Comprehensive in silico characterization of proteins associated with light responses, transport, and stress-related processes in the gametophyte of the fern Dryopteris affinis ssp. affinis

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Abstract

Background

. Fern gametophytes represent a key phase in the fern life cycle and play an essential role in establishment and persistence. Despite their ecological and developmental relevance, the molecular basis of gametophyte function remains poorly characterised, particularly in apogamous ferns. The growing availability of transcriptomic resources offers new opportunities to investigate the functional organisation of this life stage.

Results

. We conducted an *in silico* analysis of a previously published RNA-seq dataset obtained from gametophytes of the apogamous fern *Dryopteris affinis* ssp. *affinis*, focusing on the functional annotation of high-confidence protein candidates. Following stringent filtering, 1,160 proteins were identified and assigned to light-related processes, transport functions and stress responses. Light-associated proteins were primarily linked to photosynthesis, photorespiration, xanthophyll metabolism and photomorphogenesis, including components involved in photosystem assembly, chloroplast function and photoreceptor-mediated signalling. Transport-related proteins comprised a diverse set of membrane- and cytoplasm-associated transporters mediating the movement of sugars, amino acids, ions and other metabolites. In addition, numerous proteins associated with biotic and abiotic stress responses and plant immunity were detected. Protein–protein interaction analysis identified a small number of highly connected nodes, with MODIFIED TRANSPORT TO THE VACUOLE 17 (MTV17) emerging as the most connected protein, mainly supported by database- and text-mining-based evidence.

Conclusions

. This study presents a curated, high-confidence protein dataset that extends current knowledge of fern gametophyte molecular organisation. The functional categories and interaction patterns identified highlight core processes underlying gametophyte physiology and stress adaptation, and provide a resource for future functional, comparative and experimental studies in ferns and other early-diverging land plants.

1. Background

Ferns represent an early-diverging lineage of vascular plants and constitute an important component of terrestrial ecosystems worldwide [1, 2]. Their evolutionary relevance has made them key systems for understanding the origin and early diversification of vascular plant shoots and leaves [2]. A distinctive feature of the fern life cycle is the free-living gametophyte, a physiologically autonomous stage that develops independently and is directly exposed to environmental conditions [3]. Early developmental studies have shown that the transition from spore to gametophyte involves coordinated regulation of photosynthate production, ion uptake, and cellular polarity [3–6], highlighting the functional complexity of this life stage. Subsequent physiological and molecular studies have expanded this view, revealing conserved and divergent mechanisms governing germination, polarized cell growth, and early development in fern gametophytes [4–8]. Photosynthetic performance varies widely among ferns and fern allies, reflecting adaptation to diverse light environments and reinforcing the importance of light-dependent processes during gametophyte growth [9]. Environmental factors such as light, temperature, and nutrient availability strongly influence spore germination and gametophyte establishment, underscoring the high degree of functional plasticity characteristic of this stage [12].

Despite these advances, the molecular basis underlying gametophyte function remains comparatively underexplored, particularly in non-model fern species. Progress in fern molecular biology has historically been constrained by large genome sizes, high chromosome numbers, and complex karyotypes [31–33]. Recent work has emphasized the extraordinary scale of genome expansion in some fern lineages [34]. Although transcriptional analyses and genome assemblies are now available for a limited number of species [35–38], most ferns still lack reference genomes. In this context, transcriptome-based approaches have emerged as a practical and powerful alternative for investigating gene content and functional pathways in ferns, enabling comparative analyses in the absence of complete genomic resources [16, 19, 39].

The fern *Dryopteris affinis* (Lowe) Fraser-Jenk. ssp. *affinis* provides a suitable experimental system for gametophyte studies due to its efficient in vitro culture, rapid development, and amenability to microscopic and molecular analyses [14, 15]. This species reproduces by obligate apogamy, producing diploid gametophytes that give rise to sporophytes without fertilization, a reproductive strategy that has been discussed in the context of apomixis evolution in ferns [40–42]. Experimental studies have also examined the influence of growth regulators and culture conditions on apogamy in *D. affinis* [43]. Proteomic and transcriptomic approaches applied to this system have expanded available molecular information and provided insights into proteins associated with development, reproduction, and stress-related processes [14, 16–20].

A transcriptomic resource derived from D. *affinis* gametophytes has been generated and made publicly available, offering a valuable foundation for further in silico analyses [16, 20]. Building on this resource, the present study focuses on a curated and high-confidence subset of protein candidates associated with key functional processes operating in the gametophyte. Specifically, we targeted proteins involved in light-dependent processes (including photosynthesis, photorespiration, xanthophyll metabolism, and photomorphogenesis), transport functions, and responses to biotic and abiotic stress, all of which are central to gametophyte physiology and environmental adaptation [21–23].

The aim of this work was to provide a comprehensive functional characterization of these protein groups and to explore their potential interactions through protein–protein interaction network analysis. By refining existing transcriptomic information and integrating functional and network-based approaches, this study contributes to a clearer understanding of the molecular landscape of the *D. affinis* gametophyte and provides a curated resource for future comparative and experimental studies in ferns and other early-diverging land plants.

2. Materials and methods

2.1 Plant material, spore sterilization and *in vitro* culture

Fertile fronds of *Dryopteris affinis* (Lowe) Fraser-Jenk. ssp. *affinis* were collected in Turón forest (Asturias, Spain; 43°12'10" N, 5°43'43" W; 477 m a.s.l.). Taxonomic identification of the plant material was carried out by Prof. Herminio Severiano Nava Fernández, and a voucher specimen has been deposited in the Herbarium of the Department of Organisms and Systems, FCO, under accession number FCO44441. Plant material was collected from natural populations growing in the field. The sampling did not involve protected species or protected areas, and no specific permits were required according to local regulations. Collection complied with institutional and national guidelines.

2.2 RNA extraction and transcriptome resource. Spore set, surface sterilization and in vitro culture conditions followed previously published protocols for this species and are therefore summarized here only briefly [15, 16, 43]. Spores were collected from mature sporangia, surface-sterilized and cultured on Murashige and Skoog (MS) medium [44] supplemented with sucrose under controlled temperature and photoperiod conditions. Gametophytes corresponding to spatulate and cordate developmental stages were obtained under standard in vitro culture conditions, pooled, frozen in liquid nitrogen and stored at − 80°C until RNA extraction.

2.2 RNA extraction, sequencing and transcriptome resource

RNA extraction, library preparation and sequencing procedures were performed as previously described [16]. Briefly, total RNA was isolated from pooled gametophyte samples, treated to remove genomic DNA, quality-checked, and sequenced using Illumina technology. The resulting Transcriptome Shotgun Assembly project is publicly available at the European Nucleotide Archive (ENA) under accession number PRJEB18522. The assembled transcriptome and associated annotations were deposited in Zenodo [45]. Redundant transcript sequences were collapsed and transcriptome completeness was assessed following established workflows [46, 47]. This curated transcriptome dataset constituted the basis for all subsequent in silico analyses performed in the present study.

2.3. Computational analysis and functional annotation

Because a reference genome for *Dryopteris affinis* is not yet available, transcript sequences were functionally annotated by comparison with Arabidopsis thaliana. BLASTX searches were conducted using Geneious Prime v2023.2.1 [48] against the Araport11 protein database. Only transcript annotations longer than 450 bp and displaying an E-value < 1×10^{-100} were retained to ensure high-confidence homolog identification. Redundant annotations were removed, and proteins associated exclusively with general metabolic processes were excluded from further analyses. The resulting curated dataset was subsequently filtered to focus on proteins related to light-dependent processes (including photosynthesis, photorespiration, xanthophyll metabolism and photomorphogenesis), transport functions, and responses to biotic and abiotic stress. Functional enrichment analyses were performed using STRING v12.0 [49] and UniProt-based annotations [50], including Gene Ontology (GO) biological process, molecular function and cellular component categories, as well as Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways [51]. Enrichment significance was evaluated using false discovery rate (FDR) correction according to the Benjamini–Hochberg method, and only categories with FDR < 0.05 were considered statistically significant.

Protein–protein interaction networks were explored using STRING with a high-confidence interaction threshold (0.700). Network visualization and further analyses were performed using Cytoscape v3.10.3 [53] and R v4.3.2 [52], including the generation of enrichment plots and gene–category association networks. In addition, selected *D. affinis* transcripts were queried against available fern genomes in FernBase [39] to provide comparative context within monilophytes.

3. Results

Our analysis yielded a broad set of Arabidopsis thaliana homologs expressed in the gametophyte of the non-sequenced fern *Dryopteris affinis*. After applying the E-value cutoff (≤ 1×10^{-100}) and removing redundant annotations, we excluded proteins assigned exclusively to general metabolic functions, while retaining those related to photosynthesis, photorespiration, and xanthophyll metabolism, which were central to the present study. This filtering step resulted in approximately 3,000 annotated proteins.

From this pool, we focused on 1,160 proteins associated with light-related processes, transport, and stress responses. The distribution of proteins across the selected functional groups is summarized in Fig. 2. Transport represented the largest fraction of the curated set (approximately 45%), followed by proteins involved in responses to biotic stress (11%) and photomorphogenesis (7%).

Table 1 summarizes enrichment statistics for the main biological categories, including protein counts, enrichment strength, and adjusted significance (FDR), as provided by STRING. Collectively, these results indicate a strong enrichment in light-dependent functions (photosynthesis, photorespiration, photomorphogenesis, and wavelength-specific light responses), as well as in transport processes and stress-response pathways.

Table 1
Functional enrichment of the protein dataset, in the gametophyte of *Dryopteris affinis*, highlighting light-dependent processes, transport functions, and responses to biotic and abiotic stresses. Columns show protein counts, enrichment strength, and adjusted significance (FDR).

Biological function	Protein count	Strength	FDR
Photosynthesis	54 of 250	0.71	$4.43 \cdot 10^{-61}$
Photorespiration	16 of 53	0.85	$1.51 \cdot 10^{-29}$
Xanthophyll metabolism	9 of 15	1.15	0,000407
Photomorphogenesis	23 of 67	0.97	$3.24 \cdot 10^{-9}$
Response to red or far-red light	50 of 210	0.75	$1.9 \cdot 10^{-16}$
Response to blue light	17 of 90	0.65	0.0023
Response to UV	30 of 113	0.8	$3.79 \cdot 10^{-10}$
Transport	663 of 2,659	0.77	0
Response to the bacterium	103 of 473	0.71	$1.52 \cdot 10^{-33}$
Response to nematode	25 of 82	0.86	$3.01 \cdot 10^{-9}$
Response to wounding	38 of 228	0.6	$4.74 \cdot 10^{-8}$
Response to water deprivation	71 of 368	0.66	$5.68 \cdot 10^{-60}$
Response to oxidative stress	68 of 434	0.57	$2.26 \cdot 10^{-82}$
Response to salt stress	86 of 444	0.66	$3.52 \cdot 10^{-166}$
Response to starvation	52 of 207	0.77	$4.79 \cdot 10^{-18}$
Response to cold	96 of 391	0.76	$1.12 \cdot 10^{-34}$
Response to heat	72 of 254	0.83	$1.28 \cdot 10^{-28}$

3.1. Proteins associated with light: photosynthesis, photorespiration and xanthophyll metabolism

The proteins associated with light were categorised according to the Gene Ontology (GO). Regarding the biological function, two main groups are deemed: one involved in the processes of photosynthesis, photorespiration and xanthophyll metabolism, while the other plays a significant role in photomorphogenesis. As for the first group, the molecular function of most of the proteins was linked to the binding of molecules such as ions and nucleic acids, followed by catalytic and oxidoreductase activities. In terms of the GO classification of cellular components, many of the proteins were found within the cytoplasmic space and plastid organelles, as well as in the cell membrane. The most prevalent protein domains included chlorophyll A-B binding and aldolase-type TIM barrel.

The enrichment analysis identified several biological processes significantly associated with the corresponding genes. To illustrate these associations, gene–category interaction networks (Cnetplots) were generated for this group of proteins (Fig. 3). Each network displays the relationships between enriched GO terms and their corresponding genes. Only categories that remained statistically significant after multiple testing correction using the false discovery rate (FDR < 0.05) were retained, ensuring that the visualized processes reflect robust functional associations.

Upon examining the biological functions of the *A. thaliana* homologue proteins devoted to photosynthesis, it was evident that they primarily participate in the electron transport chain. Key proteins in this process are PROTON GRADIENT REGULATION 6 (PGR6), a kinase that regulates the function of plastoquinone, β-carotene, and xanthophyll lutein homeostasis, LEAF-TYPE CHLOROPLAST-TARGETED FNR 1 (LFNR1) and 2 (LFNR2), which manage electron flow to fulfil the plant's requirements for ATP and reducing power, and K (+) EFFLUX ANTIPORTER 3 (KEA3), which promotes photosynthesis when the chloroplast ATP synthase activity is low, by reducing the pH gradient across the thylakoid membrane. In addition, proteins involved in the formation, assembly and stabilisation of photosystem II were identified, such as HIGH CHLOROPHYLL FLUORESCENCE 136 (HCF136) along with two auxiliary proteins: HIGH CHLOROPHYLL FLUORESCENCE PHENOTYPE 173 (HCF173) and 244 (HCF244), and CYCLOPHILIN 38 (CYP38). Besides, a significant number of subunits of the light-harvesting complex, which captures and transfers excitation energy to photosystems, were present, including LIGHT-HARVESTING CHLOROPHYLL B-BINDING 2 (LHCB2), PHOTOSYSTEM I LIGHT HARVESTING COMPLEX GENE 1 (LHCA1), and LIGHT HARVESTING COMPLEX PHOTOSYSTEM II (LHCB4). Associated with this, a protein necessary to maintain the efficiency of the light-harvesting complex, SUPPRESSOR OF QUENCHING 1 (SOQ1), was annotated. Likewise, three subunits of the magnesium-chelatase complex involved in chlorophyll biosynthesis were found. In support of a proper chloroplast development, two proteins are reported: ENHANCER OF VARIEGATION 3 (EVR3) and FTSH PROTEASE 1 (FTSH1). Furthermore, proteins related to various other categories, including carbon fixation, as well as regulators of photosynthesis and stomatal movement, were also noticeable.

