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EXPRESSION PATTERNS OF SCOTS PINE DEFENSIN GENES UNDER ENVIRONMENTAL STRESSES

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Background. Scots pine (*Pinus sylvestris* L.) is a highly adaptable forest species, yet it faces increasing threats from climate-induced droughts despite its natural stress tolerance. To cope with environmental stressors, plants deploy defense mechanisms, such as antimicrobial peptides (AMPs). However, the role of defensins, a key AMP class, in pine stress responses remains insufficiently explored.

Materials and Methods. To investigate the responsiveness of *PsDef1–4* genes to environmental stimuli, Scots pine seedlings were exposed to biotic stress (phytopathogenic fungi *Fusarium verticillioides* F3 and *Ophiostoma clavatum* B0922, and a beneficial endophytic bacterium *Pseudomonas putida* P57) and various abiotic stressors, including acidification, cold, heat, drought, salinity, flooding, and heavy metals. Gene expression was analyzed via quantitative RT-PCR. Orthologs of *PsDef1–4* were identified in the genome assemblies of *Pinus tabuliformis* and *Pinus taeda*. Promoter regions (2 kb upstream) were analyzed for *cis*-acting regulatory elements using the PlantCARE database.

Results and Discussion. *In silico* analysis of the promoter regions of *PtbDef1–4* and *PtaDef1,2,4* (orthologs of *PsDef1–4*) revealed a high proportion (44–57 %) of *cis*-acting regulatory elements associated with stress responsiveness, suggesting their involvement in plant protection. *In vivo* gene expression analysis showed that both pathogenic fungi and the endophytic bacterium induced upregulation of *PsDef1–4* at 48 h post-inoculation. The responses to abiotic stress varied: drought and flooding increased expression of all four defensin genes, while zinc treatment and cold stress caused strong downregulation. Expression responses to heat, salinity, acidification, and cadmium were gene-specific.

Conclusion. Overall, Scots pine defensin genes are responsive not only to pathogenic and beneficial microbes but also to a wide range of abiotic stresses, indicating their broader role in adaptive responses to environmental challenges. These findings highlight defensins as promising candidate genes for breeding programs aimed at developing climate-resilient pine genotypes.

Keywords: *Pinus sylvestris* L., defensin gene expression, *cis*-acting regulatory elements, biotic and abiotic stress

INTRODUCTION

Scots pine (*Pinus sylvestris* L.) is native to Eurasia, an introduced species in North America, valued for its high adaptability, ability to grow on nutrient-poor soils, and tolerance to both frost and drought (Brichta *et al*., 2023). However, prolonged droughts driven by global climate change threaten many tree species, potentially exceeding their adaptive capacities and causing forest dieback through xylem cavitation, carbon starvation, and increased susceptibility to pathogens and insects (Hartmann *et al*., 2022; Brichta *et al*., 2024). Since 2010, drought-induced stress combined with bark beetle outbreaks has caused widespread mortality of *P. sylvestris* in Ukraine, affecting thousands of hectares (Davydenko *et al*., 2021).

To withstand environmental challenges, plants have evolved diverse protective and adaptive mechanisms against both biotic and abiotic stresses. One such strategy is the induction of specific defense-related genes, particularly those encoding antimicrobial peptides (AMPs) (Ruszczyńska & Sytykiewicz, 2024).

Plant defensins, one of the major AMP classes, are found across both the plant and animal kingdoms, and play a central role in innate immunity (Stotz *et al*., 2009). They share a conserved cysteine-stabilized αβ (CSαβ) fold, which confers stability under extreme temperatures and pH. Despite this structural conservation, plant defensins exhibit high amino acid sequence variability, which likely underlies their functional diversity.

Genome-wide analyses reveal that defensin-like (DEFL) genes form highly polymorphic multigene families (Silverstein *et al*., 2005). While mainly linked to immunity, their extensive diversification and broad distribution suggest additional, yet uncharacterized, roles in plant physiology. Transcriptomic approaches, particularly RNA-seq, enable the study of such large and complex families. Comparative analyses across tissues, developmental stages, and stress conditions have revealed functional specialization within DEFL families and linked their expression to responses to both biotic and abiotic stressors (Domingo *et al*., 2024). These findings support breeding strategies for multi-stress-resistant crops.

In conifers, and especially in the Scots pine, the role of defensins in stress responses remains poorly understood. Key obstacles include the absence of a complete 24 Gb genome assembly, high repeat content that complicates annotation, and limited RNA-seq resources. Consequently, only a few studies have examined defensin expression in *Pinaceae* under biotic stress (Pervieux *et al*., 2004; Germain *et al*., 2012; Jaber *et al*., 2014; Chano *et al*., 2023).

Previously, we cloned four Scots pine defensin genes (*PsDef1–4*), expressed across multiple tissues and developmental stages of pine, along with two seed-specific defensins (Shalovylo *et al*., 2021). Recombinant *Ps*Def1, *Ps*Def2, and *Ps*Def5.1 showed

antimicrobial activity and inhibited insect amylases. Expression of *PsDef1–4* increased in pine seedlings under biotic stress from damping-off fungi. However, their involvement in abiotic stress remains unexplored.

The present study aims to investigate *PsDef1–4* expression under diverse biotic and abiotic stresses using qPCR to clarify their role in conifer defense. We also identify orthologs in other pine genomes and analyze *cis*-regulatory elements to reveal potential regulatory mechanisms and broader functional roles of pine defensins.

