

The cytotoxic properties of a plant lipid transfer protein involve membrane permeabilization of target cells

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ABSTRACT

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Aims: To determine whether Ha-AP10, a member of the plant lipid transfer proteins (LTPs) family produces a direct cytotoxic effect on fungal cells mediated by membrane permeabilization. LTPs can inhibit fungal growth and are considered members of the ubiquitous class of antimicrobial peptides. However, the way they exert their effects on target cells is not yet understood.

Methods and Results: Viability assays demonstrate that Ha-AP10 acts as a fungicidal compound but no harmful effect is observed on plant cells. Liposome leakage assays show that the protein induces a moderate release of fluorescent probes encapsulated in model membranes, indicating its ability to interact with phospholipids. Using a fluorescent indicator of damage at the membrane level, we demonstrate that Ha-AP10 is able to induce the permeabilization of intact fungal spores in a dose-dependent manner.

Conclusion: The results presented here demonstrate the permeabilization of fungal spores caused by Ha-AP10.

Significance and Impact of the Study: To our knowledge, this is the first demonstration of fungal membrane damage by an LTP, giving a clue to elucidate the basis of its antimicrobial properties.

Keywords: antifungal, antimicrobial, antimicrobial peptide, fungi, lipid transfer protein, plant defence.

INTRODUCTION

Plants as well as animals can defend themselves against pathogenic microbes utilizing cationic antimicrobial peptides (AMPs) and proteins which can kill micro-organisms without any harmful effect on the host (Hancock and Lehrer 1998). The mode of action of the hydrophobic and cationic AMPs from animals has been studied in detail, the cytoplasmic membrane being the main site of action (Hancock and Lehrer 1998).

The AMPs from plants have been classified into families according to their consensus motif and basically include thionins, defensins, lipid transfer proteins (LTPs), hevein and knottin-type peptides, and snakins (Broekaert *et al.*

1997; García-Olmedo *et al.* 1998; Segura *et al.* 1999). A common characteristic of these peptides is the presence of an even numbers of cysteines (four to 12) which are involved in disulphide bridges conferring high stability (Broekaert *et al.* 1997; García-Olmedo *et al.* 1998). Compared with animal AMPs only limited knowledge is available on their plant counterparts, but studies on the mode of action of thionins and defensins have been performed (Thevissen *et al.* 1996, 2003). Accumulated evidence supports the contribution of certain AMPs to the complex plant defence mechanisms but in other cases their role is still a matter of controversy. This is the case for LTPs which, distinct from other AMPs, have been implicated in many other functions *in planta* (see Kader 1996 for review). Initially, the defence function for LTPs was proposed taking into account the ability of certain members of this family to inhibit the growth of fungal and bacterial pathogens *in vitro* (Terras *et al.* 1992; Molina *et al.* 1993) but this role has further been supported by other

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experimental evidence. First, their expression patterns and apoplastic localization are consistent with a defence role (Kader 1996). Secondly, transgenic *Arabidopsis* and tobacco plants with constitutive overexpression of an antimicrobial LTP gene displayed a reduction in the symptoms caused by the bacterial pathogen *Pseudomonas syringae* (Molina and García-Olmedo 1997). These observations suggest that at least certain LTPs may control the growth of microbial pathogens. However, two recent reports have demonstrated the possible existence of novel roles for LTPs in plant defence. Particularly, it has been reported that an LTP from wheat binds to a plant receptor involved in the control of defence responses (the elicitor receptor) and it has been postulated that LTPs may be involved in the signalling pathways leading to the hypersensitive response triggered by elicitors (Buhot *et al.* 2001). In addition, an LTP-like gene from *Arabidopsis* is involved in the production/transmission of the mobile signal in the systemic acquired resistance response (Maldonado *et al.* 2002). Taking into account these reports, the previous evidence concerning localization and transgenic expression of LTPs must be re-interpreted, as both results are compatible with LTPs acting either as antimicrobial proteins of direct effect (AMPs) or as signalling molecules. By now, the analysis of LTPs as AMPs has been limited in determining whether they inhibit the growth of fungi and bacteria, and preliminary studies addressing their mode of action based on their effect on model membranes (Caaveiro *et al.* 1997; Tassin *et al.* 1998). Hence, the question arises, is the microbial cytoplasmic membrane the site of action of antimicrobial plant LTPs.

