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Structural basis of the antifungal activity of wheat PR4 proteins

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ABSTRACT

PR4 proteins possess antifungal activity against several pathogenic fungi suggesting a pivotal role in defence reactions against plant pathogen attack. We already showed that wheatwin1, a wheat PR protein of class 4, is endowed with ribonuclease activity. In this study we produced three mutants altering the active site and performed comparative analysis with the native protein also in the presence of the ribonuclease inhibitor 5′-ADP. We characterized the RNA binding site and its interaction with 5′-ADP by 3D modelling and docking studies. Moreover, in vitro antifungal assays have been carried out in order to study the relationship between antifungal and ribonuclease activities. Finally, localization of wheatwin1 in *Fusarium culmorum* spores was evaluated using fluorescence light microscope.

1. Introduction

In their natural environment plants are challenged by several pathogenic agents such as viruses, viroids, fungi and bacteria [1]. For their survival plants have developed a complex variety of defence mechanisms that activate a highly co-ordinated resistance response upon recognition of a potential pathogen. Among these mechanisms are the synthesis and accumulation of a set of host-encoded pathogenesis-related (PR) proteins [2]. These proteins, defined as proteins encoded by the host plant but induced specifically in pathological situations, not only accumulate locally at infection sites, but are also systemically induced concomitantly with the development of Systemic Acquired Resistance (SAR) against further infection by fungi, oomycetes, bacteria and viruses [3]. It has been suggested that the collective set of PR proteins may be effective in inhibiting pathogen growth and multiplication and that they may, at least in part, be responsible for the state of SAR [4].

At present, a large number of PR-proteins have been characterized and grouped into 17 families based on their structural and functional features [5] showing antifungal activity against specific pathogens, and possessing enzymatic or inhibitory activity such as glucanases (PR2), peroxidases (PR9), chitinases (PR3, PR8, PR11), proteinase inhibitors (PR6) and ribonuclease-like (PR10). Their wide-ranging modes of action make these molecules a complete tool in defence strategies.

In the last years, we have extensively studied wheat PR proteins of class 4, one of the less known family of PRs. We isolated and sequenced four PR4 proteins from wheat kernels, named wheatwin1 to wheatwin4, that inhibit host specific pathogens (e.g. *Fusarium culmorum* and *F. graminearum*) and phytopathogenic fungi with a wide host range (e.g. *Botrytis cinerea*) [6–8]. These proteins and their genes are specifically induced in wheat seedlings infected with *F. culmorum* and upon treatment with systemic acquired resistance activators [9,10]. Based on the knowledge of the tertiary structure in solution of barwin (PDB code 1BW3) [11,12], a highly homologous protein from barley [13] showing a six-stranded double-psi β barrel [14], we designed and validated the three-dimensional model of wheatwin1 (PDB code 1C2Z) [15]. We also compared the 3D structures of the four wheatwin proteins by homology modelling and related their micro differences to the different antifungal activity [16]. More recently, we have characterized five new PR4 genes that proved to be induced upon *F. culmorum* infection, SAR chemical inducer treatments and wounding showing different spatial and temporal induction pathways [10]. Moreover, we also reported that both native and recombinant wheatwin1 (r-win1) endowed with ribonuclease activity that cannot be related to the action mechanism of the PR10 family proteins [17], but rather to the classical acid–base mechanism of ribonucleases A and T1 involving two His residues [18]. In this paper, we studied the relationship between the ribonuclease activity of wheatwin1 and its antifungal properties by site-directed mutagenesis. We especially pointed out, for the first time, that His11 and His113, known to be critical for RNase activity, play

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a pivotal role also for the antifungal capacity of wheatwin1. We performed comparative ribonuclease and antifungal analyses using either wild-type or mutant proteins even in the presence of 5′-ADP that is a powerful inhibitor of ribonuclease activity [19]. Also, molecular modelling and docking studies allowed us to define the RNA binding site and its interaction with 5′-ADP. Finally, cellular localization of wheatwin1 in fungal cells was also investigated.

