In the non-insect-transmissible line of onion yellows phytoplasma (OY-NIM), the plasmid-encoded transmembrane protein ORF3 lacks the major promoter region

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‘Candidatus Phytoplasma asteris’, onion yellows strain (OY), a mildly pathogenic line (OY-M), is a phytopathogenic bacterium transmitted by Macrosteles striifrons leafhoppers. OY-M contains two types of plasmids (EcOYM and pOYM), each of which possesses a gene encoding the putative transmembrane protein, ORF3. A non-insect-transmissible line of this phytoplasma (OY-NIM) has the corresponding plasmids (EcOYNIM and pOYNIM), but pOYNIM lacks orf3. Here we show that in OY-M, orf3 is transcribed from two putative promoters and that on EcOYNIM, one of the promoter sequences is mutated and the other deleted. We also show by immunohistochemical analysis that ORF3 is not expressed in OY-NIM-infected plants. Moreover, ORF3 protein seems to be preferentially expressed in OY-M-infected insects rather than in plants. We speculate that ORF3 may play a role in the interactions of OY with its insect host.

INTRODUCTION

Phytoplasmas are phytopathogenic bacteria that cause more than 700 diseases in over 300 plant species (Lee et al., 2000). They lack cell walls and are pleomorphic in shape, with an average diameter of 200–800 nm (Doi et al., 1967; Hogenhout et al., 2008). Phytoplasmas belong to the Mollicutes, and are phylogenetically closely related to achloplasmas (Hogenhout et al., 2008). Because culture of phytoplasmas in vitro has not been achieved, knowledge of their biology is limited (Christensen et al., 2005; Firrao et al., 2007; Hogenhout et al., 2008). Phytoplasmas inhabit phloem sieve elements in infected plants and are transmitted by sap-sucking insect vectors, in particular leafhoppers (Weintraub & Beanland, 2006). After a leafhopper feeds on a phytoplasma-infected plant, the phytoplasmas multiply and spread throughout the insect’s body. After a latency period of 7–80 days, the infected insect can transmit phytoplasmas to healthy plants (Hogenhout et al., 2008; Weintraub & Beanland, 2006). Although some phytoplasmas may be transmitted at a low rate via plant seeds or insect eggs (Botti & Bertaccini, 2006; Cordova et al., 2003; Khan et al., 2003), they are mainly spread via direct inoculation by the insects. Thus, it is crucial to identify factors involved in the host–microbe interaction for understanding their physiology and the mechanism of infection, and developing a strategy for controlling infection.

Onion yellows phytoplasma (OY) is a strain of ‘Candidatus Phytoplasma asteris’ (Oshima et al., 2004). Three lines of OY have been isolated: a severely pathogenic line (OY-W), a mildly pathogenic line (OY-M) and a non-insect-transmissible line (OY-NIM) (Oshima et al., 2001b; Shiomi et al., 1996). Because OY-NIM is derived from

Abbreviations: Amp, antigenic membrane protein; EcOY-DNA, extrachromosomal DNA of OY; OY, onion yellows phytoplasma; OY-M, mildly pathogenic line of OY; OY-NIM, non-insect-transmissible line of OY; OY-W, severely pathogenic line of OY; pOY plasmid, plasmid of OY; RACE, rapid amplification of cDNA ends; Rep, replication initiator protein.

Three supplementary figures are available with the online version of this paper.
OY-M, comparing their genomes should provide clues to identify genes involved in insect transmissibility.

Plasmids often carry genes that are important for bacterial survival, such as antibiotic-resistance genes or genes involved in responses to environmental changes (Thomas, 2004). Many bacteria can adapt to specific environments by acquiring plasmids. In phytopathogenic bacteria, genes involved in pathogenicity, virulence and host specificity are often carried on plasmids (Vivian et al., 2001). Because many phytoplasmas contain plasmids, genes borne on these plasmids may serve important functions (Bai et al., 2006; Liefting et al., 2004, 2006; Nishigawa et al., 2003; Tran-Nguyen & Gibb, 2006).

