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Apogamy in the fern *Dryopteris affinis* ssp. *affinis*. Physiological and transcriptomic approach.

Apogamia en el helecho *Dryopteris affinis* ssp. *affinis*. De la fisiología a la transcriptómica.

TESIS DOCTORAL

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RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

1.- Título de la Tesis	
Español/Otro Idioma: Apogamia en el helecho *Dryopteris affinis* ssp. *affinis*: de la fisiología a la transcriptómica	Inglés: Apogamy in the fern *Dryopteris affinis* ssp. *affinis*: a physiological and transcriptomic approach

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RESUMEN (en español)

Esta memoria de tesis aborda un estudio sobre el desarrollo vegetativo y reproductivo del gametofito helecho *Dryopteris affinis* ssp. *affinis*, cuyo ciclo vital cuenta con un caso peculiar de apomixis. En concreto, se informa de un enfoque fisiológico y transcriptómico, revelando un papel de las fitohormonas, ya sea mediante el análisis de los efectos causados por la adición al medio de cultivo, o midiendo los niveles endógenos, así como a través de perfiles transcriptómicos comparativos entre gametofitos unidimensionales y bidimensionales.

La tesis consta de cinco capítulos: una introducción, tres trabajos experimentales y una discusión general. En la introducción, se ofrece una visión general de la investigación llevada a cabo en helechos en los últimos años, para que el lector conciba una nueva idea sobre la contribución que este grupo vegetal puede ofrecer para profundizar en la reproducción de plantas, y en el concreto, en la apomixis.

En cuanto a los capítulos experimentales, el primero de ellos, hace referencia a los efectos que tiene la adición al medio de cultivo del gametofito, de una selección de fitohormonas e inhibidores de biosíntesis y transporte, reflejando una modulación del desarrollo por parte de auxinas, giberelinas y poliaminas. Además, se ofrece un protocolo válido para inducir y proliferar tejido de callo. En el segundo capítulo, se analizan los niveles endógenos de auxinas, citoquinas, ácido abscísico, giberelinas, ácido salicílico y los brasinosteroides, por cromatografía líquida-espectrometría de masas de ultra-rendimiento (UHPLC), en gametofitos apogámicos en tres estados de desarrollo (filamentosos, espátulas y corazón), y en gametofitos acorazonados femeninos, de su progenitor sexual D. oreades, revelando variaciones en el contenido. Finalmente, en el tercer capítulo experimental, se generó un transcriptoma de novo y utilizando un enfoque RNA-seq, se compararon los perfiles de expresión génica de gametofitos unidimensionales y bidimensionales. Genes implicados en la regulación del desarrollo del meristemo, señalización de auxinas, reproducción y metabolismo de la sacarosa se regularon al alza en gametofitos bidimensionales, y genes implicados en respuesta a estímulo y defensa, así como en regulación epigenético y degradación de la ubiquitina, sobreexpresaban en gametofitos unidimensionales. Los resultados obtenidos proporcionan nueva información sobre la reproducción vegetativa y apogámica en el gametofito de vida libre, y representan un recurso útil para nuevas investigaciones en plantas no modelo.

RESUMEN (en Inglés)

This work represents a step forward to gain a better knowledge on the apogamous process in the fern *Dryopteris affinis* ssp. *affinis*, which represents a peculiar case of apomixis, in ferns. In concrete, a physiological and transcriptomic approach are reported, revealing a role of phytohormones, either by analyze the effects caused by the addition to the culture medium, or by measuring their endogenous levels, as well as through comparative transcriptomic profiles between of one- and two-dimensional gametophytes.

The thesis comprises five chapters: an introduction, three experimental works, and a general discussion. In the introduction, a general view on the research driven on ferns for the last years is provided, so that the reader could conceive a new idea about the contribution this plant group might offer to deepen on plant reproduction, and in concrete on apogamy. Regarding the experimental chapters, the first one dealt with the effects caused by a wide sort of the major group of phytohormones and inhibitors of their biosynthesis and transport, reflecting a modulation of the vegetative and apogamous development by auxins, gibberellins and polyamines. Additionally, a valid protocol is available to induce proliferating callus when cultured in presence of 2,4D (2,4-dichlorophenoxyacetic acid), showing bodies of lipid accumulation is reported. In the second chapter, the endogenous levels of auxins, cytokinins, abscisic acid, gibberellins, salicylic acid and brassinosteroids were assessed by ultra-performance liquid chromatography-mass spectrometry (UHPLC) in filamentous, spatula and heart-shaped gametophytes of the apogamous fern *Dryopteris affinis* ssp. *affinis*, and in female heart-shaped gametophytes of its sexual progenitor *D. oreades*, revealing different profiles comparing intra an inter species. In the third experimental chapter, a gametophyte *de novo* transcriptome using an RNA-Seq approach was done and used to compare the gene expression profiles of one- and two-dimensional gametophytes. Genes implicated in the regulation of meristem growth, auxin signaling, reproduction, and sucrose metabolism were upregulated in two-dimensional gametophytes, and genes enriched in stimulus and defense genes as well as genes involved in epigenetic gene regulation and ubiquitin degradation, in one-dimensional gametophytes. Our results provide insights into the vegetative and apogamic reproduction in the free-living gametophyte, providing a useful resource for further investigations of asexual reproduction in non-model plants.

SR. PRESIDENTE DE LA COMISIÓN ACADÉMICA DEL PROGRAMA DE DOCTORADO EN BIOGEOCIENCIAS_____

"*Es de bien nacidos, ser agradecidos*."

-Refrán español-

No se puede hacer nada en solitario en esta vida. Para cualquier cosa necesitamos una familia, un equipo, unos amigos, unos rivales, alguien con en quien apoyarnos, competir e incluso alguien a quien querer.

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Abbreviations

Short name	Definition
NPA	N,1 naphtylthalamic acid
2,4-D	2,4-dichlorophenoxiacetic acid
ABA	Abscisic acid
AF	*D. affinis* filamentous-shaped
AH	*D. affinis* heart-shaped
ANOVA	Analysis of Variance
AOC4	Allele Oxide Cyclase
ARATH	*Arabidopsis thaliana*
AS	*D. affinis* spatula-shaped
BA	6-Bencylaminopurine/Bencyl adenine
BA-d6	Deuterated bencyladenine
BBM	Baby Boom complex
BK / CAST	Castasterone
BLAST	Basic Local Alignment Search Tool
BLASTX	Basic Local Alignement Tool nucleotide to protein
BR	Homo-brassinolide
BUSCO	Benchmarking Universal Single-Copy Orthologue
C18	HPLC chromatography column with 18 carbon
CHA	Cyclohexylamine
CPM	Counts per million
CUC	Cup shaped cotyledon gene
DA	*Dryopteris affinis*
DHRZ	Zeatine dihydroriboside
DHZ	Dyhidrozeatine
DO	*Dryopteris oreades*
DTT	Dithiothreitol
EPI	Epibrassinolide
EDTA	Ethylenediaminetetraacetic acid
EGTA	Egtazic acid
ENA	European Nucleotide Archive
F	Flurprimidole
FDR	False Rate Discovery
FLC	Flowering locus C
FLD	Flowering locus D
FRL / FRL-1*	Frost Related, Frost tolerance gene
FT	Flowering promotion , equivalent to florigen
FW	Fresh Weight
GA´s	Gibberellins
GA_3	Gibberellic acid
GA_4	Gibberellin 4

GASA/GAST/SNAKIN	Gibberellic Acid Stimulated genes
GlcNAc	b-1,4-N-acetylglucosamine
GO	Gene Ontology
GRAS	Giberellin Related transcription factor family
HBTI	Hormone Biosynthesis and Transport Inhibitors
IAA	Indol acetic acid
IAA-d5	Deuterated indole acetic acid
IBA	Indole 3-butyric acid
IP	Propidium ioide
iPA	Isopentenil-adenosine
iP	Isopentenil-adenine
LAT52	Late embryogenesis abundant 52
LC-MS/MS	Liquid chromatography–mass spectrometry
LEA	Late embryogenesis abundant
MADS	MCM-AGAMOUS-DEFICIENT-SRF
MMC	Megaspore mother cell
MS	Murashige and Skoog (1962) basal medium
MudPIT	Multidimensional Protein Identification Technology
MYB	Myeloblastome family
NAA	Naphthalene 1-acetic acid
NAC	Acronym composed of NAM, ATAF and CUC
NAM	Acronym for No Apical Meristem
NEC1	Nectarine 1
NO	Nitric Oxide
OH	D. oreades heart
PCA	Principal Components Analysis
PCR2	Polycomb Repressive Complex
PEG	Polyethylene glycol
PGR	Plant Growth Regulators
PMSF	phenyl-methyl-sulfonyl fluoride
PpCLF	*Physcomythrella patens* Curly Leaf
PpFIE	*Physcomythrella patens* Fertilization Independent
PVP	Polyvinyl pyrrolidone
RHL	Root hair Defective
RNAseq	RNA sequencing
RZ	Zeatine riboside
S o SPE	Spermidine
S/SU	Sucrose
SA	Salicilic acid
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SERK	Serine receptor Kinase

SPLS	Sparse Least Square
STM	Shoot Meristemless
S-W	Shapiro-Wilk test
TIBA	Triiodobenzoic acid.
tZ	*trans*-zeatine
WUS	WUSCHEL
YUCCA	Gene of curling root induced by auxins

INDICE

Chapter 1. General Introduction

1.1. Why is important to study ferns and other non-seed plants.

Looking at the evolution history of plant kingdom, we can esteem the concatenated successes happened until how they are at present, among others the conquest of land from the fresh water streptophyte green algae, during the Ordovicean period, over 470 million years ago. Then colonizing and dominating terrestrial (Becker and Marin 2009). Flora expansion between the Silurian and Permian periods leads to the origin of taxonomic groups represented today by bryophytes (which comprise hornworts, mosses and liverworts), lycophytes, and euphyllophytes, which include monilophytes (ferns) and spermatophytes (seed plants), and the more primitive plant groups are expected to hide clues capable to shed light on how plant development took place (Fig. 1.1).

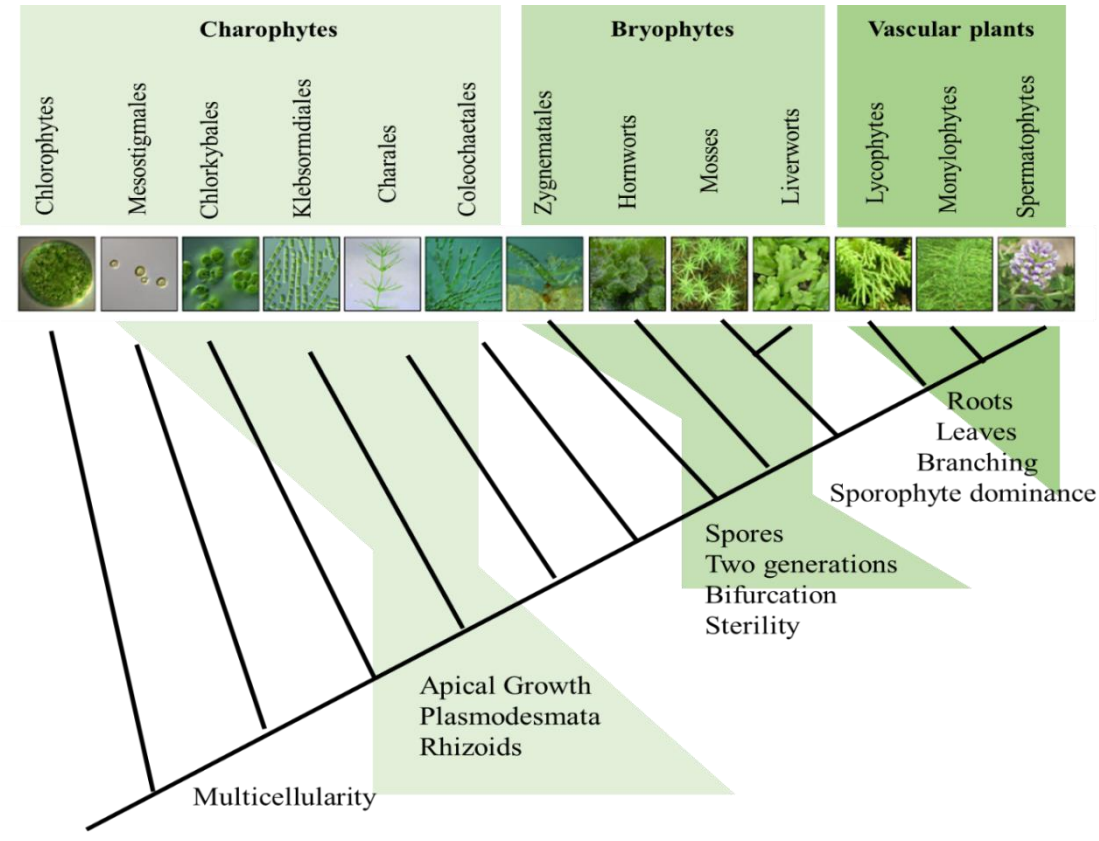

Figure 1.1. Scheme showing some important hits on plant evolution (Harrison, 2017).

Ferns are among the pioneer vascular plants coping with the new environmental conditions, which imposed variations in water availability and temperature, as well as increased exposure to radiation, thus demanding changes in body plant and modifications to cellular, physiological, and regulatory processes (Rensing et al. 2008; Pires and Dolan 2012). Consequently, ferns represent a critical clade for comparative evolutionary studies in land plants (Harrison, 2017). They keep traits of an ancestral life history such as the lack of secondary growth, homospory, motile sperm, and independent free-living gametophyte and sporophyte generations. Moreover, ferns represent an unexplored genetic diversity that could be taken advantage for improving plants by means genetic transfer technologies (Rathinasabapathi 2006). Moreover, ferns represent an unexplored genetic diversity that could be taken advantage for improving plants by means genetic transfer technologies.

For decades, ferns have received less attention than other groups. Only few species such as *Adiantum capillus-veneris*, *Anemia phyllitidis*, *Blechnum spicant*, *Dryopteris affinis* ssp. *affinis*, *Ceratopteris richardii*, *Marsilea vestita*, *Matteuccia strupthiopteris*, *Onoclea sensibilis* or *Pteridium aquilinum* have been used to study basic developmental processes such as photomorphogenesis (Wada 2007), germination (Salmi et al. 2005, 2007; Suo et al. 2015), cell polarity (Salmi, Bushart 2010), cell wall composition (Eeckhout et al. 2014), or asexual and sexual reproduction, over fern gametophyte that is an autonomous-living organism, easily for in vitro culture and sample collection (Whittier and Steeves 1960; Aderkas 1984; Wen et al. 1999; Fernández and Revilla 2003; Cordle et al. 2007; Kazmierczak 2010; Lopez and Renzaglia 2014; Valledor et al. 2014; Grossmann et al. 2017a).

Apart from the previously mentioned processes, we assist recently to the use of ferns to resolve interesting problems in the plant world caused by abiotic and biotic stress. Drought is one of the most severe abiotic stress factors affecting plant growth and productivity. It has caused considerable reduction in crop yield worldwide (Ludwig-Müller 2000; McAdam and Brodribb 2012). Several fern and fern-ally species of Actiniopteridaceae, Sinopteridiaceae, Pteridaceae and Selaginellaceae, have been associated to desiccation tolerance. Concretely, the fern-ally *Selaginella* is one of the most primitive vascular resurrection plants, which can survive a desiccated state and recover when water becomes available, by morphological adaptations, hormonal regulation, antioxidant protection and accumulation of osmolites, which could serve to

cope with drought in crops (Wang et al. 2010). Other important adaptations of ferns to extreme environments such as salinity, heavy metal, epiphytism, or invasiveness tolerance are summarized by Rathinasabapathi (2006). More recently, it was published a fascinating paper based upon the feature that ferns and mosses are rarely infested by phytophagous insect in the field (Hendrix 1980; Markham et al. 2006), and in which an insecticidal protein from the fern species *Tectaria macrodonta* (Fee) C. Chr. was identified and expressed in transgenic cotton lines, conferring protection against whitefly, a sap-sucking pest (Shukla et al. 2016).

1.2. The gametophyte: born to reproduce either sexually or asexually

During its life cycle, a fern exists in two distinct forms: the small, sample, haploid gametophyte, and the large, morphologically complex, diploid sporophyte (Fig. 1.2). In ferns, the gametophyte lives separately from sporophyte (Klekowski 1969; Schuettpelz et al. 2016) excluding the time after fertilization takes place, when gives support at the beginning of sporophyte development. As a rule, sporophytes are perennial plants with different life spans, while the growth and development of gametophytes proceeds faster (Vedenicheva and Kosakivska 2018). In fact, as sporophyte develops, the gametophyte disappears in most cases, reflecting to have a role purely involved on reproduction. In *Thelypteris palustris* (Salisb.) Schott, sporophytes are supported by gametophytes until the first leaves develop; then sporophytes grow on the organic matter they produce themselves (Sakamaki and Ino 2007).

In brief, the vital cycle of a fern might be described as follows (Fig. 1.2). When a spore has the right humidity and temperature conditions, it germinates resulting in a simple structure that is the young gametophyte or *prothalus*. At first, it develops one-dimensionally and acquires filamentous form, which then derives into a two-dimensional structure, spatulated in shape. From this moment on, in the apical part begins the differentiation of a group of cells, which are smaller than the rest and that define a meristematic zone active of cell divisions. At the margins of the gametophyte the cells are small, so that there is a differential growth between the meristematic zone and the margins, thus forming lobes and acquiring the gametophyte a heartened shape. In general, it is then that the formation of the female reproductive organs or archegonia takes place, in the middle area, mainly on the lower face of the gametophyte; male reproductive organs

or antheridia have been able to form in earlier stages, forming at the same time as archegonia or later.

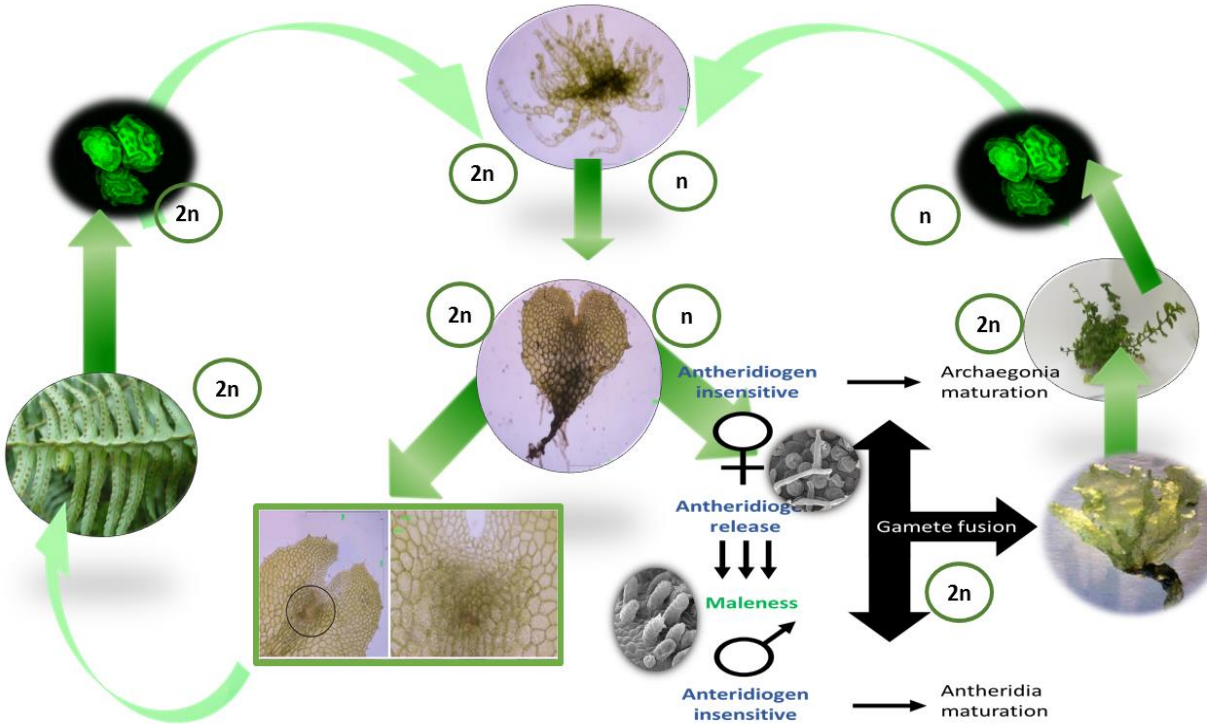

Figure 1.2. Life cycle of ferns, showing the reproduction mechanisms.

Two mating systems are described in ferns: intergametophytic and intragametophytic (Klekowski 1969). Intergametophytic mating is the fusion of gametes from two different gametophytes, which may have originated from the same sporophyte or from different sporophytes. Intragametophytic mating is the fusion of gametes produced by the same gametophyte, and necessarily leads to the formation of a homozygous individual.

While there is an initial tendency in most homospore ferns to experience intragamethic mating, there are adaptations that increase or decrease that probability. According to Klekowski (1969) these adaptations can be morphological, population and genetic. Among the first would be the sequence in which the gametangia are formed. In this sense, the initial formation of antheridia followed by a prolonged hermaphrodite phase would be an adaptation for intragametophytic mating and possibly the most common in these plants. However, the possibilities of intergametophytic mating increase with an ontogeny sequence where archegonia would initially form, followed by a

hermaphrodite phase, as is the case with many species studied in the family *Blechnaceae* (Klekowski 1969), among others.

Apart from a sexual pattern of reproduction, in ferns it has been described another mean to form a sporophyte, bypassing gamete fusion, and which will be further treated.

1.3. Reproduction in plants

The evolution of sexual reproduction represents a major transition in the evolution of life, and occurred well before plants first ventured onto land some 470 million years ago, when the ancestors already had differentiated male and female gametes (spermatozoids and eggs), inside the sex organs archegonia and antheridia. The first land plants had also already evolved an alternation of haploid and diploid generations, gametophyte and sporophyte, being the gametophyte, the generation when the differentiation of sex organs into egg- or sperm-producing tissues occurs. Actually, in seedless plants, gametophytes may be male or female, with separate individuals producing either antheridia or archegonia, or they may be hermaphroditic, with both antheridia and archegonia present in the same individuals (Okada et al. 2001).

Sex determination is a matter of the differentiation of cells and tissues in different parts of the same individuals. In many bryophytes, sex is determined in gametophytes by (U and V) sex chromosomes. To my knowledge, no sex chromosomes have been described for any ferns or lycophytes so far. In these plant groups, sex determination differs between ‘homosporous’ species, in which sporophytes produce spores of the same size (as in bryophytes), and ‘heterosporous’ species, in which sporophytes produce both small ‘microspores’ and larger ‘megaspores’. In some homosporous ferns, gametophytes are all functionally hermaphroditic, with both antheridia and archegonia. In these cases, sex determination is a question of cellular and tissue differentiation into different male and female ‘gametangia (Klekowski 1969). In other homosporous species, gametophytes may develop a unisexual function (producing either only antheridia or only archegonia) (Klekowski 1969). In ‘heterosporous’ ferns and lycophytes, sex determination acts through the size of the spore from which the gametophytes germinate. The gametophytes do not differ genetically, and the sporophyte controls the sex of its gametophytes by regulating the spore-producing ‘sporangia’ to produce either small ‘microspores’, which develop into male (micro-) gametophytes, or larger ‘megaspores’, which develop into female (mega-) gametophytes. Heterospory has evolved several times in vascular land

plants, in ferns and lycophytes, as well as independently in the lineage that gave rise to seed plants. In angiosperms (flowering plants) and gymnosperms, the gametophytic phase has become even more highly modified and reduced, and differentiation between the male and female structures takes place in sporophytic structures in which the gametophytes form. In contrast to non-seed plants, seed-plant gametophytes are always either male or female, and it is the sporophyte that determines their sex (Pannell 2017).

1.4. Apomixis in Angiosperms

Sexual reproduction generates new genetic individuals by combining the genetic material of two parental individuals while asexual reproduction is limited to one genetic entity. Most angiosperms reproduce sexually through seeds, where a single generative cell (archesporial cell or megaspore mother cell) undergoes meiosis to produce four chromosomally reduced cells (megaspores). After significant cellular enlargement, the nucleus of the functional megaspore usually undergoes three rounds of mitosis before giving rise to a gametophyte composed of seven cells: two companion synergids, the egg cell, a binucleated central cell and three antipodals. Double fertilization of both the egg and central cell is necessary to trigger embryogenesis and endosperm development, respectively (Rodriguez-Leal and Vielle-Calzada 2012).

In plants, as in animals, transition to asexuality may occur, however, despite of its importance little is known about the mechanisms that cause these transitions and why some taxa experience much higher transition rates than others, especially in plants, where more intensively it has been studied, due to the potential use of asexuality in crop plants to improving agriculture. Asexual reproduction in plants can occur either through budding and vegetative growth (e.g. shoots and runners) or by seeds, referred as apomixis. Apomictic process was a great obstacle for Mendel, when he decided to corroborate his results of genetic inheritance in peas (*Pisum*), in hawkweed (*Hieracium*) and saw astonish that *Hieracium* offspring were morphologically identical to their mother plants (Neiman et al. 2014).

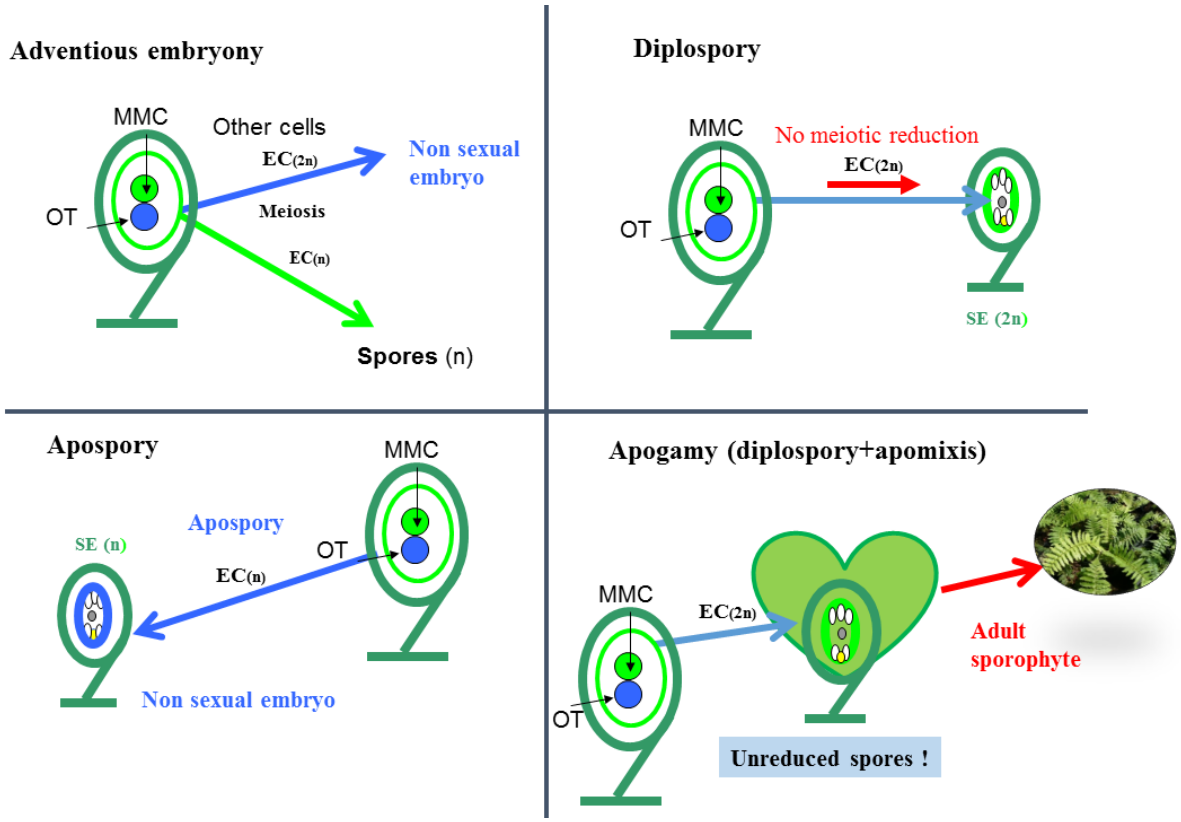

Figure 1.3. Scheme showing the three types of apomixis in Angiosperms and Apogamy in ferns. EC, egg cell; MMC, megaspore mother cell.