MATERIALS AND METHODS

Plant material and stress treatments. Scots pine seeds were obtained from the Lviv Forestry Breeding Seed Center (Lviv region, Ukraine). Before sowing, seeds were sterilized in 70% ethanol for 1–2 min, rinsed with sterile water, placed on moist sterile filter paper in Petri dishes, and germinated in the dark at 26 °C for 7 days.

Fungal cultures *of Fusarium verticillioides* F3 and *Ophiostoma clavatum* B0922, and the bacterium *Pseudomonas putida* P57 were from the collection of the Ukrainian National Forestry University. For inoculations, fungal cultures were grown in malt extract broth for 10 days at 22 °C, washed with sterile water, and homogenized using an Ultra-Turrax T-25 homogenizer. *P. putida* P57 was grown in LB broth on a shaker for 48 h at 30 °C, harvested by centrifugation (3000 × g, 15 min at room temperature), washed in sterile 0.9% NaCl, and resuspended in water to 10^7 CFU/mL. These suspensions were used to treat 7-day-old seedlings.

For biotic stress assays, twenty seedlings were placed on moist, sterile filter paper in Petri dishes with roots toward the center, treated with 3 mL fungal homogenate or bacterial suspension, and covered with another moist sterile filter paper layer. Control seedlings received 3 mL of sterile water. Plants were incubated at 26 °C in the dark. Seedlings from both groups were collected at 6, 24, and 48 h post-inoculation (hpi), immediately frozen in liquid nitrogen, and stored at -86 °C.

For abiotic stress assays, seven-day-old seedlings (twenty per treatment) were exposed for 24 h to heavy metals (2.5 mM Zn^{2+}; 4.4 μM Cd^{2+}), salinity (250 mM NaCl), acidity (10 mM HCl), drought (100 mM mannitol), cold (+4 °C), heat (+37 °C), or flooding. Untreated seedlings maintained at 26 °C served as controls. Samples were stored at -86 °C until RNA extraction.

Identification of *Ps*Def1–4 orthologs in pine genomes. Protein sequences of Scots pine defensins *Ps*Def1 (GenBank: KX690107.1), *Ps*Def2 (EF455617.1), *Ps*Def3 (JN980401.1), and *Ps*Def4 (OR050664.1) were used as queries in BLAST searches (version 2.9.0+) against the NCBI Whole Genome Shotgun (WGS) contigs database, restricted to *Pinus tabuliformis* (Chinese red pine; PRJNA784915) and *P. taeda* (loblolly pine; PRJNA207631) genome projects. Searches applied an E-value cutoff of 1e−50 and query coverage >80 %, identifying sequences similar or identical to *Ps*Def1–4. Signal peptides were predicted with SignalP5.1 (https://www.cbs.dtu.dk/services/SignalP) using “Eukarya” settings. Chromosomal positions of *Pinus tabuliformis* defensin orthologs were visualized with the PhenoGram Plot tool (https://visualization.ritchielab.org/phenograms/plot).

Analyses of promoter regions. Putative *cis*-acting regulatory elements (CAREs) were identified in 2 kb upstream sequences of each orthologous gene. Sequences were manually extracted from *P. tabuliformis* and *P. taeda* genome assemblies and analyzed

with PlantCARE (https://bioinformatics.psb.ugent.be/webtools/plantcare/html). CAREs were visualized with PyGenomeTracks (https://github.com/deeptools/pyGenomeTracks).

Quantitative RT-PCR analyses. Total RNA was extracted from frozen Scots pine seedlings (100 mg FW; 3 biological replicates) using Quick-RNA Kits (Zymo Research, USA) following the manufacturer's instructions. RNA concentration and purity were measured with a Nanodrop™ spectrophotometer (Fisher Scientific), and integrity was verified by the presence of 28 S and 18 S bands on a 1% agarose gel. cDNA was synthesized from 1 µg of total RNA using RevertAid Premium (Thermo Fisher Scientific, USA). The cDNA was diluted to 5 ng/µL and amplified with gene-specific primers designed using the primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast (**Table 1**), and Luna® Universal qPCR Master Mix (New England Biolabs, Ipswich, MA, USA). *P. sylvestris* GAPDH gene (Šķipars *et al*., 2011), and 60S ribosomal protein L44 gene served as internal controls for relative expression of *PsDef1–4* genes.

Table 1. **Primers used in this study**

Gene	Primer	Sequence	Reference
PsDef1	RTDef1F	GGTTCAAGTTGCAGAGGGC	this study
	RTDef1R	CGTTGGAAATCCCTCAGTG	
PsDef2	RTDef2F	GAATGTGCAAAACCCCAAGT	this study
	RTDef2R	GTAGCACTTTCGGCTGGTGAT	
PsDef3	RTDef3F	GATGATGGCGGTTCAAGTG	this study
	RTDef3R	GGAAATCGCAGCTTCCCGA	
PsDef4	RTDef4F	TCAAGTTGCAGAGGGTCGT	this study
	RTDef4R	CCGTTGGAAACCCTTCAGTA	
RPL44	RPL44F	TGCTCAAGGGAAACGACGG	this study
	RPL44R	TTCCCCTTCTTGTCTCCACC	
GAPDH	GAPDH-F	ACGGTTTTGGTCGAATTGGA	Šķipars *et al*., 2011
	GAPDH-R	CCCCACGAGCTCGATATCAT	

RT-qPCR was performed on the Tianlong Gentier 96R (Tianlong Science & Technology, China) under the following conditions: 95 °C for 1 min; 40 cycles of 95 °C for 20 s, 60 °C for 1 min. Primer specificity was confirmed by melting curve analysis (50–95 °C, 0.5 °C increments at 2–3 s/step). Relative expression was calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001) and expressed as fold change compared with controls (Giménez *et al*., 2011).

