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Proteome and Interactome Linked to Metabolism, Genetic Information Processing, and Abiotic Stress in Gametophytes of Two Woodferns

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Abstract: Ferns and lycophytes, now known as monilophytes, have received scant molecular attention in comparison to angiosperms. The advent of high-throughput technologies allowed an advance towards a greater knowledge of their elusive genomes. In this work, proteomic analyses of heart-shaped gametophytes of two ferns were performed: the apomictic species *Dryopteris affinis* ssp. a*ffinis* and its sexual relative *Dryopteris oreades*. In total, a set of 218 proteins shared by these two gametophytes were analyzed using the STRING database, and the proteome associated with metabolism, genetic information processing, and responses to abiotic stress is discussed. Specifically, we report proteins involved in the metabolism of carbohydrates, lipids, and nucleotides, the biosynthesis of amino acids, and secondary compounds, energy, oxide-reduction, transcription, translation, protein folding, sorting and degradation, and responses to abiotic stress. Looking at the interactome of this set of proteins, it represents a total network composed of 218 nodes and 1,792 interactions, obtained mostly from databases and textmining. The interactions among the identified proteins of the ferns *D. affinis* and *D. oreades*, together with the description of their biological functions, might contribute to a better understanding of the function and development of ferns as well as to fill knowledge gaps in plant evolution.

Keywords: *Dryopteris affinis* ssp. *affinis*; *Dryopteris oreades*; fern; gametophyte; non-seed plants; proteome; STRING database

1. Introduction

Ferns and lycophytes represent a genetic legacy of great value, being descendants of the first plants that evolved vascular tissues about 470 million years ago. Compared to angiosperms, they have received scant attention, relegating them to the background after a splendid past. The aesthetic appeal of their leaves and using them to alleviate ailments in traditional medicine is all that these plant groups have traditionally inspired. Only a handful of species have been used to delve into basic developmental processes, such as photomorphogenesis (1), spore germination (2–4), cell polarity (5), cell wall composition (6), or reproduction. These studies focused on the gametophyte generation, an autonomously growing organism, well-suited for in vitro culture and sample collection (7–8). Although the gametophytes of ferns possess a very simple structure consisting of one cell layer, they display some degree of complexity: apical-basal polarity, dorsoventral asymmetry, rhizoids, meristems in the apical or lateral parts, reproductive organs (male antheridia and female archegonia), and trichomes distributed over the entire surface.

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From a metabolic point of view, ferns and lycophytes contain many secondary metabolites: flavonoids, alkaloids, phenols, steroids, etc., and exhibit various bioactivities such as antibacterial, antidiabetic, anticancer, antioxidant, etc. (9). The therapeutic use of both plant groups is changing, from its use in the traditional medicine of different peoples to current applications, in which these plants are used to generate nanoparticles (10). Finally, the use of ferns and lycophytes was recently advocated to address problems caused by biotic and abiotic stresses. Drought is one of the most severe abiotic stresses affecting plant growth and productivity, and fern and lycophytes could contribute to managing it (11). Other important adaptations of ferns to extreme environments, such as salinity, heavy metals, epiphytes, or a low invasion of its habitats were summarized by Rathinasabapathi (12). Likewise, Dir (13) highlights the high efficiency of many species of aquatic and terrestrial ferns in extracting various organic and inorganic pollutants from the environment.

Increasingly, researchers have become more interested in these plants, made possible by the advent of high-throughput technologies, such as transcriptomics, proteomics, and metabolomics, providing greater knowledge of the functions encoded by their elusive genomes. Changes in gene expression, induced by either environmental or developmental conditions, can now be examined in non-model organisms because the required techniques have become more affordable as automation and efficiency have reduced costs. To date, some transcriptomic and proteomic datasets have been published for ferns, e.g., *Pteridium aquilinum* (14), *Ceratopteris richardii* (5,15), *Blechnum spicant* (16), *Lygodium japonicum* (17), *Dryopteris affinis* ssp. *affinis* (18–20), and *Dryopteris oreades* (20,21). For the last species, both transcriptomic and proteomic analyses were performed by RNA-sequencing and shotgun proteomics by tandem mass spectrometry.

This work expands our knowledge of proteomic data in non-seed plants, which is far less explored than in seed plants. We present a continuation of previous work (21), in which proteins of heart-shaped gametophytes from two ferns, the apomictic species *D. affinis* spp. *affinis* (referred to as *D. affinis* hereafter) and its sexual relative *D. oreades*, were extracted and identified using a species-specific transcriptome database established in a previous project (18,19). The functional annotation was inferred by blasting identified full length protein sequences. We report the categorization of proteins that are shared by both sexual and apomictic gametophytes. Specifically, our analysis reveals new proteomic information involved in the metabolism of carbohydrates and lipids, the biosynthesis of amino acids, the metabolism of nucleotides and energy, as well as of secondary compounds, such as flavonoids, terpenoids, lignans, etc., important in plant defence against stress. In addition to oxide-reductive processes, it also reveals proteins related to transcription, translation, as well as protein folding, sorting, transport, and degradation.

2. Results

A set of 218 proteins shared by the gametophytes of the apomictic fern D. affinis and its sexual relative D. oreades were analyzed using the software programs STRING version 11.5 and CYTOSCAPE version 3.9.1. proteomic data are available on line https://www.frontiersin.org/articles/10.3389/fpls.2021.718932/full#supplementary-material.

To gain insights into their biological functions, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) classifications provided by the STRING platform were analyzed (Figures 1 and 2). Based on GO classification, most of the identified proteins are involved in biological functions linked to the primary metabolism, and more specifically to other cellular processes, such as response to stimulus, protein degradation, translation, etc.

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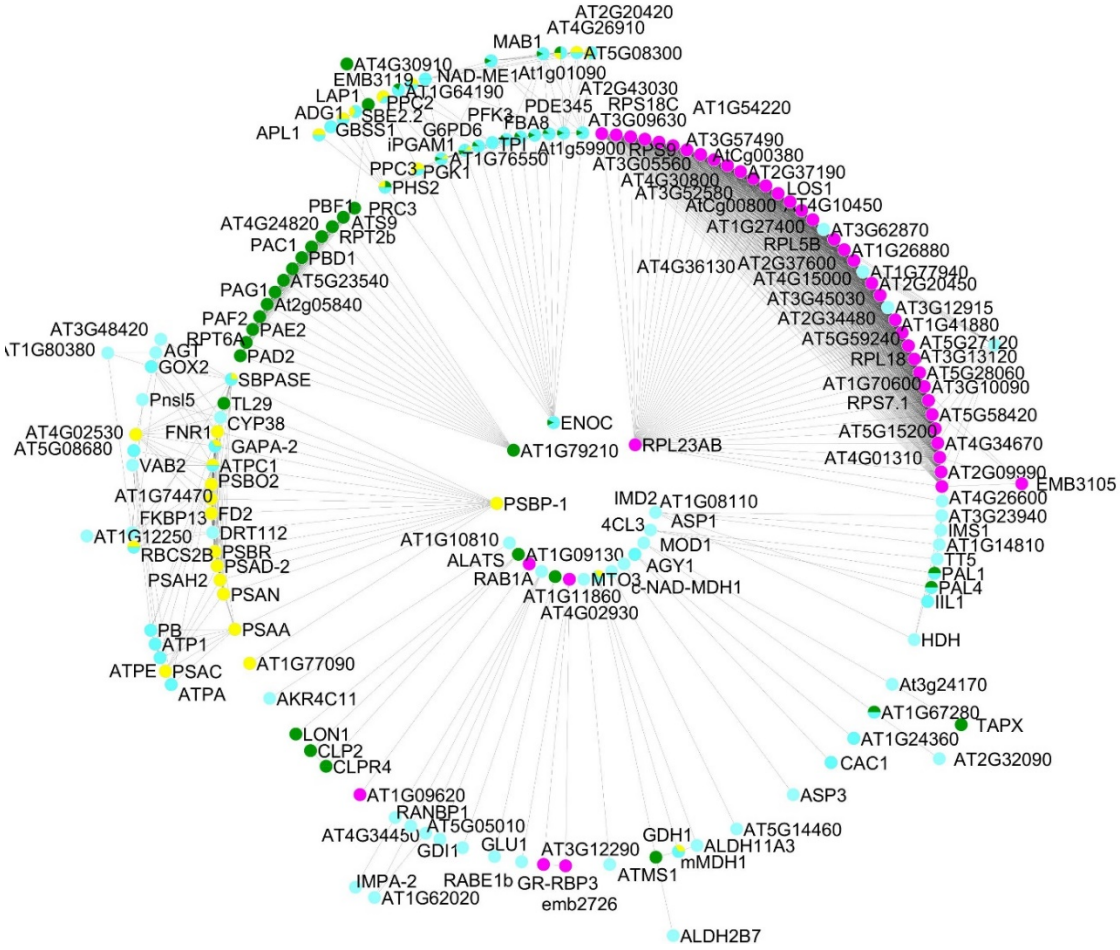

Figure 1. GO enrichment terms of the shared proteomes obtained from gametophytes of *Dryopteris affinis* and *D. oreades* according to the category biological function, analyzed by STRING and CYTOSCAPE. Turquoise indicates metabolism of carbohydrates, yellow metabolism of energy, pink transcription and translation, and green protein degradation.

