ISSN 0233-7657. Biopolymers and cell. 2007. vol. 23. N 5. Translated from Ukrainian.

Molecular cloning and characterization of defensin 1 from Scots pine

V. A. Kovalyova, I.T. Gout[1], R. G. Kiyamova[2], V. V. Filonenko[2], R. T. Gout

Department of Forestry, National Forestry University of Ukraine, Chuprynka St., 103, Lviv, 79057, Ukraine

[1]University Collage London, Gower Street, London WC1E 6BT, United Kingdom

[2]Institute of molecular biology and genetics NAS of Ukraine
Academicain Zabolotnog str., 150, Kyiv, 03680 Ukraine

vakovaleva@mail.ru

A protein with antifungal activity and a molecular weight of 5.6 kDa was purified from 7-day-old Scots pine seedlings. The LC-MS/MS analysis revealed that purified protein is highly homologous to plant defensins. The obtained sequences and database searches allowed us to design a set of primers for molecular cloning of a full length coding sequence, corresponding to Scots pine defensin 1 (PsDef1). This was achieved by a PCR approach, using Pinus sylvestris cDNA library as a template. The specific product of PCR amplification was cloned into pET 23d(+) vector and sequenced. Bioinformatic analysis of generated sequences showed that the coding sequence of PsDef1 cDNA has the potential to encode a protein of 83 amino acids in length. The first 33 amino acids correspond to the N-terminal signal peptide, which is removed after processing. The mature protein possesses conserved residues which are common to all plant defensins. Based on antifungal properties and sequence similarities PsDef1 was assigned to group 1 of plant defensins.

Key words: Scots pine, defensin PsDef1, molecular cloning

Introduction. In the last decade, significant progress has been made in understanding the mechanisms which coordinate the response of plants to environmental changes and pathogenic organisms, in particular.

The activation of transcription of antimicrobial proteins (AMP), which increase the resistance of plants to pathogenic organisms or inhibit the growth of pathogens, is considered to be crucial in driving the immune response to pathogenic factors [1]. The family of plant AMPs includes thionins, lipid-transport proteins, heveins, snakins, defensins *etc*. AMPs are small (2–9 kDa) secretory proteins, whose compact structure is stabilised by disulphide bridges. The number and localisation of -Cys-Cys- pairs is a specific feature of each AMPs group. Antimicrobial proteins exhibit high activity against many pathogenic bacteria and fungi both *in vitro* and in transgenic plants [2, 3].

Among AMPs, plant defensins possess a very broad spectrum of biological activity. Their molecular weight is 4–6 kDa, a tertiary structure is formed by an -helix, 3 anti-parallel -sheets and 4 intramolecular disulphide bridges [2, 4]. The protective function of plant defensins is defined by their antifungal [5, 6], antibacterial [7], and insecticidal [8] activities. It is important to note that some of these proteins are known to act as pro-

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tease inhibitors [7, 9]. The heterogeneity of properties and structure/function determinants created the basis for the classification of defensins into four groups [6]. The representatives of group I (morphogenic group) are known to inhibit the growth of a wide range of phytopathogenic fungi and induce morphological changes in mycelium. The antifungal activity of group II defensins is not associated with morphological changes in fungi. The antibacterial features of group I and II defensins are either not significant or absent at all. Plant defensins of group III are active against bacteria, but lack any fungistatic properties. They were found to inhibit -amylases and proteases *in vitro* as well as protein synthesis in a cell-free system. Defensins belonging to group IV possess both antifungal activity without any morphogenic effect and high antibacterial activity against gram-positive and gram-negative pathogens [10].

