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Effect of *Urtica dioica* agglutinin and *Arabidopsis thaliana* Chia4 chitinase on the protozoan *Phytomonas françai*

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Abstract

The genus *Phytomonas* is responsible for many diseases in different crop plant species. The finding that chitin is an exposed cell surface polysaccharide in *Phytomonas françai* and the observation that chitinases can inhibit fungal growth raises expectations about the potential effect of plant chitinases on the *P. françai* cell membrane surface. The plant chitinases *Urtica dioica* agglutinin (UDA) and *Arabidopsis thaliana Chia4* (ATCHIT4) proteins were over-expressed in bacteria and the interaction between these proteins and *P. françai* surface was analyzed by immunocytochemistry. We showed that UDA and ATCHIT4 proteins can interact with surface-exposed chitin from *P. françai*.

Keywords: Chitinase; Plant defense; Agglutinin; *Arabidopsis thaliana*; *Manihot esculenta*; *Phytomonas françai*

1. Introduction

The empty root is a disease associated with *Phytomonas françai*, a parasite flagellate of the family Trypanosomatidae. It was observed affecting certain cultivars of cassava (*Manihot esculenta*) in the Espírito Santo state of Brazil [1,2]. Usually, the roots of affected plants remain small and slender, containing little or no starch. The aboveground parts of infected plants show chlorosis and decline. The empty root disease can be transmitted by grafting and/or by insect vectors of the *Lincus* or *Ochlerus* genera (Hemiptera Pentatomidae). Diseased plants contain numerous *Phytomonas* in the lacifer ducts but not in the phloem [1]. Another member of the genus (*Phytomonas staheli*) has become one of the most serious pathogens of oil palm and coconut crops in South America. The control of such diseases has been limited to the elimination of affected plants and the bug responsible for the transmission (*Lincus* sp). This treatment is based on the use of organochlorines that have dangerous cumulative effects and concentrate along the food chains. Its employ in agriculture is therefore forbidden in most of the countries where the disease is rife [3]. Chitin is an important structural component of the cell wall of fungi and of the exoskeleton of many invertebrates, such as insects and nematodes. It has also been demonstrated that the *P. françai* presents chitin exposed in the membrane surface [4]. In this work the authors characterized chitin by its insolubility in hot alkali and chromatographic immobility, as well as by the release of glucosamine on hydrolysis with strong acid and of *N*-acetylglucosamine (Glc*N*Ac) on hydrolysis with chitinase. The presence of chitin was also shown directly by binding of wheat-germ agglutinin (WGA) [4]. Chitin is also present as a structural component of *Trichomonas vaginalis* and *Tritrichomonas foetus* [5] and in the cyst wall of *Entamoeba* [6,7]. Chitin may be important in the maintenance of cell integrity by providing protection from mechanical and chemical environmental stress.

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Chitinases alone, or acting synergistically with glucanases, can inhibit fungal growth in vitro or in vivo when over-expressed in transgenic plants [8–10]. Two proteins with distinct characteristics of this group might be used against trypanosomatids: the *Urtica dioica* agglutinin (UDA) and the *Arabidopsis thaliana Chia4* (ATCHIT4). Stinging nettle lectin or UDA is a single-chain protein containing two homologous chitin-binding domains [11,12], which independently bind to *N*,*N*′,*N*″-triacetylchitotriose or larger oligomers [13,14]. UDA is present in stems and in the outer exodermis cell layer of roots, but it is absent from leaves [12,15,16]. UDA possesses both antifungal and insecticidal activities [15,17]. It exhibits growth-inhibiting activities against several plant pathogenic fungi, which contain chitin in their cell walls [15]. UdaI cDNA comprises an N-terminal signal peptide, two chitin-binding domains, a small hinge region, and a C-terminal chitinase domain [18,19]. The signal peptide, the hinge region, as well as the chitinase domain are processed from the precursor to yield mature UDA. Because of the presence of two chitin-binding domains and the homology of the chitinase domains with the other plant chitinases (45% identity with chitinase domains of plant class I chitinases), the precursor to UDA was classified as a *Chia5* chitinase [20,21].

ATCHIT4 protein contains an N-terminal signal peptide, one chitin-binding domain and one C-terminal chitinase domain separated by a hinge region. Deletions in the catalytic domain, characteristic of all *Chia4* chitinases, are also present. The specific physiological role of ATCHIT4 remains unclear. Apparently, ATCHIT4 is involved in both plant–pathogen interaction and embryo development [22]. ATCHIT4 transcripts are detected in seedpods, but not in roots, inflorescence stems, leaves or flowers of healthy plants. However, analysis of ATCHIT4 transcripts expression showed that this chitinase is involved in a rapid defense response to bacteria, wounding, UV light and salicylic acid treatment [23].