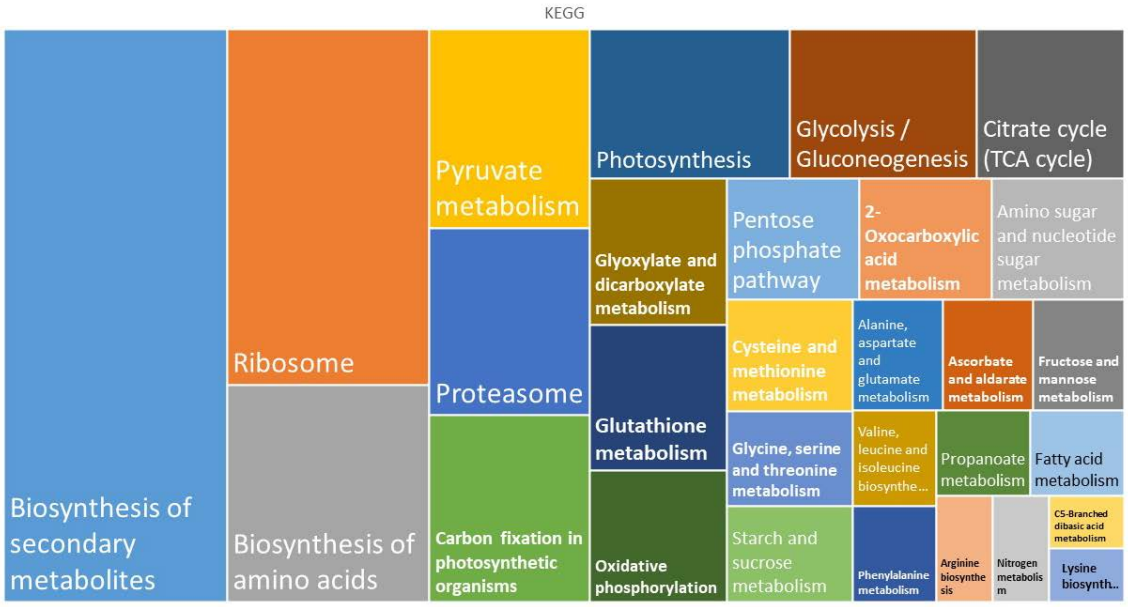

Figure 2. KEGG enrichment terms of the shared proteomes obtained from gametophytes of *Dryopteris affinis* and *D. oreades,* analyzed using the STRING platform.

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In turn, KEGG classification revealed that common proteins are mostly associated with the biosynthesis of secondary metabolites, the ribosome, and the biosynthesis of amino acids. These processes include the building of cellular organelles such as ribosomes (Figure 3) or proteasomes (Figure 4). Related to ribosomes, there were several protein classes, such as nucleic acid-binding proteins, ribosomal proteins, translation elongation factors, etc. On the other hand, proteasomes mediate the degradation of proteins, and we found proteins of 20S particle, the proteolytic core, but also regulatory factors.

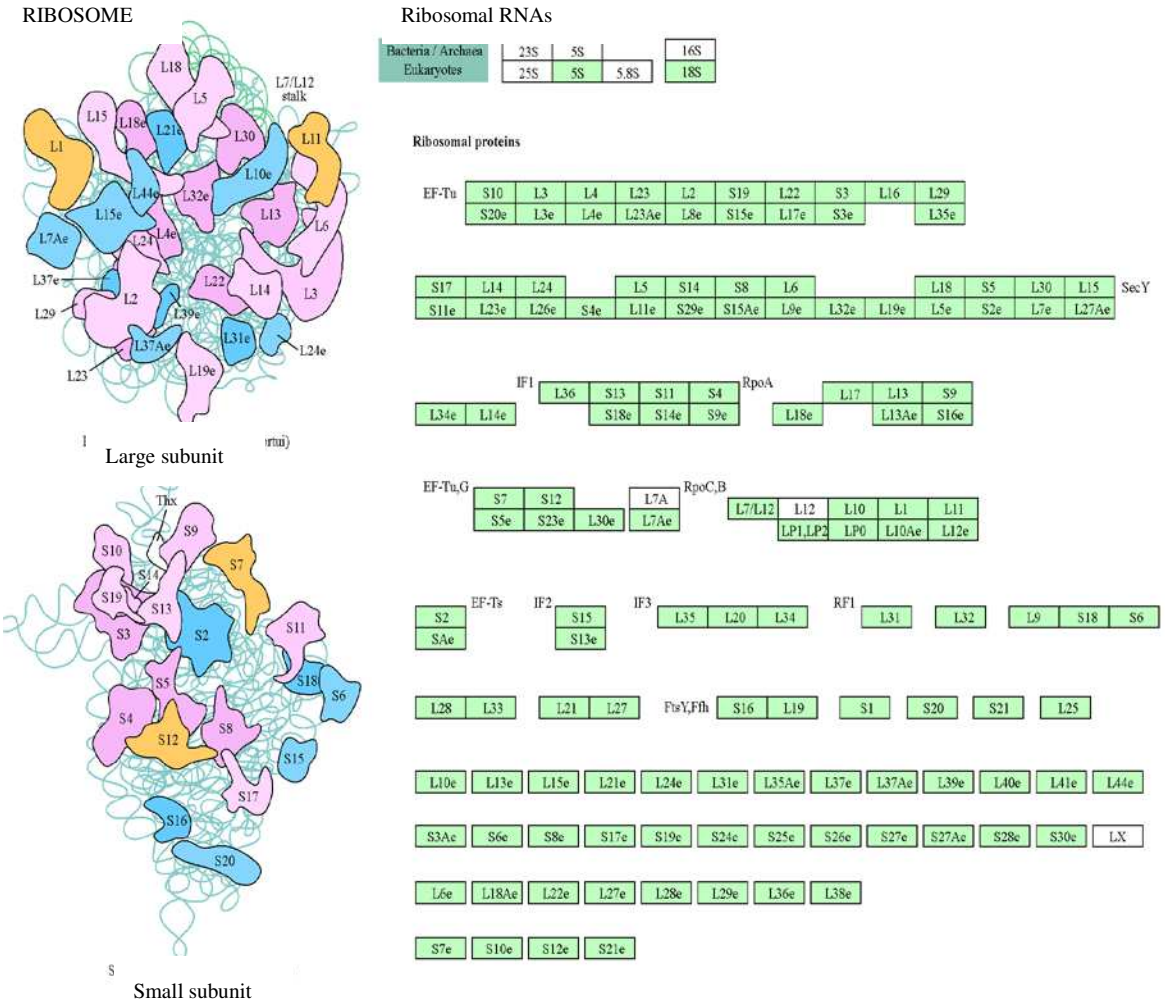

Figure 3. Proteins involved in ribogenesis found in the gametophyte of the ferns *Dryopteris affinis* and *D. oreades*. Imaged provided by STRING platform according to KEGG dataset. "Light-green" highlighted boxes are the identified proteins.

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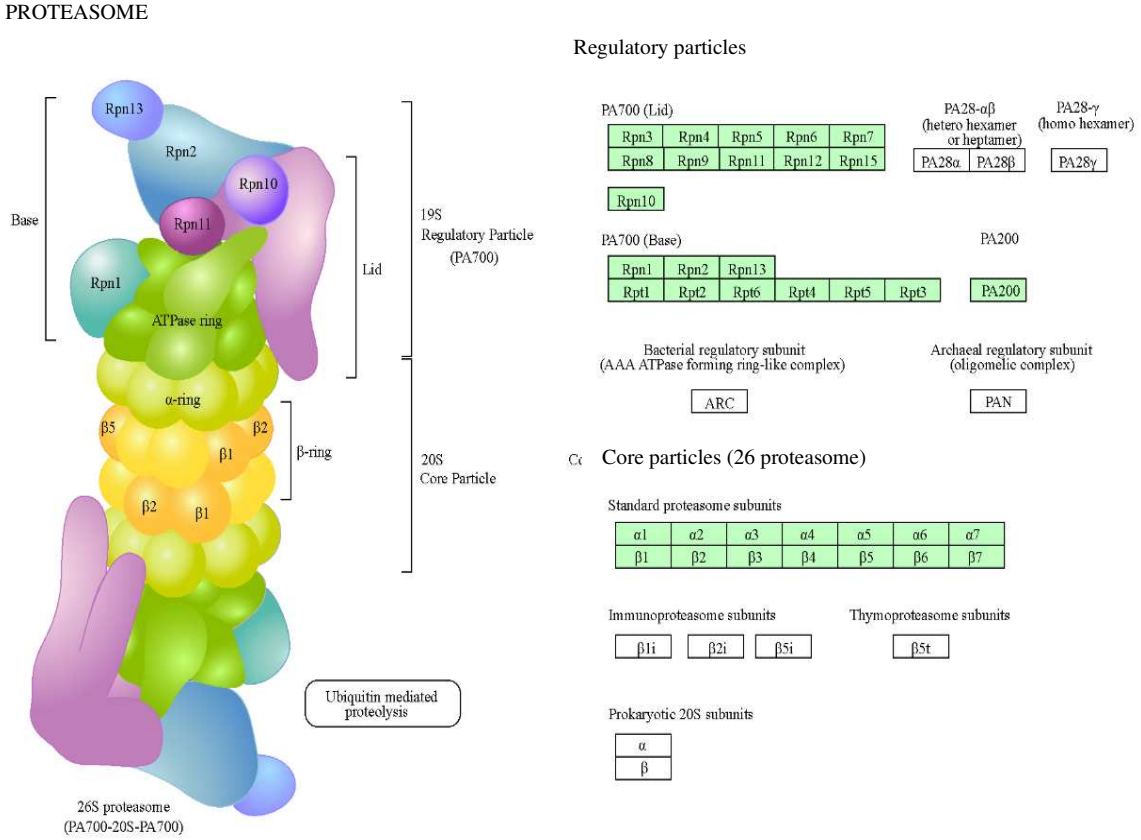

Figure 4. Proteins of the proteasome found in gametophytes of *Dryopteris affinis* and *D. oreades*. Images provided by the STRING platform according to KEGG annotations. Boxes highlighted in green represent identified proteins.

