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Alt a 1 from *Alternaria* interacts with PR5 thaumatin-like proteins

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ABSTRACT

Alt a 1 is a protein found in *Alternaria alternata* spores related to virulence and pathogenicity and considered to be responsible for chronic asthma in children. We found that spores of *Alternaria* inoculated on the outer surface of kiwifruits did not develop hyphae. Nevertheless, the expression of Alt a 1 gene was upregulated, and the protein was detected in the pulp where it co-localized with kiwi PR5. Pull-down assays demonstrated experimentally that the two proteins interact in such a way that Alt a 1 inhibits the enzymatic activity of PR5. These results are relevant not only for plant defense, but also for human health as patients with chronic asthma could suffer from an allergic reaction when they eat fruit contaminated with *Alternaria*.

Structured summary of protein interactions:
Alt a 1 binds to **PR5-thaumatin-like protein** by pull down (1, 2) **Alt a 1** binds to **peach PR5** by pull down (View interaction) **Alt a 1** binds to **banana PR5** by pull down (View interaction) **Alt a 1** physically interacts with **PR5-thaumatin-like protein** by pull down (View interaction).

1. Introduction

The fungal genus *Alternaria* includes many saprobic and endophytic species, but is well known for containing many notoriously destructive plant pathogens [1,2]. *Alternaria* species have been classified and identified based on conidial characters and molecular-genetic data and comprise more than 280 species [3]. With the exception of studies regarding pathogen-derived host-specific phytotoxins, the physiological and molecular mechanisms underlying the interactions between saprobic and endophytic fungi and their respective host plants remain largely unexplored [4]. It is known that insidious fungal infections of postharvest mould remain quiescent as biotrophs during fruit growth and harvest, but actively develop and transform into saprobes during ripening and senescence [1]. Exposure of unripe hosts to the fungus quickly initiates defensive signal-transduction cascades that limit fungal growth and development, but exposure to the same fungus during ripening activates a very different signalling cascade that facilitates fungal colonization [5].

Strains of *Alternaria alternata* are ubiquitous in nature and exist predominately as saprobes. They are frequently found on fruits, vegetables and cereals. They can produce mycotoxins and other signals described as pollutants of plant-flavoured products such as juices, sauces and preservatives, thereby entering the human food chain without any fungal development having been observed [6].

A. alternata is considered to be one of the most prolific producers of fungal allergens. In particular, Alt a 1 (AAM90320.1, NCBI Protein Database), its principal allergen, has been associated with asthma, and sensitivity to this allergen was recently shown to be a risk factor for life-threatening asthma [7–9]. Alt a 1 is a heat-stable, 28 kd dimer, which dissociates into 14.5- and 16-kd subunits under reducing conditions [10]. Until now, the function of Alt a 1 in fungal metabolism or ecology is unknown [11–13]. Recently, its homologue in *Alternaria brassicicola* was found in *Arabidopsis thaliana* to be highly expressed during the infection process of *A. thaliana*, suggesting that the protein may be involved in plant pathogenicity [7,14]. It is possible that *Alternaria* spores are present in foods/fruits without developing hyphae, with the spores producing components involved in allergenicity, such as Alt a 1 protein.

Thus, we studied the behaviour of *Alternaria* spores in a non-host fruit—kiwifruit. Our results suggested that *A. alternata* in kiwi

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behaved as a saprobic fungus. Alt a 1 was detected, despite the fact that hyphae development was not observed after 14 days post-inoculations (dpi). In order to search for a kiwi receptor for Alt a 1, pull-down assays were performed. Alt a 1 interacted with the kiwi PR 5-TLP, and both proteins co-localized in the kiwi pulp by immunofluorescence in confocal microscopy. The interaction resulted in the inhibition of the PR5 enzymatic activity. This effect was not limited to kiwi PR5; PR5 from other fruits, such as peach and banana, also interacted with Alt a 1. Thus, Alt a 1 was characterized as an enzymatic inhibitor of the PR5 family.

2. Materials and methods

2.1. Plant material and fungus growing conditions

Alternaria spores were isolated from kiwifruits by scraping the surface and cultured on PDA medium (potato dextrose agar; Difco™ Becton Dickinson and Company, Sparks, MD, USA) with cefotaxime (200 μg/ml) (Calbiochem®, Merck KGaA, Darmstadt, Germany). After one week, isolated fungi were re-cultured separately on PDA. Isolated fungi were identified by ITS rDNA sequencing. For this purpose, genomic DNA was extracted using a Plant DNA Preparation Kit (Jena Bioscience GmbH, Jena, Germany). The region of interest was amplified with oligonucleotides ITS1F 5′-CTTGGTCATTTAGAGGAAGTAA-3′ and ITS4 5′-TCCTCCGCTTATTGATATGC-3′ [15]. The amplified sequences were then sent for identification by alignment/comparison with the NCBI database. After 8 days, identified *Alternaria* spores were recovered with sterile water and stored at −80 °C in 20% glycerol.