The aim of this work was to characterize the effect of these two plant chitinases against the pathogenic trypanosomatid *P. françai*. For this purpose we over-expressed in *Escherichia coli* the ATCHIT4 and UDA chitinases fused to maltose-binding protein – MBP. We show that both protein fusions interacted with surface carbohydrate residues and were able to trim chitin present on the surface of *P. françai*.

2. Materials and methods

2.1. Cloning procedures, production and purification of fusion proteins

The cDNAs of ATCHIT4 and UdaI were introduced into the polylinker of vector pMAL-2c, downstream from the MalE gene of *E. coli*, resulting in expression of the target gene product as a fusion to MBP, a water-soluble protein with a molecular mass of 42.7 kDa. The molecular masses of MBP-UDA and MBP-ATCHIT4 are 82.5 and 72.5 kDa, respectively. Expression of the fusion proteins was induced by growing the bacteria BL21 for further 4 h in the presence of IPTG (isopropylthio-β-galactoside – final concentration 0.0003 mM). After this time, the cells were harvested by centrifugation ($4000\times g$ for 10 min), and re-suspended in STE buffer (150 mm NaCl, 1 mm EDTA, 10 mm Tris, pH 8.0) containing 0.1 mg ml^{-1} lysozyme, and incubated on ice for 2 h. Dithiothreitol (final concentration 5 mm) and *N*-laurylsarcosinate (sarcosyl – final concentration 0.5%) were then added [24]. The cells were lysed by cycles of quick freezing in liquid nitrogen and thawing at 37°C. The lysate was centrifuged at $12\,000\times g$ for 30 min. The supernatant was centrifuged once more at $12\,000\times g$ for 30 min to minimize clogging of the amylose resin column and Triton X-100 (final concentration 1.5%) was added. An enriched fraction was then obtained through affinity chromatography onto amylose resin, according to the manufacturer's specification (Protein Fusion and Purification System – BioLabs). Protein concentration was determined using a bicinchoninic acid Protein Assay Reagent kit (Pierce). Purity of the fusion protein was checked by 7.5% SDS–polyacrylamide gel electrophoresis (PAGE). Protein bands were visualized with Coomassie brilliant blue R-250.

Trypsin (2 μg ml^{-1}, 37°C; 1.30 h) was used for cleavage of MBP-UDA. Reaction was stopped by the addition of leupeptin to a final concentration of 1 mM. The samples were analyzed by SDS–PAGE. After cleavage, the UDA protein was purified using affinity chromatography onto amylose resin.

2.2. Chitinase assay

For quantitative assays of chitinase activity, measurements were carried out using chitin (Sigma) as a substrate as described [8,25]. For qualitative assays, the chitinase activity was detected using SDS–PAGE in combination with glycol chitin substrate [26].

2.3. Microorganism

P. françai was maintained by weekly transfer [27]. For the experiments, the cells were grown in Warren's complex medium (37 g l^{-1} brain heart infusion, 10 mg ml^{-1} folic acid and 10 mg ml^{-1} hemin). After 96 h of incubation, the cells were collected by centrifugation ($2000\times g$) for 10 min at 4°C, and were washed three times in 0.01 mM phosphate-buffered saline (PBS, pH 7.2).

2.4. Binding analysis of protein fusions and P. françai

For the experiments, 25 μl of PBS containing 1×10^7 protozoa ml^{-1} was placed on a glass slide, air-dried, and

