI and HindIII. Electrophoresis of DNA digests with these enzymes confirmed that this fraction was derived mainly from chloroplasts; nevertheless, this fraction contained a small amount of the nuclear DNA (Fig. S6). Neither PvEV-1 nor PvEV-2 was found in the cytosol fraction (Fig. 5, lanes 11–12). These results suggest different intracellular localizations for PvEV-1 and PvEV-2.

Presence of PvEV-1 and PvEV-2 in common bean cultivars

Some common bean cultivars analysed were co-infected with both viruses, while others were virus-free (Table 1). Two cultivars, Bellagio and Majesty, were found to be
infected with only a 14 kbp dsRNA. None of the cultivars tested was infected with the 15 kbp dsRNA alone. A virus-specific duplex RT-PCR, designed and developed in the framework of this study, confirmed that the 14 kbp dsRNA present in cultivars Bellagio and Majesty represents the genome of the same virus, PvEV-1, as was detected in double infections in other cultivars (Fig. 6, lanes 2–3). In the case of double infections, the test yielded two PCR products of distinct size, indicating the presence of two target viruses in reverse-transcribed RNA extracts (Fig. 6).

**DISCUSSION**

We have isolated and sequenced two dsRNAs of approximately 14 and 15 kbp from the common bean cultivar BTS and determined that they represent the genomes of two distinct species of endornavirus. Phylogenetic analyses of the putative Hel-1, UGT and RdRp showed that these two dsRNA viruses are members of the family *Endornaviridae* (Fig. 3). Therefore, we propose that both viruses be classified in the genus *Endornavirus* of the family *Endornaviridae* and propose the names *Phaseolus vulgaris* endornavirus 1 (PvEV-1) and *Phaseolus vulgaris* endornavirus 2 (PvEV-2) for the 14 and 15 kbp dsRNAs, respectively. The partial sequences (630 bp) of a dsRNA virus from the *P. vulgaris* cultivar BTS have been published (Wakarchuk & Hamilton, 1990) and, based on the sequences and other properties of the dsRNA, it was placed in the genus *Endornavirus* and named *Phaseolus vulgaris endornavirus* (PvEV) (Fukuhara et al., 2006). The genome sequences of PvEV-1 and PvEV-2 revealed that the partial sequences reported by Wakarchuk & Hamilton (1990) were derived from PvEV-1. Therefore, we propose that PvEV should be renamed PvEV-1 in order to clarify the nomenclature of these two endornaviruses co-infecting the same host.

With the exception of VfEV and TaEV, a UGT domain has been found in all other sequenced endornaviruses. Hacker et al. (2005) reported that the putative UGT is probably a UDP-glucose:sterol glycosyltransferase, as it shows high similarity to those found in yeasts, fungi and plants. Both PvEV-1 and PvEV-2 have a conserved UGT domain, which is highly unusual for RNA viruses. Although the exact function of the UGT in endornaviruses remains to be elucidated, it has been suggested that it could modify and enforce their vesicle membranes surrounding the naked dsRNAs to protect them against cellular enzymes such as nucleases (Roossinck et al., 2011). The putative CRR domain with multiple CXCC signatures, conserved among
endornaviruses, was found in both viruses. This region has been suggested to be a candidate for a viral protease that can process the polyprotein to functional units (Hacker et al., 2005; Tuomivirta et al., 2009; Okada et al., 2011).

While conserved domains of Hel-1, UGT and RdRp were found in both viruses, a viral MTR domain was only found in PvEV-2. Among the endornaviruses identified to date, the MTR domain is only found in BPEV and GaBRV-XL. The MTR domain is characteristic of alpha-like viruses and, along with Hel-1 and RdRp, is implicated to be part of the replication machinery (Koonin et al., 1993). The order of these three domains in PvEV-2 supports the hypothesis that endornaviruses share a common ancestor with alpha-like viruses (Gibbs et al., 2000). The MTR in some ssRNA viruses is shown to have methyltransferase and guanylyltransferase activities and to be involved in formation of a cap structure at the 5′ end of viral mRNAs, cooperating with Hel-1 (Huang et al., 2004). Conservation of these important domains in PvEV-2 may suggest that PvEV-2 would have the cap structure at the 5′ end(s) of its dsRNA. In contrast to MTR, a CPS domain was found in PvEV-1, but not in PvEV-2. CPS is commonly produced by bacteria to help them adhere to surfaces and to prevent from desiccation. It is also a major virulence factor in Streptococcus pneumoniae (Jiang et al., 2001).

