

Full Length Research Paper

# Cloning and characterization of the immunodominant membrane protein of mulberry yellow dwarf phytoplasma

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Mulberry yellow dwarf (MYD) disease is a quarantine disease in China and the causal agent is a phytoplasma. The antigenic membrane protein (Amp) of the “*Candidatus Phytoplasma asteris*” was hypothesized to play an important role in insect transmission specificity. Here, we firstly cloned and characterized *Amp* gene from the “*Candidatus Phytoplasma asteris*”, MYD strain. The results showed that the MYD *Amp* gene consisted of 702 nt encoding a predicted protein of 233 aa which was highly similar to Amp of “*Candidatus Phytoplasma asteris*”, sumac witches'-broom phytoplasma (SWB) strain and mulberry dwarf phytoplasma (MD) strain. Prediction of protein structure showed that the MYD-Amp protein possess an N-terminal export leader sequence (Amp-E), a large central hydrophilic domain (Amp-H) and a C-terminal transmembrane domain with a short hydrophilic domain (Amp-T).

**Key words:** Mulberry yellow dwarf phytoplasma, Immunodominant membrane protein.

## INTRODUCTION

Mulberry yellow dwarf (MYD) disease, which was recorded in an ancient agricultural book written as far back as 1131 to 1162 and commonly referred to as “Jinsang”, is one of the most serious diseases on mulberry (*Morus alba* L.) in China. The symptoms of the diseased mulberry consisted mainly of yellowing, crinkling and downward rolling of leaves, stunting, and dwarf. It has been reported that the causal agent of the

MYD disease was a phytoplasma (The group of virology and The group of mulberry protection, 1974) which parasitize the phloem tissue of the infected mulberry and are naturally transmitted by leaf hopper vectors, *Hishimonoides sellatiformis* Ishihara and *Hishimonus sellatus* Uhler, the former of which is stronger transmission capability than that of the latter (The group of mulberry protection and The group of virology, 1977). Based on 16S rDNA phylogenetic analysis, MYD phytoplasma has been classified into 16Srl-B subgroup (“*Candidatus Phytoplasma asteris*”) (Qiu et al., 1998; Lu, 2010).

Plant pathogenic phytoplasmas are wall-less, endo-cellular, Gram-positive bacteria with a small, low G+C content genome ranging from 530 to 1350 kb (Marcone et al., 1999; Hogenhout et al., 2008). Because no phytoplasma has yet been successfully cultured *in vitro*, our knowledge about its physiology and biology is limited. It is largely unknown how phytoplasmas cause disease in plants and how they interact with their plant host. However, previous studies have shown that a surface

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**Abbreviations:** MYD, Mulberry yellow dwarf; IDP, immunodominant membrane protein; Imp, immunodominant membrane protein A; Amp, antigenic membranes protein; AY, aster yellows; CPh, clover phyllody; JHP, Japanese hydrangea phyllody; OY, onion yellows; MD, mulberry dwarf; SWB, witches'-brood; CYP, chrysanthemum yellows phytoplasmas; ORF, open reading frame; PCR, polymerase chain reaction; RBS, ribosome-binding site; BLAST, Basic Local Alignment Search Tool.

membrane protein, termed the immunodominant membrane protein (IDP), possibly plays important roles in host-phytoplasma interactions and the IDPs are classified into three distinct types: type I was termed immunodominant membrane protein (Imp); type II was termed immunodominant membrane protein A (Idp A); and type III was termed antigenic membranes protein (Amp) (Barbara et al., 2002; Kakizawa et al., 2006a). These three types are nonhomologues to each other, are located on different parts of genome and their predicted transmembrane structures differ greatly (Barbara et al., 2002; Kakizawa et al., 2006a; Arashida et al., 2008). Genes encoding Amp have been isolated from several phytoplasmas, such as aster yellows (AY) and clover phyllody (CPh) (Barbara et al., 2002), Japanese hydrangea phyllody (JHP) (Arashida et al., 2008), onion yellows (OY) (Kakizawa et al., 2004), mulberry dwarf (MD) (Kakizawa et al., 2006b, 2009) and sumac witches'-brood (SWB) (Kakizawa et al., 2006b).