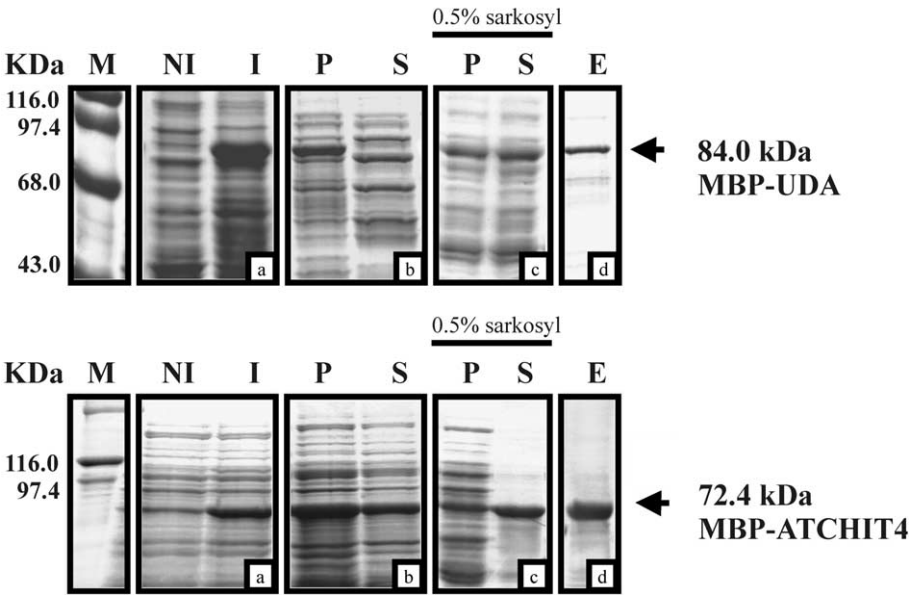


Fig. 1. SDS–PAGE (7.5% acrylamide) analysis: expression and solubilization of MBP-UDA and MBP-ATCHIT4 fusion proteins. a: Comparison of IPTG-induced *E. coli* clones harboring cDNAs of *uda* or *atchit4*. Cells were harvested just before induction with 0.3 mm IPTG (NI) and 4 h after induction (I). b: Solubilization analysis of the MBP-UDA and MBP-ATCHIT4 fusion proteins using lysozyme and the freeze–defreeze method. c: Solubilization of the MBP-UDA and MBP-ATCHIT4 fusion proteins using lysozyme, sarcosyl and the freeze–defreeze method. After the freeze–defreeze method, the lysate was subjected to centrifugation. d: The proteins MBP-UDA and MBP-ATCHIT4 were purified by affinity chromatography onto amylose resin after lysis of bacteria. Samples taken of supernatant (S) and insoluble pellets (P). The lane M shows prestained marker with the molecular masses indicated. E, eluate.

fixed in methanol for 10 min at room temperature. Slides were pre-incubated in PBS with 1% bovine serum albumin (BSA) for 1 h, and then incubated for 1 h at 4°C in a moist chamber in PBS with: 20 μg ml^{-1} of MBP-UDA, MBP-ATCHIT4 or MBP proteins. As a control for the proteins, cells were incubated with equal volumes of PBS. The slides were washed three times with PBS, incubated first with 25 μl of anti-MBP antibodies for 1 h, and then with PBS containing 1% BSA for 1 h. After that, the slides were incubated with 20 μl of fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG (Sigma) at a concentration of 100 μg ml^{-1} for 1 h at room temperature. The slides were then washed in PBS, and examined under a fluorescence microscope with standard filter for observing fluorescence from fluorescein (Zeiss Axioplan equipped with epifluorescence).

2.5. FITC/WGA-binding studies

Slides were prepared as described above, and incubated in a moist chamber with any one of the following proteins dissolved in PBS in a final concentration of: 5 μg of chitinase from *Streptomyces griseus* (5 mU – Sigma); 5 μg, 10 μg or 20 μg of MBP-UDA, MBP-ATCHIT4, UDA or MBP proteins, for 12 h, 24 h, 36 h, and 48 h at 4°C. Control cells were incubated with equal volumes of PBS for similar periods. The slides were washed twice with PBS and incubated with 15 μl of FITC-labeled WGA (Sigma) at a concentration of 0.1 μg ml^{-1} for 1 h at room temperature. The slides were then washed in PBS, and examined under a fluorescence microscope with standard filter for observing fluorescence from fluorescein (Zeiss Axioplan equipped with epifluorescence).

2.6. FACS (fluorescent-activated cell sorter) analysis

The protozoa were collected by centrifugation ($2000 \times g$) for 10 min at 4°C, and washed three times in PBS. Cells were fixed in 4% formaldehyde for 30 min at room temperature, washed three times in PBS and re-suspended in 1% BSA in PBS. Experiments were performed with 1×10^7 protozoa ml^{-1} in PBS incubated with MBP-UDA, MBP-ATCHIT4, UDA or MBP proteins at a final concentration of 20 μg ml^{-1} for 48 h and 72 h at 37°C. *S. griseus* chitinase was used as positive control. Residues of *N*-acetyl-D-glucosamine on the cell surface of *Phytomonas* were labeled by FITC WGA (Sigma) at a concentration of 10 μg ml^{-1} for 1 h at room temperature. The cells were washed and re-suspended in PBS. Flow cytometry of *P. françai* treated with proteins was performed on a dual beam instrument (Coulter EPICS® Elite). Positive or negative staining with FITC-WGA was defined as the emission of a level of fluorescence that exceeded or did not exceed, respectively, levels obtained by 99% of the cells from the same starting population when these were incubated only with PBS. Data were analyzed using WinMDI software (Joseph Trotter, Scripps Research Institute, La Jolla, CA, USA). Fluorescence levels derived from the FACS analysis were plotted versus cell number.