Within the group of homologs involved in photorespiration, various enzymes were identified as key players in the photorespiratory core cycle, including HYDROXYPYRUVATE REDUCTASE (HPR) and HYDROXYPHENYLPYRUVATE REDUCTASE 2 (HPPR2). Additionally, the regulation of amino acid biosynthesis and catabolism during photorespiration involves GLUTAMATE: GLYOXYLATE AMINOTRANSFERASE 1 (GGAT1). Proteins associated with protein homotrimerization, biosynthesis of hydrogen peroxide, and tetrahydrofolate interconversion were also observed.

Regarding xanthophyll metabolism, several homologs involved in its biosynthesis were detected in the transcriptome, including β-CAROTENOID HYDROXYLASE 1 (BCH1) and LUTEIN DEFICIENT 5 (LUT5). In addition, VIOLAXANTHIN DE-EPOXIDASE 1 (VDE1), a protein that regulates the concentration of zeaxanthin in chloroplasts as part of the xanthophyll cycle, was also identified.

3.2. Proteins associated with light: photomorphogenesis

The enrichment analysis highlighted key biological processes related to the genes of interest. In particular, photomorphogenesis emerged as significantly represented. Among the proteins reported here, a considerable number were associated with this process, which describes the effects of light on plant development. It is well-established that plants respond to various light wavelengths, including red, far-red, blue, and UV. Ion and DNA binding, as well as catalytic and transferase activities, were the predominant molecular functions, mainly associated with the nucleus and cytoplasm, and being WD-40 repeats and PAS the most abundant domains.

The analysis of enriched GO terms of biological functions in this group of proteins associated with photomorphogenesis (Fig. 4) reveals proteins involved in processes mediated by continuous far-red light, such as LONG AFTER FAR-RED 3 (LAF3), and by red and far-red light, such as PHYTOCHROME B (PHYB). In addition, two transcription factors implicated in phytochrome signalling were identified: VASCULAR PLANT ONE ZINC FINGER PROTEIN (VOZ1), which acts on phytochrome B, and PHYTOCHROME A SIGNAL TRANSDUCTION 1 (PAT1), which acts on phytochrome A. Besides, several serine/threonine-protein phosphatases and kinases are linked to the regulation of phytochromes. For the response to blue light, notable proteins implicated included CRYPTOCHROME 1 and 2 (CRY1 and 2), PHOTOTROPIN-2 (PHOT2), and G-PROTEIN COUPLED RECEPTOR 1 (GCR1). Additionally, ADAGIO 1 (ADO1), a component of an E3 ubiquitin ligase complex involved in the blue light-dependent circadian cycle, was identified. Regarding the response of gametophytes after being exposed to UV light, several homologs were identified involved in repairing DNA damage, including UV RESISTANCE 2 and 3 (UVR2 and UVR3). Related to the response to UV-B specifically, homologs that confer resistance and tolerance to this wavelength were found, such as HOMOLOG OF REVERSIONLESS 1 (REV1) and WD REPEAT-CONTAINING PROTEIN CSA-1 (CSA1). Also, some proteins focused on root protection against UV-B were found: ROOT UV-B SENSITIVE 1 (RUS1) and 2 (RUS2). Likewise, the numerous subunits of the COP9 signalosome complex, relevant in photomorphogenesis, are highlighted. Particularly, seven components of this complex were identified in the transcriptome. Moreover, several additional proteins were detected, including ROOT PHOTOTROPISM PROTEIN 3 (JK218), a signal transducer involved in the phototropic response; DAMAGED DNA BINDING PROTEIN 1A (DDB1A), a component of the light signal transduction machinery; and EMBRYO DEFECTIVE 168 (EMB168), an E3 ubiquitin-protein ligase. The latter two proteins are implicated in the repression of photomorphogenesis in darkness. Besides, a protein related to the repression of photomorphogenesis in light, SPA1-RELATED 3 (SPA3), was found.

As well as photomorphogenesis, gravitropism also affects plant growth, in which the gravity modulates its direction. In our protein set, some homologs related to this process were identified, such as those implicated in the amyloplast sedimentation in the endodermis during shoot gravitropism, where they act as statoliths, named SHOOT GRAVITROPISM 1 (SGR1) and 2 (SGR2). As it is well known, the gametophyte of ferns lacks endodermis.

3.3. Proteins associated with transport

In this work, a total of 659 different proteins associated with transport were found. Functional enrichment analysis revealed that several transport-related GO terms were significantly overrepresented (Fig. 5). The dot plots highlight the main biological processes (BP) and molecular functions (MF) associated with transport (Fig. 5a, b), including a strong enrichment for ATP-binding cassette (ABC) transporter activity, being the primary protein domains the ABC transporter transmembrane region and the mitochondrial carrier. To further explore these associations, an interaction network of ABC transporter genes was constructed using Cytoscape (Fig. 5c).

The classification of biological functions points out that the most abundant categories were associated with transmembrane transport, cellular localisation, protein and ion transport, and vesicle-mediated transport. These proteins bind to a pleiad of compounds like anions and cations, hormones, proteins, lipids, carbohydrates, nucleic acids, nitrogenous bases, etc., and move them through the cell. In terms of cellular components, nearly all the proteins were localised in the cell membrane and the cytoplasm.

Notably, as shown in Fig. 5c, a significant number of ATP-binding cassette (ABC) transporters were identified. Specifically, the gene count revealed a total of 47 different proteins belonging to this family, which were type A, B, C, D, E or G. This group included members involved in hormone transport, such as ATP-BINDING CASSETTE B1 (ABCB1) and G40 (ABCG40); in fatty acids transport, such as D1 (ABCD1); as well as those responsible for ion transport, like C5 (ABCC5), which regulates K^+ and Na^+ levels in the cell.

As mentioned above, the proteins involved in transport encompass a long archive, which is briefly commented. Then, some examples of transporters of different compounds, such as AMINO ACID PERMEASE 2 (AAP2), LYSINE HISTIDINE TRANSPORTER 1 (LHT1), and CATIONIC AMINO ACID TRANSPORTER 1 (CAT1), with affinity, as its name suggests, for cationic amino acids. Other important transporters included an ATP/ADP/AMP exchanger in the inner mitochondrial membrane: ADENINE NUCLEOTIDE TRANSPORTER 1 (ADNT1); a protein involved in ammonium uptake from the soil and transport to the shoots: AMMONIUM TRANSPORTER 1 (AMT1); other relative that facilitates ammonium transport between the apoplast and symplast of the cells: AMMONIUM TRANSPORTER 2 (AMT2); and a sugar proton-coupled antiporter located in the vacuole: TONOPLAST SUGAR TRANSPORTER 2 (TST2).

Another important group of transporters are represented by all those involved in the endocytosis and exocytosis traffic. In line with it, multiple subunits of the clathrin-associated adaptor protein complex and the exocyst complex were reported. In terms of nuclear import, components of the nuclear pore complex, such as NUCLEOPORIN 155 (NUP155), were identified. Furthermore, a protein essential for the transport of adenine, guanine and uracil was also noted: PLASTIDIC NUCLEOBASE TRANSPORTER (PLUTO). Another remarkable result was the obtaining of various aquaporins, i.e. channels in the cell membrane that facilitate water permeability, among them NAMED PLASMA MEMBRANE INTRINSIC PROTEIN 1;2 (PIP1;2) and 2;1 (PIP2;1). In the gametophyte of *D. affinis*, some mechanosensitive ion channels were also observed, such as MECHANOSENSITIVE ION CHANNEL PROTEIN 1 (MSL1), which opens in response to stretch forces in the membrane lipid bilayer.

Following with ion transport, some additional examples can be given, such as a boron transporter from roots to shoots: BORON TRANSPORTER 1 (BOR1); a calcium-dependent protein that catalyses the import of ATP co-transported with metal divalent cations across the mitochondrial inner membrane in exchange of phosphate: ATP/PHOSPHATE CARRIER 2 (APC2); a K^+/H^+ antiporter that regulates K^+ uptake and homeostasis in the cell: CATION/H+ EXCHANGER 17 (CHX17); and a homolog needed in copper import into the cell: HEAVY METAL ASSOCIATED PROTEIN 51 (HMP51). Finally, it is worth emphasizing the identification of numerous transporters for molecules such as nitrate, magnesium, zinc, sulphate, sucrose, citrate, and phosphate.

3.4. Proteins associated with stress

The proteins related to stress were categorised into two groups based on the type of stressor: biotic stress (resulting from bacteria, nematodes, or viruses) and abiotic stress (resulting from factors such as water deprivation, oxidative stress, salt stress, starvation, cold, or heat). The classification of molecular functions predominantly featured binding and catalytic activities, while the cellular component classification was predominantly represented by the cytoplasm and the cell membrane. Identified protein domains included chlorophyll A-B binding and the DnaJ central domain. The set of proteins extracted from *D. affinis* gametophytes related to biotic stress is shown in Fig. 6.

Many *A. thaliana* homologous proteins reported were involved in resistance to both bacterial and fungal attacks, including CERAMIDASE (ACER), AMINOTRANSFERASE ALD1 (ALD1), L-TYPE LECTIN RECEPTOR KINASE S.4 (LECRK-S.4), GUANINE NUCLEOTIDE-BINDING PROTEIN SUBUNIT β (AGB1), and β-GLUCOSIDASE 42 (BGLU42). Additionally, two proteins involved in regulating defence against bacteria — HOPW1-1-INTERACTING 1 (WIN1) and 2 (WIN2) — were detected, along with a homolog of the receptor FLAGELLIN-SENSITIVE 2 (FLS2), which recognises bacterial flagellin, a pathogen-associated molecular pattern that triggers plant defence responses. This was complemented by a protein involved in fungal defence that also restricts bacterial growth: LYSM-CONTAINING RECEPTOR-LIKE KINASE 1 (LYK1). It is worth mentioning homologs that play roles in defence against insects, such as SIGNAL RESPONSIVE 1 (SR1), and against nematodes, such as EMBRYO DEFECTIVE 2728 (EMB2728).

Related to biotic stress, some proteins involved in plant immunity were annotated, such as BRASSINOSTEROID-SIGNALING KINASE 1 (BSK1), NON-RESPONDING TO OXYLIPINS 7 (NOXY7), SERINE/THREONINE-PROTEIN KINASE/ENDORIBONUCLEASE IRE1A (IRE1A), PLANT U-BOX 59 (PUB59), SUPPRESSOR OF CPR5 44 (SCPR44), and ASPARTYL PROTEASE APCB1 (APCB1). A few proteins essential for the induction, establishment, and activation of systemic acquired resistance, such as TRANSCRIPTION FACTOR TGA2 (TGA2), FLAVIN-DEPENDENT MONOOXYGENASE 1 (FMO1), and SUPPRESSOR OF FATTY ACID DESATURASE DEFICIENCY 1 (SFD1), were also identified. Additionally, a homolog involved in chitin-triggered immune signalling, PBS1-LIKE 34 (PBL34), and another regulating the hypersensitive response, RESPIRATORY BURST OXIDASE PROTEIN F (RBOH F), were found. Several proteins linked to immunity signalling, such as MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE 1 (ARAKIN) and PBS1-LIKE 39 (PBL39), were also observed.

In addition, a protein related to cell death as a defence mechanism was included: PLEIOTROPIC DRUG RESISTANCE 8 (PDR8). E3 UBIQUITIN-PROTEIN LIGASE NLA (SYG1) was associated with general defence responses, while CALLOSE SYNTHASE 12 (CALS12) was linked to callose formation after wounding. Similarly, other homologs that regulate the biosynthesis of metabolites responsible for defence, such as GREENING AFTER EXTENDED DARKNESS 1 (GED1); related to disease resistance, such as NRL PROTEIN FOR CHLOROPLAST MOVEMENT1 (NCH1); and to non-host disease resistance, such as ORNITHINE-Δ-AMINOTRANSFERASE (Δ-OAT), were notable findings.

Transitioning from biotic to abiotic stress, we observed that *A. thaliana* homolog proteins associated with various stressors, such as water deprivation, oxidative stress, salt stress, starvation, cold, and heat, were well-documented. Some networks can be seen in Fig. 7.

Focusing first on water deprivation, several proteins involved in the response to dehydration were identified, including one which enhances its tolerance by improving ion retention, called PYROPHOSPHATE-ENERGIZED VACUOLAR MEMBRANE PROTON PUMP 1 (VHP1), and another which regulates drought responses, named CALCINEURIN B-LIKE PROTEIN 1 (CBL1).

The gametophyte also contains homologs related to oxidative stress. Some examples were an enzyme which restores the functionality of proteins that have been inactivated through methionine oxidation: METHIONINE SULFOXIDE REDUCTASE A4 (MSRA4); two proteins involved in resistance to oxidative stress by intervening in reactive oxygen species production: ACTIVITY OF BC1 COMPLEX KINASE 7 (ABC1K7) and 8 (ABC1K8); other that contributes to maintain the reactive oxygen species homeostasis in mitochondria: ALKALINE/NEUTRAL INVERTASE A (A/N-INVA); and LIPOCALIN IN THE PLASTID (LCNP), which prevents thylakoidal membrane lipids peroxidation, conferring it protection against oxidative stress. Other noteworthy achievements were a protein that helps plants to perceive singlet oxygen in plastids, avoiding any possible photooxidative damage that can take place: EXECUTER 2 (EXE2); the protein Γ-GLUTAMYL TRANSPEPTIDASE 1 (GGT1), which prevents oxidative stress by metabolizing extracellular oxidized molecules of glutathione; and GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE C SUBUNIT 1 (GAPC1), responsible for plant adaptation to conditions generated by oxidative stress. Other noteworthy findings included a protein that intervenes in redox homeostasis, named GLYOXYLATE/SUCCINIC SEMIALDEHYDE REDUCTASE 1 (GLYR1), and another that promotes oxidative stress resistance, called NEUTRAL CERAMIDASE 1 (NCER1), along with several ascorbate peroxidases that eliminate hydrogen peroxide.

Homologs associated with salt stress resistance and tolerance included α-MANNOSIDASE 2 (GMII), PRE-mRNA SPLICING FACTOR SR-LIKE 1 (SRL1), PROTEIN PHOSPHATASE 2C GROUP 1 (PP2CG1), and BEACH-DOMAIN HOMOLOG A1 (BCHA1). Moreover, two proteins essential for generating responses to salt stress were DEAD-BOX ATP-DEPENDENT RNA HELICASE 3 (RH3) and MA3 DOMAIN-CONTAINING TRANSLATION REGULATORY FACTOR 3 (MRF3); whereas PROTEIN KINASE 2 (PK2) is involved in plant adaptation to high salinity conditions.

