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Production of polyclonal and monoclonal antibodies specific against membrane proteins of '*Candidatus* Phytoplasma asteris', chrysanthemum yellows isolate

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Abstract

Three genes encoding membrane proteins of chrysanthemum yellows (CY) phytoplasma were cloned and sequenced: *Sec*Y, *Amp* and *Art*I encoding the protein translocase subunit *Sec*Y, the antigenic membrane protein *Amp* and the arginine transporter *Art*I, respectively. Alignments of CY-specific *Sec*Y sequences with the corresponding genes of other phytoplasmas confirmed the 16SrDNA-based classification, while *Amp* sequences resulted highly variable. Five CY partial sequences were cloned into pRSetC expression vector and two (*Amp* 64-224 and *Art*I 131-512) were expressed as fusion antigens in *Escherichia coli* for the production of CY-specific polyclonal antisera (A416 against *Amp* 64-224; A407 against *Art*I 131-512) and monoclonal antibodies. A416 recognized, in Western blots, the full length *Amp* from CY-infected plants (periwinkle, daisy) and insect vectors (*Euscelidius variegatus*, *Macrosteles quadripunctulatus*). A416 also reacted to European aster yellows (EAY) and primula yellows (PY) phytoplasmas and to Northern Italian strains of '*Candidatus* Phytoplasma asteris' from lettuce (LY163) and gladiolus (GLA), but not to American aster yellows (AAY) and clover phyllody (CPh) phytoplasmas.

Key words: Chrysanthemum yellows, membrane-proteins, *Sec*Y, *Amp*, *Art*I, antibodies.

Introduction

Phytoplasmas are plant pathogenic uncultivable, endocellular, wall-less bacteria (Firrao *et al*., 2007). These characteristics allow phytoplasmal membrane proteins to contact host plant and insect cells directly. The importance of membrane proteins is also suggested by studies on other *Mollicutes*, in which the adhesion to the host cell, mediated by pathogen membrane proteins, plays an important role in infection.

Several proteins must be present in the phytoplasma membrane. *Amp* is the most abundant among them and it exposed to the extracellular environment. *Amp* genes have been identified in several phytoplasmas (Kakizawa *et al*., 2006). The function of this protein is unknown, but it is supposed to be involved in the phytoplasma transmission specificity by insects (Suzuki *et al*., 2006). The three Sec components (SecA/Y/E) have been chacterized in OY, suggesting that the Sec secretion system exists in phytoplasmas and is supposed to mediate the secretion of *Amp* (Kakizawa *et al*., 2004). Phytoplasmas have a very minimal gene set (Oshima *et al*., 2004) and their survival is probably due to the uptake of solutes from the host cells, as suggested by the presence, in their genomes, of different transporter systems (Bai *et al.* 2006), including Art, a family of ABC transporters involved in the uptake of arginine (Wissenbach *et al*., 1995).

Chrysanthemum yellows (CY) is associated with a disease in ornamental plants in the Italian Riviera and is transmitted by *Macrosteles quadripunctulatus* (Kirschbaum), *Euscelidius variegatus* (Kirschbaum) and *Euscelis incisus* (Kirschbaum) (Bosco *et al.*, 1997). CY belongs to the same ribosomal group (16SrI-B) as onion yellows (OY) and aster yellows witches' broom (AY-WB) phytoplasmas, which have been completely sequenced (Oshima *et al.*, 2004; Bai *et al.*, 2006). A CY-based model system has been useful to study some aspects of the relationships between the phytoplasma and the plant hosts (Saracco *et al*., 2006).

The aim of this work was to obtain molecular reagents to study the interactions among phytoplasmas, host plants and insect vectors.