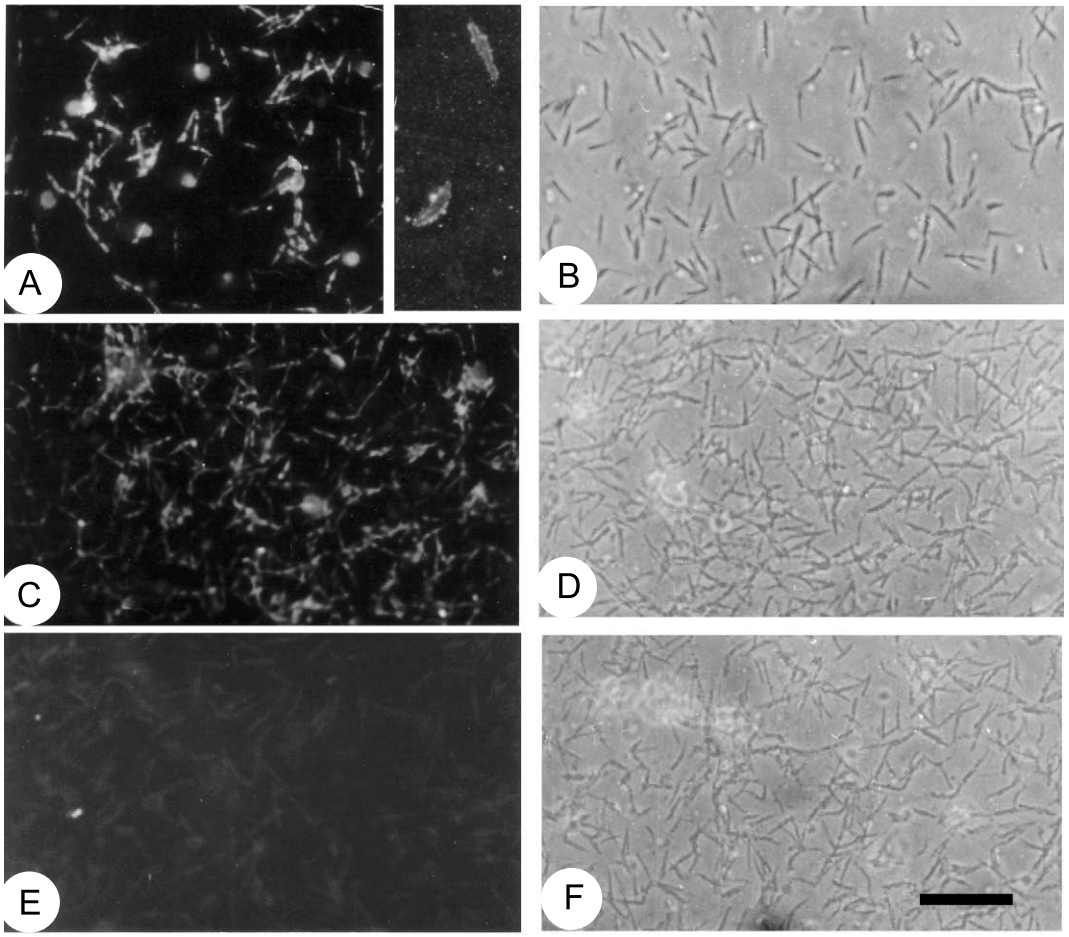


Fig. 2. Binding of MBP-UDA, MBP-ATCHIT4 and MBP to the cell surface of *P. françai*. Cells were incubated for 1 h with MBP-ATCHIT4 (A), MBP-UDA (C) and MBP (E) proteins and then anti-MBP antibodies were used for the immunolabeling. Visualization was performed using FITC-labeled anti-rabbit IgG. For MBP no labeling was observed. B, D and F: phase-contrast of the same fields shown in A, C and E, respectively. Bar equals 30 μm.

