Bulletin of Insectology ***60*** *(2): 105-107, 2007*
ISSN 1721-8861

Host-parasite interaction of phytoplasmas from a molecular biological perspective

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Abstract

Phytoplasmas are plant pathogenic bacteria that are transmitted by insects. We examined several molecular biological findings involved in both plant–phytoplasma interactions and insect–phytoplasma interactions. (i) The complete genomic sequence of the phytoplasma suggested that the genome encodes very few metabolic functions, implying that the consumption of metabolites by phytoplasmas in plants may cause disease symptoms. (ii) The approximately 30-kb region, including glycolytic genes, was tandemly duplicated in the genome of a severe pathogenic phytoplasma. The presence of two glycolytic gene clusters suggested that a higher consumption of the carbon source may affect the growth rate of the phytoplasma and may also directly or indirectly cause more severe symptoms. (iii) Positive selection was recognised on Amp, a surface membrane protein of the phytoplasma. This positive selection may reflect an interaction between the phytoplasma and the host cytoplasm. (iv) An affinity column assay showed that Amp formed a complex with insect microfilament proteins. This interaction was correlated with the phytoplasma-transmitting capability of leafhoppers.

Key words: immunodominant membrane protein, phytoplasma, positive selection, reductive evolution.

Introduction

Phytoplasmas comprises a group of phytopathogenic bacteria in the class *Mollicutes*, which are transmitted from plant to plant by sap-feeding insect vectors (leafhoppers), infect more than 700 plant species and cause symptoms such as stunting, yellowing, witches' broom (proliferating shoots), phyllody (leaf-like petals and sepals), virescence (greening of floral organs), and sometimes withering of plants (Lee *et al*., 2000). They propagate within the cytoplasm of both insects and plants. In plants, they exclusively inhabit nutrient-rich phloem tissues. Phytoplasmas are cell wall-free prokaryotes, and both their cell size (0.1–0.8 μm in diameter) and genome size (0.5–1.3 Mbp) are the smallest among the bacteria. Their metabolic pathways and host interactions are of interest for the agricultural and basic sciences. However, the inability to culture phytoplasmas *in vitro* has hindered their characterisation at the molecular level. To shed light on these microorganisms, we determined and annotated the genomic DNA of '*Candidatus* Phytoplasma asteris' OY strain, OY-M line (Oshima *et al.*, 2001), and compared the results with other bacteria whose genomes have been sequenced. We also investigated the function of the phytoplasmal membrane protein in host–parasite interactions.

Reductive evolution of metabolic pathways

The complete genomic sequences of '*Ca.* P. asteris' OY strain and AY-WB strain have been determined (Oshima *et al.*, 2004; Bai *et al.*, 2006). The genome of OY phytoplasma consists of one chromosome of 860 kbp and two plasmids, and that of AY-WB phytoplasma consists of one chromosome of 707 kbp and four plasmids. In general, small-genome pathogenic bacteria have lost genes for many biosynthetic pathways; this is probably because many metabolites are available within the host cell environment (Moran, 2002). Mycoplasmas lack the genes for the tricarboxylic acid cycle, sterol biosynthesis, fatty acid biosynthesis, nucleotide *de novo* synthesis, and most amino acid biosynthesis, and depend totally on the host cell to supply these functions (Razin *et al.*, 1998). Similarly, no genes for these biosynthetic pathways have been identified in phytoplasma genomes. However, the phytoplasmas have lost more genes for metabolic pathways than the mycoplasmas (Oshima *et al.*, 2004; Bai *et al.*, 2006), e.g., genes of the pentose phosphate pathway. Instead, phytoplasma genomes encode multiple copies of transporter-related genes, which are not found in mycoplasma genomes (Oshima *et al.*, 2004). These genomic features imply that phytoplasmas are highly dependent on metabolic compounds from the host cell.

The most distinct feature of phytoplasma genomes is the absence of F_0F_1-type ATP synthase. Most bacteria, including reduced-genome bacteria, produce F_0F_1-type ATP synthase and synthesise ATP through a reverse proton discharge reaction. However, none of the eight ATP synthase subunits have been identified in phytoplasma genomes (Oshima *et al.*, 2004). This suggests that the set of known metabolic pathways of phytoplasmas is even smaller than that of mycoplasmas, representing the minimal genome in living organisms. This may be the result of reductive evolution as a consequence of life as an intracellular parasite in a nutrient-rich environment.

