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A comprehensive characterization and expression profiling of defensin family peptides in *Arabidopsis thaliana* with a focus on their abiotic stress-specific transcriptional modulation

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ABSTRACT

In addition to defensins, plants possess an array of defensin-like peptides that share many of their characteristics, as well as a role in plant's innate immunity. Their involvement in the response to pathogens is well-known but the contribution in the plant response to abiotic stimuli is not fully understood. We have undertaken an *in silico* analysis to characterize all defensin family genes hitherto found in Arabidopsis, including genes encoding for defensin-like peptides, by detecting several peptides as candidates for further studies aiming to decipher specific responses to biotic and abiotic stresses, as well as to their crosstalk. We performed several analyses, including co-expression and cis-regulatory elements analyses, using transcriptomic data obtained from the ARS database, which integrates more than 20,000 Arabidopsis RNA-seq libraries.

In silico analysis showed that jasmonates and ABA, together with transcription factors belonging to WRKY and AP2/EREBP families, modulate defensin and defensin-like gene expression. Indeed, the analysis performed in this study allowed to extract and organize omics data, which finally supported the inducible nature of defensins under both abiotic and biotic stresses. Moreover, *in vivo* expression analyses confirmed the heat and drought responsiveness of *PDF1.4*, *ATTI1*, *PDF1.1*, *DEFL 206*, defensin family genes selected for being upregulated by several abiotic conditions, at transcriptional level. Finally, the co-expression analysis provided information on other biological processes that may be correlated to the defensin induction, such as maintaining ROS homeostasis. Combining the comprehensive analysis of different transcriptional datasets with the integration of *in vivo* analyses emerged as a robust methodological approach to assess the proposed multi-stress responsive nature of defensin family genes.

1. Introduction

As sessile organisms, plants must constantly cope with a variety of abiotic and biotic stressors in order to survive. For this reason, they have developed complex mechanisms including the expression of defense-related genes that play a key role in plant innate immunity. Among the pathogenesis-related (PR) proteins, antimicrobial peptides (AMPs) are essential weapons in the fight against plant microbial pathogens [1]. Defensins are cationic AMPs with a surprising conservation of structure and function across the animal and plant kingdoms [2,3]. The tertiary structure of plant defensins is characterized by the presence of a single alpha-helix and three antiparallel beta-sheets, which are stabilized by four disulfide bridges formed by eight conservative cysteine residues [4,5]. Although plant defensins share a common tertiary structure, there is considerable variation in the amino acid sequences and length of the peptides [5,6].

Defensins have been reported to be highly active against bacteria [7,8], fungi [2,9–12], insects [13,14], and viruses [15]. The leakage caused by the interaction of defensins with the negatively charged membranes of pathogens is considered to be the main reason for their antibacterial and antifungal properties [3], but some authors have also suggested an active role played by the reactive oxygen species (ROS) produced inside the pathogen [2,5,12].

Originally, 15 defensin genes have been identified in the Arabidopsis

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genome [16]. In addition to defensins, several plant species also contain larger amounts of defensin-like peptides (DEFLs), which exhibit all the characteristics of defensins, including motifs, tissue-specific expression, gene structure, and genome organization. According to literature, *Arabidopsis thaliana* contain 317 DEFLs [17]. The number and arrangement of cysteines in DEFLs may differ from the eight residues defined for "true" plant defensins [5,18]. DEFLs can also presents an additional N-terminal signal sequence.

Since many DEFLs have similar three-dimensional structures to the proper defensins, a role in the response to biotic stress is expected [5,19] and supported by recent evidences. In this regard, the overexpression of specific DEFL genes enhanced the resistance against *Fusarium oxysporum* in *A. thaliana* [20]. The involvement of the defensin-like protein 206 (AT3G59930) in resistance to the nematode *H. schachtii* has also been reported although no direct nematicidal activity has yet been observed [21].

The large number of defensin family peptides suggests functional redundancy due to their organ- and tissue-specific expression [4,22]. Plant defensins are promiscuous peptides [5,6] that play a role in processes not directly related to biotic stress, such as pollen-pistil interaction and response to abiotic stimuli [23–25]. The inducibility of defensin genes after different types of stress suggests a possible transversal role of these peptides, which has not yet been fully elucidated.

This study aimed to provide a basis for a deeper empirical functional analysis of the multifaceted role of defensins and DEFLs in *Arabidopsis thaliana*. To this aim, we conducted an *in silico* gene expression analysis based on data retrieved from the *Arabidopsis* RNA-seq database (ARS; http://ipf.sustech.edu.cn/pub/athrna/). ARS integrates 20068 publicly available *Arabidopsis* RNA-seq library data deposited at the Gene Expression Omnibus, the Sequence Read Archive, the European Nucleotide Archive, and the DNA Data Bank of Japan databases [26]. The extensive amount of data within the ARS database has enabled us to overcome the limitations often encountered in *in silico* studies regarding the lack of accessible and reliable large datasets. This approach allowed us to characterize the expression patterns of defensin and DEFL genes under various biotic and abiotic stress conditions.

Among environmental stresses, heat and drought have a huge impact on agricultural productivity that will be exacerbated by ongoing climate change [27]. Therefore, to validate the *in silico* predictions with experimental data, the heat and drought responsiveness of defensins and DEFLs was investigated by analysing the expression profiles of specific genes in *A. thaliana* seedlings using qRT-PCR.

2. Materials and methods

2.1. Phylogenetic analysis of the Arabidopsis defensin family proteins

The list of peptides classified as defensins in the Uniprot database (www.uniprot.org; January 10, 2023) was retrieved by searching terms such as "defensin", "defensin-like peptides" and "*Arabidopsis thaliana*". To reduce redundancy and provide more robust data, unreviewed proteins were removed. The complete list of defensin-related peptides/genes was hereafter referred to as "defensin family peptides/genes".

A phylogenetic tree was constructed based on non-redundant amino acid sequences annotated in Swissprot using MEGA 11 software and the maximum likelihood (ML) method with 1000 bootstraps. First, the CLUSTAL-O program of MEGA 11.0 was used to generate the multiple sequence alignment, and then the maximum likelihood method (ML) with the Jones-Taylor-Thornton (JTT) model was used to construct a phylogenetic tree with 1000 bootstrap replicates. The phylogenetic tree was visualized and edited using iTOL v6. To display pattern in sequence conservation, sequence logos have been obtained by WebLogo tool (https://weblogo.berkeley.edu/logo.cgi).

Multiple sequence alignments of peptides encoded by defensin family genes and induced by biotic and abiotic stressors were performed using mature protein sequences and the Clustal Omega online tool (https://www.ebi.ac.uk/Tools/msa/clustalo/) [28]. Neighbor-joining trees were constructed from the percentage of sequence divergence and without distance corrections.

2.2. Identification of defensin genes expressed in Arabidopsis thaliana

For selected defensin family genes, normalized expression levels expressed in fragments per kilobase of transcript per million mapped reads (FPKM) were retrieved from the Arabidopsis RNA-seq database (ARS; http://ipf.sustech.edu.cn/pub/athrna/; June 10, 2023). According to ARS libraries classification, data about the expression levels in different tissues were extrapolated and grouped as seed-related (embryo, endosperm, seed, silique), shoot-related (leaf, shoot, stem, meristem), flower-related (flower and pollen), while root libraries were considered separately. Abiotic stress-related libraries were divided into genes induced by cold, heat, drought, osmotic stress, salinity, oxidative stress and nutrient deficiency.

Biotic stressor-related libraries present in the ARS database were grouped into bacteria-related (*Microbacterium sp, Pseudomonas syringae, Rhizoctonia solani*) and fungi-related (*Blumeria graminis, Sclerotinia sclerotiorum, Botrytis cinerea, Fusarium graminearum, Hyaloperonospora arabidopsidis, and Verticillium dahlia*). Fungi-related data were further separated according to the fungus lifestyle (necrotrophic, biotrophic, and hemibiotrophic). Libraries from experiments conducted with Cabbage leaf curl virus (CaLCuV), *Heterodera schachtii* and *Plutella xylostella* were collected separately. Libraries related to *Enterobacter sp. SA187* [29], *Colletotrichum tofieldiae* [30], *Pseudomonas simiae WCS417* [31] and *Rhizobium sp* [32] were grouped in the beneficial organism category.

Average FPKM values were then calculated for each gene in each category. Genes with average FPKM values below 2 were filtered out. Library numbers, stress types and fold changes for selected up-regulated genes of the defensin family were retrieved from the 'Data Plot' page of the Arabidopsis RNA-seq database (ARS; http://ipf.sustech.edu.cn/pub/athrna/; June 10, 2023).