Regarding starvation, the *D. affinis* gametophyte exhibited a range of *A. thaliana* homologous proteins: PEROXISOME UNUSUAL POSITIONING 2 (PEUP2), essential for autophagosome formation under nutrient-limited conditions; 14-3-3-LIKE PROTEIN GF14 PSI (GRF3), which regulates nutrient metabolism; 2-OXOISOVALERATE DEHYDROGENASE SUBUNIT β 2 (DIN4), required during sugar starvation; SOS2-LIKE PROTEIN KINASE 17 (PKS17), conferring tolerance to low potassium conditions; and PROTEIN KINASE 6 (PKS6), required for potassium homeostasis under limited availability of this ion. Significant findings were observed in an activator of the amino acid biosynthesis pathway required during adaptation to amino acid starvation: GENERAL CONTROL NON-DEREPRESSIBLE 2 (GCN2), and in a protein which alleviates the stress generated during copper-limiting periods: KIN17-LIKE PROTEIN (KIN17). In addition, we detected PHOSPHATIDIC ACID PHOSPHOHYDROLASE 1 (PAH1), an enzyme involved in galactolipid synthesis in the endoplasmic reticulum, required for membrane lipid remodelling, as an essential adaptation to phosphate starvation.

For combating cold stress, the fern gametophyte relied on diverse proteins, including several calmodulin-binding transcription activators that contribute to freezing resistance. Related to this, we annotated 3-HYDROXYISOBUTYRYL-COA HYDROLASE 1 (CHY1), involved in cold signalling and tolerance; SENSITIVE TO FREEZING 2 (SFR2), which also plays a role in freezing tolerance; and ENOLASE 2 (ENO2), a regulator of cold-responsive gene transcription. In addition, we found Δ (8)-FATTY-ACID DESATURASE 2 (SLD2), an enzyme required for sphingolipid formation, necessary for cold resistance; other proteins crucial for maintaining chloroplast membrane integrity during low temperatures, such as β-KETOACYL-ACP SYNTHETASE 2 (KAS2); and other needed in chloroplast biogenesis in frigid conditions: PALEFACE 1 (PFC1). The set of proteins associated with cold stress is completed by LONG-CHAIN BASE KINASE 2 (LCBK2), which is implicated in reduced root growth under freezing conditions.

On the other hand, the response to heat stress involved several noteworthy homologous proteins, such as a FORGETTER 1 (FGT1), which facilitates the acquisition of heat stress memory; HEAT INTOLERANT 4 (HIT4), a protein needed for basal thermotolerance, regulating transcriptional gene silencing; and SERINE/THREONINE-PROTEIN PHOSPHATASE 7 (PP7), which confers thermotolerance to high temperatures. Likewise, a high number of chaperones and heat shock proteins expanded the annotations.

After performing a general analysis of the entire set of proteins related to light, transport and stress, approximately 180 proteins were selected (see Table 2). Their sequences are provided in **Supplementary Table 1**. The sole criterion for selection criterion was the availability and abundance of information.

Table 2
Selected proteins found in gametophytes of *Dryopteris affinis* associated with light, transport and stress.

Category	UniProtKB/ Swiss-Prot	Gene name	Protein name	MW (kDa)	Amino acids	E-value	Number of transcripts	Transcriptom identifier
Photosynthesis	Q8RWG1	*PGR6*	PROTON GRADIENT REGULATION 6	77	682	0	1	165950-172
	Q9FKW6	*LFNR1*	LEAF-TYPE CHLOROPLAST-TARGETED FNR 1	40	386	$1.42{\cdot}10^{-173}$	2	544–2004
	Q8W493	*LFNR2*	LEAF-TYPE CHLOROPLAST-TARGETED FNR 2	41	369	$1.03{\cdot}10^{-180}$	2	189389-147
	Q9M0Z3	*KEA3*	K (+) EFFLUX ANTIPORTER 3	84	776	0	4	242805-104
	O82660	*HCF136*	HIGH CHLOROPHYLL FLUORESCENCE 136	44	403	0	1	170491-166
	Q8W4D6	*HCF173*	HIGH CHLOROPHYLL FLUORESCENCE PHENOTYPE 173	66	598	0	1	191979-145
	O65502	*HCF244*	HIGH CHLOROPHYLL FLUORESCENCE PHENOTYPE 244	44	395	0	1	153419-190
	Q9SSA5	*CYP38*	CYCLOPHILIN 38	48	437	$9.72{\cdot}10^{-148}$	1	165189-173
	Q9S7J7	*LHCB2*	LIGHT-HARVESTING CHLOROPHYLL B-BINDING 2	29	265	$1.38{\cdot}10^{-159}$	7	80207-282
	Q01667	*LHCA1*	PHOTOSYSTEM I LIGHT HARVESTING COMPLEX GENE 1	26	241	$2.76{\cdot}10^{-100}$	2	26916-486
	Q9XF88	*LHCB4*	LIGHT HARVESTING COMPLEX PHOTOSYSTEM II	31	287	$1.98{\cdot}10^{-133}$	1	86125-270
	Q8VZ10	*SOQ1*	SUPPRESSOR OF QUENCHING 1	114	1,055	0	1	10132-749
	Q949Y5	*EVR3*	ENHANCER OF VARIEGATION3	60	548	0	1	865–1735
	Q39102	*FTSH1*	FTSH PROTEASE 1	77	716	0	1	168–2720
Photorespiration	Q9C9W5	*HPR*	HYDROXYPYRUVATE REDUCTASE	42	387	0	1	342338-47
	Q9CA90	*HPPR2*	HYDROXYPHENYLPYRUVATE REDUCTASE 2	34	313	$7.57{\cdot}10^{-124}$	9	346800-45
	Q9LR30	*GGAT1*	GLUTAMATE:GLYOXYLATE AMINOTRANSFERASE 1	53	481	0	3	62491-320
Xanthophyll metabolism	Q9SZZ8	*BCH1*	β-CAROTENOID HYDROXYLASE 1	34	310	$2.61{\cdot}10^{-112}$	4	59405-329
	Q93VK5	*LUT5*	LUTEIN DEFICIENT 5	67	595	0	2	10333-743
	Q39249	*VDE1*	VIOLAXANTHIN DE-EPOXIDASE 1	52	462	0	1	273162-84
Photomorphogenesis	A0A1I9LN01	*LAF3*	LONG AFTER FAR-RED 3	63	576	$2.32{\cdot}10^{-175}$	1	216051-124
	P14713	*PHYB*	PHYTOCHROME B	129	1,172	0	9	339708-48
	Q9LDL7	*PAT1*	PHYTOCHROME A SIGNAL TRANSDUCTION 1	55	490	$1.58{\cdot}10^{-149}$	5	19326-565
	Q9SGQ0	*VOZ1*	VASCULAR PLANT ONE ZINC FINGER PROTEIN	54	486	$1.22{\cdot}10^{-147}$	1	7950 – 830
	P48484	*TOPP4*	TYPE ONE SERINE/THREONINE PROTEIN PHOSPHATASE 4	36	321	0	4	3684 – 1120
	Q2MHE4	*HT1*	HIGH LEAF TEMPERATURE 1	44	390	$2.32{\cdot}10^{-106}$	3	198537-139
	Q9FKP4	*KAC2*	KINESIN-LIKE PROTEIN KIN-14B	140	1,264	0	1	161494-178
	P93025	*PHOT2*	PHOTOTROPIN-2	102	915	0	3	1171–1634

Category	UniProtKB/ Swiss-Prot	Gene name	Protein name	MW (kDa)	Amino acids	E-value	Number of transcripts	Transcriptom identifier
	Q43125	*CRY1*	CRYPTOCHROME 1	77	681	0	8	247818-101
Category	**UniProtKB/ Swiss-Prot**	**Gene name**	**Protein name**	**MW (kDa)**	**Amino acids**	**E-value**	**Number of transcripts**	**Transcriptom identifier**
Photomorphogenesis	Q96524	*CRY2*	CRYPTOCHROME 2	69	612	$2.37 \cdot 10^{-175}$	2	91249-262
	O04714	*GCR1*	G-PROTEIN COUPLED RECEPTOR 1	37	326	$1.85 \cdot 10^{-121}$	1	128995-216
	Q94BT6	*ADO1*	ADAGIO 1	66	609	0	5	42715-390
	Q9SQI2	*FB*	GIGANTEA	128	1,173	0	1	407541-21
	Q9SYX2	*SPA1*	SUPPRESSOR OF PHYA-105 1	115	1,029	0	5	282059-79
	Q9SB00	*UVR2*	UV RESISTANCE 2	56	490	0	1	258621-94
	O48652	*UVR3*	UV RESISTANCE 3	64	556	$1.29 \cdot 10^{-163}$	2	10126-750
	O04716	*MSH6*	MUTS HOMOLOG 6	147	1,324	0	3	169778-167
	Q9FLF7	*HAM1*	HISTONE ACETYLTRANSFERASE OF THE MYST FAMILY 1	51	445	0	4	153569-190
	A3EWL3	*REV1*	HOMOLOG OF REVERSIONLESS 1	122	1,105	0	3	56998-336
	Q93ZG3	*CSA1*	WD REPEAT-CONTAINING PROTEIN CSA-1	50	450	$2.3 \cdot 10^{-122}$	2	215636-125
	Q9FN03	*UVR8*	UV RESISTANCE LOCUS 8	47	440	0	7	335586-50
	Q7X6P3	*RUS1*	ROOT UV-B SENSITIVE 1	66	608	$8.23 \cdot 10^{-133}$	1	192337-144
	Q9SJX7	*RUS2*	ROOT UV-B SENSITIVE 2	48	433	$2.5 \cdot 10^{-175}$	1	210429-129
	P45432	*CSN1*	COP9 SIGNALOSOME COMPLEX SUBUNIT 1	51	441	0	1	635–1906
	Q8W207	*CSN2*	COP9 SIGNALOSOME COMPLEX SUBUNIT 2	51	439	0	3	65101-313
	Q8W206	*CSN6A*	COP9 SIGNALOSOME COMPLEX SUBUNIT 6A	36	317	$2.35 \cdot 10^{-139}$	1	66102-311
	Q9M0V3	*DDB1A*	DAMAGED DNA BINDING PROTEIN 1A	121	1,088	0	1	228–2495
	P43254	*EMB168*	EMBRYO DEFECTIVE 168	76	675	0	1	189856-147
	Q9LJR3	*SPA3*	SPA1-RELATED 3	93	837	$1.09 \cdot 10^{-174}$	2	292671-72
	Q9FMF5	*JK218*	ROOT PHOTOTROPISM PROTEIN 3	82	746	0	6	326476-54
	Q9M384	*SGR1*	SHOOT GRAVITROPISM 1	72	653	$3.06 \cdot 10^{-170}$	3	317100-59
	Q8W5R2	*SGR2*	SHOOT GRAVITROPISM 2	106	933	0	1	380210-31
Transport	Q9ZR72	*ABCB1*	ATP-BINDING CASSETTE B1	141	1,286	0	5	13378-662
	Q9SRU2	*CRM1*	CORYMBOSA1	568	5,098	0	1	170710-166
	Q96247	*AUX1*	AUXIN TRANSPORTER PROTEIN 1	54	485	0	5	270726-86
	Q9M9E1	*ABCG40*	ATP-BINDING CASSETTE G40	161	1,423	0	19	18622-575
	Q94FB9	*ABCD1*	ATP-BINDING CASSETTE D1	150	1,337	0	4	153527-190
	Q7GB25	*ABCC5*	ATP-BINDING CASSETTE C5	169	1,514	0	9	86261-270

Category	UniProtKB/ Swiss-Prot	Gene name	Protein name	MW (kDa)	Amino acids	E-value	Number of transcripts	Transcriptom identifier
	O65718	*CNGC2*	CYCLIC NUCLEOTIDE GATED CHANNEL 2	83	726	0	2	256364-95
	Q94AS9	*DND2*	DEFENSE, NO DEATH 2	80	694	$2.88 \cdot 10^{-172}$	1	274100-84
	Q9SRH5	*VDAC1*	VOLTAGE DEPENDENT ANION CHANNEL 1	29	276	$8.01 \cdot 10^{-102}$	5	27884-478
Category	**UniProtKB/ Swiss-Prot**	**Gene name**	**Protein name**	**MW (kDa)**	**Amino acids**	**E-value**	**Number of transcripts**	**Transcriptom identifier**
Transport	Q8VZL4	*MSL1*	MECHANOSENSITIVE ION CHANNEL PROTEIN 1	54	497	$5.91 \cdot 10^{-118}$	3	187440-149
	Q9FN18	*NRAMP4*	NATURAL RESISTANCE ASSOCIATED MACROPHAGE PROTEIN 4	56	512	0	1	178438-158
	Q8VYR7	*BOR1*	BORON TRANSPORTER 1	79	704	0	2	4056 – 1078
	Q9FI43	*APC2*	ATP/PHOSPHATE CARRIER 2	56	498	$2.67 \cdot 10^{-175}$	3	311606-62
	Q9SUQ7	*CHX17*	CATION/H+ EXCHANGER 17	89	820	0	3	340679-48
	Q9S7J8	*HMP51*	HEAVY METAL ASSOCIATED PROTEIN 51	107	1,001	0	3	181448-155
	Q06611	*PIP1;2*	NAMED PLASMA MEMBRANE INTRINSIC PROTEIN 1;2	31	286	$4.51 \cdot 10^{-160}$	2	82340-277
	P43286	*PIP2;1*	NAMED PLASMA MEMBRANE INTRINSIC PROTEIN 2;1	30	287	$2.66 \cdot 10^{-153}$	5	352913-42
	Q42510	*MIZ2*	MIZU-KUSSEL2	163	1,451	0	4	77921-286
	Q84K25	*COG8*	CONSERVED OLIGOMERIC GOLGI COMPLEX 8	63	569	0	1	206819-132
	Q9FY61	*TRAPPC9*	TRANSPORT PROTEIN PARTICLE C 9	130	1,186	0	4	186312-150
	Q0WQF4	*VPS53*	VPS53 HOMOLOG	93	828	0	3	87649-268
	Q9FGK9	*MAG5*	MAIGO 5	146	1,350	$5.78 \cdot 10^{-156}$	1	128216-217
	Q84WI4	*SEC23G*	PROTEIN TRANSPORT SEC23 G	89	794	0	1	308268-64
	F4HXV6	*NUP155*	NUCLEOPORIN 155	160	1,464	0	1	133551-212
	Q9C811	*NUP160*	NUCLEOPORIN 160	169	1,500	0	1	172756-164
	Q8LPR8	*TIC100*	TRANSLOCON AT THE INNER ENVELOPE MEMBRANE OF CHLOROPLASTS 100	100	871	$5.18 \cdot 10^{-149}$	1	196196-141
	Q9SK50	*TIC55*	TRANSLOCON AT THE INNER ENVELOPE MEMBRANE OF CHLOROPLASTS 55	61	539	$2.73 \cdot 10^{-170}$	4	336135-50
	Q7Y1W1	*TIC56*	TRANSLOCON AT THE INNER ENVELOPE MEMBRANE OF CHLOROPLASTS 56	62	527	$2.37 \cdot 10^{-127}$	1	17489-592
	Q9STE8	*TOC75-3*	PROTEIN TOC75-3	89	818	0	2	63672-317
	Q38967	*AAP2*	AMINO ACID PERMEASE 2	54	493	$1.79 \cdot 10^{-158}$	3	49644-361
	Q39134	*AAP3*	AMINO ACID PERMEASE 3	52	476	$8.24 \cdot 10^{-101}$	3	45759-376
	Q9FKS8	*LHT1*	LYSINE HISTIDINE TRANSPORTER 1	50	446	$1.9 \cdot 10^{-157}$	4	70343-301

