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Transmission of a dsRNA in bell pepper and evidence that it consists of the genome of an endornavirus

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Abstract A double-stranded (ds) RNA from bell pepper (BP-dsRNA) cv Yolo Wonder was inherited maternally and paternally after crossing Yolo Wonder with Jalapeño M or Hungarian Wax pepper. Partial sequence information was obtained from two cDNA clones derived from the BP-dsRNA and based on sequence similarity was related to members of the genus *Endornavirus*. Clones of the BP-dsRNA hybridized with similar dsRNAs from four other pepper cultivars, suggesting that all five dsRNAs tested are related. One of the cDNA clones contained a region that had significant similarity with UDP-glucose:glycosyltransferases from fungi, bacteria, plants, and three endornaviruses. Data presented indicate that the BP-dsRNA is the genome of a distinct species of the genus *Endornavirus*.

Keywords *Capsicum annuum* · dsRNA · Endornavirus · Glycosyltransferases · Pollen transmission · Ovule transmission

Introduction

In virus-infected plants, dsRNAs are found in the form of genomic segments of dsRNA viruses and replicative forms of single-stranded RNA viruses. The family *Partitiviridae* and the genus *Endornavirus* contain members with a dsRNA genome that infect plants, are transmitted at high rate through seed, and are not associated with disease symptoms [1, 2]. However, unlike endornaviruses, members of the *Partitiviridae* contain virions and two unrelated dsRNA segments [1]. Endornaviruses resemble hypoviruses (*Hypoviridae*) because, no true virions have been identified in infected tissue and both consist of a linear dsRNA genome with a single open reading frame with recognizable helicase and polymerase domains [2, 3]. The full sequence of the genome of endornaviruses from cultivated rice [*Oryza sativa endornavirus* (OSV)] [4], wild rice [*Oryza rufipogon endornavirus* (ORV)] [5], broad bean [*Vicia faba endornavirus* (VFV)] [6], *Phytophthora* sp [*Phytophthora endornavirus* 1 (PEV1)] [7], and *Gremmeniella abietina* [*Gremmeniella abietina* type B RNA virus (GenBank accession no. DQ399290)] have been reported. These endornaviruses encode a single open reading frame (ORF). The proteins encoded by endornaviruses are presumed to be processed by virus-encoded proteinases, although evidence of proteinase activity is lacking. Conserved motifs for RNA-dependent RNA polymerase (RdRp) and RNA helicase have been identified among endornaviruses [2]. Recently, a UDP-glucose:sterol glucosyltransferase-like motif have been found in PEV1, OSV, and ORV [7]. Partial sequences of an endornavirus from common bean (*Phaseolus vulgaris endornavirus* (PVuV) [2], putative endornaviruses from melon (*Cucumis melo*), bottle gourd (*Lagenaria siceraria*), barley (*Hordeum vulgare*), Malabar spinach (*Basella alba*), sea grass (*Zostera marina*), and the full sequence of the fungus *Helicobasidium mompa* have been reported [8, 9]. Sequence analyzes support placing them in the *Endornavirus* genus.

The GenBank accession numbers of the sequences reported in this paper are DQ242514 and DQ667684.

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A dsRNA of approximately 12 kbp has been reported in 13 bell pepper (*Capsicum annuum*) cultivars [10]. This dsRNA (BP-dsRNA) is similar in some physical and biological properties to dsRNAs of members of the *Endornavirus* genus. Molecular hybridization experiments did not reveal detectable nucleic acid similarities between BP-dsRNA and similar dsRNAs from rice, bean, and melon (11). It has been suggested that the BP-dsRNA could be the genome of an endornavirus [8]. The purpose of this investigation was to determine the relationship of the BP-dsRNA with members of the genus *Endornavirus*.

