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A secreted effector protein of AY-WB phytoplasma accumulates in nuclei and alters gene expression of host plant cells, and is detected in various tissues of the leafhopper *Macrosteles quadrilineatus*

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Abstract

Aster yellows phytoplasma strain witches' broom (AY-WB) is transmitted by the polyphageous aster leafhopper, *Macrosteles quadrilineatus*. AY-WB is abundantly present in cells of various aster leafhopper tissues, including midgut and salivary glands. In another study, we mined the complete genome sequence of AY-WB for genes encoding secreted proteins based on the presence of N-terminal signal peptides. This resulted in the identification of 56 secreted AY-WB proteins (SAPs), which are candidate effector proteins potentially involved in modulating the physiology of plant and insect hosts. Indeed, one SAP (SAP11) was shown to accumulate in nuclei of plant host cells and to change gene transcription profiles of plants. In this study, we show that SAP11 is present in AY-WB-infected aster leafhopper, and appears to be particularly abundant in some salivary gland cells.

Key words: Immunolabeling, confocal laser-scanning microscopy, virulence proteins, genomics, homoptera, cicadellidae.

Introduction

The genome of aster yellows phytoplasma strain witches' broom (AY-WB) was sequenced to completion (Bai *et al*., 2006; http://www.jic.ac.uk/staff/saskia-hogenhout/genome.htm). AY-WB belongs to subgroup 16SrI-A of '*Candidatus* Phytoplasma asteris'. The vector of AY-WB is the polyphageous aster leafhopper, *Macrosteles quadrilineatus* (Forbes). AY-WB has a broad host range, including but probably not restricted to China aster (*Callistephus chinensis* Nees), lettuce (*Lactuca sativa* L.), *Nicotiana benthamiana* L., tomato (*Solanum lycopersicum* L.) and *Arabidopsis thaliana* (L.) Heynhold. AY-WB is mainly limited to the phloem of its plant hosts, but can invade and replicate in most organs of *M. quadrilineatus* (Ammar and Hogenhout, 2006).

The AY-WB genome was mined for the presence of membrane-targeted proteins by determining whether deduced protein sequences carry N-terminal signal peptide (SP) sequences. This resulted in the identification of 76 AY-WB proteins with SPs. Of these 76, 20 proteins contained a SP and additional transmembrane domains. Thus, these 20 proteins are apparently initially secreted but remain associated with the membranes. The remaining 56 proteins contain only SPs and no additional transmembrane domains, and hence, are probably secreted into the extracellular environment of AY-WB. These 56 proteins were named secreted AY-WB proteins (SAPs), and are potential effector proteins involved in the manipulation of plant and insect physiology (Bai *et al*., unpublished).

Additional independent evidence that the SAPs are effector proteins was obtained (Bai *et al*., unpublished). Two phytoplasma effectors, SAP11 and SAP30, contain eukaryotic nuclear localization signals (NLSs) that are functional in plant cells. Indeed, SAP11 localizes in host nuclei of AY-WB-infected China aster plants, and needs the NLS domain for this nuclear localization. In *N. benthamiana*, nuclear localization of SAP11 requires a host factor, importin α. Also, SAP11 alters gene transcription, notably of several transcription factors, in tomato (Bai *et al*., unpublished). Here we report the detection of SAP11 in *M. quadrilineatus*.

Materials and methods

The SAP11 protein was produced as a FLAG-tagged fusion in *Escherichia coli*. To this end, the portion of the open reading frame (ORF) corresponding to the SAP11 protein, but excluding the SP, was amplified using specific primers, cloned into the pFLAG-ATS vector (Sigma-Aldrich, St. Louis, MO), and electro-transformed into *E. coli* XL1-blue cells. At 6 h after induction with 0.4 mM IPTG, the supernatants of the *E. coli* cultures were collected by centrifugation at 6,000 rpm at 4 °C for 30 min, filtered through a 0.45 μm stringe, and affinity purified with anti-FLAG M2 agarose affinity gel columns (Sigma-Aldrich). Polyclonal antibodies to FLAG-SAP11 were produced in 6-week-old female balb/c mice.

Protein samples were separated in 10% - 15% SDS-PAGE gels in 1x Tris-glycine-SDS buffer and stained by Coomassie Brilliant Blue R-250 for visualization or electrophoretically transferred to nitrocellular membrane for Western blot hybridization. The protein-antibody hybridizations on Western blots were visualized by color reaction catalyzed by alkaline phosphatase (AP) or chemiluminescent reaction catalyzed by horseradish peroxidase (HRP).

Reverse transcription polymerase chain reactions (RT-PCR) were conducted using total RNA isolated from insects as template.