2.2. Controlled infection of kiwifruits with Alternaria spores

Kiwifruits (*Actinidia deliciosa,* commercial variety Hayward), washed with 20% bleach solution for 10 min, were infected by placing 1×10^6 *A. alternata* spores in one drop (20 μl) onto the outer surface. The fruit was covered with plastic film and incubated at 24 °C. To quantify Alt a 1 expression by Real Time PCR, samples were collected at days 1–7 post-infection, and then homogenized and lyophilized. RNA was extracted using an RNeasy® Mini Kit (Qiagen GmbH, Hilden, Germany) and quantified by NanoDrop® (NanoDrop Technologies, Wilmington, DE, USA). cDNA was obtained and amplified with Power SYBR Green PCR Master Mix (Applied Biosystems, Life Technologies Ltd., Paisley, UK) according to the manufactureŕs recommendations, and run on an Applied Biosystems 7300 Real-Time detection system (Applied Biosystems, Life Technologies Ltd., Paisley, UK). The following primers were used: Alt a 1 (5′-AGGAACCTACTACAACAGCC-3′; 5′-GTACCACTTGTGGTCCTCAA-3′) and 18S rRNA (5′-GTCGTAACAAGGTTTCCGTAGGT-3′; 5′-CAAAGGGAAGAAAGAGTAGGGTT-3′) as an endogenous control, as described by Li et al. [16]. The fold change of the corresponding mRNA of these genes was normalized with the endogenous control 18S, and relative quantification was performed using the comparative threshold cycle method ($2^{-\Delta\Delta Ct}$), as described by Livak and Schmittgen [17]. The amplification was carried out in quadruplicate.

2.3. Extracts and purified proteins

A mixture of spores and mycelia of *A. alternata* (ALK-Abello, Madrid, Spain) and lyophilized kiwifruit were extracted with phosphate-buffered saline (0.1 M sodium phosphate, pH 7.0 and 0.5 mol/L NaCl; 1:5 (w/v), 1 h, 4 °C). After centrifugation (12 000×*g*, 30 min, 4 °C), the supernatant was dialyzed (cut-off point 3.5 kDa) and freeze-dried. Protein concentration was quantified according to the Bradford method. Thaumatin-like proteins (Kiwi-PR5-P81370 (Uniprot), Pru p 2.0202-ACE80955.1 (NCBI Protein), and *Musa* 4-AFK29763.1 (NCBI Protein)) were purified as previously described [18]. Recombinant Alt a 1 was purchased from Bial Aristegui (Bilbao, Spain) [19].

2.4. Immunohistochemistry assays

To detect the presence of Kiwi-PR5 and Alt a 1 proteins, samples were recovered after 7 days post-infection (dpi). Small, hand-cut pieces of infected kiwifruits were fixed with 4% formaldehyde in PBS pH 7.4 at 4 °C overnight. After PBS washing, the tissues were cut into 30–40 μm sections with a vibratome under water and dried down on 10-well multiwell slides (Fisher Scientific Inc., Pittsburgh, USA). The sections were permeabilized by dehydration in a series of methanol (30%, 50%, 70% and 100%, 5 min each) and re-hydrated (70%, 50%, 30% methanol, PBS; 5 min each). After washing with PBS, tissue sections were incubated overnight at 4 °C with specific antibodies: monoclonal anti-Alt a 1-Alexa 488-conjugated (1:50, Bial Aristegui, Bilbao, Spain) and polyclonal anti-PR5-Alexa 550-conjugated (1:50, [20]). Sections were mounted with glycerol:PBS (1:1) and observed with a Leica TCS-SP8 confocal microscope, using the laser excitation lines of 488 and 561 nm. Collection of 3D stacks was optimized to the maximum Z resolution.

2.5. Immunoprecipitation assays

Dynabeads® M-280 Tosylactivated (10^7 Dynabeads; approximately 20 μg/ml; Invitrogen, Oslo, Norway) were resuspended by vortexing, and washed with PBS (10 mM pH 7.4). Purified protein (20–50 μg/ml) was incubated overnight with the activated Dynabeads at 37 °C with slow rotation. After incubation, the excess protein was recovered in the supernatant fraction. The coated beads were then washed four times alternately with PBS pH 7.4 BSA 0.1% (2×), 0.1 M borate buffer pH 9.5 (1×) and PBS pH 7.4 (1×). After that, protein extract (1000 μg/ml) or purified protein (20 μg/ml) was incubated with the coated beads in PBS pH 7.4 BSA 0.1% overnight at 4 °C with slow rotation. After extensive washing, coated beads were resuspended in Laemmli buffer and separated in 15% SDS–PAGE, following the immunodetection assay protocol outlined below.

