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Recombinant expression, affinity purification and functional characterization of Scots pine defensin 1

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Abstract Plants produce a variety of molecules to defend themselves from fungal pathogens. Defensins belong to the family of antimicrobial peptides that play a central role in innate immunity in all species of plants. We have previously reported the purification of antimicrobial peptides from Scots pine seedlings and the identification of some of them, including defensin, by mass spectrometry. In this study, we extend our original study on molecular cloning of *Pinus sylvestris* defensin 1 (*Ps*Def1) by presenting the expression and affinity purification of recombinant defensin 1 (r*Ps*Def1). The full-length coding sequence of *Ps*Def1 has an open reading frame capable to encode a protein of 83 amino residues, including a signal peptide of 33 aa, followed by a characteristic defensin domain of 50 amino acids representing its active form. The calculated molecular weight of the mature form of PsDef1 is 5,601.6 Da. We have employed pET system to express mature form of *Ps*Def1 fussed to GST. As GST-*Ps*Def1 fusion protein was not biologically active, we removed GST moiety from the mature defensin 1 peptide by proteolytic cleavage with Factor Xa. The resulting r*Ps*Def1 protein exhibited strong antifungal activity against a panel of pathogenic fungi which is comparable to that of endogenous Scots pine defensin 1. In addition, r*Ps*Def1 was used to produce specific polyclonal antibodies. Using generated antibodies, we found that the level of *Ps*Def1 is significantly increased in Scots pine seedlings during germination and in their response to pathogenic infection with *Heterobasidion annosum.*

Keywords Antimicrobial peptides · Scots pine defensin · Recombinant *Ps*Def1 · Antifungal activity · *Ps*Def1 specific antibodies

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Introduction

Plants produce a diverse range of molecules that have the potential to inhibit the growth of microbial pathogens. These include hydrogen peroxide, phenolics, terpenoids, alkaloids, polyacetylenes and a diverse array of pathogenesis-related (PR) defence proteins (Broekaert et al. 1997; Garcia-Olmedo et al. 1998; Osbourn 1999; van Loon et al. 2006; Benko-Iseppon et al. 2010). Defensins belong to the PR defence proteins and form a family of small cationic peptides of 45–54 amino acid residues with a conserved signature of cysteines, which form four disulfide bridges. Therefore, plant defensins exhibit a very similar tertiary structure composed by a triple-stranded anti-parallel β-sheet and one α-helix (Almeida et al. 2002; Fant et al. 1998). Despite similarity of their three-dimensional struc-

tures, plant defensins have low homology in their primary amino acid sequences. It has been speculated that an observed high variability of primary sequences stipulates the diversity of biological activities reported for plant defensins (Thomma et al. 2002; Lay and Anderson 2005; Carvalho and Gomes 2009).

Defensins are widely spread in the plant kingdom and have been purified from seeds, leaves, fruit, roots, tubers and floral tissues of many angiosperm species. Defensins are secretory proteins, which are produced as precursors with an endoplasmic reticulum signal sequence and a mature defensin domain. Most plant defensins inhibit the growth of broad spectrum of phytopathogenic fungi at micromolar concentrations *in vitro*. Moreover, some of them were found to possess antibacterial properties (Osborn et al. 1995). In addition, other biological activities such as inhibition of insect gut α-amylase (Bloch and Richardson 1991), blocking the activity of ion channels (Kushmerick et al. 1998; Spelbrink et al. 2004), mediation of metal tolerance (Mirouze et al. 2006), as well as the inhibition of protein synthesis have been documented for some of plant defensins (Mendez et al. 1996).

The exact mechanism of action of plant defensins is not completely understood, but there is evidence that plant defensins interact with sphingolipids on fungal cell membrane and integrate into the phospholipids bilayer of the membrane, resulting in the permibilization and membrane destabilization (Thevissen et al. 2003; Thevissen et al. 2004). It was recently demonstrated that plant defensins can be also internalized into the cytoplasm and be involved in specific interactions with intracellular targets (Lobo et al. 2007; van der Weerden et al. 2008).

The above biological properties of plant defensins make them attractive targets in biotechnology, especially for the development of plants resistant to phytopathogens and pests. To date, several transgenic plants have been developed with stable overexpression of plant defensins. For example, constitutive expression of pea defensin enhanced resistance of canola plants against blackleg (*Leptosphaeria maculans*) disease (Wang et al. 1999), while the expression of *Medicago sativa* defensin in potatoes provided high level of field resistance against pathogenic fungus of *Verticillium dahliae* (Gao et al. 2000).

In addition, plant defensins have also been used in pharmaceutical industry for the development of novel antibiotics directed against pathogenic microorganisms. Drug-development programs, pre-clinical studies and clinical trials require large quantities of defensins. However, purification of endogenous defensins from plants is laborious, and the yield is low. Therefore, the most attractive approach for the production of large quantities of biologically active defensins is the application of recombinant DNA technology, based on diverse expression systems.

We have recently developed a highly efficient protocol for the purification of antimicrobial peptides from Scots pine seedlings, which allowed us to identify with the use of mass spectrometry several proteins with antifungal properties. Based on these findings, molecular cloning of cDNA corresponding to *Pinus sylvestris* defensin 1 (*Ps*Def1, Acc. No. EF455616.1) was reported for the first time (Kovaleva et al. 2009). Furthermore, we observed strong antifungal activity of endogenous *Ps*Def1 towards a broad spectrum of phytopathogenic fungi and *Candida albicans*. These results reveal the potential of *Ps*Def1 as a novel fungicide agent. To explore this potential and to advance our studies on molecular mechanisms of *Ps*Def1 action, we decided to establish an expression system which would allow the production of large quantities of biologically active recombinant *Ps*Def1. Here, we report a highly efficient protocol for bacterial expression and affinity purification of biologically active *Ps*Def1. The availability of large quantities of recombinant *Ps*Def1 allowed us the production of polyclonal antibodies, which recognize specifically both recombinant and endogenous *Ps*Def1. We also found that the expression of *Ps*Def1 in Scots pine seedlings increases significantly during germination and in response to fungal infection.

