Cloning and Characterization of the Antigenic Membrane Protein (Amp) Gene and In Situ Detection of Amp from Malformed Flowers Infected with Japanese Hydrangea Phyllody Phytoplasma

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Accepted for publication 20 February 2008.

ABSTRACT

Arashida, R., Kakizawa, S., Ishii, Y., Hoshi, A., Jung, H.-Y., Kagiwada, S., Yamaji, Y., Oshima, K., and Namba, S. 2008. Cloning and characterization of the antigenic membrane protein (Amp) gene and in situ detection of Amp from malformed flowers infected with Japanese hydrangea phyllody phytoplasma. *Phytopathology* 98:769-775.

A Japanese hydrangea phyllody (JHP) disease found throughout Japan causes economic damage to the horticultural industry. JHP phytoplasma-infected Japanese hydrangea plants show several disease symptoms involved in floral malformations, such as virescence, phyllody and proliferation. Here, we cloned and characterized the antigenic membrane protein (Amp) gene homolog from the JHP phytoplasma (JHP-amp),

expressed the JHP-Amp protein in *Escherichia coli* cells, and then obtained an antibody against JHP-Amp. The antibody against JHP-Amp had no cross-reactions with the antibody against the Amp protein from a closely related onion yellows phytoplasma. This serologic specificity is probably due to the high diversity of the hydrophilic domains in the Amp proteins. The in situ detection of the JHP-Amp protein revealed that the JHP phytoplasma was localized to the phloem tissues in the malformed flower. This study shows that the JHP-Amp protein is indeed a membrane protein, which is expressed at detectable level in the JHP phytoplasma-infected hydrangea.

Additional keywords: immunodominant membrane protein, mollicutes.

Phytoplasmas are economically important plant pathogens that are associated with diseases in over hundreds of plant species (20,25). They produce a wide variety of symptoms in host plants, including virescence (greening of floral tissue), chlorosis, phyllody (changing of floral parts into leafy structures), stunting and proliferation (witches'-broom) (22,36). Phytoplasmas are intracellular within both plant phloem and insect cells, and are transmitted from plant to plant by insect vectors. Despite their economic importance and unique biological features, phytoplasmas remain among the most poorly characterized plant pathogens, primarily because efforts at in vitro culture, gene delivery, and mutagenesis have been unsuccessful. However, the complete genomic sequences of '*Candidatus* Phytoplasma asteris' OY (onion yellows) strain and AY-WB (aster yellows witches'-broom) strain have shed light on their unique genomic evolution (2,29). The phytoplasma genomes encode even fewer metabolic proteins than mycoplasmas. Phytoplasmas probably lost many biosynthesis genes as a result of reductive evolution following adaptation to an intracellular environment.

The Japanese hydrangea phyllody (JHP) phytoplasma-infected Japanese hydrangea plants show several disease symptoms involved in floral malformations, such as virescence, phyllody, and proliferation. Kanehira and co-workers (19) reported the occurrence of JHP disease in Japan, and suggested that the causal agent was a phytoplasma. The JHP disease occurs all over Japan and damages the horticultural industry. However, it is difficult to prevent the dissemination of the disease because the insect

vector(s) of the JHP phytoplasma have not been identified. To date, the JHP phytoplasma has only been found in Japan. Phyllody and virescence diseases of the hydrangea plants have been reported in many countries, such as Italy (6), Belgium (40), France (11), and Canada (13). However, the causal phytoplasmas of these diseases were classified into the aster yellows type (subgroup Ia) phytoplasmas based on 16S rDNA gene sequences, whereas the JHP phytoplasma was classified into subgroup Id (34). From this phylogenetically unique feature, the JHP phytoplasma was designated as a new species, '*Candidatus* Phytoplasma japonicum' (34).