Materials and methods

Bell pepper cv. Yolo Wonder (YW) that was previously reported to contain a 12 kb dsRNA was grown under standard greenhouse conditions and used as source of BP-dsRNA for transmission and cloning experiments. Jalapeño M (JM) and Hungarian Wax (HW), two *C. annuum* cultivars that belong to two different horticultural types, were used in transmission experiments. In previous studies, it was shown that these two cultivars were infected with two distinct cryptic viruses (*Partitiviridae*) but BP-dsRNA was not detected [12]. Four *C. annuum* cultivars from four horticultural pepper types [pimento (Pimento Perfection), cayenne (Tam Cayenne), Cuban (Cubanelle), and Anaheim chili (Tam Sweet Chile)] reported to contain a dsRNA similar to that found in YW, were used in molecular hybridization experiments [13].

Transmission experiments

Transmission of the BP-dsRNA was conducted by making reciprocal crosses between YW and JM, and YW and HW. At least four selected flowers from individual plants (maternal parent) were pollinated and tagged. Twenty plants (five from each of four successful crosses) from the F1 progeny of each cross were tested for the presence of the BP-dsRNA. As previously described, dsRNA was extracted from 3.5 g of foliar tissue [12]. Purified dsRNA was treated with RNAse A and DNAse I and resolved in 6% polyacrylamide gels. Graft transmission experiments were conducted using YW rootstocks infected with Louisiana isolates (from pepper) of *Tobacco etch virus* (TEV) or *Cucumber mosaic virus* (CMV) and healthy (non-inoculated) JM as scions. Conventional viruses (TEV and CMV) were used as transmission controls and to test them for their ability to assist the systemic spread of BP-dsRNA. Four rootstocks infected with TEV and four infected with CMV were wedge–grafted with healthy JM scions. One month after grafting, scions were tested for the presence of BP-dsRNA by electrophoretic analysis in polyacrylamide gels.

DsRNA purification and cloning

DsRNA was gel purified (from agarose gels) using the MinElute gel extraction kit (Qiagen, Valencia, CA, USA). DsRNA was denatured with dimethyl sulfoxide and used as template to construct a cDNA library using random hexadeoxynucleotide primers. cDNA was ligated into pGEM-T Easy Vector (Promega, Madison, WI, USA) and used to transform JM 109 competent cells. Several cDNA clones that ranged from 500 to 1,300 bp were obtained and selected clones were sequenced. The nucleotide sequences were determined by automated sequence analysis at Genomics Technology Support Facility of Michigan State University, East Lansing using a Perkin Elmer/Applied Biosystems 3100 capillary sequencer (Perkin Elmer, Foster City, CA). Derived amino acid sequences of two clones (BP-17 and BP-22) were compared with sequences in databases using the Basic Local Alignment Search Tool (BLAST) [14]. Multiple sequence alignment was conducted using the clustal series of programs available at the European Bioinformatics Institute Website (http://www.ebi.ac.uk) [15].

Molecular hybridization

Purified dsRNAs from YW and four other pepper cultivars belonging to four horticultural types were denatured by boiling in 50 mM NaOH for 5 min (11). Aliquots of approximately 10 µl (estimated by ethidium bromide staining to be 100 ng of dsRNA) were spotted onto nylon membranes and fixed with UV light.

Molecular hybridization was conducted using Amersham's ECL kit (Arlington Heights, IL, USA) following the manufacturer's procedures. Two cDNA clones (BP-17 and BP-22) were used as probes. Clone preparation, labeling, and hybridization was carried out under previously described conditions [11].