Amp is an immunodominant membrane protein in phytoplasmas, present on the surface of the phytoplasma and apparently abundant portion of the total cellular membrane proteins (Yu et al., 1998; Berg et al., 1999). The Amp protein might play biologically important roles in the phytoplasma-host interactions. Owing to the Amp protein locating on the surface of the phytoplasma, it is likely that positive selection on Amp is caused by the interaction between the phytoplasma and its extracellular environment, the host cytoplasm. However, it remains unclear whether the positive pressure was derived from either insects or plants (Kakizawa et al., 2006b). Interaction between the Amp of OY-W phytoplasma and its vector insect microfilament formed Amp-microfilament complex. The formation of the Amp-microfilament complex is correlated with the phytoplasma-transmitting capability of leafhoppers (Suzuki et al., 2006). The latest research demonstrated that the Amp of "Ca. P. asteris", the chrysanthemum yellows phytoplasmas (CYP) strain, interacted specifically with ATP synthase of vector insect *Euscelidius variegatus*. ATP synthase acts as a receptor of phytoplasma Amp in the transmission of CYP (Galetto et al., 2011).

In this study, the *amp* gene from "Ca. P. asteris", MYD strain was cloned, sequenced and analysed. The result data will provide reference for further studies such as interaction of phytoplasma-host, production of antiserum against MYD-Amp for the detection of this phytoplasma in mulberry.

## MATERIALS AND METHODS

### Healthy and phytoplasma-infected mulberry

The MYD phytoplasma-infected mulberry branches used for cloning the MYD-*amp* gene were obtained from a field in Anhui province, China. The "Ca. P. asteris", MYD strain were tubular budding-transmitted to healthy mulberry and maintained in greenhouses without the rhombic-marked leafhopper vector, *H. sellatus* Uhler,

and mulberry caltrop-marked leafhopper vector, *H. sellatiformis* Ishihara.

### Extraction of total DNA

Total DNA from healthy and infected mulberry was extracted according to Kollar et al. (1990). Total DNAs extracted from 0.1 g barks of the epicormic branches were suspended in 60 µl of TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0).

### Polymerase chain reaction (PCR) amplification and sequencing of MYD-*amp* gene

PCR primers were designed based on the partial genomic sequence of mulberry dwarf phytoplasma deposited in GenBank (accession No. AB124809). To clone accurately the entire open reading frame (ORF) of MYD-*amp* gene, various primer pairs that flank the ORF of MD-*amp* gene were tested and a successful amplification was achieved by primers ampF (5-GATTGTCTTTTAACTCTTTGTTAG-3) and ampR1 (5-TAATAAAGATAAATAAAAAAACTAGGT-3) (the position of ampF and ampR1 are shown in the Figure 2). The resulting total DNA samples were 1:5 diluted before using them for polymerase chain reaction (PCR) amplification. PCR was performed in 50 µl reactions containing 50 ng template DNA, 200 µM of each dNTP, 0.5 µM of each primer, 1× PCR reaction buffer containing MgCl<sub>2</sub> and 2 U *Taq* DNA polymerase (Takara). The amplification was carried out under the following conditions: 94°C for 3 min followed by 35 cycles of 94°C for 30 s, 45°C for 1 min and 72°C for 1 min, followed by a final 7 min extension at 72°C. 5 µl of PCR product was analyzed by electrophoresis in 1% agarose gel with ethidium bromide and visualized by a UV transilluminator. The amplified fragment was purified using EZ Spin Column DNA Gel Extraction Kit (BBI) and then ligated into plasmid vector pUCm-T (Sangon). The plasmid was transformed into *Escherichia coli* strain Top 10 cells. Transformants were selected on LB medium containing 100 µg/ml ampicillin, five positive clones identified by colony PCR were sequenced. Primer synthesizing and sequencing was performed by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd.