OY contains several extrachromosomal DNAs that can be classified into two types depending on their replication protein gene (rep). The extrachromosomal DNA (EcOY-DNA) encodes a replication protein homologue to that of geminivirus, whereas the replication protein of plasmid pOY is homologous to that of other bacterial plasmids (Nishigawa et al., 2002a). OY-W contains two EcOY-DNAs (EcOYW1 and EcOYW2) and a pOY plasmid (pOYW) (Kuboyama et al., 1998; Nishigawa et al., 2001, 2002a; Oshima et al., 2001a). OY-M contains both an EcOY-DNA (EcOYM) and a pOY plasmid (pOYM), and OY-NIM also has both an EcOY-DNA (EcOYNIM) and a pOY plasmid (pOYNIM) (Nishigawa et al., 2002a, b, 2003). Interestingly, pOYNIM lacks orf3, which encodes a putative transmembrane protein and is carried on pOYW and pOYM (Nishigawa et al., 2002b). Therefore, we previously hypothesized that ORF3 could be involved in the insect transmissibility of OY (Nishigawa et al., 2002b). However, sequencing EcOYNIM revealed that it included the orf3 gene (Nishigawa et al., 2003), which conflicts with our hypothesis. In this study, we found that ORF3 is not expressed in OY-NIM-infected plants. To investigate the cause of this, we identified the transcriptional start sites and two putative promoter sequences of orf3, and showed that these promoter sequences were deleted or mutated on EcOYNIM. A possible link between the non-expression of ORF3 and the lack of functional orf3 promoters is suggested.

**METHODS**

**Phytoplasma lines.** ‘Candidatus P. asteris’ OY strain was isolated from plants in Saga Prefecture, Japan (Shiomi et al., 1996). Two derivative lines of OY (OY-W and OY-M) were maintained in garland chrysanthemum (Chrysanthemum coronarium) and leafhopper vectors (Macrostelis strifrons) (Oshima et al., 2001b). OY-W-infected plants showed typical symptoms (yellowing, dwarfing and stunting), whereas OY-M-infected plants still produced many lateral shoots and showed only mild leaf yellowing and almost no stunting. OY-NIM was isolated from OY-M by plant grafting without using insect vectors for about 2 years (Oshima et al., 2001b). OY-NIM showed the same symptoms as OY-M, OY-W-, OY-M- or OY-NIM-infected plants were maintained at 25 °C in a greenhouse with a 16 h light/8 h dark photoperiod until used for analyses. OY-W- or OY-M-infected leafhoppers were prepared by feeding on OY-W- or OY-M-infected plants for 40 days. Healthy plants and leafhoppers were used as negative controls.

**Preparation of antibody against ORF3 protein and Western blot analysis.** The orf3 of OY-W is 456 bp in length and encodes a protein of 152 amino acids (17.2 kDa) containing a transmembrane domain at both the N- and C-termini (Nishigawa et al., 2002b). The central, hydrophobic region of ORF3 between the two transmembrane domains (residues 38–119) was expressed by cloning in Escherichia coli. A 246 bp fragment of orf3 was amplified from pOYW by PCR using primers ORF3a (5′-AGA ATT CCA TAT GAT TAA AGA TAA AAA TAA AGT TGA AAC CCA-3′) and ORF3b (5′-TGA GCT CGA GTA CGA CTG CGA TAT CTT TAA TTA ATT-3′). The PCR product was digested with both Ndel and Xhol, and cloned into pET-30a (Novagen). The histidine-tagged ORF3 protein was expressed in E. coli BL21-CodonPlus (DE3)-RIL cells (Stratagene). The cell extracts were applied to a nickel-NTA column (Novagen), washed with TBS buffer (20 mM Tris/HCl, pH 7.9 and 500 mM NaCl), and the fusion protein was eluted with TBS buffer containing 1 M imidazole.

**Immunohistochemical analysis.** To confirm the expression level of ORF3, three antibodies were used in immunohistochemical analyses: anti-Amp (against an antigenic membrane protein of OY-W; Kakizawa et al., 2004), anti-Rep (against the replication protein encoded on pOYW; Oshima et al., 2001a) and anti-ORF3 (this study). Concentrations of the anti-Amp, anti-Rep and anti-ORF3 antibodies used in plant tissue sections were 1, 100 and 100 μg ml⁻¹, respectively, and those used in insect tissue sections were 1, 100 and 1 μg ml⁻¹, respectively.

To avoid sampling errors (such as effects of differences in individuals, protein expression only in restricted tissues, or sampling of healthy insects), five plants that showed typical symptoms of OY-W, OY-M or OY-NIM, and 15 insects sucking on OY-W- or OY-M-infected plants were collected.