Statistical analysis. Three biological and three technical replicates per treatment were analyzed. Data analysis was performed using Microsoft Excel 2016 and Python 3.12.6. A one-way analysis of variance (ANOVA) was employed to assess statistical differences between means with statistical significance set at $P \leq 0.05$.

RESULTS AND DISCUSSION

Understanding the genetic basis of plant stress tolerance is vital for developing resilient genotypes. Among defense-related genes, defensins are key components of plant immunity, conferring resistance to fungal pathogens and enhancing stress tolerance. While well studied in herbaceous plants, this gene family is poorly characterized in conifers.

This study examined four Scots pine defensin genes (*PsDef1–PsDef4*), previously cloned in our laboratory, to evaluate their roles under biotic and abiotic stresses. Gene expression regulation, particularly under stress, is largely governed by promoter regions containing *cis*-regulatory elements crucial for gene activation. However, the absence of a complete *P. sylvestris* genome assembly precludes access to promoter sequences for these genes. To address this, we analyzed the genomes of two related species with public assemblies: a contig-level genome of *P. taeda* and a chromosome-level assembly of *P. tabuliformis*.

Identification and comparative analysis of *PsDef1–4* orthologs in pine genome assemblies. Using *PsDef1–4* coding sequences as BLAST queries, we identified orthologous defensin genes in *P. tabuliformis* and *P. taeda*. Based on sequence homology, these genes were designated *PtbDef1–4* (*P. tabuliformis*) and *PtaDef1,2,* and *4* (*P. taeda*). No ortholog of *PsDef3* was found in the *P. taeda* genome, likely due to incomplete assembly or annotation.

All identified genes encode 83-amino acid proteins with a 33-residue signal peptide and a 50-residue mature defensin domain. The mature domains are highly conserved: *PsDef2–4* are identical across three species, while *Ps*Def1 shares 96% identity with *Pta*Def1 and 94% with *Ptb*Def1 (**Fig. 1**).

Gene structure analysis showed that all *PsDef* genes and their orthologs consist of two exons separated by a single intron. *PsDef1* (KX690107.1) and its orthologs contain an 84 bp intron. *PsDef4* (OR050664.1) and *PtaDef4* have 132 bp introns, while *PtbDef4* has 131 bp. Although intron sequences for *PsDef2* and *PsDef3* are unavailable in *P. sylvestris*, their orthologs in *P. tabuliformis* and *P. taeda* possess introns of 366/426 bp and 356 bp, respectively.

Protein	Sequence	Length (aa)	Intron length (bp)
***Ps*Def1**	magkgvgsrlsalfllvllvitigmmqvqvaegRMCKTPSGKFKGYCVNNTNCKNVCRTEGFPTGSCDFHVAGRKCYCYKPCP	83	84
***Pta*Def1**	magkgvgsrlsalfllvllvitigmvqvqeaegRTCKTPSGKFKGYCVNSTNCKNVCRTEGFPTGSCDFHVAGRKCYCYKPCP	83	84
***Ptb*Def1**	magkgvgsrlsalfllvllvitigmmqvqvaegRECKTPSGKFKGYCVNTTNCKNVCRTEGFPTGSCDFHVAGRKCFCYKPCP	83	84
***Ps*Def2**	magkgvgtplsalfllvllvitigmmevqvaegRMCKTPSGKFKGYCVSSTNCKNVCRTEGFPTGSCDFHITSRKCYCYKPCP	83	Nd
***Pta*Def2**	magkgvgtrlsalfllvllvitigmtevqvaegRMCKTPSGKFKGYCVSSTNCKNVCRTEGFPTGSCDFHITSRKCYCYKPCP	83	366
***Ptb*Def2**	magkgvgtrlsalfllvllvitlgmmevqvaegRMCKTPSGKFKGYCVSSTNCKNVCRTEGFPSGSCDFHITSRKCYCYKPCP	83	426
***Ps*Def3**	magkgvgsplnaifllvllvitigmmavqvaegRMCKTPSGKFKGYCVSSNTCKNVCRTEGFPSGSCDFHVTSRKCYCYKPCP	83	Nd
***Ptb*Def3**	magkgvgtplsalfllvllvitigmmavqvaegRMCKTPSGKFKGYCVSSTNCKNVCRTEGFPSGSCDFHVTSRKCYCYKPCP	83	356
***Ps*Def4**	magkgvgtrlsalflvvllvvsigmmevqvaegRMCKTPSGKFKGYCVSSTNCKNVCRTEGFPTGSCDFHVASRKCYCYKPCP	83	132
***Pta*Def4**	magkgvgtrlsalflvvllvlsigmmevqvaegRMCKTPSGKFKGYCVSSTNCKNVCRTEGFPTGSCDFHVASRKCYCYKPCP	83	132
***Ptb*Def4**	magkgvgtrlsalflvvllvvsigmmevqvaegRMCKTPSGKFKGYCVSSTNCKNVCRTEGFPTGSCDFHVASRKCYCYKPCP	83	131

Fig. 1. Sequence alignment of defensins from *Pinus sylvestris* (*Ps*Def1-4), *P. taeda* (*Pta*Def1,2,4), and *P. tabuliformis Ptb*Def1-4. Differences in the amino acid sequences between orthologs are highlighted in color

Interestingly, *PtbDef1–4* are co-localized on chromosome 7 of *P. tabuliformis*, in close physical proximity (**Fig. 2**). This arrangement suggests that they may have originated

through segmental duplication, as reported for other multigene families (Cannon *et al*., 2004). Such clustering may reflect a shared regulation or functional linkage.

