***In planta* expression of a protein encoded by the extrachromosomal DNA of a phytoplasma and related to geminivirus replication proteins**

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A new extrachromosomal DNA, EcOYW1, was cloned from the onion yellows phytoplasma (OY-W). Southern blot and PCR analysis showed that EcOYW1 is not present in the OY-M, a mild symptom line derived from OY-W. We determined the complete nucleotide sequence of EcOYW1; it is a circular dsDNA of 7.0 kbp in length, which contains seven ORFs. ORF1 encoded a homologue of the geminivirus Rep protein. Western immunoblot analysis revealed that this Rep homologue is expressed in OY-W infected plants, suggesting that EcOYW1 replicates via a geminivirus-like rolling-circle replication mechanism. EcOYW1 is the first phytoplasmal extrachromosomal DNA shown to express encoded genes.

Keywords: phytoplasma, extrachromosomal DNA, Rep protein, geminivirus

INTRODUCTION

Plant pathogenic phytoplasmas are prokaryotic organisms that have no cell wall and are associated with numerous plant diseases (Kirkpatrick, 1992; McCoy *et al.*, 1989). Due to the difficulty in culturing phytoplasmas *in vitro*, these organisms remain one of the most poorly characterized groups of plant pathogens. Onion yellows phytoplasma (OY), belonging to aster yellows (AY)-clade phytoplasmas, is the causal agent of onion yellows disease, which produces a wide variety of symptoms in its plant hosts, including virescence (excessive greening of floral tissue), yellowing, phyllody (leaf-like petals and sepals), stunting, proliferation and witches' broom (Shiomi *et al.*, 1996). A wild-type line of onion yellows phytoplasma (OY-W) was isolated in Saga Prefecture,

Japan in May 1982 (Shiomi *et al.*, 1996). We previously reported a mild-symptom line (OY-M) that induces milder symptoms than OY-W on the host plant garland chrysanthemum (*Chrysanthemum coronarium*) (Shiomi *et al.*, 1998; Kuboyama *et al.*, 1998). OY-M was isolated from OY-W maintained in the garland chrysanthemum in a greenhouse using the insect vector *Macrostelus strifrons* Anufriew.

Extrachromosomal DNA, including ss- and dsDNA, associated with some spiroplasmas, mycoplasmas and achleplasmas, has been described (Maniloff, 1988; Razin, 1985; Renaudin & Bove, 1994). Extrachromosomal DNA was also found in phytoplasmas (Davis *et al.*, 1988; Sears *et al.*, 1989; Bertaccini *et al.*, 1990; Harrison *et al.*, 1991; Goodwin *et al.*, 1994). The nucleotide sequences of some extrachromosomal DNA from the AY group of phytoplasmas (partial sequence; approx. 200 bp), *Vaccinium* witches' broom (VAC) phytoplasma (approx. 1.5 kbp) and sugarcane white leaf (SCWL) phytoplasma (approx. 2.7 kbp) have been reported (Rekab *et al.*, 1999; Kuske & Kirkpatrick, 1990; Nakashima & Hayashi, 1997). Previously, we

Abbreviations: AY, aster yellows; OY, onion yellows; OY-M, mild symptom line of onion yellows; OY-W, wild-type onion yellows phytoplasma; SCWL, sugar cane white leaf; TGMV, tomato golden mosaic virus; VAC, *Vaccinium* witches' broom.

The GenBank accession number for EcOYW1 is AB010426.

reported a 3.6 kbp DNA fragment of the dsDNA, pOYW1, from OY-W phytoplasma and suggested that pOYW1 is a plasmid replicating by a rolling-circle replication mechanism (Kuboyama *et al.*, 1998).

In other plant-pathogenic bacteria, it is well known that plasmid-encoded genes play important roles in the pathogenicity and virulence (Panopoulos & Peet, 1985). We previously reported that the copy number of pOYW1 was higher in OY-W phytoplasma than OY-M and suggested that this difference might have some relationship to the differences in pathogenicity. However, nucleotide sequence information on pOYW1 alone is not sufficient to deduce its biological significance in the pathogenicity of phytoplasmas.