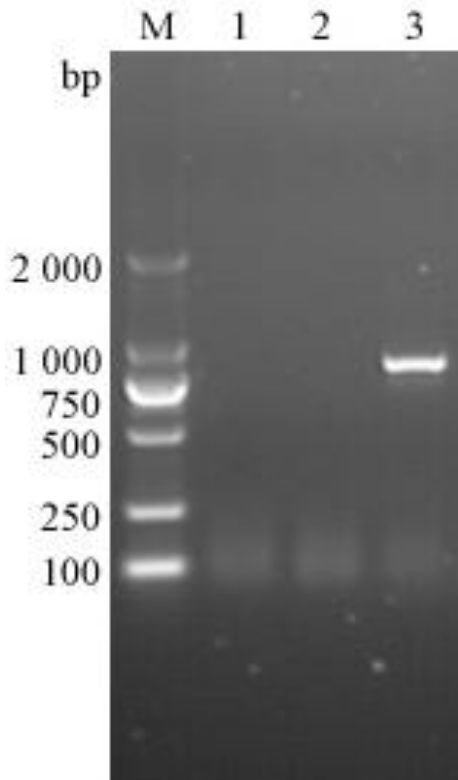
### Sequence alignment and protein prediction

Sequence assembly was completed using DNASTar software. The sequence was compared with those of reference in GenBank by BLAST. The putative transmembrane domain in the predicted protein sequence was identified with TMHMM Server v. 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>). The N-terminal signal sequence was predicted using the SignalP 3.0 program ([www.cbs.dtu.dk/services/SignalP/](http://www.cbs.dtu.dk/services/SignalP/)) (Bendtsen et al., 2004).

## RESULTS

### Cloning and characterization of the MYD-*amp*-containing gene fragment

Because of the low G+C content of the phytoplasma genome, many of the PCR primers would not be good sequencing primers. To maximize the chances of generating fragments that contain MYD-*amp* gene, several primer pairs were synthesized and tested in PCR with DNA extracted from the MYD phytoplasma-infected



**Figure 1.** PCR amplification products using primer pairs (ampF/ampR1). M: DNA marker; Lane 1: H<sub>2</sub>O; Lane 2: Healthy mulberry; Lane 3: Diseased mulberry.

mulberry as a template simultaneously and one primer pairs (ampF/ampR1) resulted in successful amplification. As shown in Figure 1, a specific band of approximate 840 bp was amplified from the DNAs extracted from mulberry infected MYD phytoplasma by PCR using primer pairs ampF/ampR1 and No PCR amplification occurred in the negative control (H<sub>2</sub>O and healthy mulberry) (Figure 1).