Results

Transmission

Electrophoretic analyses of dsRNAs clearly identified the presence or absence of BP-dsRNA as well as the dsRNAs of the cryptic viruses in the F1 plants (Fig. 1). Results of transmission experiments are shown in Table 1. The BP-dsRNA was transmitted both maternally and paternally. Transmission of BP-dsRNA from YW appears to be more

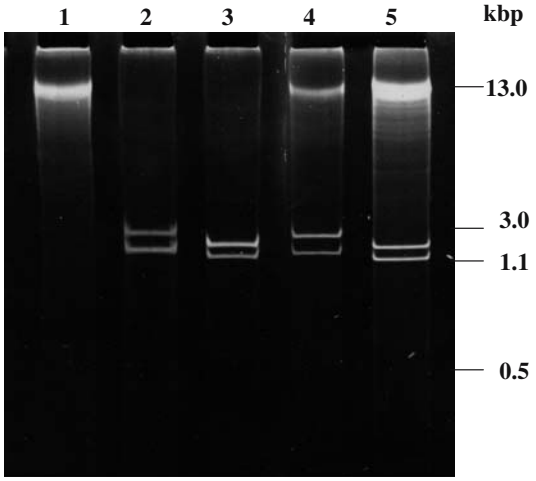

Fig. 1 Polyacrylamide gel electrophoresis of dsRNA extracted from pepper (*Capsicum annuum*). Lane 1, Yolo Wonder (YW); lane 2, Jalapeño M (JM); lane 3, Hungarian Wax (HW); lanes 4 and 5, dsRNAs extracted from F1 plants that resulted from crossing YW with JM (lane 4) and YW with HW (lane 5)

efficient when reciprocal crosses were conducted with JM than with HW pepper. In all cases, higher levels of transmission were obtained through the ovule than through pollen.

Graft transmission experiments were successful for CMV and TEV. Typical symptoms caused by these viruses on pepper were observed on all the JM scions two weeks after grafting. However, dsRNA analyzes of the scions, one and two months after grafting, did not yield the BP-dsRNA. These results indicate that CMV or TEV infections did not assist the transmission of BP-dsRNA.

Molecular hybridization

Molecular hybridization using cloned viral DNA as probes confirmed that the clones were derived from the BP-dsRNA. Positive signals with similar intensities were obtained with dsRNA extracts from all four horticultural types of peppers.

Table 1 Maternal and paternal inheritance of BP-dsRNA after reciprocal crosses of Yolo Wonder with Jalapeño M and Hungarian Wax peppers

♂		♀	Positive plants/plants tested[a]	%
Yolo Wonder	×	Hungarian Wax	7/20	35
Hungarian Wax	×	Yolo Wonder	15/20	75
Yolo Wonder	×	Jalapeño M	12/20	60
Jalapeño M	×	Yolo Wonder	18/20	90

Presence or absence of dsRNA in the F1 generation was determined by dsRNA extraction and electrophoretic analyses

[a] Twenty individual plants from the F1of each cross were analyzed

Clones and sequence analyses

Two cDNA clones, BP-17 (1273 bp) and BP-22 (610 bp) were sequenced (GenBank accession no. DQ242514 and DQ667684 respectively). These clones contained open reading frames predicted to encode proteins of 423 (BP-17) and 197 (BP-22) amino acids. A BLASTP search of the Uniprot database using derived amino acid sequences from BP-17 resulted in significant alignments with predicted polyprotein sequences encoded by plant viruses in the genus *Endornavirus*. The highest scores were obtained with VFV (32% identity) followed by PEV1 (27% identity), ORV (31% identity), OSV (30% identity), PVuV (30% identity) and *Gremmeniella abietina* type B RNA (27% identity). Putative endornaviruses with significant alignments included: *Cucumis melo* (30% identity) and *Helicobasidium mompa* endornavirus (28% identity), A 155 amino acid of the helicase-like region present in this clone was aligned with corresponding sequences of other endornaviruses and two putative endornaviruses (Fig. 2). This region contained motifs (a–d) typical of RNA helicases. The highest score was with corresponding regions from ORV.

A BLASP search using the 197 amino acids coded by the ORF of clone BP-22 produced significant alignments with a conserved domain of plant, bacterial, viral, and fungal multigenic family UDP-glucose:glycosyltransferases (UGTs). A motif located near the C terminus of this region contained a 39 amino acid sequence motif characteristic of all UGTs. A multiple alignment of this motif and corresponding amino acid sequences of several organisms is shown in Fig. 3.