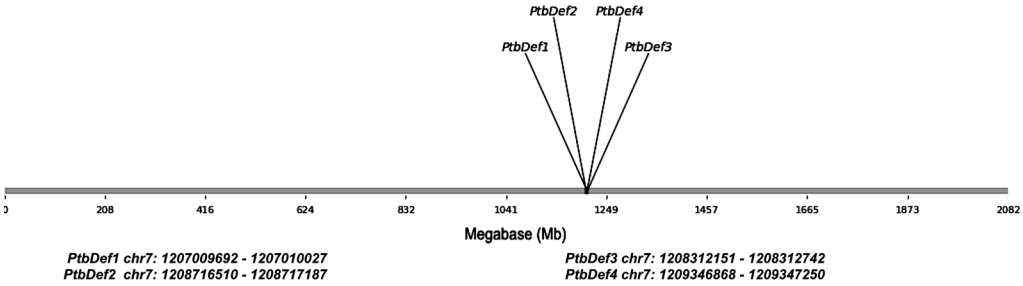

Fig. 2. Locations of the four *PtbDef* genes on *P. tabuliformis* chromosome 7. The scale at the bottom represents the length of the chromosome. Mb = million base pairs

***Cis*-regulatory element analyses.** CAREs are essential for gene regulation, particularly in plant responses to environmental stresses (Shariatipour & Heidari, 2020). Identifying them is essential for elucidating regulatory networks across biological levels. *In silico* analysis of 2-kb upstream regions of *PtbDef1–4* and *PtaDef1,2,4* revealed that orthologous genes have highly similar CARE profiles (**Fig. 3**). Functional annotation of these elements was performed using the NEW PLACE database (**Table 2**).

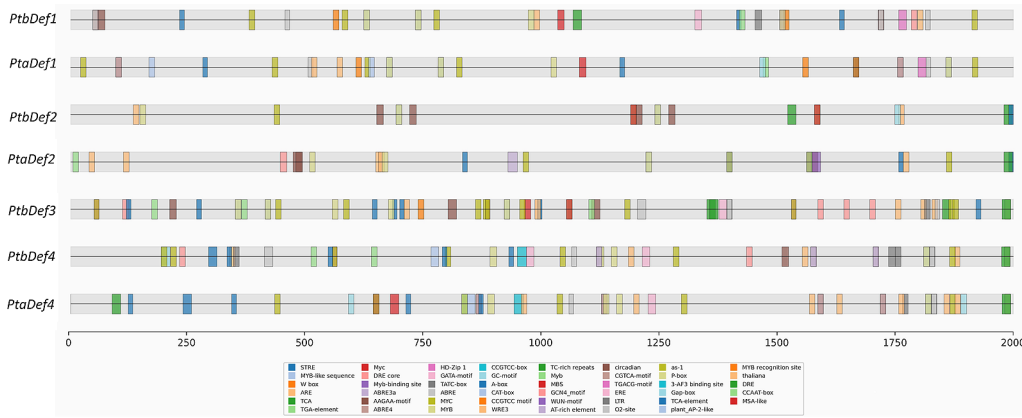

Fig. 3. Distribution of frequently occurring CAREs in four *PtbDefs* and three *PtaDefs*. Different colors represent different types of motifs. The bottom box shows the names of the motifs. The scale at the bottom indicates the length of the promoter region

Table 2. ***Cis*-acting regulatory elements in promotors of *PtbDef1–4* genes**

Motif	Function	*PtbDef1*	*PtbDef2*	*PtbDef3*	*PtbDef4*
1	2	3	4	5	6
3-AF1 binding site	light responsive element	0	0	0	1
AAGAA-motif	involved in secondary xylem development	1	4	1	1
ABRE	*cis*-acting element involved in the abscisic acid responsiveness	4	0	1	6

Continued Table 2

1	2	3	4	5	6
ABRE3a	*cis*-acting element involved in the abscisic acid responsiveness	2	0	1	3
ABRE4	*cis*-acting element involved in the abscisic acid responsiveness	2	0	1	3
ACE	*cis*-acting element involved in light responsiveness	1	0	0	0
AE-box	part of a module for light response	1	0	0	0
ARE	*cis*-acting regulatory element essential for the anaerobic induction	2	1	4	1
as-1	SA and Auxin responsive element	0	0	1	1
AT~TATA-box	promoter binding element	0	0	33	10
AT-rich element	binding site of AT-rich DNA binding protein (ATBP-1)	0	0	1	0
Box 4	part of a conserved DNA module involved in light responsiveness	0	1	4	4
CAAT-box	common *cis*-acting element in promoter and enhancer regions	35	37	40	35
CGTCA-motif	*cis*-acting regulatory element involved in the MeJA-responsiveness	0	0	1	1
dOCT	development related element	0	0	1	0
DRE core	dehydration responsive element	1	0	0	2
ERE	*cis*-acting regulatory element involved in ethylene responsiveness	0	0	1	2
GA-motif	part of a light responsive element	1	0	0	0
Gap-box	part of a light responsive element	0	0	0	1
GATA-motif	part of a light responsive element	1	0	0	0
G-box	*cis*-acting regulatory element involved in light responsiveness	7	1	2	9
GC-motif	enhancer-like element involved in anoxic specific inducibility	0	1	0	0
GT1-motif	light responsive element	0	1	0	1
GTGGC-motif	light responsive element	1	0	0	0
HD-Zip 1	element involved in differentiation of the palisade mesophyll cells	1	0	0	0
I-box	part of a light responsive element	1	4	1	1
LAMP-element	part of a light responsive element	1	0	0	1
LTR	*cis*-acting element involved in low-temperature responsiveness	0	0	0	2
MBS	MYB binding site involved in drought-inducibility	0	2	0	0