3. Results

3.1. Over-expression and purification of MBP-UDA and MBP-ATCHIT4

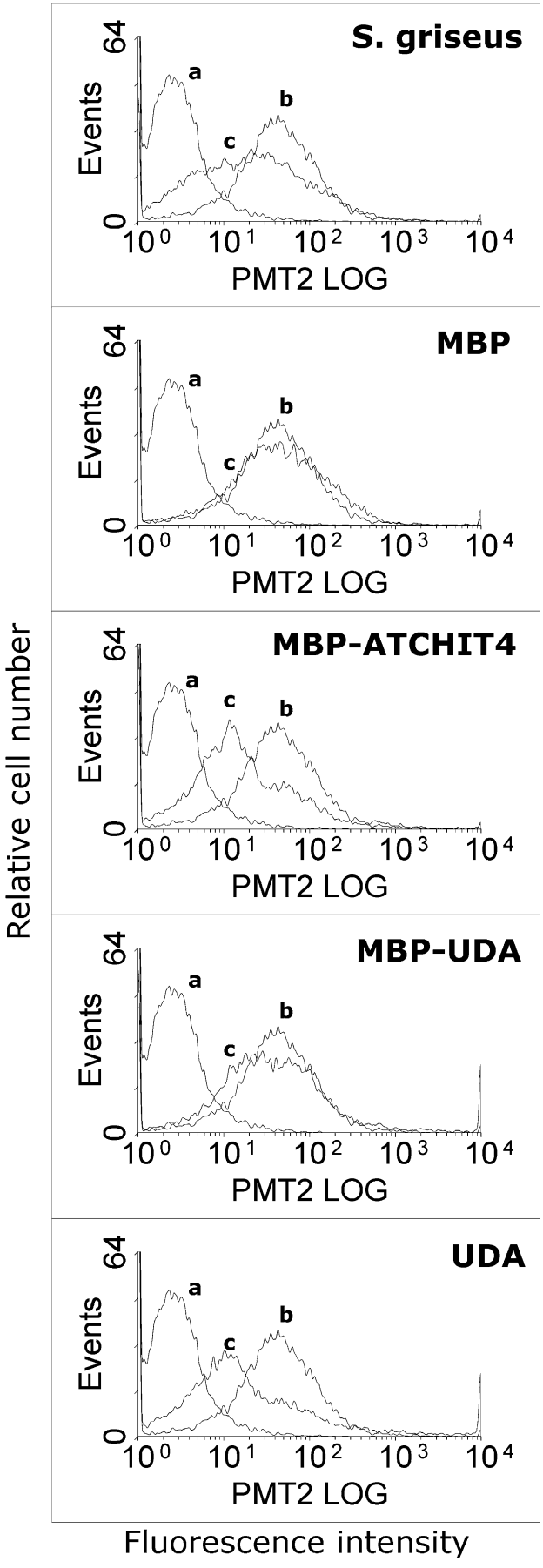
Protein fractions analysis showed that the chimeric proteins were localized in the bacterial-insoluble fractions. A standard protocol associated with a sarcosyl and Triton X-100 treatment was used to solubilize the chimeric MBP-ATCHIT4 and MBP-UDA proteins (Fig. 1, top) [24]. An enriched fraction was then obtained after affinity chromatography onto amylose resin (Fig. 1, bottom).

3.2. Functional analysis of the chimeric MBP-UDA and MBP-ATCHIT4 proteins

Colorimetric assay using chitin as substrate demonstrated that the chimeric MBP-UDA and MBP-ATCHIT4 displayed chitinase activity. The chitinase activities of MBP-UDA and of MBP-ATCHIT4 were approximately 10–15-fold lower than that of *S. griseus* chitinase. To obtain 1 mU of chitinase activity 2.5 μg, 25.8 μg and 37.7 μg of *S. griseus* chitinase, MBP-UDA and MBP-ATCHIT4 are necessary, respectively. Using SDS–PAGE in combination with glycol chitin 0.01% substrate we observed that over-expressed UDA without MBP protein also displays chitinase activity (data not shown). Lysis zones were visualized by UV illumination as non-fluorescent dark bands corresponding to the localization of the UDA in SDS–PAGE in contrast to the fluorescent intact glycol chitin. No lysis zone was observed when MBP protein was used (data not shown).

Immunolabeling assays were performed to check the binding competence of the chimeric proteins to the cell surface of *P. françai*. After incubation with the chimeric proteins, the cells were incubated with antibodies against MBP protein. These antibodies were obtained from rabbits by inoculation with MBP (LGMV, Departamento de Genética, UFRJ). The cells were finally incubated with FITC-labeled anti-rabbit IgG (Sigma) and were seen by fluorescence microscopy.

Only the cells incubated with MBP-UDA or MBP-ATCHIT4 proteins were labeled. No labeling was observed when the cells were incubated with MBP protein,



indicating a specific interaction between UDA, ATCHIT4 and *P. françai* cell surface (Fig. 2).