In this paper, we report another extrachromosomal DNA from OY-W. This replicon was revealed to encode a homologue of geminivirus replication protein Rep; it was not found in OY-M, suggesting that it might also be related to the pathogenicity of OY-W.

METHODS

Phytoplasmas and phytoplasma-infected plants. OY-W and OY-M were used for these experiments. Healthy garland chrysanthemum plants were infected with OY-W or OY-M using the insect vector *M. striifrons* as described previously (Shiomi *et al.*, 1998; Kuboyama *et al.*, 1998).

Extraction of DNA from infected plants. The phytoplasma-enriched fraction of infected garland chrysanthemums was prepared as reported previously (Lee & Davis, 1983). Total DNA was extracted from this fraction following the method of a previous report (Kuboyama *et al.*, 1998).

Cloning and sequencing the extrachromosomal DNA. The total DNA of the phytoplasma-enriched fraction of infected garland chrysanthemum plants was electrophoresed and stained with ethidium bromide. As shown in Fig. 1, several

DNA bands were observed from OY-W and OY-M DNA. In addition to the chromosomal DNA band (indicated by an asterisk), at least four major extrachromosomal DNA bands were observed in lane W, whilst only two bands were observed in lane M.

The largest extrachromosomal DNA band observed in OY-W DNA (lane W), close to a 7.0 kbp open circular DNA size marker, was extracted from agarose gel, digested with *EcoRV* and cloned into the *SmaI* site of pUC18. A clone containing a 2.5 kbp insert (p2.5) was sequenced by the dideoxynucleotide chain termination method with an automatic DNA sequencer (Applied Biosystems 377; Perkin Elmer). Sequence analysis was performed with the BLAST algorithm (Altschul *et al.*, 1990) available through the NCBI server (National Center for Biotechnology Information). The ORFs that encode over 100 aa were used for BLASTP searches of all non-redundant peptide databases (GenBank CDS translations + PDB + SWISS-PROT + PIR + PRF).

To obtain the remaining part of the possibly circular extrachromosomal DNA, we utilized the sequence information of p2.5 to design inverse PCR primers in outward orientations. A pair of inverse PCR primers, P1 (5'-TCT CTT TCT TGT CAA AGC CCC TA-3') and P2 (5'-TCT TCG GGT TCT TGT TTT TCA GTT-3'), and OY-W DNA as a template, were used for inverse PCR. Amplification was performed in a thermal cycler (Perkin Elmer 9700) for 30 cycles, under the following conditions: denaturation for 15 s at 94 °C, annealing for 30 s at 55 °C, and extension for 3 min at 60 °C. The amplified 4.5 kbp fragment was cloned (p4.5) and sequenced. Complete sequencing of p2.5 and p4.5 suggested that the original DNA was a circular 7.0 kbp DNA possessing a single *HindIII* restriction site. Subsequently, from a shotgun library of *HindIII*-digested total DNA of an OY-W phytoplasma-enriched fraction, a single 7.0 kbp clone (p7.0), which hybridized to both p2.5 and p4.5, was obtained. Sequence analysis of p7.0 was performed as described above.

PCR amplification to detect the circular extrachromosomal DNA. To detect EcOYW1 from OY-W or OY-M infected plants, each PCR primer set for the 2.5 kbp or the 4.5 kbp DNA fragment of EcOYW1 was used. The primers P1 and P2 were used for the 4.5 kbp DNA fragment amplification. The primers P3 (5'-AAC CCT GAA ATC TCA TTT GA-3') and P4 (5'-AAC TTT AAC CAC AGG TGC A-3') were synthesized and used to amplify the 2.5 kbp DNA fragment.