Defensins have been identified in various taxonomic groups of plants. The expression of four defensin-like proteins has been demonstrated in gymnosperm plants, including defensin precursor (GbD) *Ginkgo biloba* (Ginkgoaceae family) [11], defensin (PgD1) *Picea glauca* [12], putative plant defensin SPI1 and SPI1B *Picea abies* (Pinaceae family) [13]. To date, plant defensins were isolated from seeds, vegetative and generative organs of gymnosperm. Recently, we have purified defensin-like protein from gymnosperm which was similar to AMP in its antifungal properties [14]. Molecular cloning and comparative analysis of primary sequences of *Scots pine* defensin 1 (PsDef1) with other plant defensins are presented in this study..

Materials and Methods. In this study we used selected viable seeds of *Pinus sylvestris L.* obtained from Busk State Forestry, L'viv region, Ukraine. The cDNA sequence of *Pinus sylvestris* Defensin 1 was cloned into bacterial expression vector *pET 23d* (+) (*Novagen*, USA).

The purification of Defensin from *Pinus sylvestris* seedlings was carried out as previously described [14–16]. The purity of preparations was analysed by gradient (5–22%) gel electrophoresis at denaturating conditions in Laemmli system [17] or in tris-tricine buffer system [18]. Separated proteins were visualised by silver staining. The identification of purified proteins was performed by mass-spectrometry at University College London. The bands of interest were cut out from the gel, reduced with 10 mM dithiothreitol and alkalined with 100 mM of iodine acetamide. The samples were then hydrolysed with trypsin. The mixture of generated proteolytic peptides was fractionated on PepMap C18 column (*LC Packings*, the Netherlands) for 30 min in density gradient (5–40%) of acetonitrile and 0.1% formic acid. Mass-spectrometry analysis was performed using Q-TOF I (*Micromass*, UK). Peptide sequences generated by Mascot (*Matrixscience*, UK) were used to search Swissprot and EST GenBank databases.

The set of primers used for the amplification of PsDef1 cDNA was designed using the results of mass-spectrometry analysis and the alignment of nucleotide sequences corresponding to Defensins from *Pinus pinaster* (maritime pine) and *P. taeda* (loblolly). The amplification of PsDef1 cDNA was carried out in two separate PCR reactions using as templates 1 l of primary ($1.2 \cdot 10^6$ pfu/ml), or amplified ($2 \cdot 10^9$ pfu/ml) *P. sylvestris* cDNA expression library. The library was produced in our laboratory from mRNA of *Pinus sylvestris* root seedlings as previously described [19]

For PCR amplification, we used oligonucleotides CR763 (5'-CCATTCCATGGCGGGCAAGGGAGT-3') and CR764 (5'-CATGAGAATTCTCAAGGGCAGGGTTTGTA-3'), which contained *NcoI* and *EcoRI* cloning sites. PCR reactions were carried out using *Proteus* amplifier (*Helena BioSciences*, UK), and Tag polymerase from *Fermentas* (Lithuania). The following conditions were used for amplification: 94°C for 3 min and then 30 cycles (94°C, 1 min; 60°C, 1 min; 72°C, 1 min) and finally 5 min at 72°C.

PCR products were analysed in 1.5% agarose gel in Tris-borate buffer, pH 8.3 (50 mM tris-H_3BO_3, 2 mM EDTA) at 20 V/cm^2. Amplified DNA of the expected size was eluted from the gel using DNA extraction kit (*Qiagen*, USA).

The product of amplification and *pET23d*(+) plasmid were digested with *NcoI* and *EcoRI*, separated by gel electrophoresis and purified as described above. Ligation reaction was performed for 2 hours at room temperature using T4 phage DNA ligase (*Fermentas*) according to the manufacturer's protocol. XL-1 Blue competent cells were transformed with the ligation mixture by a standard method [20]. The presence of the DNA in-