End of the Table 2

1	2	3	4	5	6
Myc	light, abscisic acid (ABA), and jasmonic acid (JA) signaling pathways	1	0	0	0
Myb	drought responsive element	0	2	0	1
MYB	water response, drought response	4	3	1	4
Myb-binding site	cell cycle and cell proliferation response	1	0	0	0
MYB-like sequence	involved cell cycle regulation	2	2	0	2
O2-site	*cis*-acting regulatory element involved in zein metabolism regulation	0	0	0	1
MYC	dehydration responsive element	4	1	3	6
P-box	gibberellin-responsive element	0	0	0	1
plant_AP-2-like	involved in endosperm expression	0	0	0	1
Sp1	light responsive element	1	0	0	0
STRE	stress responsive element	3	1	2	4
TATA	core promoter element around -30 of transcription start	1	0	1	3
TATA-box	core promoter element around -30 of transcription start	24	55	109	64
TATC-box	*cis*-acting element involved in gibberellin-responsiveness	1	0	0	1
TCA	salicylic acid-responsive element	1	0	1	0
TCA-element	*cis*-acting element involved in salicylic acid responsiveness	0	0	1	1
TC-rich repeats	*cis*-acting element involved in defense and stress responsiveness	0	2	0	1
TCT-motif	part of a light responsive element	1	1	0	0
TGACG-motif	*cis*-acting regulatory element involved in the MeJA-responsiveness	0	0	1	1
TGA-element	auxin-responsive element	1	0	2	1
Unnamed__1	60K protein binding site	2	0	0	6
Unnamed__10	Function unknown	0	0	0	1
Unnamed__12	Function unknown	0	0	0	1
Unnamed__14	Function unknown	0	0	0	1
Unnamed__2	Function unknown	0	1	0	0
Unnamed__4	Function unknown	0	7	9	10
Unnamed__8	Function unknown	0	0	0	1
W box	Fungal elicitor responsive element	2	0	0	0
WRE3	wound-responsive element	0	1	1	3

Given the close phylogenetic relationship between *P. tabuliformis* and *P. sylvestris,* and the more distant link to *P. taeda* (Xia *et al*., 2023), we have characterized the *PtbDef1–4* promoters to extrapolate findings to *PsDef1–4.*

We identified 31 CARE types in *PtbDef1*, and 20, 26, and 42 in *PtbDef2–4*, respectively (**Table 2**), which were grouped into seven functional categories (**Fig. 4**): light responsiveness, growth and development, abiotic stress, biotic stress, hormonal regulation, core promoter elements, and unknown functions.

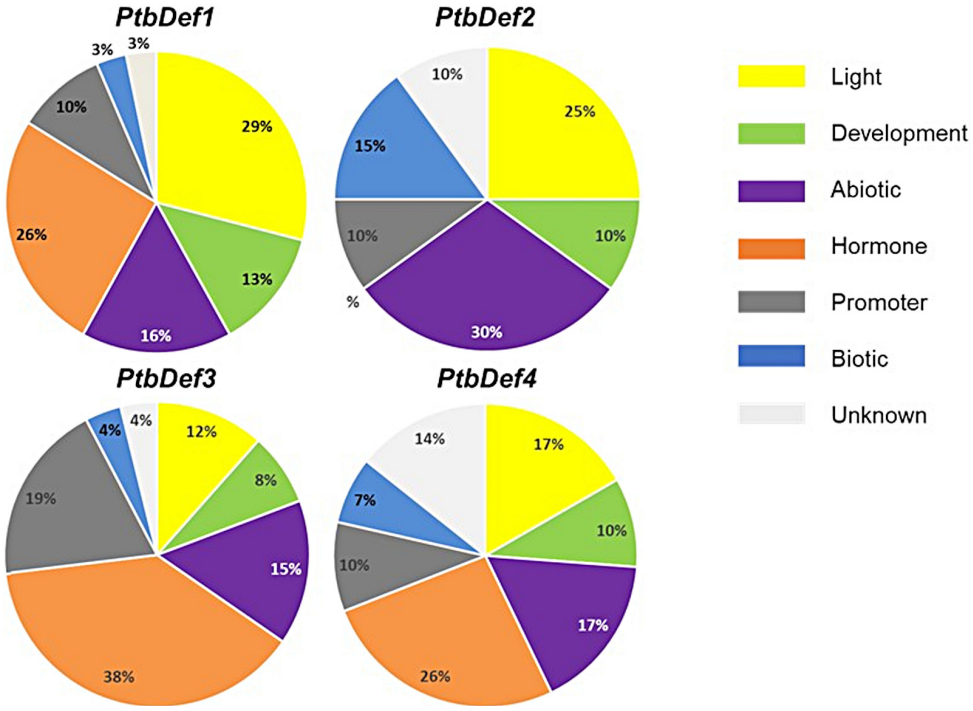

Fig. 4 Functional classificatin of predicted *cis*-elements in *PtbDef1-4* promoter regions. Different colors indicate the frequency of *cis*-elements for every functional category in percentages

CARE distribution varied among promoters. Light-responsive elements were most abundant in *PtbDef1* (29%) and least in *PtbDef3* (12%), whereas *PtbDef3* was enriched in core promoter elements (19%). Development-related CAREs were consistently ~13% across promoters.