We previously isolated and characterized a basic antifungal peptide, Ha-API0 (*Helianthus annuus* antifungal peptide of 10 kDa), belonging to the family of plant LTPs (Regente and de la Canal 2000). Molecular characterization of its cDNA clone (Regente and de la Canal 2003) shows that it is homologous to *ltp4* from *Arabidopsis thaliana* (Arondel *et al.* 2000) and presents a signal peptide that directs it to the secretory pathway. The aim of this work was to determine whether the LTP Ha-API0 produces a direct cytotoxic effect on fungal cells mediated by membrane permeabilization. The results presented here provide insight into the complex mode of action of LTPs.

MATERIALS AND METHODS

Biological materials

Spores of *Fusarium solani* f. sp. *eumartii*, isolate 3122 (INTA Collection, Balcarce, Argentina), were harvested from cultures growing on potato dextrose agar (PDA) plates as described previously (Regente and de la Canal 2000). Cells of *Solanum tuberosum* ssp. *tuberosum* cv. Spunta (kindly provided by C. Tonón) were obtained from callus cultured

on Murashige-Skoog (MS) solid medium (Murashige and Skoog 1962) and maintained in MS liquid medium at 25°C with continuous agitation performing re-inoculations every week.

Protein purification and analysis

Ha-API0 (SWISS-PROT accession number P82007) was purified to homogeneity from *H. annuus* dried seeds as previously described (Regente and de la Canal 2000). Chromatographic methods used, included gel filtration, cation exchange and reverse phase HPLC. The purity of the protein was verified on reverse phase HPLC and SDS-PAGE followed by silver nitrate staining. Protein concentration was determined by the bicinchoninic acid assay (Smith *et al.* 1985) using bovine serum albumin as standard.

Viability assays

Fusarium solani spores at the final concentration of 6×10^4 spores ml⁻¹ were incubated overnight at 25°C in the presence of different concentrations of Ha-API0 in water, in a low ionic strength (LIS) medium (Cammue *et al.* 1995), or LIS medium supplemented with 1 mM CaCl₂. Then, 50 µl of the incubation mix was plated on PDA plates and incubated for 3 days at 25°C. Control assays for each incubation medium were performed replacing the protein sample by sterile water. The percentage of fungal viability was calculated counting the CFUs taking each control as 100% of viability. The results from three independent repetitions were analysed.

Potato cells from 4-day-old cultures were transferred from MS medium to the same volume of sterile water. The cells were incubated overnight at 25°C in 1.5-ml tubes with continuous agitation in the presence of Ha-API0 at the indicated concentrations. Control cells were incubated in the same conditions but without Ha-API0. A quantity of 10 µl of the incubation mix was tested for cell viability with Evans blue and microscopic observation (Gaff *et al.* 1971).

Leakage experiments

The assay for leakage of liposomal contents was performed as detailed in Contreras *et al.* 2001. Briefly, large unilamellar vesicles (LUVs) containing 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and *p*-xylene-bis-pyridinium bromide (DPX) (Molecular Probes, Eugene, OR, USA) as fluorescent probes were used to study vesicle leakage. LUVs were prepared by the extrusion method (Mayer *et al.* 1986). Nonencapsulated probes were separated from the vesicle suspension using a gel filtration chromatography.

Assay for fungal membrane permeabilization

Fusarium solani spores (1.7×10^7 spores ml^{-1}) were mixed with the protein sample at the indicated doses in water, LIS medium or LIS medium supplemented with 1 mM CaCl_2 . Appropriate controls without Ha-API0 were performed. After incubation at 18°C during the indicated times SYTOX Green probe (Molecular Probes) was added at a final concentration of 1 μM and fluorescence was viewed with a Nikon Eclipse E200 fluorescence microscope (Nikon, Tokyo, Japan) equipped with a B-2A fluorescein filter set. Positive controls included spores treated with 0.5% (w/w) Triton X-100. After the treatment a quantitative test was performed by counting the fluorescent and nonfluorescent spores on a Neubauer camera. The results from three independent experiments were analysed to calculate the percentage of permeabilized spores.