Protein domains that are abundant in the gametophytes of both ferns were the pyruvate dehydrogenase E1 component and the histidine and lysine active sites of the phosphoenolpyruvate carboxylase, which is involved in carbohydrate metabolism. Regarding the biosynthesis of amino acids, the most abundant domains were aspartate aminotransferase and pyridoxal phosphate-dependent transferase. Among proteins involved in the metabolism of energy, the HAS barrel domain and the F1 complex of the alpha and beta subunits of ATP synthase were abundant. Related to the metabolism of secondary compounds, aromatic amino acid lyase, phenylalanine ammonia-lyase, and the N-terminus of histidase were enriched. Finally, the frequently found domains in proteins involved in transcription and translation were the GTP-binding domain and domain 2 of elongation factor Tu as well as conserved sites of the ribosomal proteins S10 and S4.

The interactome of the proteins common to *D. affinis* and *D. oreades* represented a network composed of 218 nodes and 1,792 interactions (p-value < 1 x e-16). The proteins with the highest number of interactions among the identified proteins are shown in Table 1, i.e., SUPPRESSOR OF ACAULIS 56 (SAC56), RIBOSOMAL PROTEIN US11X (US11X), and RIBOSOMAL PROTEIN US17Y (US17Y), each having 44 interactions.

Table 1. Biological functions and number of interactions exhibited by proteins common to apomictic *D. affinis* and sexual *D. oreades*.

Biological functions	*Protein*	*Nº Interactions*
Metabolism of carbohydrates	ENOC	12
	PGK1	12
Biosynthesis of amino acids	AT1G14810	6
Metabolism of energy	ATPC1	20
Secondary metabolism	4CL3	3

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	SAC56	44
Transcription & Translation	US11X	44
	US17Y	44
Transport	RAB1A	4

The strength of the interactions can be weak or strong (Table S1) based on a scale from 0 to 1, where a weak interaction will have a score close to 0 and a strong one a score close to 1. Taking into account only interactions with a score equal to or greater than 0.99 foreach group of proteins studied, proteins involved in transcription and translation are those with the highest number of interactions (554), followed by proteins involved in energy (29), carbohydrate metabolism (16), and finally biosynthesis of amino acids (5), and transport (3). According to the STRING software, the evidence of interactions between proteins can be of various types: (a) Experiments: these refer to proteins that have been shown to have chemical, physical, or genetic interaction in laboratory experiments; (b) Databases: describes interactions of proteins found in the same databases; (c) Textmining: the proteins are mentioned in the same PubMed abstract or the same article of an internal selection of the STRING software; (d) Co-expression: indicates that the gene expression patterns of the two proteins are similar; (e) Neighbourhood: the genes encoding the proteins are close to each other in the genome; (f) Gene fusion: indicates that at least in one organism the orthologous genes encoding the two proteins are fused into a single gene; and (g) Co-occurrence: refers to proteins that have a similar phylogenetic distribution. Next, we consider some of these type of interaction between proteins (Figure 5): text mining, experiments, co-expression, and databases.

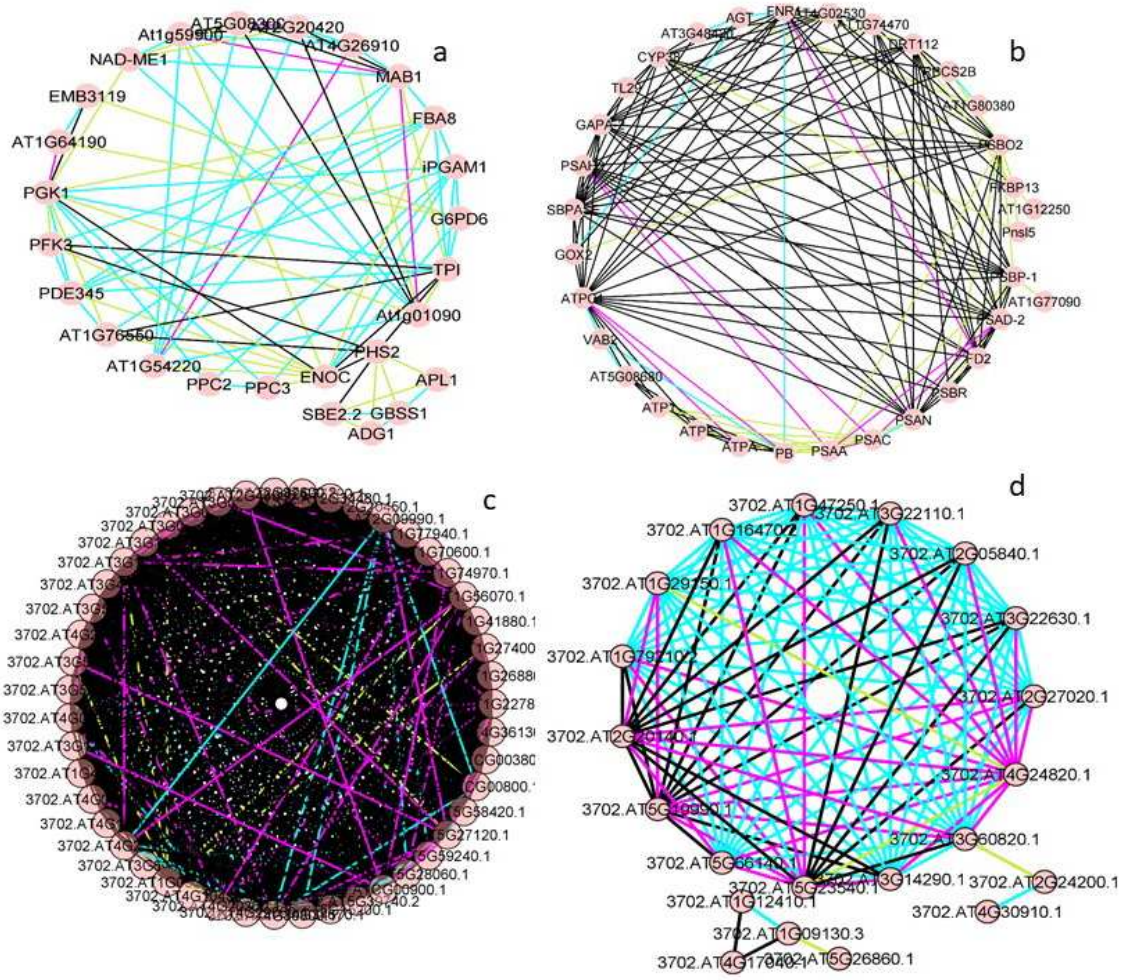

Figure 5. Circular representations obtained by STRING and CYTOSCAPE for proteins detected in the gametophytes of both *Dryopteris affinis* and *D. oreades*: **(a)** metabolism of carbohydrates, **(b)** metabolism of energy, **(c)** ribogenesis, and **(d)** protein degradation. Pink lines refer to evidence from

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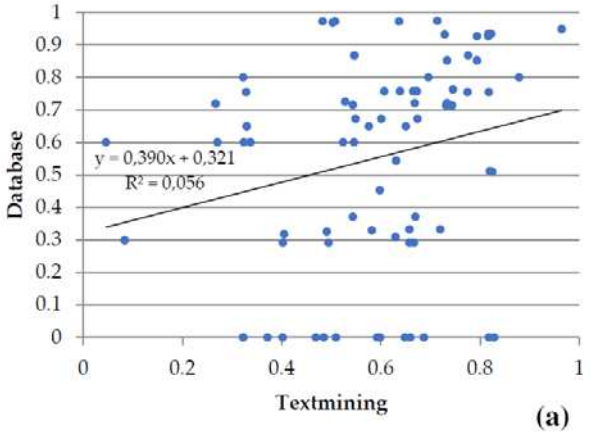
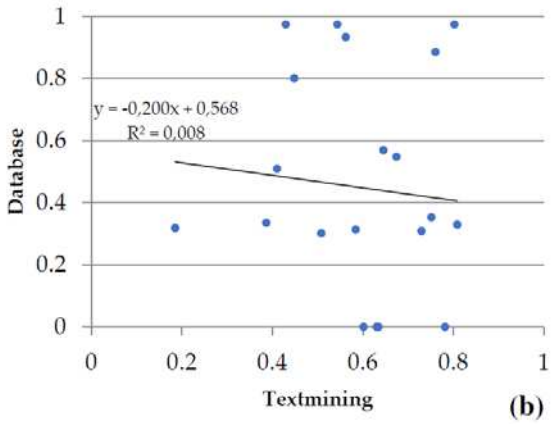
experiments, green lines from textmining, black lines from co-expression, and blue lines from databases.