It is interesting to study the molecular mechanisms involved in the symptoms of phytoplasma infections, especially symptoms with floral abnormalities, such as virescence, phyllody, and proliferation. In a recent study on flower malformations caused by a phytoplasma, Pracros and co-workers (32) demonstrated that the five flower development genes were misregulated in the stolbur phytoplasma-infected tomatoes. However, the mechanisms of flower abnormalities induced by a phytoplasma are still unknown, and it is also unclear through which tissue phytoplasmas influence the flower development. To answer this question, several attempts have been made to examine the distribution of a phytoplasma in plant tissues. For example, lethal yellowing phytoplasma in fruits of coconut was detected by in situ PCR to examine the possible potential for seed transmission of the phytoplasma (10), and the mulberry dwarf (MD) phytoplasma in the reproductive organs of mulberry trees was detected by direct or nested PCR to clarify the possibility of seed transmission (14). In these previous works, the PCR method was used to investigate the presence of phytoplasmas in the organs of the host plants. However, immunolocalization analysis with an antibody against phytoplasma is also useful in examining the distribution patterns of phytoplasmas in infected tissues.

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doi:10.1094/PHYTO-98-7-0769

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For the sensitive detection of phytoplasmas, an antibody raised against a phytoplasma membrane protein can be a useful tool. In most phytoplasmas, a single membrane protein, termed the immunodominant membrane protein (IDP), exists abundantly in cell preparations (35). Genes encoding IDPs have been isolated from several groups of phytoplasmas, and it has been suggested that the IDPs are classified into three distinct types: (i) immunodominant membrane protein (Imp); (ii) immunodominant membrane protein A (IdpA); and (iii) antigenic membrane protein (Amp) (17). These IDPs are nonhomologous because they show no amino acid similarity, are located on different parts of the genome and their predicted transmembrane structures differ greatly (17). Antigenic membrane protein (Amp), one of the IDP types, is composed of three domains: an N-terminal signal leader domain that is processed by the secretory translocation system, a large hydrophilic domain that is exposed to the host cytoplasm, and a C-terminal transmembrane domain (3,16). Since the specific and sensitive detection of phytoplasmas was performed with antibodies against the IDPs, the antibody against Amp is expected to be a good tool for detection, diagnosis, and other experiment techniques for phytoplasmas (3,5).

In this study, we cloned and characterized the *amp* gene homolog from the JHP phytoplasma, expressed the JHP-Amp protein in *Escherichia coli* cells, and then obtained an antibody against JHP-Amp. With the use of this antibody, we performed the in situ detection of the JHP-Amp protein in infected floral tissues. Our results confirmed that the JHP phytoplasmas inhabit exclusively in phloem cells. The relationship between the localization of phytoplasma in phloem cells and the abnormal flower development is discussed.

MATERIALS AND METHODS

Plant materials. The healthy and the JHP phytoplasma-infected hydrangea plants (Fig. 1) used for cloning the JHP-*amp* gene and for western blot analysis were obtained from a field in Saitama Prefecture, Japan. The JHP phytoplasma-infected hydrangea plants used for morphological observations and immunolocalization analysis were provided by the Nippon Hydrangea Association. The OY-W used in this study was isolated in 1982 in Saga Prefecture, Japan (36). Healthy and the OY-W-infected garland chrysanthemum (*Chrysanthemum coronarium*) plants were maintained in greenhouses with the leafhopper vector, *Macrostelus striifrons*. Total DNA was extracted from tissues of the hydrangea plants using previously described methods (27).

Cloning and sequence analysis of the JHP-*amp* gene. A series of previously designed primers for the amplification of the OY *amp* gene (15) was used to clone the JHP-*amp* gene. The PCR amplification for the JHP-*amp* gene was performed under previously described conditions (15). As a result, DNA fragments containing the JHP-*amp* gene were amplified with the several primer pairs. Amongst these, the largest fragment was amplified using the ES-1 primer: 5'-TTG AGC TCG CGG CCG CAT GAA ACA AAA AAC AAT TAT C-3' and Nad-2 primer: 5'-TTT GCC TGA TTG AAA CCA TCT-3'. This amplified fragment was purified using ExoSAP-IT(R) (GE Healthcare, Milwaukee, WI) and then sequenced directly with the EL-3 primer (5'-TAT CAA GAA TTA AAA GAT ACT-3') and Nad-2 primer. The JHP-*amp* sequence was completed by the primer-walking method using the JHP-Amp-F primer (5'-AGA ATT CCA TAT GCA AAA CGT TAA AAC TAA AAA ATC-3') and the JHP-Amp-R primer (5'-GAA AAA GCT AGA TTA AAA ACC TAG C-3'). Each ORF was analyzed with the standard genetic code, and annotated with the BLAST algorithm (1). The multiple alignments of the partial *groEL*, the intergenic sequence 1 (IGS1), the *amp* gene, the Amp protein, the intergenic sequence 2 (IGS2), the partial *nadE* from OY-W, OY-M, AY-C, AY-WB, CP, and JHP phytoplasmas