Sections (~2 cm in length) of stems were excised from OY-W-, OY-M- or OY-NIM-infected garland chrysanthemum, respectively. We used the OY-NIM-infected plants sampled in 1999. The stem pieces were fixed, embedded in paraffin, and cut into 8 μm sections with a PR-50 microtome (Yamato Scientific). OY-W- or OY-M-infected insects were sectioned by the same methods. The continuation of this method was performed according to Oshima et al. (2001b).

An Axio Imager Z1 microscope equipped with an AxioCam HRc camera controlled by AxioVision Rel. 4.6 software (equipped and software from Carl Zeiss MicroImaging) was used to collect images. Signal intensity was quantified using Adobe Photoshop version 7.0 (Adobe Systems) and ImageJ software (National Institutes of Health). Results are expressed as the mean ± standard error. The significance of the difference between the mean values of the groups was evaluated by Student’s t test with Stactel software (OMS Publishing).

**RNA isolation.** Isogen reagent (Nippon Gene) was used to isolate total RNAs from OY-M-infected and healthy insects. To eliminate DNA contamination, the total RNAs were treated with DNase I (Takara) prior to using for cDNA synthesis, RT-PCR or 5’-RACE.

**RT-PCR.** Reverse transcription was performed using random primers and Reverse Transcriptase XL-AMV (Takara). Subsequent PCR amplification was performed using LA-Taq DNA polymerase and primers (Table 1). The amplified PCR products were visualized by agarose gel electrophoresis.
Table 1. Oligonucleotide primers for RT-PCR and 5΄-RACE

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Annealing site</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcOYM/ORF1(F)</td>
<td>AAGATTCCTATGAGATTTCAATTATACATCAA</td>
<td>5΄ end of orf1</td>
</tr>
<tr>
<td>EcOYM/ORF1(R)</td>
<td>CGAGCTGAGGAGGTGTTTTTTATAATCTTTTTTTA</td>
<td>3΄ end of orf1</td>
</tr>
<tr>
<td>EcOYM/ORF1-nested(R)</td>
<td>TGAGCTGAGTGTGTTTTTTTAATTCTGTTAGAG</td>
<td>Within orf1</td>
</tr>
<tr>
<td>EcOYM/ORF2(F)</td>
<td>AGAATTTGATGAAAGAAAATATAATTTTTA</td>
<td>5΄ end of orf2</td>
</tr>
<tr>
<td>EcOYM/ORF2(R)</td>
<td>CGAGCTGAGAATATTAAATTTCTTTTTACAGT</td>
<td>3΄ end of orf2</td>
</tr>
<tr>
<td>EcOYM/ORF2-nested(R)</td>
<td>TGAAGCTGAGGACCTGCGGTGTTAATCCTTAGTATAG</td>
<td>Within orf2</td>
</tr>
<tr>
<td>EcOYM/ORF3(F)</td>
<td>AGAATTTGATGAAAGAAAATATAATTTTTA</td>
<td>5΄ end of orf3</td>
</tr>
<tr>
<td>EcOYM/ORF3(R)</td>
<td>CGAGCTGAGAGCTAATAGAGACAGAGGAGGAGCT</td>
<td>3΄ end of orf3</td>
</tr>
<tr>
<td>EcOYM/ORF3-nested(R)</td>
<td>TGAAGCTGAGGAGCTGAGCTGTGATGAGCAGTTACT</td>
<td>Within orf3</td>
</tr>
<tr>
<td>EcOYM/ORF3-nested2(R)</td>
<td>TGAAGCTGAGGAGCTGAGCTGAGCTGAGCTGCT</td>
<td>5΄ end of orf4</td>
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<tr>
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<td>AAGATTTGATGAAAGAAAATATAATTTTTA</td>
<td>5΄ end of orf4</td>
</tr>
<tr>
<td>EcOYM/ORF4(R)</td>
<td>TGAGCTGAGGAGCTAATAGAGACAGAGGAGGAGCT</td>
<td>3΄ end of orf4</td>
</tr>
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5΄-RACE and promoter prediction. To identify the 5΄ end of mRNA derived from orf3, 5΄-RACE analysis was performed using the Gene Racer kit (Invitrogen). First, 7 μg total RNA from OY-M-infected insects was treated with tobacco acid pyrophosphatase to eliminate two of the three RNA 5΄-phosphates. Gene Racer 5΄-RNA oligonucleotides (5΄-CGA CUG GAG GAC GAG ACU GAC AUG GAC UGA AGG AGU AGA AA-3΄) were ligated to the total RNA using T4 RNA ligase, and the total RNA was reverse-transcribed using Cloned Avian Myeloblastosis Virus Reverse Transcriptase and random primers. GeneRacer 5΄-primers (5΄-CGA CTG GAG CAC GAG GAC GAC ACT GA-3΄) and gene-specific primers (Table 1) were used for initial PCRs. GeneRacer 5΄-nested primer (5΄-GGA CAC TGA CAT GGA CTC GAG TA-3΄) and gene-specific nested primers (Table 1) were used for nested PCRs. The nested PCR products were visualized by agarose gel electrophoresis. They were cloned using the pGEM-T Easy vector system 1 (Promega). Nucleotide sequences were determined by the dideoxynucleotide chain-termination method using an ABI Prism 3130 DNA sequencer (Applied Biosystems). We identified the 5΄ end of each nested PCR product as the putative transcriptional start site of orf3. The −10 and −35 regions of the orf3 and amp gene promoters were predicted by the Mulligan method (Mulligan et al., 1994) using GENETYX-MAC software (GENETYX).

