An Antibody Against the SecA Membrane Protein of One Phytoplasma Reacts with Those of Phylogenetically Different Phytoplasmas

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ABSTRACT

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Antisera raised against phloem-limited phytoplasmas generally react only with the phytoplasma strain used to produce the antigen. There is a need for an antiserum that reacts with a variety of phytoplasmas. Here,

we show that an antiserum raised against the SecA membrane protein of onion yellows phytoplasma, which belongs to the aster yellows 16S-group, detected eight phytoplasma strains from four distinct 16S-groups (aster yellows, western X, rice yellow dwarf, and elm yellows). In immunoblots, an ~96-kDa SecA protein was detected in plants infected with each of the eight phytoplasmas. Immunohistochemical staining of thin sections prepared from infected plants was localized in phloem tissues. This antiserum should be useful in the detection and histopathological analysis of a wide range of phytoplasmas.

The phytoplasmas are diverse, economically important, prokaryotic phytopathogens that are transmitted by insects, primarily leafhoppers. Like other mollicutes, phytoplasmas lack cell walls and reside endocellularly within insects and plant phloem (16,20). Phytoplasmas cause more than 700 diseases in several hundred plant species, representing about 100 families (16,19,22). Phylogenetic analysis of 16S ribosomal DNA sequences indicates that phytoplasmas are most closely related to achleplasmas, and are currently classified into 10 16S-groups (12,13,21,26).

Several techniques have been used to detect phytoplasmas, including polymerase chain reaction (PCR) (23), DNA hybridization (17), and electron microscopy (8), but each of these methods has inherent disadvantages. PCR and DNA hybridization require specialized equipment but do not offer histological views of the infected tissues. In contrast, serological detection using a specific antiserum is an economical and convenient method that allows for both analysis of many samples in a short time and immunohistological observation of infected tissues.

Phytoplasmas have not been cultured *in vitro*. The first antibody that recognizes phytoplasmas was produced in 1974, when a partially purified phytoplasma preparation was used as an immunogen (27). Since then, enriched or partially purified phytoplasma preparations from infected plants have been used to raise polyclonal and monoclonal antibodies against several phytoplasma isolates, including aster yellows (AY) (10), clover phyllody (CP) (11), primula yellows (7), grapevine flavescence dorée (3), maize bushy stunt (5), elm yellows (4), and ash yellows (6). However, this approach can result in polyclonal antibodies with insufficient titers, low specificity to the target phytoplasma, and

cross-reactivity with plant components. The application of monoclonal antibody technology has eliminated several of these problems, but monoclonal antibodies often react only with the original phytoplasma isolate used as the immunogen.

Recently, a novel strategy (14) including the following steps was reported: (i) cloning of the phytoplasma gene fragment into an *Escherichia coli* expression vector; (ii) expression in *E. coli* and purification of the protein; and (iii) preparation of antibodies in rabbit. This technique has been used to develop antibodies to the immunodominant membrane protein of several phytoplasmas including apple proliferation (AP) (1), CP, western X (WX) disease (2), and onion yellows (OY) (15). These antibodies represent important tools in the detection, localization, and purification of phytoplasmas and the target antigen. However, immunodominant membrane proteins from different strains usually have limited amino acid sequence identity, such that antibodies raised against each immunodominant membrane protein are generally strain-specific. Although an antiserum raised against the gyrase A protein of *Achleplasma laidlawii* was reported to react with phytoplasmas of three different 16S-groups (18), it also reacts with achleplasmas.

The SecA protein is one of only a few phytoplasmal membrane proteins for which a function has been determined. As a component of the Sec protein translocation system, SecA with its ATPase activity drives protein secretion in a stepwise manner (9). The Sec system is universally present in bacteria and is indispensable for cell viability. Amino acid sequence motifs are highly conserved among SecA proteins from widely ranging species of bacteria (14). Therefore, SecA is a viable candidate immunogen for the production of antibodies that react with many different phytoplasmas. We previously isolated and cloned the *secA* gene from the OY phytoplasma genome, prepared anti-SecA rabbit antibodies using a partially purified SecA protein that had been expressed in *E. coli*, and confirmed that the SecA protein is pro-

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duced in plants infected with OY phytoplasma (14). The aim of the present study was to evaluate the reactivity of different phytoplasmas with the antiserum raised against OY-SecA.