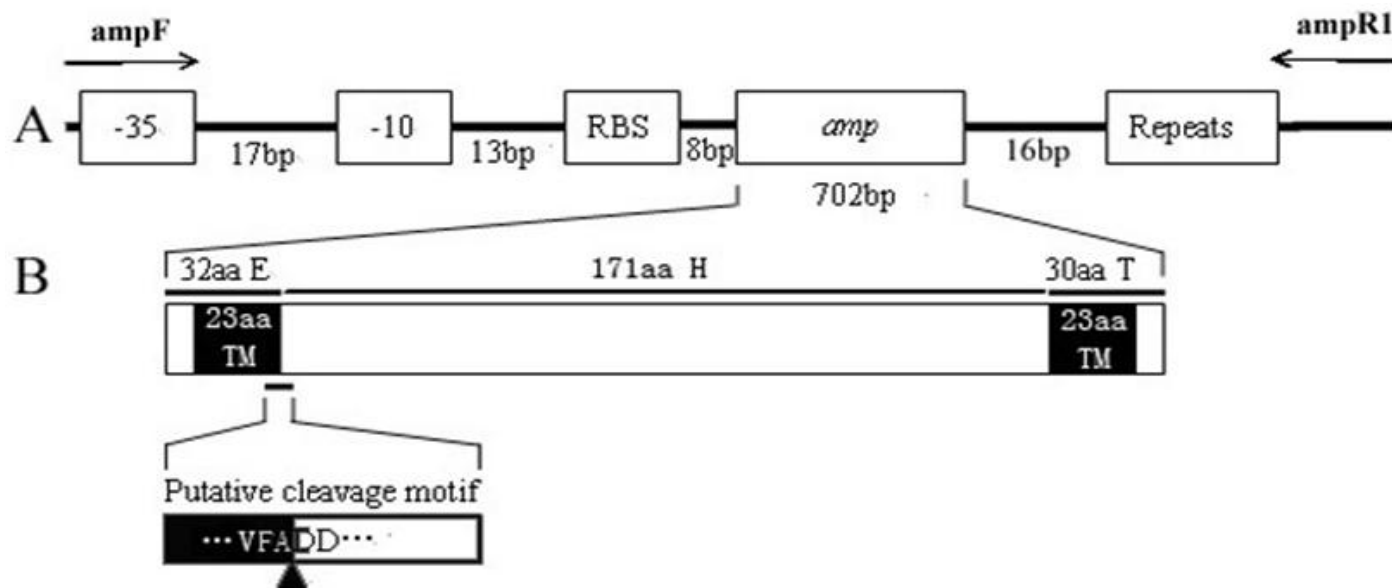
PCR products were purified and the amplicon was cloned and sequenced. The results indicated that the fragment consisted of 841 bp (GenBank accession No. HQ153155) and contained one complete ORF. This ORF was preceded by a putative ribosome-binding site (RBS) sequence (5'-AAAGGAG-3') which was complementary to the core sequence (5'-CTCCTTT-3') of 3'-end region of the MYD-Anh 16S rRNA (GenBank accession No. GQ249410) and RNA polymerase binding sites (-10: TATAAT; -35: TTGTTA). A short, slightly imperfect inverted repeats sequence (5'-TTTAAAAGCT-AGG-TTTTTTAA-3') which may act as a putative transcription terminator was found in the downstream of the complete ORF (Figure 2A).

The complete ORF which began with ATG start codon and terminated with TAA stop codon is composed of 702 bp with a G+C content of 31.91% and predicted to encode a 25.064 kDa protein (233 amino acid residues

in length) with an isoelectric point (pI) value of approximately 9.27. A normal BLAST search revealed that the nucleotide sequence and amino acid sequence of the complete ORF shared significant identity with those of Amps from Sumac witches'-broom (SWB) phytoplasma, Mulberry dwarf (MD) phytoplasma, Onion yellows (OY) phytoplasma and other phytoplasma strains belonging to "Ca. P. asteris", and thus was identified and designated as MYD-Amp. The most abundant amino acid of MYD-Amp was valine (16.3%) followed by lysine (14.2%), leucine (10.7%) and alanine (9.9%), and with two cysteine residues. There were no arginines and histidines. Two transmembrane regions were predicted at aa 9 to 32 and 204 to 226 in the N-terminal and C-terminal regions of MYD-Amp, respectively. The N-terminal 32 aa was predicted to be a N-terminal signal sequence. Like the consensus sequence of bacterial Sec system signal peptides, the signal peptide also have a N-terminal domain with at least one positively charged aa (two lysine residues in MYD-Amp), followed by a hydrophobic core domain and a C-terminal domain containing neutral amino acids, the amino acid which is the nearest signal-sequence cleavage sites invariably is one with small side chain such as alanine, glycine (one alanine in MYD-Amp) (Tjalsma et al., 2000; Kakizawa et al., 2004). The location of the max cleavage site probability is between residues 32 and 33 (VFA-DD). The 171 aa domain between the two transmembrane regions was predicted to be hydrophilic and relatively neutral (predicted pI 6.63). A short, strongly hydrophilic C-terminal region (at amino acids 227 to 233) followed the C-terminal transmembrane domain. These results indicate that, as previously reported for other Amp proteins, MYD-Amp is also composed of three domains: an N-terminal export leader sequence (Amp-E), a large central hydrophilic domain (Amp-H), and a C-terminal trans-membrane domain with a short hydrophilic domain (Amp-T) (Figure 2 B).