RESULTS

Immunohistochemical analysis of ORF3

To compare expression levels and distributions of Amp, Rep and ORF3 proteins in phytoplasma-infected plants, immunohistochemical analyses were performed using individual antibodies.

In the OY-W-infected and the OY-M-infected plants, blue signals were observed in the phloem tissues of the main stems by anti-Amp, anti-Rep and anti-ORF3 antibodies (at concentrations of 1, 100 and 1 μg ml−1, respectively) (Fig. 1a, b, d, e, g and h). The signal intensities of ORF3 were significantly lower than those of Rep (P<0.0005; Fig. 1j) and approximately equal to background level (data not shown), suggesting that OY-NIM does not express ORF3 protein. Similar results were also obtained from the OY-W-infected plants (20/20 sections; 100 %), the OY-M-infected plants (19/20 sections; 95 %), and the OY-NIM-infected plants (20/20 sections; 100 %).

Immunohistochemical analyses were also performed using phytoplasma-infected insects. In the OY-W-infected and the OY-M-infected insects, blue signals were observed in the guts, salivary glands and fat bodies by anti-Amp, anti-Rep and anti-ORF3 antibodies (at concentrations of 1, 100 and 1 μg ml−1, respectively) (Fig. 2b, c, e, f, h and i). The signal intensities of ORF3 were higher than those of Rep despite the lower antibody concentration (P<0.005; Fig. 2j). Similar results were also obtained from the OY-W-infected insects (20/20 sections; 100 %) and the OY-M-infected insects (19/20 sections; 95 %).

Analysis of the transcriptional units of orf3

To detect transcription products of EcOYM-encoded genes (orf1–5), RT-PCR analysis was performed using total RNA isolated from OY-M-infected insects as the template. All orf fragments were amplified (Supplementary Fig. S2), indicating that all of these genes were transcribed in phytoplasma cells. We also checked that no fragment was amplified without reverse transcription, to prove the absence of DNA contamination in the RNA preparations (Supplementary Fig. S2). To identify a transcriptional unit of orf3, we performed RT-PCR analyses of intergenic regions of orf1–2, orf2–3 and orf3–4 (Fig. 3a). All orf fragments were amplified (Fig. 3b), suggesting that each orf was co-transcribed with its neighbouring genes. However, the orf1–2 fragment was amplified at lower levels than the others. These results were repeatedly observed in all of three experiments, suggesting that orf1 would be transcribed alone while orf3 would be co-transcribed with orf2 and orf4.
Identification of the putative transcriptional start sites of orf3 by 5’-RACE

To identify the 5’ end of mRNA derived from orf3, 5’-RACE analysis was performed using total RNA isolated from OY-M-infected insects. Random primers were used for reverse transcription, and adaptor primers and gene-specific primers were used for initial and nested PCRs (Table 1). The nested PCR products were cloned and sequenced.

PCRs were performed using an initial primer and a nested primer that anneal to the 3’ end and within orf3, respectively (Fig. 4b, row 4). Two fragments were amplified (Fig. 4a, lane 4), suggesting that orf3 is transcribed from two putative transcriptional start sites.