Hormone-, abiotic-, and biotic stress-related CAREs, which are central to environmental stress signaling, showed notable variation. *PtbDef2* lacked hormone-responsive elements, whereas they accounted for 38% in *PtbDef3*. Abscisic acid (ABA)-responsive elements (ABRE, ABRE3a, ABRE4) were common in *PtbDef1* and *PtbDef4* (10 and 12, respectively), but only three in *PtbDef3.* Salicylic acid (SA), methyl jasmonate (MeJA), and ethylene (ET)-responsive elements, known to regulate plant defense (Verma *et al*., 2016), were also detected. MeJA motifs (CGTCA, TGACG) occurred in *PtbDef3*, and *PtbDef1* carried a Myc (TCTCTTA) element. SA-responsive elements (TCA-element, TCA, as-1) appeared in *PtbDef1* and *PtbDef3*; while *PtbDef3* and *PtbDef4* each contained an ET-responsive element (ERE).

These patterns are consistent with prior findings indicating that conifer defensins are up-regulated by jasmonic acid treatment (Pervieux *et al*., 2004), through either direct regulation via JA-responsive CAREs (in *PtbDef1,3,4*) or indirect activation by transcription factors (TFs) such as MYC2. In *Arabidopsis*, MYC2 binds G-box motifs to regulate JA- and SA-responsive genes (Du *et al*., 2017) and to enhance pathogen resistance (Gautam *et al*., 2021).

ABA, a key abiotic stress signal, induces gene expression through ABRE-binding proteins (AREBs) under drought or salinity (Tuteja, 2007; Rai *et al*., 2024). Abiotic stress-related motifs (11 types) occurred in all promoters, with *PtbDef2* having the highest proportion (30%). Drought-responsive elements (DRE, MBS, MYB, MYB recognition site, MYC) dominated in *PtbDef1,2,4*, whereas *PtbDef3* was enriched in anaerobic induction motifs (ARE, GC-motif). Salt-responsive GT-1 elements were present in *PtbDef2* and *PtbDef4* (Park *et al*., 2004). These motifs serve as binding sites for TFs such as DREBs (dehydration responsive element binding proteins), NAC (N-acetylcarnosine), MYB (myeloblastosis)/MYC (myelocytomatosis), and WRKYs, which mediate ABA-independent stress responses. In *Arabidopsis*, PDF1.2 is regulated by DREB2A, ERF1, WRKY18/40, and ABF3 through DRE, GCC-box, W-box, MBS, and ABRE motifs (Ou *et al*., 2011).

All *PtbDef* promoters contained biotic stress-related CAREs: WRE3 (Wound Responsive Element 3) in *PtbDef2–4*, a W-box in *PtbDef1*, and TC-rich repeats in *PtbDef2,4*. These motifs are involved in responses to fungi, bacteria, insects, and viruses (Gao *et al*., 2014; Viswanath *et al*., 2023). W-box motifs with consensus sequence TTGAC(C/T) interact with WRKY TFs, mediating responses to *Pseudomonas syringae*, *Botrytis cinerea*, ABA, and abiotic stress (Chen *et al*., 2010; 2025). WRE3 also contains W-box sequences targeted by WRKYs such as AtWRKY6 and AtWRKY11. TC-rich repeats (TTCTC/TCTCT) are bound by WRKY and MYB TFs, including OsWRKY45 in rice, SIMYB1 in tomato (Huangfu *et al*., 2016; Yin *et al*., 2023).

Growth hormone-related CAREs were also present, including auxin-responsive motifs (TGA in *PtbDef1*, as-1 in *PtbDef3*) and gibberellic acid motifs (TATC-box in *PtbDef1,4*; P-box in *PtbDef4*). Developmental elements for meristem activity (CAT-box, CCGTCC-box), xylem development (AAGAA), and cell cycle control (HD-Zip1, MYB-binding sites) were also identified, indicating their potential roles in growth and tissue differentiation (**Table 2**).

Transcriptional response of *PsDef1–4* genes to environmental stress in Scots pine seedlings. To assess the responsiveness of *PsDef1–4* genes to environmental stimuli, Scots pine seedlings were exposed to biotic (phytopathogenic fungi and endophytic bacteria) and abiotic stresses, including acid exposure, cold, heat, drought, salinity, flooding, and heavy metals. Gene expression was measured by qRT-PCR (**Fig. 5**).

To assess transcriptional changes in response to pathogens, seedlings were inoculated with two fungi differing in lifestyle: *Ophiostoma clavatum* (necrotrophic, wood-infecting) and *Fusarium verticillioides* (hemibiotrophic seedling pathogen). Both induced *PsDef1–4* expression, peaking at 48 hpi, with *F. verticillioides* eliciting a faster, stronger response. At 6 hpi, *Fusarium* triggered early expression of all defensins, especially *PsDef3* (9-fold), while at 48 hpi, *PsDef1* and *PsDef2* showed the greatest induction (11- and 14-fold). *Ophiostoma* caused a moderate 3–5-fold increase only at 48 hpi.