There are examples of asexual seed formation (apomixis), where seeds form without meiosis and fertilization (Nogler GA 1984; Koltunow et al. 1995; Koltunow and Grossniklaus 2003; Bicknell and Koltunow 2004; Ozias-Akins 2006; Rodriguez-Leal and Vielle-Calzada 2012; Barcaccia and Albertini 2013). Different types of apomixis are shown in Fig.1.3. In its simplest form (adventitious embryony, also called sporophytic apomixis), apomictic plants form embryos directly from a somatic cell from nucellus or inner integuments, and in species with endosperm development, the endosperm is formed by polar nuclei. Apomictic plants can also form embryos directly from a chromosomally unreduced female gametophyte (apomeiosis) in which the egg cell develops autonomously into an embryo by parthenogenesis (gametophytic apomixis), by means of two types, apospory and diplospory. In apospory, the embryo and endosperm develop in unreduced embryo sac in the ovule. In this case, the megaspore mother cell in the sexual ovule starts to develop but stops at some stage, and one or more somatic cells in the ovule and their nuclei start to develop, resembling megaspore mother cells. Before the mature

embryo sac formation, the megaspore or young embryo is aborted and replaced by developing aposporous sacs. Apospory is by far the most common mechanism in higher plants and has been reported in *Beta*, *Brachiaria*, Cenchrus, *Chloris*, *Compositae*, *Eriochloa*, *Heteropogon*, *Hieracium*, *Hyparrhenia*, *Hypericum*, *Panicum*, *Paspalum*, *Pennisetum*, *Poaceae*, *Ranunculus*, *Sorghum*, *Themeda*, and *Urochloa* (Barcaccia and Albertini 2013). In diplospory, the embryo and endosperm develop in an unreduced embryo sac derived from the megaspore mother cell, which differentiates as in sexual ovules but does not undergo meiosis. Diplospory is found in *Tripsacum*, *Eragrostis*, and *Taraxacum* (Kandemir and Saygili 2015). Apomictic female gametes (2n) undergo embryogenesis autonomously, without fertilization. Apomictic plants produce functional pollen, which they sometimes need for endosperm formation as the formation of endosperm is still dependent on fertilization of the central cell (pseudogamy) (Rodriguez-Leal and Vielle-Calzada 2012, Kandemir and Saygili 2015). Observation of apomixis is difficult since it is generally accompanied by sexual reproduction or facultative apomixis (Kandemir and Saygili 2015).

Because apomixis allows the fixation of complex genotypes, including that of highly productive F1 hybrids, many researchers have extolled the tremendous potential that apomixis holds for plant improvement, whose benefits could surpass those of the green revolution (Grossniklaus et al. 1998a, b; Spillane et al. 2004; Marimuthu et al. 2011). Apomixis combines the advantages of propagation by seed (higher multiplication rate, easier storage and planting, suitability for machine planting, less seed material use, and less bearing of diseases) with those of propagation by clone (maintaining genetic structure and hence fixing superior genotypes after crossing) (Kandemir and Saygili 2015).

Over the last decades, several studies focusing on apomixis in model species of angiosperms concluded that sexual and apomictic pathways share gene expression profiles and, thus, common molecular regulatory features, indicating that they are not distinct pathways (Grossniklaus et al. 2001; Tucker et al. 2003). The initiation of apomixis invariably occurs during early ovule ontogeny; sexual and apomictic development can coexist within the same ovule, or within different ovules of a same individual, suggesting that apomixis could have originated as a modified form of sexual reproduction that has undergone deregulation of key developmental steps during gametogenesis (Koltunow and Grossniklaus 2003). So far it is a truth that very few crop species are apomictic and attempts to introduce this trait by crossing have failed. The

alternative would be to de novo engineer apomixis but for this strategy to be applied, the genes that confer elements of apomixis must be identified.

Apomixis research can be faced by different approaches in each of its three major stages: apomeiosis, parthenogenesis, and seed formation. In most species under study, the basic components of apomixis can be explained by a few genes that control unreduced gamete formation and parthenogenesis, respectively, however, polyploidy, segregation distortion, suppressed recombination, epistatic interactions, naturally active modifiers and environmental effects complicate their genetic analyses (Rodriguez-Leal and Vielle-Calzada, 2012).

There are various ways of converting crop plants into apomictic ones: a) wide crosses with apomictic wild relatives, b) mutation, and c) genetic transformation. Transfer of apomixis from wild relatives via sexual hybridization depends on the presence of relatives with which interspecific hybridizations can be made but this is not possible for most cultivated species. Although significant developments have been done recently, it is generally accepted that apomixis transfer via wide crosses has been unsuccessful so far (Spillane et al. 2004). Cloning of LOA and LOP genes of *Hieracium* is underway and, after their cloning and transfer, apomixis could be introgressed into other crop species (Kotani et al. 2014). On the other hand, studies in the model plant *Arabidopsis* revealed that apomixis can also be achieved through artificial mutations. One of them involves the gen Osd1, which controls entering into the second meiotic division (d'Erfurth et al. 2009). By combining a mutation in this gene with two other mutations: one that eliminates recombination and pairing (Atspo11-1) and another that modifies chromatid segregation (Atrec8), a new genotype was created in which meiosis is totally replaced by mitosis without affecting subsequent sexual processes, and called MiMe for ''mitosis instead of meiosis'' (d'Erfurth et al. 2009; Marimuthu et al. 2011). The induction of apomeiosis by the creation of the MiMe genotype is an important step towards understanding and engineering apomixis, giving place to a genotype called MiMe in which meiosis was replaced with mitosis. In another mechanism, a mutation in the Arabidopsis SWI1 gene leads to apomeiosis and diploid egg formation (Ravi et al. 2008). Chaudhury et al. (1997) reported seed development in mutations of Arabidopsis FIS1, FIS2, and FIS3 genes in the absence of fertilization (Chaudhury et al. 1997).

1.5. Apomixis in Ferns: Diplospory + Apogamy

In ferns, apomixis is an important mode of asexual reproduction (Manton, 1950), which has evolved several times independently and being its frequency at least 3 %, a value much higher than in other major plant groups (Ekrt and Koutecký 2016). However, most apomictic fern species are concentrated in just four families (Liu et al. 2012). Apomixis in ferns (Fig.1.4) includes apogamy, the formation of sporophytes from somatic cells of the prothallium, and agamospory (or diplospory), and the production of unreduced (diplo) spores (Manton 1950; Ekrt and Koutecký 2016). The archesporial cell of sexual fern species usually undergoes four mitoses to produce 16 spore mother cells that suffer regular meiosis, resulting in 64 reduced spores in 16 tetrads. Under the prevailing type of agamospory (Döpp-Manton scheme) the last (premeiotic) mitosis fails, resulting in eight spore mother cells that undergo regular meiosis, producing 32 diplospores in eight tetrads (Dopp 1939; Manton 1950). Rarely, the first meiotic division fails, which results in 32 diplospores in 16 diads (Braithwaite 1964). Genetic variation among apomictic offspring has been documented (Peredo et al. 2013).

In contrast to flowering plants, the fern apomictics are obligate (Lovis 1978), being under study some sporadic case as *Asplenium hallbergii* (Ekrt and Koutecký 2016). Other authors suggest that obligate apomixis as an evolutive alternative to keep a certain degree of genetic variation. Apomictic ferns are prone to show apomixis when flowering plants start to compete against them (Schmidt 2020).

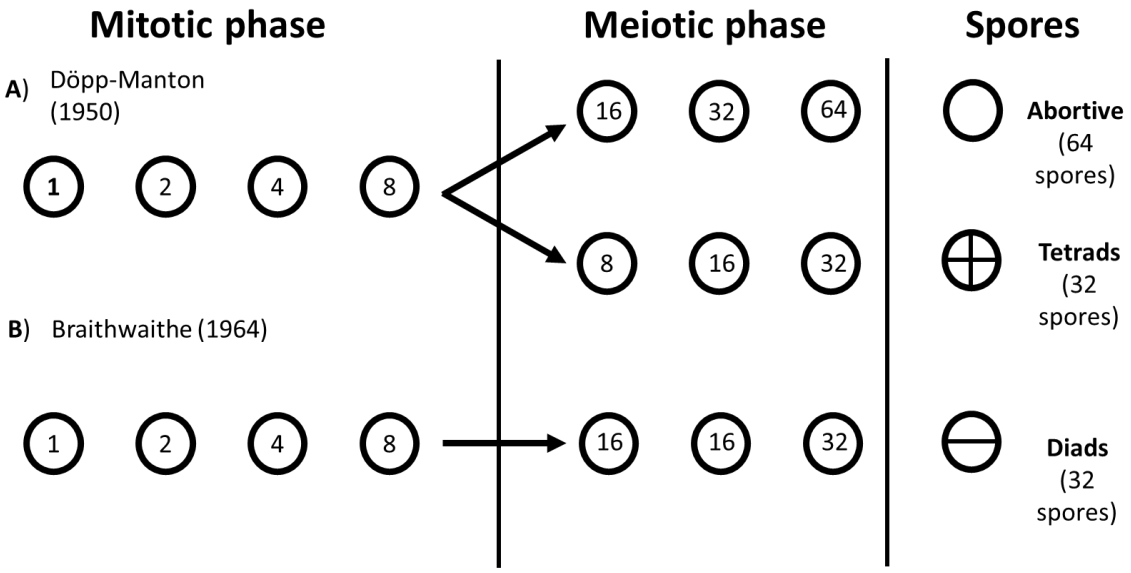

Figure 1.4. Proposed mechanisms to bypassing meiosis in fern sporogenesis (Manton, 1950; Braithwaithe 1964).

Regarding apogamy, in ferns may be obligate, when gametophytes produce nonfunctional gametes, and facultative or induced by exogenous factors (Menéndez et al. 2006; Cordle et al. 2007). Contrary to ferns, apogamy does not occur naturally in angiosperms (Yang and Zhou 1992), but apogamous sporophytes can be induced by the culture of pollen or embryo sacs, indicating that the developmental plasticity necessary to overcome meiosis and fertilization barriers is not restricted to ferns (Seguí-Simarro 2010; Germanà 2011). In apogamy, somatic cells of the gametophyte are reprogrammed to start the sporophytic developmental program (Okano et al. 2009).

1.6. Could apogamy in ferns contribute to understand apomixes in Angiosperms?

According to that mentioned above, in both apomixis and apogamy, unreduced cells form an embryo without fertilization, and furthermore it might be expected they share some common features. In addition, apogamy in ferns can be seen as an opportunity to investigate on embryogenesis, one of the more powerful tools in plant biotechnology. Moreover, the mechanism of asexual reproduction in lower and higher plants appears to be controlled by overlapping sets of genes (Cordle et al. 2012).

How somatic cells, either of sporophytic or gametophytic origin become and develop as embryogenic is still poorly understood (Radoeva and Weijers 2014). Somatic embryogenesis research in ferns is scarce so far and limited to a few reports (Mikuła et al. 2015; Domżalska et al. 2017), and even minor the number of reports involving embryogenesis linked to apogamy (Cordle 2012;, Bui et al. 2017; Grossmann et al. 2017). In general terms, we can assume that the molecular basis of embryogenesis by sexual or asexual means, in plant kingdom is far to be understood (De Smet et al. 2010). In seed plants, several groups of genes, mostly encoding transcription factors, such as BABBY BOOM, AINTEGUMENTA-like 5, FUSCA3, LEAFY COTYLEDON, etc, have been associated to embryogenesis although if they are required either for embryogenesis itself or plant cell viability would need still further explanation (Radoeva and Weijers 2014). Exploring apogamy in fern species could represent a possibility to increase our understanding those processes that circumvent sexual reproduction.

1.7. Approach and objectives

Behind the apogamy process undergoing in many species of ferns, a complex interaction of cellular events is expected to be working and thereafter waiting to be investigated. The state-of-art about the knowledge accumulated so far on apogamy bring us to focus this research on two main strategies: physiological and molecular.

The processes of growth and development of gametophyte and sporophyte of ferns, as well as representatives of other taxa, are controlled by a multicomponent hormonal system (Haufler et al. 2016). The determining factor in the action of phytohormones is their concentration and localization in individual organs and tissues of plants (Davies 2010). Due to hormonal regulation and the influence of exogenous factors, the stages of the genetic program of a plant organism can be accelerated or decelerated (Bradford and Trewavas 1994).

Apogamy has been reported to be activated by using of exogenous phytohormones (Whittier 1966; Kwa et al. 1995; Menéndez et al. 2006). Auxins such as IAA induced the apogamous development of sporophyte on sterile gametophytes of *Platycerium coronarium*, and endogenous ethylene with the addition of IAA depressed the apogamous development of sporophytes on gametophytes of *P. coronarium*, which could be caused by a deterioration in the transport of IAA in gametophyte cells (Kwa et al. 1995). The formation of apogamous sporophytes on *P. aquilinum* gametophytes was initiated by exogenous NAA and GA_3 (Whittier 1966), and these compounds were also effective for the development of somatic sporophytes of *Dryopteris affinis* (Fernández, et al. 1996). The addition of cytokinin BAP completely inhibited the formation of sporophyte and activated callus formation (Menéndez et al. 2006).

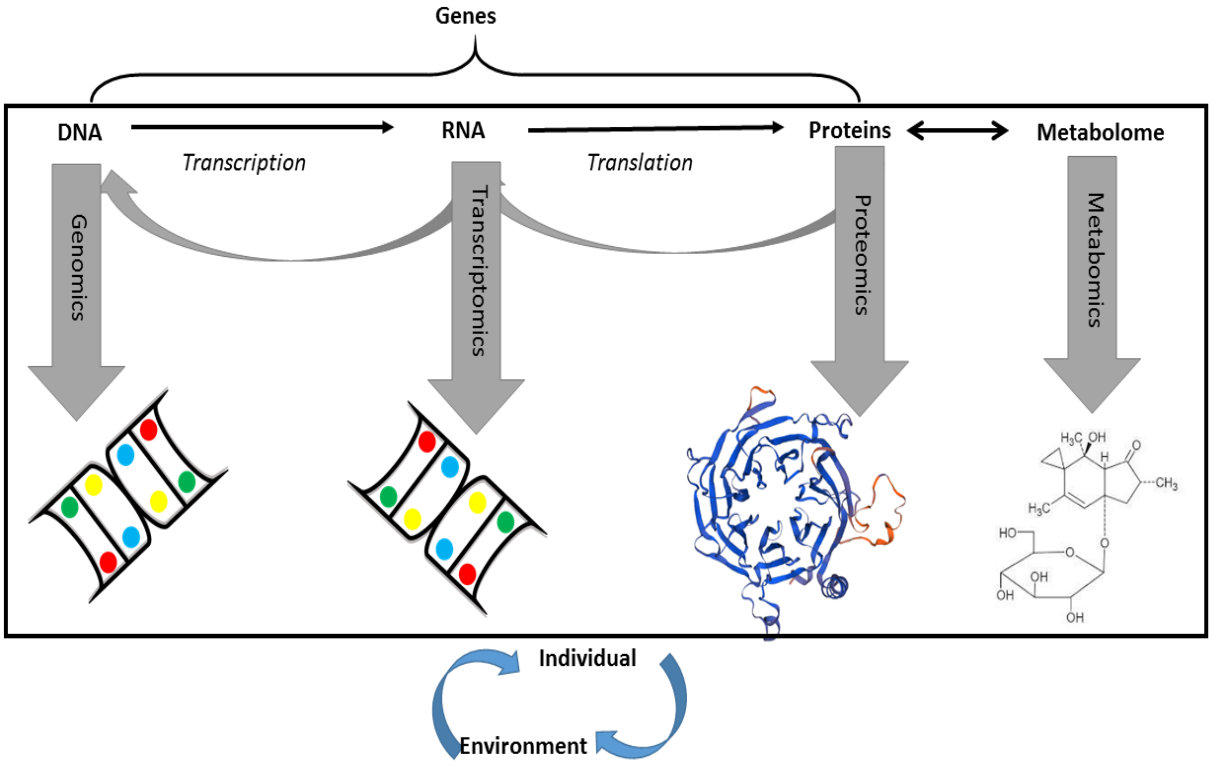

Figure 1.5. New *omic* technologies.

For a better understanding of the functions of the phytohormones and the interactions between them, exhaustive determination of the hormone contents is thus of great importance (Bai et al. 2010). The fact that different hormones have diverse chemical and structural properties makes their co-quantification difficult, and their low active concentrations, usually at parts per billion (ppb) or nanomolar level. Thanks to the advent on last years, of rapid, sensitive, accurate and efficient methods for the analysis of phytohormones from biological samples, and especially when only small amounts of sample are available for analysis, the research on this field of plant physiology has made possible to wider the number of compounds analyzed. During the years, analyses of phytohormones have turned around auxins, gibberellins, cytokinin, abscisic acid, or ethylene, being others like jasmonates or brassinosteroids more recently added, as standards and protocols were available. At present day, HPLC–MS is the most spread method to made quantitative analysis of low quantity compounds with a good performance and a low price. As a result, it was optimized to detect small amounts of hormones without derivatization and subsequent sample loss. In addition, HPLC–MS is the most accurate method to perform quantitative analysis of endogenous phytohormones

or plant regulators which are also short-life compounds (Pan et al. 2008; Delatorre et al. 2017). Related to it, some authors had been tested UHPLC to detect those small amounts of PGR in fresh tissue. This technique had shown better results than HPLC-MS with a spectra of plant hormones. As a result, the use of a UHPLC-MS system allows us to detect smaller quantities that the ones we could detect ten years ago.

Regarding a molecular approach, certainly, ferns are reported to have higher chromosome numbers and larger genomes than mosses and seed plants (Barker and Wolf 2010), making difficult to stablish genetic resources for them such as genomic and transcript sequence data. However, the advent of the next-generation sequencing (NGS) technologies, such as Roche'454 GS-FLX Titanium and Illumina HiSeq sequencers, by means of which is possible to characterize the transcriptome in plants, represents a small but information reach-target compared to complete genome (Ward et al. 2012). The variation in gene expression induced by whatever environmental or inner condition can be examined in non-model organisms because these techniques have become more feasible as automation and efficiency has reduced the cost. Until present and recently, some NGS transcriptome data sets have been published for ferns, which include the species *Pteridium aquilinum* (Der et al. 2011), *Ceratopteris richardii* (Salmi ML, Bushart TJ 2010), *Lygodium japonicum* (Aya et al. 2015) *Dryopteris affinis* ssp. *affinis* (Grossmann et al. 2017a) and some others resulting from the OneKP project (Matasci et al.). Moreover, one of the major genomics centers in the world, BGI (in Beijing) and the China National GeneBank (CNGB) have announced 10KP, their plan to sequence 10,000 genomes or more, crossing every major plant clade and eukaryotic microbes, which will build on oneKP project.

Recently, both transcriptomic and proteomic analyses were performed to increase our knowledge on the molecular basis of apogamy in *D. affinis* ssp. *affinis*, by using next-generation sequencing (NGS) and shotgun proteomics by tandem mass spectrometry (Grossmann et al. 2017a). This transcriptome represents the first attempt done in this species. The data reflected 1,397 protein clusters with 5,865 unique peptide sequences identified, from several taxa, including homologs of proteins involved in several activities, on reproduction of higher plants, as well as proteins with a potential role on apogamy. Furthermore, in this study, we detected some fern protein homologous to ARGONAUTE10/PINHEAD/ZWILLE, and also to the A. thaliana SERRATE (SE) RNA effector protein which could participate in the meristematic activity of the incipient

apogamic embryo or unknown roles in the switch between sexual and asexual reproduction and perhaps in the regulation of apogamy in ferns (Grossmann et al. 2017). Additionally, proteins involved in gene silencing, or enzymes involved in cell wall modifications such as pectinesterases were also identified, and which could hide some role on apogamy (Li et al. 2011). Besides, we have done an RNA-Seq approach to compare gene expression profiles of one and two-dimensional gametophytes of this species, finding several thousands of genes differentially expressed, and related to different aspect of either vegetative or reproductive behavior of the gametophyte (Wyder et al. 2020). In summary, with the increasing availability of genomic data from non-model species, better approaches will improve the sensitivity in protein identification for species distantly related to models.

In conclusion, the main goal of the present study is to gain insight about the physiological and molecular clues operating behind this process. To accomplish this main goal, the following partial objectives were defined:

1. To test the possible role, the plant growth regulators might havel on apogamy, either by adding them or their inhibitors (IPGRs) to the culture medium where gametophyte are growing, (Chapter 2), or measuring the endogenous levels of the phytohormones by using UHPLC, (Chapter 3).

2. To carry out comparisons of the proteomic profiles between two developmental stages of gametophytes, (one versus two-dimensional), (Chapter 4).

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Chapter 2. Modulation of gametophyte development and apogamy by the addition of phytohormones and inhibitors of their biosynthesis or transport (HBTIs) in the fern *Dryopteris affinis* ssp. *affinis*

Abstract

The fern *Dryopteris affinis* ssp. *affinis* exhibits a case of apomixis, in which an embryo develops from somatic cells of the prothallium (apogamy). The effect of the following phytohormones and inhibitors of their biosynthesis or transport (HBTIs), on vegetative and reproductive development of homogenized gametophytes, when added to Murashige and Skoog liquid media (MS), was evaluated: two balances of indole butyric acid (IBA)+6-benzylaminopurine (BA) (5-0.5 µM, and 2.5-2.2 µM); two balances of naphthaleneacetic acid (NAA)+BA (2.5-2.2 µM and 0.5-4.4 µM); gibberellic acid (GA_3) (0.3 and 3 µM); spermidine (S) (0.7 and 7µM); the inhibitor of auxin polar transport N,1-naphthylthalamic acid (NPA) (0.35 and 3.5µM); the inhibitor of GAs biosynthesis, flurprimidol (F) (0.3 and 3 µM); and the inhibitor of spermidine biosynthesis, cyclohexylamine (CHA) (1 and 10 µM). The auxin IBA exhibited a wide-ranging effect on morphogenesis, including length/width ratio, apogamy, embryo size and rhizoid localization. Auxin polar transport could be involved as wider gametophytes and bigger and conical embryos were observed with the inhibitor NPA. Gametophyte elongation was clearly promoted by GA_3, being this result reinforced by the inhibitor flurprimidol. Gametophyte growth in length or width, and embryo size resulted also affected by the polyamine spermidine. A valid protocol is available to induce proliferating callus in *D. affinis* ssp. *affinis*, when cultured in presence of 2,4D (2,4-dichlorophenoxyacetic acid), showing bodies of lipid accumulation. The findings would be useful to lead further understanding of the mechanisms related to apogamy, and this peculiar case of somatic embryogenesis.

2.1. Introduction

Plant research mostly focuses into angiosperms models. Historically, ferns had been forgotten, being regarded basically by their ornamental, conservational or therapeutical applications (Fernández and Revilla 2003). To date, a few fern species have been used to study basic plant developmental processes such as photomorphogenesis (Wada 2007), germination (Salmi et al. 2005, 2007; Suo et al. 2015), cell polarity (Salmi et al. 2010), cell wall composition (Eeckhout et al. 2014), or reproduction (Fernández and Revilla 2003; Menéndez et al. 2006c, a, 2009; Cordle et al. 2007, 2010; Kazmierczak A 2010; Valledor et al. 2014; Lopez and Renzaglia 2014; de Vries et al. 2015; Grossmann et al. 2017).

Asexual reproduction by clonal seeds, would allow to maintain agriculturally important phenotypes, including that of highly productive F1 hybrids, and many researchers have emphasized the tremendous potential that apomixis represents for plant improvement, higher than the green revolution (Grossniklaus et al. 1998). In ferns, apomixis has evolved several times independently, being its frequency at least 3%, a value which is higher than in other key plant groups (Grusz 2016). Apomixis in ferns includes apogamy, the formation of sporophytes from somatic cells of the prothallium, and agamospory (or diplospory), the production of unreduced (diplo) spores (Ekrt and Koutecký 2016). Indeed, the absence of sexual reproduction, in most of apogamous ferns, makes reproduction less complex than in Angiosperms, where often coexists apomixis and sexuality (Liu et al. 2012; Kandemir and Saygili 2015).

The gametophyte of ferns is a free-living organism, being easy either for in vitro culture and sample collection (Fernández and Revilla 2003). Given its characteristics of living separated from sporophyte, it results suitable to deal with studies on reproduction (asexual and sexual) (Rivera et al. 2018). In addition, apogamy could be regarded as a somatic embryogenesis process from gametophytic cells, which can be indeed induced *in vitro*, playing with environmental factors such as carbohydrates (Whittier and Steeves 1960, 1962; Whittier 1964a; Kawai et al. 2003; Kawakami et al. 2003; Ekrt and Koutecký 2016), osmotic conditions Elmore and Whittier 1975, Aderkas (1984), and growth regulators if supplied with sucrose (Whittier 1964b; Kato 1967; Elmore and Whittier 1975; Aderkas 1984). Both apogamy and somatic embryogenesis are scarce reported in ferns (Aderkas 1984; Makowski et al. 2015; Rybczynski et al. 2018).

Homogenized gametophytes represent an interesting culture system to take advantage of the proven capacity of regeneration exhibited by them (Finnie and van

Staden 1987; Fernandez et al. 1993; Menéndez et al. 2006c, 2010; Somer et al. 2010; Rivera et al. 2018). The reactivation of cell division and subsequent differentiation processes of organogenesis and embryogenesis in homogenized gametophytes, are influenced by phytohormones such as auxins, gibberellins, cytokinins, and polyamines (Menéndez et al. 2006c, a; De-La-Peña et al. 2008; Menéndez et al. 2009; Grossmann et al. 2017; Rivera et al. 2018). Indeed, their biosynthesis and transport are subject to enzymatic reactions, which can be blocked by using inhibitors, such as NPA, flurprimidol and cyclohexylamine, interfering on PIN auxin transport, ent-kaurene oxidation or spermidine biosynthesis, respectively by Rivera et al. 2018 (Klíma et al. 2016; Rivera et al. 2018). Hormonal biosynthesis and transport inhibitors (HBTIs) are useful compounds for elucidating the effect of phytohormones on plant growth and development (Grzyb et al. 2018).