To investigate whether orf3 transcription starts at the upstream region of orf1, PCRs were performed with an initial primer at the 3’ end of orf3 and a nested primer at the 3’ end of orf1. Two fragments, of ~300 bp and ~1.8 kbp, were amplified (Fig. 4a and b, lane 1). Sequencing these fragments revealed that the 300 bp fragment was orf1 while the 1.8 kbp fragment was a non-specific product, identified as insect rDNA. PCR amplification with an initial primer at the 3’ end of orf2 and a nested primer at the 3’ end of orf1 yielded a ~500 bp fragment identified as orf1 (Fig. 4a and b, lane 5). Similarly, amplification with an initial and a nested primer located respectively at the 3’ end of orf1 and within orf1 yielded two products, of ~400 bp and ~600 bp, respectively (Fig. 4a and b, lane 7). The 400 bp sequence was identical to that of orf1 whereas the 600 bp fragment was similar to insect rDNA. As a whole these results showed that all mRNA transcripts carrying orf1 sequences start within orf1 and suggest that one of the putative transcriptional starts of orf3 is located within orf1.

To investigate whether orf3 transcription starts at the upstream region of orf2, PCRs were performed using an initial primer at the 3’ end of orf3 and two nested primers at the 3’ end of orf2 and the 5’ end of orf3, respectively (Fig. 4b, rows 2 and 3). Both PCR amplifications yielded a ~650 bp product (Fig. 4a, lanes 2 and 3). Sequences of these 650 bp fragments were identical to that of orf2, and their 5’ ends started at the orf1–2 intergenic region. This
result suggests that a putative transcriptional start site of orf3 is located in the orf1–2 intergenic region in addition to the one within orf1.

Finally, PCRs were performed using an initial primer and a nested primer that anneal to the 3' end of orf2 and within orf2, respectively (Fig. 4b, row 6). As a result, ~600 bp and ~1 kbp fragments were amplified (Fig. 4a, lane 6). The sequence of the 600 bp fragment was identical to that of orf2, and its 5' end started at the orf1–2 intergenic region. The sequence of the ~1 kbp fragment was identical to that of orf1–2, with its 5' end starting within orf1. Taken together, these results indicate that orf3 transcription starts from two distinct promoters located at the 5' end of orf1 and within the orf1–2 intergenic region, respectively.

We sequenced 37 clones encoding inserts whose 5' ends started at the inside of orf1 (Fig. 4a, lane 1) and 59 clones encoding inserts whose 5' ends started at the orf1–2 intergenic region (29 and 30 clones in Fig. 4a, lanes 2 and 3, respectively). The positions of the 5' ends of nested PCR products (putative transcriptional start sites) are shown in Fig. 5(a). Although multiple 5' ends of the orf3 mRNA were detected, residues A located 46 bp downstream of the orf1 5' end, and C located 49 bp downstream of the 5' end of the orf1–2 intergenic region were considered as the main transcriptional starts by a majority decision (Fig. 5a, asterisk).

Promoter prediction and comparative analysis

We predicted orf3 promoters using a promoter-prediction program, and compared their sequences between EcOYM and EcOYNIM. The putative promoters were identified in the upstream region of the two putative transcriptional start sites of orf3 (Fig. 5a and b). We designated the promoter located within orf1 as ORF3-pro1, and that located in the orf1–2 intergenic region as ORF3-pro2. In the ORF3-prol sequences, there were two nucleotide differences between EcOYM and EcOYNIM (accession nos AB076263 and AB097150; Fig. 5b). Surprisingly, the 157 bp sequence containing ORF3-pro2, present in EcOYM, was deleted in EcOYNIM (accession nos AB076263 and AB097150; Fig. 5b).

Sequence comparisons of the −10 and −35 regions of ORF3-pro2 with those of the 16S rDNA (Jung et al., 2003) and amp gene promoters of OY-M predicted with the same program revealed five nucleotide differences (Fig. 5b).