This difference reflects pathogen–host interaction modes. Necrotrophs typically activate JA/ET-mediated defenses via CAREs such as CGTCA/TGACG, ERE, and Myc-elements, regulated by TGA, ERF, and MYC TFs. In contrast, SA-mediated pathways dominate defense against biotrophs, involving WRKY TFs binding TCA-elements (Glazebrook, 2005). WRKY proteins play central roles in coordinating defense responses (Phukan *et al*., 2016). WRKYs integrate SA- and JA/ET-signaling, binding W-box motifs and TC-rich repeats in defensin promoters. Some, like WRKY70, act as switches between SA and JA pathways to optimize energy expenditure (Proietti *et al*., 2018)

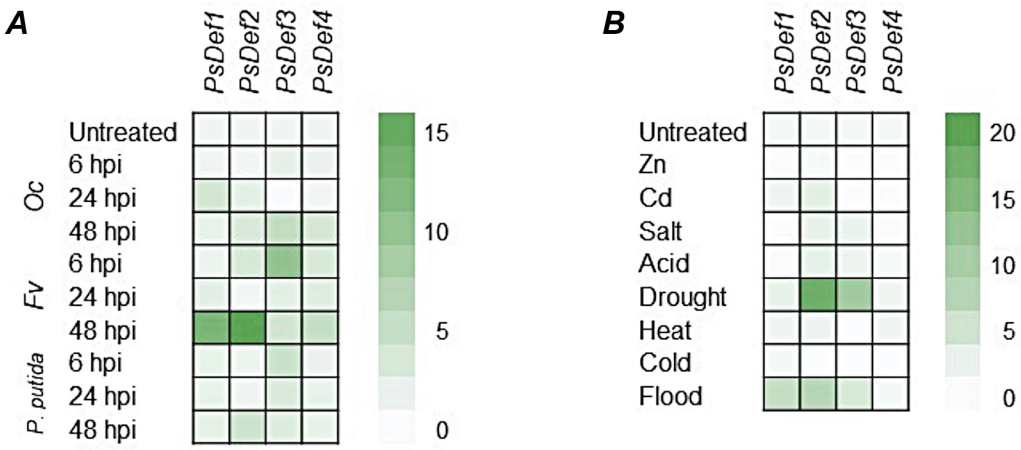

Fig. 5. Expression patterns of *PsDef1–4* genes. (**A**) Heatmap showing the expression levels of target genes in pine seedlings inoculated with *Ophiostoma clavatum* (Oc), *Fusarium verticillioides* (Fv), or *Pseudomonas putida* for 6, 24, and 48 h post-inoculation (hpi). (**B**) Heatmap of defensin gene expression profiles in seedlings subjected to heavy metals (2.5 mM Zn^{2+}; 4.4 μM Cd^{2+}), salinity (250 mM NaCl), acidity (10 mM HCl), drought (100 mM mannitol), cold (+4 °C), heat (+37 °C), or flooding for 24 h. The *GAPDH* and *60S RPL44* genes were used as internal controls to calculate relative expression levels. Relative expression values are presented as fold changes compared to control conditions. Three biological replicates per treatment were analyzed, each with three technical replicates

Promoters of *PtbDef2–4* contain WRE3-like motifs targeted by JA/ET-responsive TFs (MYC2, ERF1, WRKY33) and pathogen-responsive WRKYs (WRKY18, WRKY40), enabling signal integration. Stronger *PsDef* activation during *Fusarium* infection likely reflects a combined pathogen-associated molecular pattern (PAMP) recognition and toxin-induced JA/ET signaling, whereas *Ophiostoma* may fail to efficiently trigger these pathways.

Endophytic bacteria *P. putida* P57 caused a 2-4-fold upregulation within 6 h, sustained thereafter. Endophyte-induced systemic resistance (ISR) occurs via JA and ET signaling, activating defensin genes (Oukala *et al*., 2021). Similar effects have been observed in the model plant *Arabidopsis* (PDF1.2) and *Withania somnifera* upon inoculation with beneficial *Bacillus* and *Pseudomonas* strains (Conn *et al*., 2008; Mishra *et al*., 2018), enhancing pathogen resistance and seedling survival in field trials.

Although plant defensins are primarily known for antifungal defense, they also contribute to abiotic stress tolerance. Cold and zinc treatments strongly downregulated

all *PsDef* genes, whereas drought induced them markedly, especially *PsDef2* and *PsDef3* (18-fold and 11-fold). Promoters of the corresponding orthologs contain numerous drought-responsive elements, including ABRE, MBS, MYB, and MYC motifs.

Heat stress caused a modest increase in *PsDef1, PsDef2, and PsDef4,* and a reduction in *PsDef3*. The absence of classical heat shock elements (HSEs) in pine defensin promoters suggests an indirect activation via shared pathogen/oxidative stress pathways (WRKY, AREB, MYB/MYC). Notably, heat may also repress defensins through JA–SA antagonism – for example, PDF1.2 is inhibited at 37 °C via WRKY25 suppression of JA signaling (Gao *et al*., 2011).

Flooding leads to strong upregulation of *PsDef1–3*, likely via anaerobic response elements (AREs) that mediate low-oxygen adaptation (Sadeghnezhad *et al*., 2014).