(GenBank accession nos. AB124806, AB124807, AF244540, AF244540, and JHP AB355409, respectively) were performed using ClustalW (38). Sequence identities were then calculated using GeneDoc (28). The putative transmembrane domain in the predicted protein sequence was identified with the SOSUI program (12). The N-terminal signal sequence was predicted using the SignalP 3.0 program (4).

Preparation of antiserum against the JHP-Amp protein.

The pET system (Novagen, Madison, WI) was used for the expression of the histidine-tagged JHP-Amp in *E. coli*. The JHP-Amp gene consisted of an N-terminal export signal sequence (*amp*-E), a central hydrophilic domain sequence (*amp*-H) and a C-terminal transmembrane sequence with a short hydrophilic domain (*amp*-T) (Fig. 2). For the expression of the *amp* gene with a six-histidine tag at the C-terminal end, we designed three constructs: an intact *amp* gene (*amp*-E/H/T), an *amp* gene without *amp*-T (*amp*-E/H) and an *amp* gene (*amp*-H) with only the hydrophilic domain. The *amp*-E/H/T constructs were amplified by PCR using the JHP-Amp-F primer and the JHP-Amp-R2 primer (5'-AAG AGC TCG AGC GCT TGT TGT CTT TTT TTA TC-3'). The *amp*-E/H construct was amplified by PCR using the primers JHP-Amp-F and JHP-Amp-CR (5'-AAG AGC TCG AGT TTT TTG TAA AAC GGC GTT GC-3'). The *amp*-H construct was amplified by PCR using the primers JHP-Amp-NF (5'-AGA ATT CCA TAT GTT TGG TGA TGC AAA ATT ACC ATC-3') and JHP-Amp-CR. Following a previously described procedure (17), a soluble fraction of crude extract of *E. coli* expressing JHP-Amp-H was applied to a nickel column to purify the histidine-tagged protein. The purity of the JHP-Amp-H protein was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). One milligram of the purified JHP-Amp-H protein was used to immunize a rabbit. Antibodies against the JHP-Amp protein were emulsified with 1:1 volume of Freund's complete adjuvant (Difco, Detroit, MI). The rabbit was boosted with a second injection after 2 weeks. The IgG fractions were purified from the crude serum with a Protein A column (Bio-Rad, Kanagawa, Japan).

SDS-PAGE and western blot analysis. Fresh leaf tissue from healthy or the phytoplasma-infected plants was frozen in liquid nitrogen and ground with a mortar and pestle. Extraction buffer (30 mM Tris/HCl buffer (pH6.8), 2% SDS, and 6% mercaptoethanol) was added, and then boiled at 95°C for 5 min. After centrifugation (18,000 g, 15 min), the supernatant was separated by SDS-PAGE. Western blotting was then applied following a previously described method (16) with some modifications. A 15% polyacrylamide gel was used for SDS-PAGE with an ECL(R) plus western blotting detection system (GE Healthcare). After incubation with the first antibody, the membranes were washed with TBST (20 mM Tris/HCl (pH7.9), 500 mM NaCl, and Tween 20), and reacted with a horseradish peroxidase-linked anti-rabbit IgG (from donkey) (GE Healthcare) diluted 1:5,000 in TBST. The proteins were detected with ECL(R) plus western blotting detection reagents (GE Healthcare) according to the manufacturer's instructions, and visualized with a luminescence image analyzer (LAS1000; FujiFilm, Tokyo, Japan).