Materials and methods

Biological material

Scots pine (*P. sylvestris* L.) seeds were obtained from Busk State Forestry (Lviv region, Ukraine). Surface-sterilized seeds were germinated on Whatman paper soaked with distilled water in Petri dishes at 26 °C in the thermostat. Seedlings were taken away daily during germination for the period of 7 days. All plant samples were frozen in liquid nitrogen and stored at −70 °C until further usage.

Cultures of fungal pathogens *Fusarium oxysporum* UKM F-52897, *Fusarium solani* UKM F-50639, *Botrytis cinerea* UKM F-16753 and *Alternaria alternata* UKM F-16752 were obtained from D.K. Zabolotny Institute of Microbiology and Virology, Ukraine. The culture of necrotrophic fungus *Heterobasidion annosum* was kindly provided by Dr. V. Kramarec (Ukraine). Fungal cultures were grown on potato dextrose agar at 22 °C. Cultures of *H. annosum* were grown for 8 days in potato dextrose broth and then blended with Ultra-Turrax T-25 homogenizer.

Inoculation of pine seedling roots with *H. annosum*

The fungal homogenate (3 ml) was spread onto sterile filter paper pre-laid on 1.2% *w/v* potato dextrose agar. Twelve seedlings of *P. sylvestris* (6 days old) were asceptically

transferred on the plates, with only the root region in contact with the filter paper. Control seedlings were treated with sterile distilled water. After inoculation, pine seedlings were grown at 22 °C in darkness. Seedling root samples were collected at 48 h after inoculation, immediately frozen in liquid nitrogen and stored at −70 °C until use. The experiments were repeated in triplicates.

Construction of the pET42a/*Ps*Def1 expression plasmid

Molecular cloning was carried out in *Escherichia coli* XL-1 Blue strain, while bacterial strain *E. coli* BL21 (DE3) was used for protein expression. Bacteria were grown in Luria-Bertani (LB) medium.

Cloning of the full-length PsDef1 cDNA into vector pET23d was previously described (Kovaleva et al. 2009). A fragment of cDNA encoding mature Scots pine defensin 1 (m*Ps*Def1) was amplified by polymerase chain reaction (PCR) using pET23d/*Ps*Def1 as a template and a set of primers: sense primer CR765 (5′-CCATTCCATGGGAA TGTGCAAAACCCCGAG-3′) with *Nco*I restriction site and antisense primer CR764 (5′-CATGAGAATTCTCAA GGGCAGGGTTTGTA-3′) with *Eco*RI. The PCR conditions were 94 °C for 3 min followed by 30 cycles (94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min) and finally 72 °C for a 5-min extension. The amplified fragment was digested with *Nco*1 and *Eco*R1 restriction enzymes and ligated into pET-42a vector (Novagen) in frame with the N-terminally located S-transferase (GST). The resulting pET-42a/*Ps*Def1 plasmid was transformed into *E. coli* XL-1 Blue cells. The event of cloning of m*Ps*Def1 cDNA into the expression vector was confirmed by purifying the plasmid from kanamycin-resistant colonies and digesting it with endonucleases *Nco*I and *Eco*RI as well as by PCR analysis with primers CR765 and CR764. The sequence of the pET-42a/m*Ps*Def1 plasmid was verified by DNA sequence analysis.

The expression of recombinant *Ps*Def1

E. coli BL21 (DE3) competent cells were transformed with the pET42a/m*Ps*Def1 plasmid, and bacterial colonies were selected by plating onto LB agar with 50 mg/ml kanamycin. A single colony was inoculated into 5-ml LB medium with antibiotic and grown overnight at 37 °C. The overnight cultures were diluted 1:100 (*v*/*v*) into 500 mL of the same medium and grown in the shaking incubator at 37 °C and 200 rpm until an absorbance of 0.6 at 600 nm. Protein expression was induced by adding 0.4 mM IPTG (Sigma) and subsequent culturing at 37 °C for 2 h. Cells were harvested by centrifugation at 7,000×*g* for 20 min at 4 °C, washed twice 1× PBS pH 7.4 and stored at −70 °C until protein extraction.

Purification of recombinant GST/*Ps*Def1

The cell pellet (2.5 g) was thawed and re-suspended in 5 ml of ice-cold buffer A, consisting of 20 mM Tris, pH 7.5, 50 mM NaF, 150 mM NaCl, 5 mM EDTA and 1% Triton X-100 in the presence of cocktail of protease inhibitors (Roche). Cells were destroyed by mechanical grinding in a homogenizer, and the lysate was passed three times with a syringe through a 21-gauge needle. The suspension was left on ice for 30 min, cell extract was centrifuged at 15,000×*g* for 15 min at 4 °C and the cleared supernatant was passed through a 0.22-μm filter (Millipore). Recombinant protein was batch purified with glutathione sepharose resin (Amersham). Briefly, the resulting supernatant was added to a 50% suspension of glutathione sepharose, equilibrated with buffer A and the fusion protein allowed to bind for 2 h on a wheel at 4 °C. Unbound proteins were removed by washing beads three times with 50 mM Tris–HCl, pH 7.5, 150 mM NaCl and 0.05% Twin-20 and twice with 50 mM Tris–HCl, pH 8.0. Bound proteins were eluated from glutathione sepharose with 50 mM reduced glutathione in 50 mM Tris–HCl, pH 8.0, 150 mM NaCl. Eluted fractions were then analysed by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis and separated proteins visualized with Coomassie G-250. Fractions containing recombinant GST-*Ps*Def1 were dialyzed against buffer containing 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM DTT and stored at −20 °C in the presence of 50% glycerol.

In order to remove the GST moiety, the GST-*Ps*Def1 fusion protein was immobilized on glutathione sepharose and then subjected to Factor Xa (Sigma) digestion in a cleavage buffer (20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 2 mM $CaCl_2$). The reaction was performed under room temperature for 5 h on a rotating wheel. Beads were then collected by centrifugation at 3,000×*g* for 1 min. The supernatant was loaded onto Centricon YM 30 (Millipore), and protease was removed by centrifugation. The filtrate was collected and dialyzed against distilled water and then concentrated by ultracentrifugation (Centricon YM3). The protein concentration was measured by Bradford assay. The quality of purified preparations of *Ps*Def1 was analyzed by SDS electrophoresis in 15% polyacrylamide gel using a Tris-tricine buffer system (Schägger and von Jagow 1987).