2.3. Arabidopsis defensin expressions during stress conditions and phytohormones treatment

To confirm and evaluate the defensin's over-expression under abiotic and biotic stress conditions and after phytohormone treatment, FPKM mean values of mocks and treatments, with their relative standard deviations (STDs), were retrieved from the ARS database. Studies including multiple stress conditions were not considered. Fold changes (expressed as logFC) were calculated and genes without a positive logFC were filtered out. Venn diagrams created using InteractiVenn (http://www.interactivenn.net) were used to detect overlapping genes across considered groups.

2.4. Cis-regulatory elements and co-expression analysis

The TFBS-Discovery Tool Hub (TDTHub) web server tool [33](http://acrab.cnb.csic.es/TDTHub/; July 2, 2024) was used to analyse transcription factor binding sites in the upstream regions of upregulated defensin family genes. Parameters have been set as follows: *A. thaliana* as reference species, FIMO as the TFBS mapping algorithm, upstream region size of 1000 bp, minimum S-Score threshold of 5 %.

Gene co-expression analysis was performed to find expression patterns closely related to the queried defensin family genes. For this purpose, cold- and heat-related libraries have been combined into temperature-related libraries. The osmotic-related data set used for co-expression analysis included drought-, osmotic stress-, and salt-related libraries. For each set of genes, the top 50 co-expressed genes were obtained using the Conserved and Comparative Co-expression Networks (CoCoCoNet) tool (https://milton.cshl.edu/CoCoCoNet; June 15, 2023) which comprises 39517 samples across 14 species [34]. Co-expression networks were constructed by computing Spearman's correlation

between every pair of genes. Gene descriptions were obtained from the Arabidopsis Information Resource (TAIR) database (www.arabidopsis.org). The enrichment analysis of the top 50 highly co-expressed genes was performed using ShinyGO v0.61 [35] with a P-value cutoff (FDR) of 0.05. Networks were then visualized using Cytoscape software.

2.5. Protein-protein networks and hub genes identification

The Search Tool for Retrieval of Interacting Genes (STRING) database (https://string-db.org, September 20, 2023) was used to search for potential interactions between defensin family peptides and to visualize the co-expression networks. [36]. Parameters have been set as follows: co-expression as active interaction sources and medium confidence (>0.4). Disconnected nodes have been hidden in the network. A Markov clustering (MCL) algorithm was used to identify modules of highly interconnected genes using an inflation factor of 2.1. Cytoscape software version 3.6.1 was used to visualize the protein-protein interaction (PPI) network [37]. The maximal Clique Centrality (MCC) algorithm, present in the CytoHubba plugin and reported to be the most effective method to find hub nodes [38], was used to detect the top hub genes in co-expression networks.

2.6. Plant material and growth and stress conditions

Arabidopsis thaliana (genetic background Col-0) seeds were surface-sterilized and sown out on Murashige and Skoog medium (Duchefa, Haarlem, The Netherlands) supplemented with 2 % (w/v) sucrose and 1.5 % (w/v) Phyto-Agar (Duchefa, Haarlem, The Netherlands). After 2 days of stratification at 4°C in the dark, seedlings were grown for 7 days (22°C, 80 mmol m_{-2} sec_{-1} on 16 h/8 h light/ dark cycles). Heat treatments were performed on day 8 by incubating the Petri dishes in dark at 42°C for 1 h [39,40]. Under control conditions, seedlings were kept in dark at 22°C. For drought treatment, 1-week-old seedlings were gently removed from the agar medium and placed on low-water agar at 22°C for 2 hours [41]. Under control conditions, seedlings were placed on classic agar medium at 22°C. After heat and drought treatments, seedlings were immediately frozen in liquid nitrogen for subsequent analyses.

2.7. RNA extraction and relative gene expression by qRT-PCR

Total RNA was isolated from frozen seedlings (100 mg FW; 3 biological replicates) as described elsewhere [42]. The RNA concentration and purity were measured with a Nanodrop™ spectrophotometer (Fisher Scientific), while RNA integrity was verified by checking the presence and the quality of 28 S and 18 S ribosomal RNA (rRNA) bands on a 1 % agarose gel. cDNA was synthetized starting from one microgram of RNA by using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). According to *in silico* characterization, the expression analysis of 4 defensin family genes (*AT1G19610, AT2G43510, AT1G75830, AT3G59930*), randomly selected from those induced by multiple stresses, including heat and drought, was studied in *A. thaliana* seedlings. The actin-2 gene (*ACT2; AT3G18780*) and the ubiquitin-conjugating enzyme E2 (*UBC21; AT5G25760*) were used as internal controls to calculate the relative expression of the selected target genes [43,44]. The primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/; accessed on 1 February 2023) [45] was used for the primers design. Sequences of primers are listed in Table S1. RT-qPCR analyses were performed on a CFX96 Real-Time system (Bio-Rad Laboratories, Hercules, CA, USA), using SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, CA, USA).

The efficiencies of target and internal control genes were verified by qRT-PCR as described elsewhere [46]. Data obtained from three biological and three technical replicates were analysed with the CFX Maestro software (Bio-Rad Laboratories, Hercules, CA, USA) in order to extract cycle threshold (Ct) values. The data analysis and the calculations for relative quantitation were calculated as described elsewhere [47]. Relative expression of selected defensin family genes was finally reported as the fold increase of the transcript level with respect to the control condition.

3. Results

3.1. Characterization of defensin family genes in Arabidopsis thaliana

A detailed *in silico* analysis was performed considering *Arabidopsis thaliana* defensins and DEFL peptides. A total of 297 reviewed peptides, encoded by 297 defensin family genes, were retrieved from the Uniprot database (Table S2). Defensin-related peptides were aligned with Clustal Omega and, according to the defensins classification proposed by De Coninck and collaborators [5], 16 defensin-related peptides were categorized as “true” defensins, 2 as “true defensins with an extra-domain”, and 279 as DEFLs (Table S2; Fig. S1).

In order to investigate the evolutionary conservation and relationship among the defensin family peptides known in *Arabidopsis thaliana*, a multiple alignment of protein defensin sequences was built. It is known that, despite the strongly conserved tertiary structure, defensin family peptides only share few conserved residues [5]. Indeed, the amino acid sequence conservation of Arabidopsis defensin proteins was limited to a small number of residues (WebLogo; Fig. S2), as it was also confirmed by a general low identity percentage (Table S3). The resulting phylogenetic data showed that Arabidopsis defensin family peptides, including the originally identified defensins (Thomma et al., 2002), were divided into four main clades, represented in different colors, with the second and fourth being the major expanded clades. Clade I (red) consists of 31 leaves and 29 nodes, clade consists of II of 99 leaves and 98 nodes (green), clade III (blue) consists of 47 leaves and 44 nodes, clade IV (yellow) consists of 120 leaves and 116 nodes (Fig. 1).

In order to identify potential novel interconnections between plant defensin peptides, the STRING online tool was used to explore protein-protein interactions (PPI). The obtained network consisted of 291 nodes and 477 edges (Fig. 2). The PPI enrichment p-value was lower than 1.0 e^{-16} indicating that the network has significantly more interactions than expected and that a good connection among genes has occurred. The MCL algorithm has identified 5 main clusters composed by 42, 20, 18, 18 and 4 genes/proteins (Fig. 2; Table S4).

3.2. Analysis of expression pattern of Arabidopsis defensin genes

The expression patterns of *Arabidopsis thaliana* defensin family genes were investigated in plant tissues and organs. According to the ARS classification, 1122 libraries were collected and grouped as tissue-related. A total of 242 defensin family genes were found expressed (FPKM>2) in all plant tissues with a large part of them expressed in the silique and flowers (64 % and 55 %, respectively; Table S5). Overall 181 and 40 genes were highly expressed in seed-related and shoot-related tissues, respectively, while 31 genes in root libraries and 139 genes in flower-related libraries. An overview of the expression profiles of the five most expressed defensin genes in each tissue group is represented in Table I.

Venn diagrams were used to identify commonly and specifically expressed genes among groups (Fig. S3). A total of 48, 15 and 32 genes were found expressed in all seed-related (Fig. S3A), shoot-related (Fig. S3B) and flower-related tissues (Fig. S3C), respectively. Two genes were expressed in all types of tissues (*AT2G02100, AT2G02130*; Fig. S3D).