Immunolocalization analysis. Using an antibody against JHP-Amp, immunolocalization analysis was carried out following a previously described procedure (18). Briefly, approximately 1-cm² pieces of carpels with stamens and petals were excised from the healthy and the JHP phytoplasma-infected hydrangea plants. Tissues were fixed in 4% (wt/vol) paraformaldehyde and 0.25% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) overnight at 4°C, dehydrated first through a graded ethanol series and then a 2-methyl-2-propanol series (33). Fixed tissues were embedded in paraffin and sliced to 8-μm thickness with a microtome (PR-50; Yamato Scientific, Tokyo, Japan). The localization of JHP-Amp in each tissue was detected with an alkaline phosphatase-mediated reporter system (18).

RESULTS

Flower abnormalities in the JHP phytoplasma-infected hydrangea. It has been reported that the hydrangea plants infected with the JHP phytoplasma show the floral organ abnormalities (19). The part generally called the flower of hydrangea plants is an ornamental flower, not a true flower. The organ that looks like a petal is actually a sepal, and the true petal is a central small organ (Fig. 1A, E, and I). The JHP phytoplasma-infected hydrangeas have three distinct kinds of the disease symptoms: virescence, phyllody, and proliferation. In the infected hydrangea flowers that showed virescence, although the sepals were green,

other floral organs remained normal (Fig. 1B, F, and J). In the infected hydrangea flowers that showed phyllody, the sepals changed into leaf-like structures, and the petals were green (Fig. 1C). Moreover, the stamens were filamentous (Fig. 1G), the stigma changed into a leaf and the ovary disappeared (Fig. 1K). In the infected hydrangea flowers that showed proliferation, in addition to features of phyllody, the pistil changed into the shoot apex (Fig. 1D, H, and L, arrow), suggesting that the flower development reverted to a vegetative developmental stage.

Cloning and characterization of the JHP-*amp* gene. To clone the *amp* gene homolog from the JHP phytoplasma, various pairwise combinations of the primers designed for amplification

Fig. 1. Healthy ornamental flower of Japanese hydrangea (A, E, and I), and Japanese hydrangea phyllody (JHP) phytoplasma-infected plants (B, C, D, F, G, H, J, K, and L). Sepal greening and normal corolla (virescence: B, F, and J), leaf-like sepal and greening petal (phyllody: C, G, and K), and stem-like pistil (proliferation: D, H, and L). Immunolocalization analysis of transverse sections through carpels, petals, and stamens of M, healthy and N, Japanese hydrangea phyllody-phytoplasma-infected plants. O to R, Magnified images in N. An arrowhead indicates the signals in a leaf-like stigma. Abbreviations: ov = ovary; st = stigma; an = anther; pe = petal; se = sepal; ph = phloem; x = xylem. Scale bars: M, N, 1.0 mm; O, P, Q, 200 μ m; R, 50 μ m.

of the OY *amp* genes (15) were tested in PCR with DNA extracted from the JHP phytoplasma-infected hydrangea plants as a template. As a result, the DNA fragments containing the JHP-*amp* gene were amplified with several primer pairs, and the largest fragment was directly sequenced. The 1,551-bp fragment contained one complete ORF and two incomplete ORFs. BLAST search analysis suggested that the first incomplete ORF encoded a protein that was similar to part of the molecular chaperonin, GroEL (*groEL*), and the third incomplete ORF encoded a protein that was similar to the part of an NAD synthase (*nadE*) (Fig. 2A). The second ORF (474 bp) was identified as an *amp* gene from the JHP phytoplasma (JHP-*amp*) because BLASTP analysis showed a weak similarity (48%) to the *amp* gene from AY-WB, and the gene organization around *amp*, 5'-*groEL*-*amp*-*nadE*-3', was

Fig. 2. Schematic representation of the open reading frames (ORF) and other features for the Japanese hydrangea phyllody (JHP) phytoplasma sequences (A) and putative antigenic membrane protein (Amp) translation products (B). -35 and -10, possible transcription signals; RBS, putative ribosome-binding site; Repeats, short imperfect inverted repeats; *groEL*, molecular chaperonin GroEL gene; *amp*, antigenic membrane protein gene; *nadE*, NAD synthase gene; IGS1 and IGS2, intergenic sequences 1 and 2; Amp-E, N-terminal export leader sequences; Amp-H, major hydrophilic domain; Amp-T, transmembrane/short C-terminal hydrophilic domain; TM, transmembrane predicted by the SOSUI program (12). Putative cleavage motif was predicted by the SignalP 3.0 program (4) and is indicated by an arrowhead.

highly conserved in several strains of the AY subclade phytoplasmas (2,3,17,30).