Antifungal activity assay

Antifungal activity of Scots pine recombinant defensin 1 against *A. alternata* was determined as previously described (Lam et al. 2000). For the assay, a piece of *A. alternata* mycelia was placed in the centre of the Petri dish containing about 15 ml potato dextrose agar. The plates were incubated at 23 °C. When the diameter of fungal

colony attained 3 cm, sterile paper disks (3 MM, Whatman) were placed at a distance of 0.5 cm around the frontal mycelia. Different amounts of filter-sterilized recombinant protein were added to each disk. The plates were incubated at 23 °C for approximately 48 h until mycelial growth had enveloped discs containing sterile distilled water (control) and had formed growth inhibition zones around the discs containing active preparations of antimicrobial peptides.

To determine the IC_{50} value, fungal spore suspension (2×10^4 spores ml^{-1}) was cultivated in 100 μl of potato dextrose broth containing serial dilutions of filter-sterilized recombinant *Ps*Def1. Control wells contained no peptide. After 48 h of incubation at 23 °C in darkness, the optical density of fungal suspension was measured in every well of a 96-well microtitre plate under 595 nm. Antifungal activity of *Ps*Def1 was expressed as a percentage of growth inhibition. Growth inhibition percentage is defined as 100× the ratio of the A_{595} of the control minus the A_{595} of the sample over the A_{595} of the control (Broekaert et al. 1990). All experiments were repeated in three independent set ups.

Production of *Ps*Def1 polyclonal antibodies

Polyclonal antibodies directed against *Ps*Def1 were raised by immunizing rabbits with GST-*Ps*Def1. Two rabbits were immunized by subcutaneous injection of 200 μg of recombinant GST-*Ps*Def1 fusion protein with equal amount of complete Freund's adjuvant (Sigma). In subsequent immunizations, 100 μg of GST-*Ps*Def1 was mixed with incomplete Freund's adjuvant and injected with a 2-week interval. Final immunization was carried out with 100 μg GST-*Ps*Def1 in PBS given 2 weeks before the terminal bleed. The fraction of immunoglobulins was precipitated from the immune serum with 50% ammonium sulphate and used for affinity purification by chromatography on protein A–sepharose CL-4B (Amersham). Fractions containing eluted IgGs were dialyzed in 1× PBS, pH 7.2. The aliquots of purified antibodies were stored at −70 °C.

Western blot analysis

Scots pine seeds and seedlings (100 mg) were homogenized in 0.3 ml of 50 mM sulphuric acid. The suspension was left on ice for 40 min and centrifuged at 15,000×*g* for 15 min at 4 °C. The supernatant was collected, and protein concentrations were determined by the protein assay kit (Bio-Rad), following the manufacturer's instructions.

Samples of GST-*Ps*Def1 (100 ng), recombinant *Ps*Def1 (100 ng), purified Scots pine defensin 1 from seedlings (0.5 μg) and total protein extracts from pine seedlings (20 μg) were separated by 15% Tricine SDS-PAGE. One gel was stained with Coomassie Brilliant Blue G-250, and the other was electroblotted (2 h in 48 mM glycine, 39 mM Tris, 20% methanol, pH 9.4 at 1.5 mA/cm^2) onto Immobilon-P^{SQ} membrane (Millipore) using Transblot SD apparatus (Bio-Rad). After blotting, the membrane was blocked in buffer containing 7% skim milk in TTBS (20 mM Tris, 0.15 M NaCl, 0.1% *w/v* Tween-20, pH 7.4) for 1 h at room temperature and then incubated with *Ps*Def1-specific antibodies (1:2,000) for 2 h at RT. Bound antibody was detected using goat anti-rabbit IgG conjugated to horseradish peroxidase (1:2,500 dilution; Promega) and enhanced chemiluminescence detection reagents (Amersham) before being exposed to Super RX film (Fujifilm Corporation).

The level of *Ps*Def1 in germinating seedlings was measured by scanning the exposed immunoblot film membranes using the UMAX vistascan 3.5.2 (UmMAX Data Systems, Inc.) program and analyzed with Gel-Pro Analyzer 4.0 (Media Cybernetics). The *Ps*Def1 level detected by Western blot analysis was calculated based on the optical density of all pixels within the band boundary. The results are the average of three separate immunoblots.

Results

High-level expression of soluble GST-*Ps*Def1 fusion protein in *E. coli* is driven by the pET-42a/m*Ps*Def1 plasmid. The expected 174-bp fragment encoding the mature form of Scots pine defensin 1 obtained via PCR amplification was cloned into pET42a(+) expression vector. The resulting construct, named pET-42a/m*Ps*Def1, was used to transform competent BL21 (DE3) cells. The expression of recombinant GST-*Ps*Def1 was induced by the addition of 1 mM IPTG to bacterial cultures at OD_{600} approximately 0.6. The SDS-PAGE analysis of total lysates from induced and non-induced cells revealed the presence of recombinant protein of an expected molecular weight, 35.5 kDa. The fusion protein consists of 220 amino acids of GST at the N-terminus and 52 aa corresponding to the mature form of *Ps*Def1 (Fig. 1a). There is also a 56 aa insert between GST and *Ps*Def1, which contains Factor X restriction site and some sequences from the pET42a vector. In addition, this analysis showed that GST-*Ps*Def1 fusion protein is soluble in buffer containing 1% Triton X-100. No peptide bands corresponding to the recombinant protein were observed in non-induced cultures (Fig. 1b, lanes 1 and 2). We also found that under this experimental setup, some amount of the GST-*Ps*Def1 protein was present in inclusion bodies. Therefore, we focused our efforts on optimizing the conditions for achieving the maximal yield of soluble GST-*Ps*Def1. Expression of soluble GST-*Ps*Def1 was optimized by varying IPTG concentration (0.1–1 M), temperature (23 °C, 28 °C and 37 °C) and time of induction. We found