Dryopteris affinis ssp. *affinis* is a diploid fern with an obligated apomictic life cycle, originated from the crossing of *D. oreades* and some sexual ancestor of the species *D. wallichiana*, or *D. caucasica* (Salvo 1990). It is widely distributed in the regions of the Mediterranean, Macaronesia and West of Eurosiberia. Given that the gametophyte of this subspecies is male but not female reproductive organs are formed, apogamy becomes obligate. Usually, when the gametophyte draws the typical heart shape, it is observed near the apical notch, a group of small and dark brown cells, which finally will arise an embryo (Menéndez et al. 2006c) (Fig. 2.1).

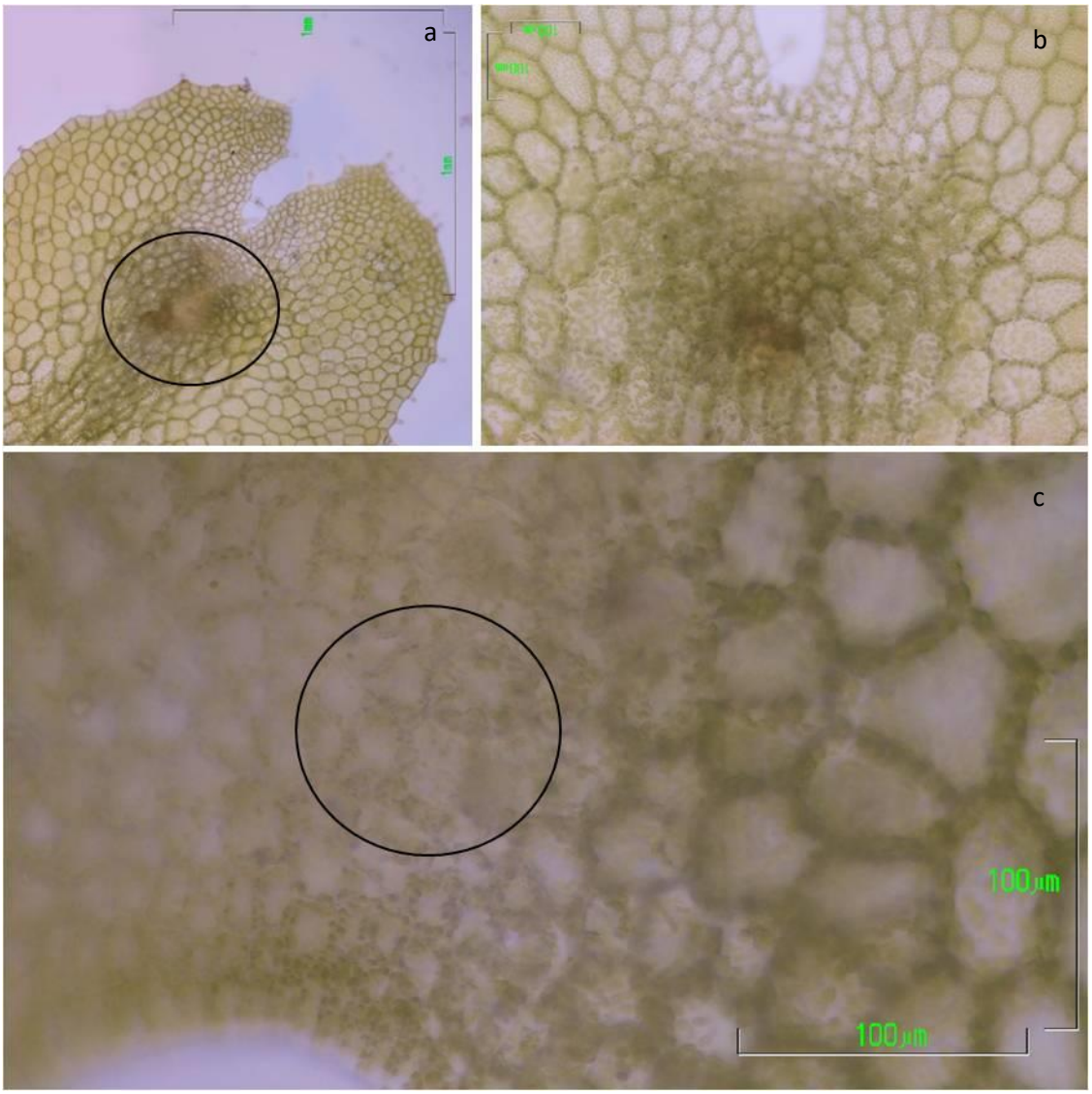


Figure 2.1. Apogamous centre in *Dryopteris affinis* ssp. *affinis*. a) Placement near to the apical notch. b) Composed by isodiametric cells dividing in all planes, with dense cellular content, c) A very initial stage of four cells.

The goal of this research work was to test the effect of the phytohormones auxins, cytokinins, gibberellins, polyamines, and the HBTIs N-1-naphthylthalamic, flurprimidol, acid and cyclohexylamine, on both vegetative development and asexual reproduction (apogamy) in homogenized gametophytes of *D. affinis* ssp. *affinis* cultured *in vitro*.

2.2. Materials and Methods

2.2.1. Collection, cleaning, and culture of spores and gametophytes

Spores of *D. affinis* ssp. *affinis* were collected from mature fronds obtained in the Valley of Turón (Asturias, Spain), coordinates 43° 12´ N and 5° 43´ W. Removed fronds were placed between sheets of paper, in a dry environment. After that, spores and sporangia were sieved to separate spores from the rest of plant material, and spores were kept in vials and stored at 4 °C until using. Spores (10 mg) were soaked in water for 2 h, disinfected for 10 min with a solution of NaClO (0.5% w/v) containing Tween 20 (0.1% w/v), and rinsed three times with sterile distilled water. Spores were centrifuged at 700g for 3 min between rinses. Prior *in vitro* culture of spores, density was adjusted to around 4000 spores per flask, by using an optical microscope (Nikon Eclipse E-600) and a Füsch-Rosenthal (Brand) chamber.

Spores were cultured in 100 mL flasks containing 20 mL Murashige and Skoog medium (MS) supplemented with 2% (w/v) sucrose and 0.7% (w/v) agar and, unless otherwise noted, the pH was adjusted to 5.7 with 1 or 0.1 N NaOH (Murashige and Skoog 1962). The cultures were maintained at 25 ºC under cool-white fluorescent light (70 µmol $m^{-2}s^{-1}$) with a 16:8 h light: dark photoperiod. After spore germination, gametophyte development took place and they were subcultured into the same medium composition, monthly.

2.2. 2. Homogenized gametophyte cultures

Gametophytes (0.2 g) were mechanically fragmented using a Waring blender for 15 s under aseptic conditions at 9500 rpm. The homogenized tissue samples were cultured in 250 ml Erlenmeyer flasks, containing 50 mL of liquid MS medium with 2% (w/v) sucrose, and supplemented with the following concentration of phytohormones and HBTIs, added in grouping (IBA 2.5 µM+BA 2.2 µM; IBA 5 µM+BA 0.5 µM; NAA 0.5 µM+BA 4.4 µM; NAA 2.7 µM+BA 2.2 µM) or alone (GA_3 0.3 and 3 µM; S 0.7 and 7 µM; NPA 0.3 and 3.5 µM; F 0.3 and 3 µM; CHA 1 and 10 µM). Cultures were placed on an orbital shaker and kept at the same conditions cited above for spore cultures.

2. 2.3. Callus culture

Cellular aggregates obtained from homogenized gametophytes cultured in MS medium without or with IBA 5µM+BA 2.2µM, or GA_3 0.3 µM, were transferred to MS medium with the auxin 2,4D 2.3 µM plus cytokinin (BA 2.2 µM or kinetin 2.3 µM). In

other experiment, gametophytes were wounded with a scalpel and placed on MS media supplied with 2,4D 2.3 µM + BA 2 .2 µM.

2.2.4. Microscopical examination

Regenerated and fresh gametophytes, derived from the above-mentioned treatments, were observed under an optical microscope (Nikon Eclipse E-600), after 50 days of culture initiation. Data about vegetative development, the gametophyte shape (filamentous, spatula and heart), and length/width ratio, and apogamy (percentage of apogamous gametophytes, and embryo size) were scored. One hundred individuals from each treatment were taken randomly to determine the frequency of morphotype and the length/width ratio. Apogamy data were determined from 50 gametophytes heart-shaped, at which the embryo is usually well defined, although in some cases, spatulas were also considered as well, with those treatments accelerating the apogamic process.

2.2.5. Statistical analysis

The Chi-square (χ^2) test was applied to non-parametric data, such as morphotype and number of apogamous embryos. The parametric data, i.e. rate length/width and the embryo size, were analysed by ANOVA, using the Levene and Bartlett test for homogeneity of variances, and Shapiro-Wilk for normality. The tests *post hoc* Tukey-HSD and Duncan where used in the case there were differences among treatments, in an ANOVA test. Analyses were carried out by R-Studio (Team 2016) and set to a significance level of α= 0.05.

2.2.6. Histological examination

Callus tissue was fixed using 4% paraformaldehyde-PBS (v/v) (phosphate buffer, pH=5.8), at 4 ºC, and transferred, after 48 hours, to a solution of 0.1% paraformaldehyde-PBS (v/v), to keep them until use. Callus were dehydrated in a series of tertiary-buthyl alcohol solutions, embedded in *Paraplast* (Fisher Scientific Co.), as described by Jensen (1962) and sectioned into 10µm slides using a microtome (JUNG, mod. 1130). Sections were placed in a microscope slide, covered with albumin until dried at 40 ºC. Then, they were stained with Nile red, to bind lipids inside the callus, for 10 minutes, in a dark chamber. Gametophyte samples were stained with O-Toluidine blue for one hour (Jensen 1962).

2.3. Results

2.3.1. Effects of assayed treatments on vegetative development

Vegetative development of regenerated gametophytes was annotated in terms of their shape or morphotype, i.e., one-dimensional (filamentous) versus two-dimensional (spatulate and heart), and the length/width ratio (Fig. 2.1).

a) Morphotype. The transition from one to two-dimensional growth of gametophytes, were favoured either by the addition of phytohormones such as the NAA/BA balances (χ^2=91, p-value<0.001), by GA_3 and its inhibitor flurprimidol, at the lowest dose (χ^2=10, p-value=0.001, and χ^2=5, p-value=0.021), respectively, and also the polyamine spermidine at the highest concentration (χ^2=35, p-value<0.001). The inhibitor CHA impeded two-dimensional transition at the lowest doses (χ^2=12, p-value<0.001, and induction at the highest (χ^2=35, p-value<0.001).

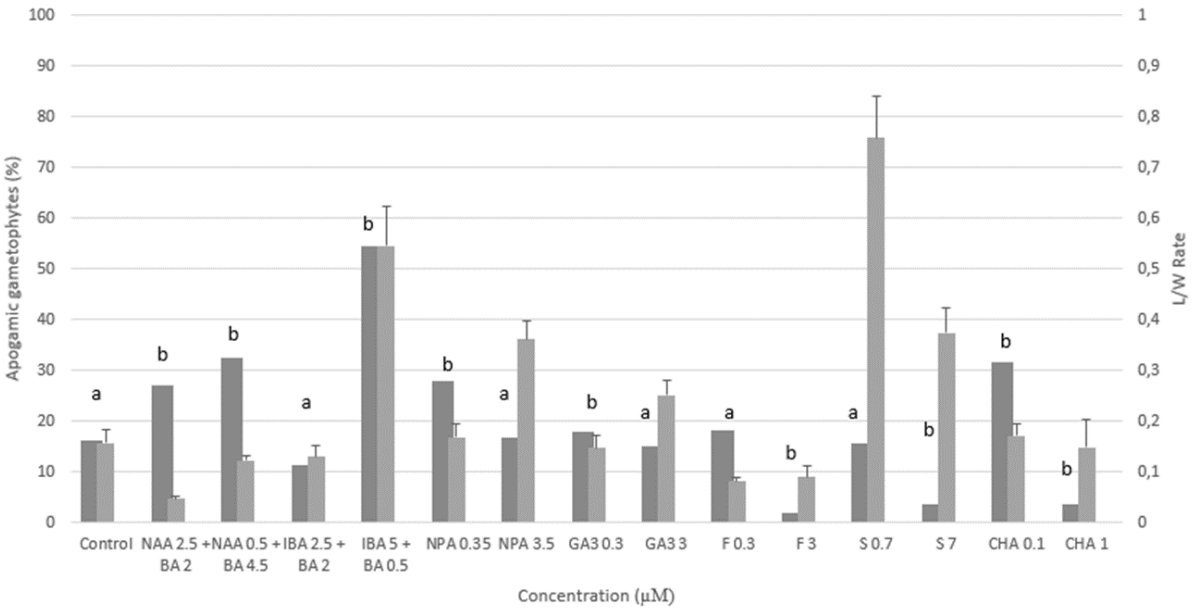


Figure 2.2. Effect of phytohormones and inhibitors of their biosynthesis or transport, on two-dimensional transition and length/width rate, in regenerated gametophytes of *Dryopteris affinis* ssp. *affinis*. Data after 50 days.

b) Length/width ratio. This relation indicates in what direction (apical-basal or lateral) expands the gametophyte as it is growing up. Gametophyte elongation, following the apical-basal polarity, was promoted by IBA (p-value<0.001), and GA_3 (p-value<0.001), being most of them spatula-shaped (Fig. 2.2). This phytohormone favoured the antheridia formation in filamentous shape gametophytes (Fig. 2.2). Respect to the auxin inhibitor transport NPA, gametophytes were longer than wider at the lowest dose,

and wider than longer at the highest, drawing a circle in the last case (p-value<0.001) (Fig. 2.2). The inhibitor of the biosynthesis of GAs, flurprimidol, at 0.3 μM, induced the regeneration of gametophytes wider than longer, decreasing the proportion of spatulas to 18% (Fig. 2.2). As occurred with NPA, the gametophytes growing in presence of the inhibitor of spermidine, cyclohexylamine, were also longer than wider at the lowest dose, and wider than longer at the highest.

c) Aberrations in the morphotype.

In almost all the cultures, were observed gametophytes that deviate from the standard form of spatula (greater length than width) and heart (two symmetrical lobes, presence of notch and length/width rate slightly less than 1), and they were annotated as irregular morphologies, when exhibiting: a) two asymmetrical lobes (Fig. 2.3 e), b) more than two lobes, as in (Fig. 2.3 c), presenting up to 8 wings, and c) amorphous or not easily descriptive as occurred with flurprimidol at the highest dose, showing cellular protrusions like spikes (Fig. 2.3 f), and gametophytes following an undefined growth pattern (Fig. 2.3 g). Another irregularity was the presence of rhizoids far from basal areas of regenerated gametophytes (Fig. 2.3 h).

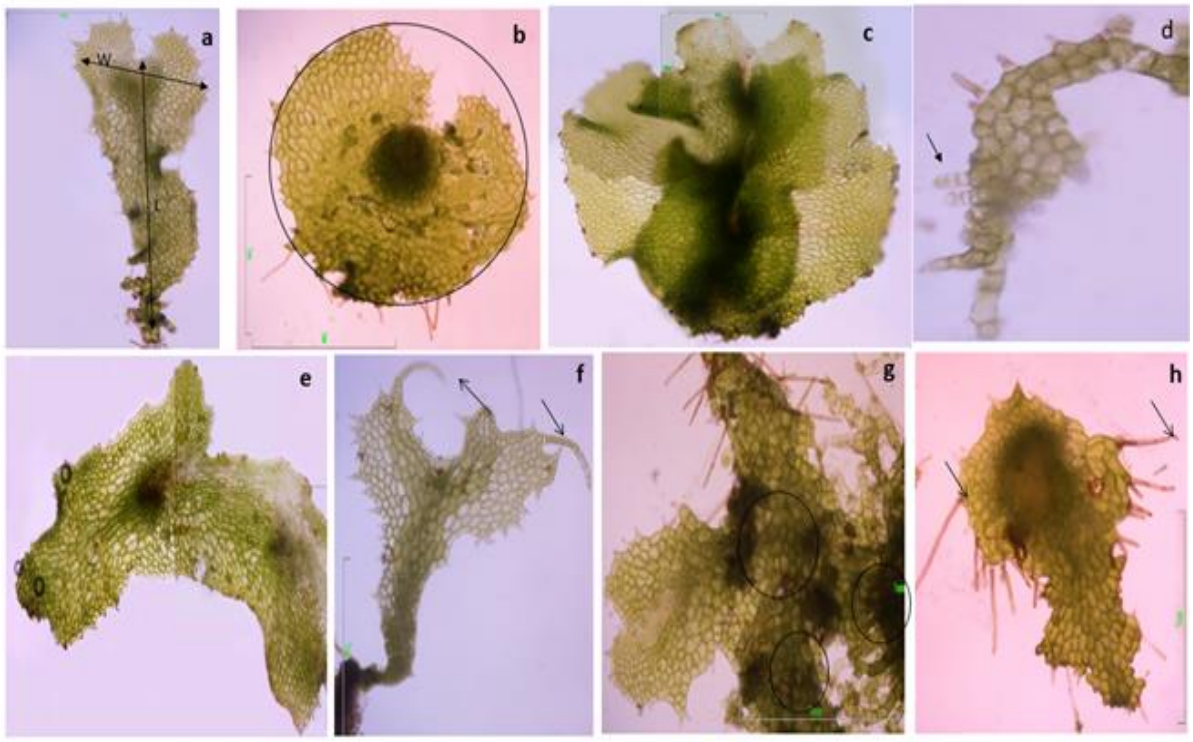


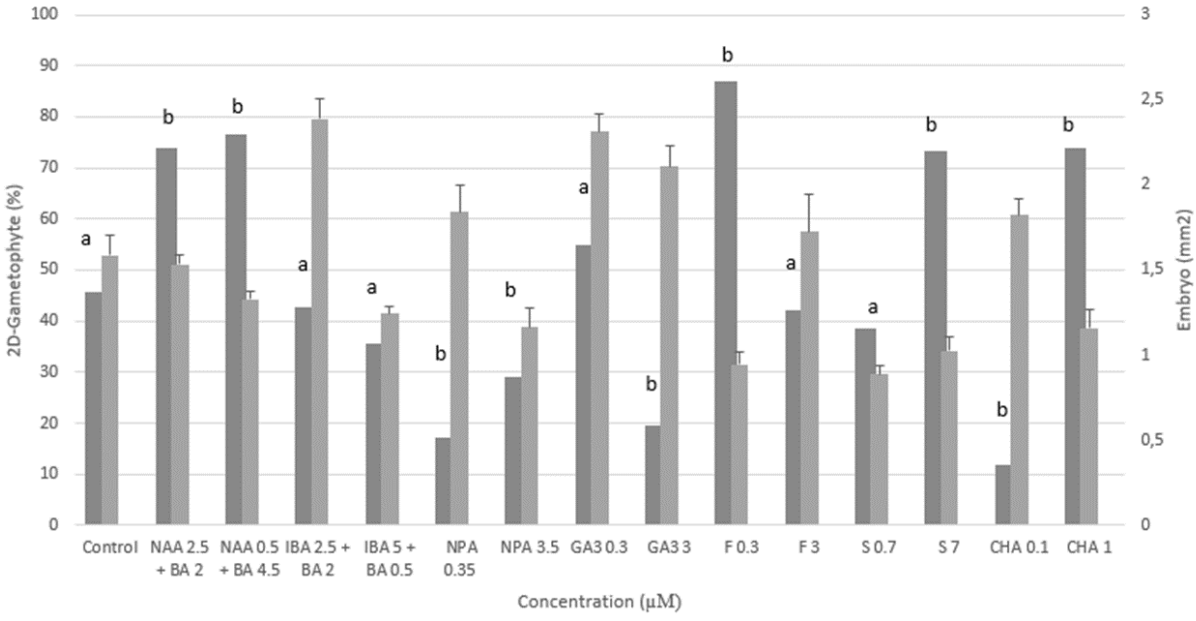
Figure 2.3. Morphological aspects in regenerated gametophytes of *Dryopteris affinis* ssp. *affinis*, cultured in MS liquid medium with the following phytohormones or inhibitors of

their biosynthesis or transport: a) elongation with GA_3 0.3 µM (w=width; l=length); b) circular shape with NPA, 3.5 µM; c) growing in width flurprimidol 0.3 µM; d) antheridia observed with GA_3 3 µM. e) asymmetrical lobes in free-hormone or inhibitors medium; f) thorn-like protrusions (arrows), and g) undefined growth pattern, when growing in MS liquid medium (circles) with flurprimidol 3 µM; h) rhizoids placed far from the basal region (arrows) in presence of IBA 2.5 µM+2.2 µM.

2.3.2. Effect of assayed treatments on apogamy

Regenerated gametophytes formed asexual embryos from vegetative cells, and data about frequency of apogamy, i.e. number of two-dimensional gametophytes with apogamous embryos, and the area of embryo, are shown in Fig. 2.3.

a) Apogamy rate. With the exception of the balanced combination IBA/BA (χ^2=2, p-value <0.197), the rest of auxin/cytokinin treatments significantly increased the percentage of apogamous gametophytes, reaching a maximum of 50% with IBA 5 µM+ BA 0.5 µM. Apogamy decreased with the addition of flurprimidol 3 µM to only 2% of the total number of regenerated gametophytes forming embryos (χ^2=8, p-value=0.004),



and also with spermidine at the highest concentration (χ^2=7.08, p-value=0.01). In opposite, apogamy increased with CHA 0.1 µM, to 30% (χ^2=11, p-value<0.001), dropping with the highest concentration, to only 4% of gametophytes forming apogamous embryo (χ^2 =5, p-value=0.033).

Figure 2.4. Effect of phytohormones and inhibitors of their biosynthesis or transport, on apogamy frequency and embryo size, in regenerated gametophytes of *Dryopteris affinis* ssp. *affinis*. Data after 50 days.

b) Embryo size. The size of apogamous embryos enlarged respect to the control by the addition of the balanced combination IBA/BA to the culture medium, (p-value=0,003) (Fig. 2.4 a), with NPA 3.5 μM (p-value< 0.001), adopting a conic aspect in this case (2.4 b), or by adding spermidine 0.7 μM (p-value=0,001). In contrast, the size significantly decreased by the addition of flurprimidol (p-values 0.023 and 0.034), and CHA 1 μM (p-value=0.007).

b) Aberrations in apogamy. A notable alteration of apogamy, was the presence of more than one embryo in the regenerated gametophytes (polyembryony), (Fig. 2.4 c) but it is not very frequent. The emergence of gametophytes with embryos placed at the tip of lobes was recurrent at the lowest concentration of the inhibitor of gibberellins, flurprimidol (χ^2=6; p-value=0.014) (Fig. 2.4 d).

2.3.3. Callus induction

a) Homogenized gametophytes. Cellular aggregation was observed in a MS hormone or inhibitor-free medium and also in MS medium with the following treatments: IBA 5 μM+BA 0.45 μM or GA_3 0.3 μM (Fig. 2.5a), that proliferated when transferred to MS solid medium supplemented with the auxin 2, 4-D 2.3 μM plus the cytokinin BA 2.2 μM or kinetin 2.3 μM.

(b) Hand-cut gametophytes. Gametophytes wounded manually with a scalpel, showed also cellular aggregation and proliferation when cultured on solid MS medium with the hormonal treatments mentioned in the preceding paragraph.

In both types of cultures, *calli* presented a friable texture and a yellowish-green colour (Fig. 2.6 a). Histological examination revealed abundant bodies of lipids (2.6. b).

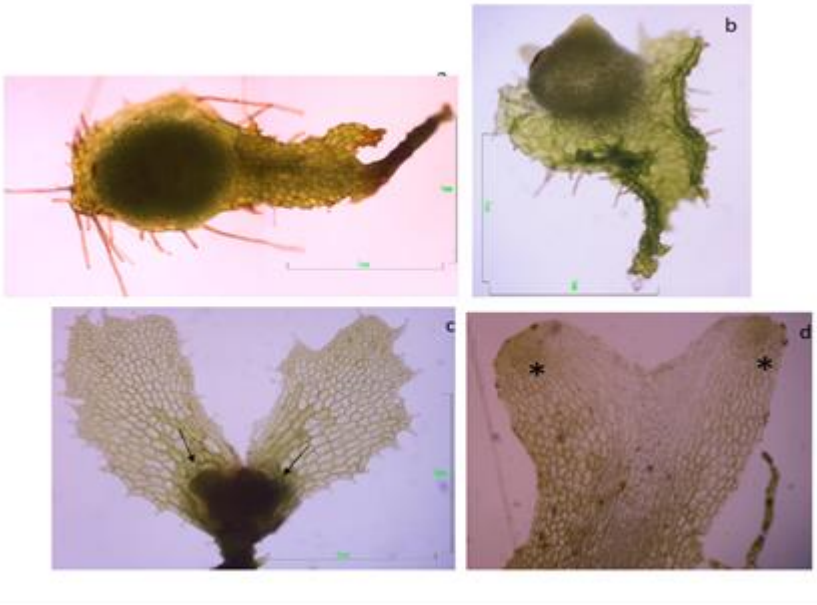


Figure 2.5. Apogamy peculiarities in homogenized gametophytes, cultured in liquid MS medium with the following treatments: a) embryo developing from a spatulate gametophyte with IBA 2,5µM+BA2,2µM; b) conical aspect of embryo with NPA 3.5µM; c) and d) polyembryony and displacement of embryos at the tip of lobes, observed with flurprimidol 0.3µM.

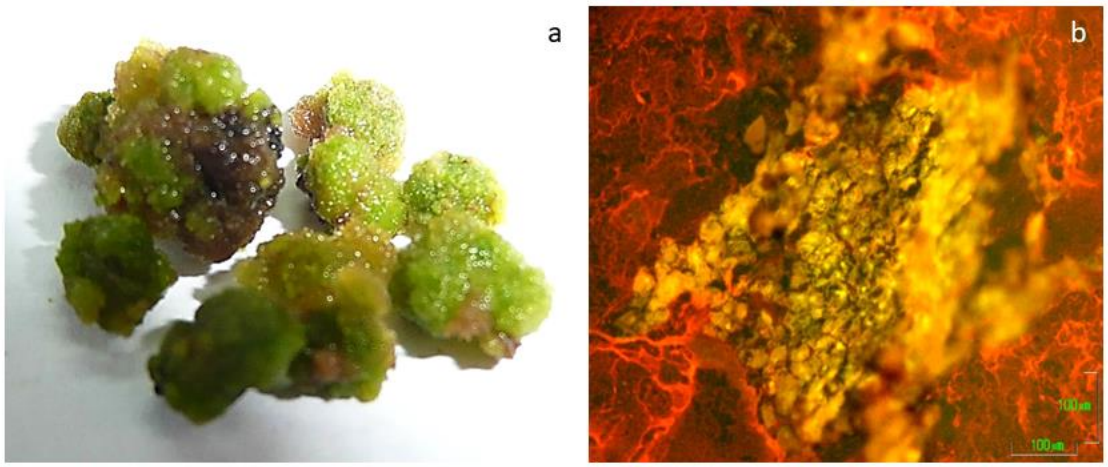


Figure 2.6. Callus derived from regenerated gametophytes of *Dryopteris affinis* ssp. *affinis*, showing, a) compact aspect and yellowish-green colour; b) lipidic bodies as spots of green colour.