### Sequence comparisons of the *amp* genes and putative translation product

The sequence identity of the complete gene and each domain of MYD-*amp* were compared with the *amp* genes from six phytoplasmas belonging to 16Srl group (Table 1). The results revealed that, at nucleic acid sequence level, the complete MYD-*amp* gene shared the highest identity with that of SWB which is closely related with MD based on 16S rRNA gene sequence analysis (Lee et al., 2009), followed by MD, AY and OY which belong to the same 16Srl subgroup as MYD. Lower identity was shown with CPh and JHP which belong to the different subgroups of 16Srl. At the amino acid sequence level, the putative products of the complete MYD-*amp* gene also shared the highest identity with that of SWB, followed by MD, AY and OY. In addition, MYD-Amp is the same length as SWB, MD, AY and OY and different



**Figure 2.** Schematic representation of the open reading frames (ORF) and other features for the mulberry yellow dwarf (MYD) phytoplasma sequence (A) and putative antigenic membrane protein (Amp) translation products (B). The arrowheads indicate the position and direction of the primer pairs used in this study. -35 and -10, putative -35 and -10 promoter region; RBS, putative ribosome-binding site; *amp*, antigenic membrane protein gene; Repeats, short inverted repeats; E, predicted N-terminal export leader signal sequence; H, central hydrophilic domain; T, C-terminal transmembrane with short hydrophilic intracellular sequence; TM, transmembrane region; ▲, the cleavage point.

**Table 1.** Sequence identities (%) of the complete and three parts of the *amp* gene and their putative translation products from mulberry yellow dwarf (MYD) and six 16Srl phytoplasmas.

Strain <sup>a</sup>	Domain <sup>b</sup>	SWB	MD	AY	OY	JHP	CPh
MYD	C	95.9 (90.2)	92.5 (85.5)	91.9 (82.9)	91.9 (83.3)	15.8 (25.9)	46.9 (41.2)
	E	100 (100)	100 (100)	99 (96.9)	100 (100)	56.3 (40.6)	93.8 (84.4)
	H	94.5 (87.7)	89.9 (81.3)	89.1 (77.8)	89.3 (78.9)	23.4 (14.9)	43 (10.7)
	T	98.9 (96.7)	98.9 (96.7)	100 (100)	97.8 (93.3)	69 (39.3)	83.3 (62.1)
16SrDNA <sup>c</sup>		99.8	99.8	99.9	99.8	95.7	89.1

<sup>a</sup>AY, Aster yellows (AF244540); CPh, Clover phyllody (AF244541); JHP, Japanese hydrangea phyllody (AB355409); MD, Mulberry dwarf (AB124809); OY, Onion yellows (AB110271); SWB, Sumac witches'-broom (AB242236). <sup>b</sup>C, the complete *amp* gene; E, N-terminal export leader domain; H, central hydrophilic domain; T, C-terminal transmembrane sequence with a short hydrophilic domain. Nucleic acids in plain type; amino acids in parentheses.

<sup>c</sup>AY (HQ646367); CPh (HQ589196); JHP (AB010425); MD (AB693124); MYD (GQ249410); OY (D12569); SWB (AB693125).

length from CPh and JHP (233 aa encoded by MYD, SWB, MD, AY and OY *amp* gene, 164 and 157 aa encoded by CPh and JHP *amp* gene, respectively).

Amongst the Amp-E, Amp-H, and Amp-T, the sequence of Amp-E and Amp-T showed relatively high identity between MYD and other six phytoplasmas. Amp-E had 56.4 to 100% identity, and Amp-T had 69 to 100% identity. However, the Amp-H domains were variable and considerably lower than the other domains in sequence identity which had only 23.4 to 94.5%, 10.7 to 87.7% at nucleic acid and amino acid sequence level, respectively. These data indicate that, as previously described for OY, AY and JHP Amps, both the Amp-E and the Amp-T

domains of MYD-Amp were also well conserved, but the Amp-H domain was diverse (Kakizawa, 2004; Barbara et al., 2002; Arashida et al., 2008).