The inducible nature of Arabidopsis defensins was also investigated. For this purpose, the ARS database has been examined and 844 abiotic-related libraries were retrieved. Overall, 46 defensin family genes were over-expressed under abiotic stimuli (Fig. 3A). Among them, 27 and 26 genes showed an over-expression after exposure to high and low temperatures stress, respectively. A total of 27 genes were over-expressed

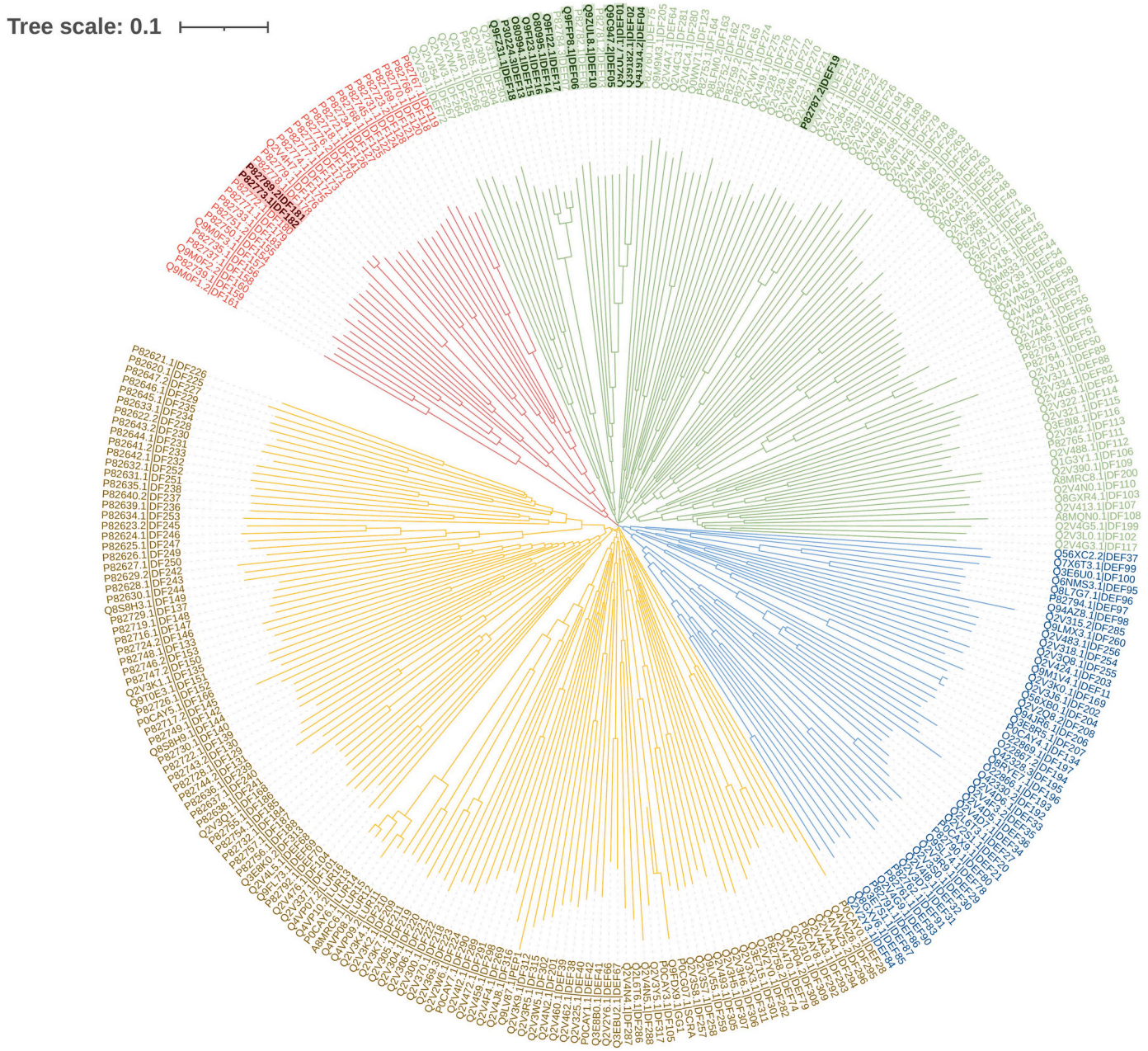


Fig. 1. Phylogenetic tree of defensins and defensin-like peptides in *A. thaliana*. The phylogenetic tree was constructed using MEGA 11.0 with the neighbor-joining (NJ) method and 1000 bootstrap replicates. Proteins are represented by their accession number followed by protein name. The originally identified defensins were marked. Clade I (red); Clade II (green); Clade III (blue); Clade IV (yellow).

under drought, while 11 genes were induced by salt treatment. The osmotic stress led to the over-expression of 11 defensin family genes. Nutrient deficiency and oxidative stress triggered the expression of 29 and 16 genes, respectively. Two genes (*AT2G43510, AT2G43530*) resulted over-expressed under all types of abiotic stimulus considered (Fig. 3A). The shared genes among those induced by drought, salt and osmotic stress conditions were 5 (*AT2G43510, AT2G43530, AT1G60989, AT2G02120, AT1G13609*). A total of 20 genes were commonly upregulated by high and low temperatures.

According to the ARS classification, 418 biotic-related libraries were also collected. A total of 37 defensin genes were found over-expressed following biotic stress (Fig. 3B). Pathogen fungal and bacterial exposure induced the expression of 14 and 27 genes, respectively. Among fungi, the largest inductions were observed with biotrophs and necrotrophs (10 and 9 overexpressed genes, respectively).

Six genes were induced by the Cabbage leaf curl virus, while *Heterodera schachtii* and *Plutella xylostella* triggered the expression of 6 and 2 genes, respectively (Fig. 3B). Most of the over-expressed defensin genes (17; 63 %) found in libraries related to Arabidopsis-bacteria interactions were also induced by the beneficial organisms considered. A total of 11 defensin family genes were shared between libraries related to bacterial- and fungal- plant interactions (Fig. 3B); while two genes (*AT3G59930, AT1G19610*) were found commonly over-expressed after the exposure to bacteria, fungi, viruses and beneficial organisms. On the other hand, 4 defensin family genes (*AT4G22217, AT4G13235, AT3G63360, AT4G22214*) were specifically expressed following inoculation with beneficial microbes.

Phylogenetic tree analysis revealed 3 distinct clades in biotic-related defensins (Fig. 3A). Defensin family genes included in groups 1 and 2 were mainly induced by bacteria, viruses, nematodes and beneficial

