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 *Macrostelus 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.

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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 achleplasmas (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, extra-chromosomal 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; Liewing *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 chrysanthemums (*Chrysanthemum coronarium*) and leafhopper vectors (*Macrostelus striifrons*) (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, hydrophilic 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 *Nde*I and *Xho*I, and cloned into pET-30a (Novagen). The histidine-tagged ORF3 construct 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. The molecular mass of the purified ORF3 protein was estimated by SDS-PAGE as approximately 11 kDa (see Supplementary Fig. S1, available with the online version of this paper), which is approximately equal to that of the central domain. An antibody against the partial ORF3 protein was generated as previously described (Kakizawa *et al.*, 2001). The reactivity of the anti-ORF3 antibody was confirmed by Western blotting analysis against the purified ORF3 protein by a previously reported method (Kakizawa *et al.*, 2004).

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 (equipment 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 Statcel 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

Primer	Sequence	Annealing site
EcOYM/ORF1(F)	AGAATTCATATGCAGATATTCAATTATAACATAA	5' end of <i>orf1</i>
EcOYM/ORF1(R)	CGAGCTCGAGGGGGTTTTTTTATAATCTTTTTTA	3' end of <i>orf1</i>
EcOYM/ORF1-nested(R)	TGAGCTCGAGTGGTTGGGTTAATTCTGATTTAGGA	Within <i>orf1</i>
EcOYM/ORF2(F)	AGAATTCATATGAAAACCAATAATCAAAAAAAT	5' end of <i>orf2</i>
EcOYM/ORF2(R)	CGAGCTCGAGATAAATTAATCTTGTTTAACAGTT	3' end of <i>orf2</i>
EcOYM/ORF2-nested(R)	TGAGCTCGAGACCCCTCGGGGTTGAACCTCCCTAATAG	Within <i>orf2</i>
EcOYM/ORF3(F)	AGAATTCATATGAATAAAAAAGAAAAATTATATTAA	5' end of <i>orf3</i>
EcOYM/ORF3(R)	TGAGCTCGAGAGCTAATAAAGCAAGAGGAGCTGCT	3' end of <i>orf3</i>
EcOYM/ORF3-nested(R)	TGAGCTCGAGAGCTGCACCTGTGGCAAGAGTTACT	Within <i>orf3</i>
EcOYM/ORF3-nested2(R)	TGAGCTCGAGTGCAATAAAGATAGTCCACATTATT	Within <i>orf3</i>
EcOYM/ORF4(F)	AGAATTCATATGTTAAATAAAGTACAATAATCG	5' end of <i>orf4</i>
EcOYM/ORF4(R)	TGAGCTCGAGAGCTAATAAAGCAAGAGGAGCTGCT	3' end of <i>orf4</i>

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 CAC GAG GAC 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 ACT GA-3') and gene-specific primers (Table 1) were used for initial PCRs. GeneRacer 5'-nested primer (5'-GGA CAC TGA CAT GGA CTG AAG 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 I (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 a 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.*, 1984) 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 100 µg ml⁻¹, respectively) (Fig. 1a, b, d, e, g and h). The signal intensities of ORF3 were approximately equal to those of Rep at the same antibody concentrations (Fig. 1j). In the OY-NIM-infected plants, specific signals were also observed by anti-Amp and anti-Rep antibodies (Fig. 1c and f), but not by anti-ORF3 antibody (Fig. 1i). 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*.

Fig. 1. (a–i) Immunohistochemical analyses of phytoplasma-infected garland chrysanthemum tissue sections. (a, d, g) OY-W infected plants, (b, e, h) OY-M infected plants, (c, f, i) OY-NIM infected plants. (a–c) Anti-Amp antibody, (d–f) anti-Rep antibody, (g–i) anti-ORF3 antibody. Each phytoplasma-infected plant was serially sectioned at 8 µm thickness. X and P indicate xylem and phloem. Blue signals represent the presence of phytoplasma. Scale bars, 0.1 mm. (j) Comparison of relative signal intensities detected by immunohistochemical analyses of phytoplasma-infected plants. The relative signal intensities are means of 20 sections, with standard error of the mean. Black and grey bars indicate relative signal intensities of Rep and ORF3, respectively. Their values are normalized to that of Amp. ** indicates a value that is statistically significant ($P < 0.0005$, Student's *t* test).