## DISCUSSION

In this study, a 841 bp fragment containing a complete ORF (702 bp) with additional flanking DNA sequence was amplified and sequenced from DNA extracted from mulberry infected by MYD phytoplasma. Analysis of the DNA sequence by BLAST search suggested that the complete ORF was phytoplasmal immunodominant

membrane protein (IDP) gene with translation product of 233 aa. The phytoplasmal immunodominant membrane proteins were divided into three distinct types, all of which possess a central hydrophilic region with different length that is probably located on the exterior of phytoplasma cell. The organization of the hydrophilic transmembrane anchor is a distinguishing feature of the different types. Type I (Imp) is anchored by only N-terminal transmembrane; type II (Ildp A) has N-terminal and C-terminal transmembrane regions, and neither of them is cleaved; type III (Amp) also has two transmembrane regions, but the N-terminal one is cleaved and only the C-terminal one serves as an anchor (Kakizawa, 2004; Barbara et al., 2002). The structural properties of MYD Amp are consistent with type III of immunodominant protein. The predicted properties of type III of immunodominant protein suggested that when MYD Amp was expressed, the N-terminal leader sequence of MYD Amp will be cleaved by enzymolysis during pre-protein export to give mature extracellular protein anchored to the cell membrane by the transmembrane domain near the C-terminal.

The putative amino acid sequence of the MYD-*amp* gene showed significant high identity with that of those from the same subgroup phytoplasmal *amp* gene, but extremely low identity with that of those from the different subgroup phytoplasmal *amp* gene. The putative amino acid sequence of MYD Amp shared the highest identity with SWB Amp, 22 (1 in Amp-T and 21 in Amp-H) of the 233 aa was different, and the second highest identity with MD Amp, 33 (1 in Amp-T and 32 in Amp-H) of the 233 aa was different. These comparisons further showed that the Amp proteins are highly divergent in their central hydrophilic domains. It has been proposed that the high divergence in hydrophilic domain is probably mainly due to the strong selective pressure (Barbara et al., 2002).

Based on 16S rRNA gene sequence analysis of phytoplasma, MYD was found to be most related phylogenetically to MD (Lu, 2010), SWB and MD isolates were clearly related (Lee et al., 2009). In addition, MYD, MD and SWB were naturally transmitted and spread by the same insect vectors *H. sellatiformis* and *H. sellatus* (The group of mulberry protection and The group of virology, 1977; Ishijima, 1971; Han and Cha, 2002). The structural resemblance among MYD, MD, and SWB Amps are therefore in agreement with phylogenetic relationship among these three phytoplasmas. However, the AA sequence identity of Amps between MYD and SWB, infecting different plants respectively, is higher than that between MYD and MD, despite MYD and MD phytoplasma both can infect mulberry (*M. alba*). Such result prompt us to study further which selective pressure affects the divergence of Amp of MYD phytoplasma occurred in China and MD phytoplasma occurred in Japan.

The MYD-*amp* gene, to our knowledge, was firstly cloned. Further study is necessary to understand its

significance, such as the interaction between MYD-Amp and microfilament proteins of its vector insect would shed light on the different of transmission possibility of both *H. sellatiformis* and *H. sellatus*, understanding the role of Amp is important for us to better describe the biology and also will extend our knowledge of phytoplasma evolution. In addition, it was previously reported that monoclonal antibodies were produced to detect MYD phytoplasma by the purified MYD phytoplasma (Xia, 1993; Xia et al., 1998), but the concentration of phytoplasma cells in the phloem of infected plants is often very low in woody hosts (Berges et al., 2000). Production of high purity phytoplasmas was difficult and the titer of antisera was low. Expressing MYD Amp in *E. coli* and subsequent production of polyclonal or monoclonal antibodies against MYD Amp could be a good approach to develop a high titer and sensitive antiserum for the detection of MYD phytoplasma in general.

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