Specifically, we analyzed the groups metabolism of carbohydrates (Figure 5a), metabolism of energy (Figure 5b), ribogenesis (Figure 5c), and protein degradation (Figure 5d). Paying attention only to the two main types of evidence for each of these groups, their relationships were analyzed (Figure 6). Evidence from databases and text mining were the most relevant for metabolism of carbohydrates (Figure 6a), biosynthesis of amino acids (Figure 6b), the metabolism of secondary compounds, and transport; textmining and co-expression data for the metabolism of energy (Figure 6c), and experiments and co-expression data for transcription and translation (Figure 6d). The associations between variables statistically were as follows: in metabolism of carbohydrates and in transcription and translation highly significant in both (p-value < 0.001), in biosynthesis of amino acids no significant (p-value > 0.05), and in metabolism of energy marginally significant (p-value slightly greater than 0.05).

Alternatively, when comparing the same type of evidence among the different groups of proteins, we observed that the neighborhood interaction was the most important for biosynthesis of amino acids and transcription and translation; gene fusion for metabolism of carbohydrates and biosynthesis of amino acids; co-occurrence for biosynthesis of amino acids and metabolism of secondary compounds; co-expression for metabolism of energy and transcription and translation; experiments for transcription and translation, and transport; evidence from databases for metabolism of carbohydrates and transport; and, finally, textmining for metabolism of secondary compounds and transport.





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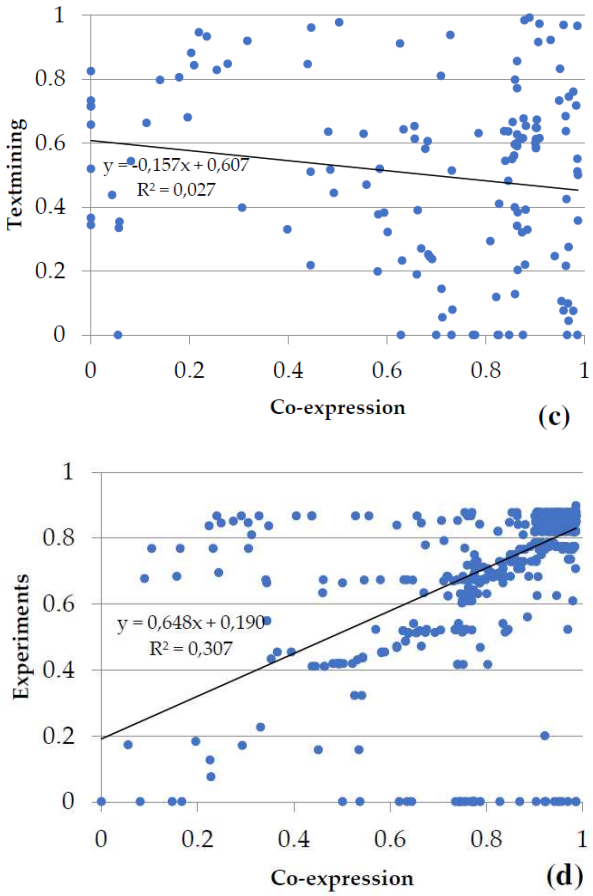

Figure 6. Plots of the two main types of evidence for interactions in the groups of proteins shared by the gametophytes of *Dryopteris affinis* and *D. oreades*: **(a)** metabolism of carbohydrates, **(b)** biosynthesis of amino acids, **(c)** metabolism of energy, and **(d)** transcription and translation. Each spot represents the intersection of the type of evidence for interactions between two proteins. The linear regression and the coefficient of correlation are provided for each pair of evidence for the interaction.

3. Discussion

The current work provides novel information on the proteome shared by gametophytes of the apomictic fern *D. affinis* and its sexual relative *D. oreades*, and provides continuity to previous studies in these species (18–21). Specifically, for ease of discussion of biological functions and interaction, the proteins discussed here after were grouped into two major categories: metabolism and genetic information processing (Table 2).

Table 2. Selected proteins found in gametophytes of both apomictic *D. affinis* and sexual *D. oreades*.

Category	Accession Number	UniProtKB/ Swiss-Prot	Gene Name	Protein Name	MW (kDa)	Amino Acids
Carbohydrates	58787-330_2_ORF2	Q94AA4	*PFK3*	Phosphofructokinase 3	53	489
Carbohydrates	135690-210_1_ORF2	Q9ZU52	*PDE345*	Pigment defective 345	42	391
Carbohydrates	tr|A9NMQ0|A9NMQ0_PICSI	Q9LF98	*FBA8*	Fructose-bisphosphate aldolase 8	38	358
Carbohydrates	38153-411_5_ORF2	Q38799	*MAB1*	Macchi-bou1	39	363
Carbohydrates	83096-276_3_ORF2	Q5GM68	*PPC2*	Phosphoenolpyruvate carboxylase 2	109	963
Carbohydrates	54280-344_1_ORF1	Q84VW9	*PPC3*	Phosphoenolpyruvate carboxylase 3	110	968

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Carbohydrates	113756-233_2_ORF1	Q9SIU0	*NAD-ME1*	NAD-dependent malic enzyme 1	69	623
Carbohydrates	102811-246_6_ORF2	O04499	*iPGAM1*	2,3-biphosphoglycerate-independent phosphoglycerate mutase 1	60	557
Carbohydrates	70011-302_2_ORF1	O82662	*AT2G20420*	-	45	421
Carbohydrates	8279-816_3_ORF2	P68209	*AT5G08300*	-	36	347
Carbohydrates	222487-119_2_ORF2	P93819	*c-NAD-MDH1*	Cytosolic-NAD-dependent malate dehydrogenase 1	35	332
Carbohydrates	156827-185_4_ORF1	Q9SH69	*PGD1*	6-phosphogluconate dehydrogenase 1	53	487
Carbohydrates	12493-682_6_ORF2	Q9FJI5	*G6PD6*	Glucose-6-phosphate dehydrogenase 6	59	515
Carbohydrates	20760-547_4_ORF1	Q9LD57	*PGK1*	Phosphoglycerate kinase 1	50	481
Carbohydrates	69882-302_6_ORF2	Q9LZS3	*SBE2.2*	Starch branching enzyme 2.2	92	805
Carbohydrates	tr|Q5PYJ7|Q5PYJ7_9MONI	Q9MAQ0	*GBSS1*	Granule bound starch synthase 1	66	610
Carbohydrates	tr|A9SGH8|A9SGH8_PHYPA	P55228	*ADG1*	ADP glucose pyro-phosphorylase 1	56	520
Carbohydrates	181563-155_3_ORF2	P55229	*APL1*	ADP glucose pyro-phosphorylase large subunit 1	57	522
Carbohydrates	tr|D7MQA6|D7MQA6_ARALL	Q9LUE6	*RGP4*	Reversibly glycosylated polypeptide 4	41	364
Carbohydrates	162660-176_6_ORF1	P83291	*AT5G20080*	-	35	328
Lipids	20213-554_2_ORF1	Q9SLA8	*MOD1*	Mosaic death 1	41	390
Lipids	387953-27_4_ORF1	Q9SGY2	*ACLA-1*	ATP-citrate lyase A-1	49	443

Category	**Accession Number**	**UniProtKB/ Swiss-Prot**	**Gene Name**	**Protein Name**	**MW (kDa)**	**Amino Acids**
Lipids	211149-128_1_ORF1	Q9LXS6	*CSY2*	Citrate synthase 2	56	514
Amino acids	47558-369_4_ORF2	P46643	*ASP1*	Aspartate aminotransferase 1	47	430
Amino acids	72506-296_4_ORF1	Q94AR8	*IIL1*	Isopropyl malate isomerase large subunit 1	55	509
Amino acids	125905-219_3_ORF2	Q9ZNZ7	*GLU1*	Glutamate synthase 1	179	1,648
Amino acids	393073-25_4_ORF2	Q94JQ3	*SHM3*	Serine hydroxymethyltransferase 3	57	529
Amino acids	tr|D8RLH8|D8RLH8_SELML	Q9C5U8	*HDH*	Histidinol dehydrogenase	50	466
Amino acids	294436-71_4_ORF2	Q9LUT2	*MTO3*	Methionine over-accumulator 3	42	393
Nucleotides	2121-1366_3_ORF2	Q9SF85	*ADK1*	Adenosine kinase 1	37	344
Nucleotides	59309-329_5_ORF1	Q96529	*ADSS*	Adenylosuccinate synthase	52	490
Nucleotides	152024-193_3_ORF2	Q9S726	*EMB3119*	Embryo defective 3119	29	276
Energy	164104-175_1_ORF1	Q9FKW6	*FNR1*	Ferredoxin-NADP (+)-oxidoreductase 1	40	360
Energy	sp|Q7SIB8|PLAS_DRYCA	P42699	*DRT112*	DNA-damage-repair/toleration protein 112	16	167
Energy	154679-189_1_ORF2	Q9S841	*PSBO2*	Photosystem II subunit O-2	35	331