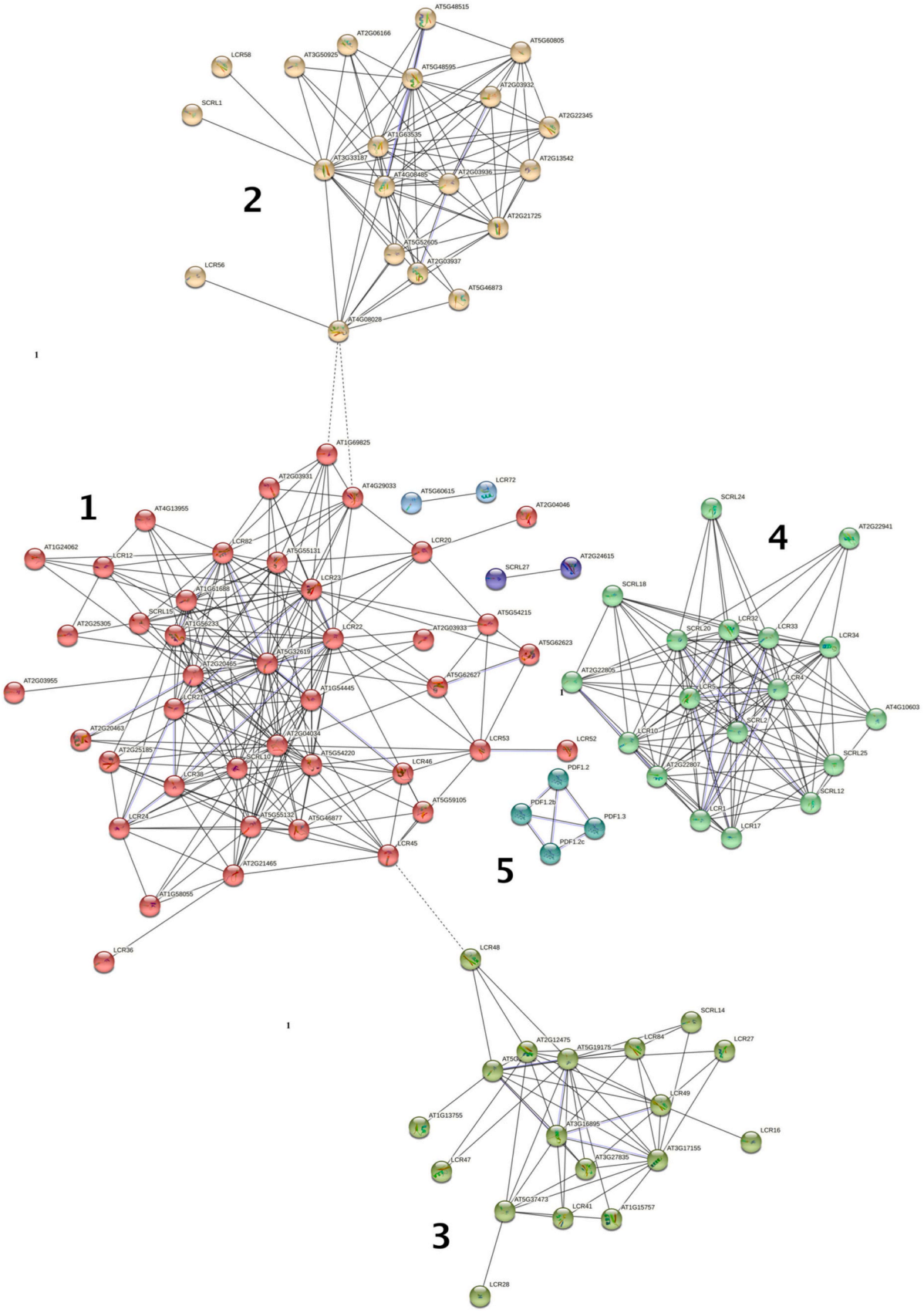


Fig. 2. Protein-protein interaction network constructed using search tool for retrieval of interacting genes (STRING) database. The Markov clustering (MCL) algorithm was used to identify modules of highly interconnected genes/proteins. Dots with the same color belong to the same cluster. Co-expression evidence (black line); co-occurrence evidence (blue line).

Table I
Overview of the expression profiles of the ten most expressed Arabidopsis defensin family genes in each tissue considered. A sequential red scale indicates different average FPKM values.

Gene name	Predominance	FPKM values											
		Seed-related				Shoot-related					Flower-related		Root
		Embryo	*Endosperm*	*Seed*	*Silique*	*Shoot*	*Leaf*	*Stem*	*Meristem*	*Seedling*	*Pollen*	*Flower*	
AT1G47540	Seed	138.76	183.91	176.16	29.55	0.03	0.05	5.28	0.00	0.47	0.00	0.58	0.12
AT2G02120		78.26	149.74	186.41	24.40	5.43	2.17	10.84	0.99	19.94	0.59	3.63	29.80
AT2G02100		63.16	231.88	82.07	71.63	92.61	41.65	53.49	140.50	88.69	2.58	68.16	53.23
AT3G27835		8.74	180.31	11.24	18.42	0.00	0.00	0.70	0.00	0.00	0.00	0.42	0.13
AT1G75830		24.33	0.05	239.09	0.43	2.15	3.84	4.55	1.42	9.28	0.00	1.35	1.14
AT2G43530	Shoot	44.74	104.33	20.42	79.08	119.79	88.25	115.20	114.79	62.15	0.31	87.34	21.74
AT3G05730		7.27	0.73	1.23	0.82	41.16	36.68	7.67	51.96	44.80	0.00	1.30	2.65
AT1G13609		12.12	48.92	4.46	24.64	36.69	25.86	4.35	13.36	23.98	0.00	2.98	2.60
AT3G05727		5.22	5.17	0.09	1.32	70.94	21.21	42.48	166.38	62.54	4.82	2.62	4.30
AT2G43550		6.00	2.83	1.72	66.05	108.99	82.54	110.44	156.83	72.97	0.99	89.77	28.97
AT5G60615	Flower	5.51	1.91	7.29	15.03	0.02	0.35	3.61	0.00	0.01	323.51	25.65	0.23
AT2G02140		4.04	2.57	4.00	10.43	0.02	0.23	2.03	0.00	0.00	200.05	18.96	0.16
AT4G19038		0.98	0.32	1.07	3.64	0.00	0.09	0.90	0.00	0.01	227.64	10.79	0.10
AT5G40155		7.54	96.99	16.82	14.67	0.05	0.06	0.75	0.00	0.05	187.80	8.36	0.05
AT2G22805		0.45	0.09	0.22	0.53	0.00	0.03	0.19	0.00	0.00	158.15	13.35	0.03
AT4G22212	Roots	0.30	0.00	4.18	0.29	3.54	2.38	5.57	4.06	22.10	0.00	0.61	55.46
AT4G22230		5.12	2.85	9.09	0.97	1.67	1.01	0.67	0.35	11.27	0.00	0.84	48.05
AT2G02130		59.92	8.44	36.64	42.73	65.76	64.07	47.98	91.60	74.62	3.43	54.47	79.06
AT2G43535		33.56	26.86	22.11	18.10	34.31	22.56	26.54	17.56	47.09	0.00	15.44	65.50
AT4G22235		30.19	3.40	12.82	2.38	0.36	0.38	0.21	2.25	9.16	0.00	3.24	32.07

microorganisms, while those included in group 3 were largely induced by fungi. No clear functional distinction was observed among the 3 phylogenetic clades into which the defensins family genes induced by abiotic stress have been grouped (Fig. 3B). Venn diagram showed that abiotic and biotic stress shared 34 defensin (Fig. S4).

According to the PPI network analysis (Fig. 2), all genes included in clusters 1 and 3 belonged to the seed-related group. Cluster 4 was entirely composed of genes expressed in flower and pollen, while both abiotic and biotic stress induced the expression of genes belonging to Cluster 5. Cluster 2 included genes that were not found in common with any category.

The inducible nature of defensin genes would depend on regulative mechanisms, possibly involving phytohormones' signaling pathways and transcription factor activation. Therefore, the expression of defensin family genes related to phytohormones was investigated by comparing genes commonly over-expressed after (a)biotic stimuli and hormone treatments. Several phytohormones triggered the expression of defensin family genes involved in both abiotic and biotic stress responses (Fig. 4), in particular, jasmonates and ABA. The highest number of the phytohormone-related defensin family genes were induced by high and low temperatures and nutrient deprivation, among the abiotic stresses (Fig. 4A). The 91 % and 82 % of defensin family genes over-expressed following osmotic stress were also induced by ABA and jasmonates, respectively.

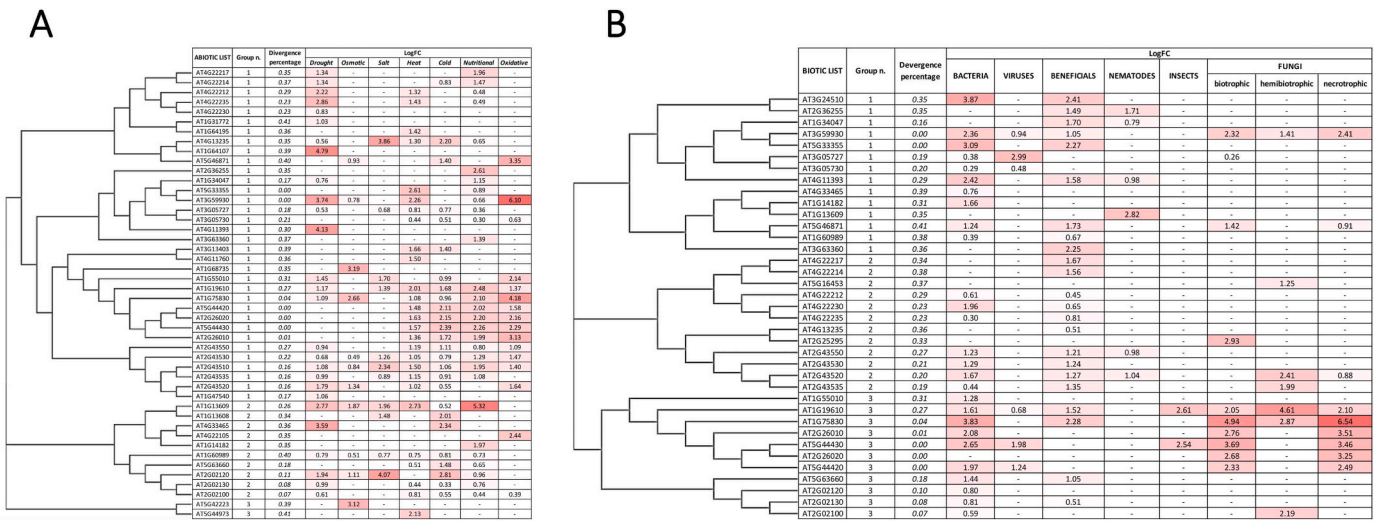
Among biotic stimuli, 18 and 19 defensin family genes, which represented 67 and 70 % of all over-expressed bacteria-related genes, were also induced by ABA (Fig. 4B).