Discussion

Inheritance studies revealed that OSV is transmitted to F1 hybrids via pollen and ovule [5]. The copy number of the OSV dsRNA increased more than ten fold in pollen grains when compared with other tissues. Reciprocal crosses have been conducted between Nipponbare (a *Japonica* rice variety infected with OSV) and IR-26 or Kasalath (two *Indica* type varieties free of OSV) [16]. When Nipponbare and IR-26 were used high percentage dsRNA transmission through both pollen (89%) and ovules (93%) was obtained. However, when Nipponbare and Kasalath were used, the OSV transmission to the F1 progeny was 20% through pollen and 68% through the ovule. We obtained similar results using two pepper cultivars belonging to two different horticultural types. Transmission of BP-dsRNA was

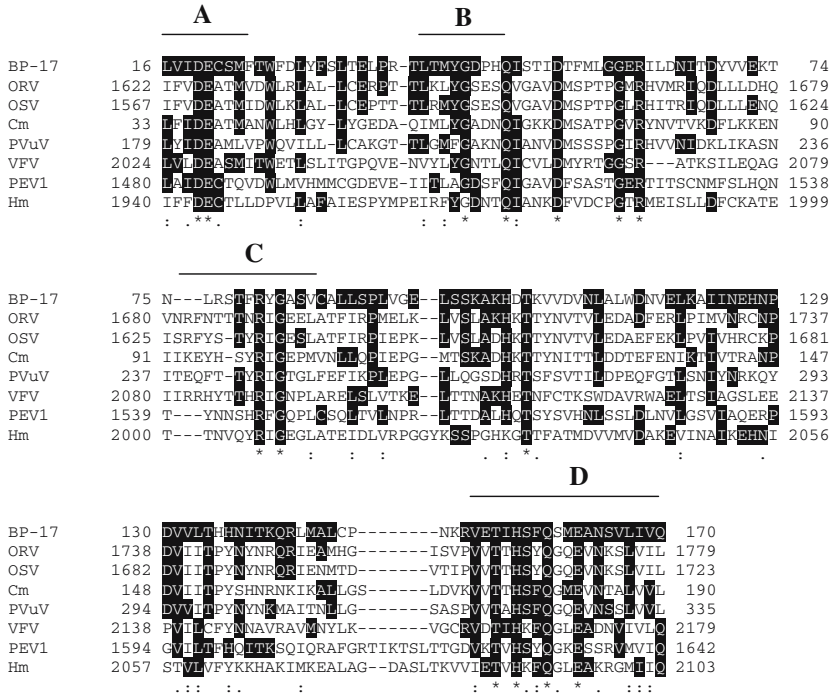

Fig. 2 Multiple alignment of the amino acid sequences of the helicase-like regions encoded by cDNA clone BP-17 from bell pepper dsRNA and other endornaviruses. Abbreviations with Uniprot database accession number in parenthesis are: BP-17 (Q306M8); ORV, *Oryza rufipogon endornavirus* (GenBank YP_438202); OSV, *Oryza sativa endornavirus* (Q40712); Cm, *Cucumis melo* (Q50HX8); PVuV, *Phaseolus vulgaris endornavirus* (Q5KSD1); VFV, *Vicia faba endornavirus* (O82731); PEV, *Phytophthora endornavirus* 1 (Q53I73); Hm, *Helicobasidium mompa* (Q1HAY3). The position of the motifs A–D are indicated by lines above the sequences. Identical residues in all the sequences are shown by asterisks (*) below the sequences. Residues identical to those in the BP-17 are highlighted with a black background and white lettering. The alignment was carried out using the program CLUSTAL W 1.83. (:), conserved substitutions; (.), semi-conserved substitutions