We characterized the nucleotide sequence and the deduced amino acid sequence of the JHP-*amp* gene in detail. The G + C content of the *amp* ORF (31.7%) was high compared to the average G + C content of the 1,551-bp whole fragment (26.0%). A putative ribosome-binding site (AAAGGAA) and transcription signals (-35; TTGTTT: -10; ACAAAT) were found in the upstream region of the translation start codon (IGS1: intergenic sequence 1). A putative transcription terminator was found as the short imperfect inverted repeats (5'-AAAAAGCTAGGTT-TTT-AATCTAGCTTTTT-3') in the intergenic sequence 2 (IGS2) (Fig. 2A). The putative amino acid sequence encoded by the JHP-*amp* gene (JHP-Amp) was 157 aa in length, which is approximately the same length as CP Amp (164 aa) and AY-WB Amp (164 aa), but smaller than AY Amp (233 aa), OY-M Amp (234 aa), and OY-W Amp (233 aa). JHP-Amp was rich in lysine (14.6%), valine (12.7%), and alanine (12.4%). Two transmembrane regions were predicted in the N-terminal and the C-terminal regions of JHP-Amp (Fig. 2B). The putative cleavage motif of the Sec transport system was predicted at the border between the N-terminal transmembrane domain and the hydrophilic domain (Fig. 2B). These results indicate that JHP-Amp consists of three domains: an N-terminal export leader sequence (Amp-E), a large hydrophilic domain (Amp-H), and a transmembrane C-terminal hydrophobic domain (Amp-T) (Fig. 2B). This secondary structure is the same as that of other previously reported Amp proteins (3,16).

Sequence comparisons of the *amp* genes and flanking DNA sequences. To describe the protein sequence identities, we then compared the sequence identity of each domain of JHP-Amp with the Amps from five AY subclades phytoplasmas (Table 1). As previously described, the percent identities of the three domains of Amp were different from each other (3,26). Amongst the five AY subclade phytoplasmas, sequence identities of Amp-E, Amp-H, and Amp-T ranged from 72 to 100%, 30 to 97%, and 67 to 100%, respectively (Table 1). These data indicate that both the Amp-E and the Amp-T domains were well conserved, but the Amp-H domain was diverse. The comparison of the Amp between

TABLE 1. Sequence identities (%) for three parts of the *amp* gene orthologs and their putative translation products from Japanese hydrangea phyllody (JHP) and five aster yellows (AY) group phytoplasmas (below, left), nucleotide sequence identities (%) for intergenic sequence 1 (IGS1), IGS2, and *nadE* (above, right)

Strain ^a	Gene	Domain	JHP	OY-W	OY-M	AY-C	AYWB	CP	
JHP			—	60	59	59	61	61	IGS1 ^b
			—	49	49	49	47	46	IGS2 ^b
			—	80	78	78	81	79	<i>nadE</i> ^b
OY-W	<i>amp</i> (Amp)	E ^c	66 (55) ^d	—	96	96	93	92	IGS1
		H ^c	25 (14)	—	100	100	89	91	IGS2
		T ^c	65 (48)	—	98	98	96	94	<i>nadE</i>
OY-M	<i>amp</i> (Amp)	E	63 (51)	90 (88)	—	100	95	92	IGS1
		H	25 (15)	96 (97)	—	100	89	91	IGS2
		T	54 (45)	97 (93)	—	100	97	95	<i>nadE</i>
AY-C	<i>amp</i> (Amp)	E	63 (51)	90 (88)	100 (100)	—	95	92	IGS1
		H	26 (14)	95 (93)	96 (97)	—	89	91	IGS2
		T	59 (45)	89 (93)	91 (100)	—	97	95	<i>nadE</i>
AYWB	<i>amp</i> (Amp)	E	60 (46)	85 (80)	90 (80)	90 (80)	—	92	IGS1
		H	44 (24)	31 (37)	31 (35)	31 (35)	—	91	IGS2
		T	65 (50)	79 (76)	81 (83)	74 (83)	—	97	<i>nadE</i>
CP	<i>amp</i> (Amp)	E	65 (52)	88 (78)	90 (82)	90 (82)	84 (72)	—	
		H	44 (21)	29 (37)	37 (30)	30 (37)	74 (67)	—	
		T	70 (43)	75 (67)	67 (77)	70 (74)	90 (82)	—	

^a OY, onion yellows; AY-C, chlorante isolate of aster yellows; AYWB, aster yellows witches'-broom; CP, clover phyllody.