Southern blot hybridization. Total DNA extracted from phytoplasma-enriched fractions of OY-W and OY-M infected plants was digested with restriction endonucleases, and Southern hybridization analysis was performed. Transblotting was done using the method described by Sambrook *et al.* (1989). Detection was done with a digoxigenin-labelled probe and a DIG DNA labelling and detection kit (Roche Diagnostics) following the manufacturer's guidelines. Fragments of 306 bp and 354 bp, corresponding to nt 5033–5338 (probe A, Fig. 2) and 6583–6936 (probe B, Fig. 2), respectively, of EcOYW1 were used as probes.

Preparation of anti-ORF1 protein serum. The ORF1 coding region (1041 bp) was amplified by PCR with primers Rep-N (5'-AGAATTCCATAT GAA AAA AAC AAA TAA AGT TAA AAA AGA-3') and Rep-C (5'-TGAGCTCGAGGT TTC GTG GTT TAT ATT TCA TAT TGA-3'), carrying restriction enzyme sites at their 5' ends (underlined). This PCR product was digested with *EcoRI* and *XhoI*, and inserted into pGEX-4T-1 vector plasmid (Amersham Pharmacia Biotech). The 2.1 kbp *PvuII*–*XhoI* fragment of this plasmid was ligated

Fig. 1. Agarose gel electrophoresis of the phytoplasma-enriched total DNA extracted from plants infected with OY-W and OY-M. The extrachromosomal DNA regions are indicated as EC-DNAs. An asterisk indicates the phytoplasma chromosomal DNA band. 7 kbp ocDNA and 7 kbp cccDNA indicate the migration distances of 7 kbp open- and closed-circular DNA size markers, respectively, electrophoresed under the same conditions. W, OY-W; M, OY-M.

Fig. 2. Schematic physical map of EcOYW1. *EcoRV* and *HindIII* cleavage sites are indicated. Putative ORFs are shown on the circle as thick arrows. The repeat sequences R1 and R2 are shown inside the circle as small arrows. A and B denote the regions used as the probe for the Southern blot hybridization shown in Fig. 3(b). Arrowheads P1–P4 indicate the positions and orientations of the PCR primers.

into the *SphI*–*XhoI* site of pET30a vector (Novagen), of which *SphI* site had been filled-in with Klenow. The resulting plasmid encodes a (glutathione *S*-transferase)–(Rep)–(6 × histidine-tag) fusion protein, which was expressed in *Escherichia coli* BL21 after IPTG induction and purified with a His-Bind resin column following the manufacturer's guidelines (Novagen). A rabbit was immunized with the purified protein and antiserum was obtained. IgG was purified from the anti-ORF1 protein antiserum using a Protein A column (Bio-Rad).

SDS-PAGE and Western blot analysis. Total protein was extracted from healthy and OY-W infected garland chrysanthemum plants. The phytoplasma-enriched fraction, prepared as described above, was suspended in 1 ml sonication buffer (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM 2-mercaptoethanol) (Sambrook *et al.*, 1989) and sonicated for 5 min, before separation into soluble and insoluble fractions by centrifugation (20000 g, 15 min); the insoluble fraction was suspended in 1 ml sonication buffer. These samples were electrophoresed into SDS-PAGE gels and analysed using Western immunoblotting (Sambrook *et al.*, 1989).

The fractionated proteins were transferred onto PVDF membranes. These membranes were first reacted with rabbit anti-ORF1 protein IgG, and then with a mouse anti-rabbit Ig conjugated with alkaline phosphatase. Signals were detected using the ECF Western Blotting Reagent Pack (Amersham Pharmacia Biotech) and a Fluoro Imager (Molecular Dynamics). Pre-immune IgG was used as a control. Prestained SDS-PAGE Standards (Bio-Rad) were used as molecular mass markers.