The genome organizations and nucleotide sequences of PvEV-1 and PvEV-2 strongly support the notion that they are distinct endornavirus species. Whilst PvEV-1 is related closely to OsEV, PvEV-2 appears to be a close relative of BPEV. As the topology of the trees from endornaviruses does not seem to follow the taxonomic relationship of their hosts (Fig. 3), it is probable that endornaviruses have a common origin, most likely in fungi, and have been transmitted horizontally at some time in the past (Roossinck et al., 2011).

A unique molecular feature of members of the family Endornaviridae is the presence of a site-specific nick in the 5′ region of the coding (plus) strand RNA molecule. The 5′ region of the coding strands of OsEV, OrEV, VfEV, PEV-1, HmEV-1 and BPEV contains a site-specific nick at nt 1211, 1197, 2735, 1215, 2552 and 880 from the 5′ end, respectively (Fukuhara et al., 1995; Moriyama et al., 1999a; Pfeiffer, 1998; Hacker et al., 2005; Osaki et al., 2006; Okada et al., 2011). Similarly to other endornaviruses, PvEV-1 and PvEV-2 contain a nick in the 5′ region of their genome at nt 1111 and 881 from the 5′ end, respectively.

In this investigation, both PvEV-1 and PvEV-2 were transmitted to the progeny plants at rates close to 100%. However, virus-free plants were detected in a BTS accession. This is not surprising, as similar results were obtained with BPEV, which infects bell pepper cv. Marengo (Okada et al., 2011). This supports previous data on the highly efficient transmission of other endornaviruses through seed (Moriyama et al., 1996; Valverde & Gutierrez, 2007). Like other endornaviruses (Valverde et al., 1990; Fukuhara et al., 1993), both viruses were detected in every tissue tested (Figs 1 and S7). This property of endornaviruses is not observed with conventional plant viruses, but it is common in fungal viruses (mycoviruses). Nevertheless, unlike many mycoviruses (Rogers et al., 1986; Anagnostakis, 1988; Coenen et al., 1997; Chun & Lee, 1997), endornaviruses can be maintained efficiently during meiosis via pollen and egg cells. Research to elucidate the mechanism of transmission of fungal and plant endornaviruses during meiosis will help to explain the efficient seed transmission of plant endornaviruses. This unique property of endornaviruses can lead to the establishment of a symbiotic relationship with host plants (Roossinck, 2010).

The results of our investigation show that these two endornaviruses are capable of maintaining stable co-infection in common bean. Although it is known that multiple partitiviruses can infect the same plant (Sabanadzovic & Valverde, 2011), this is the first report of co-infection of a host by two distinct endornaviruses. Testing common bean cultivars with the virus-specific one-step RT-PCR method developed in this study confirmed that dsRNAs present in various bean cultivars consist of the genome of the same viruses. Furthermore, the results confirmed that the 14 kbp detected in the cultivars Bellagio and Majesty is the genome of the same virus (PvEV-1) detected in several double-infected cultivars. Testing more bean cultivars and other Phaseolus species, together with pedigree analyses of infected cultivars, could help to unravel the origin of these viruses in common bean.

In the case of rice, two endornaviruses infecting cultivated rice and wild rice (OsEV and OrEV, respectively), which share 85% identical amino acid sequence, have been reported (Moriyama et al., 1995, 1999a). Attempts to co-infect the rice with these two viruses by interspecific crosses failed because apparently the two viruses were incompatible with each other in hybrid rice progenies (Moriyama et al., 1999b). In contrast, PvEV-1 and PvEV-2 seem compatible in common bean, although they are apparently localized in different areas of the cell. VfEV, OsEV and OrEV are mainly found in the microsomes of host cells (Lefebvre et al., 1990; Moriyama et al., 1996), while BPEV was reported to be detected mainly
in the chloroplast fraction of bell pepper (Valverde et al., 1990). Cell-fractionation experiments showed that PvEV-1 was associated mainly with the microsomes and PvEV-2 was found mainly in the chloroplast and mitochondrial fraction (Fig. 5). The subcellular localizations of PvEV-1 and PvEV-2 appeared to be similar to those of their phylogenetically related species, OsEV and BPEV, respectively (Fig. 3). Endornaviruses seem to have functional proteins, such as Hel-1, UGT and RdRp. In the case of PvEV-1 and PvEV-2, these proteins are distantly related to each other (Fig. 3). The MTR domain was present only in PvEV-2, while the CPS domain was present only in PvEV-1. The differences in the presence of these proteins might reflect the different subcellular localization of PvEV-1 and PvEV-2. The UGT in endornaviruses may have roles in membrane modification and membrane binding. Future studies related to the intracellular localization of common bean endornaviruses should involve these proteins.