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Energy	218625-122_1_ORF2	O22773	*MPH2*	Maintenance of photosystem II under high light 2	23	216
Energy	6036-926_2_ORF1	Q9ASS6	*Pnsl5*	Photosynthetic NDH subcomplex l 5	28	259
Energy	250817-99_2_ORF2	Q94K71	*AT3G48420*	-	34	319
Energy	tr|A9RDI1|A9RDI1_PHYPA	Q944I4	*GLYK*	Glycerate kinase	51	456
Energy	297118-70_2_ORF2	Q56YA5	*AGT*	Alanine:glyoxylate aminotransferase	44	401
Energy	33137-439_6_ORF2	O48917	*SQD1*	Sulfoquinovosyldiacylglycerol 1	53	477
Energy	227095-115_1_ORF2	Q84W65	*CPSUFE*	Chloroplast sulfur E	40	371
Energy	311596-62_2_ORF2	Q9ZST4	*GLB1*	GLNB1 homolog	21	196
Energy	318906-58_1_ORF1	Q39161	*NIR1*	Nitrite reductase 1	65	586
Secondary compounds	156331-186_3_ORF2	P41088	*TT5*	Transparent testa 5	26	246
Secondary compounds	230420-113_2_ORF2	P34802	*GGPS1*	Geranylgeranyl pyrophosphate synthase 1	40	371
Secondary compounds	85783-271_1_ORF2	Q9T030	*PCBER1*	Phenylcoumaran benzylic ether reductase 1	34	308
Secondary compounds	153413-190_1_ORF2	P42734	*CAD9*	Cinnamyl alcohol dehydrogenase 9	38	360
Secondary compounds	156554-185_2_ORF1	Q9S777	*4CL3*	4-coumarate:coA ligase 3	61	561
Secondary compounds	223603-118_1_ORF1	P05466	*AT2G45300*	-	55	520
Oxido -reduction	133847-212_2_ORF2	Q9SID3	*GLX2-5*	Glyoxalase 2-5	35	324
Oxido -reduction	tr|E1ZRS4|E1ZRS4_CHLVA	Q9ZP06	*mMDH1*	Mitochondrial malate dehydrogenase 1	35	341
Oxido -reduction	34437-432_2_ORF1	Q9M2W2	*GSTL2*	Glutathione transferase lambda 2	33	292
Oxido -reduction	115571-230_4_ORF1	Q9LZ06	*GSTL3*	Glutathione transferase L3	27	235
Transcription	tr|A2X6N1|A2X6N1_ORYSI	Q96300	*GRF7*	General regulatory factor 7	29	265
Transcription	287872-75_1_ORF1	Q9C5W6	*GRF12*	General regulatory factor 12	30	268
Translation	209284-130_2_ORF2	Q9FNR1	*RBGA7*	MA-binding glycine-rich protein A7	29	309
Translation	293356-72_1_ORF1	Q9LR72	*AT1G03510*	-	47	429
Translation	26795-487_6_ORF2	Q0WW84	*RBP47B*	MA-binding protein 47B	48	435
Translation	20230-554_5_ORF2	Q9LES2	*UBA2A*	UBP1-associated protein 2A	51	478
Translation	174433-162_1_ORF1	Q9ASR1	*LOS1*	Low expression of osmotically responsive genes 1	93	843
Folding	26640-489_1_ORF2	Q9M1C2	*GROES*	-	15	138
Folding	189606-147_1_ORF2	Q9SR70	*AT3G10060*	-	24	230
Folding	149253-199_6_ORF2	O22870	*AT2G43560*	-	23	223
Folding	2524-1285_6_ORF2	Q9SKQ0	*AT2G21130*	-	18	174

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Transport	19573-562_5_ORF2	Q9SYI0	*AGY1*	Albino or glassy yellow 1	117	1,042
Transport	248569-101_3_ORF1	P92985	*RANBP1*	RAN binding protein 1	24	219
Transport	146969-201_2_ORF1	F4JL11	*IMPA-2*	Importin alpha isoform 2	58	535
Transport	151836-193_1_ORF2	P40941	*AAC2*	ADP/ATP carrier 2	41	385

Category	Accession Number	UniProtKB/ Swiss-Prot	Gene Name	Protein Name	MW (kDa)	Amino Acids
Transport	161087-178_2_ORF2	Q8H0U5	*Tic62*	Translocon at the inner envelope membrane of chloroplasts 62	68	641
Transport	82340-277_1_ORF2	Q39196	*PIP1;4*	Plasma membrane intrinsic protein 1;4	30	287
Transport	154825-188_3_ORF2	Q9SMX3	*VDAC3*	Voltage dependent anion channel 3	29	274
Transport	272341-85_2_ORF2	Q94A40	*alpha1-COP*	Alpha1 coat protein	136	1,216
Transport	29489-466_3_ORF1	Q0WW26	*gamma2-COP*	Gamma2 coat protein	98	886
Transport	38639-409_2_ORF3	Q93Y22	*AT5G05010*	-	57	527
Transport	43675-385_1_ORF2	Q67YI9	*EPS2*	Epsin2	95	895
Transport	68824-304_5_ORF2	Q9LQ55	*DL3*	Dynamin-like 3	100	920
Transport	-	F4J3Q8	*GET3B*	Guided entry of tail-anchored proteins 3B	47	433
Transport	3434-1154_1_ORF2	Q96254	*GDI1*	Guanosine nucleotide diphosphate dissociation inhibitor 1	49	445
Degradation	141778-205_4_ORF2	Q8L770	*AT1G09130*	-	40	370
Degradation	172993-163_5_ORF1	Q9XJ36	*CLP2*	CLP protease proteolytic subunit 2	31	279
Degradation	72587-296_2_ORF2	Q8LB10	*CLPR4*	CLP protease R subunit 4	33	305
Degradation	17420-593_1_ORF2	P93655	*LON1*	LON protease 1	109	985
Degradation	tr|A9SF86|A9SF86_PHYPA	Q9LJL3	*PREP1*	Pre-sequence protease 1	121	1,080
Degradation	186732-150_2_ORF2	Q9FL12	*DEG9*	Degradation of periplasmic proteins 9	65	592
Degradation	170504-166_2_ORF2	P30184	*LAP1*	Leucyl aminopeptidase 1	54	520
Degradation	170504-166_2_ORF2	Q944P7	*LAP3*	Leucyl aminopeptidase 3	61	581

3.1. Metabolism

Metabolism comprises two main branches: primary and secondary. Primary metabolism concerns essential metabolites that are directly involved in plant growth (carbohydrates, lipids, amino acids, nucleotides), as well as those reactions that fuel their biosynthesis, such as photosynthesis, glycolysis, tricarboxylic acid cycle, etc. In plants, there is also an important secondary metabolism, which concerns non-essential metabolic routes that govern, for instance, defence and stress responses.

Metabolism of carbohydrates

Proteins linked to metabolism of carbohydrates centres around glycolysis, pyruvate metabolism, citrate/tricarboxylic acid cycle, pentose phosphate pathway, starch, and biosynthesis of nucleotide sugars. Glycolysis converts glucose into pyruvate, and in the gametophytes under study, we found enzymatic proteins, such as ATP-DEPENDENT 6-PHOSPHOFRUCTOKINASE 3 (PFK3), involved in the first reaction; two enzymes participating in glycolysis and gluconeogenesis: FRUCTOSE-BISPHOSPHATE ALDOLASE 3 (FBA3), and FRUCTOSE-BISPHOSPHATE ALDOLASE 8 (FBA8); and others catalysing the decarboxylation of pyruvate to acetyl-CoA, such as the mitochondrial

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component of pyruvate dehydrogenase MACCHI-BOU1 (MAB1). Linked to *pyruvate metabolism, we* identified two phosphoenolpyruvate carboxylases (PPC2 and PPC3), which supply oxaloacetate for the tricarboxylic acid cycle; and the protein NAD-DEPENDENT MALIC ENZYME 1 (NAD-ME1), which is involved in regulating the metabolism of sugars and amino acids during the night (22). Worth mentioning is also 2,3-BIPHOSPHOGLYCERATE-INDEPENDENT PHOSPHOGLYCERATE MUTASE 1 (iPGAM1), important for the functioning of stomatal guard cells and fertility in *A. thaliana* (23).

Citrate/tricarboxylic acid cycle

Likewise, we identified some proteins associated with the citrate/tricarboxylic acid cycle: AT2G20420 and AT5G08300, involved in the only phosphorylation step at the substrate level of this cycle. Another protein we identified is the cytosolic MALATE DEHYDROGENASE 1 (c-NAD-MDH1), which catalyses a reversible NAD-dependent dehydrogenase reaction involved in central metabolism and redox homeostasis between organelle compartments (24).

Pentose phosphate pathway

In parallel to glycolysis, the pentose phosphate pathway generates NADPH and pentoses. This metabolic pathway is represented in our dataset by the proteins 6-PHOSPHOGLUCONATE DEHYDROGENASE 1 (PGD1), GLUCOSE-6-PHOSPHATE DEHYDROGENASE 6 (G6PD6), and PGK1. A mutation in the gene of the first protein may decrease cellulose synthesis, thus altering the structure and composition of the primary cell wall (25). G6PD6 is important for the synthesis of fatty acids and nucleic acids involved in membrane synthesis and cell division (26). PGK1 contributes to trigger the phosphorylation of the proteins FTSZ2-1 and FTSZ2-2, required for chloroplast division (27).