MATERIALS AND METHODS

Plant materials and phytoplasma isolates. Eight phytoplasma isolates of four distinct 16S-groups (13) were used in this study (Table 1). Five of the phytoplasmas were transmitted to periwinkle (*Catharanthus roseus* L.) and maintained therein using their insect vectors: water dropwort yellows (WDY), lettuce yellows (LeY), and adzuki yellows (AdY) by *Macrostes striifrons* Anufriev; gentian witches' broom (GWB) by *Scleroracrus flavopictus* Ishihara; and the jujube witches' broom (JWB) phytoplasmas by *Hishimonoides sellatiformis* Ishihara. Mulberry dwarf (MD) phytoplasma was transmitted by *H. sellatiformis* Ishihara and maintained in garland chrysanthemum (*Chrysanthemum coronarium* L.). MD-infected garland chrysanthemum and naturally infected mulberry (*Morus alba* L.) were used for immunohistochemical analysis and immunoblot analysis, respectively. Japanese hydrangea phyllody (JHP) and rice yellow dwarf (RYD) phytoplasmas were collected from naturally infected Japanese hydrangea (*Hydrangea macrophylla* Thunb.) and rice (*Oryza sativa* L.) host plants. In addition, OY phytoplasma was transmitted by *M. striifrons* to garland chrysanthemum and maintained therein. OY-infected garland chrysanthemum showing typical symptoms (yellowing, stunting, and virescence) was used as a positive control, and healthy garland chrysanthemum, mulberry, Japanese hydrangea, rice, and periwinkle were used as negative controls.

Immunoblot analysis. Leaves (0.1 g) of phytoplasma-infected plants and control plants were homogenized in 1 ml of 2× sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, and 2% [wt/vol] mercaptoethanol), transferred to a tube, boiled in water for 5 min, and centrifuged at 15,000 rpm for 10 min. The solubilized proteins present in the supernatant were separated on 8% (wt/vol) SDS-polyacrylamide gels using Laemmli buffer. Proteins were electroblotted from gels (Bio-Rad Laboratories, Hercules, CA) onto polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA). Immunological detection of blotted proteins was performed as described previously (14). Membranes were blocked with TBST (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.1% Tween 20 [vol/vol]) and incubated with 3 µg of anti-SecA immunoglobulin G (IgG) per ml. After incubation with this primary antibody, the membranes were washed with TBST and incubated with a horseradish peroxidase-tagged secondary antibody (Amersham Bioscience, Piscataway, NJ) that had been diluted 1:5,000 in TBST. Reacted proteins were visualized with an enhanced chemiluminescence (ECL) Plus Kit (solution A/solution B = 4:1 [vol/vol]) according to the manufacturer's protocol (Amersham Bioscience).

TABLE 1. Classification status and host plant sources of phytoplasmas used in this study

Name of phytoplasma	Acronym	16S-group/ subgroup ^a	Plant material
Water dropwort yellows	WDY	AY/AY	Periwinkle
Lettuce yellows	LeY	AY/AY	Periwinkle
Mulberry dwarf	MD	AY/AY	Garland chrysanthemum/ mulberry
Onion yellows	OY	AY/AY	Garland chrysanthemum
Japanese hydrangea phyllody	JHP	AY/JHP	Japanese hydrangea
Adzuki yellows	AdY	WX/WX	Periwinkle
Gentian witches' broom	GWB	WX/WX	Periwinkle
Rice yellow dwarf	RYD	RYD/RYD	Rice
Jujube witches' broom	JWB	EY/JWB	Periwinkle

^a AY, aster yellows; WX, western X; and EY, elm yellows.

Immunohistochemical analysis. Immunohistochemical analysis of infected plants was carried out using anti-SecA IgG as described by Oshima et al. (24). A main stem or a root about 5 mm in length was excised from each phytoplasma-infected plant, fixed, embedded in paraffin, and cut into 8-µm sections with a microtome. Anti-SecA IgG was allowed to react with the cross sections, and phytoplasma was detected with an alkaline-phosphatase reporter system according to the manufacturer's protocol (Promega, Madison, WI). To prevent rice root sections from floating off, the slides were treated with 2% (wt/vol) 3-aminopropyltriethoxysilane before use.