The virus concentration of PvEV-2 in seedlings was lower than that in mature leaves (Fig. 1). In the case of OsEV, it has been demonstrated that the concentration of virus decreases or is lost completely in some Dicer-like protein (OsDCL2) knock-down plants during their development and, therefore, the rate of transmission of it is lower in these plants than in the wild-type rice plants. This suggests that OsDCL2 regulates host factor(s) for maintenance of OsEV in somatic and meiotic division (Urayama et al., 2010). In contrast, the maintenance system for PvEV-2 may depend on developmental gene expression of common bean, which could be elucidated if the exact subcellular localization for this virus is demonstrated.

Biological properties, genome organization and phylogeny indicate that the dsRNAs co-infecting the common bean cultivar BTS represent the genomes of two novel, distantly related species of the genus *Endornavirus*. As reported previously, none of the double-infected BTS plants exhibited disease symptoms (Wakarchuk & Hamilton, 1985; Mackenzie et al., 1988). Moreover, phenotypic observations of virus-free and virus-infected BTS lines did not reveal detectable differences, although comparative studies between virus-infected and virus-free lines need to be conducted. The information reported here will be helpful to understand the relationships among endornaviruses and to gain a better insight into the origin and evolution of this group of viruses.

### METHODS

**Plant materials.** Seeds of common bean from various genotypes were obtained from various sources (seed sources and cultivar names are listed in Table 1). Plants were grown in a greenhouse at an average temperature of 28 °C.

**Extraction and electrophoresis of dsRNAs.** Plant tissues were pulverized with a mortar and pestle after being frozen in liquid nitrogen, and total nucleic acids were extracted with 2 x STE buffer (200 mM NaCl, 20 mM Tris/HCl and 2 mM EDTA, pH 8.0). Nucleic acids were further purified with phenol and treated with DNase I (Takara). Alternatively, dsRNAs were fractionated from purified total nucleic acids by column chromatography on CF-11 cellulose (Whatman) as described by Morris & Dodds (1979) or as modified by Valverde et al. (1990). For preliminary screening of dsRNAs in common bean cultivars, purified dsRNAs were resolved in 0.8% agarose gels (TAE buffer) at 50 V for 6 h or by PAGE (5% acrylamide) at 100 V for 3 h. However, to determine and discriminate the presence of the individual viruses, 0.5% agarose gels were run at 20 V for 40 h at room temperature. The dsRNAs from BTS accession no. 48724 (National Institute of Agrobiological Sciences, Tsukuba, Japan) were extracted and analysed from seed and at two different developmental growth stages (10 and 60 days after emergence); 20 mg foliar tissue was firstly cut off from a 10-day-old plant, and then 20 mg foliar tissue was cut off again from the same individual plant after 50 days.

**Transmission experiments.** Eight BTS plants (accession no. 48724, National Institute of Agrobiological Resources, Tsukuba, Japan), which were co-infected with PvEV-1 and PvEV-2, were self-pollinated and seed pods were collected. Seeds from each parental plant were germinated and grown. Leaves from 2-month-old progeny plants were harvested to determine the presence or absence of the dsRNAs. Extraction of dsRNAs was performed as described above.