2.4. Discussion

In this work, it is shown the homogenates cultures of the fern gametophyte as an interesting tool to deepen on apogamy, a peculiar case of apomixis. The homogenized gametophyte of *D. affinis* ssp. *affinis*, exhibits a great plasticity, being able to vary its vegetative and reproductive behavior by means of the addition of phytohormones or HBTIs, to the culture medium. Moreover, the high regeneration capacity of the fern gametophyte, represents an excellent supply of plant material to carry out further analyses for different purposes coping with plant development.

The mechanical disruption caused to the prothallus, breaks somehow the connection between cells, and the interrelationships between many structural and functional elements. Despite the stress involved in the mechanical fragmentation exerted, some cells retake the capacity of division and differentiation, generating a new individual, demonstrating, once again, the plasticity that characterizes the plant organisms, and how the fern gametophyte has evolved and retained the ability for reprogramming cell identity to facilitate tissue repair and developmental plasticity (Shin and Seo 2018). In line with it, the gametophyte of *D. affinis* ssp. *affinis* exhibits a great regenerative potential comparable to other species that have been previously cultivated in our laboratory (Fernández and Revilla 2003; Menéndez et al. 2006c, b; Somer et al. 2010; Rivera et al. 2018). In general terms, basal land plants display high regenerative capacities (Ikeuchi et al. 2016).

The fragmentation and culture of the gametophyte, gave us the opportunity that from an individual, which had reached a certain degree of organization (apical-basal polarity, apical meristem, rhizoids, trichomes, antheridia, lateral wings, dorso-ventral symmetry, an asexual embryo), might be possible to reset each of these differentiation patterns, and rebuild a new individual, thanks to the remaining totipotency or pluripotency of some cells, resting alive after being fragmented.

In previous work with cultures of gametophytes derived from spores, the addition of compounds like those used here, to the medium, did not prove to cause major variations or changes, in terms of gametophyte morphology and the apogamy process (Rivera et al. 2018). The reason could be found in the explant itself; both spore and the whole gametophyte, represent a more differentiated and organized structure than just a piece of few gametophyte cells, which, after the disorganization caused by the mechanical disruption, could be more influenced by these compounds. Therefore, homogenized cultures of gametophytes, provided a wider range of variations, affecting both the

reproductive and vegetative growth as a result of either the fragmentation of tissue as the presence in the medium of plant growth regulators or compounds inhibitors of their biosynthesis and transport. However, we must state that gametophyte development in homogenized cultures experience some delay respect to those originated from spores disseminated on solid medium, at the end of the same period, as each piece of tissue must face a longer way until regenerating a new gametophyte. Also, apogamy decreased from 70% to just a 30%, as we discuss following.

Apogamy can be regarded as a case of asexual embryogenesis, in which some vegetative cells in the gametophyte, form an embryo, maintaining the same ploidy level gametophyte and sporophyte. In this work, a balanced ratio IBA/BA increased either the formation of apogamic embryos as well as their size. In general, auxins have shown an important role in the somatic embryogenesis (Johri 2008) and, in particular, the inducer effect of IBA has been demonstrated (Jha et al. 2007; Johri 2008). On the other hand, differences among the auxins IBA and NAA were evident, so that while IBA seems to have a major role on embryogenesis, NAA resulted more effective to favour the transition from one- to two-dimensional growth, which finally could also mean more apogamous embryos if have considered a longer period of culture. It has been reported a positive effect of the same concentrations of NAA on the number of (apogamous sporophytes) after three months of culture (Menéndez et al. 2006 c). The variations found in our research among NAA and IBA, compounds belonging to the same family of chemicals, may be due to various factors. On the one hand, the fact that these processes might be controlled by genetic mechanisms, as it was reported in rib1 mutants of *Arabidopsis*, which are resistant to the induction of adventive roots by IBA but not NAA, and not finding variations between them in the wild genotype (Ludwig-Müller 2000). On the other hand, they might experience differences in the absorption by cells; being favoured the IBA intake. In this sense, it has been found that NAA is not a good substrate for influx auxin transports (AUX1) (Klíma et al. 2016).

Rhizoids are present in the gametophyte of ferns and clubmosses, and it is thought they may have a function similar to the root hairs from the roots of Angiosperms, favouring the intake of nutrients and also serving as anchor of the gametophyte to substrate, although they are not formed neither in pollen grain or embryo sac (Jones and Dolan 2012). Rhizoids and root hairs seem to share genetic mechanisms, in particular, transcription factors of the family of the ROOT HAIR DEFECTIVE (RHD), and ROOT HAIR DEFECTIVE LIKE (RSL) (Vijayakumar et al. 2016), having been identified

members of these families, in the gametophytes of this species (Wyder et al. 2020). Auxins control the formation of roots and hairs root in Angiosperms (Ludwig-Müller 2000), and the formation of rhizoids on algae, ferns and liverworts (Hickok 1984; Klämbt et al. 1992; Jones and Dolan 2012; Atallah et al. 2018), regulating the expression of these transcription factors. Observing the anomalous position of rhizoids in the regenerated gametophytes cultured in presence of IBA+BA, we can deduce, that effectively, auxins can mediate the development of these structures, and which might be misplaced by what of alteration represent the regeneration process itself.

The inhibitor of auxin transport, NPA, is classified as a phytotropin, having a controversial mode of action on polar auxin transport (Teale and Palme 2018). In our work, it did not seem to affect neither the two-dimensionality transition nor the percentage of apogamous gametophytes, but the L/W ratio of gametophytes, and the embryo size and shape, when added at 3.5 µM, promoting wider gametophytes and bigger and conical embryos.

The effect of the chemical compounds added to the culture medium, on embryo size, was underestimated as it was calculated by the area of the apogamous centre. The embryos grown in presence of NPA displayed a prominent size, and the measures may have been more affected, as occurred also with highest dose of IBA and the lowest of spermidine. The auxin polar transport has a profound effect on plant development as stem and root growth, the initiation of lateral buds, vascular pattern and embryogenic polarity, are influenced by auxins (Su et al. 2011). In studies with stem corn (Scanlon 2003), the use of NPA altered the transport of the auxin IAA at the root, causing decrease in elongation and a globular form in *Picea abies*. The same authors, working with stalks of corn grown with NPA, reported an elongation of the apical meristem of the stem, as well as a delay in the initiation of the leaves. The circular form observed in regenerated gametophytes of *D. affiis* ssp. *affinis* (and the lack of multilobes), as the bigger size and elongated form of embryos, could reflect an alteration in the transport of auxins and their involvement on gametophyte elongation and on embryogenesis.

GA_3 has an important role on both vegetative and reproductive plant development, including the fern gametophyte (Menéndez et al. 2006c; Valledor et al. 2014; Rivera et al. 2018). In this work, the effect on gametophyte elongation caused by GA_3 was reinforced by the addition to the culture medium of the biosynthesis inhibitor flurprimidol, which causes the opposite effect, stimulating growth in width. Flurprimidol, at the lowest concentration, caused irregularities in the gametophyte morphology, such as

the presence of several lobes, -which has been previously reported (Menéndez et al. 2006c), in the gametophyte of *Blechnum spicant*-, and also were observed amorphous gametophytes, growing without a well-defined pattern, at the highest concentration of this inhibitor. The combined action of the stress caused by the mechanical tissue disruption and the inhibitor could lead to these anomalies, which were absent with GA_3 treatments. Thus, gibberellins are involved in the vegetative development of the gametophyte in *D. affinis* ssp. *affinis*.

The importance of the GAs on apogamy, was revealed also by flurprimidol, whose addition to the medium decreased the size of the embryo. Moreover, the highest concentration of flurprimidol, inhibited apogamy, in agreement with that reported by our lab (Menéndez et al. 2006a, b). However, it is not clear the role of GAs on apogamy since the exogenous addition of GA_3 seems to have not affect in the process, not discarding that another gibberellin might have a more decisive function. It has been stated that GA_4 could have a more active role on apogamy, scoring higher endogenous levels of this compound when the apogamous centre start developing (Menéndez et al. 2006a).

The polyamines are involved in vegetative growth, promoting division and differentiation (Hickok 1984). In our work, it was noted that the addition of spermidine stimulates two-dimensional transition and gametophytes wider than longer, being these results unmodified by CHA addition, remaining not clear the role of these compounds.

Polyamines have been considered essential for the embryogenic development in *Arabidopsis* and somatic embryogenesis (De-La-Peña et al. 2008; Dutra et al. 2013). In our species, spermidine influenced embryo development, and its inhibitor, CHA, on the induction of apogamy. All these results agree with previous studies reporting the presence of several proteins related to the action of the classical phytohormones as auxins, cytokinins, gibberellins or polyamines, among others (Grossmann et al. 2017).

Homogenized gametophyte cells were able to divide and differentiate a new gametophyte, and to form disorganized cell masses, like callus, which were observed also in cultures derived from gametophytes wounded with scalpel. This response was induced spontaneously, probably caused by culturing under a stressful situation, also when pieces of gametophyte tissue grew in presence of phytohormones like GA_3 0.3µM or the balance IBA 5µM +BA 5µM. Although the auxin IBA favoured the formation of these cellular aggregates, it was ineffective to keep them proliferating, and it was only possible when cultured on solid MS medium with the auxin 2,4-D, which is otherwise a classical compound for this purpose (Rybczynski et al. 2018). Initially, the callus was compact and

green, and after being *subcultured* for several times, it becomes brown and softer, showing symptoms of toxicity. The presence of lipid bodies in the histological sections of callus, represents a line of research about the profile of fatty acids that comprise them, due to the interest that exists today to replace more polluting energy (Jouzani et al. 2018).

In brief, **t**he experimental system used in this work along with the addition of certain phytohormones and inhibitors of their synthesis or transport, provided a better understanding of these chemicals on both vegetative development and apogamy, in the gametophyte of *Dryopteris affinis* ssp. *affinis*, resulting to be more efficient than previous studies, starting from spore or the entire gametophyte. A balance auxin/cytokinin favourable to IBA, increases elongation, the frequency of apogamy and the size of the embryos. GA_3 shows an important role in vegetative growth, promoting elongation, and evidenced also by the inhibitor of GAs biosynthesis, flurprimidol. Although the application of flurprimidol, inhibited apogamy, is not clear the possible involvement of GAs since their addition did not give any positive or negative result. The role of the polyamine spermidine on both vegetative development and apogamy remains more controversial. Now, a valid protocol is available to induce and proliferate callus of *D. affinis* ssp. *affinis*, which can lead to a biological system to deepen on apogamy and to conduct further molecular analyses on this topic.

2.5. References

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Chapter 3. Phytohormones profiles in apogamous and sexual gametophytes of *Dryopteris affinis* ssp. *affinis,* and its relative *D. oreades*

Abstract

Physiological and biochemical processes in plants are regulated by phytohormones. The phytohormone profiles are scarce in ferns. The endogenous levels of indol-3-acetic acid (IAA), cytokinins (CKs), abscisic acid (ABA), gibberellins (GA_3 and GA_4); salicylic acid (SA) and brassinosteroids were assessed by ultra-performance liquid chromatography-mass spectrometry (UHPLC) in filamentous, spatula and heart-shaped gametophytes of the apogamous fern *Dryopteris affinis* ssp. *affinis*, and in female heart-shaped gametophytes of its sexual progenitor *D. oreades*. The hormonal profiles are different comparing either the three apogamous stages, or apogamous versus sexual heart-shaped gametophytes. In the first case, it was noticed that the cytokinin BA and salicylic acid, increased at the filamentous stage, the gibberellin GA_3 at the spatulate, and finally a slight variation is observed of RDHZ in the cordiforme gametophytes. In the second case, up to seven out fourteen phytohomones accumulated more in the sexual female gametophyte respect to the apogamous one, which are the auxin AIA, the cytokinins Z, RZ, IPA, the gibberellin GA_4, the brassinosteoids castosterona, and ABA. In the light of these data, we might suppose a coordinate action of phyhormones on gametophyte growth as well on reproduction either by sexual or asexual means, in this non-model species.

3.1. Introduction

Phytohormones take part in the regulation of almost all phases of plant development, and also mediate the responses to various environmental stresses (Hicks 1894; Rademacher 2000; Dobrev and Kamínek 2002). In particular, competition of ferns with flowering plants for resources turned out to be an evolution factor that resulted in many adaptive traits as to carry out photosynthesis at a low light intensity, a high resistance to intensive moistening, tolerance to a substrate with a poor mineral content, spore resistance to air quality injuries, poikilohydry of gametophytes in some species, potential longevity of sporophytes, etc. (Page 2002). Such adaptation advantages would not have been possible without the formation of an evolutionarily perfect mechanism of metabolic process regulation. It is the complex, multi-component hormonal system that provides the coordination and regulation of such basic physiological processes as growth, development, photosynthesis, respiration, tolerance to environmental factors (Kosakivska et al. 2016).

For a better understanding of the functions of the phytohormones and the interactions between them, exhaustive determination of the hormone contents is thus of great importance (Bai et al. 2010). The fact that different hormones have diverse chemical and structural properties, makes their co-quantification difficult, and also their low active concentrations, usually at parts per billion (ppb) or nanomolar level. Thanks to the advent on last years, of rapid, sensitive, accurate and efficient methods for the analysis of phytohormones from biological samples, and especially when only small amounts of sample are available for analysis, the research on this field of plant physiology has made possible to wider the number of compounds analysed (Du et al. 2012; Porfírio et al. 2016; Delatorre et al. 2017). During the years, analyses of phytohormones have turned around auxins, gibberellins, cytokinin, abscisic acid, or ethylene, being others like jasmonates or brassinosteroids more recently added, as standards and protocols were available. At present day, HPLC–MS is the most spread method to made quantitative analysis of low quantity compounds with a good performance and a low price. As a result, it was optimized to detect small amounts of hormones without derivatization and subsequent sample loss. In addition, HPLC–MS is the most accurate method to perform quantitative analysis of endogenous phytohormones or plant regulators which are also short-life compounds (Pan et al. 2008; Delatorre et al. 2017). One step forward, some authors had tested UHPLC to detect those small amounts of PGR in fresh tissue. This technique had shown better results than HPLC-MS with a spectra of plant hormones. As a result, the use

of a UHPLC-MS system allows us to detect smaller quantities that the ones we could detect ten years ago.

The typical life cycle of ferns involves the alternation of generations, which ensures the independent development of a nonsexual (sporophyte) and sexual (gametophyte) generations. Gametophytes are small, multicellular autotrophs, and can be grown *in vitro* culture to supply material to analyse growth and morphogenesis of gametophyte thallus, formation of archegonia and antheridia, differentiation of gametes, and the formation of sporophytic zygote and embryo (Banks JA 1999). Somehow, the gametophyte is the "Achilles heel", in which pivots the successful establishment of a future sporophyte, being a difficult task in many cases, given the need of free water to cope with sexual fertilization. This peculiarity of free-living gametophyte of ferns, made to consider the Pteridophytes (now *Monilophyta*), as the plant Kingdom's equivalent of the Amphibia (Page 2002).

As occurs in seed plants, in ferns, growth and development of gametophyte and sporophyte, are controlled by plant growth regulators, receiving especial attention their applications on morphogenesis, and its great repercussion on the sporophyte multiplication (Amaki and Higuchi 1992, Fernández and Revilla 2003, Somer et al. 2010, Rybczynski et al. 2018, Singh and Johari 2018). In the gametophyte, the role of phytohormones have received minor attention. Nonetheless their impact on gametophyte morphogenesis, sexual dimorphism and apomixis has been studied in a no small number of species (Romanenko et al. 2020). It must be remarked here that the way and intensity of the effect of phytohormones on fern gametophytes growth and development is determined by the hormone concentration and depends of the fern species (Romanenko et al. 2020).

Regarding the weight of phytohormones on the reproduction behaviour of fern gametophytes, we can review some notes. To start with, there are sexual species in which an antheridiogen system plays an important role on reproduction, promoting genetic exchange (Chiou and Farrar 1997, Korpelainen 1998, Yamane 1998, Tanaka et al. 2014). Anteridiogens are linked chemically to gibberellin-related diterpenoids (Tudzynski et al. 1998), which are synthesized and released into the environment at the latter stages of gametophyte morphogenesis, and it activates germination of fern spores and induces the development of antheridia on young gametophytes, as occurs in *Blechnum spicant* (Fernández et al. 1997, Menéndez et al. 2006a, Kazmierczak 2010, Valledor et al. 2014). In previous work with this species, it was noteworthy the levels of the gibberellins GA_4,

GA_7 and GA_{20} in male and female gametophytes (Menéndez et al. 2006b), and a significant increase in the content of the cytokinins iP and iPA in the female gametophytes (Menéndez et al. 2009). Otherwise, ABA has been reported to act as an antheridiogen antagonist, in *Ceratopteris richardii* (Warne and Hickok 1989). In the sexual fern *Asplenium nidus* L., qualitative differences in the content of gibberellins were noted between gametophyte and sporophyte generations, which absences an antheridiogen influence (Menéndez et al. 2011). Lately, and working with two other fern species, such as *Polystichum aculeatum* and *Dryopteris filix-mas*, either qualitative and quantitative changes in the arsenal of growth regulators present in the gametophyte, associated to the regulation of growth processes and the development of reproductive structures, were stated (Kosakivska et al. 2019, 2020). As well, variations in the content of phytohormones, have been noticed in the apogamous species *D.affnis* ssp. *affinis* (Lowe) Fraser-Jenkins, in which embryos evolved from somatic cell, bypassing fecundation (Menéndez et al. 2006a), and therefore it pointed out apogamy to be regulated by phytohormones. Minor attention has received so far other phytohormones like salicylic acid or brassinosteroids in basal branching vascular plants (i.e., lycophytes and ferns) (Sun et al. 2010, Choudhary et al. 2012). The former traditionally associated to pathogen defense, together jasmonic acid (de Vries et al. 2018), and the latter, in junction with auxins and gibberellins, form part of a key subset of plant hormones which are major determinants of plant growth and development (Ross and Reid 2010, Gómez-Garay et al. 2018).

Dryopteris affinis (Lowe) Fraser-Jenkins ssp. *affinis* is a diploid fern which probably originates from the crossing of *D. wallichiana* and *D. oreades* (Salvo 1990) but there is not certainty regarding *D. wallichiana*. At present research, the objective aimed to quantify a wide range of plant hormones in *D. affinis ssp. affinis* at different developmental stages (filament, spatula and heart or cordate), and in female heart-shaped gametophytes of its relative *D. oreades*, by using UHPLC techniques, to evaluate the yield in terms of number of compounds and the existing differences between the three apogamous stages of *D. affinis*, and comparing sexual and asexual cordate gametophytes.

3.2. Material and methods

3.2.1. Collection, cleaning, and culture of spores and gametophytes

Spores of *D. affinis* ssp. *affinis* were collected from mature fronds of ferns growing in the Valley of *Turón* (Asturias, Spain), coordinates 43° 12´ N and 5° 43´ W. Removed fronds were placed between sheets of paper, in a dry environment. After that, spores and sporangia were sieved to separate spores from the rest of plant material, and spores were kept in vials and stored at 4 °C until using. In the case of *D. oreades*, spores were collected from sporophytes growing in *Neila* lagoon (Burgos, Spain), at 1920 m. a.s.l., coordinates 42º02'48"N and 3º03'44"W. Spores (5 mg) were soaked in water for 2 h, disinfected by inmersion in a solution of NaClO (0.5% w/v) containing Tween 20 (0.1% w/v) for 10 min. Then, they were rinsed three times with sterile distilled water. Spores were centrifuged at 700 g for 3 min between rinses. Prior to *in vitro* culture of spores, cell density was adjusted from 80 to 130 spores/ml and they were cultured in flasks from 250 or 500 ml volume by an optical microscope (Nikon Eclipse E-600) and a Füsh-Rosenthal (Brand) chamber. Then, they were cultured in 500-mL Erlenmayer flasks containing 100 mL of Murashige and Skoog (MS) medium (Murashige and Skoog 1962), supplemented with 2% sucrose (w/v). Unless otherwise noted, the pH would be adjusted to 5.7 with 1 or 0.1 N NaOH. The cultures were maintained at 25 ºC under cool-white fluorescent light (70 µmol $m^{-2}s^{-1}$) with a 16:8 h light: dark photoperiod.

Gametophytes from *D. affinis* ssp. *affinis*, at three developmental stages – filamentous, spatula, and heart -in the last case with visible signs (under microscope) of an evolving apogamic center- were collected (Figure 3.1). On the one hand, filamentous gametophytes were obtained by maintaining the spores in liquid cultures placed on an orbital shaker (75 rpm) for 35 days. On the other hand, spatula and heart-shaped gametophytes, were obtained transferring 30-days filamentous gametophytes to 200 mL flask containing 25 mL of MS medium supplemented with 2% sucrose (w/v) and 0.7% agar, being collected after 20 or 30 days, respectively. The gametophytes of *D. oreades* needed around six months to accomplish sexual maturity, being collected when archegonia were visible under microscope. Samples of gametophytes were weighted before and after being lyophilized for 48h (Telstar-Cryodos) and stored in *Eppendorf* tubes on a freezer at -20 ºC until they required. Present essay was done using three

biological samples for every apogamous stages of *D. affinis* and the sexual gametophytes of *D. oreades.*

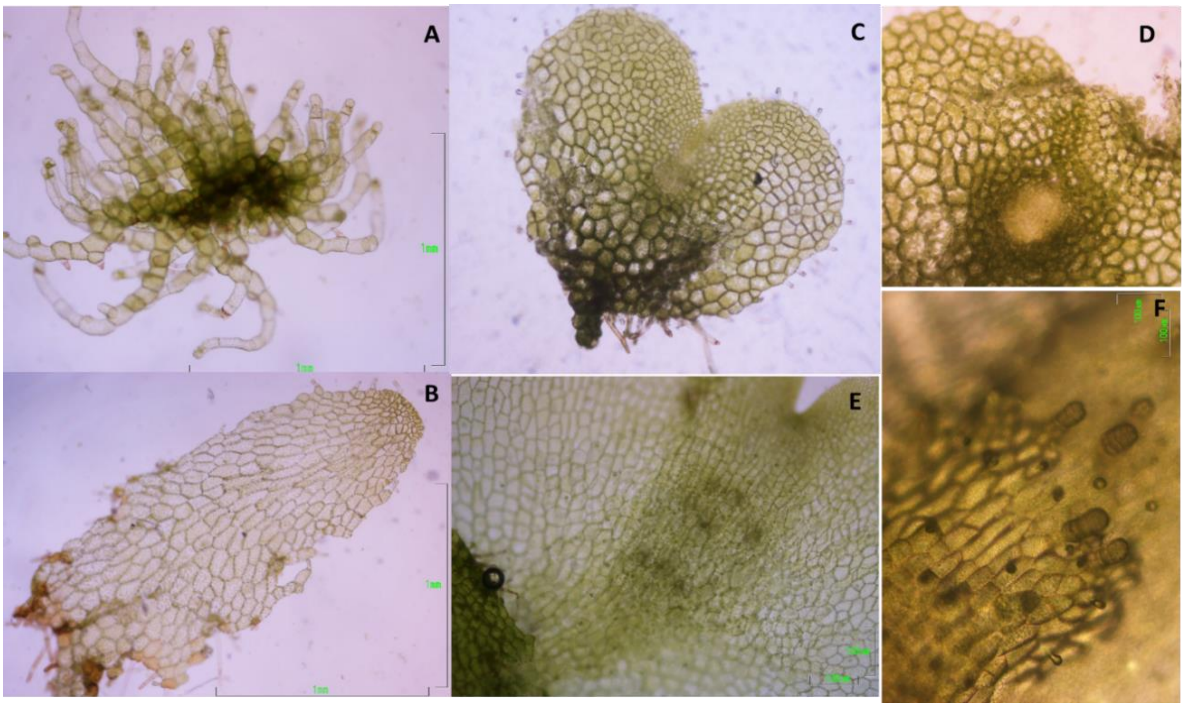

Figure 3.1. Developmental stages of gametophytes of *Dryopteris affinis* ssp. *affinis:* filament (A), spatula (B), heart (D), showing an apogamous center (D); and female heart-shaped gametophytes of *D. oreades* (E), detail of archegonia (F).

3.2.2. Endogenous phytohormone analyses

The extraction was done according to Pan *et al*. protocol, with modifications from Delatorre (Pan et al. 2008, 2010; Delatorre et al. 2017), which avoid derivatization, and purification of the samples. Changing 1-propanol by 2-propanol, contributed to speed up the process and increase accuracy. The phytohormones analyzed were ABA, IAA, the cytokinins isopentenyl adenine (iP), isopentenyl adenosine (iPA), trans-zeatin (tZ), zeatin riboside (RZ), dihydrozeatin (DHZ), dihydrozeatin riboside (DHRZ), the gibberellins GA_3 and GA_4, salicylic acid (SA), and the brassinosteroids epibrassinolide (EPI) and castosterone (BK) (Sigma-Aldrich, St. Louis, MO, USA). The following deuterated standards were employed: DHZ-d3 and ABA-d6, GA_9-d2, the homo-brassinolide (BK-d2) and castasterone; AIA-d5, BA-d7, and SA-d6. Starting from these standards, known samples are prepared and dissolved in methanol (HPLC grade) with the help of formic acid, hydrochloric acid, 2-propanol, ammonium formate, dichloromethane and finally, deionized water (Milli-Q).

After centrifugation, dried pellets were re-suspended in a final volume of 200µl of 100% methanol. Then, samples were filtered through a cellulose filter of 0.2 µm and loaded with 15 mg of silica gel (SiO_2). The chromatographic separation takes place on a reverse phase chromatographic column (*Zorbax* SB-C18 2.1X50 mm) coupled to a *Zorbax* plus eclipse pre-column (C18 2.1x5mm) at 40 °C. Two solvents are used as mobile phases with a flow rate of 0.45ml / min. MeOH is acidified with 0.1% formic acid (solvent A) and by using mili-Q water and formic acid to adjust the pH to 4 (solvent B). To avoid sudden changes in pH, ammonium formate is used as a buffer.

To quantify PGR levels, calibration curves were used to calculate analytical standards before estimate samples. This essay performed next standard values: 0.038, 0.076, 0,175, 0,305, 0,61, 1, 2, 4, 9, 19, 39, 78, 156, 312, 625, 1250 ng/ml as IDS, (*Internal Deuterated Standards*). Apart from that, 0.25 ng de BA-d_7; 0,5 ng de ABA-d_6, DHZ-d_3, IAA-d_5 y SA-d_6, 1ng de Bk-d_5 y GA_7-d_2 were referred as deuterated standards for curves.