RESULTS

Immunoblot analysis. An antiserum raised against SecA of OY phytoplasma was evaluated for detection of different phytoplasmas. Crude protein preparations of WDY, LeY, MD and JHP (AY 16S-group), AdY and GWB (WX 16S-group), RYD (RYD 16S-group), and JWB phytoplasmas (EY 16S-group) were prepared from infected plants (Table 1) and separated on 8% (wt/vol) SDS-polyacrylamide gel electrophoresis. Western blot analysis using the anti-SecA IgG revealed an abundant polypeptide in fractions prepared from phytoplasma-diseased plants (Fig. 1, lanes 1, 3, 5, 7, and 9 to 13) that was not present in the extracts of healthy plants (Fig. 1, lanes 2, 4, 6, 8, and 14). The estimated molecular weight of the detected polypeptides (≈96 kDa) corresponded well with that of OY SecA (95.7 kDa), as observed previously (14). In the JHP-diseased plant and RYD-diseased plant, a nonspecific polypeptide also was detected (Fig. 1, lanes 5 and 7), respectively.

Immunohistochemical analysis. To confirm that the anti-SecA antibody raised against SecA of OY phytoplasma also can be used to detect different phytoplasmas in diseased plants, and to compare their distribution patterns in the infected phloem cells, immunohistochemical localization of the detected protein was performed. Blue signals representing the SecA protein were detected in all phytoplasma-infected plants (Fig. 2A, B, D, F, and H), whereas no signal was detected in healthy plants (Fig. 2C, E, G, and I). Moreover, the SecA-specific signals were restricted to the phloem tissues (Fig. 2A, B, D, F, and H), where phytoplasmas are exclusively localized in plants (17,19,22). Unlike garland chrysanthemum and Japanese hydrangea, periwinkle and rice have two distinct (internal and external) phloem tissues, and the phytoplasmas were observed in both (Fig. 2D and H).

Internal morphological changes also were observed in host plants infected with phytoplasmas. In OY-infected garland chrysanthemum, phloem hyperplasia and sieve-cell necrosis (Fig. 2A) (24) were apparent. Necrotic cells were misshapen and dark

Fig. 1. Western immunoblot detection of the SecA protein in phytoplasma-infected plants using an anti-OY-SecA antiserum. Lane 1, garland chrysanthemum infected with onion yellows phytoplasma; lane 2, healthy garland chrysanthemum; lane 3, mulberry infected with mulberry dwarf phytoplasma; lane 4, healthy mulberry; lane 5, Japanese hydrangea infected with Japanese hydrangea phyllody phytoplasma; lane 6, healthy Japanese hydrangea; lane 7, rice infected with rice yellow dwarf phytoplasma; lane 8, healthy rice; lanes 9 to 13, periwinkle plants infected with water dropwort yellows, lettuce yellows, adzuki yellows, gentian witches' broom, and jujube witches' broom phytoplasmas, respectively; and lane 14, healthy periwinkle.

brown, and no blue signal indicative of phytoplasmas was observed in these cells (Fig. 2A). In addition, vascular bundles lost their normal shape and the phloem tissues of vicinal vascular bundles were connected with each other, due to phloem hyperplasia (Fig. 2A). In contrast, in MD-infected garland chrysanthemum, no phloem hyperplasia or necrosis was observed (Fig. 2B), its internal symptoms were similar to those of garland chrysanthemum infected with OY-M, a variant derived from OY that causes only mild proliferation, mild leaf yellowing, and no stunting of the plant (24). Vascular bundles in RYD-infected rice were barely distinguishable from neighboring bundles, due to structural breakdown (Fig. 2H). No histopathological changes except for phloem necrosis were observed in periwinkles infected with WDY (Fig. 2D), LeY, AdY, GWB or JWB (data not shown), or in JHP-infected Japanese hydrangea (Fig. 2F).