Materials and methods

For the identification and sequencing of CY membrane-associated genes, five primer pairs were designed based on the sequence of the homologous genes of the closely related OY and AY-WB phytoplasmas, and used to amplify total DNA extracted from CY-diseased periwinkles. For the expression of fusion proteins, five primer pairs were designed based on the CY-specific sequences and directionally cloned into the pRSetC expression vector. The expression of the 5 CY inserts was induced with IPTG 1 mM in transformed *Escherichia coli* BL21 pLysS and evaluated by SDS-PAGE. The expressed fusion antigens *Amp* 64-224 and *Art*I 131-512 were electroeluted from gel and used to immunize rabbits (4 injections of 0.5 mg each, at two-week intervals). BALB/c mice were immunized with 0.5 mg of the two fusion antigens mixed together. Supernatants from hybridoma clones were screened by standard indirect ELISA and dot Western blots against the antigens.

For Western blots, proteins were extracted from 1 g of leaves from CY-infected daisy or periwinkles infected with European aster yellows (EAY), primula yellows (PY), American aster yellows (AAY), clover phyllody

(CPh) and two '*Ca.* P. asteris' strains isolated from lettuce (LY163) and gladiolus (GLA) in North Western Italy and from 10 CY-infected *M. quadripunctulatus* or 5 *E. variegatus* individuals. The samples were macerated in protein extraction buffer, sonicated, centrifuged and the supernatants were fractionated with Triton-X114 in aqueous and detergent phases. Proteins were separated by SDS-PAGE, blotted and membranes were incubated in (1: 1,000) primary antibodies, then in AP-conjugated secondary sera (Sigma) and developed with NBT and BCIP reagents.

Results

The complete CY sequences encoding *Sec*Y, *Amp* and *Art*I membrane proteins were obtained. While 16Sr-DNA gene sequence homology between CY, OY and AYWB is 99%, CY is closer to OY than to AY-WB when similarity is calculated on these three genes. *Amp* genes showed the highest variability: sequence identities comparing CY *Amp* with homologous ORFs from other '*Ca*. P. asteris' phytoplasmas (AAY, OY, AYWB, CPh) were 97, 96, 51, 30%, respectively.

Five CY constructs were cloned into the expression vector pRSetC, but only *Amp* 64-224 (14 kDa) and *Art*I 131-512 (25 kDa) fusion peptides were stably expressed. The antiserum A416 recognized in Western blots *Amp* 64-224 fusion antigen and the full length *Amp* (25 kDa) from CY-infected plant and insect membrane proteins, whereas A407 reacted only with *Art*I 131-512 fusion antigen. A416 reacted also with EAY-, PY-, LY163- and GLA-infected periwinkle membrane proteins, whereas no signal was produced from AAY- and CPh-infected samples.

Sixty out of 1248 supernatants from hybridoma clones recognized the fusion antigens in indirect ELISA and seven out of those sixty reacted in dot Western blots against the antigens (two against *Amp* 64-224 and seven against *Art*I 131-512). These monoclonal antibodies in Western blots reacted only with the fusion antigens.

Discussion

Sequence identities for all three genes indicated that CY is more similar to OY than to AY-WB, confirming the 16SrDNA-based classification. *Amp* sequence resulted highly variable even within the '*Ca*. P. asteris'. The possible requirement of concomitant *Sec*E and *Sec*Y expression may explain our failure to produce CY *Sec*Y fusion protein in *E. coli*. This is in line with the fact that only OY S*ec*A was expressed as partial fusion protein (Kakizawa *et al*., 2004). *Art*I protein could be easily detected in Western blots of transformed *E. coli* cells grown in minimal medium, while growth in reach medium inhibited its expression (Wissenbach *et al*., 1995). We therefore suggest that phytoplasmas colonizing plants and vectors may express low levels of *Art*I protein, which cannot be detected with our antibody.

A416 antibody reacted with all isolates of '*Ca*. P. asteris' but AAY and CPh. Antigenic differences between CY and the Florida isolate AAY may be explained by positive selection occurring in two separate geographic areas. CY and LY163 have been recognized by IgG raised against EAY purified cells; PY and EAY were already known to cross react between them. The molecular reagents produced in this work are currently used to study the role of membrane proteins in the interaction between CY and its plant and vector hosts.

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