Samples were injected into UHPLC System (1290 Infinity binary LC system, Agilent Technologies, Madrid Spain). This system was equipped with *autosampler*, thermostat, double vacuum pump and diode array detector (DAD). The UHPLC was coupled to a Triple Quadrupole (6460 Triple Quad, LC/MS equipped with ESI-Ion Source). Phytohormone levels were determined by peak area measures. Quantification limits were related to/depending on every compound ad varying from compound, from 10:1 y 3:1 rate, respectively. UHPLC conditions were set at 300 ºC for standard temperature, gas flow at 10 l/min, nebulization pressure at 20 psi and capillarity (+/-) of 4000 V. Every compound cycle was calculated by the software due to retention times in an automated way (with a media of 700 m/s). and previously determined by the previous targets and their retention time (Delatorre et al. 2017). Those area or proportions reported for endogenous hormones were obtained with the support software *Masshunter Workstation* (Agilent Technologies, Madrid).

3.2.3. Statistical analyses

Statistical analysis were performed following the procedures described by Menéndez et al. (2006c). Deviation from normality and homogeneity of variance were tested respectively with Shapiro-Wilk and Levene test. One-way ANOVA was carried out with a Tukey-HSD test to group samples. To obtain the PCA-biplot graphs and SPLS were performed with R, 3.7.2 and Past. All statistical analysis were completed in R

environment with R Studio (R Team 2016) or the free software from ***Past.uio 3.26***. (Hammer 2001). The level of significance was set at α=0.05 for all tests.

3.3. Results

The endogenous levels of the plant growth regulators analyzed, are shown in the figures 3.2a, b. Next, we are going to describe the results obtained comparing; a) the three developmental stages (filamentous, spatulate and heart-shaped) of the apogamic species *D. affinis),* and b) the heart-shaped gametophyte of both species, *D. affinis* and *D. oreades*.

At first glance, some general impressions about the amount of these compounds in which they are present in the gametophyte tissue, might be considered. The concentration of the two metabolites of brassinosteroids (castasterone and epibrassinolide) yielded above 5 ng, being especially high the levels of the gibberellin GA_4 and also with castasterone.

The auxin IAA was found in a greater quantity in *D. oreades* gametophytes than in any other sample of *D. affinis*, and in spatula respect to the filamentous and heart-shaped gametophytes. Regarding all the cytokinin analyzed, BA peaked in the filamentous of *D. affinis,* and no significant differences were found among the rest of gametophyte stages and species. tZ and RZ exhibit a similar tendency, showing low levels in *D. affinis* (<0.1 ng/ng FW) and meaningfully higher in the sexual counterpart.

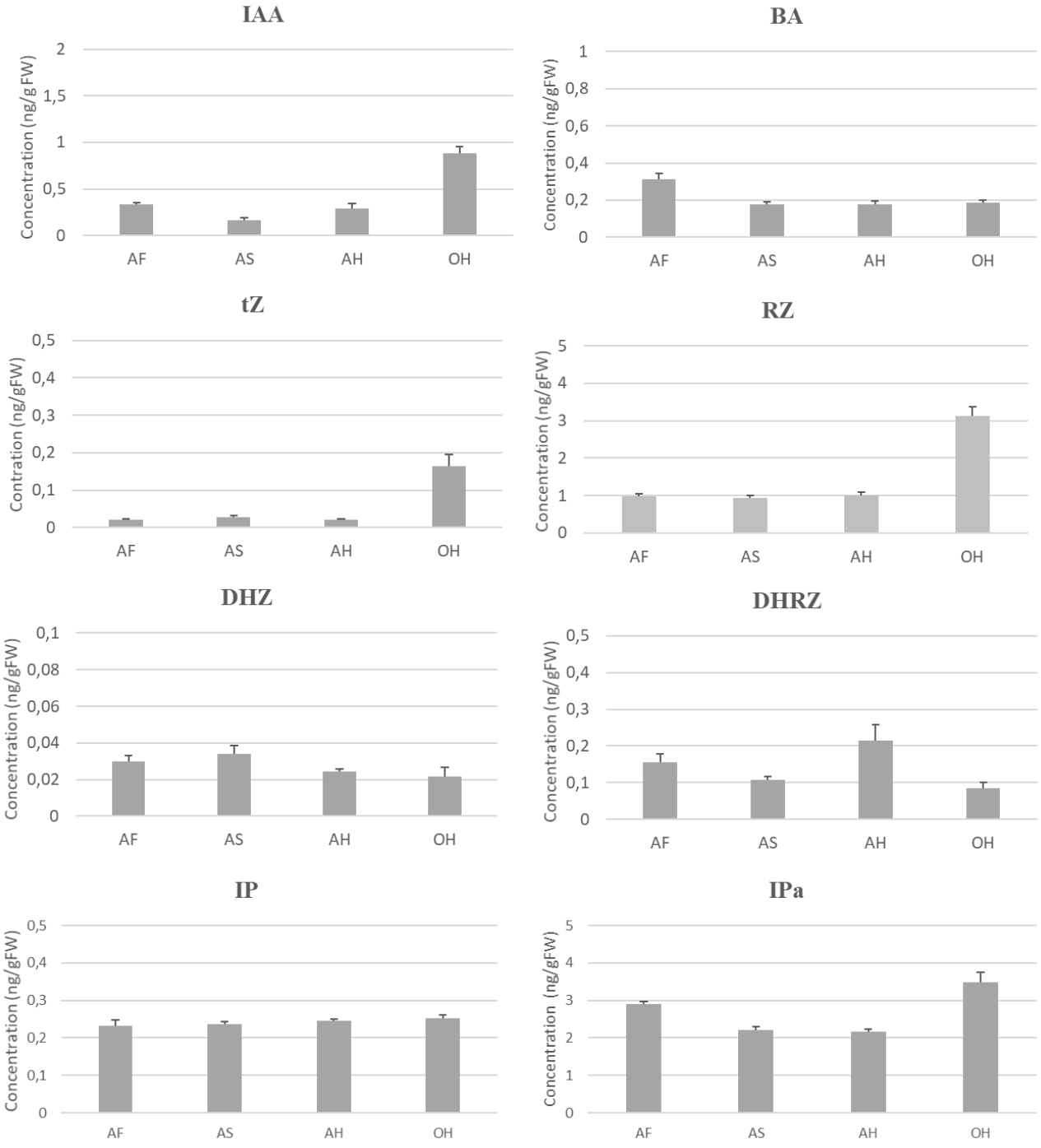

Figure 3.2.a. Endogenous content of phytohormones, in gametophyte of *Dryopteris affinis* (AF, filamentous; AS, sptula; AH, heart), and female cordate gametophytes of *Dryopteris oreades*. Data are means ± standard error.

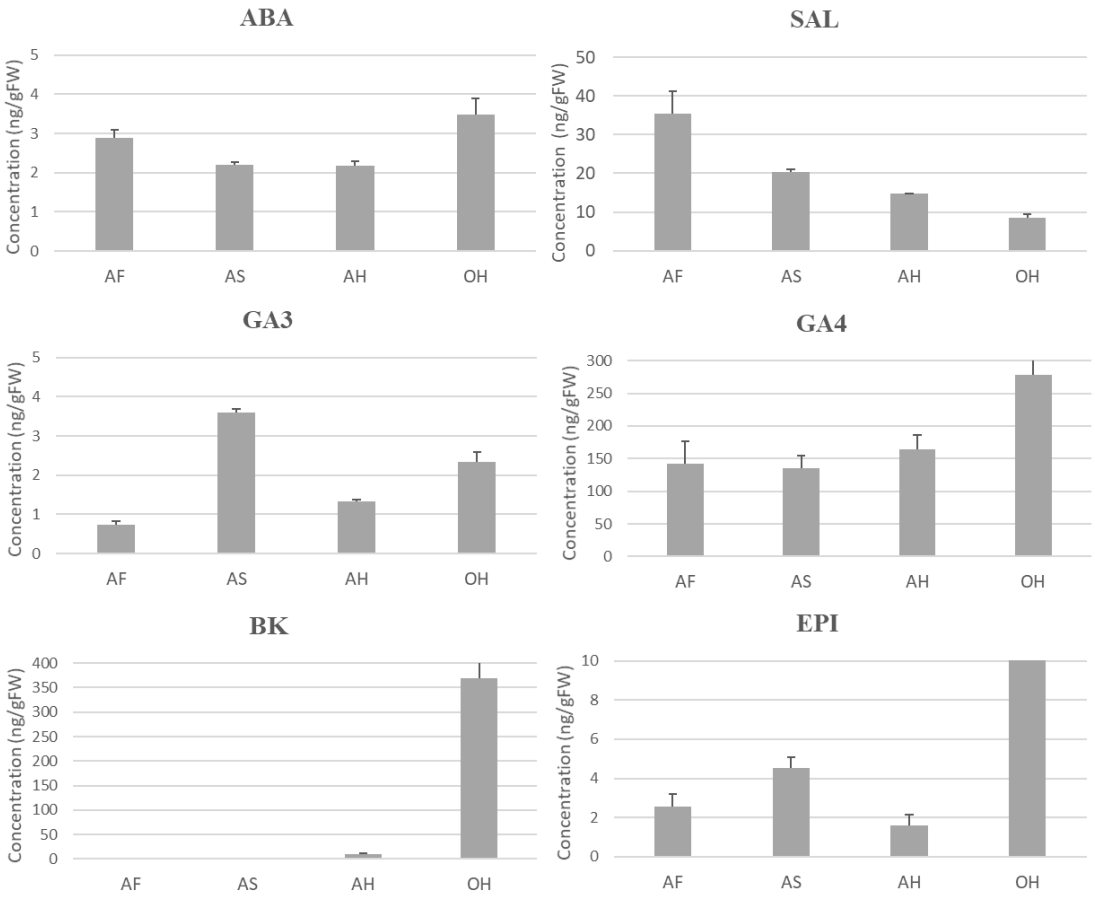

Figure 3.2.b. Endogenous content of phytohormones, in gametophytes of *Dryopteris affinis* (AF, filamentous; AS, spatula; AH, heart), and female cordate gametophytes of *Dryopteris oreades*. Data are means ± standar error. IAA=indol-3-acetic acid, BA, 6-benzylaminopurine, tZ= trans-Zeatin, RZ= Zeatin riboside (RZ), DHZ= dihydrozeatin, DHRZ= dihydrozeatin riboside, iP= isopentenyl adenine, iPA= isopentenyl adenosine, ABA=abscisic acid, SA= salicylic acid, GA_3, gibberellic acid, GA_4 = gibberellin 4, EPI= epibrassinolide and B=castosterone

No differences were found in the content of DHZ, while the levels of its riboside, RDHZ, significantly shrank in spatula gametophytes of *D. affinis* respect to heart gametophytes, dropping considerably in the sexual parent. Finally, no differences were found regarding the isoprenoid cytokinin IP, while the levels of its riboside, isopentenyladenosine, was lower in the spatula and heart shapes respect to filamentous, and also to the sexual female gametophytes of *D. oreades*.

The levels plant growth inhibitor ABA increased in the sexual prothalli of *D. oreades* and no differences were observed among the three developmental stages of *D. affinis.* Salicylic acid showed substantial differences between treatments. Levels up to 35 ng per grams of fresh weight, were detected in filamentous gametophytes of the apogamic fern *D. affinis,* falling significantly in *D. oreades*. In relation to the tested gibberellins, sharp levels of GA_4 were noticed in *D. oreades,* being also significant in the apogamous gametophytes. Aside from it, GA_3 reached a maximum in spatulate gametophytes of *D. affinis*. Finally, on one hand, the brassinosteroid castasterone was undetected from *D. affinis* filament and spatula samples, and weak in the heart ones. On the other hand, it was ten times more abundant in *D. oreades* gametophytes than *D. affinis* reaching values astonishing high. Epibrassinolide is 25 times more abundant in *D. oreades* compared to *D. affinis* (but just one samples was successful*). In addition, significant differences were noted between spatula and the two other gametophyte stages of the apogamous species.

Principal Component Analyses (PCA) was done and results revealed that one component (PC1) included the most part of the variance, splitting sexual and asexual gametophytes (Fig. 3.3). The Biplot shows the phytohormones grouping into the four analyzed samples. DHZ and DHRZ, are common to heart and spatula gametophytes, GA_3 is the hormone which differentiate both groups of samples. Filaments show variation with a great dispersion; they share differences with both spatula and heart shapes by the cytokinin BA and salicylic acid. *D. oreades*, have shown differences at most of the regulators.

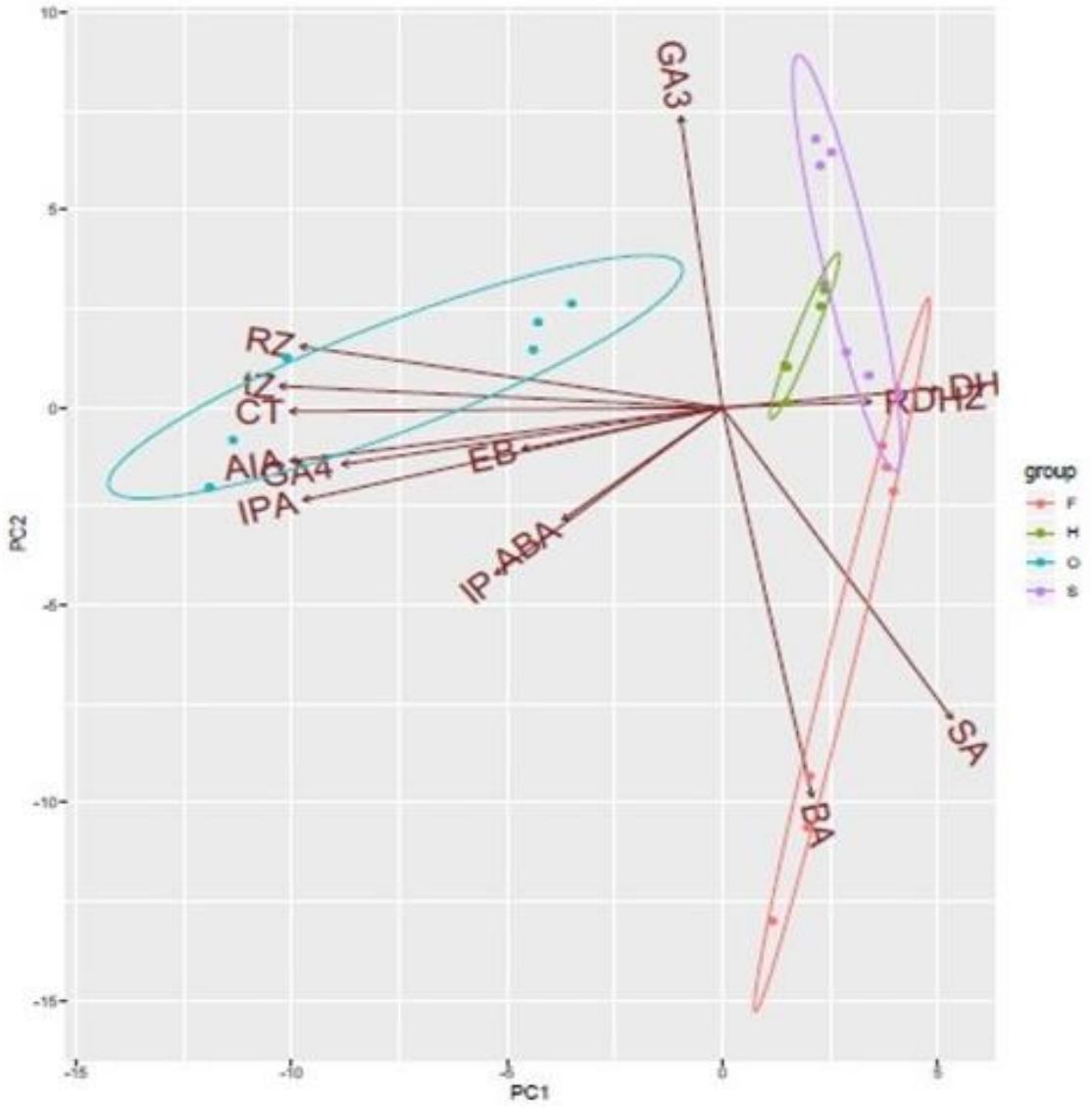

Figure 3.3. PCA biplot showing the contribution of all variables. Ellipses encircled samples from the same origin: *D. affinis* filament, spatula, heart gametophytes, and *D. oreades* heart gametophytes.

Finally, a heatmap of endogenous phytohormones is shown in the figure 3.4. which is performed on the variations of endogenous hormones and seeks to show the differences in a more visual way than a table. In addition, the heatmap allows cluster-grouping of treatments among those with the greatest similarity. That is, we can see which samples can see each other the most. Our heatmap presents results related to PCA analysis. On the one hand separated from the *D. oreades* sample from the *D. affinis*

samples, on the other hand from the sample of the great similarities in the SA and BA samples in the samples.

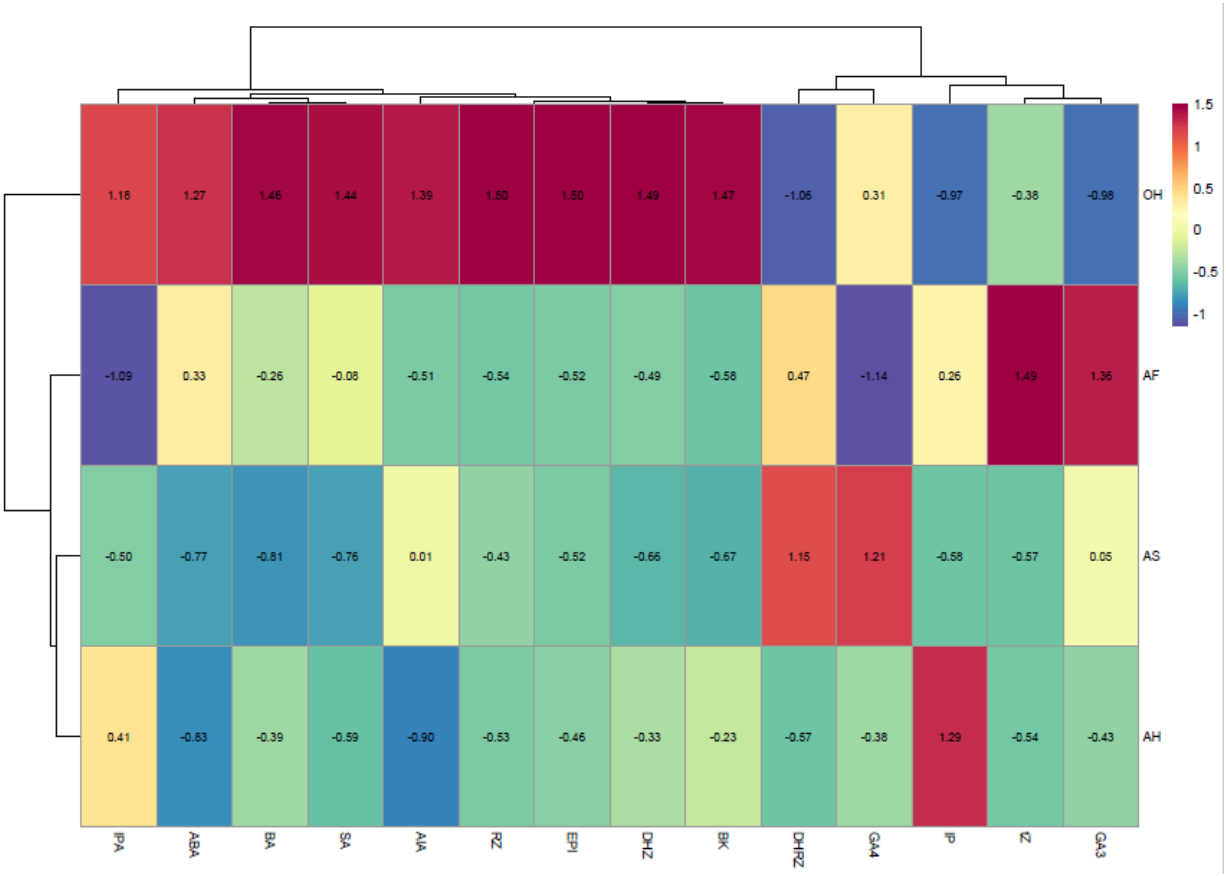

Figure 3.4. Heatmap cluster showing phytohormone endogenous content. Manhattan distance and Ward's aggregation method were used to calculate sample distances.

3.4. Discussion

The phytohormonal profiles observed in the filamentous, spatula and heart-shaped gametophytes of the apogamous fern *D. affinis*, and in the heart-shaped gametophytes of its relative *D. oreades*, were analyzed by using ultra-performance liquid chromatography-mass spectrometry (UHPLC). Certainly, small amount of information was acquired so far about the physiology and molecular events operating inside this free-leaving fern's generation. As far as our knowledge, the present study represents a first attempt to assess an ambitious number of phytohormones, in these two fern species, and which can contribute to gain insight on the role of phytohormones on the reproductive behavior in non-model species, still needing to receive more attention. By using this technique, it was

possible to detect and quantify from the same sample, up to fourteen compounds, representatives of six major families of plant growth regulators: auxins, cytokinins, gibberellins, abscisic acid, salicylic acid and brassinosteroids. No doubts, the hormonal content is usually an uneven balance partially due to the complex interactions displayed among phytohormones, sensitivity, the effects that exogenous factors might exercise, and their biological time (Bradford and Trewavas 1994), making a very difficult task to attempt inferring too many conclusions from the snapshot that represent a quantitative estimation.

The most part of approaches to the role on phytohormones on fern's development, passed for testing their effects when applied to the gametophyte or sporophyte being cultured (Fernández and Revilla 2003; Menéndez et al. 2011; Rivera et al. 2018). Nevertheless, although this kind of information could give us important acquaintance, overall from a practical point of view, it lacks the freshness supplied by the outcome derived from the quantification of the endogenous levels of the phytohormones in any tissue at any time, and which could give us clues about possible tendencies or patterns shown by these chemicals on plant development, so that we can infer possible roles on biological processes (Elmore and Whittier 1974; Cordle et al. 2007; Bai et al. 2010). As aforementioned, it is difficult to infer conclusions from the diversity of responses phytohormones can exhibit depending on concentration and species studied.

At first sight, it seems obvious that the level of the detection was not the same in all cases. In this procedure, we must to pass the phytohormones in the column in a single step instead of using different samples to obtain each phytohormone, separately. This assumption could make an increase of the losses of these compounds at some extend. Saying that, the differences noted between compounds belonging to the same chemical family, for instance GA_3 and GA_4, the two brassinosteoids castasterone and epibrassinolide, or among the analyzed cytokinins, talk about a possible role on gametophyte growth and reproduction (Fernandez et al. 1997; Šimura et al. 2018).

Next, we are going to discuss the quantitative differences found comparing firstly the three stages of apogamic gametophytes, and secondly, between sexual and asexual gametophytes. From our results, qualitative and quantitative differences were observed intra-species (filamentous, spatulate and heart-shaped gametophyte) and inter-species (hear-shaped gametophytes from the apogamous and sexual species). A different patterning in the distribution of the assayed phytohormones is identified from the PCA analyses.

a) Phytohormone profile in *D. affinis*, When we focused on the three apogamous gametophyte stages of this species, we noticed that salicylic acid, and the cytokinin BA increased at the filamentous, the gibberellin GA_3 at the spatulate-shaped, and finally a slight variation is observed of RDHZ in the cordiform or hear-shaped gametophytes. In our work, the presence of salicylic acid in higher levels in the filamentous, could be related with a stressful environment as could be the *in vitro* culture itself, especially at the beginning of the gametophyte development. We can think also of the existence of a natural response that could be superfluous once adapted to the growing conditions, decreasing in the succeeding developmental stages. Similar results were observed in the gametophyte of *D. filix-mas* when cultured *in vitro*, as reported by Kosakivska et al. (2019). Salicylic acid is a phenolic compound bearing a hydroxyl group, and influences a wide range of processes, including seedling establishment and responses to abiotic and biotic stresses, (Vlot et al. 2009). At this point, salicylic acid has been found to be induced in the moss *Physcomytrella patens* by infection with the pathogen *Botrytis cinerea* (Ponce de León et al. 2012) being one of the first features in plant-microbe interaction, already present in basal-branching algae (Pieterse et al. 2012; Hori et al. 2014). The salicylic acid response pathway is typically (but not exclusively) effective against microbial biotrophic pathogens (Glazebrook 2005). Recent studies about differences in gene expression between one and two- dimensional gametophytes of *D. affinis*, revealed to be upregulated in filamentous or one-dimensional gametophytes, genes involved in stimulus and defence (Wyder et al. 2020). Certainly, the independent gametophyte generation is a critical exploratory stage for ferns. Gametophytes, in most all cases, are more stress tolerant than sporophytes and can grow in areas where sporophytes cannot (Haufler et al. 2016). Given that, we can suggest a role of this phytohormone in the plant-microbe interaction, apart from other strategies such as the phytochemical armament, widespread in ferns and other monilophytes, as a defence mechanism of sporophyte but also gametophyte, which needs to be well protected, overall at the beginning of its development.

An important part of our research was devoted to explore the endogenous content of phytohormones in the three aforementioned stages of the apogamous gametophyte. Although very simple morphologically, the gametophyte accounts for successive developmental changes until reach maturity, and there are no doubts that the phytohormones are candidates to governing the morphological form and gender expression in leptosporangiate fern gametophytes. An early crucial event in the formation of the prothallus involves the re-orientation of a cell plate, from a transverse to a

longitudinal alignement (Racusen 2002). Filamentous comprise some few cells long, arisen by divisions in a single plane or periclinal. Then, divisions proceed in more than one plane, drawing a two-dimensional structure named spatulate, which continue evolving until defining a mature heart-shaped gametophyte, characterized by a meristematic area placed in the apical notch, broad wings separated by a midrib or cushion, of approximately equal length and width, and bearing the sexual organs archegonia and antheridia (Nayar and Kaur 1971). Undoubtedly, all these changes and inherent processes, are susceptible to be regulated by phytohormones. A possible connection between the cytokinin BA and the filamentous morphology, and between GA_3 and the spatulate shape, might derive from the results. In the first case, it has been reported that cytokinins can affect the rate and pattern of cell division, cell elongation and cell differentiation in ferns. Cytokinins added to the culture medium induced gametophyte growing in light to become shorter, widened and lacking meristem, in *B. spicant* and *Osmunda regalis* (Menéndez et al. 2006c, Greer et al. 2012). On top on it, Spiro et al. (2004) demonstrated that cytokinins can mimic in grown-dark gametophytes of *Ceratopteris*, the normally light-induced transition from filamentous to prothallial growth. Although all these findings remark a role of cytokinins in gametophyte development, their effect on light are not very comparable in the studied species.

In relation to the increase of GA_3, recorded in the spatulate gametophytes, it could be linked to the important cellular expansion happening in this stage. Gibberellins usually coordinate the process of cell division and enlargement among other biological processes, and it has been stated in some sexual fern species such as *B. spicant*, *Anemia phillitidis* and *P. aculeatum* (Fernández et al. 1997, Kaźmierczak 2003, Menéndez et al. 2006b, 2009; Kosakivska et al. 2020). In previous work done in this apogamous species, significant levels of GA_3 were also found in spatulate–stage samples of gametophytes (Menéndez et al. 2006c).