2. Materials and methods

2.1. Mutants production and recombinant protein expression

To generate mutation(s) on *wPR4a* gene, coding for wheatwin1 protein, Splice Overlap Extension (SOE) PCR strategy [20] was used. *wPR4a* gene cloned into EcoRI site of pGEX-2T vector was used as template in PCR reactions performed to produce single mutant gene. To construct each mutant two couples of oligonucleotides were designed. One couple contained pGEX-5′ (5′-gggctggcaagccacgtttggtg-3′) as forward primer and an oligonucleotide perfectly matching with the template except for a mismatch corresponding to the wanted mutation as reverse primer. The last primer was also used in opposite orientation as forward primer in the second couple along with pGEX-3′ (5′-ccgggagctgcatgtgtcagagg-3′). pGEX-5′ and pGEX-3′ annealed to the vector upstream and downstream of *wPR4a* gene, respectively. The following oligonucleotides were used to generate mutations: His11Gly, 5′-cgacgta**cgg**ctactacc-3′ (forward) and 5′-cggtagtag**ccg**tacgtcg-3′ (reverse); His11Leu, 5′-cgacgtac**ctc**tactacc-3′ (forward) and 5′-cggtagta**gag**gtacgtcg-3′ (reverse). The two amplified products obtained for each mutant were mixed and denaturated and then used in the subsequent PCR reaction. The single-stranded DNA molecules having complementary sequences annealed and acted as primer for each other. The amplification of the whole gene was then obtained using pGEX-5′ and pGEX-3′ primers. To construct double mutant (His11Leu/His113Leu), the mutant His11Leu-*wPR4a* was used as template in PCR reactions with the above external primers and the following forward and reverse primers, respectively: 5′-gtaccagcagggc**ctc**cttaacg-3′ and 5′-acgttaag**gag**gccctgctggta-3′. Mutated codon is bold underlined in all primers. DNA sequencing of the entire sequences was accomplished in order to verify the insertion of all mutations. The mutants were cloned in frame into EcoRI site of pGEX-4T-2 vector, which contains the sequence coding the glutathione-S-transferase (GST) upstream the multiple cloning sites.

The recombinant vectors harbouring either single or double mutants were inserted into *Escherichia coli* strain BL21 for production of GST fusion proteins. Recombinant protein expression, purification from inclusion bodies and refolding were performed as previously described [17]. Refolded protein purification by gel filtration chromatography and removal of the N-terminal GST-tag by digesting the fusion protein by thrombin were also performed as previously described [17].

2.2. Ribonuclease activity assays

RNase activity of mutant proteins was assayed in comparison with RNase activity of native and r-win1. Total RNA was isolated from wheat coleoptiles according to Prescott and Martin [21]. The reaction mixture (20 μl) contained 12 μg of wheat coleoptile RNA and 4 μg of each protein in 10 mM Tris–HCl, pH 7.5, containing 10 mM imidazole and 5 mM NaCl. After 1 h incubation, the proteins were removed by phenol–chloroform (1:1) extraction and the results were observed on 1.2% agarose gel. The same heat-inactivated proteins were used as a control.

RNase activity of the above proteins was also tested by spectrophotometric assay. RNase type XII-A was used as a control. The reaction mixture (200 μl) contained 80 μg of wheat coleoptile RNA and 0.7 μg of each protein in 50 mM Tris–HCl, pH 7.4. After incubation for 30 min at 37 °C, the residual not degraded RNA was recovered by ethanol precipitation, whereas free oligonucleotides remained in the supernatant. The pellet was resuspended in 1 ml of 0.5% SDS and the absorbance at 260 nm was read in comparison with the absorbance of control RNA treated as above. The ratio between ΔA_{260} and mg of protein in the reaction mixture was used to calculate enzymatic activity (U/mg).

In order to evaluate putative ability of 5′-ADP to inhibit RNase activity, the same spectrophotometric assay was carried out adding 5 mM 5′-ADP to the reaction mixture. Inhibition assays were carried out using r-win1 and RNase type XII-A as a control. Each experiment was carried out three times independently.

2.3. Antifungal activity assays

In vitro antifungal activity assays were performed comparing the activity of mutant proteins with native and r-win1. *F. culmorum* macroconidia (4×10^4 spores/ml in 0.05× PDB) were grown in the presence of each protein at a concentration of 75 μg/ml using 0.05× PDB as a control. One hundred microliters of each sample were placed in 96-well microtiter plates and incubation was carried out for 5 h at 25 °C. Thereafter, 10 μl of the suspension were placed on sterile microscope slides and hyphal growth inhibition was evaluated microscopically with a light microscope.

To correlate ribonuclease and antifungal activity, hyphal growth inhibition of r-win1 was also tested adding to the reaction mixture 5 mM 5′-ADP, as inhibitor of ribonuclease activity. *F. culmorum* macroconidia (4×10^4 spores/ml in 0.05× PDB) in the presence of 5 mM 5′-ADP were used as a control of spores vitality. Each experiment was carried out three times independently.

2.4. Molecular modelling

3D structure models of wheatwin1 were based on the availability of the NMR three-dimensional co-ordinates of the homologous protein barwin [11–13] (PDB code 1BW3) and performed as previously described [15,16]. The alignment of wheatwin1 and barwin did not require deletion or insertion of gap. 3D models of wheatwin1 mutants were constructed using the co-ordinates of the native protein as template. The program MODELLER [22] and Quanta (Accelrys Inc.) were used to build protein models according to the comparative protein modelling methodology. 5′-ADP co-ordinates were downloaded from the Protein Data Bank as "pdb" file. 5′-ADP docking was performed using the program Molegro Virtual Docker (http://www.molegro.com/). Figures were drawn with Swiss PDB Viewer [23] and Pymol (Delano scientific, http://pymol.sourceforge.net) programs.