Category	UniProtKB/ Swiss-Prot	Gene name	Protein name	MW (kDa)	Amino acids	E-value	Number of transcripts	Transcriptom identifier
	Q84MA5	*CAT1*	CATIONIC AMINO ACID TRANSPORTER 1	65	594	0	2	1290–1584
	Q9LFB8	*NPF8.2*	PROTEIN NRT1/ PTR FAMILY 8.2	63	570	$1.12 \cdot 10^{-173}$	19	2295 – 1330
	O04619	*ADNT1*	ADENINE NUCLEOTIDE TRANSPORTER 1	38	352	$1.27 \cdot 10^{-173}$	2	13512-659
	Q7DNC3	*MPT1*	MITOCHONDRIAL PHOSPHATE TRANSPORTER 1	34	309	$7.73 \cdot 10^{-141}$	2	283415-78
	P54144	*AMT1*	AMMONIUM TRANSPORTER 1	54	501	$1.09 \cdot 10^{-175}$	2	3618 – 1132
	Q9M6N7	*AMT2*	AMMONIUM TRANSPORTER 2	51	475	$6.51 \cdot 10^{-120}$	6	434232-1
	Q9LZD0	*PLUTO*	PLASTIDIC NUCLEOBASE TRANSPORTER	65	599	0	10	208937-130
	Q9SYD6	*MATE*	MULTI-DRUG AND TOXIC COMPOUND EXTRUSION	55	509	$1.01 \cdot 10^{-138}$	2	173408-163
Category	**UniProtKB/ Swiss-Prot**	**Gene name**	**Protein name**	**MW (kDa)**	**Amino acids**	**E-value**	**Number of transcripts**	**Transcriptom identifier**
Transport	Q9SAH0	*MGT1*	MAGNESIUM TRANSPORTER 1	50	443	$1.39 \cdot 10^{-168}$	2	17188-596
	Q9M9S6	*UTR3*	UDP-GALACTOSE TRANSPORTER 3	37	331	$1.16 \cdot 10^{-163}$	1	223712-118
	Q8LPQ8	*TST2*	TONOPLAST SUGAR TRANSPORTER 2	80	739	0	7	259050-94
	Q9FE59	*SUC4*	SUCROSE TRANSPORTER 4	55	510	$3.54 \cdot 10^{-152}$	1	207965-131
Biotic stress	Q8GSA7	*SR1*	SIGNAL RESPONSIVE 1	116	1,032	$9.46 \cdot 10^{-121}$	2	6592 – 892
	Q94IB9	*ACER*	CERAMIDASE	30	255	$3.21 \cdot 10^{-105}$	1	16708-604
	Q9ZQI7	*ALD1*	AMINOTRANSFERASE ALD1	51	456	$9.11 \cdot 10^{-122}$	9	47145-370
	Q9FIW4	*BGLU42*	β-GLUCOSIDASE 42	57	490	0	18	307697-64
	Q9M2S4	*LECRK-S.4*	L-TYPE LECTIN RECEPTOR KINASE S.4	76	684	$4.37 \cdot 10^{-107}$	5	6471 – 900
	P49177	*AGB1*	GUANINE NUCLEOTIDE-BINDING PROTEIN SUBUNIT β	41	377	$2.52 \cdot 10^{-141}$	2	299586-69
	Q8RXD6	*HUB1*	HISTONE MONO-UBIQUITINATION 1	100	878	$1.37 \cdot 10^{-164}$	1	9828 – 759
	F4IS56	*ILK1*	INTEGRIN-LINKED KINASE1	54	479	$1.31 \cdot 10^{-105}$	4	220314-121
	Q9FL28	*FLS2*	FLAGELLIN-SENSITIVE 2	128	1,173	$1.21 \cdot 10^{-122}$	28	24072-511
	Q9M8M7	*WIN1*	HOPW1-1-INTERACTING 1	49	457	0	4	329467-53
	Q8RXV3	*WIN2*	HOPW1-1-INTERACTING 2	33	311	$1.38 \cdot 10^{-100}$	6	14367-639

Category	UniProtKB/ Swiss-Prot	Gene name	Protein name	MW (kDa)	Amino acids	E-value	Number of transcripts	Transcriptom identifier
	A8R7E6	*LYK1*	LYSM-CONTAINING RECEPTOR-LIKE KINASE 1	67	617	$9.74 \cdot 10^{-148}$	16	6248 – 915
	Q9LQV2	*RDR1*	RNA-DEPENDENT RNA POLYMERASE 1	126	1,107	0	10	113558-233
	Q9LDX1	*SGS3*	SUPPRESSOR OF GENE SILENCING 3	72	625	$1.68 \cdot 10^{-106}$	2	91884-261
	Q9SAU2	*EMB2728*	EMBRYO DEFECTIVE 2728	30	281	$3.42 \cdot 10^{-157}$	1	133033-213
	Q944A7	*BSK1*	BRASSINOSTEROID-SIGNALING KINASE 1	57	512	$2.86 \cdot 10^{-170}$	3	264986-90
	F4I893	*NOXY7*	NON-RESPONDING TO OXYLIPINS 7	285	2,610	0	1	578–1970
	Q9C5S2	*IRE1A*	SERINE/THREONINE-PROTEIN KINASE/ENDORIBONUCLEASE IRE1A	95	841	$7.95 \cdot 10^{-143}$	1	1817 – 1446
	Q94BR4	*PUB59*	PLANT U-BOX 59	57	523	$2.93 \cdot 10^{-179}$	3	2450 – 1297
	Q42384	*SCPR44*	SUPPRESSOR OF CPR5 44	54	486	0	1	49883-360
	Q9M9A8	*APCB1*	ASPARTYL PROTEASE APCB1	65	583	$1.52 \cdot 10^{-122}$	1	270619-86
	O24412	*AE3*	ASYMMETRIC LEAVES ENHANCER 3	35	308	$6.49 \cdot 10^{-171}$	1	18957-570
	Q8LGI8	*NAA20*	NATB CATALYTIC SUBUNIT	20	174	$6.71 \cdot 10^{-115}$	1	148530-200
	Q39008	*ARAKIN*	MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE 1	66	608	$7.6 \cdot 10^{-100}$	3	302787-67
	Q9SF86	*PBL39*	PBS1-LIKE 39	47	418	$1.14 \cdot 10^{-127}$	1	73785-294
	P43273	*TGA2*	TRANSCRIPTION FACTOR TGA2	37	330	$2.32 \cdot 10^{-136}$	6	109675-237
	Q9LMA1	*FMO1*	FLAVIN-DEPENDENT MONOOXYGENASE 1	60	530	$2.7 \cdot 10^{-112}$	2	86204-270
Category	**UniProtKB/ Swiss-Prot**	**Gene name**	**Protein name**	**MW (kDa)**	**Amino acids**	**E-value**	**Number of transcripts**	**Transcriptom identifier**
Biotic stress	Q949Q0	*SFD1*	SUPPRESSOR OF FATTY ACID DESATURASE DEFICIENCY 1	45	420	0	1	227667-115
	Q9LFP7	*PBL34*	PBS1-LIKE 34	55	493	$6.72 \cdot 10^{-168}$	11	350918-43
	O48538	*RBOH F*	RESPIRATORY BURST OXIDASE PROTEIN F	108	944	$5.5 \cdot 10^{-118}$	15	504–2053
	Q9SRX9	*SYG1*	E3 UBIQUITIN-PROTEIN LIGASE NLA	38	335	$3.35 \cdot 10^{-105}$	5	12407-684
	F4KCC2	*GED1*	GREENING AFTER EXTENDED DARKNESS 1	224	2,006	$5.61 \cdot 10^{-109}$	1	93271-259
	Q66GP0	*NCH1*	NRL PROTEIN FOR CHLOROPLAST MOVEMENT1	68	604	$2.32 \cdot 10^{-134}$	1	33906-435
	Q9FNK4	*Δ-OAT*	ORNITHINE-Δ-AMINOTRANSFERASE	52	475	0	1	349934-44
	Q9XIE2	*PDR8*	PLEIOTROPIC DRUG RESISTANCE 8	165	1,469	0	17	31071-454

Category	UniProtKB/ Swiss-Prot	Gene name	Protein name	MW (kDa)	Amino acids	E-value	Number of transcripts	Transcriptom identifier
	Q9ZT82	*CALS12*	CALLOSE SYNTHASE 12	207	1,780	0	12	157371-184
Abiotic stress	P31414	*VHP1*	PYROPHOSPHATE-ENERGIZED VACUOLAR MEMBRANE PROTON PUMP 1	80	770	0	5	262929-91
	Q9LRR7	*SIS7*	SUGAR INSENSITIVE 7	66	599	$2.87 \cdot 10^{-132}$	1	53767-346
	O81445	*CBL1*	CALCINEURIN B-LIKE PROTEIN 1	25	213	$1.32 \cdot 10^{-103}$	5	223297-119
	P54150	*MSRA4*	METHIONINE SULFOXIDE REDUCTASE A4	29	258	$9.41 \cdot 10^{-107}$	1	78297-285
	O65378	*ACO3*	ACONITASE 3	37	320	0	2	124896-220
	Q9STS7	*LCNP*	LIPOCALIN IN THE PLASTID	39	353	$2.37 \cdot 10^{-122}$	1	6712 – 886
	Q94AT5	*EXE2*	EXECUTER 2	72	651	$2.22 \cdot 10^{-131}$	1	140509-206
	B9DGY1	*ABC1K7*	ACTIVITY OF BC1 COMPLEX KINASE 7	78	695	0	1	295638-71
	Q93Y08	*ABC1K8*	ACTIVITY OF BC1 COMPLEX KINASE 8	86	761	0	1	396367-24
	Q9FXA8	*A/N-INVA*	ALKALINE/NEUTRAL INVERTASE A	70	616	$9.14 \cdot 10^{-112}$	5	178580-158
	Q9SAK4	*ENF1*	ENLARGED FIL EXPRESSION DOMAIN 1	57	528	0	3	8905 – 792
	Q8VYW6	*GGT1*	Γ-GLUTAMYL TRANSPEPTIDASE 1	61	572	$4.12 \cdot 10^{-174}$	2	398871-23
	P25858	*GAPC1*	GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE C SUBUNIT 1	37	338	$6.22 \cdot 10^{-103}$	2	283048-78
	Q9LSV0	*GLYR1*	GLYOXYLATE/SUCCINIC SEMIALDEHYDE REDUCTASE 1	31	289	$6.78 \cdot 10^{-146}$	1	328888-53
	F4HQM3	*NCER1*	NEUTRAL CERAMIDASE 1	87	779	0	1	3227 – 1185
	Q9LFR0	*GMII*	α-MANNOSIDASE 2	135	1,173	0	1	210526-129
	F4HZB2	*BCHA1*	BEACH-DOMAIN HOMOLOG A1	401	3,601	0	1	49395-362
	Q8L7S8	*RH3*	DEAD-BOX ATP-DEPENDENT RNA HELICASE 3	81	747	0	3	134221-212
	Q8W4Q4	*MRF3*	MA3 DOMAIN-CONTAINING TRANSLATION REGULATORY FACTOR 3	77	702	$4.53 \cdot 10^{-151}$	1	190469-146
	Q8RWB1	*SRL1*	PRE-mRNA SPLICING FACTOR SR-LIKE 1	46	393	$8.58 \cdot 10^{-121}$	2	298824-69
Category	**UniProtKB/ Swiss-Prot**	**Gene name**	**Protein name**	**MW (kDa)**	**Amino acids**	**E-value**	**Number of transcripts**	**Transcriptom identifier**
Abiotic stress	P93006	*PP2CG1*	PROTEIN PHOSPHATASE 2C GROUP 1	42	380	$3.8 \cdot 10^{-121}$	2	313144-61
	Q38933	*SZL1*	SUPPRESSOR OF ZEAXANTHIN-LESS 1	56	501	0	1	223062-119
	Q39030	*PK2*	PROTEIN KINASE 2	53	471	$3.96 \cdot 10^{-169}$	11	394481-25
	F4J0W4	*CBG*	CALCIUM BINDING GTP-ASE	71	643	$5.39 \cdot 10^{-113}$	4	430063-4
	Q9SY59	*NFXL1*	NF-X-LIKE 1	131	1,188	0	1	364228-38