Starch

Starch is the main reserve form of carbohydrates and energy in plants, being accumulated in chloroplasts during the day, and transported and degraded to provide energy and nutritional substances for growth and metabolism. Gametophytes of *D. affinis* and *D. oreades* produce proteins involved in its synthesis, including STARCH BRANCHING ENZYME 2.2 (SBE2.2) and GRANULE-BOUND STARCH SYNTHASE 1 (GBSS1), the latter, together with STARCH DIRECTED PROTEIN (PTST), required for amylose synthesis (28).

Biosynthesis of nucleotide sugars

Apart from to the proteins mentioned above, we found some that are associated with the biosynthesis of nucleotide sugars, such as two pyrophosphorylases (ADG1 and APL1); the protein REVERSIBLY GLYCOSYLATED POLYPEPTIDE 4 (RGP4), involved in the synthesis of non-cellulosic polysaccharides of the cell wall (29); and AT5G20080.

Metabolism of lipids

Regarding the metabolism of lipids, three proteins were identified in this study. The first protein is MOSAIC DEATH1 (MOD1), which catalyses the last reduction step of the *de novo* fatty acid synthesis cycle and the fatty acid elongation cycle. A mutation causing a decreased activity of this protein, reduces the number of fatty acids, which triggers mosaic premature cell death and changes in the plant's morphology, such as chlorotic and curly leaves, distorted siliques, and dwarfism (30). The second protein is ATP-CITRATE LYASE A-1 (ACLA-1), necessary for the normal growth and development of plants because it synthesizes acetyl-coA, a key compound for many metabolic pathways (fatty acids and glucosinolates in chloroplasts; flavonoids, sterols, and phospholipids in the cytoplasm; and ATP and amino acids carbon skeletons in mitochondria). Moreover, it is the substrate for histone acetylation and transcription factors in the nucleus and regulates their function

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to maintain or alter chromosome structure and transcription (31,32). The third protein is CITRATE SYNTHASE 2 (CSY2), which synthesizes citrate in peroxisomes for the respiration of fatty acids in seedlings and is required for seed germination (33).

Biosynthesis of amino acids

Involved in the biosynthesis of amino acids, we found the proteins aminotransferase ASP1; the ISOPROPYL MALATE ISOMERASE LARGE SUBUNIT 1 (IIL1), which acts in glucosinolate biosynthesis involved in the defence against insects (34); GLUTAMATE SYNTHASE 1 (GLU1), required for the re-assimilation of ammonium ions generated during photorespiration (35); and SERINE HYDROXYMETHYLTRANSFERASE 3 (SHM3), HISTIDINOL DEHYDROGENASE (HDH), and METHIONINE OVER-ACCUMULATOR3 (MTO3), which catalyse the formation of glycine, L-histidine, and methionine, respectively (36–38).

Metabolism of nucleotides

We also identified proteins associated with the metabolism of nucleotides, specifically with AMP synthesis, such as ADENOSINE KINASE 1 (ADK1) and ADENYLOSUCCINATE SYNTHASE (ADSS). Worth mentioning is the protein EMBRYO DEFECTIVE3119 (EMB3119), essential for the synthesis of numerous compounds such as purines, pyrimidines, aromatic amino acids, NAD, and NADP (25).

Metabolism of energy

As it is well known, metabolism demands energy in the form of ATP derived from nutrients, and it comprises a series of interconnected pathways that can function in the presence or absence of oxygen. In the fern gametophytes studied here, several mitochondrial, chloroplastic, and vacuolar ATP synthases engaged in the oxidative phosphorylation process were found. Additionally, chemical energy can be obtained through photosynthesis. The list of annotated proteins includes the protein FERREDOXIN-NADP (+)-OXIDOREDUCTASE 1 (FNR1), which regulates the flow of electrons to meet the demands of the plant for ATP and reductive potential (39); and others involved in repairing DNA damage, such as DNA-DAMAGE-REPAIR/TOLERATION PROTEIN 112 (DRT112) (40), PHOTOSYSTEM II SUBUNIT O-2 (PSBO2), which regulates the replacement of the protein D1 impaired by light (41), and the protein MAINTENANCE OF PHOTOSYSTEM II UNDER HIGH LIGHT2 (MPH2). This protein is necessary to carry out photosynthesis correctly and efficiently under two conditions: controlled photoinhibitory light and fluctuating light; in nature, plants experience rapid and extreme changes in sunlight, requiring rapid adaptation (42). Involved in photosynthesis, we found the protein PHOTOSYNTHETIC NDH SUBCOMPLEX L 5 (Pnsl5), which modulates the conformation of the protein BRASSINAZOLE-RESISTANT 1 (BZR1) (43). This protein binds to the promoter of the *FLOWERING LOCUS D* (*FLD*) gene and represses its expression, leading to the expression of *FLOWERING LOCUS C* (*FLC*), a repressor of flowering (43). Finally, AT3G48420 degrades xylulose-1,5-bisphosphate, a potent inhibitor of the protein RUBISCO (44). On the other hand, photorespiration represents a waste of the energy produced by photosynthesis. The enzyme GLYCERATE KINASE (GLYK) catalyses the final reaction of photorespiration (45). Another important protein for photorespiration is ALANINE GLYOXYLATE AMINOTRANSFERASE (AGT), which also participate in primary and lateral root development (46).

Sulfur and nitrogen metabolism

We also detected proteins involved in sulphur and nitrogen metabolism. The first one is represented by two proteins: SULFOQUINOVOSYLDIACYLGLYCEROL 1 (SQD1), which converts UDP-glucose and sulphite to the precursor of the main group of sulfolipids, UDP-sulfoquinovose, thus preventing sulphite from accumulating as it is toxic to the cell (47); and CHLOROPLAST SULFUR E (CPSUFE), a sulphur acceptor that activates cysteine desulfurases in plastids and mitochondria, essential for embryogenesis (48). Regarding nitrogen metabolism, there are the

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proteins GLNB1 HOMOLOG (GLB1), which is a nitrogen regulatory protein and intervenes in glycosaminoglycan degradation (49); and NITRITE REDUCTASE 1 (NIR1), which catalyses the reduction of nitrite to ammonium (50). As the amount of this protein in the cell increases, tolerance and assimilation of nitrogen dioxide by the plant improve. As nitrogen dioxide is an air pollutant produced largely by motorized vehicles, plants could act as a sink for this substance, i.e., NIR1could be used in biotechnological applications for bioremediation (50).

Metabolism of secondary compounds

Concerning the metabolism of secondary compounds**,** proteins related to flavonoid biosynthesis are represented in this work such as TRANSPARENT TESTA5 (TT5), responsible for the isomerization of chalcones into naringenin (51). We also found enzymes involved in the biosynthesis of terpenoids: GERANYLGERANYL PYROPHOSPHATE SYNTHASE 1 (GGPS1); the biosynthesis of lignans: PHENYLCOUMARAN BENZYLIC ETHER REDUCTASE 1 (PCBER1); and the biosynthesis of phenylpropanoids: CINNAMYL ALCOHOL DEHYDROGENASE 9 (CAD9), very important to cell wall formation (52). Also, the protein 4CL3, which produces coA-thioesters of hydroxy- and methoxy-substituted cinnamic acids, is used to synthesize anthocyanins, flavonoids, isoflavonoids, coumarins, lignin, suberin, and phenols (53); and AT2G45300, which is involved in the synthesis of chorismate, the precursor of the amino acid phenylalanine, tryptophan, and tyrosine (54). Linked to oxide-reduction processes, we found the proteins: GLYOXALASE 2-5(GLX2-5), involved in cell detoxification in mitochondria (55); the mitochondrial MALATE DEHYDROGENASE 1 (mMDH1), participating in redox homeostasis between organelle compartments and possibly limiting photorespiration, and which is also required for partitioning of carbon dioxide and energy in leaves (56); and the transferases GSTL2 and GSTL3, which catalyze the glutathione-dependent reduction of S-glutathionyl quercetin to quercetin (57).

3.2. Genetic Information Processing

Transcription and translation

The processing of genetic information comprises transcription, translation, and protein folding, sorting or transport, and degradation. In the gametophytes of *D. affinis* and *D. oreades*, we identified two proteins involved in transcription, specifically the 14-3-3-like proteins: GF14 nu (GRF7) and GF14 iota (GRF12), which are associated with a DNA-binding complex that binds to the G-box, a *cis*-regulatory DNA element (58). Related to translation, we found: RNA-BINDING GLYCINE-RICH PROTEIN A7 (RBGA7), which has a role in RNA processing during stress, specifically in editing cytosine to uracil in mitochondrial RNA and thus controlling 6% of all mitochondrial editing sites (59); and others, such as AT1G03510; RNA-BINDING PROTEIN 47B (RBP47B); and UBP1-ASSOCIATED PROTEIN 2A (UBA2A), which regulates mRNA and stabilizes RNA in the nucleus (60). Apart from several ribosomal subunits, there were others involved in elongation, like the protein LOW EXPRESSION OF OSMOTICALLY RESPONSIVE GENES1 (LOS1), which is also involved in the response to cold (61).