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

Fig. 2. (a–i) Immunohistochemical analyses of leafhopper (*Macrostelus striifrons*) tissue sections. (a, d, g) Healthy insects, (b, e, h) OY-W infected insects, (c, f, i) OY-M infected insects. (a–c) Anti-Amp antibody, (d–f) anti-Rep antibody, (g–i) anti-ORF3 antibody. Each phytoplasma-infected insect was serially sectioned at 8 µm thickness. Blue signals represent the presence of phytoplasma. Scale bars, 0.5 mm. (j) Comparison of relative signal intensities detected by immunohistochemical analyses of phytoplasma-infected insects. Details as for Fig. 1(i). * and ** indicate values that are statistically significant ($P < 0.005$ and $P < 0.0005$, respectively, Student's t test).

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' ends 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-pro1 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

Fig. 3. Analysis of the transcriptional units of *orf3*. (a) Schematic representation of the intergenic regions on EcOYM amplified by RT-PCR. Lines and numbers represent the regions amplified by RT-PCR and the expected lengths, respectively. (b) Transcriptional analysis of the intergenic regions on EcOYM. RT-PCR of the intergenic regions on EcOYM was performed using total RNA isolated from OY-M-infected insects. To prove the absence of DNA contamination in the total RNA, PCR analysis of the OY-M *amp* gene was performed without reverse transcription (negative control).

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

Fig. 4. Analysis of the 5' ends of *orf3* mRNA transcripts. (a) 5'-RACE analysis using total RNA isolated from OY-M-infected insects. Lane numbers and symbols (open and filled circles, squares, triangles and diamonds) to the right of fragments correspond to those in (b). (b) Schematic representation of the EcOYM regions amplified by 5'-RACE. Each number on the left corresponds to a lane in (a). Black and white arrowheads indicate initial and nested primers, respectively. Symbols correspond to those in (a). The solid line and solid line plus dotted line correspond to the regions amplified by nested and initial PCRs, respectively. The fragments to the left of the filled circle in lane 1 and the filled diamond in lane 7 of (a) were non-specific bands (rDNA of insects); these two symbols are not shown in (b).

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.

Fig. 5. Identification of the putative transcriptional start sites and promoters of *orf3*. (a) Schematic representation of the putative transcriptional start sites of *orf3*. Nucleotide sequences indicate the regions surrounding the 5' ends of mRNA derived from *orf3*. The two graphs represent numbers of 5' ends of the clones resulting from cloning and sequencing of 5'-RACE products, and the 5' ends correspond to the nucleotide sequences indicated under the graphs. Asterisks indicate the nucleotide positions corresponding to the 5' ends of the most abundant clones. Underlined sequences indicate the putative -35 and -10 regions of ORF3-pro1 and ORF3-pro2. (b) Comparison of the OY gene promoters. The promoter sequences of *orf3* are compared with those of the 16S rRNA and *amp* genes. Nucleotide differences are indicated in bold.

Relationship between ORF3 and insect transmissibility

We found that promoter sequences of *orf3* were mutated or deleted on EcOYNIM (Fig. 5b). It has been previously demonstrated that both *orf3* and *orf4* are absent from pOYNIM (Nishigawa *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 *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 *Spiroplasma citri*. Among them, P58 and SARP1 are thought to play a role in adherence and invasion of insect cells (Berg *et al.*, 2001; Ye *et al.*, 1997; Yu *et al.*, 2000). Also, spiralin is required for efficient transmission by the vector insect (Duret *et al.*, 2003; Killiny *et al.*, 2005). P32 encoded on a *S. citri* plasmid has been associated with insect transmissibility (Berho *et al.*, 2006a, b; Killiny *et al.*, 2006). It has also been reported that the plasmids of *Borrelia*

burgdorferi have important functions for survival of the bacteria in the arthropod vector (Hovius *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 phytoplasmal 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; Liefting *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 *fliA*, 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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