Category	UniProtKB/ Swiss-Prot	Gene name	Protein name	MW (kDa)	Amino acids	E-value	Number of transcripts	Transcriptom identifier
	Q93VB2	*PEUP2*	PEROXISOME UNUSUAL POSITIONING 2	47	425	$6.15 \cdot 10^{-146}$	4	147053-201
	Q9LDY2	*DIN4*	2-OXOISOVALERATE DEHYDROGENASE SUBUNIT β 2	39	358	0	1	15480-622
	Q93VD3	*PKS17*	SOS2-LIKE PROTEIN KINASE 17	54	482	0	21	104893-243
	Q9MAM1	*PKS6*	PROTEIN KINASE 6	51	447	0	6	341709-47
	Q9LX30	*GCN2*	GENERAL CONTROL NON-DEREPRESSIBLE 2	140	1,241	0	4	355181-41
	P42644	*GRF3*	14-3-3-LIKE PROTEIN GF14 PSI	29	255	$4.09 \cdot 10^{-139}$	1	5364 – 974
	Q9ZVU5	*KIN17*	KIN17-LIKE PROTEIN	47	411	$1.44 \cdot 10^{-163}$	5	326712-54
	Q9SF47	*PAH1*	PHOSPHATIDIC ACID PHOSPHOHYDROLASE 1	101	904	$4.57 \cdot 10^{-124}$	2	86819-269
	Q9C9P4	*KAS2*	β-KETOACYL-ACP SYNTHETASE 2	58	541	0	1	58520-331
	Q949X0	*ADS3*	PALMITOYL-MONOGALACTOSYLDIACYLGLYCEROL Δ-7 DESATURASE	43	371	$3.15 \cdot 10^{-138}$	1	332769-51
	O65090	*PFC1*	PALEFACE 1	38	343	$2.49 \cdot 10^{-101}$	7	189738-147
	O23553	*BAM3*	β-AMYLASE 3	61	548	0	3	63967-316
	Q9FI53	*FUM2*	FUMARASE 2	56	510	0	2	96553-255
	Q9LKJ1	*CHY1*	3-HYDROXYISOBUTYRYL-COA HYDROLASE 1	42	378	$4.15 \cdot 10^{-123}$	3	144795-203
	Q93Y07	*SFR2*	SENSITIVE TO FREEZING 2	71	622	0	2	92262-261
	P25696	*ENO2*	ENOLASE 2	48	444	0	1	135093-211
	Q3EBF7	*SLD2*	Δ (8)-FATTY-ACID DESATURASE 2	51	449	$3.39 \cdot 10^{-167}$	1	287288-76
	O82359	*LCBK2*	LONG-CHAIN BASE KINASE 2	40	364	$9.89 \cdot 10^{-109}$	4	100853-248
	F4IF36	*FGT1*	FORGETTER 1	144	1,295	0	2	196756-140
	Q8W4B1	*KH28*	RNA-BINDING KH DOMAIN-CONTAINING PROTEIN RCF3	71	652	$1.32 \cdot 10^{-106}$	4	220235-121
	A2RVJ8	*HIT4*	HEAT INTOLERANT 4	51	434	$4.18 \cdot 10^{-101}$	1	47835-368
	Q9FN02	*PP7*	SERINE/THREONINE-PROTEIN PHOSPHATASE 7	47	413	$2.25 \cdot 10^{-128}$	1	211686-128

3.5. Protein-protein interactions

The interactome of the 1,160 proteins studied associated with light, transport and stress was analysed using the String program. A network consisting of 1,160 nodes and 3,168 edges was obtained, which surpassed the expected number of edges (1,327). The Protein-Protein Interaction enrichment p-value was less than $1 \cdot 10^{-16}$. A comparison between some types of interactions (neighbourhood, gene fusion, homology, co-occurrence and co-expression) in each group of proteins studied is represented in **Fig. 8**. Neighbourhood interactions peaked in photorespiration, whereas gene fusion in abiotic stress, especially heat. In homology, co-occurrence and co-expression interactions, the highest rate was found in those proteins associated to photosynthesis.

Figure 8. Estimation of the protein-protein interaction types in the selected groups of proteins extracted from the gametophytes of *Dryopteris affinis*.

To further investigate this initial analysis, the interactome was thoroughly examined. It revealed that 135 proteins had an interaction strength score above 999, where 1,000 represents the maximum possible score and 0 the minimum. Some examples of proteins with the strongest interactions were

PHOTOSYSTEM I LIGHT HARVESTING COMPLEX GENE 2 (LHCA2), SUPPRESSOR OF PHYA-105 1 (SPA1), and EMBRYO DEFECTIVE 2817 (EMB2817). Looking at the type of interaction in each group of proteins, the analysis revealed that the strongest interaction in photosynthesis came from experiments between the proteins PHOTOSYSTEM I LIGHT HARVESTING COMPLEX GENE 1 (LHCA1) and CHLOROPHYLL A-B BINDING PROTEIN 4 (CAB4); in photorespiration, from databases, between GLUTAMATE: GLYOXYLATE AMINOTRANSFERASE 1 (GGAT1) and GLYCERATE DEHYDROGENASE HPR (HPR1); in the response to UV, from experiments, between HOMOLOG OF REVERSIONLESS 1 (REV1) and 3 (REV3); in xanthophyll metabolism, from databases, between LUTEIN DEFICIENT 5 (LUT5), VIOLAXANTHIN DE-EPOXIDASE 1 (VDE1), and β-CAROTENOID HYDROXYLASE 1 (BCH1) with each other; in the case of transport, from text-mining, with the proteins EXOCYST COMPLEX COMPONENT SEC10 (SEC10) and SUBUNIT OF EXOCYST COMPLEX 8 (SEC8); in the response to biotic stress, from text-mining, with REGULATOR OF NONSENSE TRANSCRIPTS 1 HOMOLOG (UPF1) and UPF2 (UPF2); in the response to water deprivation, salt stress and starvation, from text-mining, between SOS3-LIKE CALCIUM BINDING PROTEIN 5 (SCABP5) and SOS2-LIKE PROTEIN KINASE 17 (PKS17); in the response to oxidative stress, from experiments with ELONGATOR COMPLEX PROTEIN 1 (ELP1) and 2 (ELP2); and finally, in the response to cold, the strongest interaction came from experiments between 3-KETOACYL-COA SYNTHASE 6 (CER6) and 9 (KCS9), while in the response to heat, it was from text-mining, between the annotations KU70 HOMOLOG (KU70) and KU80 HOMOLOG (KU80). Specifically, protein-protein interactions due to co-expression relationships seem to be more frequent in photosynthesis. Additionally, several cases of gene fusion were observed among several proteins associated with heat stress, which are red coloured (Fig. 9a). Finally, proteins with the highest values of co-occurrence interactions are shown (Fig. 9b).

As for the number of nodes, the proteins that had the most were MODIFIED TRANSPORT TO THE VACUOLE 17 (MTV17) with 19, CONSERVED OLIGOMERIC GOLGI COMPLEX 6 (COG6) with 18, and EXOCYST COMPLEX COMPONENT SEC6 (SEC6) with 16.

3.6. Comparison with fern genomes

As explained above, the *D. affinis* genome is not yet sequenced, so the transcriptome dataset was compared with other species for information. Some BLASTX searches were performed with the transcripts of around 180 proteins discussed here, associated with light, transport and stress against the fern species whose genomes are sequenced and deposited on the FernBase site (https://fernbase.org), showing high homology. The identification code of the proteins and, in some cases, their name, are provided in **Supplementary Table 2**.

4. Discussion

This study provides a refined in silico characterization of proteins expressed in the gametophyte of *Dryopteris affinis* ssp. *affinis*, based on a curated subset of high-confidence candidates derived from an existing transcriptomic resource [16, 20]. By applying stringent filtering criteria and focusing on specific functional categories, the present analyses emphasize biological processes directly related to gametophyte physiology, including light-dependent metabolism, transport functions, and responses to biotic and abiotic stress. The integration of functional annotation with protein–protein interaction network analysis enables a focused interpretation of molecular relationships operating at this autonomous life stage.

4.1. Light

4.1.1. Light-dependent processes and photosynthesis-related proteins

Photosynthesis plays a central role in fern gametophytes, which are free-living and rely entirely on autotrophic metabolism during development [3, 4, 9]. Ferns provide a valuable system to examine photosynthesis across two independent but genetically similar generations, sporophyte and gametophyte, that differ markedly in morphology and anatomy [59]. While sporophytes develop large fronds with stomata and intercellular air spaces, gametophytes are typically composed of a single cell layer, lack stomata and vascular tissues, and possess only a rudimentary cuticle, potentially increasing exposure to atmospheric variation [59–61].

Previous studies on fern gametophytes have shown that light-related mechanisms display substantial similarities to those operating in fern sporophytes and seed plants [62, 63]. In this context, the present study expands the repertoire of annotated homologs potentially involved in light-dependent metabolism in the gametophyte of *D. affinis* [19]. The curated dataset includes proteins associated not only with core photosynthetic processes but also with photorespiration and xanthophyll metabolism, indicating that multiple components of light-driven energy balance and photoprotection are active at this life stage.

Among the identified proteins are PROTON GRADIENT REGULATION 6 (PGR6), involved in regulation of the electron transport chain and plastoquinone, β-carotene, and lutein homeostasis [64]; LEAF-TYPE CHLOROPLAST-TARGETED FNR 1 and 2 (LFNR1 and LFNR2), which modulate electron flow to balance ATP production and reducing power [65]; and K^+ EFFLUX ANTIPORTER 3 (KEA3), which contributes to the maintenance of photosynthetic efficiency under conditions of low ATP synthase activity. These components are central to the regulation of energy conversion and redox balance within the chloroplast.

In addition, the presence of proteins associated with photorespiratory pathways suggests that mechanisms for dissipating excess reducing power and maintaining metabolic flexibility operate in the gametophyte. Together with the identification of proteins involved in xanthophyll metabolism, these findings support the existence of photoprotective strategies that modulate energy dissipation and limit photooxidative damage under fluctuating environmental conditions. Such mechanisms are likely to be particularly relevant for gametophytes developing in microhabitats characterized by rapid changes in light intensity and water availability.

Additional homologs related to photosystem II formation, assembly, and stabilization—including HCF136, HCF173, HCF244, and CYCLOPHILIN 38 (CYP38)—were also detected. Light-harvesting complex components such as LHCB2, LHCA1, and LHCB4 further indicate a broad representation of proteins involved in

excitation energy capture and transfer. Other annotated homologs, including SUPPRESSOR OF QUENCHING 1 (SOQ1), ENHANCER OF VARIEGATION 3 (EVR3), and FTSH PROTEASE 1 (FTSH1), were identified as potential contributors to chloroplast structure, function, and photoprotection [66–68].

Together, these findings support the central role of photosynthesis, photorespiration, and xanthophyll-mediated photoprotection in fern gametophytes and highlight the tight integration of light-dependent metabolism with developmental and environmental responses at this stage.

4.2. Transport related-proteins and cellular homeostasis

In this study, the *in silico* annotation of the *Dryopteris affinis* gametophyte transcriptome revealed an extensive repertoire of transport-associated proteins, with nearly 700 homologs assigned to transport-related functions. This broad representation highlights transport as one of the most prominent functional categories in the dataset and underscores the metabolic and regulatory autonomy of the gametophyte stage.

Transport processes play a central role in sustaining cellular organisation, metabolic activity and developmental progression in plant cells. In vascular plants, transport systems regulate the movement of phytohormones, ions, metabolites and macromolecules, and support intracellular trafficking between compartments such as the nucleus, mitochondria and plastids. In ferns, and particularly in the gametophyte generation, the molecular components underlying these processes remain poorly characterised, despite their functional relevance [82, 83]. Previous studies have shown that fern gametophytes exhibit dynamic cellular connectivity, including abundant plasmodesmata whose distribution varies during development, suggesting regulated intercellular transport of solutes and signals [3]. Nutrient uptake is thought to be mediated largely by rhizoids, which fulfil absorptive roles comparable to those described in bryophytes, while additional transport mechanisms contribute to internal solute redistribution and cellular homeostasis [84]. Proteomic surveys in fern gametophytes have already pointed to the relevance of transport-related proteins in environmental adjustment and developmental regulation [23]. Moreover, polar auxin transport has been proposed as a key factor coordinating cell behaviour during gametophyte development and early sporophyte formation [85].

Among the annotated proteins, ATP-binding cassette (ABC) transporters constituted a particularly abundant group. Multiple homologs from different ABC subfamilies were identified, including candidates associated with auxin transport, such as ATP-BINDING CASSETTE B1 (ABCB1) and CORYMBOSA1 (CRM1), as well as AUXIN TRANSPORTER PROTEIN 1 (AUX1) [86, 87]. Additional ABC transporters potentially involved in abscisic acid transport, lipid trafficking and ion homeostasis were also detected, including ABCG40, ABCD1 and ABCC5 [88]. The diversity of ABC transporters identified suggests the existence of complex regulatory networks controlling hormone distribution, detoxification and solute balance in the gametophyte.

Beyond hormone-related transport, numerous proteins involved in ion and metabolite transport were annotated. These include channels and carriers such as CYCLIC NUCLEOTIDE GATED CHANNEL 2 (CNGC2), DEFENSE, NO DEATH 2 (DND2), VOLTAGE DEPENDENT ANION CHANNEL 1 (VDAC1), and the mechanosensitive channel MECHANOSENSITIVE ION CHANNEL PROTEIN 1 (MSL1). Additional transporters implicated in metal homeostasis, phosphate exchange and proton-coupled transport, as well as aquaporins (PIP1;2 and PIP2;1), further support a capacity for fine-tuned regulation of ionic and osmotic balance within gametophyte cells.

Proteins associated with intracellular trafficking and vesicle-mediated transport were also well represented, including components of conserved endomembrane systems and nuclear pore complexes. These findings indicate active membrane trafficking and nucleocytoplasmic exchange, processes that are essential for coordinating growth, signalling and metabolic integration in a free-living plant generation.

Collectively, the transport-related protein landscape identified here considerably expands current molecular knowledge of fern gametophytes [18–20]. The breadth and diversity of transport functions revealed by this in silico analysis suggest that the gametophyte of *D. affinis* is equipped with an extensive and versatile transport machinery, supporting nutrient acquisition, intracellular communication and developmental coordination under variable environmental conditions. This resource provides a foundation for future functional and comparative studies addressing transport regulation in early-diverging vascular plants.

4.3. Stress-related proteins and adaptive responses

In addition to light- and transport-related proteins, stress-associated proteins were analysed in the gametophyte of *D. affinis*, providing a broad functional overview that expands and contextualises previously published data on stress responses in this developmental stage.