Cold stress suppressed all *PsDef* genes. This repression likely results from reduced biochemical activity, impaired signaling, and a strategic shift towards conserving energy over producing antimicrobial peptides. However, some defensins (e.g., *PtDef*, *MsPDFs*) are transiently upregulated during early cold shock, possibly as part of an initial protective response (Guo *et al*., 2025).

Excess Zn also suppressed *PsDefs* expression. Although zinc is essential as a cofactor for numerous enzymes and TFs, excess levels may disrupt regulatory networks or directly interfere with transcription. Recent findings in *Arabidopsis* show that reduced *PDF1* levels are linked to increased tolerance to both pathogens and zinc toxicity, while ectopic overexpression enhances stress tolerance (Nguyen *et al*., 2023).

In contrast, cadmium (Cd), toxic even at low concentrations, induced *PsDef1* and *PsDef2*. Defensins may aid Cd detoxification, as seen in species where defensin-like proteins modulate Cd uptake and accumulation (Jin *et al*., 2024).

Under salinity stress, *PsDef2* and *PsDef3* were most strongly upregulated, consistent with reports that defensin overexpression improves salt tolerance (Kumar *et al*., 2019; Hong *et al*., 2024).

Overall, *PsDef* genes are responsive to both pathogenic and beneficial microbes and to a wide range of abiotic stresses, underscoring their broad role in adaptive responses to environmental challenges.

CONCLUSION

In summary, while plant defensins are best known for their roles in biotic stress responses, our findings also highlight their significant involvement in abiotic stress tolerance in Scots pine. This study identifies defensins as promising candidates for further expression analysis in pine trees under natural forest conditions. As multifunctional peptides, defensins contribute to the resilience of Scots pine against both biotic and abiotic stressors associated with climate change.

Understanding the regulatory mechanisms and functional diversity of defensins can inform breeding programs and genetic engineering strategies aimed at developing climate-resilient pine varieties, thereby supporting forest sustainability in a rapidly changing environment. The anticipated availability of high-quality Scots pine genome assemblies will enable comprehensive identification of the full defensin repertoire and facilitate detailed functional characterization of this important gene family.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

Conceptualization, [V.K.; Y.Y.]; methodology, [V.K.; Y.Y.]; validation, [V.K.]; formal analysis, [O.K.; Y.S.]; investigation, [Y.Y.; O.K.; Y.S.; V.K.]; resources, [Y.Y.]; data curation, [V.K.; O.K.]; writing – original draft preparation, [V.K.]; writing – review and editing, [V.K.; Y.S.]; visualization, [Y.Y.; O.K.]; supervision, [V.K.]; project administration, [V.K.]; funding acquisition, [Y.Y.; O.K.; Y.S.; V.K.].

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ПАТЕРНИ ЕКСПРЕСІЇ ГЕНІВ ДЕФЕНЗИНІВ СОСНИ ЗВИЧАЙНОЇ ЗА ВПЛИВУ СТРЕСОВИХ ЧИННИКІВ ДОВКІЛЛЯ

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Обґрунтування. Сосна звичайна (*Pinus sylvestris* L.) – високоадаптивна лісова порода, проте, незважаючи на природну стійкість до стресу, вона стикається з дедалі більшими загрозами через посухи, спричинені зміною клімату. Для протидії стресовим чинникам рослини використовують захисні механізми, такі як синтез антимікробних пептидів (АМП). Однак роль дефензинів – ключового класу АМП – у стресових реакціях сосни залишається недостатньо вивченою.

Матеріали та методи. Для дослідження реакції генів *PsDef1–4* на зовнішні стимули проростки сосни звичайної піддавали дії біотичного стресу (фітопатогенні гриби *Fusarium verticillioides* і *Ophiostoma clavatum*, а також ендофітна бактерія *Pseudomonas putida* P57) та різних абіотичних чинників, включаючи закислення, холод, високу температуру, посуху, засолення, затоплення та важкі метали. Експресію генів аналізували за допомогою кількісної ЗТ-ПЛР у реальному часі. Ортологи генів *PsDef1–4* були ідентифіковані в геномних збірках *Pinus tabuliformis* і *Pinus taeda*. Промоторні ділянки (2 тис. п. н. перед геном) досліджували на наявність *цис*-регуляторних елементів за допомогою бази даних PlantCARE.

Результати. *In silico* аналіз промоторних ділянок *PtbDef1–4* та *PtaDef1,2,4* (ортологів *PsDef1–4*) виявив високу частку (44–57 %) *цис*-регуляторних елементів (CAREs), пов'язаних із відповіддю на стрес, що свідчить про певну роль їх у захисті рослин. *In vivo* аналіз експресії генів показав, що як патогенні гриби, так і ендофітна бактерія спричиняли підвищення рівня експресії *PsDef1–4* через 48 год

після інокуляції. Реакція на абіотичний стрес була різною: посуха та затоплення підвищували експресію всіх чотирьох генів дефензинів, тоді як обробка цинком і холод спричиняли сильне її пригнічення. Відповідь на високу температуру, засолення, закислення та високу концентрацію кадмію була геноспецифічною.

Висновки. Отже, гени дефензинів сосни звичайної реагують не лише на патогенні й ендофітні мікроорганізми, але і на широкий спектр абіотичних стресових чинників, що вказує на їхню важливість у адаптації до умов довкілля. Ці результати підкреслюють потенціал дефензинів як перспективних генів-кандидатів для селекції стійких до кліматичних змін генотипів сосни.

Ключові слова: *Pinus sylvestris* L., експресія генів дефензинів, *цис*-регуляторні елементи, біотичний і абіотичний стрес

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