In order to check if chimeric proteins were able to trim the chitin present on the surface of the *P. françai*, cells of this protozoan were incubated with MBP-UDA or MBP-ATCHIT4, and then with FITC-labeled WGA. The cells were seen by fluorescence microscopy. The carbohydrate specificity of WGA includes terminal sialic acid, as well as *N*-acetyl-D-glucosamine and its β-1,4-linked oligomers [28–30]. After 36–48 h of incubation with MBP-UDA and MBP-ATCHIT4, the fluorescent labeling on the *Phytomonas* cell surface was dramatically reduced compared to that of the controls, indicating a chitinase activity against *P. françai* (data not shown).

FACS analysis of the interaction between chimeric proteins and surface carbohydrate residues of *P. françai* corroborated the microscopic analysis. *P. françai* treated with chimeric proteins for 48 or 72 h, before incubation in FITC-labeled WGA for 1 h, were screened by FACS to isolate cells that bind to FITC-labeled WGA with high affinity. Purified MBP and commercial *S. griseus* chitinase were used as negative and positive controls, respectively. The initial analysis showed that MBP-UDA had lower chitinase activity when compared with the MBP-ATCHIT4. In order to make sure that the UDA chitinase activity had not been inhibited by the presence of MBP protein in the chimeric fusion, MBP was removed from recombinant UDA by trypsin digestion. The purified UDA protein was more effective on the surface of *P. françai*, when compared with MBP-UDA protein (Fig. 3).

4. Discussion

Several experiments have shown the importance of chitinases as defense mechanisms against pathogenic attacks. Their effects on fungi have been well characterized [31–35], but no information was available about the activity of plant chitinase against protozoa. We have shown for the first time that two plant chitinases (ATCHIT4 and UDA) can bind to the cell surface and trim chitin present in a phytopathogenic protozoan, suggesting that plant chitinases may represent a defense mechanism against protozoa attacks in addition to their role against fungal and bacterial infection. However, further studies are necessary to completely understand the mechanisms of plant resistance to phytoprotozoan.

In the present work, we over-expressed UDA and

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Fig. 3. FACS analysis of the interaction between proteins and surface carbohydrate residues of *P. françai*. Flow cytometric analysis of *P. françai* cells labeled with FITC WGA after treatment with *S. griseus*, MBP, MBP-ATCHIT4, MBP-UDA and UDA proteins for 48 h. Autofluorescence inherent to parasites (a); incubation of untreated (b) and protein-treated (c) *Phytomonas*.

ATCHIT4 proteins fused with MBP in *E. coli*. Both chimeric proteins show a lower level of chitinase activity, when compared with commercial chitinase from *S. griseus*. Some factors can be responsible for this low level of chitinase activity: an inadequate folding of the protein during the translation in *E. coli* cytoplasm or in the refolding step through the protein purification, or the use of an inappropriate substrate in the chitinase assay. The presence of MBP protein fused to the chitinases can also be responsible for altering the proteins three-dimensional structure and/or hiding catalytic sites inhibiting the chitinase activity. This outcome was observed when it was compared to the ability of UDA and MBP-UDA to trim chitin on the cell surface of *P. françai*. The UDA protein presented a more pronounced effect on the protozoa when compared with MBP-UDA. As mentioned before, the presence of MBP could explain the lower level of chitinase activity observed in MBP-UDA. However, the binding activity of the chimeric MBP-UDA protein to chitin was not hindered by the presence of MBP. The chimeric protein MBP-ATCHIT4 presents a higher level of activity when compared with MBP-UDA and UDA.

The MBP-UDA and MBP-ATCHIT4 proteins are capable of recognizing and binding to the surface of *Phytomonas*. This effect is not related to MBP because this protein does not bind or trim chitin residues (Figs. 2 and 3). After 1 h of incubation, the chimeric proteins were observed coupled to the *Phytomonas* surface (Fig. 2). When chitinase assay was performed with *Phytomonas* throughout different incubation periods, fluorescence loss increased through time. This observation indicates that the chimeric proteins bind to the surface of *Phytomonas* and their chitinase activity triggers the removal of chitin from the cell surface by progressive trimming. The fluorescent intensity quantification by FACS further supported the fluorescent microscopy analysis.

ATCHIT4 gene was previously characterized by our laboratory [22] and others [23], although no functional assay was performed. We have shown at this time that the ATCHIT4 protein presents chitinase activity. It has been suggested that ATCHIT4 is involved in plant protection and also in embryo development [22]. Ponstein et al. [34] also reported that tobacco chitinase of class IV presents antifungal activity by causing in vitro growth inhibition of *Trichoderma viride* and *Fusarium solani*, by causing lysis of the germ tubes and/or growth inhibition.