RESULTS

Ha-API0 is toxic for *F. solani* and harmless for plant cells

We previously reported that Ha-API0 reduces the growth of the fungus *F. solani* with an IC_{50} of $6.5 \mu\text{g ml}^{-1}$ ($0.65 \mu\text{M}$), and completely inhibits the germination of spores at a concentration of $40 \mu\text{g ml}^{-1}$ (Regente and de la Canal 2000). Preliminary experiments suggest that the protein may have a fungistatic effect. In order to characterize the cytotoxic activity of Ha-API0 we incubated *F. solani* spores in aqueous solution and after 16 h, treated spores were plated on Petri dishes to analyse colony survival. As shown in Fig. 1, the treatment with increasing concentrations of Ha-API0 reduced fungal spore viability and spores treated with $50 \mu\text{g ml}^{-1}$ did not grow on PDA medium, indicating that the protein exerts a lethal effect. Figure 1 also illustrates the similar fungitoxic effect of Ha-API0 acting in an LIS medium. When the same assay was performed in LIS medium containing 1 mM CaCl_2 the lethal effect was reduced by around a 20% but was still clearly observed at all the Ha-API0 concentrations tested. However, Ha-API0 did not modify the viability of model plant cells. In this assay, potato cells were incubated in the presence of different concentrations of Ha-API0 and after 16 h the cellular suspension was subjected to staining with Evans blue to determine cell viability. It can be seen that at all concentrations tested Ha-API0 was harmless to these cells.

Ha-API0 induces fungal membrane permeabilization

The fact that Ha-API0 was cytotoxic to fungal cells prompted us to investigate the underlying mechanism. Like

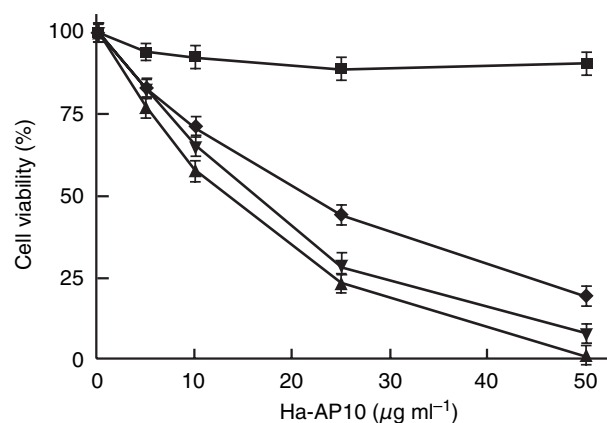


Fig. 1 Effect of Ha-API0 on fungal and plant cells. *Fusarium solani* spores were incubated for 16 h in the presence of Ha-API0 in water (▲), low ionic strength (LIS) medium (▼), or LIS medium + 1 mM CaCl_2 (●) and the spore suspension was then plated on PDA. The CFUs were counted after 3 days of incubation and the percentage of viable fungal cells was calculated relative to untreated cells. Potato cells were suspended in water and incubated in the presence of Ha-API0. After 16 h, the suspension was stained with Evans blue and the percentage of viable cells relative to total cells was calculated (■). Error bars represent the standard deviation

cationic and hydrophobic antimicrobial proteins, LTPs are thought to interact with biological membranes resulting in the inhibition of fungal growth, although this hypothesis has not been verified. As a first approach, we analysed the ability of Ha-API0 to induce the leakage of model membranes (LUVs) containing encapsulated fluorescent probes. Figure 2 shows the dependence of liposome leakage on the protein/lipid molar ratio for vesicles composed of the anionic phospholipid phosphatidylglycerol or the zwitterionic phospholipid phosphatidylcholine. Leakage was insignificant at all protein/lipid ratios tested for liposomes composed of phosphatidylcholine. However, the content of liposomes made of phosphatidylglycerol was released in the presence of Ha-API0 and this effect was dependent on the protein/lipid ratio. Despite the moderate level of liposome leakage detected under the experimental conditions used, this experiment proves the ability of Ha-API0 to permeabilize certain model membranes.