Although metabolic genes are scarce, the phytoplasma genome contains many genes encoding transporter systems such as malate, metal ion, and amino acid transporters, some of which have multiple copies, suggesting that phytoplasmas aggressively import many metabo-

lites from the host cell. The consumption of these metabolites in organisms with phytoplasma infections may greatly disturb the metabolic balance of the host cell, causing disease symptoms.

Duplication of glycolytic genes in severely pathogenic phytoplasma

We cloned and sequenced the ca. 80-kb genomic DNA from the genome of OY-W phytoplasma, which causes severe symptoms (Oshima *et al.*, 2007). Interestingly, genomic duplication of a ca. 30-kb region was observed. Almost all duplicated genes became pseudogenes by frameshift and stop codon mutations, probably because of their functional redundancy. However, five types of gene, including two glycolytic genes, retained full-length ORFs, suggesting that it is advantageous for the phytoplasma to retain these genes in its lifestyle. In particular, 6-phosphofructokinase is known as a rate-limiting enzyme of glycolysis, implying that the different number of glycolytic genes between OY-W and OY-M may influence their respective glycolysis activity. We previously reported that the phytoplasma population of OY-W was larger than that of OY-M in their infected plants. Taking this result into account, the higher consumption of the carbon source may affect the growth rate of phytoplasmas and may also directly or indirectly cause more severe symptoms.

Positive selection acting on a surface membrane protein

Previous studies have shown that immunodominant membrane protein is a major portion of the total cellular membrane proteins in most phytoplasmas (Kakizawa *et al.*, 2006b). Genes encoding immunodominant membrane protein have been isolated from several phytoplasmas. Among these, the immunodominant membrane protein from aster yellows group phytoplasma was identified as an antigenic membrane protein (Amp; Kakizawa *et al.*, 2004).

To investigate the function of the immunodominant membrane protein, we cloned and sequenced 3.6-kbp genome fragments around the *Amp* gene from 14 phytoplasmas (Kakizawa *et al.*, 2006a). Most amino acid sequence differences were only observed in Amp. The average ratio of nonsynonymous to synonymous substitutions between pairs of sequences was greater than that for all calculable pairs of Amp, suggesting that a positive selection acts on Amp. When the same analysis was performed using the maximum likelihood method, positive selection was confirmed and positively selected sites were identified. No significant evidence of positive selection was obtained for the genes located upstream or downstream of Amp. Most of the positively selected sites were located in the central domain of Amp, which is predicted to protrude outside the phytoplasma cell. Thus, this positive selection on Amp probably reflects the interaction between the phytoplasma and the host cytoplasm. The clear positive selection implies an important biological role for Amp in the host–parasite interaction.

Interaction between Amp and the insect microfilament complex

To investigate the biological role of Amp in the host–parasite interaction, we analysed the localisation of Amp in the insect intestine using immunofluorescence microscopy (Suzuki *et al.*, 2006). In the tissue of insects infected with OY, Amp was found to co-localise with the microfilaments of the visceral smooth muscle surrounding the intestinal tract.

To test the interaction of insect proteins with Amp, the total protein extracted from leafhoppers was loaded onto three affinity columns: Amp, SecA (a membrane protein of OY phytoplasma), and BSA. The proteins eluted from the columns were separated using both SDS-PAGE and two-dimensional electrophoresis. As a result, three proteins were specifically detected in the Amp affinity column, but not in the BSA or SecA affinity columns. Peptide mass fingerprints using MALDI-TOF mass spectrometry and peptide sequencing analysis showed that these proteins were insect actin and myosin proteins, suggesting that Amp forms a complex with the microfilaments of the insect cell. Moreover, Amp–microfilament complexes were detected in all OY-transmitting leafhopper species, but not in non-OY-transmitting leafhoppers. These results suggest that an interaction between the phytoplasma Amp and the microfilament complexes of the insect host is important for infection to occur (Suzuki *et al.*, 2006). Although several mammalian pathogens are known to interact with host microfilaments to infect the host, the phytoplasma is the first example of an insect-transmissible bacterium that uses microfilaments in the insect cell.

Conclusion

The distinct features of reductive evolution were observed in the genomic sequences of phytoplasmas. This implies that the consumption of metabolites by phytoplasmas may play an important role in the host–parasite interaction. In addition, our results suggest that the membrane protein of the phytoplasma has an important function in the insect–phytoplasma interaction. The phytoplasma genomes encode many membrane proteins whose functions are still unknown; further analysis of these will provide valuable insights into host–phytoplasma interactions.

Acknowledgements

This work was partly supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS; No. 09460155, 13306004, and 16108001).

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