2.6. Immunodetection assays

Coated Dynabeads incubated with protein extract or purified proteins were separated by 15% SDS–PAGE, and replica gels were electrotransferred onto polyvinylidene difluoride (PVDF) membranes. After blocking (Sigma–Aldrich, St. Louis, MO, USA), membranes were immunodetected with rabbit polyclonal antibodies raised against peach PR5 (anti-PR5; 1:10000, Carlos Pastor, Madrid, Spain) or Alt a 1 (anti-Alt a 1, 1:100000; Bial Aristegui, Bilbao, Spain), and then incubated with the detection antibody alkaline phosphatase-conjugated anti-rabbit IgG (1:5000, Sigma, St. Louis, MO, USA) and developed with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Sigma, St. Louis, MO, USA).

2.7. Endo-β-1,3-glucanase activity

Endo-β-1,3-glucanase activity was assayed using the method described by Menu-Bouaouiche et al. [21], with minor modifications, using carboxy-methylated Pachyman (CM-Pachyman, Megazyme, Wicklow, Ireland) as substrate. Kiwi-PR5 (20 μg/ml) was incubated for 0, 5, 10, 30 and 60 min 24 in 100 μL of 1% (w/v) CM-Pachyman in 50 mM sodium acetate, pH 5.0, 100 mM NaCl containing 0.05% (w/v) sodium azide. One millilitre of 0.1% (w/v) tetrazolium blue, 50 mM NaOH, and 0.5 M sodium potassium tartrate was added to samples and heated in boiling water. The

quantity of liberated D-glucose equivalents was estimated by absorbance at 660 nm. Assays were performed in triplicate. Results are expressed as nkat/mg of protein (1 kat corresponds to the formation of 1 mol of D-glucose equivalents).

2.8. Statistical analysis

SPSS 17.0, Statgraphics Centurion XVI and GraphPad Prism 6.01 software programs were used for statistical analysis. Results were compared by applying the non-parametric Kruskal–Wallis (with Dunn's correction) for multiple comparisons, contingency tables and Spearman correlation, when appropriate. *P*-values lower than 0.05 were considered significant in all the analyses.

2.9. Molecular modelling of the Kiwi-PR5–Alt a 1 complex

The crystal structures recently determined for Alt a 1 [4] and Kiwi PR5 [22] proteins were used. Poisson–Boltzmann electrostatic potentials using AMBER atomic charges and radii assigned with PDB2PQR [23] were computed with the APBS program [24] to solve