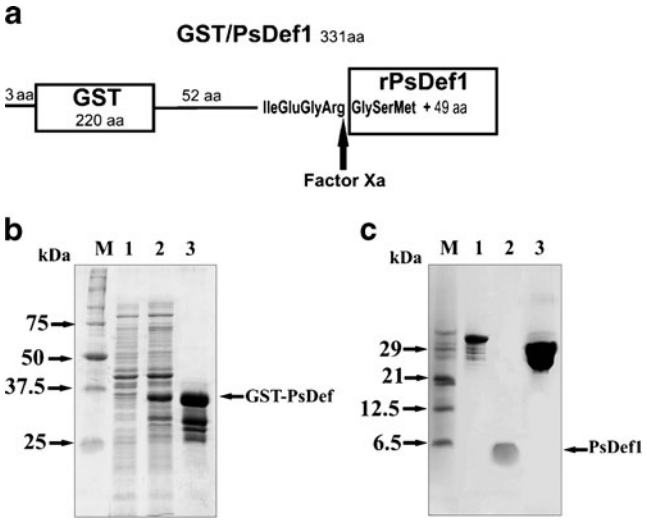

Fig. 1 Design and affinity purification of recombinant *Ps*Def1. **a** Schematic structure of recombinant GST-*Ps*Def1 fusion protein. The *arrow* indicates the location of the cleavage site for Factor Xa. **b** SDS-PAGE analysis of Triton-soluble protein fractions from *E. coli* BL21 (DE3) cells transformed with pET-42a/*Ps*Def1 expression plasmid and incubated in the absence (*lane 1*) or presence of IPTG (*lane 2*); purification of GST-*Ps*Def1 on glutathione sepharose (*lane 3*). Proteins were separated by 12% SDS-PAGE electrophoresis in Laemmli system. Lane *M*: broad-range protein standards Bio-Rad. **c** SDS-Tricine PAGE analysis of GST-*Ps*Def1 fusion protein before and after the treatment with factor Xa: *lane M*, protein test mixture 5 (Serva); *lanes 1* and 3, immobilized proteins on sepharose before and after factor Xa cleavage, respectively; *lane 2*, recombinant *Ps*Def1 after separation from factor Xa by centrifugation on Centricon YM 30 columns (Millipore)

that optimal expression of soluble GST-*Ps*Def1 is achieved by the induction of bacteria with 0.4 mM IPTG for 2 h at 37 °C. Furthermore, optimal extraction of soluble GST-*Ps*Def1 fusion protein from bacterial cells was observed in buffer containing 1% Triton X-100 (data not shown).

Affinity purification of recombinant GST-*Ps*Def1 and the production of r*Ps*Def1

Affinity purification of recombinant GST-*Ps*Def1 was carried out on glutathione sepharose as described in "Materials and methods" section. As shown in Fig. 1b (lane 3), fractions eluted from the affinity column mainly contain a protein of approximately 36 kDa, which closely correlates to the predicted molecular mass of the GST-*Ps*Def1 protein fusion. Western blot analysis with anti-GST antibodies confirmed the expression of GST-*Ps*Def1 and showed faster migrating protein bands, observed in lane 3, representing degraded forms of the fusion protein (data not shown). Purified preparations of GST-*Ps*Def1 were dialysed against the storage buffer containing 50% glycerol and stored at −70 °C until further use. To remove the GST-tag, GST-*Ps*Def1 fusion protein coupled to glutathione sepharose was subjected to proteolytic cleavage with Factor Xa. Initially, we performed a time-course digestion analysis with Factor Xa and found that the complete cleavage of the GST-*Ps*Def1 fusion protein is achieved after 5 h of incubation with the protease (data not shown). Further studies indicated that optimal cleavage of GST-*Ps*Def1 requires 20 μg of Factor Xa per 1 mg of the fusion protein. The supernatant, containing cleaved *Ps*Def1 and Factor Xa was then loaded onto a Centricon YM-30 concentrator. After the centrifugation, the filtrate was analysed by SDS-PAGE for the presence of *Ps*Def1 and stored at −70 °C in a glycerol containing storage buffer. The efficiency of Factor Xa cleavage and the quality of recombinant preparations of *Ps*Def1 are shown in Fig. 1c. As seen in lane 3, the band corresponding to the GST-*Ps*Def1 fusion protein disappears after 5 h of digestion, while the major band of approximately 30 kDa, which correspond to GST alone, is clearly detected. The developed protocol results in the production of highly homogeneous preparations of recombinant defensin 1 (r*Ps*Def1), which runs on the SDS-PAGE with a predicted molecular mass of 6 kDa (Fig. 1c, lane 2). The final output of r*Ps*Def1 was approximately 10% from total GST-*Ps*Def1 fusion protein, immobilized on glutathione sepharose.

Recombinant *Ps*Def1 exhibits antifungal activity similar to that of endogenous protein

We tested the antifungal activity of recombinant GST-*Ps*Def1 and r*Ps*Def1 against a panel of phytopathogenic fungi. We were interested to find whether GST/*Ps*Def1 fusion protein possesses any antifungal activity. No antifungal activity against several phytopathogenic fungi was observed in the presence of recombinant GST-*Ps*Def1, even when the concentration of GST-*Ps*Def1 in the culture medium was 50 μg/ml (data not shown). These findings indicate that the GST moiety interferes with antifungal properties of *Ps*Def1 in the context of the GST-*Ps*Def1 fusion protein. When the activity of r*Ps*Def1 was tested in a growth-inhibiting assay, we reproducibly observed strong antifungal properties of recombinant *Ps*Def1. It is illustrated in Fig. 2a, showing growth-inhibiting zones of *A. alternata* in areas containing sterile discs of filter paper on which different concentrations of r*Ps*Def1 were applied. In this experiment, distilled water was used as a negative control.