Auxin, salicylates and ethylene played a secondary role in the induction of defensin family genes, mainly following abiotic stimuli (Fig. 4).

Cis elements recognition analysis suggested that members of WRKY and AP2/EREBP families may modulate the defensins' induction, under both abiotic and biotic conditions (Table II; Table S6). Moreover, several promoter regions of defensin genes, induced by both abiotic and biotic stimuli, contain binding sites for common transcription factors such as the *DWARF AND DELAYED FLOWERING 2* (*DDF2; AT1G63030*) and *WRKY18* (*AT4G31800*) (Table II).

3.3. Co-expression analysis of stress-related Arabidopsis defensin family genes

To better investigate the metabolic framework in which defensins' induction occurs under both abiotic and biotic stresses a co-expression analysis was performed. For each set of genes, the top 50 co-expressed



ABIOTIC LIST	Group n.	Divergence percentage	LogFC					
			Drought	Osmotic	Salt	Heat	Cold	Nutritional	Oxidative
AT4G22217	1	0.35	1.34	-	-	-	-	1.96	-
AT4G22214	1	0.37	1.34	-	-	-	0.83	1.47	-
AT4G22212	1	0.29	2.22	-	-	1.32	-	0.48	-
AT4G22235	1	0.23	2.86	-	-	1.43	-	0.49	-
AT4G22230	1	0.23	0.83	-	-	-	-	-	-
AT1G31772	1	0.41	1.03	-	-	-	-	-	-
AT1G64195	1	0.36	-	-	-	1.42	-	-	-
AT4G13235	1	0.35	0.56	-	3.86	1.30	2.20	0.65	-
AT1G64107	1	0.39	4.79	-	-	-	-	-	-
AT5G46871	1	0.40	-	0.93	-	-	1.40	-	3.35
AT2G36255	1	0.35	-	-	-	-	-	2.61	-
AT1G34047	1	0.17	0.76	-	-	-	-	1.15	-
AT5G33355	1	0.00	-	-	-	2.61	-	0.89	-
AT3G59930	1	0.00	3.74	0.78	-	2.26	-	0.66	6.10
AT3G05727	1	0.18	0.53	-	0.68	0.81	0.77	0.36	-
AT3G05730	1	0.21	-	-	-	0.44	0.51	0.30	0.63
AT4G11393	1	0.30	4.13	-	-	-	-	-	-
AT3G63360	1	0.37	-	-	-	-	-	1.39	-
AT3G13403	1	0.39	-	-	-	1.66	1.40	-	-
AT4G11760	1	0.36	-	-	-	1.50	-	-	-
AT1G68735	1	0.35	-	3.19	-	-	-	-	-
AT1G55010	1	0.31	1.45	-	1.70	-	0.99	-	2.14
AT1G19610	1	0.27	1.17	-	1.39	2.01	1.68	2.48	1.37
AT1G75830	1	0.04	1.09	2.66	-	1.08	0.96	2.10	4.18
AT5G44420	1	0.00	-	-	-	1.48	2.13	2.02	1.58
AT2G26020	1	0.00	-	-	-	1.63	2.15	2.20	2.16
AT5G44430	1	0.00	-	-	-	1.57	2.39	2.26	2.29
AT2G26010	1	0.01	-	-	-	1.36	1.72	1.99	3.13
AT2G43550	1	0.27	0.94	-	-	1.19	1.13	0.80	1.09
AT2G43530	1	0.22	0.68	0.49	1.26	1.05	0.79	1.29	1.47
AT2G43510	1	0.16	1.08	0.84	2.34	1.50	1.06	1.95	1.40
AT2G43535	1	0.16	0.99	-	0.89	1.15	0.91	1.08	-
AT2G43520	1	0.16	1.79	1.34	-	1.02	0.55	-	1.64
AT1G47540	1	0.17	1.06	-	-	-	-	-	-
AT1G19609	2	0.26	2.77	1.87	1.96	2.73	0.52	5.32	-
AT1G13608	2	0.34	-	-	1.48	-	2.01	-	-
AT4G33465	2	0.36	3.59	-	-	-	2.34	-	-
AT4G22105	2	0.35	-	-	-	-	-	-	2.44
AT1G14182	2	0.35	-	-	-	-	-	1.97	-
AT1G60989	2	0.40	0.79	0.51	0.77	0.75	0.81	0.73	-
AT5G63660	2	0.18	-	-	-	0.51	1.48	0.65	-
AT3G02120	2	0.11	1.94	1.11	4.07	-	2.81	0.96	-
AT2G02130	2	0.08	0.99	-	-	0.44	0.33	0.76	-
AT2G02100	2	0.07	0.61	-	-	0.81	0.55	0.44	0.39
AT5G42225	3	0.39	-	-	3.12	-	-	-	-
AT5G44973	3	0.41	-	-	-	2.13	-	-	-

BIOTIC LIST	Group n.	Devergence percentage	LogFC							
			BACTERIA	VIRUSES	BENEFICIALS	NEMATODES	INSECTS	FUNGI		
								biotrophic	hemibiotrophic	necrotrophic
AT3G24510	1	0.35	3.87	-	2.41	-	-	-	-	-
AT2G36255	1	0.35	-	-	1.49	1.71	-	-	-	-
AT1G34047	1	0.16	-	-	1.70	0.79	-	-	-	-
AT3G59930	1	0.00	2.36	0.94	1.05	-	-	2.32	1.41	2.41
AT5G33355	1	0.00	3.09	-	2.27	-	-	-	-	-
AT3G05727	1	0.19	0.38	2.99	-	-	-	0.26	-	-
AT3G05730	1	0.20	0.29	0.48	-	-	-	-	-	-
AT4G11393	1	0.29	2.42	-	1.58	0.98	-	-	-	-
AT4G33465	1	0.39	0.76	-	-	-	-	-	-	-
AT1G14182	1	0.31	1.66	-	-	-	-	-	-	-
AT1G13609	1	0.35	-	-	-	2.82	-	-	-	-
AT5G46871	1	0.41	1.24	-	1.73	-	-	1.42	-	0.91
AT1G60989	1	0.38	0.39	-	0.67	-	-	-	-	-
AT3G63360	1	0.36	-	-	2.25	-	-	-	-	-
AT4G22217	2	0.34	-	-	1.67	-	-	-	-	-
AT4G22214	2	0.38	-	-	1.56	-	-	-	-	-
AT5G16453	2	0.37	-	-	-	-	-	-	1.25	-
AT4G22212	2	0.29	0.61	-	0.45	-	-	-	-	-
AT4G22230	2	0.23	1.96	-	0.65	-	-	-	-	-
AT4G22235	2	0.23	0.30	-	0.81	-	-	-	-	-
AT4G13235	2	0.36	-	-	0.51	-	-	-	-	-
AT2G25295	2	0.33	-	-	-	-	-	2.93	-	-
AT2G43550	2	0.27	1.23	-	1.21	0.98	-	-	-	-
AT2G43530	2	0.21	1.29	-	1.24	-	-	-	-	-
AT2G43520	2	0.20	1.67	-	1.27	1.04	-	-	2.41	0.88
AT2G43535	2	0.19	0.44	-	1.35	-	-	-	1.99	-
AT1G55010	3	0.31	1.28	-	-	-	-	-	-	-
AT1G19610	3	0.27	1.61	0.68	1.52	-	2.61	2.05	4.61	2.10
AT1G75830	3	0.04	3.83	-	2.28	-	-	4.94	2.87	6.54
AT2G26010	3	0.01	2.08	-	-	-	-	2.76	-	3.51
AT5G44430	3	0.00	2.65	1.98	-	-	2.54	3.69	-	3.46
AT2G26020	3	0.00	-	-	-	-	-	2.68	-	3.25
AT5G44420	3	0.00	1.97	1.24	-	-	-	2.33	-	2.49
AT5G63660	3	0.18	1.44	-	1.05	-	-	-	-	-
AT2G02120	3	0.10	0.80	-	-	-	-	-	-	-
AT2G02130	3	0.08	0.81	-	0.51	-	-	-	-	-
AT2G02100	3	0.07	0.59	-	-	-	-	-	2.19	-

Fig. 3. Phylogenetic trees (Neighbor-joining trees without distance corrections) generated with the Clustal Omega tool (https://www.ebi.ac.uk/Tools/msa/clustalo/) and heat map of the Arabidopsis defensin family genes over-expressed following abiotic (A) and biotic (B) stimuli. Percentages of divergence and fold changes (expressed as logFC) are reported. A sequential red scale indicates different over-expression levels.