4.3.1 Biotic stress

Ferns have evolved a range of strategies to limit damage caused by insects, pathogens and other biotic agents, thereby reducing disease incidence [90–92]. Insects are reported to damage ferns far less frequently than angiosperms, a pattern that has been linked to the production of compounds such as ecdysones, which can interfere with insect development or survival [93]. Consistent with this, the *D. affinis* gametophyte transcriptome includes homologs potentially associated with responses to insect attack, such as SIGNAL RESPONSIVE 1 (SR1), suggesting that defence-related mechanisms may already operate at the gametophyte stage.

With respect to microbial defence, several homologs related to bacterial and fungal resistance were annotated. Rather than indicating isolated responses, these proteins are components of conserved defence pathways, encompassing enzymes, receptor-like kinases and signalling regulators. Representative examples include CERAMIDASE (ACER), AMINOTRANSFERASE ALD1 (ALD1), β-GLUCOSIDASE 42 (BGLU42), and L-TYPE LECTIN RECEPTOR KINASE S.4 (LECRK-S.4), together with signalling components such as GUANINE NUCLEOTIDE-BINDING PROTEIN SUBUNIT β (AGB1), HISTONE MONO-UBIQUITINATION 1 (HUB1) and INTEGRIN-LINKED KINASE 1 (ILK1). In addition, homologs of FLAGELLIN-SENSITIVE 2 (FLS2), HOPW1-1-INTERACTING 1 and 2 (WIN1 and WIN2), and LYSM-CONTAINING RECEPTOR-LIKE KINASE 1 (LYK1) were identified, supporting a potential capacity for pathogen recognition and activation of downstream defence signalling [94–96].

Proteins associated with antiviral defence were also detected, including homologs of RNA-DEPENDENT RNA POLYMERASE 1 (RDR1) and SUPPRESSOR OF GENE SILENCING 3 (SGS3), which are key components of RNA silencing-based antiviral responses in plants [97–98]. In addition, homologs linked to responses against phytoparasitic nematodes, such as EMBRYO DEFECTIVE 2728 (EMB2728), were annotated, further extending the range of biotic interactions potentially addressed by the gametophyte.

Beyond pathogen-specific responses, several homologs associated with general plant immunity and defence regulation were identified. These include signalling and regulatory components such as BRASSINOSTEROID-SIGNALING KINASE 1 (BSK1), NON-RESPONDING TO OXYLIPINS 7 (NOXY7), SERINE/THREONINE-PROTEIN KINASE/ENDORIBONUCLEASE IRE1A (IRE1A), PLANT U-BOX 59 (PUB59), SUPPRESSOR OF CPR5 44 (SCPR44), and ASPARTYL PROTEASE APCB1 (APCB1), as well as transcriptional and signalling regulators including ASYMMETRIC LEAVES ENHANCER 3 (AE3), NATB CATALYTIC SUBUNIT (NAA20), MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE 1 (ARAKIN) and PBS1-LIKE 39 (PBL39). Homologs involved in systemic acquired resistance, such as TRANSCRIPTION FACTOR TGA2 (TGA2), FLAVIN-DEPENDENT MONOOXYGENASE 1 (FMO1), SUPPRESSOR OF FATTY ACID DESATURASE DEFICIENCY 1 (SFD1) and PBS1-LIKE 34 (PBL34), were also annotated, together with RESPIRATORY BURST OXIDASE PROTEIN F (RBOHF), which may participate in hypersensitive responses [99–103].

Additional defence-related homologs included E3 UBIQUITIN-PROTEIN LIGASE NLA (SYG1), GREENING AFTER EXTENDED DARKNESS 1 (GED1), NRL PROTEIN FOR CHLOROPLAST MOVEMENT 1 (NCH1), ORNITHINE-Δ-AMINOTRANSFERASE (Δ-OAT), PLEIOTROPIC DRUG RESISTANCE 8 (PDR8) and CALLOSE SYNTHASE 12 (CALS12). Callose deposition mediated by CALS12 may contribute to wound-associated barrier formation, although it remains to be established whether callose is produced constitutively in D. affinis gametophytes or is induced primarily in response to injury [104–106].

4.3.2 Abiotic stress

Marked morphological differences between sporophytes and gametophytes strongly influence their responses to environmental constraints. Gametophytes lack a cuticle and functional stomata, rendering them poikilohydric and dependent on tolerance and recovery mechanisms to cope with water stress [61, 107, 108]. Consistent with this, homologs potentially involved in dehydration responses were annotated in the *D. affinis* gametophyte transcriptome, including PYROPHOSPHATE-ENERGIZED VACUOLAR MEMBRANE PROTON PUMP 1 (VHP1), SUGAR INSENSITIVE 7 (SIS7) and CALCINEURIN B-LIKE PROTEIN 1 (CBL1), which are associated with ion homeostasis and stress-related signalling pathways [109–111].

A substantial set of proteins putatively associated with oxidative stress tolerance was also identified. These include enzymes involved in protein repair, redox regulation and protection of cellular membranes, such as METHIONINE SULFOXIDE REDUCTASE A4 (MSRA4), ACONITASE 3 (ACO3), LIPOCALIN IN THE PLASTID (LCNP) and EXECUTER 2 (EXE2), as well as components linked to mitochondrial and plastidial redox control (ABC1K7 and ABC1K8). Additional proteins involved in carbohydrate metabolism and antioxidant pathways, including ALKALINE/NEUTRAL INVERTASE A (A/N-INVA), Γ-GLUTAMYL TRANSPEPTIDASE 1 (GGT1), GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE C SUBUNIT 1 (GAPC1), GLYOXYLATE/SUCCINIC SEMIALDEHYDE REDUCTASE 1 (GLYR1) and NEUTRAL CERAMIDASE 1 (NCER1), further support the importance of redox homeostasis in gametophyte stress responses [112–118].

Proteins associated with salt stress included α-MANNOSIDASE 2 (GMII), BEACH-DOMAIN HOMOLOG A1 (BCHA1), DEAD-BOX ATP-DEPENDENT RNA HELICASE 3 (RH3), MA3 DOMAIN-CONTAINING TRANSLATION REGULATORY FACTOR 3 (MRF3), PRE-mRNA SPLICING FACTOR SR-LIKE 1 (SRL1), PROTEIN PHOSPHATASE 2C GROUP 1 (PP2CG1), SUPPRESSOR OF ZEAXANTHIN-LESS 1 (SZL1), PROTEIN KINASE 2 (PK2), CALCIUM BINDING GTP-ASE (CBG) and NF-X-LIKE 1 (NFXL1), indicating the presence of multiple regulatory layers involved in ionic balance and stress signalling [119–121].

Starvation-related homologs included proteins involved in metabolic adjustment, signalling and membrane remodelling, such as PEROXISOME UNUSUAL POSITIONING 2 (PEUP2), 2-OXOISOVALERATE DEHYDROGENASE SUBUNIT β 2 (DIN4), SOS2-LIKE PROTEIN KINASE 17 (PKS17), PROTEIN KINASE 6 (PKS6), GENERAL CONTROL NON-DEREPRESSIBLE 2 (GCN2), 14-3-3-LIKE PROTEIN GF14 PSI (GRF3), KIN17-LIKE PROTEIN (KIN17) and PHOSPHATIDIC ACID PHOSPHOHYDROLASE 1 (PAH1) [20, 122–124].

Finally, several proteins associated with temperature stress were annotated. Cold-stress-related homologs included β-KETOACYL-ACP SYNTHETASE 2 (KAS2), PALMITOYL-MONOGALACTOSYLDIACYLGLYCEROL Δ-7 DESATURASE (ADS3), PALEFACE 1 (PFC1), β-AMYLASE 3 (BAM3), FUMARASE 2 (FUM2), 3-HYDROXYISOBUTYRYL-COA HYDROLASE 1 (CHY1), SENSITIVE TO FREEZING 2 (SFR2), ENOLASE 2 (ENO2), Δ(8)-FATTY-ACID DESATURASE 2 (SLD2) and LONG-CHAIN BASE KINASE 2 (LCBK2) [125–130]. Heat-stress-related homologs included FORGETTER 1 (FGT1), RNA-BINDING KH DOMAIN-CONTAINING PROTEIN RCF3 (KH28), HEAT INTOLERANT 4 (HIT4) and SERINE/THREONINE-PROTEIN PHOSPHATASE 7 (PP7) [131–133].

Collectively, these results reinforce previous annotations of stress-related proteins in *D. affinis* [14, 18–20] while substantially expanding their functional context through an integrated in silico analysis. Given that sexual and asexual reproduction occur at the gametophyte stage, understanding stress responses —particularly under extreme conditions such as high temperatures—provides relevant insights into how ongoing climate change may affect fern reproduction and population dynamics [134].

4.4. Protein-protein interactions

The interactome of the 1,160 proteins associated with light responses, transport and stress was analysed, together with the complete interaction network. As reported in the Results section, the resulting network showed a highly significant protein–protein interaction enrichment (p-value = 1×10^{-16}), indicating that the observed interactions are far more frequent than expected for a random set of proteins of comparable size. This finding supports the existence of coordinated functional modules operating within the D. affinis gametophyte.

Within the global network, the proteins with the highest number of interactions were MTV17, COG6 and SEC6. These highly connected nodes are all involved in intracellular transport processes, highlighting vesicle trafficking and Golgi-associated transport as central hubs in the interaction network. MTV17

participates in retrograde transport from early and late endosomes to the trans-Golgi network [135], COG6 is required for proper Golgi function, and SEC6 is a core component of the exocyst complex involved in vesicle docking at the plasma membrane during secretion [136]. Their central positions in the network likely reflect the fundamental role of membrane trafficking in gametophyte cellular organisation.

Analysis of the strongest interacting protein pairs within functional categories revealed patterns consistent with shared biological roles. In the photosynthesis-related group, LHCA1 and CAB4 showed the strongest experimental interaction, which is expected given that both are components of the light-harvesting complex and function in capturing and transferring excitation energy to photosystems in Arabidopsis thaliana [137]. Similarly, in photorespiration, GGAT1 and HPR1 exhibited strong interactions, consistent with their complementary roles in regulating amino acid metabolism and maintaining the photorespiratory core cycle [138, 139].

In the photomorphogenesis-related set, REV1 and REV3 displayed the strongest interaction based on UV-response experiments, reflecting their respective involvement in resistance to UV-B and UV-C radiation [140]. For xanthophyll metabolism, the interaction among LUT5, VDE1 and BCH1 was particularly prominent in database-based evidence, likely due to their coordinated roles in xanthophyll biosynthesis and regulation of zeaxanthin levels in chloroplasts [141, 142].

Within the transport category, SEC10 and SEC8 showed the strongest interaction in text-mining analyses. Both proteins are components of the exocyst complex and are frequently co-mentioned in the literature because of their shared role in vesicle docking during secretion [143]. A similar pattern was observed for biotic stress responses, where UPF1 and UPF2, both involved in plant defence and wound responses, exhibited strong text-mining interactions [144, 145]. In responses to water deprivation, salt stress and starvation, the strongest interaction was detected between SCABP5, a key regulator under water- and salt-deficient conditions [146], and PKS17, which confers tolerance to low potassium availability [147].

Distinct interaction patterns were observed for oxidative stress and cold response, where experimental evidence highlighted the strongest associations. ELP1 and ELP2, both involved in oxidative stress signalling, showed strong interactions within the oxidative stress group [148, 149], whereas CER6 and KCS9, required for freezing tolerance, were prominent in the cold-response group [150]. In the case of heat stress, KU70 and KU80 exhibited the strongest text-mining interaction, consistent with their joint involvement in plant defence against high-temperature stress [151].

An additional feature of the interaction analysis was the high number of predicted gene fusion events, particularly among proteins associated with heat-stress responses. Gene fusion events can give rise to novel proteins with enhanced or combined functions, potentially improving tolerance to elevated temperatures through mechanisms such as increased protein stability or improved management of reactive oxygen species. Heat shock proteins (HSPs) appear especially prone to such fusion events, a pattern also highlighted in the Results section. Notably, numerous HSPs have previously been documented in the gametophyte of *D. affinis* [20], raising the possibility that their abundance and diversification contribute to heat-stress resilience in this developmental stage. Supporting this view, gene fusion strategies have been explored in crop species; for example, fusion of trehalose-6-phosphate synthase and phosphatase genes has been shown to enhance heat tolerance in tomato [152]. More broadly, gene-fusion approaches may represent a promising avenue for the development of climate-resilient crops.

Overall, the protein–protein interaction analysis offers an integrated view of the molecular organisation of the *D. affinis* gametophyte. The structure of the interaction network highlights the functional connectivity among proteins involved in transport, photosynthesis and stress responses, reinforcing the idea that these processes operate as coordinated modules rather than as isolated pathways. By linking functional annotation with interaction patterns, this analysis provides additional context for understanding how key cellular processes are organised in the gametophyte stage and establishes a framework for future functional and comparative studies in ferns and other early-diverging vascular plants.

Declarations

Funding declaration

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Conflict of interests:

The authors declare no competing interests.

Author Contribution

Conceptualisation: HF and UG; methodology: SO, JMA, and HF; formal analysis: SO, FV and NG; writing - original preparation: SO, FV, NG and HF; writing - final review: JMA, LGQ, JF, AP, and UG. All authors read and approved the manuscript.

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Data Availability

The *de novo* assembly of the transcriptome of the *D. affinis* gametophyte in FASTA format and the transcriptome annotation are available in the Zenodo research data repository ([www.zenodo.org](http:/www.zenodo.org)) with the identifier [https://doi.org/10.5281/zenodo.1040330] (https:/doi.org/10.5281/zenodo.1040330) . Specifically, the sequences studied in this work are provided in the Supplementary Table 1.