DISCUSSION

Characterization of the 5' ends of orf3 mRNA transcripts indicates that orf3 is transcribed from two distinct promoters, ORF3-pro1 and ORF3-pro2, located at the 5' end of orf1 and in the orf1–2 intergenic region, respectively. ORF3-pro2 appears to be the major orf3 promoter for three reasons: the orf1–2 intergenic region was poorly amplified
by RT-PCR (Fig. 3b, orf1–2), the orf1–3 transcription product was not always detected by 5'-RACE whereas the orf2–3 was consistently detected using all primer sets tested (Fig. 4a, lanes 2 and 3), and the transcription products from the orf1–2 intergenic region were detected more than that from within orf1 by competitive 5'-RACE for forward primer (Fig. 4a, lanes 4 and 6).

For each of the two promoter regions, multiple transcriptional starts were detected (Fig. 5a). This could result from slipping of RNA polymerase or, alternatively, from limited degradation of the mRNA 5' ends. Such heterogeneity of transcription initiation sites has also been reported in Mycoplasma pneumoniae (Sorensen et al., 1993; Wagner et al., 1990; Weiner et al., 2000; Wilson et al., 1987, 1992; Xiong & Reznikoff, 1993).

Homologues of orf1–3 on EcOYM are carried on pOYM (Supplementary Fig. S3; Nishigawa et al., 2003). Because the nucleotide sequence identities of orf1 and orf2 on EcOYM and pOYM are 89.3 % and 73.8 %, respectively, we were able to determine the targeted plasmid, i.e. EcOYM or pOYM, of the 5'-RACE products. However, the nucleotide sequences of orf3 encoded on EcOYM and pOYM are identical and could not be distinguished. When 5'-RACE was performed with initial and nested primers annealing respectively to the 3' and 5' ends of orf3, a single fragment was detected (Fig. 4a, lane 3). Although this fragment was expected to contain two distinct products, derived from EcOYM and pOYM, all of the 30 clones sequenced contained the orf2 of EcOYM. This result suggested that orf3 was mainly expressed from EcOYM rather than pOYM. It remains unclear why the homologue of orf3 is carried on pOYM despite its low expression level.
Relationship between ORF3 and insect transmissibility

We found that promoter sequences of \textit{orf3} were mutated or deleted on EcOYNIM (Fig. 5b). It has been previously demonstrated that both \textit{orf3} and \textit{orf4} are absent from pOYNIM (Nishigawa \textit{et al.}, 2002b). These results suggest that the expression level of ORF3 may be significantly lower in OY-NIM than in OY-M. This hypothesis is consistent with the fact that ORF3 was not detected in the OY-NIM-infected plants (Fig. 1i and j).

The function of ORF3 remains unclear but it is probably required for phytoplasmal survival in insects rather than plants, because OY-NIM can survive in plant hosts despite the lack of the ORF3 product. ORF3 is thought to be a transmembrane protein because two hydrophobic regions at the N- and C-terminus, and a Sec-translocation signal sequence were predicted (Nishigawa \textit{et al.}, 2002b). Therefore, ORF3 would localize to the cell surface of the phytoplasma, with its hydrophilic domain accessible to interactions with host components. In general, phytoplasmal transmembrane proteins such as ORF3 are thought to play a crucial role in the interaction with hosts, because phytoplasmas lack cell walls and reside intracellularly. It will be interesting to elucidate the interactions between ORF3 and insect proteins.

Several surface proteins involved in insect transmissibility have been reported in the phytopathogenic bacterium \textit{Spiroplasma citri}. Among them, P58 and SARP1 are thought to play a role in adherence and invasion of insect cells (Berg \textit{et al.}, 2001; Ye \textit{et al.}, 1997; Yu \textit{et al.}, 2000). Also, spiralin is required for efficient transmission by the vector insect (Duret \textit{et al.}, 2003; Killiny \textit{et al.}, 2005). P32 encoded on a \textit{S. citri} plasmid has been associated with insect transmissibility (Berho \textit{et al.}, 2006a, b; Killiny \textit{et al.}, 2006). It has also been reported that the plasmids of \textit{Borrelia
burgdorferi have important functions for survival of the bacteria in the arthropod vector (Hoviis et al., 2007). B. burgdorferi, the causative agent of Lyme disease, parasitizes animals and is predominantly transmitted by arthropod vectors. The surface protein OspA encoded on the B. burgdorferi linear plasmid is essential for the colonization and survival in tick midguts (Neelakanta et al., 2007; Stewart et al., 2005; Yang et al., 2004). The surface protein OspC encoded on the B. burgdorferi circular plasmid is involved in the invasion of and attachment to tick salivary glands (Grimm et al., 2004; Pal et al., 2004; Stewart et al., 2005). These reports suggest that bacterial plasmids often play an important role in survival in arthropod vectors. The results of this study suggest that the plasmid-encoded orf3 gene may be involved in the survival of OY in insect hosts. Although it remains unclear why determinants of insect transmissibility are carried on plasmids, our results suggest that, as in other bacteria, OY plasmids could be involved in insect transmissibility of the phytoplasma. However, an ORF3 homologue is not found in the whole genomic sequence of ‘Candidatus Phytoplasma mali’ (data not shown: Kube et al., 2008). Additionally, several strains of ‘Candidatus P. asteris’ do not contain plasmids. Further analyses are needed to elucidate the relationships between phytoplasma plasmids and insect transmissibility.