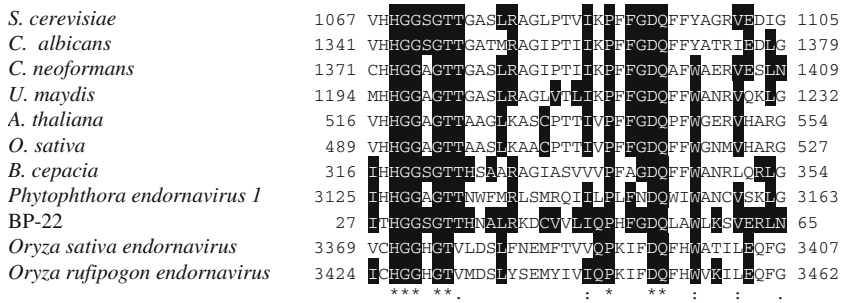

Fig. 3 Multiple alignment of the amino acid derived sequences of the UDP-like regions encoded by cDNA clone BP-22 from bell pepper dsRNA and corresponding amino acid sequences of several organisms. Included organisms with Unitprot database accession numbers in parenthesis are: *Saccharomyces cerevisiae* (Q06321), *Candida albicans* (Q5A950), *Cryptococcus neoformans* (Q5KK25), *Ustilago maydis* (Q8J1H5), *Arabidopsis thaliana* (O23649), *Oryza sativa* (Q8H5W0), *Burkholderia cepacia* (Q0B4B5), *Phytophthora endornavirus 1* (Q53I73), BP-22 (Q0PZF3), *Oryza sativa endornavirus* (Q40712), *Oryza rufipogon endornavirus* (GenBank Y_P438202). The alignment was carried out using the program CLUSTAL W 1.83. Identical residues in all the sequences are shown by asterisks (*) below the sequences. Residues identical to those in the BP-22 are highlighted with a black background and white lettering. (:), conserved substitutions; (.), semi-conserved substitutions

more efficient when reciprocal crosses were conducted between YW and JM than between YW and HW pepper. These two pepper cultivars are infected with two different cryptic viruses [12]. Nevertheless, the presence or absence of cryptic viruses in the F1 generation did not appear to affect the relative amount of BP-dsRNA observed in electrophoretic analyses. The transmission results obtained here with the bell pepper putative endornavirus are consistent with those reported for OSV in rice [16]. It is not known why these two endornaviruses are transmitted at

different rates in different plant genotypes. It has been suggested that this is due to the variable replication efficiency among plant genotypes of these dsRNAs in somatic cells during embryonic development [16]. It is well established that endornaviruses are not graft transmitted. Nevertheless, we wanted to determine if the presence of a conventional virus such as TEV or CMV which readily moves through the vascular tissues could trigger movement of BP-dsRNA as well. Our results suggest that neither, CMV nor TEV helped the movement of BP-dsRNA to JM scions. Molecular hybridization results suggest that the dsRNAs from peppers from five distinct horticultural types are related is not surprising. It is likely that crosses among various pepper genotypes by plant breeders aiming to produce new cultivars, resulted in the spread of the BP-dsRNA to different horticultural types of pepper.

Results obtained from the multiple alignments of the cDNA clone BP-17 amino acid derived sequences indicates that the BP-dsRNA is closely related to the *Endornavirus.*

Furthermore, this region of BP-17 contains motifs typical of RNA helicases and aligned with corresponding motifs of endornaviruses. After a NCBI conserved domain search, a 39 amino acid sequence motif present in clone BP-22 produced significant alignments with bacterial, fungal, plant, and endornavirus glycosyl transferases, similar to a report for PEV1 [7]. As in the case of PEV1, the highest score was obtained with the UDP-glucose:sterol glucosyltransferase of *Ustilago maydis*. This motif is typical of all UGTs and is present in OSV and ORV but not in VFV nor in *Gremmeniella abietina* type B RNA virus.

Data on the transmission and partial nucleotide sequence presented here support that the BP-dsRNA is the genome of a distinct species of the genus *Endornavirus.*

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