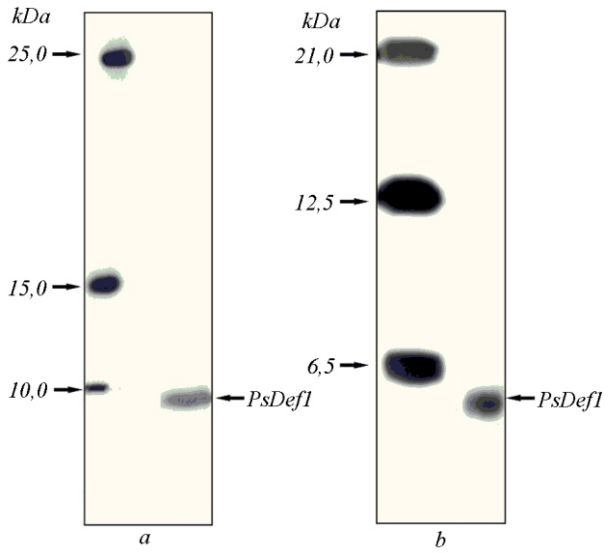

Fig.1 SDS-PAGE analysis of antifungal protein from *Pinus sylvestris*: separation of proteins in 5–22% gradient gel in Laemmle's system (*a*); separation of proteins in 15% gel with tris-tricine buffer (*b*). Separated proteins were visualized by silver staining.

sert in *pET23d*(+) plasmid was detected by restriction analysis with *NcoI* and *EcoRI,* and by PCR with primers CR 763 and CR 764. Nucleotide sequence of the cloned DNA fragment was determined by automatic DNA sequencing, ABI 73^{TM} (*Applied Biosystems*, UK).

Bioinformatic analysis and sequence alignments of the nucleotide and amino acid sequences of *Pinus sylvestris* defensin 1 were performed using electronic search service BLAST 2.0, National Centre of Biotechnological Information (NCBI), USA.

Results and Discussion. Recently we have purified low molecular weight protein, specific for high inhibitory activity towards some phytopathogenic fungi, from 7-day-old root seedlings of *Pinus sylvestris*. Biochemical properties and strong antifungal activity clearly indicated that purified protein belongs to the group of plant defensins [14]. The molecular weight of defensin-like protein from *Pinus sylvestris* was estimated to be approximately 10 kDa when analysed by 5-22% SDS-PAAG analysis (Fig.1, *a*). To determine the size of purified protein more precisely, we employed tris-tricine buffer system, which is optimal for the separation of low-molecular proteins and peptides. The separation at this conditions revealed that the molecular weight of purified protein is approximately 5–6 kDa (Fig.1, *b*), which is similar to plant defensins.

To determine the identity of purified protein with strong antifungal activity, the mass-spectrometry analysis was carried out. The protein of interest was treated with trypsin and the amounts of generated peptides analysed by Q-TOF I. In this analysis, we found one peptide of 19 amino acids in length, TEGFPTGSCDFHVAGR (Fig.2, *a*). The search for various databases in GenBank, using BLASTP electronic system (www.ncbi.nlm.nih.gov/blast/BLAST.cgi) revealed cDNA clones with high level of homology (Fig.2, *b*). The highest level of homology was observed with gymnosperm defensins: a corresponding peptide from GbD of *G. biloba* has 94% homology, while SPI1 of *P. abies* and PgD1 of *P. glauca* exhibit 87% homology. These results clearly indicate that a protein which was purified from *Pinus sylvestris* seedlings and later shown to possess antifungal activity belongs to the family of plant defensins. Therefore, we named it *Pinus sylvestris* Defensin 1 (PsDef 1).

Since nucleotide sequences of defensins from *P. abies* and *P. glauca* are only 252 bp long, it could be possible to identify EST clones from *Pinus sylvestris* cDNA libraries, which might encode the full length coding sequence of PsDef 1, using the data of the mass-spectrometry analysis. Having employed TBLASTN software to search various databases, we identified 10 clones in cDNA libraries from *P. pinaster* and 23 clones from *P. taeda* which possessed protein sequences with very high level of homology to the peptide identified by mass-spectrometry. It is noteworthy that the level of homology between their coding regions was 95%, while the N- and C-terminal fragments were completely identical. These features were taken into account when we designed the primers for molecular cloning of a cDNA clone for PsDef 1.