2.5. localization on F. culmorum spores

r-win1 protein (600 μg) was equilibrated in 100 mM $NaHCO_3$/$NaCO_3$ pH 9.0 containing 70 mM NaCl and mixed with fluorescein isothiocyanate (FITC) in a molar ratio 1:1. The mixture was incubated o.n. at 9 °C, in the dark, under gentle shaking. After centrifugation at 10 000 rpm, 4 °C for 5 min, FITC-labeled r-win1 was separated from free FITC using a small G-25 column and PBS 1× as running buffer. Eluted protein was dialyzed against 50 mM Tris–HCl, pH 8.0, containing 100 mM NaCl and used for in vitro localization.

F. culmorum macroconidia (4×10^4 spores/ml in 0.05× PDB) were mixed with FITC-labeled r-win1 protein at a concentration of 75 μg/ml in a final volume of assay of 100 μl. Following incubation for 5 h, at 25 °C in the dark, 10 μl of the suspension were placed on sterile microscope slides and 2 μl of a solution of

Fluorescent Brightener 28 (Calcofluor White M2R) (Sigma, St. Louis, MO, USA) at a concentration of 1 μg/ml was added to each sample. Following a further incubation for 10 min in the dark the samples were analyzed under fluorescence light microscope using either FITC or DAPI filters. Immunofluorescence images were acquired using a 100x plan-NEOFLUAR Zeiss objective, a digital photo camera (Axiocam MRC) connected to a Zeiss microscope equipped with epi-fluorescence, Nomarski optics, and the Zeiss Axiovision 4 software. Images acquired through the digital photo camera were sized and optimized for contrast and brightness using Adobe Photoshop (Adobe Systems, Mountain View, CA). Final images were saved at a minimum of 300 dpi.

3. Results and discussion

3.1. Production of wheatwin1 mutants

Our previous work indicated that recombinant wheatwin1 was able to inhibit the germination of *F. culmorum* spores as well as their germ tube elongation [17]. Moreover, we reported that both native and r-win1 endowed with ribonuclease activity [17] related to the classical acid–base mechanism of ribonucleases A and T1 involving two His residues (His 11 and His 113) [18]. Since these evolutionarily conserved residues are supposed to be important for ribonuclease activity, His11 and His113 were selected for point mutations in order to produce single and double mutants useful to understand their roles in the relationship between antifungal and ribonuclease activities of wheatwin1.

Two single mutants were produced, namely H11G-win1 and H11L-win1, substituting the His in position 11 with glycine or leucine, respectively. Moreover, a double mutant, H11L/H113L-win1 was also produced substituting the two histidine residues with leucine. We operated these choices to fully compare wheatwin1 and ribonuclease A activities. In fact, it is well known that substitution of just one His residue with a hydrophobic residue in ribonucleases produces only a decreasing activity. In fact, surrounding acid or basic residues can contribute to the effectiveness of the mechanism. On the contrary, the substitution of both His residues induces a quite complete lacking in the activity [18].

Recombinant wheatwin1 and all mutant proteins were individually expressed in *E. coli* strain BL21 as fusion proteins with glutathione-S-transferase (GST). All proteins were purified as previously reported from inclusion bodies and renatured by dilution in the presence of the redox couple oxidized and reduced glutathione (GSSG and GSH, respectively) [17]. After digestion with thrombin, releasing the GST-tag, all wild-type and mutant proteins were purified by gel filtration chromatography as previously described [17] (data not shown)

3.2. Ribonuclease and antifungal activity

RNA degradation assay was used to examine whether purified win1 mutants had ribonuclease activity. Total RNA isolated from wheat leaves was incubated with purified mutant proteins as well as native and r-win1 and enzymatic activity was evaluated on agarose gel as previously described [17]. Degradation of wheat RNA was not observed when incubated with the elution buffer (Fig. 1, panel A, lane 1) whereas when incubated with the purified native (lane 2) or recombinant (lane 3) wheatwin1, RNA degradation was clearly visible. On the other hand, either H11L-win1 (lane 4) or H11G-win1 (lane 5) were no longer able to completely degrade wheat RNA. Interestingly, double mutant H11l/H113L-win1 showed a reduced ability to degrade RNA (lane 6) as compared to single mutants. In addition, heat-inactivated proteins (Fig. 1, panel B) were no longer able to exhibit ribonucleolytic activity highlighting that RNase activity requires a correctly folded protein.