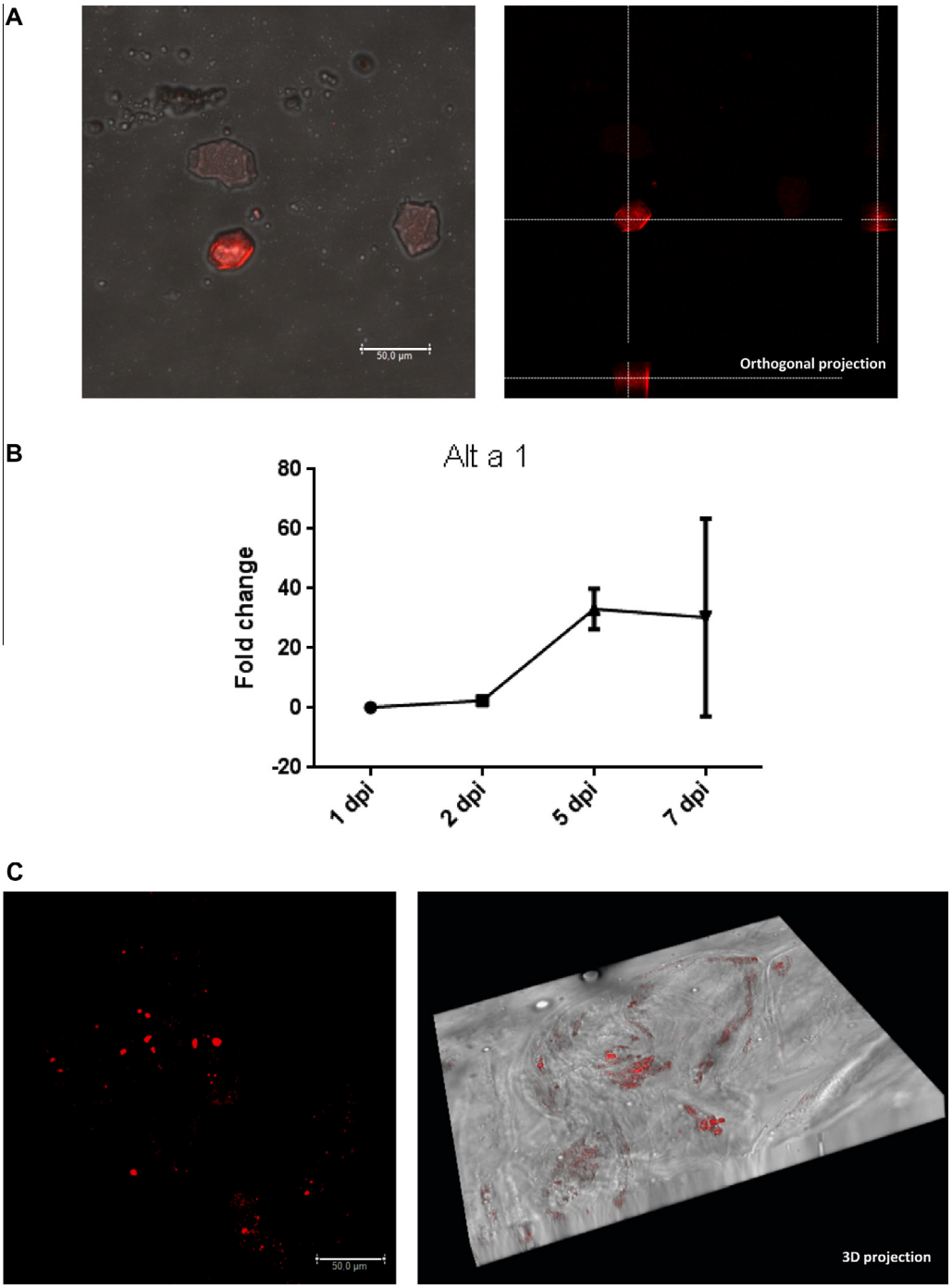


Fig. 1. (A) Immunolocalization of Alt a 1 in ungerminated spores, detected by specific monoclonal antibody, showing fluorescence signal in red (Anti-Alt a 1-Alexa 550) in an overlay with DIC (Overlay) and an orthogonal projection of a 3D stack. (B) Fold change in the Alt a 1 relative gene expression levels for kiwifruit inoculated with *Alternaria* spores (10^6/20 µl) for 1, 2, 5 and 7 days. Results are expressed in fold change compared to the expression of 18S, used as housekeeping gene. Mean values and S.D. (bars) of four independent assays are shown (*P*-value < 0.05). (C) Immunolocalization of Alt a 1 in the pulp of kiwifruits at 7 dpi with *Alternaria* spores (10^6/20 µl), by fluorescence signal (Anti-Alt a 1-Alexa 550) and the overlay with DIC in a 3D projection (3D projection).

the non-linear Poisson–Boltzmann equation in sequential focusing multigrid calculations. The potentials were obtained at 3D grids made of 161^3 points with a step size of about 0.5 Å at 298.15 K and 0.150 M ionic concentration with dielectric constants of 4 for proteins and 78.54 for water. Numerical output was processed in scalar OpenDX format and mapped onto the protein molecular surfaces and rendered with PyMOL [25].

Protein–protein docking model structures for the Kiwi-PR5–Alt a 1 complex were obtained with RosettaDock (rosettadock.graylab.jhu.edu/docking [26]) and PatchDock (bioinfo3d.cs.tau.ac.il/PatchDock/ [27]) servers. In both cases, the best-score and lowest-energy models were selected.

3. Results

3.1. Alt a 1 was detected in kiwifruits inoculated with Alternaria, despite the fact that no hyphae development was observed

Spores from *A. alternata* were isolated from kiwifruits (*Actinidia deliciosa,* variety Hayward, cultivated in an ecological field), and its identity was confirmed by ITS rDNA sequencing. The isolated fungus was used to study the role of Alt a 1 in the saprobic state in the colonization of the non-host fruit kiwifruit.

Firstly, Alt a 1 was localized inside ungerminated spores by immunofluorescence with specific monoclonal antibody in confocal microscopy (Fig. 1A), observing the most intense signal in the spore wall (Fig. 1A, orthogonal projection).

Kiwifruits from a local market were inoculated with 10^8 of the isolated spores in 20 µl drops (Supplementary Data Figure). As expected, no hyphae development could be observed until 14 dpi by staining with methylene blue (data not shown). Despite the fact that hyphae were not observed, the presence of Alt a 1 was confirmed in the kiwi pulp after 7 dpi by quantitative rtPCR (Fig. 1B), and by immunofluorescence using a specific monoclonal antibody in confocal microscopy (Fig. 1C).

Thus, Alt a 1 was found in the pulp of kiwifruit despite the fact that no hyphae development was observed.