^b IGS1, intergenic sequence 1; IGS2, intergenic sequence 2; *nadE*, partial *nadE* gene.

^c E, N-terminal export leader domain; H, major hydrophilic domain; T, C-terminal transmembrane domain.

^d Nucleic acids in plain type; proteins are in parentheses.

the JHP phytoplasma and the other AY subclade phytoplasmas also showed the diversity of the Amp-H domain. Between the JHP phytoplasma and the other AY subclade phytoplasmas, Amp-E had 46 to 55% identity, and Amp-T had 43 to 50% identity. However, Amp-H had only 14 to 24% identity, which was considerably lower than the identities of the other domains. The same results were also obtained at the nucleotide level (Table 1).

To investigate whether the sequence identities of the Amp-H domain is lower than that of intergenic regions and other genes, we calculated the nucleotide sequence identities of the putative intergenic regions (Fig. 2A; IGS1 and IGS2) and the partial sequence of *nadE* (86 bp) amongst JHP and other phytoplasmas. The IGS1 and IGS2 of JHP phytoplasma were about 20 bp and about 90 bp shorter, respectively, than those of the other AY subclade phytoplasmas. The identities of IGS1 and IGS2 were slightly lower than those of *amp-E* or *amp-T* but higher than that of *amp-H* (Table 1). The partial *nadE* was well conserved, with an identity of 78 to 81% (Table 1).

Expression and purification of JHP-Amp in *E. coli*. To obtain the antibody against the JHP-Amp protein, we attempted to express three combinations of the JHP-Amp protein domain in *E. coli*: the entire JHP-Amp (JHP-Amp-E/H/T), the N-terminal export leader domain and the major hydrophilic domain (JHP-Amp-E/H) and the hydrophilic domain alone (JHP-Amp-H). Although it has been reported that the growth of *E. coli* harboring the entire AY *amp* gene was inhibited (3), *E. coli* harboring these JHP-*amp* genes did not show a growth decline. As a result, the JHP-Amp-H protein (≈ 11.4 kDa) was expressed as a soluble protein; however, both the JHP-Amp-E/H and the JHP-Amp-E/H/T could not be successfully overexpressed (data not shown). Therefore, the JHP-*amp-H* was overexpressed, purified, and used to obtain an antibody against the JHP-Amp protein.

Western blot analysis using the antibody against JHP Amp. Crude protein extracts were prepared from an OY-infected garland chrysanthemum (OY extract), an JHP phytoplasma-infected hydrangea (JHP extract), a healthy garland chrysanthemum, and a healthy hydrangea. These four protein fractions were subjected to western blot analysis using the antibody against JHP-Amp and the previously generated antibody against OY-Amp (16). In western blot analysis using an antibody against OY-Amp, a protein band (≈ 25 kDa) was specifically detected in the OY extract, but was not detected in either the healthy extracts or the JHP extract (Fig. 3A). However, in western blot analysis using an antibody against JHP-Amp, a protein band (≈ 17 kDa) was specifically detected in the JHP extract, but not in either the healthy extracts or the OY extract (Fig. 3B). Though it seems that additional bands were found in lanes 2 and 3 in Figure 3B, these bands would probably be nonspecific signals because these bands are not the same size as the band identified with the antibody raised against OY-amp. These results suggest that JHP-Amp was expressed in the JHP phytoplasma-infected hydrangea plant. They also demonstrate that both antibodies against OY-Amp and JHP-Amp were highly specific to their respective phytoplasmal species, and did not cross-react with each other.