RESULTS

Cloning the extrachromosomal DNA

We cloned the largest extrachromosomal DNA band from the OY-W enriched fraction (Fig. 1, lane W). Clones p2.5 and p7.0, isolated from the *EcoRV*- and *HindIII*-fragment DNA libraries, respectively, and a clone p4.5, amplified by inverse PCR using p2.5 sequence information, were all sequenced. The nucleotide sequence of the extrachromosomal DNA deduced from the slightly overlapping p2.5 and p4.5 clones and that from p7.0 were revealed to be identical. We concluded that these clones are derived from the same

Fig. 3. (a) PCR amplification of a part of EcOYW1. Amplified DNA was electrophoresed in an agarose gel and stained with ethidium bromide. The template DNA is indicated above each lane: Healthy, total DNA extracted from healthy garland chrysanthemum; OY-W and OY-M, total DNA from the phytoplasma-enriched fraction of OY-W- and OY-M-infected plants, respectively. P1–P4 are the primers used for PCR indicated in Fig. 2. (b) Southern blot hybridization of the total DNA from the phytoplasma-enriched fraction of plants infected with OY-W or OY-M. The labels on the lanes indicate which restriction enzyme was used. Probes A and B are EcOYW1 fragments labelled as regions A and B in Fig. 2, respectively. The estimated size of DNA fragments is shown.

Table 1. Summary of the ORFs in EcOYW1

NS, No similarity to any functional protein in the databases.

ORF	Position	Peptide size (aa)	Putative function
ORF1	83–1126	347	Replication
ORF2	3214–3660	148	NS
ORF3	5033–5360	109	NS
ORF4	5510–6070	186	Copy number control
ORF5	6073–6519	148	NS
ORF6	6541–6930	129	NS
ORF7	4749–4348	133	NS

extrachromosomal DNA and named it EcOYW1 (extra-chromosomal DNA 1 of OY-W). EcOYW1, a circular DNA of length 7005 bp (GenBank no. AB010426), is shown schematically in Fig. 2. The G + C content of the nucleotide sequence of EcOYW1 is 21.9 mol%.

EcOYW1 was not found in OY-M

EcOYW1 was cloned from OY-W DNA. DNA extracted from OY-M (Fig. 1, lane M) suggested the absence of this molecule in OY-M. Therefore, the presence of EcOYW1 was examined by PCR using two primer sets specific for p4.5 and p2.5. As shown in Fig. 3(a), neither a 4.5 kbp nor a 2.5 kbp fragment was amplified from healthy plant DNA or OY-M infected plant DNA extracted from the phytoplasma-enriched fraction. Southern blot hybridization was then performed against total DNA of the phytoplasma-enriched fraction using 306 bp and 354 bp DNA fragments of EcOYW1 (regions A and B in Fig. 2) as probes that specifically hybridize 2.5 kbp and 4.5 kbp fragments, respectively. As shown in Fig. 3(b), when OY-W DNA was used, a 7.0 kbp *Hind*III fragment was detected with both probes, and 2.5 kbp and 4.5 kbp *Eco*RV fragments were detected with 2.5 kbp- and 4.5 kbp-fragment-specific probes, respectively. However, when OY-M DNA was used, no signal was detected. These data suggest that EcOYW1 does not exist in OY-M infected tissues.

EcOYW1 encodes a homologue of geminivirus replication protein Rep

We found seven ORFs in the complete nucleotide sequence of EcOYW1 (Fig. 2, Table 1). A search for similar sequences using BLAST showed that the deduced protein encoded by ORF1 shares a high level of similarity with Rep protein, a multifunctional replication protein found in plant viruses belonging to the family *Geminiviridae*. An overall similarity between a protein encoded by ORF1 and a Rep protein of *Tomato golden mosaic virus* (TGMV), a geminivirus, is 66%. Homologous deduced proteins were also reported for the extrachromosomal DNA from SCWL and VAC phytoplasmas (Rekab *et al.*, 1999; Nakashima & Hayashi, 1997). Some of these proteins are aligned in Fig. 4. It was reported