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Figures

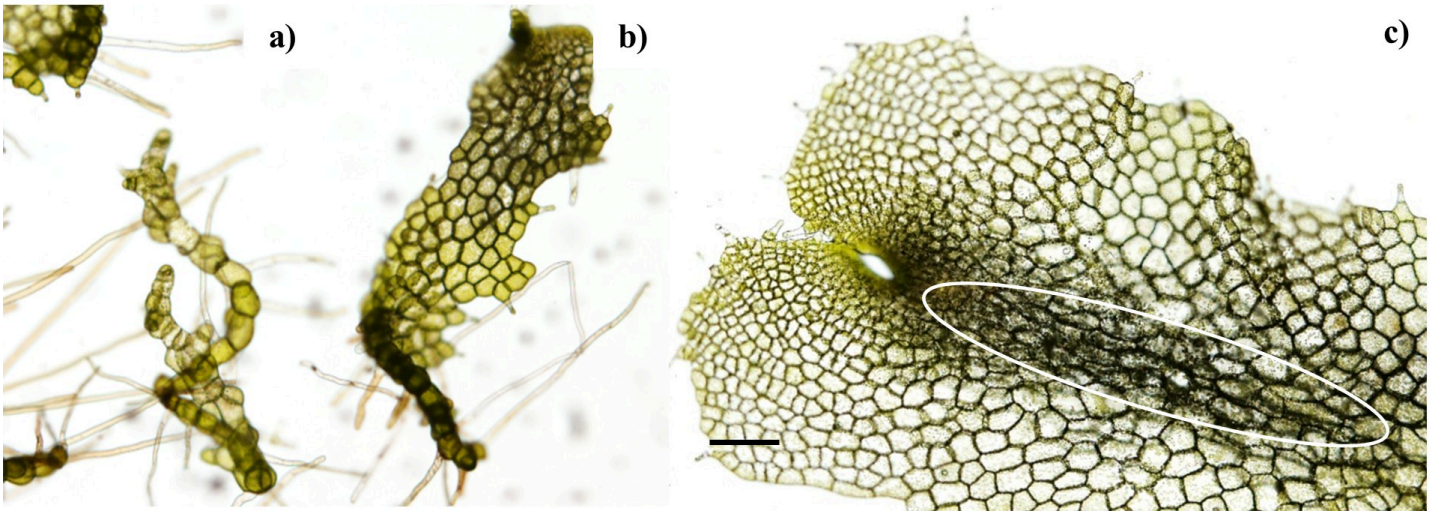

Figure 1

Developmental phases in the gametophyte of *Dryopteris affinis*: **a)** filamentous, **b)** spatulate, **c)** cordate, showing the apomictic embryo in the centre, and elongated cells, resembling a rudimentary transport system (white circle). Images were taken with brightfield microscopy (SCT Olympus BX-61). Bar = 200 µm.

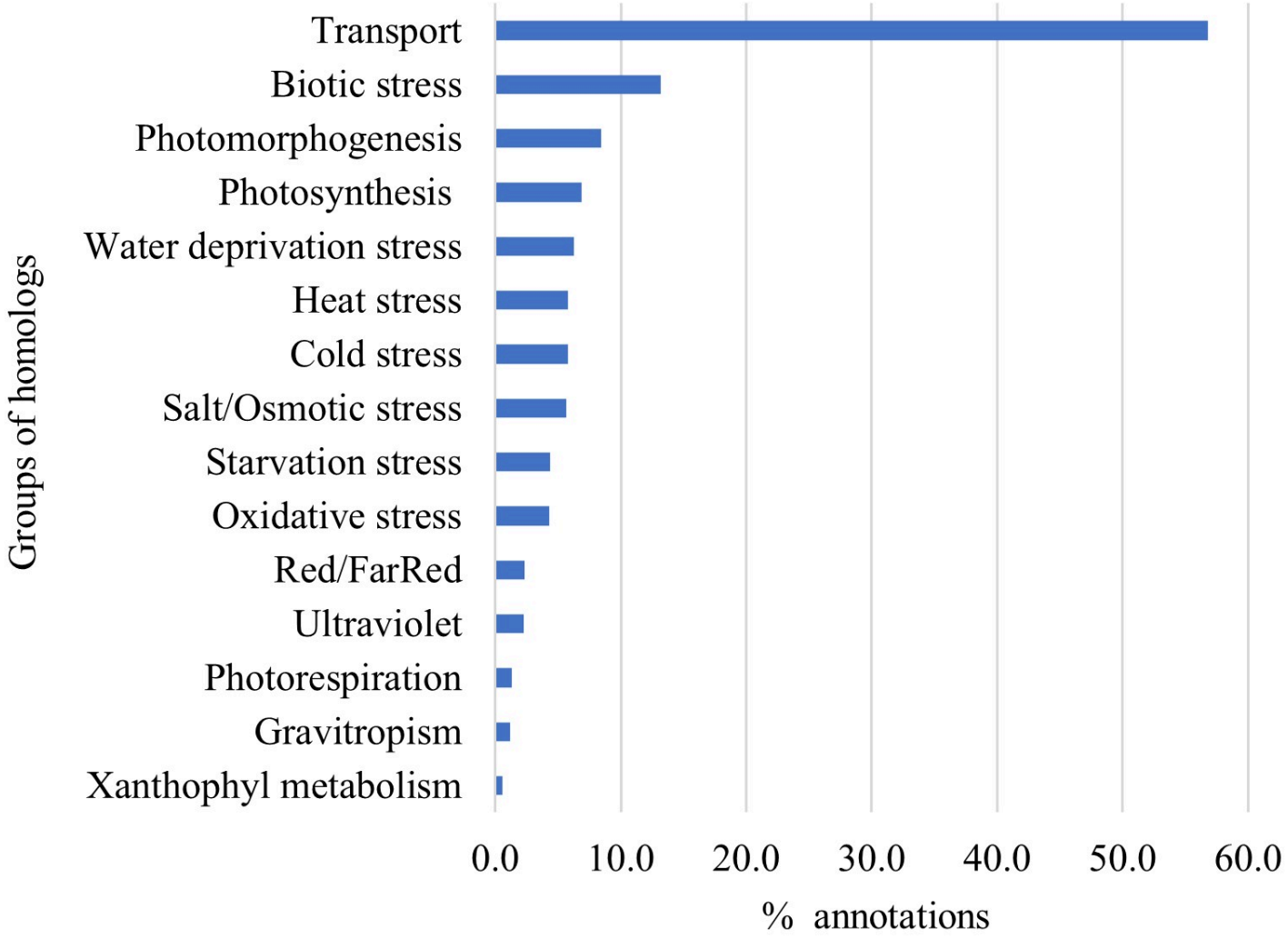

Figure 2

Bar plot showing the percentage of the selected groups of proteins extracted from the gametophyte of *Dryopteris affinis*.

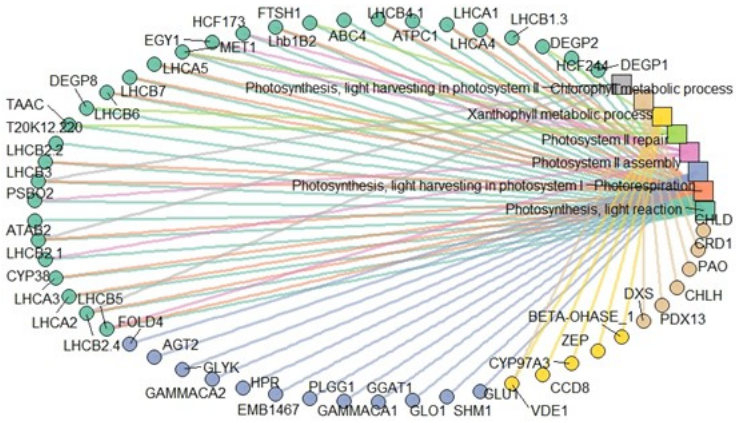

Figure 3

Circular gene–category interaction network (Cnetplot) for photosynthetic processes. Only significantly enriched categories (FDR < 0.05) are included. Node size of categories is scaled according to significance (−log10 FDR), while node colour distinguishes different categories. Circular nodes represent genes, and square nodes represent GO terms.

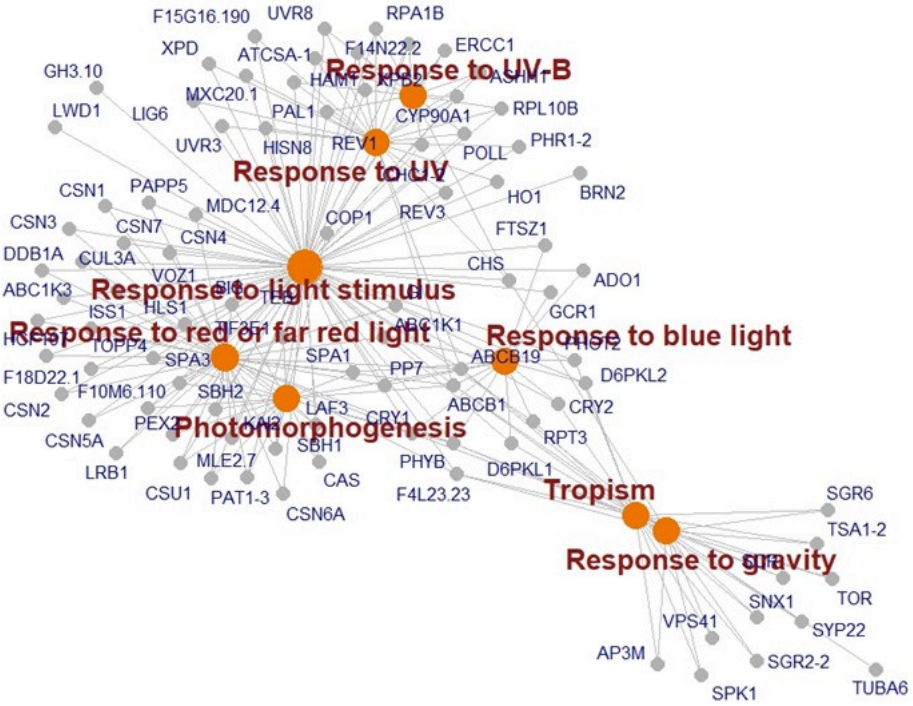

Figure 4

Gene–category interaction network (Cnetplot) related to photomorphogenesis. Only significantly enriched categories after false discovery rate correction (FDR < 0.05) are included. Grey nodes represent genes, and orange nodes represent GO terms in the gametophyte of the fern *Dryopteris affinis*.

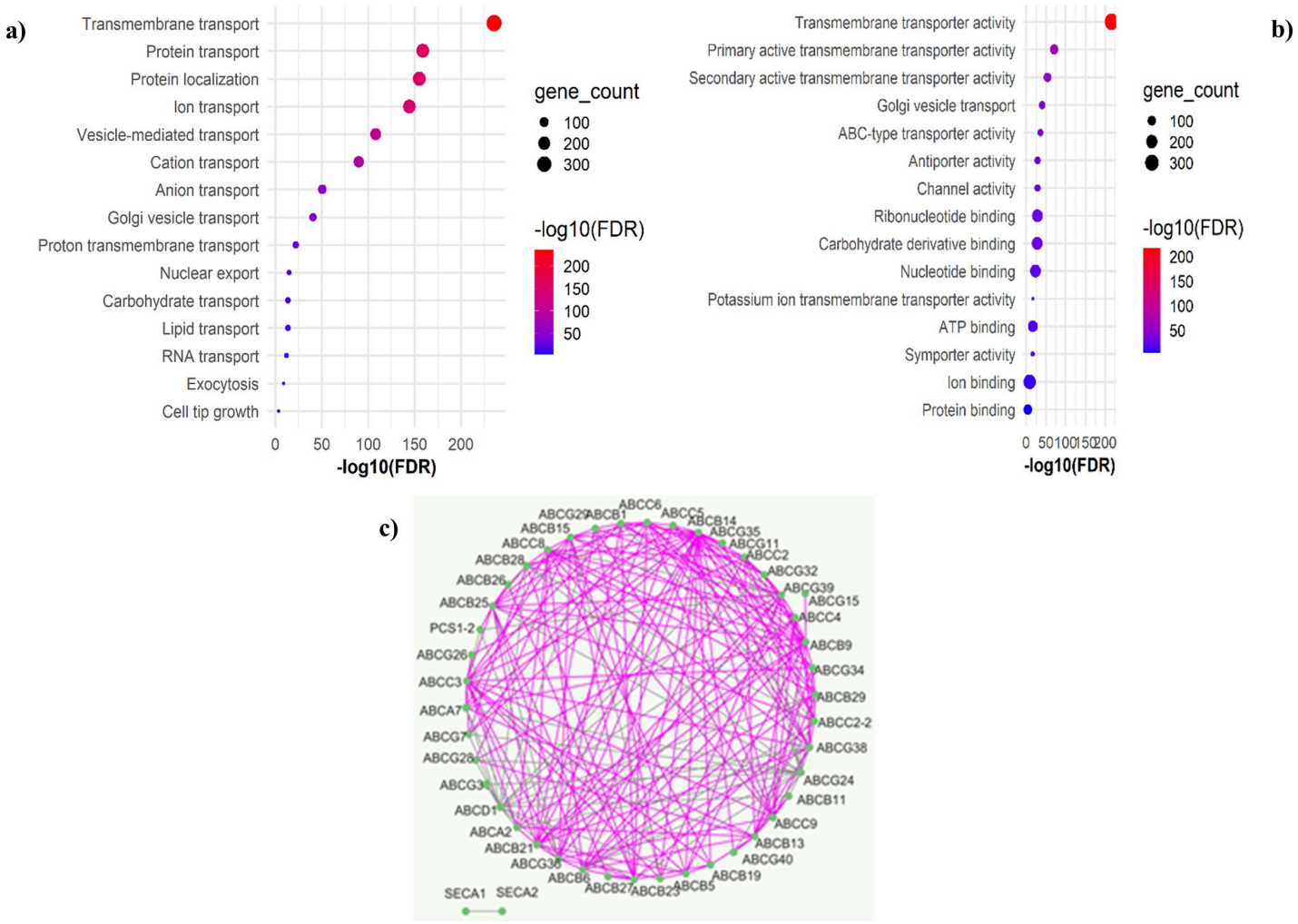

Figure 5

Functional enrichment and interaction network of transport-related proteins identified in the gametophyte of the fern *Dryopteris affinis*. **a)** and **b)**Dot plots showing significantly enriched GO terms related to biological process, and molecular function, respectively. **c)** Interaction network of ABC transporters generated with Cytoscape, where nodes represent ABC transporter proteins (mainly from the ABCB subfamily) and edges indicate functional or predicted associations among them. Pink colour refers to protein-protein interactions coming from experimental evidence.