In the following experiment, we decided to explore whether the protein was able to induce membrane damage of *F. solani* spores. This analysis was performed using SYTOX Green, a high-affinity nucleic acid stain that fluoresces upon binding to nucleic acids. As intact membranes do not allow SYTOX Green uptake in living cells, it only penetrates those cells with compromised plasma membranes. Figure 3 (panel A) shows the effect of Ha-API0 on SYTOX Green uptake by *F. solani* spores as

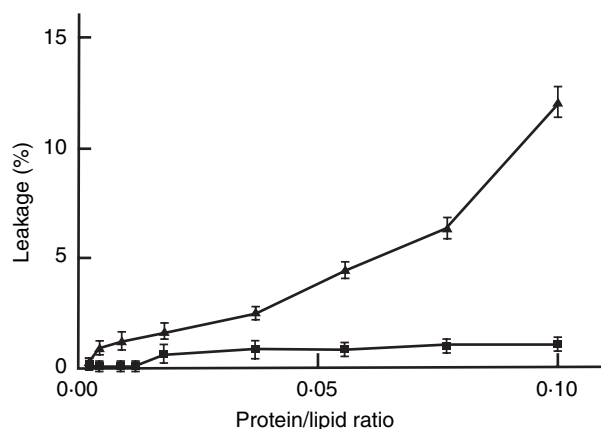


Fig. 2 Effect of Ha-AP10 on the release of the fluorescent probes 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and *p*-xylene-bispyridinium bromide (DPX) from large unilamellar vesicles (LUVs). Results are expressed as percentage of leakage relative to Triton X-100. LUVs made of egg yolk phosphatidyl glycerol (▲) and egg yolk phosphatidyl choline (■) were incubated at 25°C in the presence of different Ha-AP10 to lipid molar ratios. Ten minutes after addition of the protein, liposome leakage was measured as fluorescence release

observed under a fluorescence microscope. Intact spores did not stain with the probe (control) while those incubated in water, LIS medium or LIS medium + 1 mM CaCl_2 in the presence of Ha-AP10 incorporated the probe. These results prove the existence of alterations in fungal membrane permeability upon treatment with the antifungal peptide.

In order to establish the kinetics of the observed membrane permeabilization dose–response experiments were analysed at different times of incubation in the presence of Ha-AP10. The assays were developed as indicated previously but in this case fluorescent spores were quantified. Figure 3 (panel B) shows a modest effect of Ha-AP10 on *F. solani* membrane permeabilization during the first 30 min of incubation. After 2 h of incubation in the presence of Ha-AP10 a slight increase in the percentage of fluorescent spores is detected and this effect is clearly visible at the higher concentration used. Upon overnight incubation a clear increase in the number of permeabilized spores is detected and this permeabilization is dependent on the concentration of Ha-AP10.

To corroborate the specificity of the permeabilization effect of Ha-AP10, dose–response experiments were also performed on plant cells, analysing the membrane damage using the probe SYTOX Green. Figure 3 (panel B) shows that the treatment with Ha-AP10 does not affect membrane permeability of these plant cells even at the highest concentration tested which did permeabilize 80% of the fungal spores.

DISCUSSION

Plant LTPs have been implicated in plant defence against pathogens based on the initial finding of their ability to inhibit microbial growth *in vitro*. However, direct evidence of the basis of their antimicrobial activity is lacking and, unlike other AMPs, they are thought to have many other roles *in vivo*. In this paper, we investigated whether Ha-AP10, a member of the plant LTP family, exerts a direct effect on fungi leading to growth inhibition.