The last step in the gametophyte development is represented by the heart-shaped silhouette (Nayar and Kaur 1971). The transition from spatula to heat-shaped gametophytes implies an increase in the rate of anticlinal divisions (i.e., perpendicular to the apical surface). At the end, the formation of the apical notch characteristic of a cordate morphology takes place, and which precedes the formation of an apogamic centre, in *D. affinis* (Menéndez et al. 2006c) or female gender expression, in general, (Aderkas and Cutter 1983), and in its sexual relative, in particular. Following with the phytohormonal comparison among the apogamous stages, it has already been documented a notorious

presence of GA_4 and also GA_7 in heart-shaped gametophytes as an apogamous centre is visible. In brief, it seems that cytokinins and gibberellins regulate in some way the apogamous event, but also auxins, even though the trend is not totally correspondent with that reported before (Menéndez et al. 2006c).

b) Phytohormone profile comparison between sexual vs asexual gametophytes.

The profile of phytohormones of the sexual counterpart *D. oreades*, includes a broad range of compounds, which in some cases, as occurs with the castosterona, is almost absent in the gametophyte of the apogamous species but one of the top-detected in the sexual counterpart. Certainly, seven out of fourteen phytohomones, accumulated more in the sexual female gametophyte respect to the apogamous one, which are the auxin AIA, the cytokinins Z, RZ, IPA, the gibberellin GA_4, and the brassinosteoids epibrassinolide and its precursor castosterona, although only one sample could be analysed for the former, and this result is not statistically relevant.

Although it was not scored, we can state that the growing rate of the gametophyte of *D. oreades* is slower than most fern species cultured in our lab, and far from that exhibited by *D. affinis*. Even though the growth rate can vary among ferns, in comparison with Angiosperms, is usually slower and it has been linked to inherently slow rates of physiological processes (light and water administration among others) in this plant group (Raven 1985), and it could be the case of *D. oreades*.

As commented before, apart from the auxin IAA and the cytokinins tZ, DHZ, ZR, iP and iPA, being the levels of iP and iPA especially high, the content of gibberellin GA_4 rose in the sexual gametophytes. Cytokinins and gibberellins have been shown to influence on male and female fertility, respectively (Chailakhyan and Khrianin 1987), which has been used to manipulate those species of economic importance. In those taxa where phytohormones rather than sex-determining genes, control gender, cytokinins are associated with reproduction and femaleness (Khryanin 2002, Tanurdzic and Banks 2004). By and large, iP bases cytokinins are characteristic of mosses and ferns, being more difficult to be detected zeatin derivatives (Johri 2008). Additionally, in *D. filix-mas*, in heart-shaped gametophytes an increase in the IAA and tZ, at the formation of sexual organs archegonia and antheridia, has been reported (Kosakivska et al. 2019). These authors concluded too that GA_3 was dominant at all stages of gametophyte development, reaching the highest content during the development of both types of sexual organs. More recently, an increase of GA_3, IAA, ABA, ZR, iP and iPA in the cordiforme gametophyte of *Polystichum aculeatum* (L.) Roth, has been documented by the same authors

(Kosakivska et al. 2020), pointing out a key role of these phytohormones in the regulation of growth and development of the archegonium cushion (consisting of several cell layers necessary for the further nutrition of sporophytes) and reproductive structures.

In our species, *D. oreades*, GA_4 seems to prevail over GA_3 either on apogamic or sexual development, being especially higher in the sexual species. In *D. oreades*, the gametophyte release antheridiogen (Jiménez et al. 2008). In many fern species, antheridiogen is secreted by hermaphrodites of females gametophytes, causing their immature neighbours to develop as males as it was commented before, and it has been published that it can be converted to bioactive GA_4 once imported into fern cells (Tanaka et al. 2014). According to this model, an early-maturing prothalli in a colony, express GA biosynthetic genes, except GA3ox, thus producing a GA_9 intermediate that lacks the OH group at C3. To be effectively transmitted to the surrounding prothalli, GA_9 is modified into an antheridiogen by methyl esterification of its C6 carboxyl group before being secreted to the outside environment, probably by transporter(s) specific for antheridiogen. Secondly, antheridiogens are incorporated into neighbouring *late-maturing* prothalli in a colony, which highly express GA3ox. Imported antheridiogen is first hydrolysed to release the methyl group at C6—probably by a methyl esterase, because Lj_GA3ox1 cannot metabolize GA_9-Me into GA_4-Me —and is then metabolized by GA3ox into GA_4. Finally, GA_4 is perceived by the GID1-DELLA system with the aid of GID2. Activation of the GA signalling pathway causes specific expression to induce and suppress antheridia and archegonia formation, respectively, to maintain an appropriate population of outcrossing male and female prothalli in the colony.

In our analyses, there is also a noticeable increase of ABA in the sexual gametophytes. The only function clearly associated with ABA action in ferns involves sex determination of the free-living, haploid gametophyte generation in *C. richardii* (Banks et al. 1993) and it has been suggested recently that the origins of the core ABA signaling pathway in seed plants may lie in the sexual differentiation of ferns (McAdam et al. 2016). Experiments with *C. richardii* demonstrate that exogenous ABA can completely blocks the sex-determining effect of antheridiogen, depending on the concentration. At this point, that high levels could completely suppress the development of male gametangia but lower levels could partially inhibit the formation of antheridia, without overwhelming the development of the meristem and subsequent formation of archegonia (Hickok 1983). In female gametophytes of *D.oreades*, a possible connection of ABA with its antheridiogen-release capacity, could be to protect themselves against

the activity of these pherohormone-like compounds, preserving the female condition. Further research would be required to test this hypothesis.

On the subject of the active brassinosteroids analysed, both epibrassinolide and its precursor castasterone were detected (Nomura et al. 2005). Many processes have been linked with brassinosteroids function such as cell elongation, cell division, reproductive and vascular development, stress responses, or senescence but information about its role on ferns is scarce (Gómez-Garay et al. 2018). Interestingly, the amount of castasterone spotted were high, pointing at some a possible role of brassinosteroids on sexual development of gametophytes in *D. oreades*. Castoterone compound was identified in most of the ferns studied by Yokota et al. (2017) but not brassinolide. The levels of castasterone in ferns leaves are as low as 25 per grams of fresh weight (Yokota et al. 2017). In general, brassinosteroids are present in plants at very low concentrations but pollen and immature seeds, which are the richest sources (Yokota and Takahashi 1986, Clouse 2002). Regarding the consequence of exogenous brassinosteroids on gametophyte development, in concrete epibrassinolide, it has been reported a species-dependent effect, ranging from the promotion of the development velocity in *Polysticum lonchitis* (which would aid this species in their reproductive role under adverse environmental conditions) to the independence in *Pteridium aquilinum* and *Pteris vittata* (which, in general, live under less hard environmental conditions) (Gómez-Garay et al. 2018). Further research must be done in order to elucidate in detail the relationship between brassinosteroids and gametophyte reproduction as well as a possible crosstalk with auxins and gibberellins, among other phytohormones.

3.5. Conclusions

Our results indicate that phytohormone content vary either among the three successive gametophyte stages of the apogamous fern *D. affinis*, and between heart-shaped apogamous gametophytes and the sexual counterpart of *D. oreades*. In the former, it was noticed that the cytokinin BA and salicylic acid, increased at the filamentous, the gibberellin GA_3 at the spatulate, and finally a slight variation is observed of RDHZ in the cordiforme gametophytes. In the latter, up to seven out of fourteen phytohormones, accumulated more in the sexual (female) gametophyte, respect to the apogamic one, exhibiting the auxin IAA, the cytokinins Z, RZ, IPA, the active gibberellin GA_4, and the active brassinosteoids castasterone a more significant presence.

3.6. References

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Wyder S, Rivera A, Valdés AE, et al (2020) Plant Physiology and Biochemistry Differential gene expression profiling of one- and two-dimensional apogamous gametophytes of the fern *Dryopteris affinis* ssp . *affinis*. Plant Physiol Biochem 148:302–311. https://doi.org/10.1016/j.plaphy.2020.01.021

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Chapter 4. Differential Gene Expression Profiling of One- and Two-dimensional Apogamous Gametophytes of the Fern *Dryopteris affinis* ssp. *affinis*[1]

Abstract

Apomixis was originally defined as the replacement of sexual reproduction by an asexual process that does not involve fertilization but, in angiosperms, it is often used in the more restricted sense of asexual reproduction through seeds. In ferns, apomixis combines the production of unreduced spores (diplospory) and the formation of sporophytes from somatic cells of the prothallium (apogamy). The genes that control the onset of apogamy in ferns are largely unknown. In this study we describe the gametophyte transcriptome of the apogamous fern *Dryopteris affinis* ssp. *affinis* using an RNA-Seq approach to compare the gene expression profiles of one- and two-dimensional gametophytes, the latter containing apogamic centers. After collapsing highly similar *de novo* transcripts, we obtained 166,191 unigenes, of which 30% could be annotated using public databases. Multiple quality metrics indicate a good quality of the *de novo* transcriptome with a low level of fragmentation. Our data show a total of 10,679 genes (6% of all genes) to be differentially expressed between gametophytes of filamentous (one-dimensional) and *prothallial* (two-dimensional) architecture. 6,110 genes were upregulated in two-dimensional relative to one-dimensional gametophytes, some of which are implicated in the regulation of meristem growth, auxin signaling, reproduction, and sucrose metabolism. 4,570 genes were down-regulated in two-dimensional versus one-dimensional gametophytes, which are enriched in stimulus and defense genes as well as genes involved in epigenetic gene regulation and ubiquitin degradation. Our results provide insights into free-living gametophyte development, focusing on the filamentous-to-prothallus growth transition, and provide a useful resource for further investigations of asexual reproduction.

[1] Stefan Wyder, Alejandro Rivera, Ana E. Valdés, María Jesús Cañal, Valeria Gagliardini, Helena Fernández, Ueli Grossniklaus. Differential gene expression profiling of one- and two-dimensional apogamous gametophytes of the fern *Dryopteris affinis* ssp. *affinis* (2017). Plant Physiology and Biochemistry.

4.1. Introduction

Living organisms explore distinct strategies to secure reproduction. Angiosperms may pursue asexual reproduction through seeds, referred to as apomixis, bypassing meiosis and fertilization (Grossniklaus U et al. 2001; Koltunow and Grossniklaus 2003; Ozias-Akins 2006). An apomictic embryo in seed plants may result from: i) adventitious embryony when an asexual embryo forms from sporophytic nucellar or integumental cells adjacent to the embryo sac; ii) by diplospory, where the megaspore mother cell (MMC) either fails to initiate or complete meiosis, resulting in an unreduced embryo sac containing an egg-like cell that proceeds to form an embryo without fertilization (parthenogenesis); iii) by apospory, where one or more sporophytic cells in close proximity to the MMC differentiate into aposporous initial cells that give rise to an unreduced embryo sac containing an egg-like cell that undergoes parthenogenesis (Koltunow and Grossniklaus 2003; Tucker and Koltunow 2009). The shift to apomixis has been proposed as a possible mechanism to escape from hybrid sterility and to stabilise polyploid hybrids (Hojsgaard et al. 2014).In angiosperms, apomixis usually leads to the fixation of the genotype and thus clonal progeny, regarded as a highly desirable trait in modern agriculture (Grossniklaus et al. 1998a; Barcaccia and Albertini 2013).

The frequency of apomixis is much higher in ferns than in any other major plant group, possibly due to repeated and independent evolution. It is particularly prevalent in the largest Leptosporangiae families: Dryopteridaceae, Polypodiaceae, Pteridaceae, and Aspleniaceae (Huang et al. 2006, 2011; Lu et al. 2006; Martínez 2010; Wang et al. 2011; Dyer et al. 2012; Guo and Liu 2013; Chao et al. 2015). In ferns, the production of spores and sexual gametes is partitioned into two independent organisms, the free-living gametophytes and sporophytes. Therefore, apomixis in ferns includes apogamy (the formation of sporophytes from somatic cells of the prothallium), and agamospory (the formation of unreduced spores) that, in some way, is equivalent to diplospory in angiosperms (Dopp 1932; Manton 1950; Braithwaite 1964; Lovis 1978; Gastony and Windham 1989). Ferns are particularly prone to apogamous reproduction (Liu et al. 2012; Grusz 2016), which is often obligate due to non-functional archegonia or antheridia, making sexual reproduction impossible (Smith 1979). However, there are also some examples of facultative apogamy, such as in the *Asplenium aff. Hallbergii* specimen RD90 (Dyer et al. 2012) and the apomictic complex *Dryopteris affinis* agg (Ekrt and Koutecky 2016), in which both reduced sexual and unreduced apomictic spores can be

produced by a single individual and even within a single sporangium. It is also possible to induce apogamy *in vitro* by modifying external factors, such as water supply and light levels(Lang 1898; Steil 1939, 1951; Duncan 1941), exogenous sugars (Whittier DP and Steeves 1960; Whittier and Steeves 1962; Whittier 1964, 1975; Elmore and Whittier 1975; Aderkas 1984; Cordle et al. 2007), or growth regulators (Whittier 1966; Elmore and Whittier 1974). This facilitates the use of ferns as model organisms to study apogamy.

Molecular analyses to understand the basis of apomixis have been conducted in model species looking for mutants displaying aspects of apomixis (diplospory, apospory, parthenogenesis) (Koltunow and Grossniklaus 2003; Tucker and Koltunow 2009). Most studies followed the hypothesis that apomixis is controlled by proteins that normally function in sexual reproduction (Grossniklaus 2003; Köhler et al. 2003; Koltunow and Grossniklaus 2003; Albertini et al. 2004; Bicknell and Koltunow 2004; Sharbel et al. 2010; Hojsgaard et al. 2014). The induction of sporophyte development through the loss of *Polycomb* Repressive Complex 2 (PCR2) components in mosses and of endosperm proliferation in angiosperms, indicates that certain aspects of apomixis in seed plants and apogamy in mosses may occur through the deployment of overlapping sets of genes(Grossniklaus et al. 1998b; Luo et al. 1999; Ohad et al. 1999; Köhler et al. 2003; Okano et al. 2009; Cordle et al. 2010, 2012). Hence, the study of asexual pathways in ferns might contribute to our current understanding of apomixis in seed plants.

In ferns, the haploid gametophyte and the diploid sporophyte generations are free-living, offering a unique opportunity to easily study genes differentially expressed in the two generations (Barker and Wolf 2010; Li et al. 2018; Plackett et al. 2018; Sigel et al. 2018). However, there are only a few reports on global analyses of gene or protein expression during fern development (Salmi et al. 2005; Der et al. 2011; Cordle et al. 2012; Valledor et al. 2014; Aya et al. 2015; de Vries et al. 2015; Grossmann et al. 2017).Up to now, genes involved in apogamy commitment have remained elusive, except for the *Physcomitrella patens* orthologues of *Arabidopsis thaliana CURLY LEAF (PpCLF)* and *FERTILIZATION INDEPENDENT ENDOSPERM (PpFIE).* Both encode components of the PRC2, which acts as transcriptional repressor of target genes through histone modification(Ohad et al. 1999; Mosquna et al. 2009). In the fern *Ceratopteris richardii*, substractive hybridization identified 306 unique sequences with increased expression during apogamy commitment (Cordle et al. 2012), and an *AINTEGUMENTA* fern orthologue, which emulates *BABY BOOM*-induced somatic embryogenesis in *A. thaliana*

(Boutilier et al. 2002) by promoting apogamy in *C. richardii*, has also been reported (Bui et al. 2017). We have recently performed a proteogenomic analysis in the apogamic gametophyte of *Dryopteris affinis* (Lowe) Fraser-Jenkins ssp. *affinis*, which may have identified proteins with a potential role in apogamy (Grossmann et al. 2017).

It is known that commitment to apogamy in *C. richardii* occurs when the gametophytes become sexually mature(Cordle et al. 2007). Here, we use two stages - one- and two-dimensional - of gametophyte development in *D. affinis* ssp. *affinis* for transcriptome analyses because the apogamic center is only produced at the two-dimensional stage in this species (Fernandez et al. 1996; Menéndez et al. 2006). In the absence of a genome sequence, an RNA-Seq based *de novo* transcriptome provides a sensitive and accurate tool for transcriptome-wide expression profiling. Hence, the goal of the present study is to compare the transcriptome profiles between one- and two-dimensional gametophytes in the apogamous fern *D. affinis* ssp. *affinis* by using an RNA-Seq approach. Our results show that genes involved in reproduction, regulation of meristem growth, sucrose metabolism, and auxin signalling were upregulated in two-dimensional gametophytes, while genes involved in the response to stimuli and defence were downregulated.

4.2. Material and methods

4.2.1. Plant material and growth conditions

Spores from *D. affinis* ssp. *affinis* fronds coming from a collection of sporophytes growing *ex situ* in the forest of Turón (Asturias, Spain) 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 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 Murashige and Skoog (MS) medium(Murashige and Skoog 1962), supplemented with 2% sucrose (w/v) at pH 5.7. Gametophytes at three developmental stages: filamentous (one-dimensional), spatula and heart (two-dimensional), the latter with evolving apogamic centre, were collected to carry out molecular analyses. Cultures of filamentous gametophytes were obtained by maintaining a high density of spores in liquid cultures, placed on a gyratory shaker (75 rpm) for 50 days. Under this condition, gametophytes remained filamentous and did not initiate two-dimensional growth. Cultures of spatula and heart stage gametophytes, derived from

spores, were kept in Petri dishes with 25 mL of MS medium containing 2% sucrose (w/v) and 0.7% agar at pH 5.7 for 65 days. All cultures were maintained at 25 ºC under cool-white fluorescent light (40 μmol$m^{-2}s^{-1}$) with a 16 h light, 8 h dark photoperiod.

4.2.2. RNA extraction and sequencing

For RNA extraction, 100 mg of fresh plant material was weighed, immediately frozen in liquid nitrogen, and kept at −80 ºC until use. Three biological replicates of each one- and two- dimensional gametophytes were used for RNA-Seq. Gametophytes at specific stages were homogenized with a Silamat S5 shaker (Ivoclar Vivadent, Schaan, Liechtenstein) twice for 10 s and 5 s, respectively. Total RNA was isolated using the SpectrumTM Plant Total RNA kit (Sigma-Aldrich, Buchs, Switzerland). After DNA elimination with the TURBO DNA-free kit (Life Technologies, Carlsbad, CA), the quality of the RNA was checked using the Bioanalyser Agilent RNA 6000 Pico Kit (Agilent Technologies, Waldbronn, Germany).

Sequencing libraries were prepared using the TruSeq RNA Sample Prep Kit v2, which were sequenced on Illumina HiSeq 2000. The Transcriptome Shotgun Assembly project is available in the European Nucleotide Archive (ENA, http://www.ebi.ac.uk/ena) under the accession number PRJEB18522. The *de novo* transcriptome assembly in fasta format as well as the transcriptome annotation have been deposited in the Zenodo (www.zenodo.org) research data repository (https://doi.org/10.5281/zenodo.1040330).

4.2.3. *De novo* transcriptome improvement and annotation

Highly similar contigs assembled by Trinity were further collapsed using Corset 0.93 (Davidson and Oshlack 2014) with a distance threshold of 0.3, resulting in 166,191 transcript clusters (with a minimal contig size of 201 nt). We used BUSCO v2.0.1 with the Embryophyta odb9 dataset to assess the completeness of the transcriptome. From each cluster, the longest transcript was selected as a representative protein and subjected to BLASTX against protein databases Swiss-Prot Plants, TrEMBL Plants (UniProt Knowledgeable Release 2014_08 of 03-Sep-2014), as well as Azolla v1.1 and Salvinia v1.2 transcript FASTA sequences (both high- and low-confidence) from ftp://ftp.fernbase.org/. The annotations of the best BLASTX hit were used to assign functional annotations to the transcript clusters. In addition, the longest transcript per cluster was also annotated with GO (Gene Ontology) terms and Interpro protein domains.

A Gene Ontology Summary for the transcriptome was produced using the Agbase AutoSlim (McCarthy et al. 2011) webtool (http://www.agbase.msstate.edu/) with the Plant GOSlim set. Assignment to transcription factor families was done according to PlantTFDB (Jin et al. 2014) using the family assignment rules described on http://planttfdb. cbi.pku.edu.cn/help_famschema.php.

4.2.4. Differential gene expression

Reads were aligned to the reference transcriptome using STAR mapper 2.2.0c (Dobin et al. 2013)with default settings, and transcript abundances were estimated using Corset 0.93. Differential gene expression analyses were carried out using edgeR v3.4.2 (Robinson et al. 2009) with Corset providing transcript counts (at a stringent FDR cut-off of 10^{-5}). We performed Gene Ontology Enrichment Analyses using the topGO package 2.12.0 (Alexa et al. 2006) with the elim algorithm for differentially expressed genes at a cut-off of 5% FDR, and summarized the results using Revigo(Supek et al. 2011).

4.3. Results

4.3.1. Characterization of de novo transcriptome assembly

A total of six libraries, three each from one-dimensional and two-dimensional gametophytes, were subjected to Illumina HiSeq 2000 sequencing. As no genome sequence is available for *D. affinis* ssp. *affinis*, we assembled the combined 470 million paired-end RNA-Seq reads (2x100 bp) using Trinity (Grossmann et al. 2017) into a total of 436,707 contigs with an average median length of 721 nucleotides (nt), with half of the total assembly length in contigs > 1.5 kb (N50 = 1,460 nt). An overview of the sequencing and *de novo* transcriptome is given in Table 1. To reduce redundancy, highly similar contigs were then further collapsed into 166,191 contigs using Corset (Fig. 4.1). Half of the total assembly length belong to contigs longer than 2 kb (N50 = 1,981 nt). 72,640 contigs (43.7%) were at least 500 nt in length, and 39,560 (23.8%) were longer than 1,000 nt.

	Dryopteris affinis ssp. *affinis*
Total number of read pairs (2x100)	469,577,165
GC percentage	47
Step-wise assembly	
Trinity	
Number of contigs	436,707
Length of all contigs (nt)	507,144,609
Average length of contigs (nt)	721
N50 (nt)	1,460
Clusters	
Number of clusters	166,191
Average number of contigs per cluster (range)	1 (1-57)
Length of all clusters (nt)	136,089,984
Average length of longest transcript per cluster (nt)	433
N50 (nt)	1,981
Clusters with BLAST matches (% of total clusters)	54,925 (33.0)
Clusters with InterPro annotation (% of total clusters)	42,576 (25.6)

Table 1. Overview of transcriptome sequencing and assembly.

4.3.2. Completeness of the assembly

To assess the completeness of the transcriptome assembly, we used two different quality metrics: RNA-Seq read representation of the assembly and BUSCO benchmarking. Over 81% of the RNA-Seq reads were mappable to assembled transcripts. We also compared the set of 166,191 contigs to a set of 1,440 highly conserved plant single-copy orthologues. BUSCO benchmarking (Simao et al. 2015) showed that 71% of the single-copy orthologues could be identified in our gametophyte transcriptome (Table S1), only slightly less than with the full transcriptome before collapsing, which identified 75% of the BUSCO orthologues. Most of the identified orthologues had sequence similarity over their entire length, with only 4.6% being fragmented. Collapsing reduced the proportion of duplicated single-copy orthologues from 69% to 19% (Table S1). Schematic alignment of three selected genes with their closest BLASTX hit reveals

similar transcript structures and sequence conservation in the central portion of the transcripts (Figure S1). In summary, the lengths of assembled transcripts and their annotation results indicate a good quality of the gametophyte transcriptome.

4.3.3. Functional annotation

BLASTX searches were conducted between the predicted protein sequences and several public databases, taking the longest gene per cluster as a cluster representative (Fig.4.1). A total of 52,566 genes (of 166,191, *i.e.* 32%) had a significant hit (E-Value < 1E-5) to a known protein, and a total of 42,576 identified genes (26%) could be annotated with *InterPro* protein domains. To gain information about possible functions of these proteins, we assigned them to Gene Ontology (GO) functional categories ("biological process", "molecular function", "cellular component") based on significant BLAST hits of the transcripts. Figure 4.2 shows the GO classification of the *D. affinis* ssp. *affinis* transcriptome generated from one-dimensional and two-dimensional gametophytes. Under "biological process", the most frequently assigned GO categories were "catalytic activity", "nucleic acid binding activity", and "transferase activity". Under "molecular function" one GO category dominated, namely "cellular metabolic process", and under "cellular component", we mostly found proteins localized in "organelle", "membrane", and "intracellular".

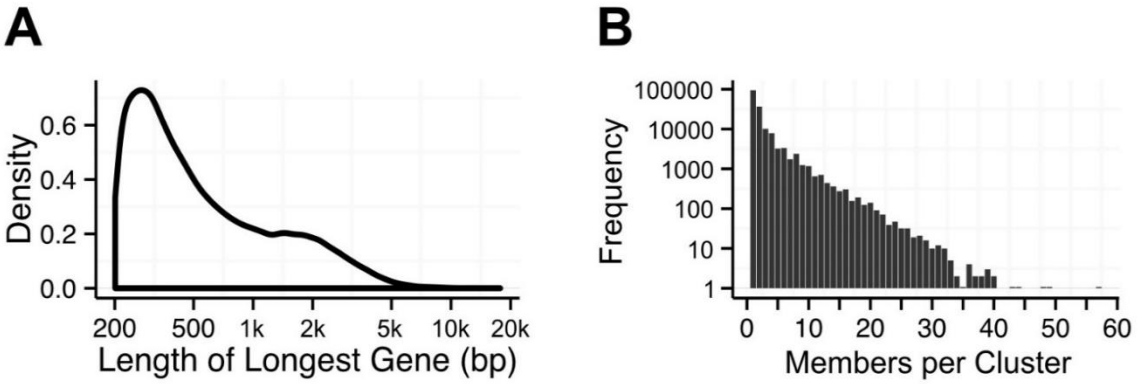

Fig. 4.1. Characteristics of the *Dryopteris affinis* spp. *affinis* gametophyte *de novo* transcriptome. a) Length distribution of genes (longest gene per Corset cluster). b) Number of Trinity-assembled contigs per Corset cluster.