that motifs 1–3 and helices 1 and 2 in the N-terminal domain of the Rep protein of TGMV were very important for replication of geminivirus genomic DNA (Orozco & Hanley-Bowdoin, 1998). These domains were highly conserved in ORF1 of EcOYW1. Specifically, motifs 1 and 3 showed higher similarities of 80% and 83%, respectively. The Tyr residue in motif 3 (indicated by an arrowhead in Fig. 4), which is considered necessary for DNA cleavage, was conserved. In addition, the nucleotide-binding site called the P-loop in the C-terminal domain was also highly conserved with 100% similarity. Therefore, we suggest that the protein encoded by ORF1 functions as a replication-initiation protein.

The Rep homologue encoded by ORF1 is expressed in a phytoplasma-infected plant

To see if ORF1 is expressed *in planta*, we performed a Western immunoblot analysis. As shown in Fig. 5, the anti-ORF1 protein IgG specifically reacted with a 42 kDa protein, which has a molecular mass consistent with the putative ORF1 protein, in the OY-W lane. This suggests that the Rep homologue encoded by ORF1 of EcOYW1 is expressed in plant cells infected by the phytoplasma. This is the first time that the expression of a functional protein encoded by the extrachromosomal DNA of a phytoplasma has been detected.

Other interesting features of the EcOYW1 structure

The deduced protein encoded by ORF4 shares a high level of similarity to *cop*, the plasmid copy number control gene of pIP404, a bacteriocinogenic plasmid of *Clostridium perfringens* (Fig. 6) (Garnier & Cole, 1988). Studies of *C. perfringens* have shown that the *cop* gene functions as a negative regulator of copy number. It is interesting that EcOYW1 encodes two proteins showing similarity to Rep and Cop proteins, which are presently recognized as being part of two different replication regulatory systems. None of the remaining ORFs showed significant similarity to any known proteins.

Similar to the VAC phytoplasma extrachromosomal DNA (Rekab *et al.*, 1999), EcOYW1 contains some

		motif 1	helix 1	helix 2
EcOYW1	1	MKKTNKVKKE	TMFOAQNIFLTYS	CCDLSKEETKNEFIINT
TGMV	1	MESEHPK	...RFOINAKNYFLTY	PCCSLSKESHSQAOINTEINKK
TYLCV	1	ME	...RSG...RSISIAKKNYFLTY	PKCDLTKENALSOHTNLOTETNKL
BCTV	1	ME	...RSP...SFOIAKKNIFLTYP	RCSVTKEDALEITKNTPCSDKL
SCWL	1	MKN	...VDN	...KINYLIIVG
		motif 2		
EcOYW1	60	HKGKHHHVF	QLNKRKTRD	ILNIENHPKNIKEK
TGMV	55	QOP	HLHLVLIQFE	GKYCCONORFEDLVSPTRSAHF
TYLCV	54	GEP	HLHLVLIQFE	GKYCCONORFEDLVSPTRSAHF
BCTV	54	GSL	HLHLVLIQFE	GKYCCONORFEDLVSPTRSAHF
SCWL	15	KKQKRLNVL	LLNLRPCFRKLE	IFNLV
		motif 3		
EcOYW1	120	ROT	TDVNRNYVKKD	GDFIEE
TGMV	96	KSSSDV	KYIDKDGDT	LVWGEF
TYLCV	95	KSSSDV	KYIDKDGDT	LVWGEF
BCTV	95	KSSSDV	KYIDKDGDT	LVWGEF
SCWL	64	SPQERY	EDIDANS	DKVCEV
		P-loop		
EcOYW1	179	NFSKTKN	ETKKL	...KLYAESLEPNYAFKNTKRFKNMAYEHIVES
TGMV	150	LIRE	KTPKML	EFPHN
TYLCV	149	LIRE	KTPKML	EFPHN
BCTV	149	LIRE	KTPKML	EFPHN
SCWL	121	TVEQ	VIDSVE	SELVYRKNYM
ORFB	1	NKNI	IPFNYI	
EcOYW1	235	KKIP	ILICIXDIL	KTKQKEQLSNVISORFKTLIVEGNSKSGKTQ
TGMV	191	TNV	PEDEMRQW	...AENYFGKSSAARPERP
TYLCV	190	DOV	PEDEMRQW	...VSENVMDAAARPWRPVSIVIEGDSRTCKT
BCTV	190	TOV	PEDEMRQW	...ADSYFGVDAAARPFRYNSIIEGDSRTCKT
SCWL	179	ROV	HEHIL	...NHKTKQIKRLN
ORFB	1	NKNI	IPFNYI	
EcOYW1	295	DDV	DES	DENYDEDKY
TGMV	244	GHLD	NSRVMS	SNKVE
TYLCV	242	GHLD	NSRVMS	SNKVE
BCTV	243	GHLD	NSRVMS	SNKVE
SCWL	234	GRIN	FSRRKRD	DDQAEID
ORFB	11	RGSL	DFS	KETIKYKNEVINVEDD
EcOYW1	346	R	...	...
TGMV	301	IPS	IVLCNPG	EGASVYKVELDKE
TYLCV	297	IPS	IVLCNPG	EGASVYKVELDKE
BCTV	300	IPS	IVLCNPG	EGASVYKVELDKE
SCWL	294	KLN	HCINP	...HNSFQKEFDEHPGSKSYLEDKSENNSI
ORFB	71	LNIF	LCNEDIS	FSVRFCKKNKEMGGK
EcOYW1				
TGMV				
TYLCV	354	EE	ASEA	
BCTV	353	QG	QSPL	
SCWL				
ORFB				