Alt a 1 was capable of interacting with a kiwi PR5-thaumatin- like protein (Kiwi-PR5)

Pull down assays were carried out to find potential plant receptors for Alt a 1. Dynabeads® M-280 Tosylactivated (Invitrogen,

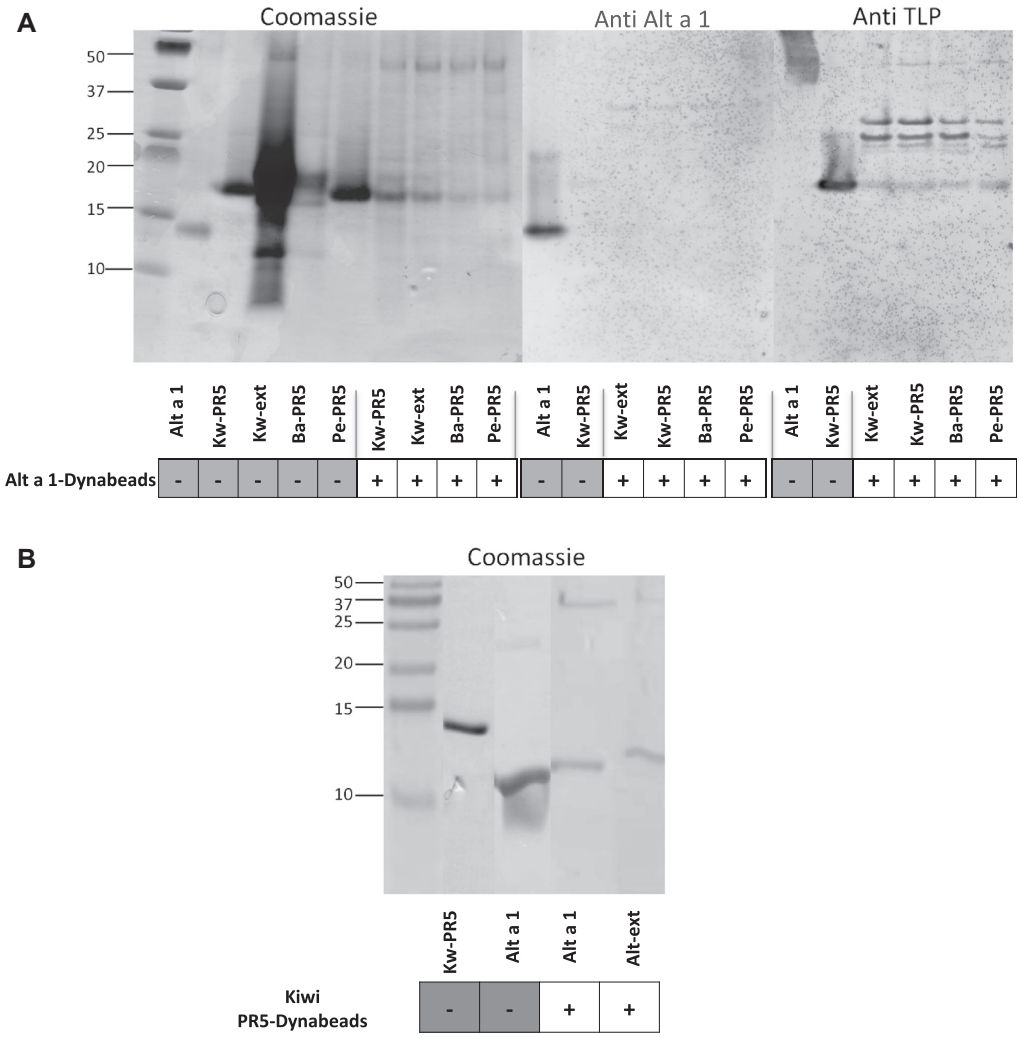
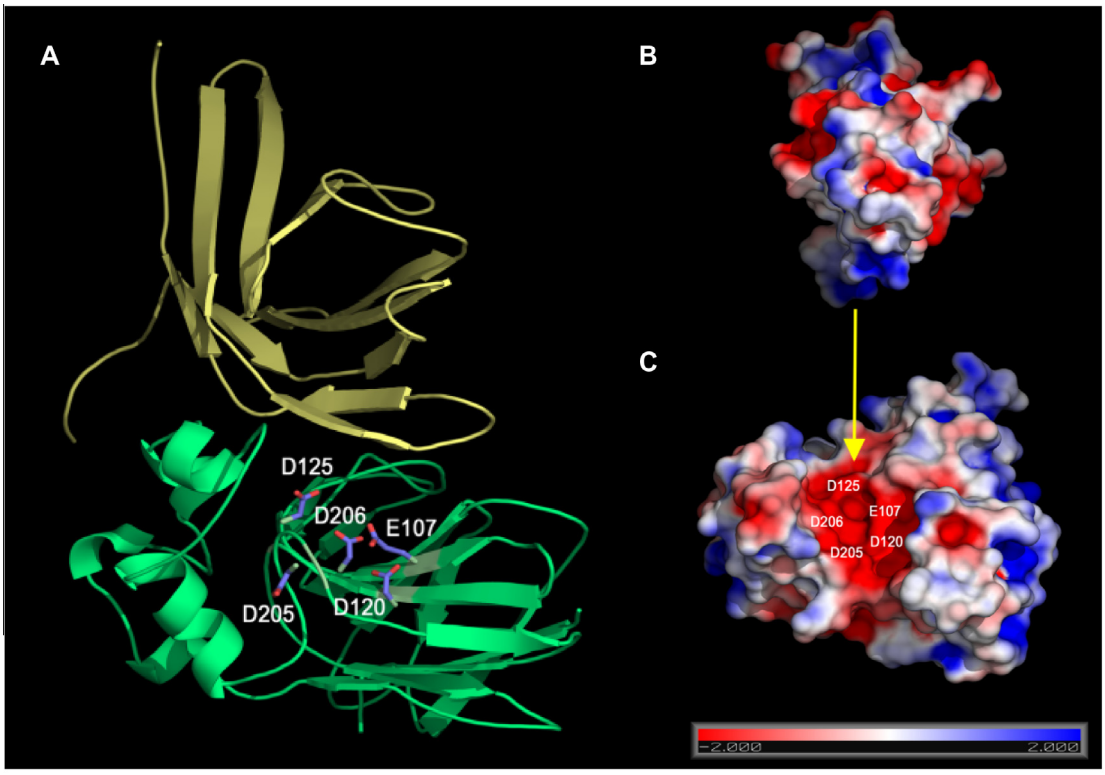