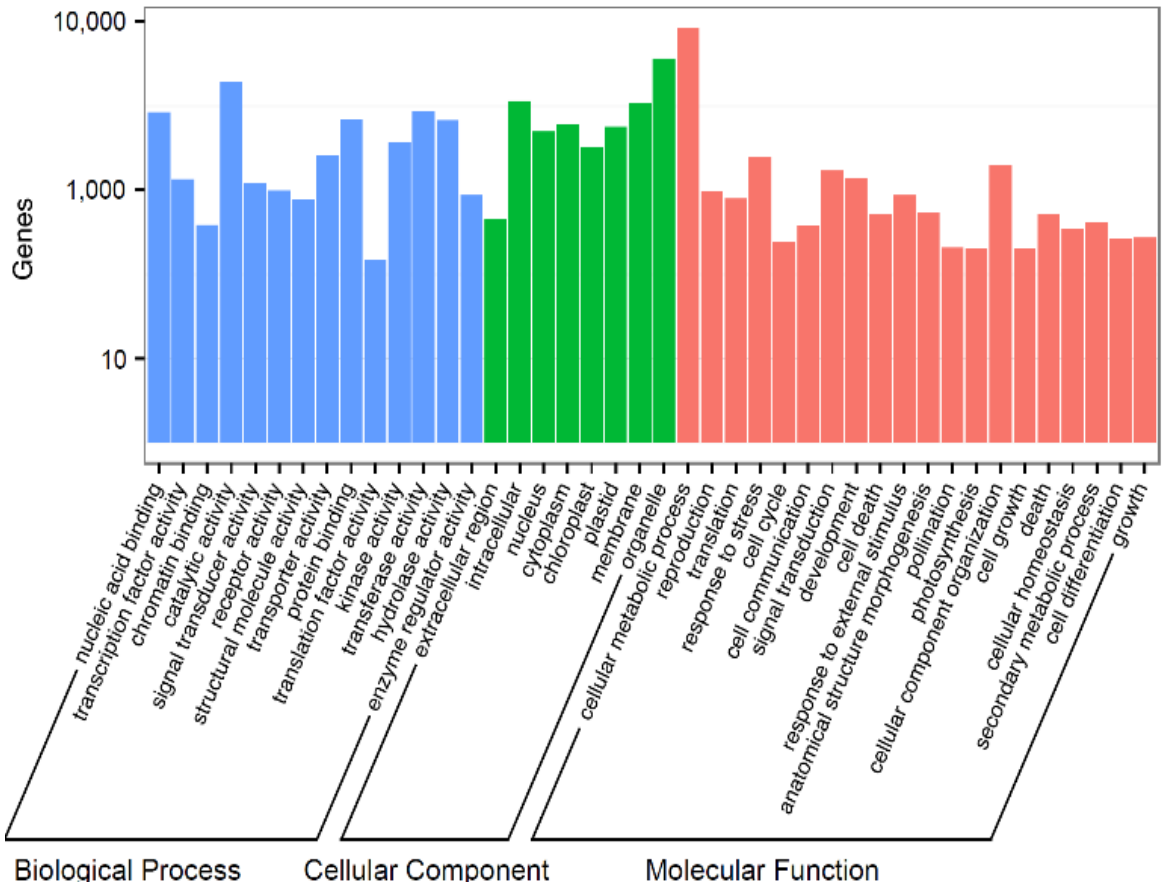

Figure. 4.2. Gene Ontology annotation of assembled transcripts.

4.3.4. Analysis of differential gene expression

Next, we compared RNA-Seq profiles between two development stages: one-dimensional (filamentous) and two-dimensional (spatula- and heart-shaped gametophytes with signs of apogamy) to identify potential candidate genes involved in apogamic processes. RNA-Seq was performed on three biological replicates for each stage. A total of 470 million paired-end reads (2x100 bp) was mapped to the *D. affinis* ssp. *affinis de novo* gametophyte transcriptome. Among the total 166,191 genes, 10,679 genes were significantly differentially expressed, including 6,109 up-regulated (3.7% of all genes) and 4,570 down-regulated (2.7%) genes in two-dimensional relative to one-dimensional gametophytes (Fig. 4.3, Table S2). Next, we analyzed whether differentially regulated transcripts were enriched in specific biological processes (Fig. 4.4, Table S3). The following GO categories were significantly enriched among genes upregulated at two-dimensional stages: "cell wall chitin metabolism", "lignin biosynthesis", and "regulation of meristem growth". Functional GO categories downregulated at two-dimensional relative to one-dimensional stages included “post-translational protein modification”, “calcium ion transport and signaling”, “response to stimulus”, and “oxidation-reduction process”. Some of the genes belonging to these GO categories are included in Table 2. Enrichment analysis of KEGG pathways revealed "starch and sucrose metabolism" and "pentose and glucuronate interconversions" as significantly upregulated (FDR<5%) in two-dimensional compared to one-dimensional gametophytes (Table S4). Among strongly regulated genes (FDR<1E-5), we identified 36 and 55 genes upregulated in two- and one-dimensional gametophytes, respectively (Table S5). Most of them were genes encoding transcription factors of the GRAS, MYB related (MYB superfamily), and MIKC (MADS) families. Additionally, SBP family transcription factors were upregulated in two-dimensional gametophytes.

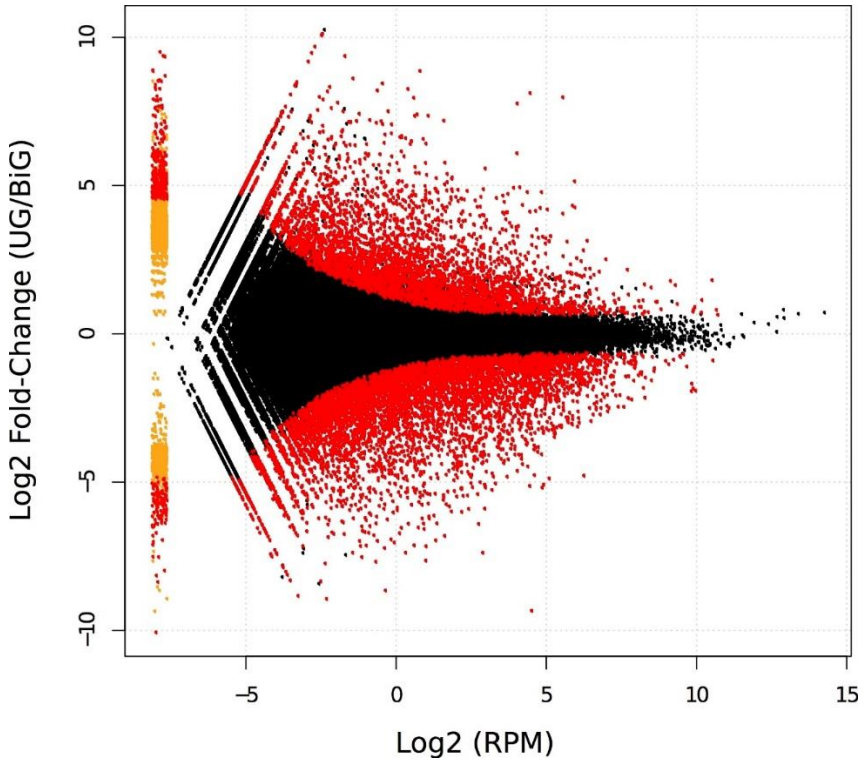

Fig. 4.3. Differentially expressed genes. The scatterplot shows changes in transcript abundance (mean of three biological replicates) based on its average expression level (in reads per million). Each dot represents an individual gene cluster. Genes showing a significant differential expression (FDR<5%) are highlighted in red.

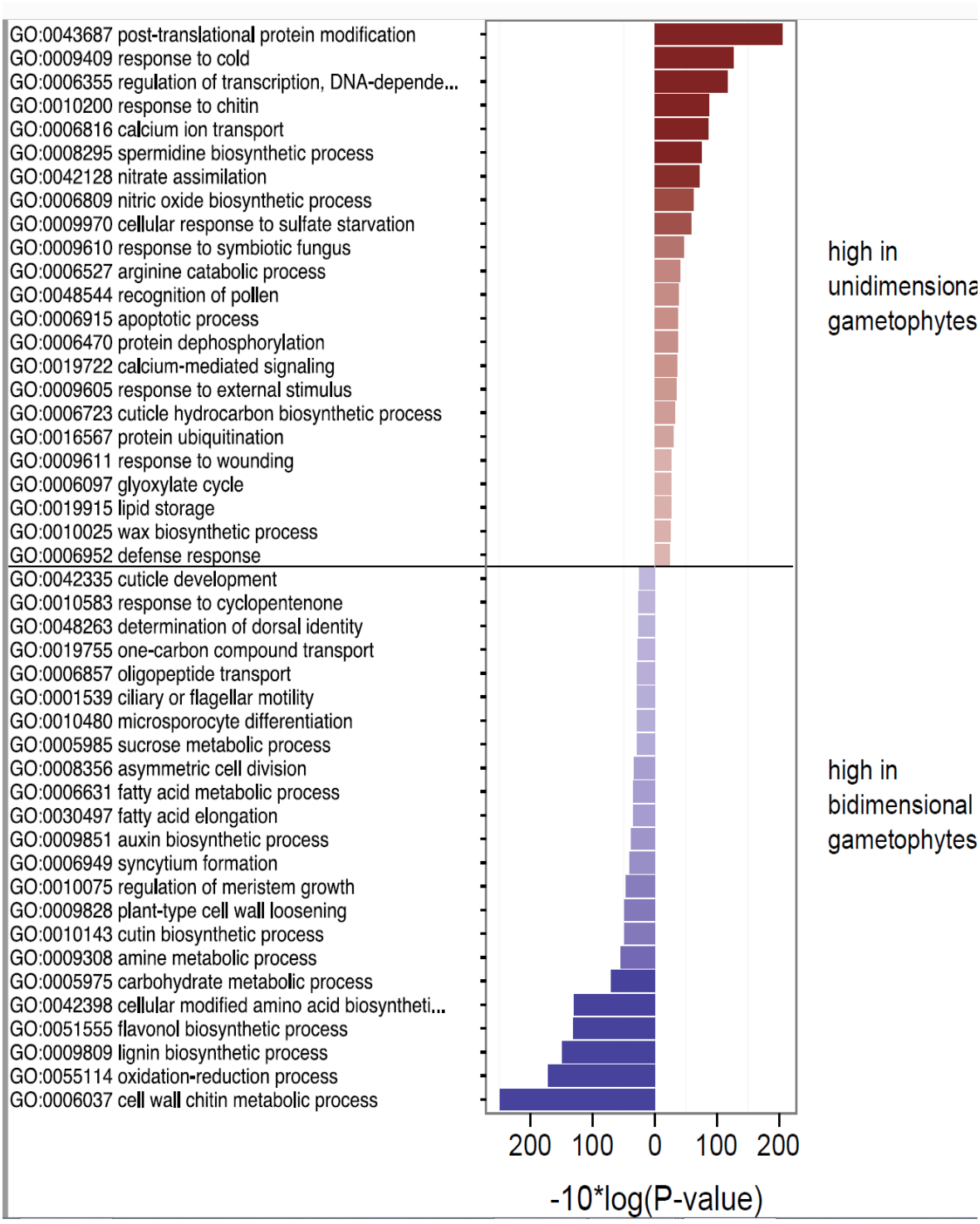

Fig. 4. 4. Gene Ontology analysis of differentially expressed genes. In red, GOs enriched in one-dimensional gametophytes. In blue, GOs enriched in two-dimensional gametophytes. Categories are sorted according to their enrichment p-value.

Category	Putative gene name	Cluster ID	Longest Transcript in Cluster	Fold change	FDR	Best blast hit accession	Score (tblastx)
Meristem growth	Shoot meristemless	Cluster-817.0	358230-40	107.6	4.0E-06	Azfi_s2342.g110698	146
Meristem growth	NAC family (no apical meristem (NAM)) proteins	Cluster-21805.1	9916-755	4.8	8.9E-08	Azfi_s0518.g075208	240
Auxin biosynthesis & signalling	Auxin responsive SAUR protein	Cluster-24741.0	40948-398	7.6	1.4E-63	Azfi_s0065.g035889	105
Auxin biosynthesis & signalling	Auxin response factor 1-like	Cluster-28966.1	186510-150	6.2	4.0E-68	Sacu_v1.1_s0003.g001668	358
Auxin biosynthesis & signalling	flavin-containing monooxygenase YUCCA8-like	Cluster-80575.0	69518-303	10.1	3.5E-07	Azfi_s0487.g073447	102
Phytohormone signalling	gibberellin-regulated GASA protein	Cluster-77869.1	337623-49	74.0	2.4E-64	Azfi_s0066.g036114	110
Carbohydrate metabolism		Cluster-67651.1	257601-95	2.5	8.8E-17	Sacu_v1.1_s0011.g005080	508
Carbohydrate metabolism		Cluster-82619.0	235152-110	1.3	0.11	Sacu_v1.1_s0100.g019648	66.1
Epigenetic regulation	multicopy suppressor of Ira1 (MSI1)	Cluster-92583.0	23371-518	1.0	0.94	Azfi_s2626.g112452	543
Epigenetic regulation	enhancer of zeste (E/Z)	Cluster-89328.0	338354-49	1.0	0.97	Sacu_v1.1_s0014.g006147	380
Epigenetic regulation	EPL1 (Enhancer of polycomb-like proteins)	Cluster-63659.1	318133-59	2.4	0.46	Azfi_s0006.g009963	216
Epigenetic regulation	Argonaute/Dicer	Cluster-48318.0	93133-259	2.1	2.8E-13	Azfi_s0001.g000070	950
Epigenetic regulation	Argonaute/Dicer	Cluster-81082.2	188176-148	2.4	4.7E-03	Azfi_s0460.g072055	53.8
Epigenetic regulation	Argonaute/Dicer	Cluster-27625.0	107482-240	2.0	0.03	Azfi_s0460.g072055	111

Epigenetic regulation	Argonaute/Dicer	Cluster-52852.0	263002-91	2.6	0.10	Azfi_s0045.g029842	106
Ubiquitin-mediated histone degradation		Cluster-61090.0	110282-236	1.0	1.00	Sacu_v1.1_s0058.g014907	274
Ubiquitin-mediated histone degradation		Cluster-96173.2	332406-52	1.0	1.00	Sacu_v1.1_s0130.g022005	327
Ubiquitin-mediated histone degradation		Cluster-2275.0	213826-126	1.0	0.98	Sacu_v1.1_s0202.g025630	101
Ubiquitin-mediated histone degradation	SKP1	Cluster-61090.0	110282-236	0.8	1.00	Sacu_v1.1_s0058.g014907	274
Reproduction	FRIGIDA-like proteins	Cluster-32131.3	30934-456	9.1	8.0E-30		
Calcium ion transport and signaling		Cluster-14514.0	61387-323	5.3	1.2E-13	Sacu_v1.1_s0065.g015990	277
Calcium ion transport and signaling		Cluster-29138.1	400773-23	82.4	1.8E-115	Sacu_v1.1_s0110.g020615	134
Calcium ion transport and signaling		Cluster-66718.0	281135-79	6.4	3.7E-48	Azfi_s0027.g023740	749
Calcium ion transport and signaling		Cluster-64285.0	333011-51	5.3	1.2E-20	Sacu_v1.1_s0013.g005844	151
Calcium ion transport and signaling	calcium-dependent protein kinase 17 (CPK17-2_1)	Cluster-92501.1	288523-75	2.4	2.3E-03	Azfi_s0038.g026325	972
Metabolism of Polyamines		Cluster-21798.0	116559-229	1.2	1.00	Azfi_s0010.g012420	350
Metabolism of Polyamines		Cluster-13196.0	352469-43	1.0	1.00		
Metabolism of Polyamines		Cluster-84417.1	248231-101	1.0	1.00	Sacu_v1.1_s0039.g012245	898

Metabolism of Polyamines		Cluster-95494.0	289468-74	3.5	8.21E-12	Sacu_v1.1_s0021.g008301	259
Metabolism of Polyamines	arginine decarboxylase (ADC)	Cluster-46196.0	262720-91	9.1	9.56E-46	Sacu_v1.1_s0020.g007991	470
Metabolism of Polyamines		Cluster-55586.0	273728-84	1.0	1.00	Sacu_v1.1_s0004.g002390	61.1
Metabolism of Polyamines		Cluster-19913.1	44517-381	1.0	1.00	Azfi_s0129.g048991	233

Table 2. List of selected genes expressed in the gametophyte of *Dryopteris affinis* ssp. *affinis*. Abbreviations. CPM: counts per million, FDR: False Discovery Rate

4.4. Discussion

Here, we describe a high-quality gametophyte transcriptome of the apogamic fern *D. affinis* ssp. *affinis* and provide the first differential expression analysis with the goal to provide a foundation to better comprehend the genetic regulation underlying sporophyte development in the absence of fertilization.

Dryopteris affinis ssp. *affinis* as a model species to study apogamy

D. affinis (Lowe) Fraser-Jenkins ssp. *affinis* is an attractive model organism for the study of the molecular basis of apogamy because it exhibits a relatively short life cycle, produces vast quantities of spores that can easily be germinated *in vitro* to supply enough gametophyte samples to carry out molecular analyses. Apogamy in this species occurs in nature and *in vitro* culture and can easily be observed under the microscope. As the gametophyte becomes two-dimensional, a brown centre composed of isodiametric, dense cells near the apical notch becomes visible, which forms an asexual embryo (Fernandez et al. 1996; Menéndez et al. 2006).

In the absence of a reference genome, we assembled and annotated a *D. affinis* ssp. *affinis* gametophyte *de novo* transcriptome. Clustering of the original trinity-generated *de novo* transcriptome resulted in 166,191 genes. It is worth noting that a relatively large portion of the genes (32%) had no hits to any known proteins at the time of this study. The number of hits increased with the inclusion of predicted fern proteins in two recently published fern genomes (Li et al. 2018).

About 81% of the reads in each of the six RNA-Seq libraries were mappable back to our *de novo* transcriptome assembly, a proportion comparable to other plant transcriptomes. This and other quality metrics indicated a high completeness and low fragmentation of the *D. affinis* ssp. *affinis* gametophyte transcriptome, making it a valuable tool for future studies.

GO analysis identified that a large number of processes are regulated during gametophyte development and differential expression analysis reveals candidate genes potentially involved in apogamy

Investigation of differential expression revealed 6,110 up- and 4,570 down-regulated genes in two-dimensional relative to one-dimensional gametophytes, some of which may play a role in apogamy and are discussed in more detail below.

Response to stimulus and defense processes are enriched in one-dimensional gametophytes

The filamentous gametophyte is a very simple morphological structure, consisting of a few cells dividing in the same orientation before switching to two-dimensional growth. In addition, its vulnerable morphology is strongly influenced by the environment(Ghosh and Quintanilla 2012). Accordingly, genes upregulated in one-dimensional gametophytes fall within GOs related to "response to environmental stimulus" and "defense" processes. Although the high spore density used in our study to retain gametophytes under a filamentous developmental program without detectable signs of apogamy could have been stressful, a global increase in stress- and defense-related pathways in the context of sexual or asexual reproduction has also been reported in the flowering plant *Hieracium praelatum* (Okada et al. 2013), the ferns *C. richardii* and *Blechnum spicant* (Cordle et al. 2010; Valledor et al. 2014), and the red seaweed *Pyropia yezoensis* (Takahashi and Mikami 2017). In any case, stressful conditions in the liquid culture were also revealed by an upregulation of genes related to biosynthesis of the polyamine spermidine and the catabolism of the precursor arginine, which is consistent with the role of polyamines in plant protection (Bouchereau et al. 1999; Kuznetsov and Shevyakova 2007). Polyamine biosynthesis and spermidine metabolism have been identified as upregulated processes in the apomictic initial cell of *Boechera gunnisoniana* (Schmidt et al. 2014),and spermidine synthesis has been reported to be essential for embryo development in *A. thaliana* and somatic embryogenesis in various plant species (Imai 2004; Wu et al. 2009). However, a possible role for polyamines in early steps of apogamy, polyamine metabolism does not seem to be upregulated in the two-dimensional gametophytes, when apogamy is noticeable.

Additional annotations found upregulated in the filamentous gametophytes of *D. affinis* ssp. *affinis* were related to "nitric oxide (NO) biosynthetic process", which plays a crucial role in plant growth and development, and "calcium ion transport" and "calcium

mediated-signalling". A close relationship between NO and calcium signalling has been described in ferns, such as in the calcium-dependent gravity response reported for *C. richardii* spores (Salmi et al. 2007). Additional reactions to stress in liquid cultures are the use of the glyoxylate cycle; a modification of the TCA cycle occurring in some plants and microorganisms to gain metabolic versatility to provide carbon sources and energy for growth (Samokhvalov et al. 2004) and stress-mediated hormonal signaling, which are both upregulated in the filamentous gametophytes of *D. affinis* ssp. *affinis*(Singh et al. 2005).

Other defence processes were, however, upregulated in two-dimensional gametophytes. We refer to chitin-binding proteins and chitinases among other glycosyl hydrolases involved in the metabolism of chitin oligosaccharides. Chitin is an abundant, insoluble polysaccharide composed of linear chains of *b*-1,4-*N*-acetylglucosamine (GlcNAc) residues, which are highly cross-linked by hydrogen bonds. Chitinase is considered as a potential biocontrol agent, degrading chitin into its monomeric or oligomeric components, thereby destroying the major component of the insect exoskeleton and the cell wall of fungi. It has recently received considerable attention because it might play a role in plant defences against chitin-containing pathogens and contribute to insect control(Cohen 1993; Pusztahelyi 2018).

Regulation of meristem growth, auxin signaling, reproduction, and sucrose metabolism are enriched in two-dimensional gametophytes

Following one-dimensional growth, gametophytes develop to form a flattened, single layer of chloroplast-containing, mesophyll-like cells forming a *prothalium* with filamentous rhizoids that have a function in nutrient uptake analogous to root hairs (Racusen 2002). As the *prothallial* architecture is formed, changing the orientation of cell division from transverse to longitudinal is crucial to achieve this higher complexity. Accordingly, we identified genes responsible for asymmetric cell division and the determination of dorsal identity, both of fundamental significance for cell fate acquisition and the generation of cellular diversity (Facette et al. 2019). Shortly after the transition from one- to two-dimensional growth, the prothallus initial cells within the meristem may differentiate into sex organs or gametangia, in which the formation of gametes does not involve meiosis (Banks JA 1999; Takahashi et al. 2012). We have identified an orthologue of SHOOT MERISTEMLESS, which is associated to the regulation of

meristem growth, and genes encoding proteins of the NAC family of plant-specific transcriptional regulators [NO APICAL MERISTEM (NAM) proteins], which are involved in development, including formation of the shoot apical meristem, the embryo, the floral organs and lateral shoots, as well as in defense and plant hormonal control (Nakashima et al. 2012). Apogamous gametophytes also showed an upregulation of genes related to auxin biosynthesis and auxin signalling which might be linked to meristem formation in the gametophyte and the development of apogamous sporophytes (Zhao 2010). Previous studies reported that auxin is capable to induce apogamy when added exogenously to the culture medium, and increased levels of endogenous indole-3-acetic acid (IAA) prior to the first visible signs of apogamy have also been described(Fernandez et al. 1996; Menéndez et al. 2006). Additionally, we observed increased expression of genes encoding auxin-responsive SAUR proteins, AUX/IAA proteins, and AUXIN RESPONSE FACTOR (ARF) family transcription factors, which have been reported to be associated with apomixis in *B. gunnisoniana* (Schmidt et al. 2014), *H. praelatum* (Okada et al. 2013) and *Paspalum simplex* (Polegri et al. 2010). Also, a homolog of the gene encoding the *A. thaliana* flavin-containing monooxygenase YUCCA8-LIKE was upregulated 10-fold in two-dimensional gametophytes. YUCCA proteins are a key enzyme in Trp-dependent auxin biosynthesis, reported to be essential for the establishment of the basal region of the embryo as well as the formation of embryonic and postembryonic organs(Cheng et al. 2007; Zhao 2014).

Furthermore, genes involved in phytohormone signalling were found upregulated in two-dimensional gametophytes, such as members of the gibberellin-regulated GASA/GAST/SNAKIN protein family. The role of these proteins in several aspects of plant development, plant responses to biotic or abiotic stress, and their participation in hormone crosstalk and redox homeostasis has been widely discussed(Herzog et al. 1995; Zhong et al. 2015).We have previously reported a role for gibberellins in apogamy, as fewer gametophytes produce apogamic sporophytes upon addition of the GA biosynthesis inhibitor flurprimidol to the culture media and there is an accumulation of the gibberellins GA_9 and GA_4 in *D. affinis* spp. *affinis* gametophytes before the onset of apogamy (Menéndez et al. 2006).

Sugar is one of the stimuli that has been found to be associated with apogamy since a long time (Whittier DP and Steeves 1960; Whittier 1964; Elmore and Whittier 1975; Aderkas 1984; Cordle et al. 2007) and, accordingly, processes related to carbohydrate metabolism, in particular sugar, were strongly enriched GO terms in two-

dimensional gametophytes among the biological process categories. Carbohydrate flux is regarded as the signal that triggers the production of apogamous sporophytes, due to the higher requirements for sporophyte as compared to gametophyte growth(Cordle et al. 2010). Consequently, in *C. richardii*, the induction of apospory, and thus the formation of unreduced gametophytes, has been observed upon lack or low levels of sugar in the medium (Raghavan 1989).

Protein domain annotations implicating epigenetic gene regulation in apogamy

Over the last years, epigenetic gene regulation was found to play a predominant role in sexual and asexual reproduction (Grossniklaus U et al. 2001; Baroux et al. 2011; Kawashima and Berger 2014; Grusz 2016; Wang and Köhler 2017; Atallah et al. 2018). *Polycomb* group (PcG) proteins mediate epigenetic silencing of genes through chromatin modifications. First discovered in fruit flies, PcG proteins are well known for silencing *Hox* genes through modulation of chromatin during development but they also control the gametophyte-to-sporophyte transition from mosses to angiosperms(Okano et al. 2009; Butenko and Ohad 2011; Bemer and Grossniklaus 2012; Holec and Berger 2012). To date, the only genes reported to be involved in apogamy commitment are two *Physcomitrella* PcG genes, *PpCLF* and *PpFIE*, encoding components of PRC2 (Mosquna et al. 2009; Okano et al. 2009). In this study, we identified orthologues of MULTICOPY SUPPRESSOR OF IRA1 (MSI1) and Enhancer of zeste [E(z)], both encoding PCR2 subunits, as well as Enhancer of Polycomb-like (Epl1 proteins), a member of a histone acetyltransferase complex involved in transcriptional activation, which is expressed at twice or more the level in two- versus one-dimensional gametophytes(Stankunas et al. 1998; Köhler et al. 2003; Grossniklaus and Paro 2014). Other genes possibly involved in the epigenetic regulation of apomixis, including components of ubiquitin-mediated histone degradation pathway, were also identified. Members of E3 ligase complexes were previously shown to be required for normal embryo sac development or found to be enriched in aposporous initial cells of *H. praelatum* (Juranič et al. 2012; Okada et al. 2013).

Increasing evidence indicates an involvement of epigenetic regulatory pathways in the discrimination between sexual reproduction and apomixis. Epigenetic regulation through small RNA pathways has been reported to be upregulated in the egg cell both in *A. thaliana* (Wuest et al. 2010) and *B. gunnisoniana* (Schmidt et al. 2014).Our analysis identified four genes in this category as upregulated in two-dimensional gametophytes,

which encode Paz, Piwi, and Dufi785 domain proteins that are associated with ARGONAUTE and DICER-LIKE proteins in *A. thaliana*.