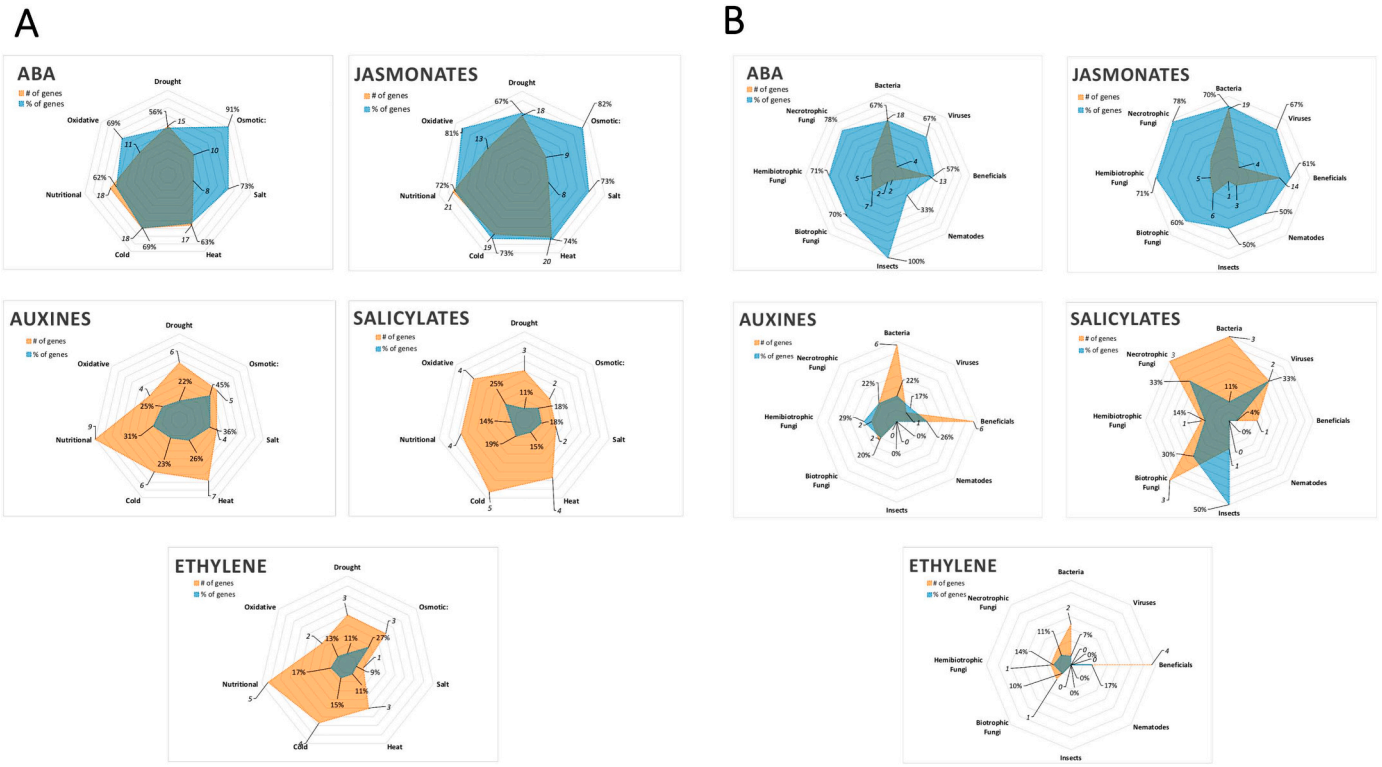


Fig. 4. Radar charts of number and percentage of Arabidopsis defensin family genes related to abiotic (A) and biotic (B) stress also induced by phytohormone treatment.

genes were listed (Table S7).

Enrichment analysis revealed that "mitochondrial electron transport" and "mitochondrial ATP synthesis coupled electron transport" were among the most enriched categories in genes co-expressed with defensins-induced abiotic stressors (Table S8). As expected, the "regulation of protein folding" category was enriched only in the temperature-related co-expression network.

The "mitochondrial electron transport" and "mitochondrial ATP synthesis coupled electron transport" were also the enriched processes in defensins induced by bacteria and beneficial organisms. The "electron transport chain" was the only enriched GO term in the fungal co-expression network. Furthermore, Cabbage leaf curl virus triggered the expression of defensins that were expressed together with genes involved in the defense response to fungi. The genes co-expressed with *Plutella xylostella*-induced defensins were mainly linked to the jasmonic acid and ethylene-dependent systemic resistance. Several genes whose expression patterns were closely related to defensins induced by *H. schachtii* showed a role in glycine catabolic process, plastid transcription and translation.

The identification of the hubs in the co-expression networks revealed the central role of genes encoding for proteins involved in plant immunity (Table S9). Indeed, the top hub genes found in virus, fungi and

Table II
Overview of the main transcription factors with binding sites in the promoter regions of Arabidopsis defensin family genes over-expressed in both abiotic and biotic stress responses, obtained using the TFBS-Discovery Tool Hub (TDTHub) web server tool (http://acrab.cnb.csic.es/TDTHub/). The type of stress has been reported.

Gene ID	Logo	TF	TF family	P-value	FDR	Log2 FoldEn	S Score	Hits in sample
AT4G31800		WRKY18	WRKY	9.64E−05	0.16394	1.4279	5.7342	16
AT5G24110		WRKY30	WRKY	0.000671	0.22123	1.1188	3.5505	17
AT1G69490		NAC029	NAC/NAM	0.001495	0.22123	1.2487	3.5279	13
AT1G63030		DREB1E	AP2/EREBP	0.003762	0.22123	1.4325	3.4732	9

bacteria co-expression networks encoded for three defensin family proteins (*AT3G59930, AT2G43510, AT1G19610*) and this also holds true for the top hub genes in temperature stress and nutritional deprivation co-expression networks (*AT2G02120, AT4G22212*). The subunit 8 protein of cytochrome b-c1 complex (*AT3G10860*) showed a central role in the osmotic stress co-expression network.

Among the genes co-expressed with biotic-induced defensins, a member of the CYP81D family of cytochrome p450s (*AT5G36220*) was the top hub in the insect co-expression network. Genes encoding for the chloroplast ribosomal protein L2 (*AT1G35680*) and the sterol carrier protein 2 (*AT5G42890*) showed high connectivity in modules composed by nematode- and beneficial co-expressed genes, respectively.

3.4. Heat and drought stress modulates the in vivo expression of Arabidopsis defensin family genes

In order to examine whether heat and drought stress affected the regulation of defensin family genes at the transcriptional level, 4 genes induced by multiple stressors according to *in silico* results were selected and their expression analysed by qRT-PCR under the *in vivo* described experimental conditions (Fig. 5; Table S10).

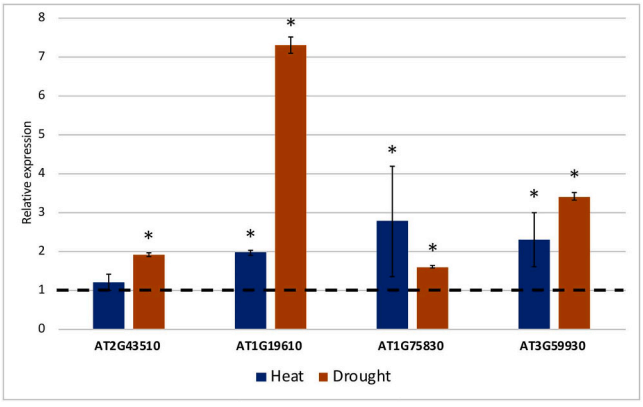


Fig. 5. Transcript levels of selected Arabidopsis defensin family genes under heat and drought stresses revealed by *in silico* analysis. The data analysis and the associated calculations for relative quantitation were calculated as described elsewhere [47]. The actin-2 gene (ACT2; AT3G18780) and the ubiquitin-conjugating enzyme E2 (UBC21; AT5G25760) were used as internal controls to calculate relative expression of the selected target genes. Relative expressions were reported as the number of fold increase of the transcript level compared to the control conditions. Three biological samples per treatment were analyzed with three technical replicates per sample. Data are means ± SE expressed as relative intensities among samples. Asterisks represents statistical significance ($P < 0.05$) compared with control.