Previous evidence suggests that Ha-AP10 arrests mycelial growth acting as a fungistatic compound (Regente and de la Canal 2000). However those tests were performed at protein concentrations in which the effect of the protein is not complete. The viability tests presented here clearly demonstrate that Ha-AP10 produces fungal spore cell death. Hence, the following experiments presented here were designed to understand the basis of this fungicidal activity. A putative site of action of antimicrobial LTPs is the plasma membrane (Kader 1996). In fact, LTPs from barley (Caaveiro *et al.* 1997) and onion (Tassin *et al.* 1998), which are not homologous to Ha-AP10, have been shown to interact with lipid model membranes. However, as LTPs have the ability to enhance the *in vitro* transfer of lipids between model membranes (Arondel *et al.* 1990) this evidence is not enough to infer their putative mode of action as AMPs. Moreover, even if LTPs could interact with phospholipid model membranes, fungi have thick cell walls that may mask or modify the interaction with target molecules. Here we provide evidence that Ha-AP10 not only induces liposome leakage but also modifies the permeability of fungal cells. The SYTOX Green experiments demonstrate that Ha-AP10 is able to produce changes in membrane permeability in *F. solani* spores. These results with intact spores validate those obtained with model membranes and constitute the first evidence that a plant LTP can alter the permeability of fungal cells. Moreover, the pattern of permeabilization observed correlates with the lethal effect of Ha-AP10 on fungal spores, as similar dose–response curves were observed. The membrane damage curves also show the same tendency as those previously obtained for the growth inhibition of *F. solani* in the presence of Ha-AP10 (Regente and de la Canal 2000). Hence, these data suggest a cause–effect relationship, indicating a possible mechanism of action for this fungicidal LTP.

It has been reported that while the activity of some antifungal LTPs is completely abolished in media supplemented with 1 mM CaCl_2 , others are only partially reduced (Cammue *et al.* 1995). We have shown in this work that the lethal effect of Ha-AP10 as well as its ability to permeabilize fungal cells persists in media containing 1 mM CaCl_2 . Therefore, at the physiological concentration of free Ca^{2+} in