For molecular cloning of *P. sylvestris* defensin 1, we used a cDNA library created in our laboratory from the 7-day-old root seedlings [19]. Primary and amplified libraries were used as templates during PCR amplification with a set of primers, specific to *P. sylvestris* defensin 1. Electrophoretic analysis of PCR products revealed the presence of a major band with an expected size of 270 bp (Fig.3, *a*). The product of amplification

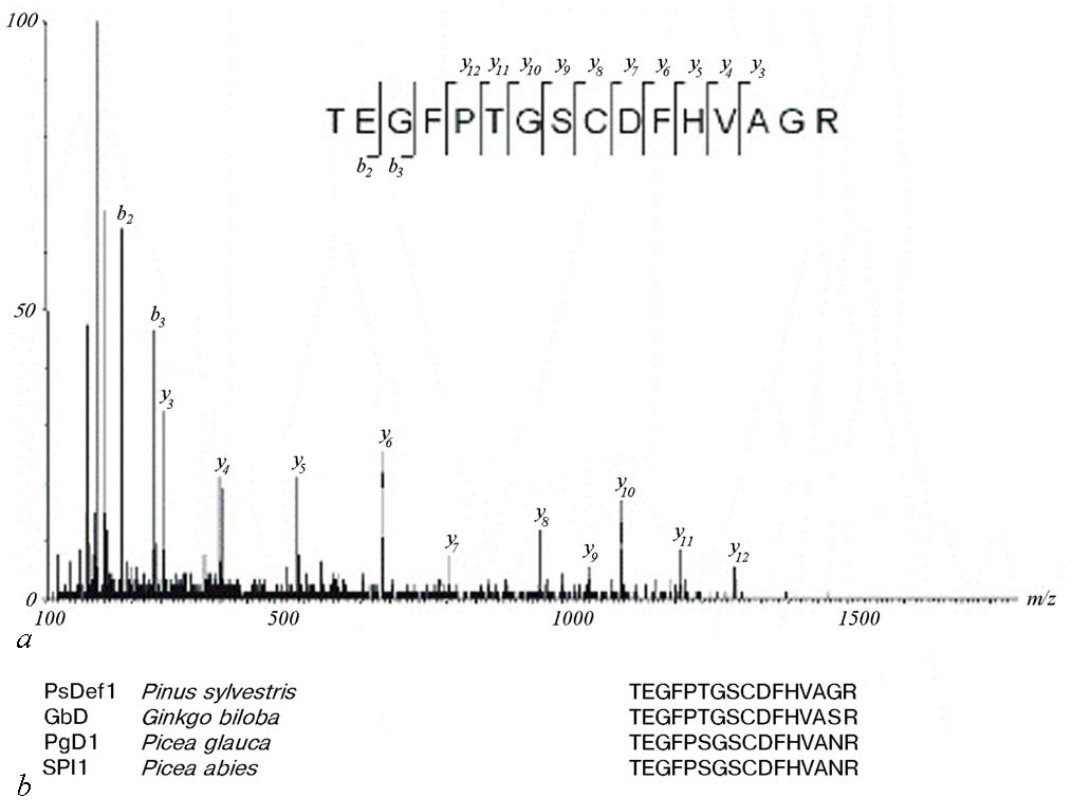

Fig.2 The identification of antifungal protein from *Pinus sylvestris* by mass-spectrometry: (*a*) the sequence of generated tryptic peptide – TEGFPTGSCDFHVAGR; (*b*) and sequence alignment of the tryptic peptide with corresponding peptides from gymnosperm defensins.