Immunolocalization analysis of JHP-Amp in JHP-infected floral tissues. To investigate the distribution patterns of the JHP phytoplasma in phyllody tissues, immunolocalization analysis was performed using an antibody against JHP-Amp. The paraffin-embedded carpels with the stamens and the petals were cut in the vertical direction. The blue signals representing the localization of the JHP-Amp protein were detected in all phytoplasma-infected plants (Fig. 1N, O, P, Q, and R), and no signal was detected in healthy plants (Fig. 1M). Interestingly, internal morphological changes were observed in the pistil of the JHP phytoplasma-infected plant. In the healthy pistil, an ovary developed at the base, and stigmas were separated into two parts and developed a

round shape (Fig. 1M). In the JHP phytoplasma-infected plant, new vascular bundles were formed at the base of the pistil instead of the ovary (Fig. 1N), and the JHP-Amp signals were detected in the newly formed vascular bundles (Fig. 1P, Q, and R). The JHP-Amp signals were exclusively detected in the phloem, which is the tubular tissue next to the xylem (Fig. 1P, Q, and R). The JHP-Amp signals were also detected in the stigmas that changed into leaf-like structures (Fig. 1O).

DISCUSSION

In this study, we showed that the Amp sequence of the JHP phytoplasma was different from that of the other phytoplasmas. It has previously been reported that the Amp sequences are highly divergent; for example, the hydrophilic domains of the Amps from AY-C and CP were highly divergent in both size and sequence (3), and a strong positive selection was observed in the central hydrophilic region of the Amp protein (15). However, JHP-*amp* showed the lowest similarity to previously known *amp* sequences (Table 1). Moreover, the hydrophilic region of JHP-Amp was more divergent than the intergenic sequences (IGS1 and IGS2) (Table 1), which is consistent with the previous reports of the other Amp proteins (3,16). Since the Amp proteins are highly divergent in their hydrophilic domains, probably mainly due to the strong selective pressure (3), it has been suggested that Amp might have a key role in phytoplasma-host interactions (15). Recently, it was reported that the Amp of OY-W formed a complex with actin and myosin proteins (components of microfilaments) of its insect vector, and the formation of this complex was correlated with insect vector specificity (37). Insect vector(s) of JHP might be different from those of other phytoplasmas because the sequence of JHP-Amp was very different from that of the Amps from other phytoplasmas. Further study involving, for example, identification of the insect vector(s) of the JHP phytoplasma and analysis of the interaction between JHP-Amp and microfilament proteins of its insect vector, would shed light on the relationships between the sequence diversity of Amp and insect vector specificity.

Although several *amp* genes have been cloned and sequenced, all previously cloned *amp* genes were derived from '*Candidatus* Phytoplasma asteris' strains (2,3,16,29). This study provides the first report of cloning and expression analysis of the *amp* gene from '*Candidatus* Phytoplasma japonicum', which is phylogenetically distinct from '*Candidatus* Phytoplasma asteris', but still belongs to the group I phytoplasmas. Since the amino acid sequence of JHP-Amp was unique as compared to the Amp sequences from other phytoplasmas, JHP-Amp was expected to have specificity as an antigen. An antibody against JHP-Amp did not react with OY-Amp, and an antibody against OY-Amp did not react with JHP-Amp; that is, no cross-reactions were observed between an antibody against JHP-Amp and an antibody against OY-Amp (Fig. 3). It has been demonstrated that monoclonal antibodies raised against IdpA of Western X-disease phytoplasma

Fig. 3. Western blot analysis using an antibody raised against OY-antigenic membrane protein (Amp) (A) and Japanese hydrangea phyllody (JHP)-Amp (B). Each lane of A and B indicates the same protein extracts. Lane 1, Extract from an OY-W phytoplasma-infected garland chrysanthemum plant; lane 2, protein extracts from a healthy garland chrysanthemum plant; lane 3, protein extracts from a JHP phytoplasma-infected hydrangea plant; lane 4, protein extracts from a healthy hydrangea plant.