Fig. 2. (A) Dynabeads® M-280 Tosylactivated were coated with Alt a 1 (Alt a 1-Dynabeads) and incubated with kiwifruit extract (Kw-ext) and purified kiwifruit PR5 (Kiwi PR5, Kw-PR5; banana PR5, Ba-PR5; peach PR5, Pe-PR5). The result was separated by SDS–PAGE. Coomassie staining (Coomassie), and replicas blotted with polyclonal antibodies against Alt a 1 (anti-Alt a 1; dilution $1{:}10^5$) and PR5 (anti-PR5; dilution $1{:}10^4$) are shown. (B) Dynabeads® M-280 Tosylactivated were coated with Kiwi PR5 (Kiwi PR5 Dynabeads) and incubated with Alt a 1 (5 µg) and *A. alternata* extract (20 µg Alt-ext). The result was separated by SDS–PAGE and stained with Coomassie (Coomassie). Grey (–) boxes indicate control proteins. White (+) boxes indicate immunoprecipitated proteins.

Oslo, Norway) were coated with Alt a 1 (Bial Aristegui, Bilbao Spain) and incubated with a kiwifruit protein extract (Fig. 2A). A principal band around 26 kDa was observed when the immunoprecipitated proteins were separated by SDS–PAGE and stained with Coomassie. This band was identified by mass fingerprinting as the PR5-thaumatin like protein (P81370). The accuracy of the retained protein identification was further supported by immunodetection using specific polyclonal antibodies against PR5 (Anti-PR5). The higher molecular weight bands of the retained which can be observed in the Coomassie staining corresponded to typical aggregates of the members of this family, as it was confirmed by the detections with PR5 antibodies.

To validate the specificity of this interaction, Kiwi-PR5 was purified from fruits and used to coat Dynabeads M-280 Tosylactivated® (Fig. 2B). Kiwi-PR5-coated beads were incubated with *Alternaria* protein extract and purified Alt a 1. When the retained proteins were separated by SDS–PAGE, a single band of 14 kDa was observed on Coomassie, confirming that Alt a 1 was the unique binding protein.

In order to assess whether the ability of Alt a 1 to bind Kiwi-PR5 was also the case with other PR5, additional pull-down assays were performed using other members of this protein family, such as peach PR5(ACE80955.1) [28] and banana PR5 (AFK29763.1) [20]. In both cases, it was confirmed that Alt a 1 was able to bind other