Antifungal activity of protein samples were assayed by microspectrophotometry as described previously (Broekaert et al. 1990). To do so, the optical density of fungi spore suspension was measured after 48 h of incubation in potato dextrose broth, containing various concentrations of r*Ps*Def1. When maximum concentration (4 μg/ml) of *Ps*Def1 was tested in this experiment, we observed significant inhibition of fungal growth: 81% for *F. solani*,

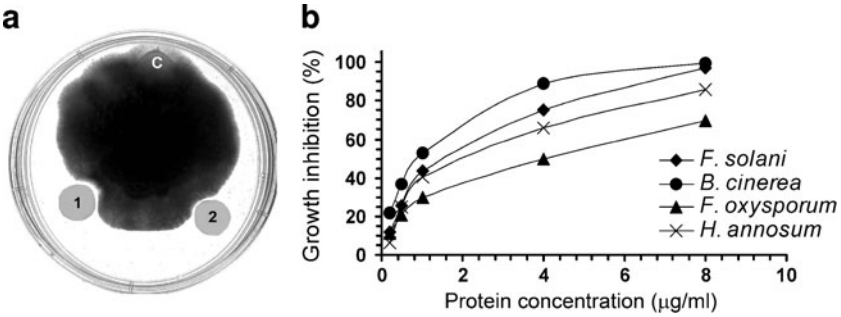

Fig. 2 Recombinant *Ps*Def1possesses antifungal activity. **a** Effect of purified recombinant *Ps*Def1 on the mycelial growth of *A. alternata*: *disk C*, sterile distilled water; *disks 1 and 2*, correspond to 10 and 5 μg of recombinant *Ps*Def1, respectively. **b** Dose-dependent growth inhibition curves. Growth inhibition of *F. solani*, *F. oxysporum*, *B. cinerea* and *H. annosum* in the presence of indicated concentrations of recombinant *Ps*Def1 was determined by measuring the cultures at OD_{595}. The measurements were taken after the incubation of fungal cultures for 48 h at 23 °C. The experiment was repeated three times

50% for *F. oxysporum*, 71% for *H. annosum* and 87% for *B. cinerea*. Furthermore, we have estimated IC_{50} values of recombinant *Ps*Def1 for *F. solani*, *F. oxysporum*, *B. cinerea* and *H. annosum* and found them as 1.4, 4.0, 0.7 and 2.6 μg/ml, respectively (Table 1). In the same experimental setup, IC_{50} values of endogenous *Ps*Def1 towards a panel of fungi was also measured and found to be comparable to that of recombinant *Ps*Def1 (Table 1).

Expression of *Ps*Def1 is induced during seed germination and in response of Scots pine seedlings to fungal infection

The availability of recombinant defensin 1 allowed us to initiate the production of polyclonal and monoclonal antibodies which can specifically recognize *Ps*Def1 in various immunological assays. Affinity-purified preparations of GST-*Ps*Def1 were used to immunize rabbits and mice for raising polyclonal and monoclonal antibodies, respectively. So far, we have not been successful in generating monoclonal antibodies towards *Ps*Def1, and the second round of hybridoma production is currently in progress. On the other hand, we succeeded in making highly specific polyclonal antibodies. The specificity of produced antibodies was initially tested by Western blot analysis of GST-*Ps*Def1 and r*Ps*Def1 (Fig. 3a, lanes 1 and 2). Further tests revealed that anti-*Ps*Def1 antibodies recognize specifically not only recombinant defensin 1 but also endogenous *Ps*Def1, purified from Scots pine seedlings (Fig. 3a, lane 3). Furthermore, a strong immunoreactive band of approximately 6 kDa was clearly detected in protein extracts from 5-day-old Scots pine seedlings (lane 4). The apparent ca. 6 kDa size of the immunoreactive protein corresponds well to the calculated molecular mass of 5,601 Da for the mature form of *Ps*Def1.

Table 1 Antifungal activity of recombinant and native *Ps*Def1

Fungi	IC_{50} values (μg/ml)	
	Recombinant *Ps*Def1	Native *Ps*Def1
F. solani	1.4	0.9
F. oxysporum	4.0	2.9
B. cinerea	0.7	0.4
H. annosum	2.6	1.4

The development of highly specific anti-*Ps*Def1 antibodies provided us with a very useful research tool for studying the expression pattern of *Ps*Def1 in various tissues at different stages of development and in response to environmental stresses and pathogenic microorganisms. In this context, we were interested to find whether the expression of *Ps*Def1 changes in the process of seed germination. At this stage of plant development, cells in the seedling roots are exposed to various environmental factors, including a variety of soil-bourn microorganisms.

We found that anti-*Ps*Def1 antibodies detected a single protein band of approximately 6 kDa in protein extracts derived from seed embryo and pine seedling roots collected at different stages of development (Fig. 3b). Furthermore, this analysis has also revealed a significant increase (50–80%) of the 6-kDa protein level at 6 and 7 days after imbibition when compared to that at day 1 (Fig. 3b, graph).

To investigate *Ps*Def1 levels in roots in response to pathogenic fungi, seedlings were exposed the necrotrophic pathogen *H. annosum*. The non-suberized seedlings have been used as a model for investigating the pattern of gene expression in the *H. annosum* conifer pathosystem because this root-rot fungus has the ability to infect the conifer roots of all ages (Asiegbu 2000; Li and Asiegbu 2004). In this study, the level of *Ps*Def1 was analyzed by immunobloting protein extracts derived from Scots pine seedling roots exposed or not exposed to the necrotrophic pathogen *H. annosum* (Fig. 3c) and quantified by scanning the optical density of immunoreactive bands (Fig. 3c, graph). In infected roots, we reproducibly observed a significant ($P \leq$