(WX) reacted with peach rosette and other strains of WX, but did not react with *Vaccinium witches'-broom* phytoplasma (VAC), even though VAC is phylogenetically related to both WX and peach rosette, based on 16S rRNA gene sequences (7). It has also been reported that the polyclonal antibody raised against Imp of the apple proliferation phytoplasma did not react with the extract from plants infected with the closely related pear decline phytoplasma (5). These previous reports and our present study indicate that a specific antibody against the IDP of a particular phytoplasma did not always react with closely related phytoplasmas. The SecA membrane protein, which is a component of the Sec protein export system, is known as another antigen for phytoplasmas. An antibody raised against SecA of the OY phytoplasma reacts with a wide range of phytoplasmas, including JHP phytoplasma (41). Since an antibody raised against JHP-Amp reacted specifically with JHP phytoplasma, this antibody has the potential to be a useful tool for specific detection and identification.

We performed immunolocalization analysis of the JHP phytoplasma in phyllody floral organs of hydrangea plants. In previous reports, radioisotope-labeled phytoplasma specific DNA probes were used to monitor colonization pattern and population dynamics of phytoplasma infection and its relationship to subsequent symptom development (21). Immunolocalization analysis with an antibody against SecA or Amp was also used to visualize the distribution patterns of phytoplasmas in a vascular bundle and a shoot apical meristem (18,30,41). Alternatively, the phytoplasma-infected plant tissue was stained with vital fluorescence dyes for bioimaging and examined under confocal laser scanning microscopy (8), and the presence of the MD phytoplasma in floral organs was confirmed by PCR (14). Transmission electron microscopy with immunolabeling (23), DAPI staining (10), or in situ hybridization employing various phytoplasma-specific oligonucleotide probes (24) were also performed for the purpose of localization of phytoplasma in infected tissues. Our results revealed that the JHP phytoplasma resided in the newly formed phloem of an abnormal pistil (Fig. 1N, O, P, Q, and R). The JHP phytoplasma was not detected in any tissues other than phloem cells. Although there are a few exceptions where the phytoplasma DNA was detected in embryo tissues other than the phloem (10), our results are consistent with previous reports that phytoplasmas do not exist in the shoot apical meristem (18).

Floral organs of the JHP phytoplasma-infected hydrangea plants showed morphological changes, such as a greening or leafy sepals (Fig. 1B, C, and D), loss of the ovary (Fig. 1K and N), leafy stigmas (Fig. 1K and N) or growth of the shoot apex instead of the pistil (Fig. 1D, H, and L). The biological mechanism of these disease symptoms is of interest, but it is still not known how phytoplasmas cause such malformations in the host plant flower. We hypothesize two possible mechanisms for these symptoms: phytoplasmas directly influence the flower development in a flower-developing organ, or uptake of phloem nutrients by phytoplasmas indirectly disturbs the metabolism and the flower development of a host plant. In this study, we observed that phytoplasmas inhabit the newly formed phloem of an abnormal pistil, but were not observed in an apical meristem, suggesting that the latter hypothesis was plausible. Previously, expression level changes in several flower development genes have been observed in the stolbur phytoplasma-infected tomato (32). Moreover, it has been proposed that these expressional changes were likely caused by a long-distance signal because phytoplasmas had never been detected in apical meristems (32). Generally, several flower-developing signals (e.g., sucrose, glutamine, and cytokinins) are produced in response to photoperiodic induction, and some of these are transported from the leaves toward shoot apical meristems, where floral morphogenesis takes place (9). In the case of the short-day plant *Impatiens balsamina*, a supply of a leaf-derived signal was needed to maintain flowering in the meristem (31), and interruption of the short-day induction

by transfer into long-day conditions caused flower reversion, i.e., a switch from the floral development back to the vegetative development (31). This switch also occurred in *Impatiens* plants by either long-day treatment or removal of leaves that unfolded during an inductive short-day treatment (39). These previous reports suggest that signals produced in leaves play important roles in flower development. Leaf-derived signals are transported to shoot apical meristems via the phloem, where phytoplasmas are found. Thus, it is possible that phytoplasmas influence the production or the transportation of leaf-derived signals in phloem cells. To test this hypothesis, further analysis is needed to obtain time-series data on the abnormal flower development or to estimate the amount of leaf-derived signals in infected plants.

ACKNOWLEDGMENTS

We thank the Nippon Hydrangea Association for providing the JHP-infected hydrangea plants used in this study. This work was supported partly by Grants-in-Aid of Scientific Research from the Japan Society for the Promotion of Science (JSPS) (No. 09460155 and 13306004).

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