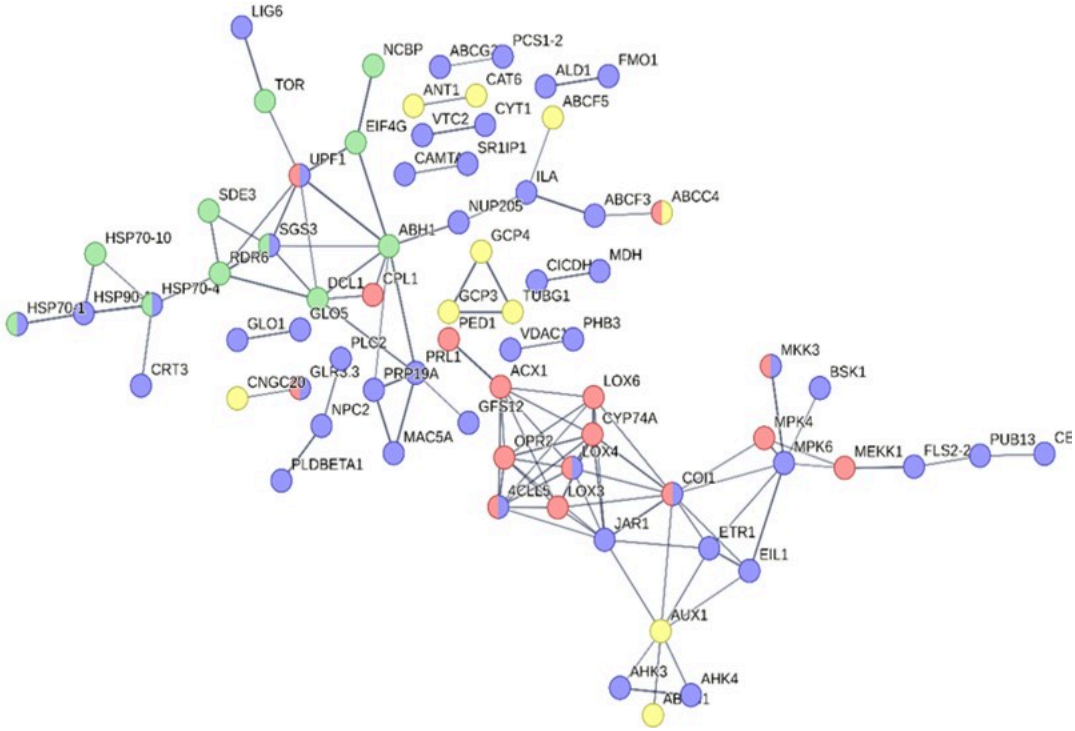

Figure 6

Network of proteins obtained from gametophytes of *Dryopteris affinis* related to response to bacterium (blue balls), nematodes (yellow balls), virus (green balls), and wounding (red balls), provided by String program v 12.0.

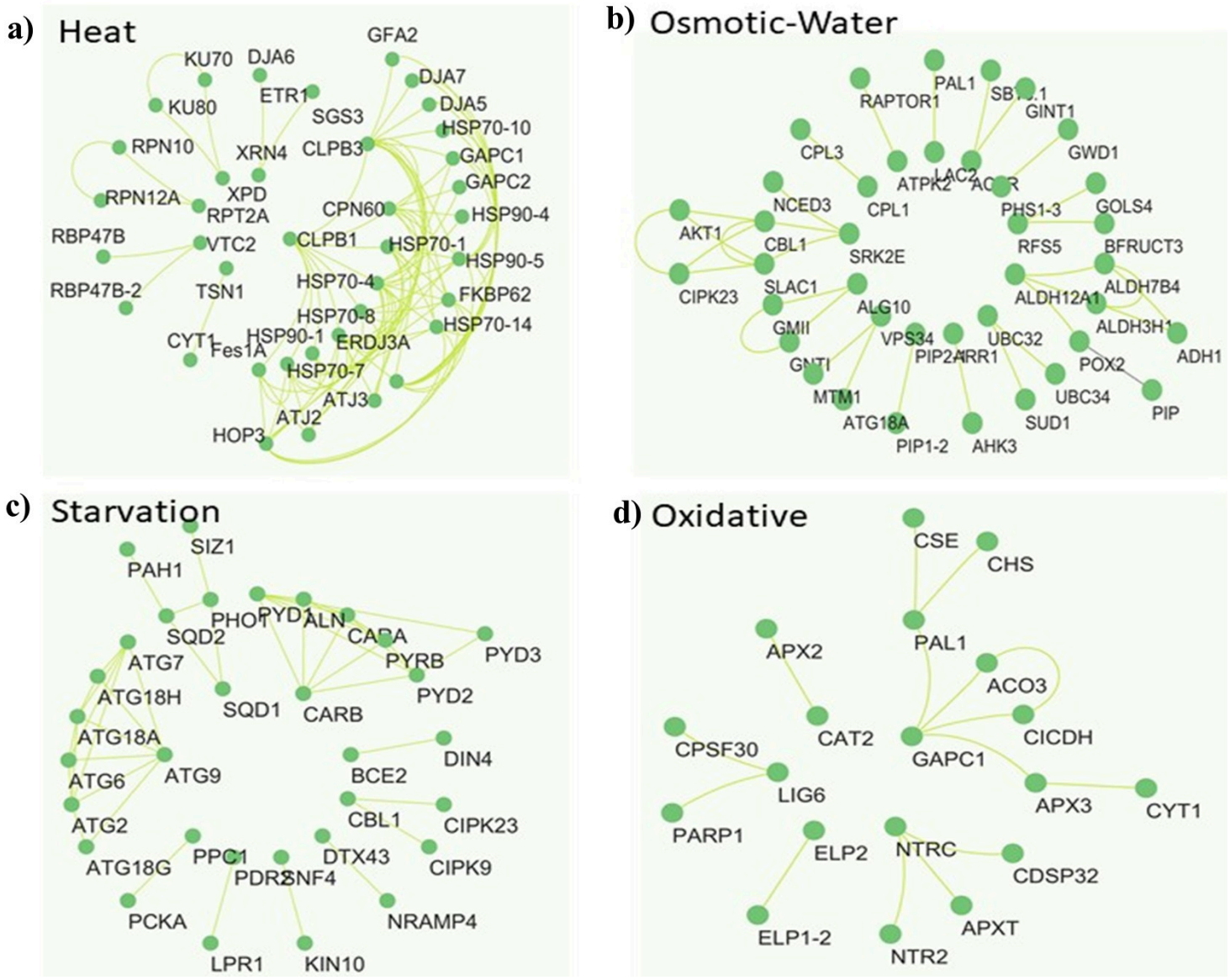

Figure 7

Networks of proteins extracted from gametophytes of *Dryopteris affinis* associated with the response to stress by: **a)** heat, **b)** osmotic/water deprivation **c)** starvation, and **d)** oxidation, provided by Cytoscape v 3.10.3. Green colour refers to protein-protein interactions coming from database evidence.

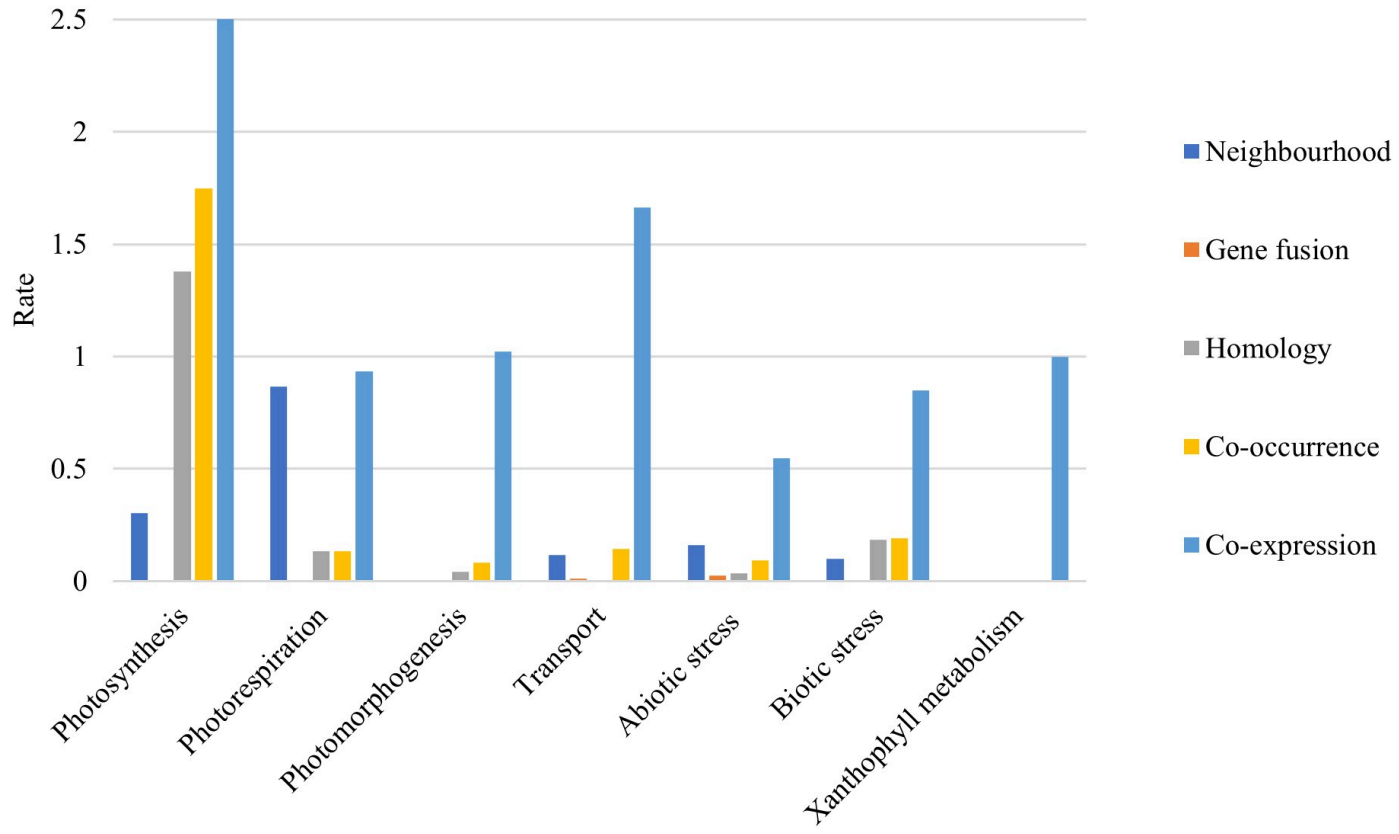

Figure 8

Estimation of the protein-protein interaction types in the selected groups of proteins extracted from the gametophytes of *Dryopteris affinis*.

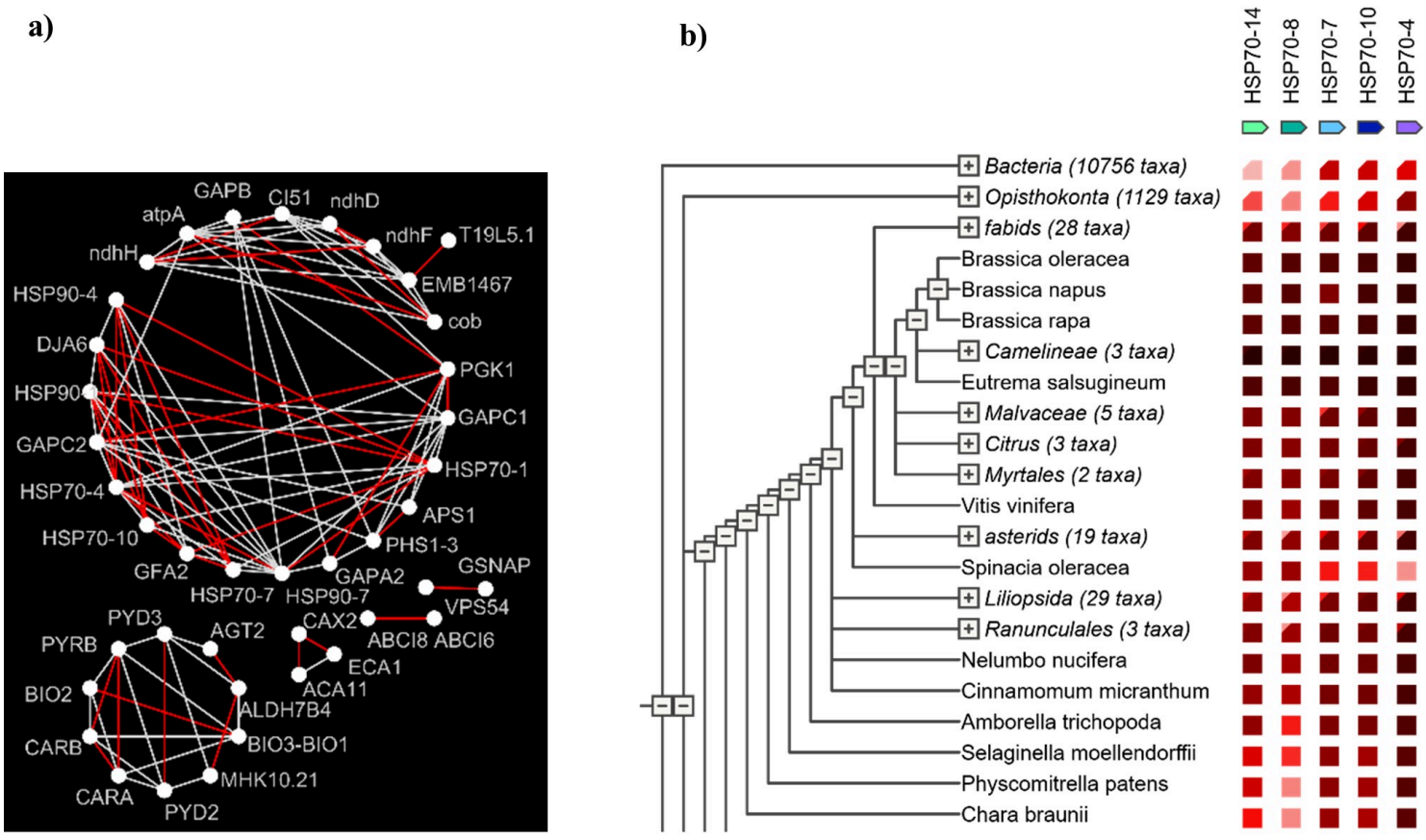

Figure 9

Protein–protein interaction network of a selected group of proteins from the *Dryopteris affinis* gametophyte, obtained using the STRING program: **a)** co-expression interaction in photosynthesis; **b)** gene fusion in response to heat.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable1.docx
- SupplementaryTable2.docx