Overall, 3 out of 4 tested transcripts *(PDF1.4, AT1G19610; PDF1.1, AT1G75830, defensin-like protein 206, AT3G59930*) were significantly induced (relative expression higher than 1.6) in seedlings subjected to heat stress with respect to unstressed ones. Gene *AT2G43510* (*ATTI1*) was not significantly induced by heat stress, although a positive trend was observed.

A positive regulation of all defensin family genes considered (*AT1G19610, AT2G43510, AT1G75830, AT3G59930*) was also recorded under water deficit.

The tertiary structures of the 4 plant defensins analyzed, generated by AlphaFold (https://alphafold.ebi.ac.uk/), are characterized by the presence of the classical single alpha-helix and three antiparallel beta-sheets (Fig. S5).

4. Discussion

4.1. Only a small percentage of Arabidopsis defensin family genes is induced by biotic stressors

Plant defensins and DEFLs are primarily known for their antifungal and antibacterial properties, however, an involvement in plant development [48], reproduction [49], heavy metal tolerance [50], and abiotic stress response has been suggested [51].

We found that the Arabidopsis genome contains 297 genes encoding for reviewed defensin-related peptides. Although there are 15 proper defensins reported in the literature, based on the classification proposed by De Coninck and collaborators [5], we identified 16 “true” defensins and 2 “true defensins with an extra-domain”, thus adding three low-molecular-weight cysteine-rich proteins (AT2G02135, AT2G31957, AT2G31953) that had not yet been classified as "true defensins" (Table S2; Fig. S1).

Of the 297 defensin family peptides detected here, 292 were in common with the Silverstein database [17], while 270 elements were also detected by Spada and collaborators [52] (Table S11).

The mechanism behind this large presence of genes is attributed to frequent internal duplications and rearrangements, often observed with AMPs [17], which leads to beneficial mutations still maintaining their native structure [53]. Moreover, besides their important role in innate host resistance, such a large number of genes supports the idea of different roles played by these AMPs. In fact, several plant peptides with novel and non-defense-related functions, including development and reproduction, derived from gene duplication and neo-functionalization events of AMPs, supporting this hypothesis [3,17,48,54–57].

Overall, defensins and DEFLs are known to be expressed in various

plant organs where they provide a first line of defense against pathogen attack. According to the literature, seeds are the most abundant source of defensins [54]. Seed defensins suppress the fungal growth enhancing seedling survival rate [7]. In addition, plant defensins and DEFLs have also been found in leaves, floral organs, tubers, fruit and roots [16,54, 58]. In this study, data from transcriptomic analyses allowed us to follow the defensin family genes tissue-specific expression showing a clear predominance in seed, silique and flower. In particular, the latter is known to be populated by defensins that act as pollen tube attractants and are involved in micropylar guidance [49]. Our findings are consistent with these data, with 4 out of 6 genes having a role in pollen–pistil interaction (*AT5G43285, AT5G43513, AT5G43518, AT5G43516*) being expressed in flower-related tissues.

Interestingly, only a small part of the detected defensin family genes (12 %) resulted over-expressed following biotic stress (Fig. 3Z). The bioinformatics analysis proposed here to decipher the publicly available transcriptomic data showed that the most of defensin family genes was induced by bacterial exposure, despite their best-known antifungal activity. Moreover, our data indicate that the induction of defensin family genes depends on the fungus lifestyle. Indeed, only a small percentage of defensins (*AT1G19610, AT3G59930, AT1G75830*) were commonly over-expressed by biotrophs, hemibiotrophs and necrotrophs (Fig. 3B).

Interestingly, 17 defensin family genes were induced during the interaction between Arabidopsis and both pathogenic and beneficial bacteria. This may be due, at least in part, to the first initial recognition by roots that does not distinguish between beneficial and pathogenic bacteria [59]. AMPs are known to be involved in the discrimination between mutualistic and pathogenic bacteria. In nodule tissues of *Medicago truncatula* several genes encoding defensins, defensin-like peptides, and glycine-rich peptides (GRPs) were up-regulated during legume–rhizobial symbiosis [60–62]. Since the development of the induced systemic resistance (ISR), which is associated with an enhanced ability to resist pathogen attack [63,64], and requires the induction of a set of pathogenesis-related proteins and PDF1.2 [65–68],

It is worth mentioning that six defensin family genes were induced by the Cabbage leaf curl virus (Fig. 3B), suggesting a potential antiviral activity of defensins and DEFLs that has not been fully explored in plant literature. Meanwhile, certain plant defensin-like peptides have demonstrated anti-HIV-1 activity [69]. Furthermore, only a mild regulation of some defensin gene expression was recorded in tomato plants infected by Cauliflower Mosaic Virus (CMV) and Potato Virus Y (PVY) [70].

4.2. *Arabidopsis defensin family genes are induced by abiotic stress*

Abiotic stressors, such as heat, drought, salinity, and nutrient deficiencies are the leading cause of crop loss worldwide [71]. The participation of AMPs in the abiotic stress response of plants is nowadays increasingly recognized [72], as is the involvement of some plant defensins [50,73,74]. However, we are still far from a complete understanding of the role of AMPs in the abiotic response of plants.

Our results showed the abiotic stress-related induction of 46 family defensin genes, of which six (*AT1G60989, AT2G43530, AT1G19610, AT1G13609, AT2G43510, AT1G75830*) were over-expressed in at least 6 of the 7 conditions considered (Fig. 3A). Among them are two members of the PDF1 multigenic family (*AT1G75830* and *AT1G19610*). PDF1 family members from *A. thaliana* and *A. halleri* conferred *in vitro* cellular tolerance to zinc [73]. Similarly, the over-expression of a defensin from *A. halleri* (*AhPDF1.1b*), under the control of the 35 S promoter, conferred zinc tolerance in *A. thaliana* seedlings [50]. We observed the over-expression of the *A. thaliana AtPDF1.1* (*AT1G75830*) paralogue of *AhPDF1.1b* following bacterial and fungal infections, as well as drought, osmotic stress, heat, cold, nutrient deprivation and oxidative stress, pointing to an important role played by this protein in response to various environmental stresses. *AtPDF1.1* expression was previously indicated as part of the local response activated by fungal attack and both local and systemic responses to the necrotrophic bacterium *Pectobacterium carotovorum subsp. carotovorum* [75]. In the latter case, the secretion of AtPDF1.1 in the apoplast was responsible for iron sequestration and consequently for the activation of an iron deficiency response associated with plant pathogen resistance. Moreover, the overexpression of *AtPDF1.1* resulted in reduced symptom development in *A. thaliana* infected by the nonhost *Cercospora beticola* [76].

According to the Intergovernmental Panel on Climate Change 2022 (IPCC 2022; http://www.ipcc.ch/), high temperatures and water deficits are two of the most frequent and impactful environmental threats to crop food production and quality. Heat and drought stress are closely related, as high temperatures cause rapid water loss from the plant and soil surface [71,77,78]. Improving plant tolerance to heat and drought stress requires an understanding of the mechanisms triggered by plants to counteract these stressors, in order to the identify candidate genes responsible for controlling the trait to be improved [79].

In our *in silico* analysis a total of 20 and 26 libraries related to osmotic stress and high temperature stress, respectively, were analysed. The results showedthat both heat and drought stress could induce the expression of several defensins and DEFLs. A link between defensins and drought has already been reported. Kumar and colleagues [74] demonstrated that the overexpression of the chickpea defensin gene enhanced the tolerance to water deficit stress in *Arabidopsis thaliana.* Furthermore, the Dhn8 defensin was found to be highly expressed in leaves of drought-resistant *Glycine max* [51].

No evidence for a role of defensins in the heat response has been reported so far, although the Tad1 defensin gene has been found to be up-regulated in cold-tolerant genotypes of *Triticum aestivum* seedlings [23].

To support the *in silico* results, we have undertaken qRT-PCR analyses on Arabidopsis seedlings exposed to high temperature and water deficit. We focused our attention on 4 defensin family genes, 2 true defensins (*AT1G19610; AT1G75830*) and 2 DEFLs (*AT3G59930; AT2G43510*), randomly selected among those induced by multiple abiotic stimuli, according to our *in silico* analysis (Fig. 3A).