On the basis of these results it can be concluded that substitution of only one histidine residue, namely His 11, is not enough to completely abolish RNase activity of wheatwin1. On the other hand, substitution of both histidine residues postulated to be involved in the acid–base mechanism of RNase activity, strongly reduce the enzymatic activity of the protein. However, with this methodology is hardly to calculate any percentage of residual ribonuclease activity of all mutants. To this end, a spectrophotometric assay of RNase activity was settled up. Total wheat RNA was incubated with both wild-type or mutant proteins and the U/mg of each protein was calculated as described in Material and Methods section. Pancreatic ribonuclease, type XII-A was used as a positive control. As shown in Table 1, in the assay condition used, r-win1 displayed a percentage of ribonucleasic activity of 93.3% with respect to RNase XII-A, thus showing a good efficiency in degrading RNA. Both single mutants H11G-win1 and H11L-win1 displayed reduced percentage of activity of 39.8 and 37.7, respectively. On the other hand, double mutant H11L/H113L-win1 retained only a little amount of ribonuclease activity, accounting for 8.5%. However, it is worthwhile to mention that this residual RNase activity is still enough to promote RNA degradation even to a little extent, as shown in Fig. 1, panel A, lane 6.

These results clearly show that both histidine 11 and 113 play a pivotal role in determining the ribonuclease activity of wheatwin1 protein. Consequently, after due consideration we can assert that ribonuclease activity of wheatwin1 can be explained by the classical acid–base mechanism common to other ribonucleases involving two His residues. It has been reported that 5′-ADP is a very powerful inhibitor of ribonuclease activity and its crystal structure in complex with ribonuclease A has been determined [19]. In order to verify whether 5′-ADP was able to inhibit ribonuclease activity

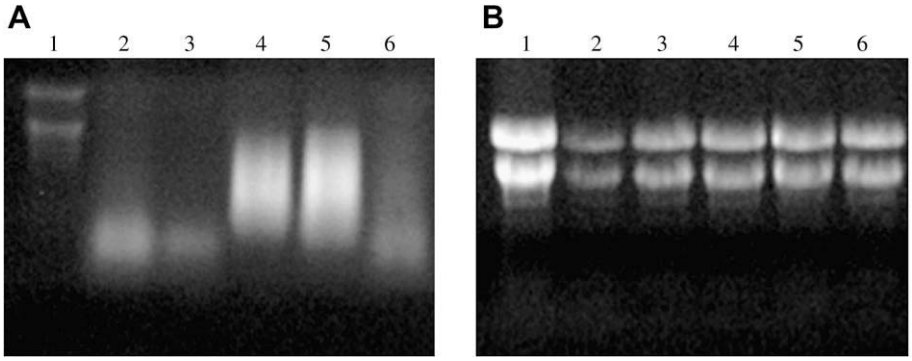

Fig. 1. Ribonuclease activity tested against wheat total RNA. Panel A: Control (1), incubation with native (2) and recombinant (3) wheatwin1, H11L-win1 (4), H11G-win1 (5), H11L/H113L-win1 (6). Panel B: Control (1), lanes 2–6 represent the same heat-inactivated proteins shown in panel A loaded in the same order.

Table 1
Percentage of enzymatic activity of recombinant Wheatwin1 (r-win1) and mutant proteins (H11G-, H11L-, H11L/H113L-win1) with respect to RNase XII-A. The enzymatic activities of RNase XII-A and r-win1 are also reported in the presence of 5′-ADP as inhibitor.

Protein	U/mg	Activity%
RNase XII-A	134.3	100
r-Win1	125.7	93.3
H11G-win1	53.4	39.8
H11L-win1	50.6	37.7
H11L/H113L-win1	11.4	8.5
RNase XII-A + 5′-ADP	50	37.2
r-Win1 + 5′-ADP	62.4	46.5

of r-wheatwin1, spectrophotometric RNase assay was also performed in its presence. RNase type XII-A was used as a positive control. As shown in Table 1, 5′-ADP is able to inhibit either RNase A or r-win1, although with less efficiency. In fact, the residual ribonuclease activity of RNase A was 37.2% whereas that of r-win1 was 46.5%.