**Cloning, sequencing and sequence analyses.** After phenol extraction, the two dsRNAs from BTS (14 and 15 kbp) that corresponded to PvEV-1 and PvEV-2 were purified by two cycles of column chromatography, and cDNA fragments were generated with random hexa-deoxyribonucleotide primers (TaKaRa). The terminal sequences of both viruses were obtained with 5'-3' RACE. 5' RACE was performed as described previously (Okada et al., 2011). For 3' RACE, dsRNAs were polyadenylated on the 3' ends of both strands by *Echerichia coli* poly(A) polymerase (TaKaRa), and first-strand cDNAs were synthesized as described previously (Isogai et al., 1998) and amplified by PCR. cDNA fragments and 5'-3' RACE products were cloned and sequenced as described by Okada et al. (2011). Obtained nucleotide sequences were analysed for ORFs, and any ORFs found were translated into amino acid sequences with GENETYX version 9 (GENETYX). Deduced amino acid sequences with similarity to PvEV-1 and PvEV-2 were searched in the NCBI database with BLAST (Altschul et al., 1997). A similarity search of protein versus protein was performed with GENETYX. Deduced amino acid sequences were aligned with CLUSTAL_X (Thompson et al., 1997) and GeneDoc v. 2.6 (Nicholas et al., 1997). Phylogenetic analyses were performed on amino acid sequences using MEGA 5.05 (Tamura et al., 2011). After testing each dataset for the best-fit substitution model, phylogenetic relationships among sequences used for analyses were inferred by the maximum-likelihood method.

**Detection and determination of the position of the nicks in PvEV-1 and PvEV-2.** For Northern blot analysis, 100 ng of both viral dsRNAs was separated on a 1.2% agarose MOPS gel with 6% formaldehyde and transferred to a nylon Zeta-Probe membrane (Bio-Rad) by capillary blotting (Okada et al., 2011). After UV cross-linking and prehybridization in hybridization (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 PvEV-1 clones YpBT44 (located between nt 34 and 633) and YpBT82 (between nt 8390 and 9374), or for PvEV-2 clones YpBT104 (between nt 258 and 692) and YpBT13 (between nt 12 417 and 14 255). Probes were synthesized with a random prime-labelling system (KuBEST labeling kit, TaKaRa). Washing and detection procedures were as described previously (Okada et al., 2011).

**Subcellular fractionation of PvEV-1 and PvEV-2.** BTS leaves (20 g) were homogenized with a mixer for 5–10 s in 80 ml STC buffer (0.3 M sucrose, 50 mM Tris, 10 mM CaCl2, 2 mM EDTA, 0.1% Triton X-100) and centrifuged at 2000 g for 20 min. Supernatants were centrifuged at 8000 g for 20 min, and the supernatant was centrifuged at 100 000 g for 60 min. The resulting pellets and supernatants were further fractionated as described previously (Okada et al., 2011).
2-mercaptoethanol, pH 7.5). The homogenate was filtered through a four-layered nylon screen (80 μm pores). The filtrate was centrifuged for 10 min at 1220 x 1000 g using a swinging bucket rotor (Tomiy, Suprema21). The resulting pellet, containing nuclei and chloroplasts, was resuspended in STC buffer and applied to a discontinuous sucrose gradient (40–55%), and centrifuged for 30 min at 74000 x 1220 g using a swinging bucket rotor (Hitachi, CP800WX). A green layer separated in the gradient was collected and considered to be the intact chloroplast fraction. Nuclei were not observed after light microscopy examinations. The pellet (from the gradient), which contained intact nuclei, was washed with 0.5 % Triton X-100 in STC buffer to remove chloroplast contaminants. After centrifugation at 1220 x 1000 g using a swinging bucket rotor, the pellet was saved and referred to as the crude nuclear fraction. The supernatant obtained after the centrifugation of the filtrate contained mitochondria, microsomes and cytosol. The supernatant was first centrifuged for 20 min at 11500 x 1000 g using a fixed-angle rotor to obtain the crude mitochondrial fraction from the resultant pellet. Then, the supernatant was centrifuged for 1 h at 100 000 x 1000 g with the RP65-733 fixed-angle rotor (Hitachi, CP800WX). The resulting pellet or the supernatant were referred to as microsomal or cytosol fractions, respectively. Nucleic acids were extracted from these subcellular fractions as described above. For enzymic analysis, total nucleic acids from the chloroplast fraction were digested with RNase A (2 μg ml⁻¹) for 16 h and with BamHI, EcoRI and HindIII.

Occurrence of the two dsRNAs in selected common bean cultivars. Seeds from common bean cultivars (listed in Table 1) were planted, and 2–4-week-old seedlings were tested for the presence of the 14 and 15 kbp dsRNAs by electrophoretic analyses. Furthermore, plants, and 2–4-week-old seedlings were tested for the presence of double-stranded RNA viruses in Aspergillus nidulans. Genet Res 69, 1–10.


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REFERENCES