The *in vivo* investigation confirmed that the transcript expression of all selected defensin family genes increased significantly after drought treatment, compared to untreated plants (Fig. 5; Table S8). Exposure to high temperatures induced the over-expression of 3 out of the 4 defensin family genes considered.

Cis-regulatory element analysis showed that the promoter regions of these defensin family genes contain binding sites for common transcription factors, including the *ABA REPRESSOR1* (*ABR1; AT5G64750*; Table S12). *ABR1* is known to be expressed in response to ABA, osmotic stress, sugar stress, and drought, and knockout mutants are more susceptible to these stresses [80]. This finding supports the involvement of ABA in the induction of defensin family genes following multiple abiotic stimuli.

According to our *in vivo* analysis, a defensin-like gene encoding the trypsin inhibitor protein ATTI-1 (AT2G43510) was up-regulated under drought treatment. Although plant trypsin inhibitors may function in defense against herbivory by inhibiting the digestive enzymes of insects [81–83], to date no role in abiotic stress response has been reported. However, the expression of several proteinase inhibitors increased in response to water deficit and was associated with improved drought tolerance [84,85]. Overall, plant proteases mitigate stress-induced effects by degrading damaged, denatured, and aggregated proteins [86]. Uncontrolled proteolytic events induced by abiotic stress can be deleterious for plants; therefore, the presence of specific endogenous inhibitors that regulate protease activities is essential for the maintaining protein homeostasis [87]. Salt and drought stress differentially affect the accumulation of extracellular proteins in plants [88,89]. The regulation of secreted proteases and protease inhibitors plays an important role also in the apoplastic space [90]. In particular, some of the proteases modulated in response to drought in plants have been reported as located in apoplast/extracellular space [91].

4.3. Common stress-responsive Arabidopsis defensin family genes regulation

The *in silico* results reported here revealed a huge overlap between abiotic and biotic responses with 74 % of the over-expressed genes in common (Fig. S4), again indicating the multi-stress responsive nature of the defensin family genes. We also found a clear crosstalk between abiotic and biotic stress signaling pathways, as highlighted by phytohormone-associated genes and cis-regulatory element analyses. Jasmonates and abscisic acid (ABA) were the major hormones involved in both abiotic and biotic regulation of defensin family genes. In addition to its important role in a variety of plant developmental processes, ABA is the most important phytohormone of response to various environmental stresses [92]. On the other hand, herbivores attack [93,94], salt stress [95], wounding, drought, osmotic alteration [96], and UV irradiation [97] induce the jasmonate signaling in plants.

Several studies have highlighted the complex nature of the interactions between biotic and abiotic stress responses [98,99] and the important role played by plant hormones such as ABA and jasmonic acid, which are closely coordinated with Ca^{2+} signaling [100,101].

The search for regulators involved in the expression of stress-related defensin family genes revealed the important role of members of the WRKY and AP2/EREBP families. Furthermore, numerous promoter regions of defensin genes induced by both abiotic and biotic stresses contained binding sites for common transcription factors. In particular, binding regions for the transcription factor DDF2 (AT1G63030) were found in promoter sequences of defensin family genes induced by heat, cold, drought, nutritional and oxidative stresses, as well as by bacteria, viruses, and fungi. DDF2 is a member of the DREB subfamily A-1. Overexpression of *SlDDF2* in tomato plants enhanced tolerance to abiotic stresses such as water deficit, salinity, and cold [102].

Similarly, upstream regions of defensin family genes induced by heat, cold, drought, nutritional, oxidative, viruses, and fungi contained binding sites for the TF WRKY18 (AT4G31800). Plant WRKYs play a crucial role in plant defense responses and are involved in regulating the expression of various defense-related genes, including pathogenesis-related (PR) genes. Arabidopsis *WRKY18*, *WRKY40*, and *WRKY60* are induced by pathogens such as the hemibiotrophic bacterial pathogen *Pseudomonas syringae* and the necrotrophic fungal pathogen *Botrytis cinereal*, but also participate in plant responses to abscisic acid and abiotic stress [103]. Constitutive expression of *WRKY18* enhanced resistance to *P. syringae* [104].

4.4. In search of evidence for mechanism of action of Arabidopsis defensins and DEFLs

To gain functional insights into the biological processes affected by defensins and DEFLs during stress responses, a co-expression analysis of genes over-expressed after each abiotic or biotic stimulus was performed. Among the genes co-expressed with defensin family genes whose expression was triggered by all abiotic stresses considered in this study, the most enriched processes were related to mitochondrial electron transport and adenosine triphosphate (ATP) synthesis. In support of a potential mitochondrial involvement in the stress response mediated by defensins and DEFLs, several components of the mitochondrial membrane respiratory chain, mainly NADH-ubiquinone oxidoreductases (*AT4G16450, AT2G47690, AT3G62790, AT5G47890*) and cytochrome c oxidase subunits (*AT3G62400, AT4G28060*), were found as hub genes in co-expression networks. The cytochrome b-c1 complex (AT3G10860), a part of the mitochondrial respiratory chain complex III, also emerged as leading hub protein in the network analysis of genes co-expressed with defensin induced by salt, drought, and osmotic stress (Table S9).

The co-expression analysis suggests a putative link between the induction of defensin-related genes and genes involved in mitochondrial electron transport and ATP synthesis, although this connection remains to be elucidated. Mitochondria play a crucial role in cell survival by generating ATP, as well as controlling apoptosis and cell cycle [105]. In addition, mitochondria generate ROS as a byproduct of aerobic respiration [105], activating specific stress-inducible pathways [106].

Transgenic tobacco lines overexpressing the NAD1 defensin gene exhibit enhanced drought tolerance attributed to the activation of various antioxidant enzymes, leading to lower oxidative damage [107]. Similar outcomes were documented in Arabidopsis plants overexpressing the *Ca-AFP* defensin gene [74]. In addition, under water-deficit conditions, these transgenic plants exhibited altered activities of the plasma membrane NADPH oxidase and apoplastic peroxidases. These enzymes are crucial for the extracellular ROS production and signaling in response to biotic and abiotic stresses [108]. To transmit the signal, apoplastic ROS can traverse the membrane and access other subcellular compartments [109]. In particular, NADPH oxidase-derived ROS have been implicated in promoting mitochondrial alkalization under salt stress, suggesting a potential role in regulating oxidative phosphorylation rates [110]. In addition, it has been demonstrated that NADPH oxidases are implicated in the ABA-increased ROS accumulation not only in the cytoplasm but also in guard cell mitochondria. Overall these results suggest a possible function for ROS to mediate the communication between apoplast and mitochondria.

Another possible role for defensin family peptides in the response to drought and high temperatures is related to their interaction with the membranes. Several abiotic stresses, including heat and drought, can disrupt the structure and function of membranes, leading to adverse effects on plant health and growth [111]. Elevated temperatures can cause the lipid bilayer of plant membranes to become more fluid, altering the organization and stability of membrane proteins. On the other hand, water scarcity leads to a reduction in cell turgor pressure, which affects the hydration status of membrane lipids. As a result, the membrane becomes more rigid, affecting the movement and activity of membrane proteins and enzymes.

The relationship between AMPs and membranes is central to their antimicrobial activity [112]. The hydrophobic regions of AMPs are attracted to the hydrophobic core of the lipid bilayer, while the positively charged regions are attracted to negatively charged components of the membrane, such as phospholipid headgroups or surface proteins. Amphipathic cationic peptides, such as defensins, may rapidly generate fluid domains in a rigid bulk membrane [113–115]. Since membrane fluidity is closely related to the plant tolerance to extreme temperatures and drought [115–117], we speculate that defensins and DEFLs peptides may play a role in modifying membrane properties under these stressful conditions.

5. Conclusions

The main focus of this study was to perform an in-depth characterization of the defensin family peptides in *Arabidopsis thaliana*, focusing on their tissue- and stress-specific expression. *In silico* approaches allowed us to hypothesize a role of defensins and DEFLs also in abiotic stress response. *In vivo* analyses confirmed their induction by high temperature and water shortage.

The delivery of this knowledge may be employed in breeding strategies; several stress responsive genes emerging from this analysis are candidates for targeted improvement of crop traits and development of multi-stress resilient plants.

Author statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

All procedures were performed in compliance with relevant laws and institutional guidelines and have been approved by the appropriate institutional committee(s).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Author Contribution

GD, CV, VL and LDG conceived the project and designed the experiments. Material preparation, data collection and analysis were performed by GD and SC. GD, LC, SC, and MM performed most of the experiments and analyzed the data. GD and VL wrote the paper and collected the contributions of all authors. CV, MB and LDG commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.cpb.2024.100376.

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