Regardless of the genus *Phytomonas* being the causal agent of diseases affecting plants of economic importance, very little attention has been assigned to understand its interaction with plants.

To our knowledge, this is the first report demonstrating the interaction between plant chitinases and chitin chains present in the cell wall of a phytoprotozoan, namely *P. françai*. The identification of plant proteins with activity against *Phytomonas* phytopathogens can provide important clues to understand the interaction between plant/protozoa and to develop strategies to improve plant resistance against this class of pathogens.

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References

[1] Agrios, G.N. (1997) Plant diseases caused by flagellate protozoa. In: Plant Pathology 4th edn., 16, pp. 599–606. Academic Press, New York.

[2] Kitajima, E.W., Vainstein, M.H. and Silveira, J.S.M. (1986) Flagellate protozoan associated with poor development of the root system of cassava in the Espírito Santo State, Brazil. Phytopathology 76, 638–642.

[3] Louise, C., Dollet, M. and Mariau, D. (1986) Research into hartrot coconut, a disease caused by *Phytomonas* (*Trypanosomatidae*), and into its vector *Lincus sp.* (*Pentatomidae*) in Guiana. Oléagineux 10, 437–449.

[4] Nakamura, C.V., Esteves, M.J.G., Andrade, A.F.B., Alviano, C.S., Souza, W. and Angluster, J. (1993) Chitin: a cell-surface component of *Phytomonas françai*. Parasitol. Res. 79, 523–526.

[5] Kneipp, L.F., Andrade, A.F.B., de Souza, W., Angluster, J., Alviano, C.S. and Travassos, L.R. (1998) *Trichomonas vaginallis* and *Tritrichomonas foetus*: Expression of chitin at the cell surface. Exp. Parasitol. 89, 195–204.

[6] Arroyo-Begovich, A. and Carabez-Trejo, A. (1982) Location of chitin in the cyst wall of *Entamoeba invadens* with colloidal gold tracers. J. Parasitol. 68, 253–258.

[7] Arroyo-Begovich, A., Carabez-Trejo, A. and Ruiz-Herrera, J. (1980) Identification of the structural component in the cyst wall of *Entamoeba invadens*. J. Parasitol. 66, 735–741.

[8] Boller, T., Gehri, A., Mauch, F. and Vögeli, U. (1983) Chitinase in bean leaves: induction by ethylene, purification, properties, and possible function. Planta 157, 22–31.

[9] Broglie, K., Chet, I., Holliday, M., Cressman, R., Biddle, P., Knowlton, S., Mauvais, C.J. and Broglie, R. (1991) Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. Science 254, 1194–1197.

[10] Zhu, Q., Maher, E.A., Massoud, S., Dixon, R.A. and Lamb, C.J. (1994) Enhanced protection against fungal attack by constitutive co-expression of chitinase and glucanase genes in transgenic tobacco. Bio-Technology 12, 807–812.

[11] Beintema, J.J. and Peumans, W.J. (1992) The primary structure of stinging nettle (*Urtica dioica*) agglutinin: a two-domain member of the hevein family. FEBS Lett. 299, 131–134.

[12] Peumans, W.J., De Ley, M. and Broekaert, W.F. (1984) An unusual lectin from stinging nettle (*Urtica dioica*) rhizomes. FEBS Lett. 177, 99–103.

[13] Hom, K., Gochin, M., Peumans, W.J. and Shine, N. (1995) Ligand-induced perturbations in *Urtica dioica* agglutinin. FEBS Lett. 361, 157–161.