It was recently reported that Amp of OY-W interacted with microfilaments of the vector insect and that the formation of Amp–microfilament complexes was required for insect transmissibility (Suzuki et al., 2006). For successful transmission, phytoplasmas must pass through the intestine, enter the haemocoel and infect several tissues including the salivary glands (Hogenhout et al., 2008; Webb et al., 1999). Besides Amp, many other factors are considered to be required in each of the steps (Purcell, 1982), and ORF3 could be one of them. The complete nucleotide sequences of several phytoplasma plasmids have been reported, including those of beet leafhopper-transmitted virescence agent (BLTVA), tomato big bud (TBB), ‘Candidatus Phytoplasma australiense’, and aster yellows phytoplasma strain of witches’ broom (AY-WB) (Bai et al., 2006; Liefing et al., 2004, 2006; Tran-Nguyen & Gibb, 2006). Among them, only the plasmids of ‘Candidatus P. australiense’ and AY-WB encode orf3 homologues. Therefore, it is likely that factors involved in the adaptation to insect hosts differ among phytoplasma strains. This may be related to the large diversity of insect vectors among phytoplasma strains (Weintraub & Beanland, 2006).

Environmental response system of phytoplasma

Immunological studies revealed that Amp protein was highly expressed regardless of the host (Kakizawa et al., 2004). Similarly, the Rep protein was equally expressed in plant and insect tissues, although to a lower level. In contrast, whereas in OY-W- and OY-M-infected plants ORF3 and Rep were equally expressed (Fig. 1j), the expression level of ORF3 in OY-W- and OY-M-infected insects was higher than that of Rep, in spite of the lower antibody concentration used (Fig. 2j). Even though quantification of the proteins cannot be achieved from these immunohistochemical analyses, the results clearly indicate that ORF3 is preferentially expressed in insects rather than in plants, suggesting the ability for the phytoplasmas to adapt to different intracellular environments. This also suggests that phytoplasmas might possess an environmental response system.

Promoters and transcriptional factors of phytoplasmas

We predicted putative promoters of both the orf3 and amp genes, and compared them with the putative promoter of the 16S rRNA gene of OY-M (Jung et al., 2003). The promoter sequence of the amp gene was similar to that of the 16S rRNA gene, and their −10 sequences were identical (Fig. 5b). However, the promoter sequence of orf3 differed from those of the 16S rRNA and amp genes, which were thought to be expressed constitutively. Our immunohistochemical analyses suggest a different expression of ORF3 between plant and insect hosts, which may be related to the differences in promoter sequence (Figs 1 and 2). Taking into account the high expression levels of ORF3 in insect cells (Fig. 2h and i), the orf3 promoter might function specifically when phytoplasma infects an insect vector.

The σ (sigma) factor of RNA polymerase targets transcription initiation to specific promoter sequences (Wade et al., 2006). Most bacteria have multiple σ factors that are required for complex cellular processes such as stress responses, morphogenesis and virulence (Gruber & Gross, 2003). Although mycoplasma and phytoplasma genomes encode few σ factors (Fraser et al., 1995), at least two σ factors, rpoD and fltA, were identified in the OY-M genome (Oshima et al., 2004). These σ factors might be involved in changing gene expression. Further analyses are necessary to elucidate the relationships between promoter sequences and σ factors in phytoplasmas.

Conclusions

We have shown that the non-insect-transmissible line OY-NIM does not express ORF3, probably due to the loss of DNA sequences including the major transcription promoter. These findings suggest that ORF3 might be a key element for the OY phytoplasma to adapt to its insect host. However, further analyses will be required to determine whether ORF3 is essential for insect transmission.

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