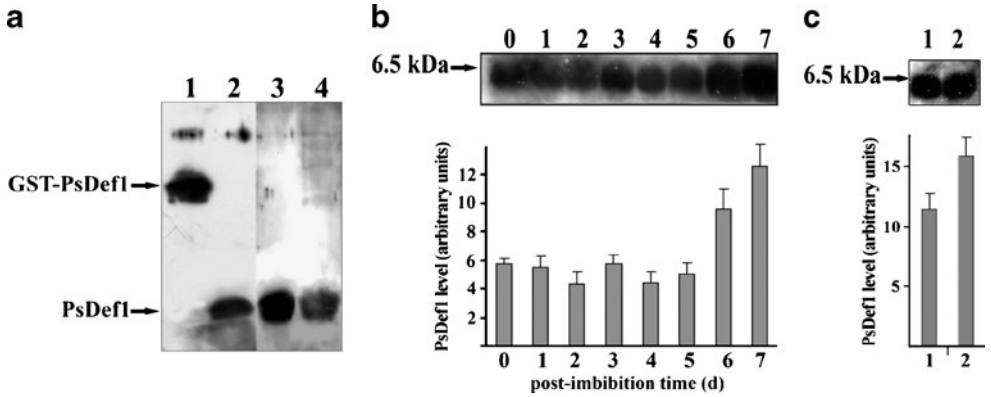

Fig. 3 Expression of endogenous defensin 1 is induced during seed germination and in response of Scots pine seedlings to fungal infection. **a** Testing the specificity of *Ps*Def1 polyclonal antibodies by Western blot analysis of GST-*Ps*Def1 (*lane 1*), recombinant *Ps*Def1 (*lane 2*), Scots pine defensin 1 purified from germinating seedlings (*lane 3*), protein extract from 5-day-old Scots pine seedlings (*lane 4*). **b** Immunoblot detection of defensin 1 in protein extracts from seed embryos (*lane 0*) and Scots pine seedling at various stages of germination (*lanes 1–7*, correspond to days 1 to 7, respectively). Total protein extracts were isolated from 0.1 g of embryos and seedling roots as described in "Materials and methods" section. Equal amount of total proteins from each sample was separated by 15% Tricine SDS-PAGE and immunoblotted with anti-*Ps*Def1 specific antibodies. The amount of endogenous *Ps*Def1 was quantified by densitometry and presented (arbitrary units) in the graphs shown *below the blot*. **c** Immunoblot detection of defensin 1 in protein extracts from healthy (*lane 1*) and infected Scots pine seedling roots (*lane 2*). The amount of endogenous *Ps*Def1 in control seedlings (*lane 1*) and seedlings infected with *H. annosum* (*lane 2*) was quantified by densitometry and presented (arbitrary units) in the graphs shown *below the blot*

0.05) increase in the amount of the *Ps*Def1 when compared to non-infected roots.

Discussion

In this study, we describe the expression system for large-scale production of recombinant *Ps*Def1, which exhibits strong antifungal activity at micromolar concentration against a broad spectrum of fungi. The mature form of *Ps*Def1 was cloned into pET-42a expression vector in the frame with GST, and the optimal conditions for the expression of soluble GST/*Ps*Def1 fusion protein was determined. Since the GST/*Ps*Def1 fusion protein was biologically inactive, we devised an efficient protocol for the removal of the GST moiety by proteolytic cleavage with Factor Xa and the production of milligram quantities of highly homogeneous and bioactive *Ps*Def1. We found that the final preparations of *Ps*Def1 exhibited strong antifungal activity against a panel of phytopathogenic fungi, which is comparable to that of endogenous Scots pine defensin 1. Furthermore, the availability of recombinant *Ps*Def1 allowed us to produce specific antibodies, which have been used successfully to study the expression profile of Scots pine defensin 1 under various experimental conditions.

To investigate the nature of defence molecules in germinating seeds, we developed an efficient protocol for the purification of antimicrobial peptides from Scots pine seedling (Kovaleva and Gout 2007). Using this protocol, we purified two proteins with antifungal activities and determined their identity by mass spectrometry as defensin and lipid-transfer protein (Kovaleva et al. 2009). Subsequently, we reported for the first time molecular cloning and characterization of the cDNA encoding Scots pine defensin, termed *Ps*Def1. In this study, we further extend these original findings by establishing bacterial expression system and the purification protocol for large-scale expression of recombinant *Ps*Def1. Bacterial expression system of *E. coli* was chosen to produce recombinant *Ps*Def1 for a number of reasons. In comparison with other expression systems, prokaryotic system of *E. coli* provides the most efficient, fast and relatively inexpensive way of producing recombinant proteins in large amounts, required for biochemical, structural and functional studies. Sequence analysis of PsDef1 cDNA clone indicated the presence of a signal peptide at the N-terminus (33 amino acid residues in length), followed by a basic domain of mature defensin of 50 aa. Since the endogenous signal peptide could not be recognized in the *E. coli* system (Da-Hui et al. 2007), we have cloned a cDNA fragment, coding the mature form of PsDef1 into pET42 expression vector in frame with the GST-tag epitope. By optimizing the conditions for expression, solubilization and affinity purification of recombinant GST-*Ps*Def1, as well as removing of the GST moiety from the fusion protein by proteolytic cleavage, we managed to produce 1 mg of r*Ps*Def1 per litre of bacterial cultures.

Importantly, the developed protocol results in the production of highly stable and biologically active *Ps*Def1. We would like to note that recombinant *Ps*Def1 shows slightly reduced activity towards a panel of pathogenic

fungi, when compared to that of native protein. This is probably the result of substituting of Arg at the N-terminal end of the native defensin by three additional amino acids Gly, Ser and Met in the recombinant peptide. Such substitution results in the decrease of total positive charge of r*Ps*Def1. Based on mutational analysis, De Samblanx et al. (1997) showed that replacement of basic amino acid residues in radish defensin *Rs*-AFP2 by neutral amino acids decreases antifungal activity. A steric hindrance effect of additional amino acids might also influence the structure/function of recombinant *Ps*Def1. In this context, Kristensen et al. (1999) reported that the AX2 defensin from sugar beet leaves had biological activity slightly lower than the natural protein. Interestingly, recombinant AX2 also have an additional amino acid at the N-terminus. In 2008, Pelegrini et al. reported that the cowpea seed defensin *Vu*D1 uses both N- and C-terminal regions to block the active site of α-amylase from the insect *Zabrotes subfasciatus*.

The use of bacterial expression system for production of bioactive plant defensins often results in the production of insoluble and/or biologically inactive proteins. To overcome this problem, plant defensins were produced as translational fusion proteins with thioredoxin in Origami *E. coli* strain (Kaomek et al. 2003; Zelicourt et al. 2007). This expression system was also used by Pervieux et al. (2004) for the production of defensin from *Picea glauca*, which exhibits 80% identity to *Ps*Def1. Recombinant *Pg*D1 inhibited the growth of *F. oxysporum* by 95.2% at 2.5 μM. We found that recombinant *Ps*Def1 inhibited growth of a broad spectrum of fungi by 50% at concentration less than 1 μM. This results suggest that pET42a/BL21(DE3) expression system is also efficient for production of bioactive plant defensins.