To address the question whether structural basis of antifungal activity relies on ribonucleasic activity of wheatwin1, in vitro antifungal assays of native, recombinant or mutant wheatwins were performed as previously described [17]. Moreover, hyphal growth inhibition of r-win1 was also tested adding 5′-ADP to the reaction mixture using *F. culmorum* macroconidia germinated in the presence of the inhibitor as a control (Fig. 2). Panel A shows the growth of *F. culmorum* in the absence of protein whereas the effect of either native or recombinant wheatwin1 is reported in panels B and C, respectively. Both proteins are able to inhibit *F. culmorum* growth and the effect is exerted either on spore germination (data not shown) or germ tube elongation combined with morphological alterations like swelling and wrinkling. r-Win1 was able to inhibit the fungal growth with the same efficiency with respect to the

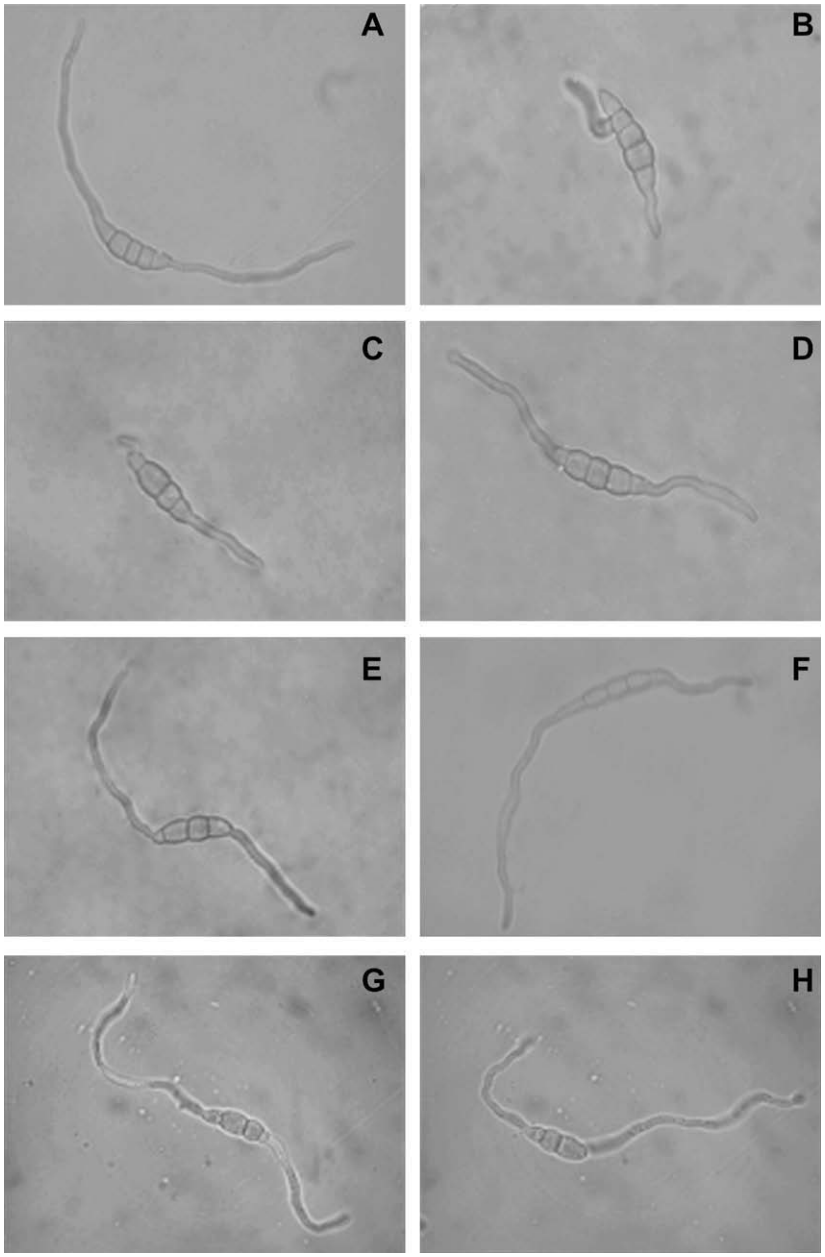

Fig. 2. Antifungal activity of native (B) and recombinant (C) wheatwin1, H11G-win1 (D), H11L-win1 (E) and H11L/H113L-win1 (F) on *F. culmorum* growth. Antifungal activity of recombinant wheatwin1 in the presence of 5′-ADP (H). *F. culmorum* macroconidia grown in the absence (A) and in the presence of 5′-ADP (G) were used as a control.