Fig. 4. An alignment of the amino acid sequence deduced from ORF1, the Rep protein from an extrachromosomal DNA of SCWL phytoplasma, and the replication proteins from some geminiviruses. TGMV, *Tomato golden mosaic virus*; TYLCV, *Tomato yellow leaf curl virus*; BCTV, *Beet curly top virus* (Worland strain); SCWL, sugarcane white leaf phytoplasma; ORFB, ORFB of VAC phytoplasma. The sequences were aligned with CLUSTAL W (Thompson *et al.*, 1994). Identical or similar residues are highlighted in black. Dots indicate gaps. Several functional motifs of the Rep protein are indicated by bars and the conserved Tyr residue in motif 3 is indicated with an arrowhead.

Fig. 5. Western immunoblot analysis. The sonicated soluble fraction of total phytoplasma-enriched protein was used. Lanes: H, healthy plant total protein (prepared by the same method as OY-W); W, OY-W infected plant. Each membrane was first reacted with pre-immune IgG or anti-ORF protein IgG.

putative prokaryotic (−35 signal) and eukaryotic (TATA box) promoter-like signals upstream from ORF1. Furthermore, two previously unreported, long

repeated sequences are found in EcOYW1 (indicated with small arrows in Fig. 2). A 111 bp sequence (R1) is repeated twice and a 109 bp sequence (R2) is repeated three times. Interestingly, two copies of R1 and R2 are located adjacent to each other, as though R1–R2 is a long inverted repeat in EcOYW1.

DISCUSSION

We characterized EcOYW1, a novel phytoplasma extrachromosomal DNA, which is the largest extrachromosomal DNA reported in phytoplasmas. Previous studies of phytoplasma extrachromosomal DNA might easily have confused the replicative forms of mollicute viruses or co-infected plant DNA viruses with the extrachromosomal DNA (Renaudin & Bove, 1994; Nur *et al.*, 1987). EcOYW1 is definitely a phytoplasma extrachromosomal DNA for several reasons. First, the results of Southern hybridization and electrophoresis were reproducible. Therefore, the OY-W-specific DNA fraction was not the product of contamination. Second, electron microscopic observations did not observe any virus particles (data not shown). Third, although the amino acid sequence of ORF1 shares a high level of similarity with that of