The availability of recombinant *Ps*Def1 has given us the options of not only examining its biochemical and antimicrobial properties but also generating specific antibodies. We were successful in producing polyclonal antibodies, which specifically recognize both recombinant and endogenous *Ps*Def1 in Western blotting and immunoprecipitation assay. With the use of generated antibodies, we showed that the level of *Ps*Def1 expression does not change significantly at earlier stages of germination, and the significant increase is detected on sixth and seventh days of imbibition (1.6- and twofolds, respectively). Similarly, Fossdal et al. (2003) also revealed the accumulation of Norway spruce defensin polypeptide (SPI1) in roots after seed germination. Studies from several laboratories indicate that the expression of defensin genes is often upregulated upon pathogen attacks. Since endogenous *Ps*Def1 possesses high antimicrobial activity towards *H. annosum* in vitro, we examined whether the infection of Scots pine seedling roots with this fungi would affect the expression of *Ps*Def1. Indeed, we found elevated levels of *Ps*Def1 in roots of Scots pine seedlings 48 h after infection with *H. annosum*. These findings suggest that the expression of this antifungal peptide in the vulnerable seedlings tissues might assist the development of resistance to pathogenic attacks.

At present, the mechanism by which plant defensins inhibit fungal growth is not well understood. It has been recently reported that plant defensins have the ability to interact specifically with membrane sphingolipids on fungal cell and to disrupt membrane integrity (Thevissen et al. 2003, 2004). Furthermore, plant defensins were also found in the cytoplasm of fungal cells, where they can interact with intracellular targets (Lobo et al. 2007; van der Weerden et al. 2008).

In conclusion, this study describes the successful heterologous expression of plant defensin with antifungal activity against a broad spectrum of fungi. The antifungal activity of the recombinant *Ps*Def1 indicates the prospect of its potential in biotechnology for protecting pine forests against infectious diseases. In addition, produced specific antibodies provide a very useful tool to study the expression profile of Scots pine defensin in response to various abiotic and biotic stress agents and to screen naturally occurring genotypes for high-level expression of defensin gene.

References

Almeida MS, Cabral KM, Kurtenbach E, Almeida FC, Valente AP (2002) Solution structure of *Pisum sativum* defensin 1 by high resolution NMR: plant defensins, identical backbone with different mechanisms of action. J Mol Biol 315:749–757. doi:10.1006/jmbi.2001.5252

Asiegbu FO (2000) Adhesion and development of the root rot fungus (*Heterobasidion annosum*) on conifer tissues: effects of spore and host surface constituents. FEMS Microbiol Ecol 33:101–110. doi:10.1111/j.1574-6941.2000.tb00732.x

Benko-Iseppon AM, Galdino SL, Calsa TJ, Kido EA, Tossi A, Belarmino LC, Crovella S (2010) Overview on plant antimicrobial peptides. Curr Prot Pept Sci 11:181–188. doi:10.2174/138920310791112075

Bloch CJ, Richardson MA (1991) A new family of small (5 kDa) protein inhibitors of insect α-amylases from seeds of sorghum (*Sorghum bicolor* (L.) Moench) have sequence homologies with wheat γ-purothionins. FEBS 279:101–104. doi:10.1016/0014-5793(91)80261-Z

Broekaert WF, Terras F, Cammue B, Vandereyden J (1990) An automated quantative assay for fungal growth inhibition. FEMS Microbiol Lett 69:55–60. doi:0.1111/j.1574-6968.1990.tb04174.x

Broekaert WF, Cammue B, DeBolle M, Thevissen K, DeSamblanx G, Osborn R (1997) Antimicrobial peptides from plants. Crit Rev Plant Sci 16:297–323. doi:10.1080/713608148

Carvalho AO, Gomes VM (2009) Plant defensins—prospects for the functional and biotechnological properties. Peptides 30:1007–1020. doi:10.1016/j.peptides.2009.01.018

Da-Hui L, Gui-Liang J, Ying-Tao Z, Tie-Min A (2007) Bacterial expression of a *Trichosanthes kirilowii* defensin (TDEF1) and its

antifungal activity on *Fusarium oxysporum*. Appl Microbiol Biotech 74:146–151. doi:10.1007/s00253-006-0631-z

De Samblanx GW, Goderis I, Thevissen K, Raemaekers R, Fant F, Borremans F, Acland DP, Osborn R, Pateli S, Broekaert WF (1997) Mutational analysis of plant defensin from radish (*Raphanus sativus* L.) reveals two adjacent sites important for antifungal activity. J Biol Chem 272:1171–1179. doi:10.1074/jbc.272.2.1171

Fant F, Vranken W, Broekaert WF, Borremans F (1998) Determination of the three dimensional solution structure of *Raphanus sativus* antifungal protein 1 by 1 H NMR. J Mol Biol 279:257–270. doi:10.1006/jmbi.1998.1767

Fossdal CG, Nagy NE, Sharma P, Länneborg A (2003) The putative gymnosperm plant defensin polypeptide (SPI1) accumulates after seed germination, is not readily released, and the SPI1 levels are reduced in *Pythium dimorphum*-infected spruce roots. Plant Mol Biol 52:291–302. doi:10.1023/A:1023915230129

Gao AG, Hakimi SM, Mittanck CA, Wu Y, Woerner BM, Stark DM, Shah DM, Liang J, Rommens CM (2000) Fungal pathogen protection in potato by expression of a plant defensin peptide. Nat Biotechnol 18:1307–1310. doi:10.1038/82436

Garcia-Olmedo F, Molina A, Alamillo JM, Rodriguez-Palenzuela P (1998) Plant defense peptides. Biopolymers 47:479–491. doi:10.1186/gb-2007-8-9-225

Kaomek M, Mizuno K, Fujimura T, Sriyotha P, Cairns JR (2003) Cloning, expression, and characterization of an antifungal chitinase from *Leucaena leucocephala* de Wit. Biosci Biotechnol Biochem 67:667–676. doi:10.1271/bbb.67.667