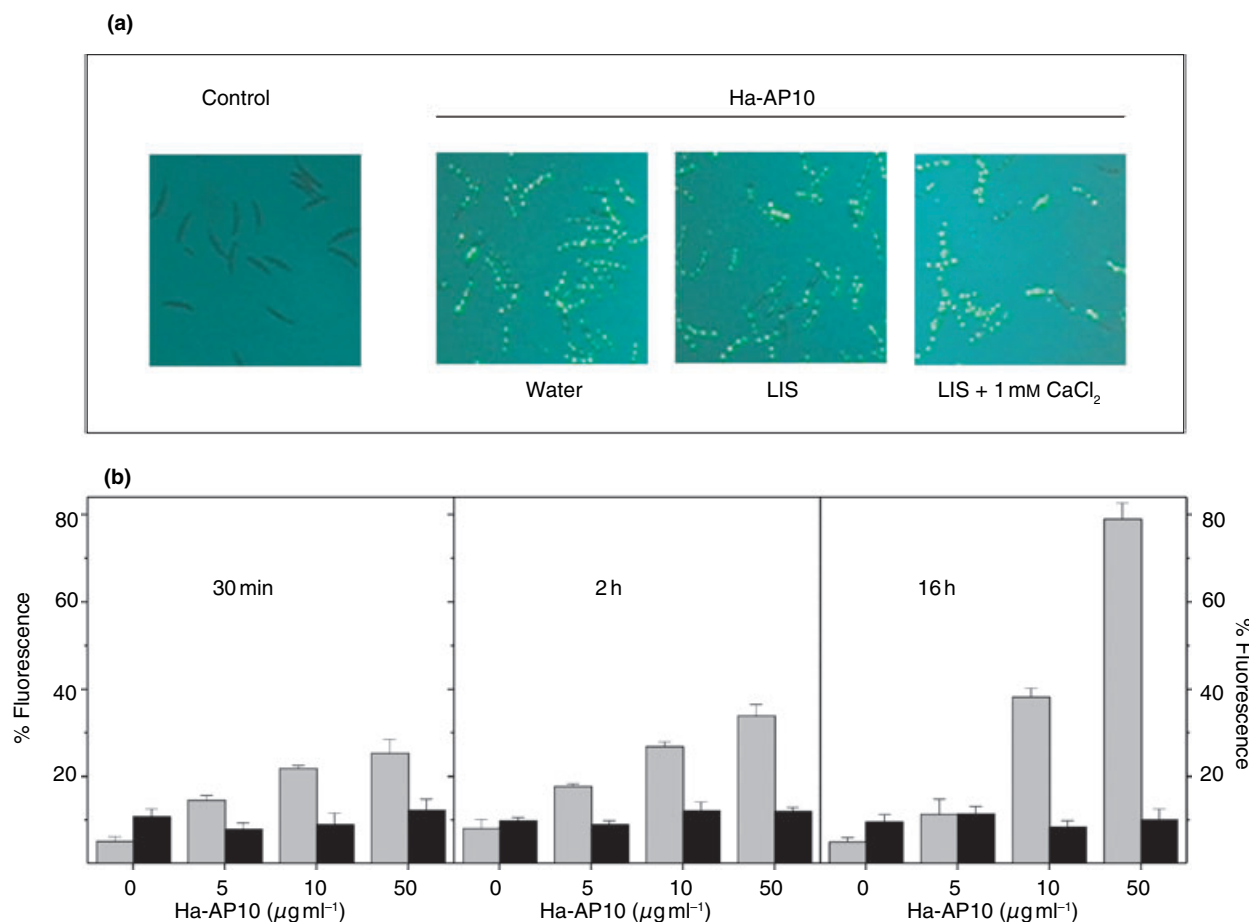


Fig. 3 Panel a: Fluorescence microscopy of fungal spores in the presence of SYTOX Green. Spores were incubated during 16 h in water (control) or $100 \mu\text{g ml}^{-1}$ of Ha-AP10 in water, low ionic strength (LIS) or LIS + 1 mM CaCl_2 media. SYTOX Green probe was added 10 min before microscopic observation. Control incubations in LIS medium and LIS + 1 mM CaCl_2 were as the water control. Magnification: 200 $\times$. Panel b: Permeabilization of fungal spores and plant cells caused by Ha-AP10. *Fusarium solani* spores (grey bar) and potato cell (black bar) suspensions were incubated in the presence of Ha-AP10 at the doses indicated during 30 min, 2 h, or 16 h. After incubation, the samples were supplemented with the SYTOX Green probe and the fluorescent cells were quantified in a Neubauer camera. Error bars represent the standard deviations

the apoplast (0.1–1 mM) where the fungal–plant contact is likely to occur, Ha-AP10 would still be active.

A relevant feature required for a direct antimicrobial activity at physiological conditions is selective toxicity, i.e. the protein should discriminate between host and microbial cells (Matsuzaki 1999). This principle has been demonstrated for AMPs from animals, although it has not been shown for plants. In this sense, an interesting result presented here is that the protein is lethal to fungal spores but is not toxic to plant cells. This observation illustrates the ability of Ha-AP10 to discriminate between cell types. Taking into account the comparative results of both fungal and plant cells, the protein should target those molecules which are abundant in fungal membrane but rarely or never present in the plant plasma membrane. Although the elucidation of the basis of this difference has not been the aim of this work, an

hypothesis may be drawn. We show that Ha-AP10 is able to induce membrane permeabilization in liposomes made of the anionic phospholipid phosphatidylglycerol whereas it is unable to release the content of model membranes made of the zwitterionic phospholipid phosphatidylcholine. As anionic phospholipids are differentially distributed in both cell types (Feofilova 2001) the ability of Ha-AP10 to differentiate among fungal and plant cells may be based on the diverse composition/distribution of phospholipids in their membranes. This observation may provide a clue to understand not only the specificity but also the basis of Ha-AP10 action: the effect of Ha-AP10, a cationic and hydrophobic peptide, may be mediated by its electrostatic interaction with anionic membrane phospholipids.

In the leakage assays, only a modest effect of Ha-AP10 was observed. This observation may be a consequence of the

lipid composition of the vesicles prepared and is also in agreement with the results reported for two other LTPs. The barley LTP2 protein only produced a modest leakage-inducing activity at LIS and was completely inactive at standard conditions in the assay for leakage of liposomal content (Caaveiro *et al.* 1997). In addition, it has already been shown that the antimicrobial LTP from onion seeds induces a weak release of the liposome content (Tassin *et al.* 1998). However, another explanation could be that the interaction with anionic phospholipids is required but not sufficient to produce the full effect observed on intact spores. In the same sense, the fact that the permeabilization of spores is not detected immediately after the addition of Ha-API0 suggests that other factors/events may also contribute to fungal cell death.

In conclusion, although many other properties have been ascribed to plant LTPs, we show here that Ha-API0 behaves as a typical AMP demonstrating its ability to induce the permeabilization of fungal spores. Future experiments will be aimed to establish the contribution of Ha-API0 to the resistance of sunflower seeds to fungal attack.

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