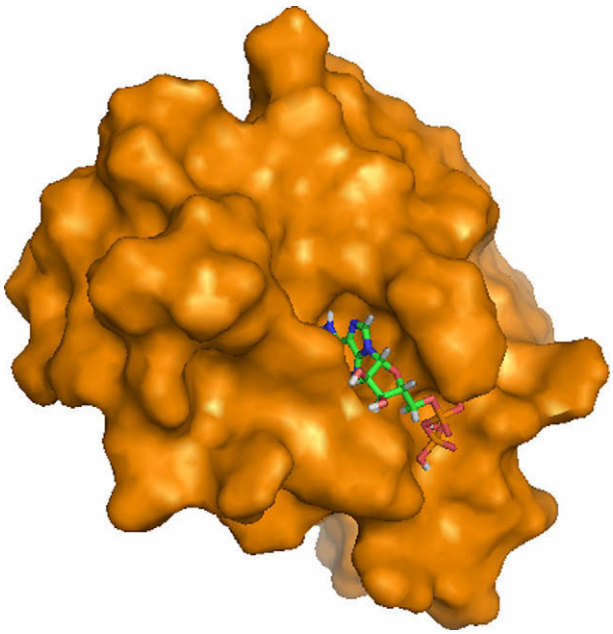

Fig. 3. 3D surface of Wheatwin1. The position of 5′-ADP into the active site cavity is shown.

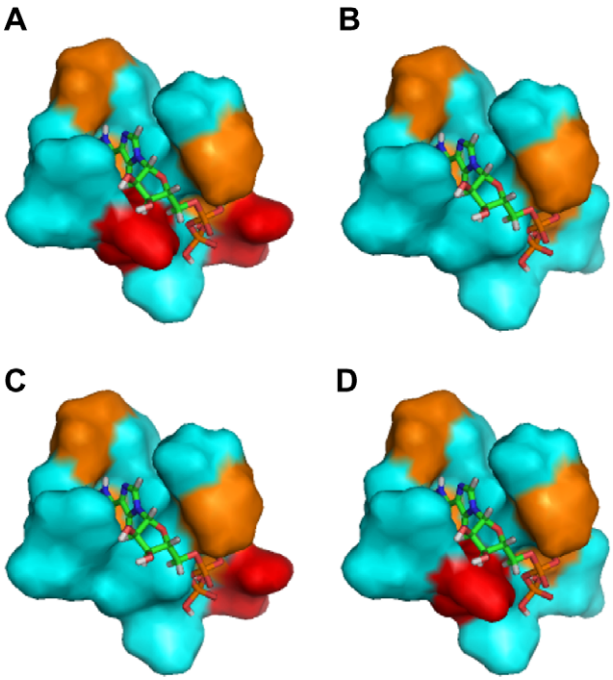

Fig. 5. Cavities of native Wheatwin1 (A), double H11L /H113L mutant (B), H11L mutant (C) and H113L mutant (D). Hydrophobic residues are coloured in cyan. His residues are coloured in red.

native protein. On the contrary, H11G-win1 and H11L-win1 mutants were found to be almost inactive either on spore germination (data not shown) or hyphal growth (Fig. 2, panels D and E, respectively) as no morphological alterations were evident like in the control (panel A) with the exception of some swelling. Even more evident is the ineffectiveness of the double mutant H11L/H113L-win1 in the inhibition of *F. culmorum* germination (data not shown) and growth (panel F). Moreover, antifungal activity assays performed in the presence of 5′-ADP also showed that ribonuclease activity inhibition correlates with antifungal activity loss (panel H). 5′-ADP alone did not interfere with *F.culmorum* growth, as shown in panel G.

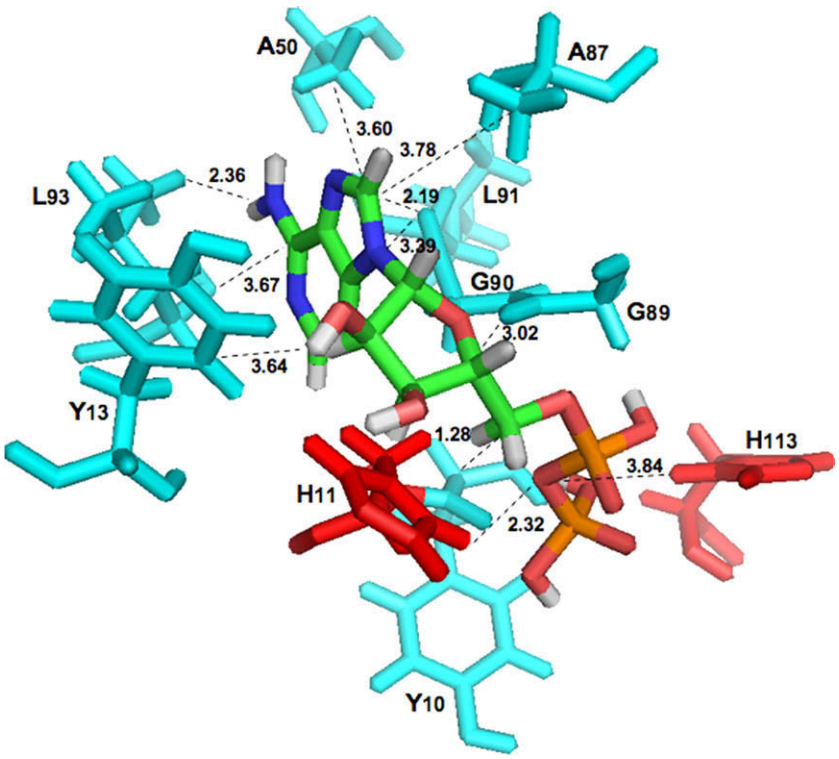

Fig. 4. Spatial positions of hydrophobic residues (cyan) in the cavity establishing interactions with 5′-ADP. The positions of His residues (red) are also shown and distances are indicated.