[14] Shibuya, N., Goldstein, I.J., Shaper, J.A., Peumans, W.J. and Broekaert, W.F. (1986) Carbohydrate binding properties of stinging nettle (*Urtica dioica*) rhizome lectin. Arch. Biochem. Biophys. 249, 215–224.
[15] Broekaert, W.F., Van Parijs, J., Leyns, F., Joos, H. and Peumans, W.J. (1989) A chitin-binding lectin from stinging nettle rhizomes with antifungal properties. Science 245, 1100–1102.
[16] Van Damme, E.J.M. and Peumans, W.J. (1987) Isolectin composition of individual clones of *Urtica dioica*: evidence for phenotypic differences. Physiol. Plant. 71, 328–334.
[17] Huesing, J.E., Murdock, L.L. and Shade, R.E. (1991) Rice and stinging nettle lectins: insecticidal activity similar to wheat germ agglutinin. Phytochemistry 30, 3565–3568.
[18] Does, M.P. and Cornelissen, B.J.C. (1999) A chimera of *Urtica dioica* agglutinin and tobacco chitinase displays both agglutination and chitinase activity. Plant Sci. 148, 121–129.
[19] Lerner, D.R. and Raikhel, N.V. (1992) The gene for stinging nettle lectin (*Urtica dioica* agglutinin) encodes both a lectin and a chitinase. J. Biol. Chem. 267, 11085–11091.
[20] Meins Jr., F., Fritig, B., Linthorst, H.J.M., Mikkelsen, J.D., Neuhaus, J-M. and Ryals, J. (1994) Plant chitinase genes. Plant Mol. Biol. Rep. 12, S22–S28.
[21] Neuhaus, J-M., Fritig, B., Linthorst, H.J.M., Meins, F., Mikkelsen, J.D. and Ryals, J. (1996) A revised nomenclature for chitinase genes. Plant Mol. Biol. Rep. 14, 102–104.
[22] de Gerhardt, L.B.A., Sachetto-Martins, G., Contarini, M.G., Sandroni, M., Ferreira, R.P., Lima, V.M., Cordeiro, M.C., Oliveira, D.E. and Margis-Pinheiro, M. (1997) *Arabidopsis thaliana* class IV chitinase is early induced during the interaction with *Xanthomonas campestris*. FEBS Lett. 419, 69–75.
[23] Passarinho, P.A., Van Hengel, A.J., Fransz, P.F. and de Vries, S.C. (2001) Expression pattern of the *Arabidopsis thaliana AtEP3/AtchitIV* endochitinase gene. Planta 212, 556–567.
[24] Frangioni, J.V. and Neel, B.N. (1993) Solubilization and purification of enzymatically active glutathione S-transferase (Pgex) fusion proteins. Anal. Biochem. 210, 179–187.
[25] Reissig, J.L., Strominger, J.L. and Leloir, I.F. (1955) A modified colorimetric method for the estimation of N-acetylamino sugars. J. Biol. Chem. 217, 959–966.
[26] Trudel, J. and Asselin, A. (1989) Detection of chitinase activity after polyacrylamide gel electrophoresis. Anal. Biochem. 178, 352–366.
[27] Nakamura, C.V., Esteves, M.J.G., Andrade, A.F.B., Alviano, C.S., De Souza, W. and Angluster, J. (1992) Cell surface saccharides in three *Phytomonas* species differing in host specificity. J. Protozool. 39, 303–309.
[28] Allen, A., Neuberger, A. and Sharon, N. (1973) The purification and specificity of wheat-germ agglutinin. Biochem. J. 131, 155–162.
[29] Goldstein, I.J., Hammarstrom, S. and Sundblad, G. (1975) Precipitation and carbohydrate-binding specificity studies on wheat germ agglutinin. Biochim. Biophys. Acta 405, 53–61.
[30] Peters, B.P., Ebisu, S., Goldstein, I.J. and Flashner, M. (1979) Interaction of wheat germ agglutinin with sialic acid. Biochemistry 18, 5505–5511.
[31] Broekaert, W.F., Van Parijs, J., Allen, A.K. and Peumans, W.J. (1988) Comparison of some molecular, enzymatic and antifungal properties of chitinases from thorn-apple, tobacco and wheat. Physiol. Mol. Plant Pathol. 33, 319–331.
[32] Mauch, F., Mauch-Mani, B. and Boller, T. (1988) Antifungal hydrolases in pea tissue. II. Inhibition of fungal growth by combinations of chitinases and β-1,3-glucanase. Plant Physiol. 88, 936–942.
[33] Melchers, L.S., Sela-Buurlage, M.B., Vloemans, S.A., Woloshuk, C.P., Van Roekel, J.S.C., Pen, J., Van den Elzen, P.J.M. and Cornelissen, B.J.C. (1993) Extracellular targeting of the vacuolar tobacco proteins AP24, chitinase and β-1,3-glucanase in transgenic plants. Plant Mol. Biol. 21, 583–593.
[34] Ponstein, A.S., Bres-Vloemans, S.A., Sela-Buurlage, M.B., Van Den Elzen, P.J.M., Melchers, L.S. and Cornelissen, B.J.C. (1994) A novel pathogen- and wound-inducible tobacco (*Nicotina tabacum*) protein with antifungal activity. Plant Physiol. 104, 109–118.
[35] Schlumbaum, A., Mauch, F., Vögeli, U. and Boller, T. (1986) Plant chitinases are potent inhibitors of fungal growth. Nature 324, 265–367.