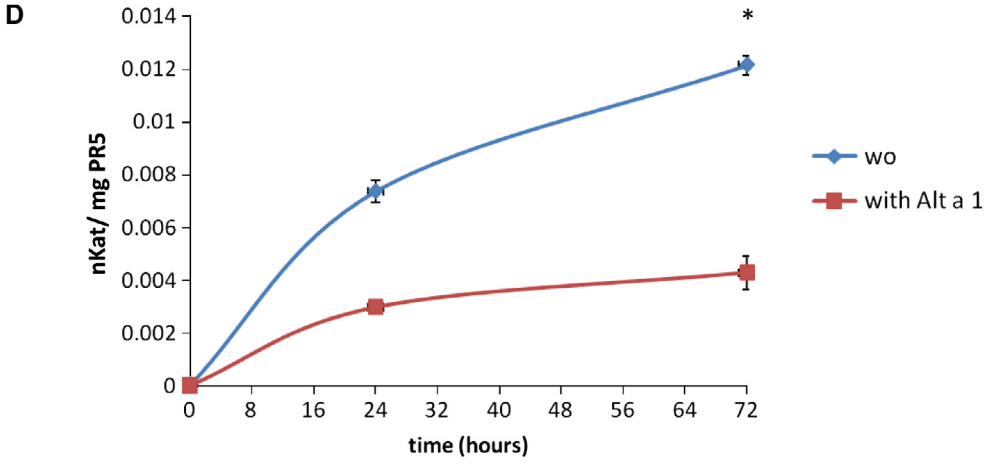


Fig. 3. Model structure of the Kiwi-PR5–Alt a 1 complex. (A) Ribbon diagram of the best RosettaDock docking structure. Alt a 1 is coloured pale yellow, Kiwi-PR5 is coloured green, and acidic residues in the catalytic cleft are shown as sticks with C atoms in violet and O atoms in red. (B and C) Poisson–Boltzmann electrostatic potential mapped onto the molecular surface of Alt a 1 (B) and Kiwi-PR5 (C) at the geometry of the complex in A. The scale bar at the bottom refers to electrostatic potential values in units of *kT* per unit charge (*k*, Boltzmann's constant and *T*, absolute temperature). (D) Inhibition of enzymatic activity of Kiwi-PR5 in the presence of Alt a 1. Endo-β-1,3-glucanase test using a carboxy-methylated Pachyman as substrate. Generated D-glucose equivalents were estimated by measuring the absorbance at 660 nm. Results are expressed as the mean of assays performed in triplicate in nkat mg^{-1} of protein.

PR5s, as shown by Coomassie and immunodetection with specific antibodies (Fig. 2A).

3.3. Alt a 1 inhibited the enzymatic activity of kiwifruit PR5

The interaction between kiwi PR5 and Alt a 1 was supported by molecular modelling (Fig. 3A–C). By using the experimental X-ray structures of Alt a 1 [4] and Kiwi-PR5 [22], different protein–protein docking calculations were performed. The best complex candidates revealed the formation of Alt a 1–kiwi-PR5 aggregates in which the electrostatically negative cleft of kiwi-PR5, where the antifungal enzymatic activity occurs, was partially blocked with Alt a 1 protein that faced an electrostatically positive prominent region.

This structural feature provided the fungal protein with an electrostatically driven affinity for the kiwi PR5 that resulted in the inhibition of its enzymatic activity. This was confirmed by measuring the kiwi PR5 β-glucanase activity in the presence of Alt a 1 (5 μg; Fig. 3D). Alt a 1 was able to inhibit the enzymatic activity of PR5, although full inhibition was not observed.

These results reveal why both proteins were visualized together in kiwi fruits (7 dpi). Kiwi-PR5 and Alt a 1 co-located in the pulp by immunofluorescence and confocal microscopy (Fig. 4).

4. Discussion

In this study, hyphal development was not observed on kiwifruits 14 dpi with spores of *A. alternata*. Spores appeared to remain dormant until fruit senescence, when the growth of *Alternaria* was induced. By contrast, the presence of Alt a 1 was detected in kiwi pulp several days after inoculation, despite the absence of hyphae. To date, the role of Alt a 1 remains unknown, although it seems to