Since we demonstrated in this study that the double mutant H11L/H113L-win1 is no longer endowed with ribonuclease activity, we can conclude that ribonuclease and antifungal activity of r-win1 are correlated. Further, we can propose that the antifungal activity of this protein is due to its ribonucleasic ability.

3.3. Structural studies

We have reported that the ribonuclease activity of wheatwin1 can be explained by the classical acid–base mechanism common to other ribonucleases involving two His and a couple of acid and basic residues. In the case of wheatwin1, the residues involved in the mechanism were His11, His113, Asp92 and Arg7 [17]. Here we describe the protein cavity involved in substrate interaction by molecular docking of 5′-ADP. Fig. 3 shows the surface 3D structure of the complex with wild-type wheatwin1. 5′-ADP penetrates the active site cavity whose molecular dimensions are very similar to that of ribonucleases. As an example, ribonuclese A cavity (PDB code 1KYA) shows a surface of 129.8 $Å^2$ and a volume of 31.7 $Å^3$ while that of wheatwin1 has a surface of 125.4 $Å^2$ and a volume of 34.5 $Å^3$, respectively. In native wheatwin1, 5′-ADP is bounded to the cavity by hydrophobic interactions with various surrounding residues, namely Tyr10, Tyr13, Ala50, Ala87, Gly89, Gly90 and Leu93. The phosphate group is located between the His residues protruding at the edge of the cavity with distances ranging from 2.32 to 3.84 Å useful to set up interactions for the effectiveness of the acid–base mechanism (Figs. 4 and 5a). These interactions can be only partially established in single L-mutants, while cannot established at all in the double L-mutant (Fig. 5b–d) explaining and validating the results obtained by enzymatic assays.

3.4. Localization of wheatwin1 in fungal cells

In order to investigate the distribution of wheatwin1 in fungal cells, the protein was labeled with FITC and incubated with *F. culmorum* spores. Antifungal effect on either spore germination or germ tube elongation has been evaluated in the presence of FITC-labeled r-win1 using fluorescence light microscope equipped with a FITC filter. Endogenous fluorescence of unlabeled r-win1 was also evaluated. Calcofluor White M2R staining was also used to verify the integrity of fungal cell wall due to its chitin- and cellulose-binding capacity. Optical brighteners of the diaminostilbene type, like Calcofluor, seem to be virtually non-toxic so that selective staining of cell walls with fluorescent dyes has been useful in morphological and developmental studies of fungi [24]. In fact, when used with wide-field fluorescence microscopy (DAPI filter) this optical brightener is a useful dye to address biological processes in fungi and other organisms.