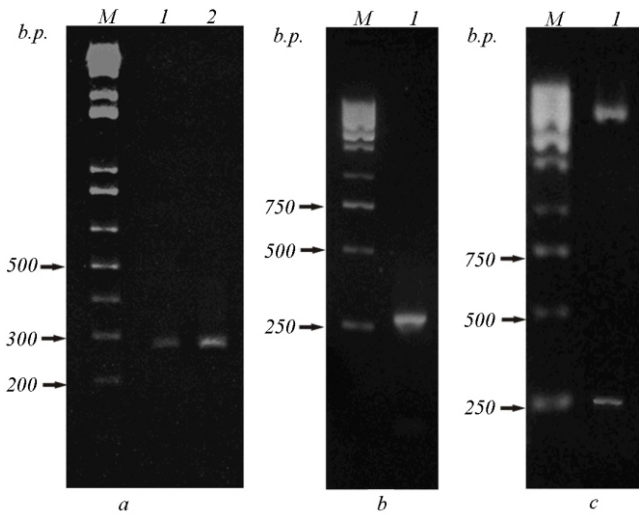

Fig.3 Cloning of *Pinus sylvestris* defensin 1 cDNA: (*a*) the products of PCR amplification from primary (*1*) and amplified cDNA library (*2*); *M* – 1 kb Plus DNA Ladder *GibcoBRL*; *b* – the analysis of *pET23d*-PsDef plasmid by PCR (*1*); *M* – 1 kb DNA Ladder *Fermentas*; *c* – restriction analysis of *pET23d*-PsDef1 plasmid using endonucleases *EcoRI* and *NcoI* (*1*); M – 1 kb DNA Ladder *Fermentas*.

was cloned into *pET 23d*(+) vector which was then transformed into XL-1 Blue competent cells. The presence of the cDNA insert in the resulting plasmid was confirmed by both PCR (Fig.3, *b*) and restriction analysis (Fig.3, *c*). DNA sequencing revealed the insert of 252 bp in length (Fig.4, *a*), which we deposited in GenBank, No.EF455616. Bioinformatic analysis showed that deducted amino acid sequence contains a peptide which is identical to that identified by mass-spectrometry. Notably, cDNA PsDef1 shows