Methods for the iCLSM (immunofluorescence confocal laser scanning microscopy) studies were similar to those described elsewhere (Ammar and Hogenhout, 2005).

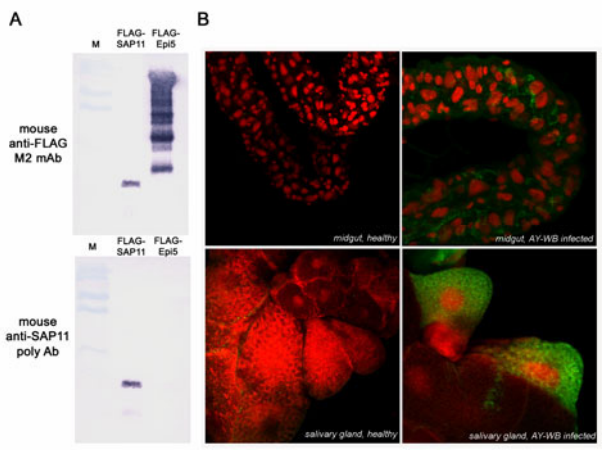

Figure 1. Detection of SAP11 in *M. quadrilineatus*. (A) Antibodies generated to FLAG-SAP11 specifically react with SAP11. The anti-SAP11 antibodies do not detect the FLAG fusion of FLAG-Epi5 (lower panel), whereas antibodies to the FLAG tag react with both proteins (upper panel). (B) ICLSM results showing the presence of SAP11 in the midgut and salivary glands of AY-WB-infected *M. quadrilineatus*. Green colors indicate SAP11 immunolabeled with Alexa Fluor-conjugated antibodies. Red colors indicate the propidium-iodide-stained nuclei in tissue cells. The SAP11 antibodies reacted with cells of AY-WB-infected insects (panels at right), but not with those of healthy insects (panels at left).
(In colour at www.bulletinofinsectology.org)

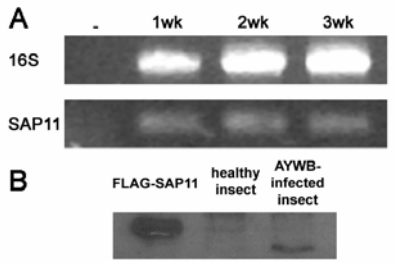

Figure 2. SAP11 is produced in insects. (A) RT-PCRs detect trans-cripts corresponding to AY-WB 16S rDNA and the SAP11 protein in AY-WB-infected *M. quadrilineatus* at one, two and three weeks (wk) after AY-WB acquisition from plants. Transcripts were not detected in insects that were not exposed to AY-WB-infected plants (-). (B) Detection of the mature SAP11 protein of predicted size in *M. quadrilineatus* protein extracts. Insect proteins were transferred to Western blots and hybridized to SAP11 antibodies (figure 1A). The SAP11 antibodies reacted with a protein of ~ 10 kDa in AY-WB-infected insects and with FLAG-SAP11. The ~ 10 kDa band was absent from healthy insects.

Results

Antibodies to FLAG-SAP11 specifically detected SAP11 (figure 1A). The iCLSM studies showed green fluorescence, indicating the presence of SAP11, in dissected guts and salivary glands of AY-WB-infected *M. quadrilineatus*, but not in those of healthy leafhoppers (figure 1B). SAP11 is apparently abundantly present in some salivary gland cells (figure 1B, lower right panel). The RT-PCR results indicated the presence of transcripts corresponding to SAP11 in *M. quadrilineatus* at several weeks after acquisition of AY-WB from plants (figure 2A). The SAP11 protein was detected in protein extracts of AY-WB-infected leafhoppers, and not in healthy leafhoppers (figure 2B). The protein is ~ 10 kDa, which corresponds to the predicted size of 10.6 kDa of the mature SAP11 protein, and as expected, is slightly smaller than the FLAG-SAP11 (figure 2B), since it does not have the FLAG tag in leafhoppers.

Discussion

The results of three independent experiments showed that SAP11 is produced during AY-WB-infection of *M. quadrilineatus*. SAP11 was detected in midgut and salivary gland cells by iCLSM. A protein corresponding to the size of the mature SAP11 was detected in protein extracts of leafhoppers. Finally, transcripts corresponding to SAP11 were detected by RT-PCR in leafhoppers at various time points after acquisition of AY-WB from plants. The iCLSM data suggest that SAP11 is not localized to insect cell nuclei, whereas this protein targets nuclei in plant cells (Bai *et al*., unpublished). The abundant presence of SAP11 in salivary gland cells provide evidence that SAP11 is introduced into plant cells along with AY-WB during insect feeding. The exact function of SAP11 in plants and insects remains to be elucidated.

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