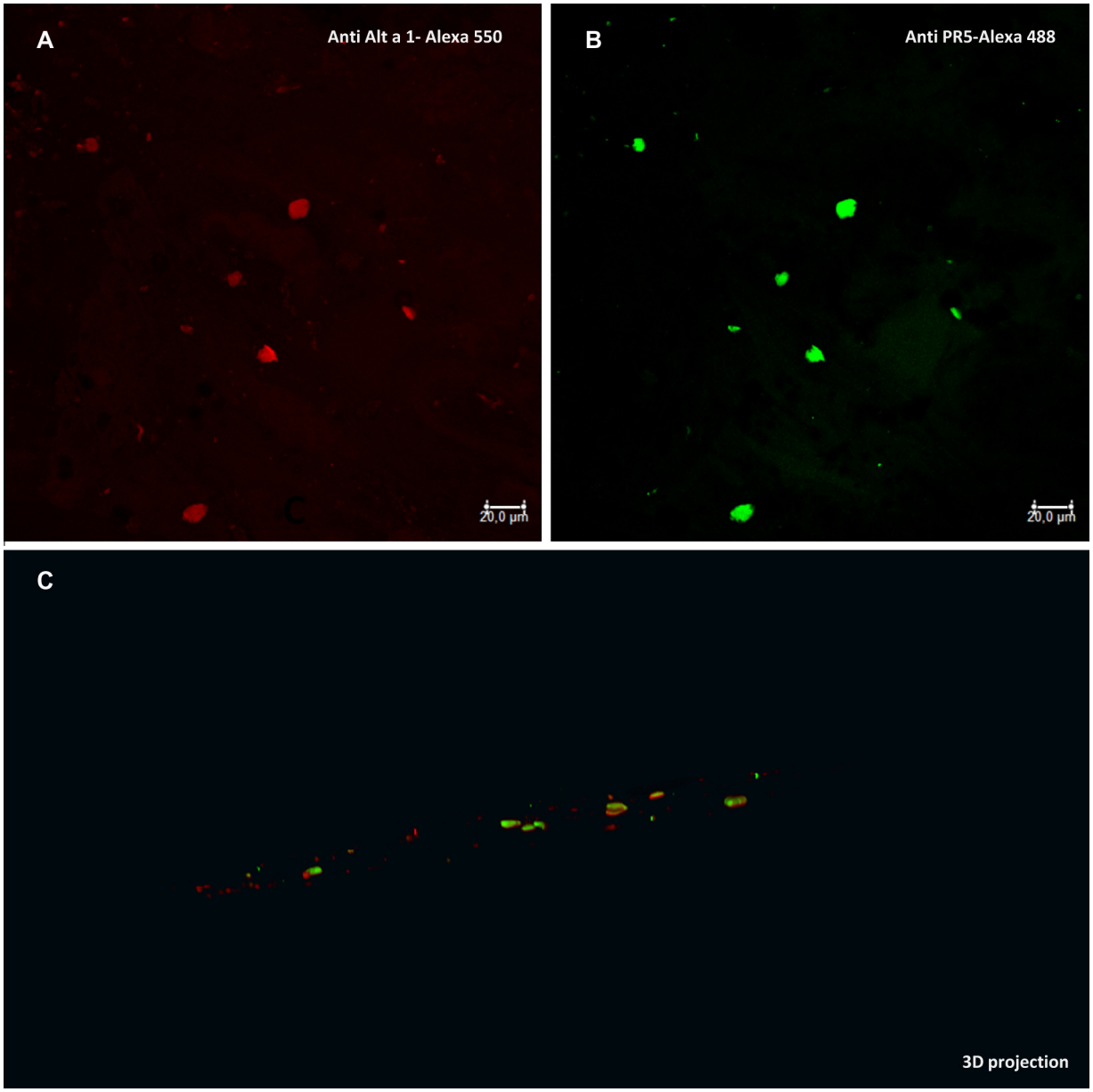


Fig. 4. Immunolocalization of Alt a 1 (A. Anti-Alt a 1-Alexa 550, 1:100) and PR5 (B. Anti-PR5-Alexa 488, 1:50) and overlay (C. 3D projection) of both fluorescence signals in kiwi pulp (7 dpi).

be related to virulence and pathogenicity [29]. Alt a 1 has also been described as the most representative secreted protein at the beginning of the germination process [19]. The present results demonstrate that, even when spores had not yet germinated, they were in fact producing Alt a 1, highlighting the importance of this protein for the development of the fungus.

The presence of the fungal spores induced the plant response by dramatically increasing the expression of plant defense proteins [30]. Hence, it is possible that the presence of Alt a 1 induces the expression of the kiwi PR5, a plant defense family, being co-located in the pulp and interacting with it.

The PR5 or thaumatin-like family compromise proteins with molecular masses around 20–30 kDa and a very stable three-dimensional structure maintained by 8 disulfide bridges [31]. PR5 protein expression is induced by fungus attack, due to the β-glucanase activity that takes place at a characteristic cleft, which is rich in acidic residues, conferring a strong negative electrostatic potential on the active site [18,20,28,31,32]. By computational modelling, Alt a 1 seemed to be bound to a surface region of the kiwi PR5 protein that had a complementary electrostatic nature. This binding resulted in the partial inhibition of PR5-enzymatic activity.

Although the role of Alt a 1 in infection has not been completely resolved by this study, the protein appears to be secreted in the early stages of colonization, before the development of hyphae. In non-host species such as kiwifruit, it may be that the physiological action of Alt a 1 is stopped by its interaction with PR5, which would in turn inhibit, partially, the anti-pathogenic activity of the PR5 proteins.

From the point of view of human health, the presence of Alt a 1 in apparently healthy kiwifruits is highly relevant. Kiwi PR5-TLP (known as Act d 2) and Alt a 1 have been characterized as major allergens from the fruit and *Alternaria*, respectively [18,33]. In fact, *Alternaria* has been described as one of the principal causes of severe asthma in the USA [34]. Thus, the results presented in this study suggest that *Alternaria*-allergic patients may experience an allergic crisis after ingesting infected kiwis. However, further experimental evidence would be required to determine whether this is indeed the case.

In summary, the present study reveals Alt a 1 as a competitive inhibitor of the PR5-TLP family, which may be particularly relevant in both fungal infection and in the processes involved in human allergic responses.

Competing interests

The authors have declared that no competing interests exist.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.02.044.

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