Protein folding and sorting

Once the proteins have been formed, there is a quality check to ensure that they have been synthesized completely folded correctly. Among the proteins playing a major role in the acceleration of folding or the degradation of misfolded proteins are GROES, AT3G10060, AT2G43560, AT2G21130, etc. The gametophyte of the ferns under study harbour proteins linked to the sorting or transport of molecules within the cell and between the inside and outside of cells. In line with this, we found ALBINO OR GLASSY YELLOW1 (AGY1), which has a role in coupling ATP hydrolysis to protein transfer across the thylakoid membrane, thus participating in photosynthetic acclimation and chloroplast formation (62); RAN BINDING PROTEIN 1(RANBP1), moving proteins into the nucleus (63); IMPORTIN ALPHA ISOFORM 2 (IMPA-2), acting in nuclear import (64); and the proteins ADP/ATP CARRIER 2 (AAC2), mediating the import of ADP into the mitochondrial matrix (65), and

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TRANSLOCON AT THE INNER ENVELOPE MEMBRANE OF CHLOROPLASTS 62 (Tic62), involved in the import of nuclear-encoded proteins into chloroplasts (66). In addition, we found proteins associated with the transport of water and small hydrophilic molecules through the cell membrane: PLASMA MEMBRANE INTRINSIC PROTEIN 1;4 (PIP1;4) (67); and VOLTAGE-DEPENDENT ANION CHANNEL 3 (VDAC3) (68). There were also proteins with dilysine motifs and associated with clathrin-uncoated vesicles that are transported from the endoplasmic reticulum to the Golgi apparatus and *vice versa*: ALPHA1 COAT PROTEIN (alpha1-COP), GAMMA2 COAT PROTEIN (gamma2-COP), and AT5G05010. In contrast, the proteins EPSIN2 (EPS2) and DYNAMIN-LIKE 3 (DL3) are related to clathrin-coated vesicles, the latter participating in planar polarity formation to correctly position the root hairs (69). Reviewing our proteomic profiles, we found GUANOSINE NUCLEOTIDE DIPHOSPHATE DISSOCIATION INHIBITOR 1 (GDI1), which regulates the GDP/GTP exchange reaction of most RAB proteins by inhibiting GDP dissociation and subsequent GTP binding (70).

Protein degradation

On the other hand, many proteins we found are related to protein catabolism or degradation, being subunits of proteasomes, i.e., complexes characterized by their ability to degrade polyubiquitinylated proteins. Others, such as AT1G09130; CLP PROTEASE PROTEOLYTIC SUBUNIT 2 (CLP2); CLP PROTEASE R SUBUNIT 4 (CLPR4); LON PROTEASE 1 (LON1); PRESEQUENCE PROTEASE 1 (PREP1), which degrades, in mitochondria, the pre-sequences of proteins that have been cut off after import, in order to prevent their export back to the cytoplasm, which is inefficient and energy-costly (71); and also DEGRADATION OF PERIPLASMIC PROTEINS 9 (DEG9), which degrades the *A. thaliana* RESPONSE REGULATOR 4 (ARR4), a regulator that participates in light and cytokinin signalling by modulating the activity of Phytochrome B (72). Plants have to cope with heat stress, and for this, the gametophytes studied here rely on the aminopeptidases LEUCYL AMINOPEPTIDASE 1 and 3 (LAP1 and LAP3), which are probably involved in the processing and turnover of intracellular proteins, and function as molecular chaperones protecting proteins from heat-induced damage (73).

3.3. Protein-Protein Interactions

Using the STRING platform, we thoroughly analyzed - one by one - the interactions of the groups of proteins studied. We observed that for metabolism of carbohydrates, evidence from co-expression, textmining, and experiments were stronger between the proteins AT2G20420 and AT5G08300, and evidence from databases between AT2G20420 and E2-OGDH1. AT2G20420 and AT5G08300 are both mitochondrial succinate-coA ligase subunits, and E2-OGDH1 catalyses the conversion of 2-oxoglutarate to succinyl-CoA and CO_2, i.e., the three proteins are involved in the tricarboxylic acid cycle (74). Among the proteins for biosynthesis of amino acids, evidence from co-expression was stronger between the proteins AT1G14810 and DIHYDROXYACID DEHYDRATASE (DHAD); while evidence from databases was stronger between DHAD and 2-ISOPROPYLMALATE SYNTHASE 1 (IMS1), IIL1 and IMS1, and IIL1 and ISOPROPYLMALATE DEHYDROGENASE 2 (IMD2). In fact, these proteins are involved in the synthesis of numerous compounds necessary for plant growth and development: AT1G14810 for the biosynthesis of lysine, threonine, and methionine (75); DHAD for isoleucine and valine (76); IMS1 and IMD2 for leucine (77,78); and IIL1 for glucosinolates (34). For metabolism of energy, evidence from co-expression was stronger between the proteins ATPC1 and GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE A SUBUNIT 2 (GAPA-2), and DRT112 and FED A; while experiments provided strong evidence for the interaction between PSAA and PSAC. In photosynthesis, the C-terminus of PSAC interacts with PSAA and other proteins, such as PSAB and PSAD for its assembly into photosystem I (79). Evidence from databases indicated the interaction between AGT and GLYCOLATE OXIDASE 2 (GOX2), both proteins being involved in photorespiration (46); while evidence from textmining suggested an interaction between PSBO2 and PHOTOSYSTEM II SUBUNIT P-1 (PSBP-1), both being chloroplastic oxygen-evolving enhancers that form part of photosystem II (41).

For metabolism of secondary compounds, evidence from textmining was the strongest, indicating an interaction between PHE AMMONIA LYASE 1 (PAL1) and PHENYLALANINE AMMONIA-LYASE 4 (PAL4). Both proteins participate in the synthesis from phenylalanine of numerous compounds based on the phenylpropane skeleton, necessary for the plant's metabolism (80). With respect to transcription and translation, co-expression evidence was stronger between ribosomal proteins, such as EL34Z and UL22Z, RPL23AB, UL11Z, EL14Z, and RPL18, among a long list of proteins forming ribosomes. Finally, for protein transport, co-expression data provided the strongest evidence for interactions between the proteins alpha1-COP and gamma2-COP; experiments for the interaction between alpha1-COP and AT5G05010; and textmining for that between AGY1 and GET3B.

As indicated in the results, in the group related to metabolism of carbohydrates, the proteins with the most interactions were ENOC and PGK1, both involved in glycolysis (81). For biosynthesis of amino acids, it was AT1G14810, which is involved in several biosynthetic pathways: lysine, isoleucine, methionine, and threonine (77). In the group metabolism of energy, ATPC1 had the highest number of interactions, likely because it is part of a chloroplastic ATP synthase (82). The protein 4CL3 had the most interactions in the group metabolism of secondary compounds. It plays a key role in the synthesis of numerous secondary metabolites, such as anthocyanins, flavonoids, isoflavonoids, coumarins, lignin, suberin, and phenols (53). In transcription and translation, the ribosomal proteins SAC56, US11X, and US17Y, necessary for the formation of ribosomes, had the highest number of interactions (83). Finally, in transport, RAB1A held to top rank; it participates in intracellular vesicle trafficking and protein transport (84).

Regarding the statistical analysis of the two highest types of interactions in the studied groups of metabolism of carbohydrates (database and textmining) and transcription and translation (experiments and co-expression), the Person's correlation coefficients, which measure the tendency of two vectors to increase or decrease together, were significant, being the pairwise interactions increased. One of the most popular types of data in databases is text, and the process of synthesizing information is known as text mining. In the case of proteins linked to the carbohydrates metabolism, it seems to have a lot of information in databases about it, and therefore textmining could be enriched as well. In the second case, we speculate that in the experiments carried out on transcription and translation processes, depending on the methodology employed, might be that more genes could be expressed at the same time.

These data on the interactions between the studied proteins and the evidence supporting it sheds new light onto the proteome shared the ferns *D. affinis* and *D. oreades*. Together with the description of the possible biological functions associated with these proteins and their interactions, this study significantly expands the scarce information on the development and functioning of fern gametophytes.

4. Materials and Methods

4.1. Plant Material and Growth Conditions

Spores of *D. affinis* were obtained from sporophytes growing in Turón valley (Asturias, Spain), 477m a.s.l., 43º 12′ 10 N−5º 43′ 43″ W. For *D. oreades*, spores were collected from sporophytes growing in Neila lagoons (Burgos, Spain), 1.920 m a.s.l., 42º 02′ 48N−3º 03′ 44″ W. Spores were released from sporangia, soaked in water for 2 h, and then washed for 10 min with a solution of NaClO (0.5%) and Tween 20 (0.1%). Then, they were rinsed three times with sterile, distilled water. Spores were centrifuged at 1,300 g for 3 min between rinses and then cultured in 500 mL Erlenmeyer flasks containing 100 mL of liquid Murashige and Skoog (MS) medium (85). Unless otherwise noted, media were supplemented with 2% sucrose (w/v), and the pH was adjusted to 5.7 with 1 or 0.1 N NaOH. The cultures were kept on an orbital shaker (75 rpm) at 25 ºC under cool-white fluorescent light (70 µmol m-2s-1) with a 16 h photoperiod.

Following spore germination, filamentous gametophytes were subcultured into 200 mL flasks containing 25 mL of MS medium supplemented with 2% sucrose (w/v) and 0.7% agar. The

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gametophytes of *D. affinis* became two-dimensional, arriving at the spatulate and heart stage after 20 or 30 additional days, respectively. Gametophytes of *D. oreades* grew slower and needed around six months to become cordate and reach sexual maturity (Figure 7). Apomictic and sexual gametophytes were collected, and images were taken under a light microscope (Nikon Eclipse E600), using microphotographic equipment (DS Camera Control, Nikon). Gametophytes of *D. oreades* had only female reproductive organs (i.e., archegonia), while cordate gametophytes of *D. affinis* had visible developing apogamic centers, composed of smaller and darker isodiametric cells. Samples of apomictic and sexual cordate gametophytes were weighed before and after lyophilization for 48h (Telstar-Cryodos) and stored in Eppendorf tubes in a freezer at -20 ºC until use.