ORF4	1	---MKINDQTKSNKKFIILGLFITGVIL-----VFLIILLLS
Cop	1	MIISKDTKDLIFSILTLFVLCLNLIVFFSYNKAVEVNQSIFILMLLN
ORF4	36	INKLQSKTDIQNQKQVETQQQKETQHVNIPSQEQKIYNDILKKIDKET
Cop	51	LSFTIISLLYLLFLKLVKFPALLNLFGLFSFSSLDLIISLNFYQI
ORF4	86	DKLTIQYPPKTIYQKDGITPKSYETVDSKCKLL-KDTYKPDGKTIYYIA
Cop	101	LSFSKEFIYIFIIFIIYYLFLSFFLTAKKKLKNKNTYSKPINKTIFIIA
ORF4	135	EYDENTNIMIKATYYKEDGKTIDYINEYTPGTGIQIKTTYNQDGTWKVK
Cop	151	ALIG--IVFSKINENIQYYIHSFLMYVFSCIFTFG--FYHIYIYINSVK
ORF4	185	NL
Cop	197	NN

Fig. 6. An alignment of the amino acid sequence deduced from ORF4 and the Cop protein from pIP404 plasmid of *C. perfringens*. The sequences were aligned the same as Fig. 4.

geminiviral Rep and has conserved amino acid motifs, the nucleotide similarity is only 26%. Finally, ORF4 is homologous to the *cop* gene of plasmid pIP404 in *C. perfringens*, which is closely taxonomically related to phytoplasma (Gundersen *et al.*, 1994). We feel that these facts confirm that EcOYW1 is a phytoplasma extra-chromosomal DNA. The G+C content of the nucleotide sequence of EcOYW1 was 21.9 mol%. An extra-chromosomal DNA found in SCWL phytoplasma (Nakashima & Hayashi, 1997) and a plasmid, pOYW1, found in OY phytoplasma (Kuboyama *et al.*, 1998) were also reported to be extremely AT-rich.

Rekab *et al.* (1999) reported geminivirus-related extra-chromosomal DNA from an X-clade phytoplasma. Although they only reported a partial sequence, their ORFB was highly homologous to the C-terminal region of geminivirus replication proteins. Our results imply that both X-clade and AY-clade phytoplasmas (containing OY-W) possess extrachromosomal DNA, which encodes a Rep gene homologue. Furthermore, we confirmed the expression of this Rep homologue protein. This suggests that ORFB of the X-clade-specific extra-chromosomal DNA might also be expressed.

It has been suggested that the geminiviruses originated from a prokaryotic plasmid containing the rolling circle DNA replication initiation protein (Koonin & Ilyina, 1992). In addition, the *Nicotiana tabacum* nuclear genome was reported to carry multiple direct repeats of a geminivirus-related DNA containing the Rep gene region of TGMV (Kenton *et al.*, 1995). These sequences are thought to have arisen during the evolution of *Nicotiana*, from illegitimate recombination following geminivirus infection. Therefore, it appears that Rep homologous sequences have been widely distributed in viruses, mollicutes and plants during evolution. Further experiments are now in progress using antibodies against Rep to clarify whether an ORF1 protein exists both in phytoplasma-infected plants and in insects.

We previously reported that the copy number of a plasmid pOYW1 from OY-W was 4.2 times greater than that from OY-M (Kuboyama *et al.*, 1998). In addition, here we showed that EcOYW1 was not found in OY-M. Thus, our results confirm differences in the extrachromosomal DNA between OY-W and OY-M. However, the relationship between the milder symptoms of OY-M and the absence of the EcOYW1 is still unknown. It

could be possible that the EcOYW1 ORFs with no similarity to any known function encode unknown pathogenicity genes. Further investigation of the extra-chromosomal DNA should enable the elucidation of the mechanism for these intriguing differences in symptoms, and the pathogenicity of phytoplasmas.

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