Kovaleva V, Gout R (2007) Isolation of the proteins with antifungal activity from *Pinus sylvestris* L. seedlings. Physiol Biochem Cultiv Plants 39:114–120

Kovaleva V, Kiyamova R, Cramer R, Krynytskyy H, Gout I, Filonenko V, Gout R (2009) Purification and molecular cloning of antimicrobial peptides from Scots pine seedlings. Peptides 30:2136–2143. doi:10.1016/j.peptides.2009.08.007

Kristensen A, Brunstedt J, Nielsen J, Mikkelsen J, Roepstorff P, Nielsen K (1999) Processing, disulfide pattern, and biological activity of a sugar beet defensin, AX2, expressed in *Pichia pastoris*. Protein Expr Purif 16:377–387. doi:10.1006/prep.1999.1085

Kushmerick C, Castro MS, Cruz JS, Bloch CJ, Beiraio PSL (1998) Functional and structural features of γ-zeathionins, a new class of sodium channel blockers. FEBS Lett 440:302–306. doi:10.1016/S0014-5793(98)01480-X

Lam YW, Wang HX, Ng TB (2000) A robust cysteine-deficient chitinase-like antifungal protein from inner shoots of the edible chive *Allium tuberosum*. Biochem Biophys Res Commun 279:74–80. doi:10.1006/bbrc.2000.3821

Lay FT, Anderson MA (2005) Defensins—components of the innate immune system in plants. Curr Prot Pept Sci 6:85–101. doi:10.2174/1389203053027575

Li G, Asiegbu FO (2004) Use of Scots pine seedling roots as an experimental model to investigate gene expression during interaction with the conifer pathogen *Heterobasidion annosum* (P-type). J Plant Res 117:155–162. doi:10.1007/s10265-003-0140-4

Lobo DS, Pereira IB, Fragel-Madeira L, Medeiros LN, Cabral LM, Faria J, Bellio M, Campos RC, Linden R, Kurtenbach E (2007) Antifungal *Pisum sativum* defensin 1 interacts with *Neurospora crassa* cyclin F related to the cell cycle. Biochemistry 46:987–996. doi:10.1021/bi061441j

Mendez E, Rocher A, Calero M, Girbes T, Citores L, Soriano F (1996) Primary structure of ω-hordothionin, a member of a novel family of thionins from barley endosperm, and its inhibition of protein synthesis in eukaryotic and prokaryotic cell-free systems. Eur J Biochem 239:67–73. doi:10.1111/j.1432-1033.1996.0067u.x

Mirouze M, Sels J, Richard O, Czernic P, Loubet S, Jacquier A, Francois I, Cammue BPA, Lebrun M, Berthomieu P, Marques L (2006) A putative novel role for plant defensins: a defensin from the zinc hyper-accumulating plant, *Arabidopsis halleri*, confers zinc tolerance. Plant J 47:329–342. doi:10.1111/j.1365-313X.2006.02788.x

Osborn RW, Desamblanx GW, Thevissen K, Goderis I, Torrekenes S, vanLeuven F, Attenborough S, Rees SB, Broekaert WF (1995) Isolation and characterization of plant defensins from seeds of Asteracea, Fabaceae, Hippocastanaceae and Saxifragaceae. FEBS Lett 368:257–262. doi:10.1016/0014-5793(95)00666-W

Osbourn AE (1999) Antimicrobial phytoprotectants and fungal pathogens: a commentary. Fungal Genet Biol 26:163–168. doi:10.1006/fgbi.1999.1133

Pelegrini PB, Lay FT, Murad AM, Anderson MA, Franco OL (2008) Novel insights on the mechanism of action of α-amylase inhibitors from the plant defensin family. Proteins 73:719–729. doi:10.1002/prot.22086

Pervieux I, Bourassa M, Laurans F, Hamelin R, Seguin A (2004) A spruce defensin showing strong antifungal activity and increased transcript accumulation after wounding and jasmonate treatments. Physiol Mol Plant Pathol 64:331–341. doi:10.1016/j.pmpp.2004.09.008

Schägger H, von Jagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1–100 kDalton. Anal Biochem 166:368–379. doi:10.1016/0003-2697(87)90587-2

Spelbrink RG, Dilmac N, Allen A, Smith TJ, Shah DM, Hockerman GH (2004) Differential antifungal and calcium channel-blocking activity among structurally related plant defensins. Plant Physiol 135:2055–2067. doi:10.1104/pp.104.040873

Thevissen K, Ferket KK, Francois IE, Cammue BP (2003) Interactions of antifungal plant defensins with fungal membrane components. Peptides 24:1705–1712. doi:10.1016/j.peptides.2003.09.014

Thevissen K, Warnecke DC, Francois IE, Leipelt M, Heinz E, Ott C, Zahringer U, Thomma BP, Ferket KK, Cammue BP (2004) Defensins from insects and plants interact with fungal glucosylceramides. J Biol Chem 279:3900–3905. doi:10.1074/jbc.M311165200

Thomma BP, Cammue BP, Thevissen K (2002) Plant defensins. Planta 216:193–202. doi:10.1007/s00425-002-0902-6

van der Weerden NL, Lay FT, Anderson MA (2008) The plant defensin, NaD1, enters the cytoplasm of *Fusarium oxysporum* hyphae. J Biol Chem 283:14445–14452. doi:10.1074/jbc.M709867200

van Loon LC, Rep M, Pieterse CMJ (2006) Significance of inducible defense-related proteins in infected plants. Annu Rev Phytopathol 44:135–162. doi:10.1146/annurev.phyto.44.070505.143425

Wang Y, Nowak G, Culley D, Hadwiger LA, Fristensky B (1999) Constitutive expression of a pea defense gene DRR206 confers resistance to blackleg (*Leptosphaeria maculans*) disease in transgenic canola (*Brassica napus*). Mol Plant Microb Interact 12:410–418. doi:10.1094/MPMI.1999.12.5.410

Zelicourt A, Letousey P, Thoiron S, Campion C, Simoneau P, Elmorjani K, Marion D, Simier P, Delavault P (2007) Ha-DEF1, a sunflower defensin, induces cell death in *Orobanche* parasitic plants. Planta 226:591–600. doi:10.1007/s00425-007-0507-1