4.2. Protein Extraction, Separation, and In-Gel Digestion

From the cordate apomictic and sexual gametophytes (three samples each), an amount of 20 mg dry weight was homogenized using a Silamat S5 shaker (IvoclarVivadent, Schaan, Liechtenstein). The protocol used for protein extraction, separation, and in-gel digestion was reported earlier (20). Samples were solubilized with 800 µL of buffer A [0.5 M Tris-HCl (pH 8.0), 5 mM EDTA, 0.1 MHEPES-KOH, 4 mM DTT, 15 mM EGTA, 1 mM PMSF, 0.5% (w/v) PVP, and 1 xprotease inhibitor cocktail (Roche, Rotkreuz, Switzerland)], and homogenized using a Potter homogenizer (Thermo Fisher Scientific, Bremen, Germany). Proteins were extracted in two steps: first, the homogenate was subjected to centrifugation at 16,200g for 10 min at 4 ºC on a tabletop centrifuge and, second, the supernatant was subjected to ultracentrifugation at 117–124 kPa (100,000g) for 45 min at 4 ºC in an Airfuge (Beckman Coulter, Pasadena, CA, USA), yielding the soluble protein fraction in the supernatant. In parallel, the pellet obtained from the first ultracentrifugation was re-dissolved in 200 µL of buffer B [40 mM Tris-base, 40 mM DTT, 4% (w/v) SDS, 1 xprotease inhibitor cocktail (Roche, Rotkreuz, Switzerland)] to extract membrane proteins using the ultracentrifuge as described above, in the supernatant. Protein concentrations were determined using a Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA). The 1D gel electrophoresis was performed as follows: 1 mg protein per each soluble and membrane fraction was treated with sample loading buffer and 2 M DTT, heated at 99 ºC for 5 min, followed by a short cooling period on ice, and then loaded separately onto a 0.75 mm tick, 12% SDS-PAGE mini-gel. Electrophoresis conditions were 150 V and 250 mA for 1 h in 1xrunning buffer.

4.3. Protein Separation and In-Gel Digestion

Each gel lane was cut into six 0.4 cm wide sections resulting in 48 slices, then fragmented into smaller pieces and subjected to 10 mM DTT (in 25 mM AmBic, pH8) for 45 min at 56 ºC and 50 mM iodoacetamide for 1 h at room temperature in the dark, prior to trypsin digestion at 37 ºC overnight. Subsequently, gel pieces were washed twice with 100 µL of 100 mM NH4HCO3/50% acetonitrile and washed once with 50µL acetonitrile. At this point, the supernatants were discarded. Peptides were digested with 20µL trypsin (5 ng/L in 10 mM Tris/2 mM CaCl2, pH 8.2) and 50µL buffer (10 mM Tris/2 mM CaCl2, pH 8.2). After microwave-heating for 30 min at 60 ºC, the supernatant was removed, and gel pieces were extracted once with 150 µL 0.1% TFA/50% acetonitrile. All supernatants were put together, then dried and dissolved in 15µL 0.1% formic acid/3% acetonitrile, and finally transferred to auto-sampler vials for liquid chromatography (LC)-tandem mass spectrometry (MS/MS) for which 5 µL was injected.

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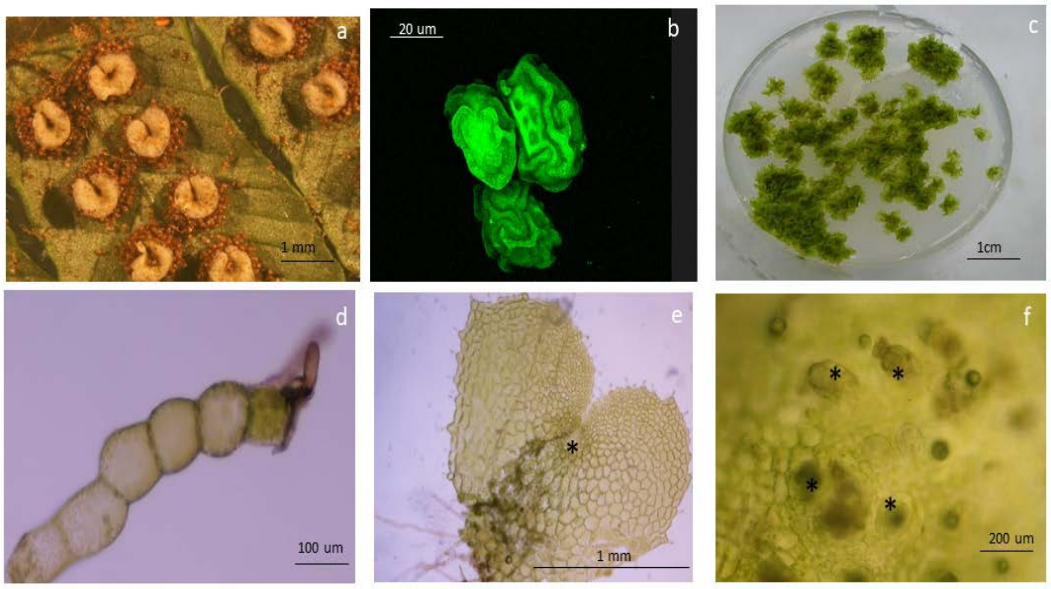

Figure 7. Morphological traits in the apomictic fern *Dryopteris affinis* and its sexual relative *D. oreades*: **(a)** typical kidney-shaped sori on the leaf underside; **(b)** confocal image of spores; **(c)** gametophytes growing in a Petri dish; **(d)** and **(e)** images taken under a light microscope of one- and two-dimensional gametophytes of *D. affinis*; asteric shows an apomictic center; and finally **(f)** refers to archegonia, indicated by asterics, in the gametophyte of *D. oreades*.

4.4. Protein Identification, Verification, and Bioinformatic Downstream Analyses

MS/MS and peptide identification (Orbitrap XL) were performed according to (18). Scaffold software (version Scaffold 4.2.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Mascot results were analyzed together using the MudPIT option. Peptide identifications were accepted if they scored better than 95.0% probability as specified by the Peptide Prophet algorithm with delta mass correction, and protein identifications were accepted if the Protein Prophet probability was above 95%. Proteins that contained the same peptides and could not be differentiated based on MS/MS alone were grouped to satisfy the principles of parsimony using the scaffolds cluster analysis option. Only proteins that met the above criteria were considered as positively identified for further analysis. The number of random matches was evaluated by performing the Mascot searches against a database containing decoy entries and checking how many decoy entries (proteins or peptides) passed the applied quality filters. The peptide FDR and protein FDR was estimated at 2 and 1%, respectively, indicating the stringency of the analyses. A semi-quantitative spectrum counting analysis was conducted. The "total spectrum count" for each protein and each sample was reported, and these spectrum counts were averaged for each species, *D. affinis* and *D. oreades*. Then, one "1" was added to each average in order to prevent division by zero and a log2-ratio of the averaged spectral counts from *D. affinis* versus *D. oreades* was calculated. Proteins were considered as differentially expressed if this log2-ratio was above 0.99. This refers to at least twice as much peptide spectrum match (PSM) assignments in one group compared to the other. Also, to provide some functional understanding of the identified proteins, we blasted the whole protein sequences of all identified proteins against *Sellginella moellendorfii* and *A. thaliana* Uniprot sequences and retrieved the best matching identifier from each of them, along with the corresponding e-value, accepting blast-hits with -values below 1E-7. These better described ortholog identifiers were then used in further downstream analysis.

4.5. Protein Analysis Using the STRING Platform

The identifiers of the genes from apomictic and sexual gametophyte samples were used as input for STRING platform version 11.5 analysis and a high threshold (0.700) was selected for positive interaction between a pair of genes.

4.6. Statistical Analyses

Regarding the two major protein-protein interactions highlighted for carbohydrates metabolism, amino acid biosynthesis, energy metabolism and transcription and translation, a Pearson's correlation test was performed using R software, and p-values lower than 0.05 were considered significant.

5. Conclusions

The analysis of a set of 218 proteins shared by the gametophytes of the apomictic fern *D. affinis* and its sexual relative *D. oreades* revealed the presence of proteins mostly involved in biological functions associated with metabolism, the processing of genetic information. and abiotic stress. Some smaller protein groups were studied in detail: metabolism of carbohydrates, biosynthesis of amino acids, metabolism of energy, metabolism of secondary compounds, transcription, translation, and transport, and abiotic stress. Possible interactions between these proteins were identified, the most common source of evidence for interactions stemming from databases and textmining information. The proteins involved in transcription and translation exhibit the strongest interactions. The description of possible biological functions and the possible protein-protein interactions among the identified proteins expands our current knowledge about ferns and plants in general.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org., Table S1. Strongest STRING interactions of proteins extracted from gametophytes of *D. affinis* and *D. oreades*, classified into the following groups: metabolism of carbohydrates, biosynthesis of amino acids, metabolism of energy, metabolism of secondary compounds, transcription, translation, and transport.

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