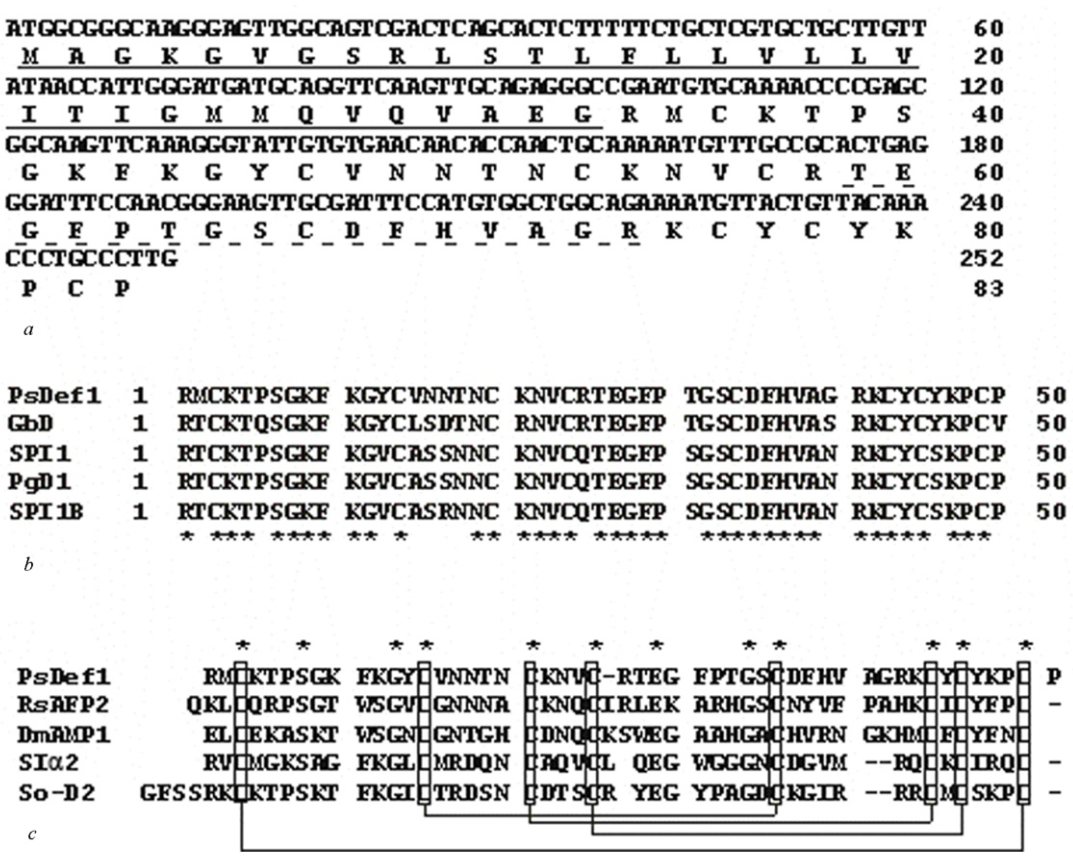

Fig.4 Nucleotide and deducted amino acid sequences of PsDef1: (a) signal peptide is underlined; the position of the tryptic peptided identified by mass-spectrometry is indicated by a dashed line; protein sequence alignment of PsFef1 with amino acid sequences of gymnosperm defensins; (*b*) and the representatives of different groups of plant defensins (*c*) the sequences of signal peptides were eliminated; the vertical line is made for maximal alignment of sequences; stars indicate identical amino acids; conservative cysteins are boxed; joining lines correspond to disulphide bridges. Abbreviations used: *Psdef1* – defensin 1 *Pinus sylvestris* (accession no.EF455616); *GbD* – defensin precursor *Ginkgo biloba* (accession no.AY695796); *SPI1* – putative gamma-thionin protein *Picea abies* (accession no.X91487); *PgD1* – defensin *Picea glauca* (accession no.AY494051); *SPI1B* – putative plant defensin *Picea abies* (accession no.AF548021); *RsAFP2* – antifungal protein 2 *Raphanus sativus* (accession no.U18556); *DmAMP1* – defensin *Dahlia merckii* (accession no.AAB34972); *SI* – inhibitor of insect -amylases 2 *Sorghum bicolour* (accession no.P21924); *So-D2* – antimicrobial peptide D2 *Spinacia oleracea* (accession no.P81571)

84% identity with nucleotide sequences of defensins from *Pinacea* family and 77% identity with *G. biloba* from *Ginkgoaceae* family.

Analysis of deducted amino acid sequence of *Pinus sylvestris* defensin 1 (83aa) using SignalP software (www.cbs.dtu.dk/services/SignalP) revealed the presence of the N-terminal signal peptide of 33aa, which is a specific feature of plant defensins. In addition, all secretory proteins are known to possess the signal peptide. The mature form of PsDef1 is 50aa in length, which is the same for other gymnosperm defensins (Fig.4, *b*). Calculated molecular weight of PsDef1 is 5601.6 Da, which is in good correlation with the results of SDS-PAGE analysis (Fig.1, *b*). The value of isoelectric point of 8.9 was obtained on the basis of amino acid sequence, using pI/Mw software [21].

The search in amino acid sequence of PsDef1 for known domains using Motif Scan software (scansite.mit.edu/motifscan) revealed that sequences between amino acid residues 35–85 form the structure, known as gamma-thionin, which provides the primary name of plant defensins – -thionins. This domain consists of an -helix and three anti-parallel -sheets, connected by four disulphide bridges [2]. The specific fea-

ture of this domain is the fact that only 23% of amino acid residues are conservative, including all cysteins (positions 4, 15, 21, 25, 36, 45, 47, and 51), two glycines (13 and 34), serine (8), an aromatic residue in position 11 and glutamic acid in position 29 (starting point is RsAFP2 – the most studied plant defensin) (Fig.4, *c*).