DISCUSSION

An antiserum that detects multiple phytoplasma strains has long been needed. The antiserum raised against OY phytoplasma

SecA (14) is a plausible candidate to detect a wide range of phytoplasmas, because the SecA protein is unique to bacteria, archaea, and organelles (25), and the sequences of the OY SecA and the putative SecA of chloroplasts are not very similar (data not shown). Thus, this antiserum should result in low levels of background signals derived from plant or organelle components. Second, reaction of anti-SecA antiserum with OY phytoplasma-infected plant tissue resulted in a specific SecA signal in the phloem (14) where phytoplasmas reside. In this study, we have demonstrated that anti-OY SecA antibody raised against SecA protein of OY phytoplasma (AY 16S-group) can be used to specifically detect a wide range of phytoplasmas, including WDY, LeY, MD, and JHP (AY 16S-group), AdY and GWB (WX 16S-group), RYD (RYD 16S-group), and JWB (EY 16S-group).

Immunoblotting analysis with anti-OY SecA antibody indicated that a disease-specific polypeptide was detected in all phytoplasma-infected plants tested, but not in uninoculated healthy plants. The estimated molecular mass of the detected protein (≈ 96 kDa) corresponds well with the calculated molecular weight of the OY SecA protein. This strongly suggests that the

Fig. 2. Immunohistochemical detection of the SecA protein in cross sections of phytoplasma-infected plants. **A and B**, Garland chrysanthemum infected by onion yellows and mulberry dwarf phytoplasma, respectively; **C**, healthy garland chrysanthemum; **D**, periwinkle infected with water dropwort yellows (WDY); **E**, healthy periwinkle; **F**, Japanese hydrangea infected with Japanese hydrangea phyllody phytoplasma; **G**, healthy Japanese hydrangea; **H**, rice infected with rice yellow dwarf phytoplasma; and **I**, healthy rice. The immunohistochemical results of periwinkle infected with WDY, lettuce yellows, adzuki yellows, gentian witches' broom, and jujube witches' broom phytoplasma were similar; therefore, here only one periwinkle sample (WDY-infected periwinkle) was shown. X, xylem; P, phloem. Scale bar = 0.1 mm. Blue pigmentation represents the localization of the SecA protein. Blue arrowhead indicates necrotic cells.

protein detected by the anti-OY SecA antibody was the SecA protein from each of the different phytoplasmas that was used to infect these plants.

Immunohistochemical analysis using the anti-SecA antibody revealed that specific signals were restricted to the phloem cells of the diseased plants. These data again strongly suggest that other phytoplasmas also synthesize SecA proteins that are specifically detectable by the anti-OY SecA antibody. Importantly, the intensities of the signals for the SecA proteins were almost identical among the different phytoplasmas, including the OY phytoplasma. This suggests that the epitope sequences of SecA proteins from different phytoplasmas are relatively conserved. In OY-infected garland chrysanthemum, no blue signal indicative of phytoplasmas was observed in necrotic phloem cells (Fig. 2A). This demonstrated that phytoplasma might not be present in these tissues, or that degradation of phytoplasmal proteins in these necrotic cells due to plant defensive reaction resulted in failure of signal detection.

The external and internal symptoms of host plants infected with phytoplasmas also were compared. Infection with the OY and MD phytoplasmas, which both belong to the AY 16S-group, resulted in different internal morphological changes in garland chrysanthemum (Fig. 2A and B), possibly due to differences in the pathogenicity of these phytoplasmas. Although a garland chrysanthemum plant infected with OY displayed typical external symptoms (yellowing, stunting, and proliferation) 4 weeks post-infection, a plant infected with MD only showed stunting without any other apparent symptoms. Internal differences also were evident in immunohistopathological analysis. However, in periwinkle plants infected through graft inoculation with the WDY (Fig. 1D), LeY, AdY, GWB, or JWB phytoplasmas (data not shown), rather than by insect transmission, only very mild histopathological changes were observed.

To our knowledge, the anti-SecA antiserum is the first phytoplasma-originated antiserum that can be used to detect a broad spectrum of phytoplasmas. This antiserum may be useful in the immunological detection of phytoplasmas. In addition, the use of the anti-SecA antiserum may facilitate the detection and identification of undiscovered phytoplasmas. Finally, the antiserum may aid in studies of the histopathological changes in infected host plants.

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