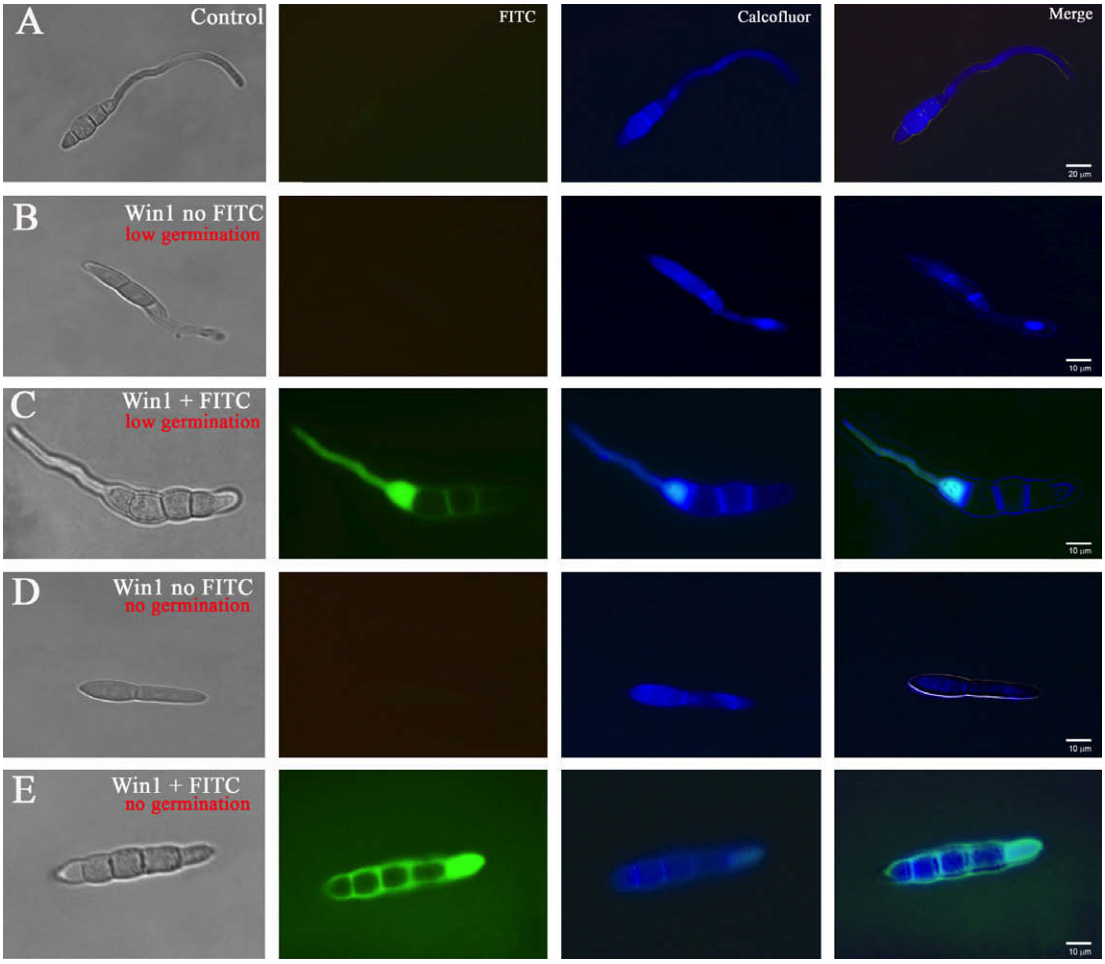

Fig. 6. Wheatwin1 localization on *F. culmorum* spores germinated in the presence of the protein. Panel A: *F. culmorum* germinated in the absence of the protein used as control; starting from the left: pictures taken in the bright field, with FITC filter, with DAPI filter (calcofluor staining) and the merged image. Panel B: Hyphal growth inhibition of *F. culmorum* treated with r-win1; Panel C: Hyphal growth inhibition of *F. culmorum* treated with FITC-labeled win1; Panel D: *F. culmorum* spore germination inhibition in the presence of r-win1; Panel E: *F. culmorum* spore germination inhibition in the presence of FITC-labeled win1. Concentration of each protein was 75 μg/ml. Immunofluorescence images were acquired using a 100× plan-NEOFLUAR Zeiss objective. Bars 20 μm in A and 10 μm in (B–E).

As shown in Fig 6, Calcofluor White M2R staining (Calcofluor panel) of control *F. culmorum* (A) highlights the living fungal cell wall as well as septa and emerging hyphae. Treatment of *F. culmorum* with unlabeled wheatwin1 results in the partial inhibition of either germ tube elongation (B) of germinated spores or spore germination (D). Calcofluor staining of the same samples demonstrated that fungal cell walls were intact as no fractures were present and no cytoplasm had been expelled. This result suggests that wheatwin1 does not exert its antifungal action on fungal cell wall. Antifungal activity was also assessed using FITC-labeled wheatwin1 (C and E). As clearly visible in the bright field panel, FITC-win1 was able to inhibit either hyphal growth (C) or spore germination (E). Moreover, FITC staining showed that the labeled protein is localized inside the cytoplasm of fungal cells and it is present also in the emerging hyphae. Calcofluor staining of the same sample showed once again the integrity of fungal cell wall. Taken together these results indicate that wheatwin1 is able to enter inside fungal cells leaving the cell wall intact. Once entered in the cytoplasm of host cells the antifungal activity of the protein can be exerted on the basis of its ribonuclease activity on endogenous RNA. Further experiments are necessary to study the mechanism by which the protein is able to cross the plasma membrane, i.e. endocytosis or receptor-mediated internalization.

4. Conclusion

To our knowledge, this is the first report correlating antifungal and ribonuclease activities of PR4 proteins and describing the RNA binding site of these proteins. We reported that mutations of the His residues involved in ribonuclease action mechanism as well as the use of 5′-ADP produce similar effects both on antifungal and enzymatic activities of wheatwin1. Furthermore, we showed the protein enters into *F. culmorum* cells. All these evidences lead to the conclusion that the antifungal activity of wheat PR4 proteins is due to enzymatic activity typical of ribonucleases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.07.045.

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