Detailed analysis of the primary structure of defensin 1 and known *Picea* defensins allowed us to reveal 81% identity between these proteins. The high level of homology points out possible similarity in their biological activity. The overexpression of SPI1 in embryonic cells of *P. abies L.* is known to increase the resistance of these cells to infection by *Heterobasidion annosum* [22]. Recently, we have demonstrated high antifungal activity of endogenous PsDef1 towards phytopathogenic fungi *Fusarium*, *Alternaria*, and *Botrytis in vitro* [14, 23].

Comparative analysis of PsDef1 amino acid sequences with that of different groups of defensins, namely RsAFP2 (group I), DmAMP1 (II), SI 2 (III), and So-D2 (IV) revealed high level of homology with RsAFP2 (58%) and So-D2 (59%). Literature data testify in favour of high antifungal activity of these plant defensins, the IC_{50} value (protein concentration, which shows 50% inhibition of fungal growth) in regards of *Fusarium* family is lower than 1 M [7, 23]. The IC_{50} for endogenous PsDef1 in the same experimental set up was found to be 0.7 M [14, 24]. One specific feature of these defensins is a high positive charge at pH 7.0: PsDef1 has the charge of +6.7 and similar values are found for RsAFP2 (group I) – +5.8 and So-D2 (IV) – +7.6. It has been previously demonstrated that antifungal activity of AMP is determined by the value of positive charge of molecule [24].

We have previously shown that endogenous PsDef1 causes morphological changes in fungi mycelium, which provided the ground for its assignment to morphogenic group I of plant defensins [14]. Experiments with site-directed mutagenesis revealed the biologic activity of each group of defensins to be determined by short conservative sequences, which are specific for each group only, or might be defined by a single amino acid. Comparative analysis of primary amino acid sequences between PsDef1 and RsAFP2 allowed us to detect a highly homologous region (2–10aa), a hydrophobic region (38–41 a.r.), which are important determinants of their antifungal activities, possibly functioning as binding sites for specific receptors on fungal membranes. The substitution of Tyr38 for Gly in RsAFP2 resulted in the change of protein conformation and the loss of its activity. Notably, this residue is highly conserved in plant defensins, which belong to groups II and III. In PsDef1 this position is occupied by homologous aromatic amino acid phenylalanine. Another conserved amino acid in both groups of defensins is Lys44 and its substitution for a neutral residue decreases antifungal activity of RsAFP2 significantly [25]. Taking into account that PsDef1 and RsAFP-2 exhibit significant structural similarities and belong to the same family of plant defensins, one might expect the similarity of molecular mechanisms coordinating their antifungal activities.

Therefore, in this study we present molecular cloning of *Pinus sylvestris* defensin 1 for the first time. The findings presented open for us the opportunities for the following: i) producing recombinant PsDef1 and investigating its biochemical, structural, and functional properties; ii) generating specific antibodies against PsDef1, which would be particularly useful to study its expression in *Pinus sylvestris* tissues at various conditions; iii) elucidating antifungal action of plant defensins.

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Клонирование и анализ кДНК дефензина 1 сосны обыкновенной

Резюме

Антифунгальный белок с молекулярной массой 5,6 кДа очищен из корней семидневных проростков сосны обыкновенной. Масс-спектрометрическим анализом показана його принадлежность к растительным дефензинам. кДНК дефензина 1 сосны (PsDef1) длиной 252 п. н. получена методом ПЦР-амплификации из кДНК библиотеки Pinus sylvestris L. и клонирована в вектор pET23d(+). PsDef1 кДНК кодирует белок из 83 аминокислотных остатков (а. о.) с N-концевым сигнальным пептидом из 33 а. о. Для зрелой формы характерно наличие специфических консервативных остатков, свойственных всем растительным дефензинам. Показано структурное и функциональное сходство между PsDef1 и дефензинами группы I.

Ключевые слова: сосна обыкновенная, дефензин PsDef1, молекулярное клонирование.

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UDC 577.112.083
Received 02.07.07