Biological functions associated with reproduction

The gametophyte of ferns is a structure committed to reproduction, and biological functions linked to reproduction are prominent in both one- and two-dimensional gametophytes of *D. affinis* spp. *affinis.* Several genes related to reproduction have previously been described in apogamous gametophytes of *C. richardii* (Cordle et al. 2012)and, similarly, we found genes involved in pollen development, crossover formation and meiotic chromosome segregation, and cell-cycle regulation among others. While the expression of genes involved in meiosis in vegetative gametophytes without meiotic cells is unexpected, some meiotic genes have been reported to be expressed in vegetative tissues of both *A. thaliana* and *B. gunnisoniana* (Kaur et al. 2006; Shah et al. 2016). Another interesting gene family that is strongly upregulated in two-dimensional gametophytes encodes FRIGIDA-like proteins similar to FRIGIDA in *A. thaliana*. FRIGIDA regulates flowering time by activating the expression of *FLOWERING LOCUS C* (*FLC*), which encodes a central repressor of flowering that becomes silenced during prolonged exposure to cold (vernalisation) (Johanson et al. 2000; Chao et al. 2013). What role FRIGIDA-like proteins play in fern gametophytes is, however, unknown.

Comparison of transcriptome data with shotgun proteomics results

We had previously used protein extracts from the same gametophyte samples prepared at the same time as those used herein, to perform shotgun proteomics in order to identify proteins expressed in one and two-dimensional gametophytes(Grossmann et al. 2017). Among the 1397 proteins we had identified in these gametophyte samples, there were also homologs of 39 proteins that had previously been associated with sexual reproduction or apomixis in angiosperms(Grossmann et al. 2017). A comparison of this list of 39 proteins with the current analysis of differential gene expression between one- and two-dimensional gametophytes, showed that 16 of them were differentially expressed (FDR < 5%). Seven genes were classified as stress response genes, four as involved in reproduction, three in phytohormone signalling, and two in embryo development. Among the genes that were most significantly upregulated in two-dimensional relative to one-dimensional gametophytes, we identified a homolog of the LATE EMBRYOGENESIS ABUNDANT1 protein (LEA1_CICAR, upregulated 17-fold), a homolog of disease

resistance response protein 206 (DR206_PEA, upregulated 14-fold), and an orthologue of Janus-B, which regulates somatic sex differentiation in the fruit fly *Drosophila melanogaster* (Yanicostas et al. 1989) (upregulated 5-fold). Among the genes that were upregulated in one-dimensional gametophytes, there was a homolog of Nectarin-1 (NEC1_NICPL, upregulated 10-fold), involved in the defense of floral reproductive tissues(Carter and Thornburg 2000), an orthologue of allene oxide cyclase 4 (AOC4_ARATH, upregulated 6-fold), involved in the biosynthesis of jasmonic acid, and a homolog of pollen-expressed LAT52 (Tang et al. 2002) (upregulated 6-fold), a ligand of a receptor-like kinase involved in pollen hydration and pollen tube growth.

Homologs of several of these differentially expressed genes do not only have potential roles in sexual or asexual reproduction but are also associated with stress responses or defense mechanisms. Indeed, many genes involved in plant reproduction are related to defense proteins, a theme that has become more and more apparent over the last years (Zhou and Dresselhaus 2018) and is also reflected in our gametophytic transcriptome dataset. What exact role these differentially expressed genes play in ferns awaits their functional characterization.

4.5. Conclusions

In conclusion, the comparative transcriptomic profiles of one- and two-dimensional gametophytes of the apogamous fern *D. affinis* ssp. *affinis* provide insights into the processes occurring after the growth pattern shifts from filamentous, one-dimensional growth to form a prothallial, two-dimensional architecture. From a total of 10,679 genes that were differentially expressed between one- and two-dimensional gametophytes (6% of all genes), 6,110 genes were up-regulated and 4,570 downregulated in two-dimensional gametophytes. Among the differentially expressed genes were many with predicted functions in defense processes, development, reproduction, phytohormone signaling, and epigenetic gene regulation. The annotation of orthologues of SHOOT MERISTEMLESS and NAC proteins supports the idea that the meristem in the prothallus of ferns shares certain molecular features with the shoot apical meristem of angiosperms. The long-known role of sugar as an inducer of apogamy is reinforced by the many identified genes associated with carbohydrate metabolism. Our results provide insights into the development of free-living gametophytes, focusing on the filamentous-to-prothallus transition, and the information will be useful for others in the field of asexual reproduction in plants in general, and apogamy in ferns in particular.

4.6. References

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Supplementary data

All of the supplementary material is included at the CD/USB unit.

Table S1. Assessment of *D. affinis* ssp. *affinis* transcriptome completeness using BUSCO

Table S2. List of differentially expressed genes with annotation.

Table S3. GO enrichment analysis between one- and two-dimensional gametophytes of *D. affinis* ssp. *affinis*.

Table S4. KEGG pathway enrichment analysis.

Table S5. Strongly up-regulated transcription factor genes in a) one-dimensional or b) two-dimensional gametophytes.

Figure S1. Schematic alignment of selected *D. affinis* ssp. *affinis* genes to their closest Blast hits.

Chapter 5. General Discussion

This work represents a step forward to gain a better knowledge on the apogamous process in the fern *Dryopteris affinis* ssp. *affinis*, which represents a peculiar case of apomixis, in ferns. In concrete, a physiological and transcriptomic approach are reported, revealing a role of phytohormones, either by analyze the effects caused by the addition to the culture medium, or by measuring their endogenous levels, as well as through comparative transcriptomic profiles between of one- and two-dimensional gametophytes.

The first commitment was to define a suitable experimental system. Given the great regeneration capacity exhibited by the gametophyte of ferns (Fernández and Revilla 2003; Menéndez et al. 2006; Somer et al. 2010; Rivera et al. 2018), and non-seed plants in general (Ikeuchi et al. 2016), the culture of homogenized gametophytes was considered a good option. Each fragment of tissue will account for some cells able to rebuilt a new gametophyte, being a useful resource of plant material to conduct, in an easy way, the physiological and molecular experiences proposed to reach the pursued goals. In regeneration experiments reported by Racusen (2002), studying the relationships between photosynthetic and ion-absorbing cells (rhizoids) in the fern gametophyte, it was found that remnants of disconnected gametophytes are able, in few cell divisions, of re-establishing the poles for a new axis basal/apical, a distance comparable with that in other systems in which a diffusible morphogen is involved in the determination of polarity (Wolpert 2011). How the apical-basal gametophytic structure is re-assumed by some cells remains to be deciphered, representing our homogenates cultures a good experimental scenery to deal with this interesting point of differentiation patterning.

Another interesting point, needing to be highlighted is the fact that homogenized gametophytes were more susceptible to respond to the presence of PGRs and HPTIs added to the medium than using spores or entire gametophytes as experimental system (Rivera et al. 2018), perhaps the spore or the complete gametophyte represent a more differentiated and organized structure than just a piece of disrupted and disconnected cells. However, the use of spores to arise gemetophytes suitable for starting the homogenized cultures, is a useful tool to avoid contamination and also being frustrated by this setback without major hindrances.

Before starting to discuss the results, it would be needed to rescue once again, the idea about what does the fern gametophyte represent from a structural point of view. Then, we could try to fit the results into the framework of gametophyte development, focusing on those crucial episodes happening from spore germination until the attainment of the cordate morphology.

The change in the morphology of fern gametophyte during growth, have attracted the attention of researches for decades (Nayar and Kaur 1971; Banks 1999; Rivera et al. 2018). Although being an individual possessing a very simple structure of one layer of cells, the gametophyte undergoes some degree of complexity. Among the events occurring during the gametophyte development, there are the achievement of an apical-basal polarity, a dorso-ventral symmetry, rhizoids differentiated directly from basal green cells (as a consequence of an unusual, highly asymmetric cell division, and designated for ion and water uptake), sexual organs (antheridia and archegonia), a meristem responsible of the expansion of the wings or lobes, and finally, the differentiation of numerous trichomes for all the surface, in our species. The structure and function of the meristem in the sporophytes of major vascular plants, are relatively well-know (Gaillochet et al. 2015). In opposition, not much is known about gametophytes of vascular plants, which only could be possible in ferns. The reason is the fact that gametophytes are strongly reduced and entirely-sporophyte dependent in seed plants, (Sundaresan and Alandete-Saez 2010) whereas in ferns, they are free-living (Rivera et al. 2018). In line with it, three main types of meristems have been described in the fern gametophytes: a) an apical cell-based meristem, placed with a distinct single apical initial cell, which is always formed during, and responsible for, the early development of the gametophyte; b) a multicellular meristem with a group of initial cells, which replaces the apical cell-based meristem and is involved in further growth of gametophyte; and c) a marginal meristem, which also contains multiple initial cells, and the activity of which is related to the expansion of the gametophyte lobes (Bartz and Gola 2018; Takahashi et al. 2015).

The question rose is whether the successive analyses done in the thesis, have contributed to throw some light over the vegetative and reproductive development in this generation of fern cycle. What did we learn when adding phytohormones and their biosynthesis and transport inhibitors to the culture medium? What did we learn from the endogenous levels of phytohormones detected in the gametophyte tissues? And finally, what have we learnt from the great effort carrying out an omic approach in a non-model species, without a sequenced genome to start with?

Next, it would be suitable to take a transverse approximation to the analyses done, recruiting the most relevant information gained on each crucial event aforesaid, during subsequent development of gametophyte. We are going to divide the discussion in the two major approached done: physiological and transcriptomic.

Certainly, from the data gathered by adding either plant growth regulators or their inhibitors of biosynthesis and transport, and also those derived from the quantification of the endogenous levels of phytohormones, we can appreciate the fern gametophyte progress to be influenced by these compounds.

This seemingly insignificant structure resembles a miniature plant, with a photosynthetic solar panel, analogous to a leaf, and rhizoids with their high surface are to volume ratios, as root hairs, otherwise experiencing similar requirements and behaviors than more complex individuals. Moreover, diversity in gametophyte form and physiology has enabled ferns to radiate into the wide range of habitats they must first colonize through gametophyte establishment, including darks, moist forest floors, extremely bright, exposed forest canopies, tropical regions never experiencing freezing temperatures, the coldest of northern latitudes and mountain tops where gametophytes formed from spores released in late summer must overwinter, and rocky desert extremes where gametophytes produce sporophytes despite the rare presence of environmentally available water (Liu et al. 2012). In some cases, they must tolerate environmental extremes beyond that of their sporophyte counterparts, and expending good part of its life testing habitat suitability (Racusen 2002). At this regard, the analyses of endogenous phytohormones revelated high levels of salicylic acid found in the filamentous, which could be related to the stressful environment the homogenized cultures represent. In fact, in the chapter 4, Wyder et al. (2020) reported enrichment in gene expression related to response to stimulus and defense in one-dimensional gametophytes of our species.

As it was aforementioned, the gametophyte accounts for successive developmental changes until reaching maturity, which are expected to be controlled by phytohormones. A possible connection between the cytokinin BA and the filamentous morphology, is suggested from the levels detected in the apogamous gametophytes. In previous works, a strong correlation between adding cytokinins to the culture medium and gametophytes remaining as filamentous or spatula-shaped was observed working with other fern species, such as *B. spicant* and *Osmunda regalis* (Menéndez et al. 2006a; Greer et al. 2012). In *B. spicant,* it was accompanied by a strong effect on differentiation

of male sexual organs or antheridia. At the beginning of gametophyte development, cellular division represents the most crucial event, in which cytokinins must play an important role; however, the addition of these compounds could surpass the threshold and cause the contrary effect, inhibiting the transition to the two-dimensional growth.

At this regard, the shifting to the two-dimensional growth, encompasses an increase in the rate of anticlinal divisions (i.e., perpendicular to the apical surface), up to the formation of the apical notch, when the meristem is place (Racusen 2002) . This event was favored by the two balances auxin-cytokinin, including the auxin NAA. The presence of this auxins seemed to be more decisive that IBA, and also than the cytokinin kinetin. The effect of IBA suggested a major role on embryogenesis, which otherwise has been reported for this auxin (Jha et al. 2007; Johri 2008). NAA resulted toxic for the gametophyte at the highest dose used here for IBA. As commented in the chapter 2, the differences noted between these two auxins could be due to the fact that IBA is better substrate for influx auxin transports (AUX1) (Klíma et al. 2016). In presence of the auxin IBA the frequency of apogamy increased and also the size of embryos. The fact that the inhibitor of auxin transport NPA, promoted wider gametophytes and bigger and conical embryos, supports a role of auxins governing gametophyte growth and embryo development. Indeed, in a previous experiment using the inhibitor of auxin transport TIBA, regenerated gametophytes remained mostly as filaments ((Rivera et al. 2018). From these results, a possible polar auxin transport could be acting of the apogamous embryo development, mainly lead by the auxins IAA (which has been detected). Some kind of movement of auxin could be governing also the organization of prothallus. In proteomic analyses done recently in *D. affinis* and *D. oreades* they have been annotated proteins involved in auxins transport like the ABC transporter B family member 19 (Grossmann et al. 2017) or clathrin which mediates endocytosis and is required for a correct polar distribution of PIN auxin transporters (Kitakura et al. 2011). PIN-mediated auxin transport drives meristem function in both life cycle stages, gametophyte and sporophyte in a moss (Bennett et al. 2014), but the existence of these carriers has not been proved in ferns so far. In the fern *Ceratopteris*, auxin mediates the activation of the lateral meristem and regulates lateral meristem function (Gregorich and Fisher 2006), and it is conceived that auxin transport may be necessary for communication between the lateral meristem and other regions of the developing gametophyte. In the gametophyte, we can distinguish a polar disposition involving an apical notch on top and the rhizoids on bottom, with the lobes expanding towards right and left, and consequently a polarity as

gametophyte develops. Intercellular transport throughout the gametophyte is extremely important as normal development depends on apical dominance, i.e. the localization of the apical cell, with concurrent suppression of this condition in all other cells. The fern gametophyte may serve as a model for apical dominance in higher plants, and the role intercellular transport might play this (Holloway and Lantin 2002).

The increase of GA_3, observed in the spatula-shaped gametophytes, could be related with the important cellular expansion happening at this stage, and which has been attributed to gibberellins in some sexual fern species such as *B. spicant*, *Anemia phillitidis* and *P. aculeatum* (Fernández et al. 1997, Kaźmierczak 2003, Menéndez et al. 2006b, 2009; Kosakivska et al. 2020). The data obtained by the comparison of the effects caused by the couple GA_3 and its biosynthesis inhibitor flurprimidol, when added to the culture medium, contributed to strengthen the influence of gibberellins on elongation. It is a response well stated in seed plants, and it is reinforced here by the inhibitor of GAs biosynthesis, flurprimidol, which causes the opposite effect, stimulating growth in width and reproductive gametophyte. This inhibitor of the gibberellins gave evidences about the importance of these phytohormones on apogamy, whose addition to the medium decreased the size of the embryo, as it has already been stated by Menéndez et al. (2006c). Nevertheless, the fact that the exogenous addition of GA_3 to the culture medium does not affect apogamy, could mean that another gibberellin might have a more critical function, as GA_4 and GA_7 whose levels increased when the apogamous center start developing (Menéndez et al. 2006c), and being supported by the results achieved in the chapter 3.

The polyamine spermidine, added to the culture medium, favored the transition and lateral expanding of lobes, being gametophytes wider than longer, but these results were not neutralized by the addition of its inhibitor to the medium, remaining not clear the role of these compounds on the vegetative development of gametophyte. Regarding apogamy, spermidine seems to influence embryo development, and its inhibitor, CHA, the induction of apogamy.

When comparing apogamous versus sexual gametophytes, up to seven out of fourteen analyzed phytohormones, accumulated more in the sexual counterpart, respect to the apogamous one, including the auxin AIA, the cytokinins Z, RZ, IPA, the gibberellin GA_4, and the brassinosteoids epibrassinolide and its precursor castosterone, although only one sample were analyzed for the former. iP bases cytokinins are characteristic of mosses and ferns (Johri 2008), and its presence seems to be relevant also in ferns, being

interesting to have a look in the at these biosynthetic pathways along the tree of plant evolution.

Increases in the main PGRs such as auxin IAA, GA_3 and cytokinins were reported in other fern species (Kosakivska et al. 2019; 2020). In our species, *D. oreades*, GA_4 seems to prevail over GA_3 either on apogamous or sexual development, being especially higher in the sexual species, and it could be related to its antheridiogen-dependent reproductive behavior as it was discussed in the chapter 2. A possible role for ABA remain uncertain.

One striking data was the significant levels found of brassinosteroids, revealing some role on sexual development of gametophytes in *D. oreades*. Further research must be done in order to elucidate in detail the relationship between brassinosteroids and gametophyte reproduction as well as a possible cross-talk with auxins and gibberellins, among other phytohormones.

In the homogenized gametophyte cultures, some cells were able to divide and differentiate a new gametophyte, and also to form disorganized cell masses, like callus, which were observed when gametophytes were wounded with a scalpel. Callus induction in this apogamic subspecies represents a good result itself. The achievement of callus from any species might represent a biotechnological tool. For instance, stable transformation by particle bombardment of callus tissue was recently reported in the fern *Ceratopteris richardii* (Plackett et al. 2015), and could be useful to validate candidate genes of apogamy.

We move now to the transcriptomic tactic, and as it is said in the chapter 4, a high-quality gametophyte transcriptome of the apogamous fern *D. affinis* ssp. *affinis* was performed, and provided the first differential expression analysis with the goal of reaching a better comprehension about the genetic regulation underlying gametophyte development and apogamy, in this species. No doubts *Dryopteris affinis* ssp. *affinis* can be considered as a model species to study apogamy even though its genetic complexity, and thanks to the novel technologies.

In this thesis, a *Dryopteris affinis* ssp. *affinis*' gametophyte *de novo* transcriptome was assembled and annotated, revealing genes down and up-regulated one and in two-dimensional gametophytes. These studies reflected an environment quite stressful linked to the filamentous cultures, funding GOs related to "response to environmental stimulus" and "defence" processes. In addition, the finding of genes related to biosynthesis of the polyamine spermidine and the catabolism of the precursor arginine, suggested a need of

plant protection (Bouchereau et al. 1999)but also a possible relationship with an apomictic event as commented in the chapter 4. Our previous data, related to the couple spermidine-inhibitor, added to the culture media, account for an effect of this polyamine favouring two-dimensional transition of gametophytes, and also affecting their shape, being gametophytes wider than longer. These responses were inhibited adding the lower dose of CHA but not the highest. The same happened regarding the role of this polyamine on apogamy. A dose-dependent effect of spermidine is observed on the percentage of apogamous gametophytes and embryo size, which once again is confirmed by adding the lower dose of CHA. Certainly, polyamines seem to have an important role on gametophyte development in general, but more studies should be addressed to determine more accurately their biological role.

Following one-dimensional growth, the gametophyte must gain in complexity, thanks to the expression of genes involved in those processes of fundamental significance for defining the cordate thallus, based on precisely established planes of sequential divisions and differentiation patterns. Among them, there were orthologues of genes involved in important aspects of asymmetric cell division, determination of dorsal identity, and transcriptional regulators of meristem growth such as SMT, and others belonging to the NAC family (NAM) involved in plant development, and including formation of the shoot apical meristem (Nakashima et al. 2012)

Phytohormones profoundly affect plant growth and development, and gametophyte development and apogamy, are expected to be strongly influenced by them. Data collected from different kind of analyses point out an important role of auxins and gibberellins on apogamy. Another important stimuli, related by longer with apogamy as it is sugar (Whittier et al. 1960; Whittier and Steeves 1962; Whittier 1964; von Aderkas 1984; Cordle et al. 2007) has been seen strongly enriched GO terms in two-dimensional gametophytes among the biological process categories, strengthening its role on this process. Finally, protein domain annotations implicating epigenetic gene regulation in apogamy were counted in this species. In this study, we identified some orthologues of MULTICOPY SUPPRESSOR OF IRA1 (MSI1) and Enhancer of zeste [E(z)], both encoding PCR2 subunits, as well as Enhancer of Polycomb-like (Epl1 proteins); a member of a histone acetyltransferase complex involved in transcriptional activation, which is expressed at twice or more the level in two- versus one-dimensional gametophytes; and others including components of ubiquitin-mediated histone degradation pathway. In addition, our analysis identified four genes related to epigenetic

regulation through small RNA pathways, upregulated in two-dimensional gametophytes, which encode Paz, Piwi, and Dufi785 domain proteins that are associated with ARGONAUTE and DICER-LIKE proteins in *A. thaliana*.

The gametophyte of ferns is a structure committed to reproduction, and we found genes involved in pollen development, crossover formation and meiotic chromosome segregation, and cell-cycle regulation among others. Another interesting gene family that is strongly upregulated in two-dimensional gametophytes encodes FRIGIDA-like proteins similar to FRIGIDA in *A. thaliana*, and some more as it was aforementioned.

The combination of different approaches, physiological or omics, is a promising way to obtain a comprehensive picture of regulatory processes. Studying the molecular mechanisms of asexual reproduction, i.e., the generation of clonal offspring, is an important topic aiming at the introduction of self-sustainable hybrids in agriculture. Hence, the introduction of apomixis has a tremendous potential for crop improvement and extending our analyses to phylogenetic branches other than those of model species may help to unravel underlying processes common to a broad range of organisms.

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Chapter 6: Conclusions

1. The experimental system used in this work along with the addition of certain phytohormones (PGRs) and inhibitors of their synthesis or transport (BTPIs), provided a better understanding of the tested chemicals on both vegetative development and apogamy, in the gametophyte of Dryopteris affinis ssp. affinis, resulting to be more efficient than previous studies, starting from spore or the entire gametophyte.
2. A balance auxin/cytokin favourable to IBA, increases gametophyte elongation, the frequency of apogamy and the size of the embryos. GA3 shows an important role in vegetative growth, promoting elongation, and evidenced also by the inhibitor of GAs biosynthesis, flurprimidol. Although the application of flurprimidol, inhibited apogamy, is not clear the possible involvement of GAs since their addition did not give any positive or negative result. The role of the polyamine spermidine on both vegetative development and apogamy remains more controversial.
3. A valid protocol is available to induce and proliferate callus of D.affinis ssp. affinis, which can lead to a biological system to deepen on apogamy, and also to conduct further molecular analyses on this topic.
4. The phytohormone content varies either among the three successive gametophyte stages of the apogamous fern D. affinis, and between heart-shaped apogamous gametophytes and the sexual ones of D. oreades. In the former, it was noticed that the cytokinin BA and salicylic acid, increased at the filamentous stage, the gibberellin GA3 at the spatula, and finally a slight variation is observed of RDHZ in the cordiforme gametophytes. In the latter, up to seven of fourteen phytohomones, accumulated more in the sexual (female) gametophyte, respect to the apogamous one, which are the auxin IAA, the cytokinins Z, RZ, IPA, the active gibberellin GA4, and the active brassinosteoid castosterona.
5. The comparative transcriptomic profiles of one- and two-dimensional gametophytes of the apogamous fern D. affinis ssp. affinis provide insights into the processes occurring after the growth pattern shifts from filamentous, one-

dimensional growth, to form a prothallial, two-dimensional architecture. From a total of 10,679 genes that were differentially expressed between one- and two-dimensional gametophytes (6% of all genes), 6,110 genes were up-regulated and 4,570 downregulated in two-dimensional gametophytes. Among the differentially expressed genes were many with predicted functions in defense processes, development, reproduction, phytohormone signaling, and epigenetic gene regulation.

6. The annotation of orthologues of SHOOT MERISTEMLESS and NAC proteins supports the idea that the meristem in the prothallus of ferns shares certain molecular features with the shoot apical meristem of angiosperms. The long-known role of sugar as an inducer of apogamy is reinforced by the many identified genes associated with carbohydrate metabolism.
7. Our results provide insights into the development of free-living gametophytes, focusing on the filamentous-to-prothallus transition, and the information will be useful for others in the field of asexual reproduction in plants in general, and apogamy in ferns in particular.

RESUMEN

Introducción

Hace unos 470 millones de años, durante el período Ordovícico, las algas carofitas empezaron a conquistar la tierra. Con el tiempo, éstas dieron paso a una sucesión de grupos vegetales, que poco a poco irían ganando en complejidad, como respuesta a los retos que el nuevo ambiente les impondría. Entre el Silúrico y el Pérmico, se gestarían los grupos taxonómicos que aún siguen hoy representados, como son los briófitos (incluyendo musgos, hepáticas y antoceros), licófitos y eufilofitos, englobando estos últimos a los monilofitos (helechos) y espermatofitos (plantas con semilla). Nuestros helechos actuales son reminiscencias de las primeras plantas vasculares, que tuvieron que hacer frente a cambios en la disponibilidad del agua, en la temperatura o en los niveles de radiación, sometiéndolas a adaptaciones permanentes de su cuerpo, a nivel celular, fisiológico y regulador. Por todo ello, los helechos son buenos candidatos para abordar estudios evolutivos comparativos, que ayuden a comprender mejor el desarrollo vegetal y completando a los estudios actuales que se realizan utilizando modelos vegetales más evolucionados.

A pesar de que, durante mucho tiempo, los helechos se han visto en una posición inferior respecto a plantas de mayor tirón agronómico o forestal. En ocasiones han servido como sistemas experimentales para ahondar en algunos procesos del desarrollo vegetal como fotomorfogénesis, germinación, polaridad, composición de la pared celular, etc. Sin duda, un aspecto trascendente del desarrollo vegetal es la reproducción, ya sea por mecanismos sexuales o asexuales. Durante las últimas décadas, se han venido publicando numerosos trabajos en revistas de prestigio, que ponen de manifiesto el esfuerzo en controlar las claves que gobiernan la apomixis, o clonación por semillas. El interés por este tipo de reproducción asexual está en su capacidad de producir descendencia clonal, permitiendo la fijación de genotipos complejos, incluyendo híbridos altamente productivos. Aquí se puede atisbar el gran potencial de este mecanismo, por ejemplo, en la agricultura, al combinar las ventajas de la propagación por semillas (una alta tasa de multiplicación, la facilidad de almacenamiento y siembra, y una menor posibilidad de transmisión de enfermedades) con las ventajas de la propagación clonal.

La apomixis se conoce en diferentes grupos de cormófitos, pero es más común en helechos, abarcando a un 10% del total de especies. La distribución dentro de ellos

tampoco es homogénea, ya que las familias *Dryopteridaceae* y *Pteridaceae*, incluyen aproximadamente el 70% de las especies apomícticas de helechos. En helechos, es muy frecuente un tipo de apomixis que combina la formación de esporas no reduccional (diplosporia) y la apogamia, o formación del embrión a partir de células gametofíticas. La apogamia en estas plantas, pudo ser una vía para escapar de la esterilidad de las hibridaciones entre helechos.

La pregunta que subyace en esta memoria es si el estudio de la apogamia en helechos podría contribuir a entender la apomixis en Angiospermas. Según lo mencionado anteriormente, tanto en la apomixis como en la apogamia, se forman embriones sin fertilización, y podría esperarse, según algunos autores, que la apomixis y la apogamia compartan algunas características comunes. La apogamia en helechos puede verse asimismo como una oportunidad para investigar sobre la embriogénesis, una de las herramientas más poderosas en biotecnología vegetal.

En las plantas de semillas, existen varios grupos de genes (en su mayoría codifican factores de transcripción) como BABY BOOM, AINTEGUMENTA-like 5, FUSCA3, LEAFY COTYLEDON, que se han asociado a la embriogénesis. No obstante, si son necesarios para la propia embriogénesis o la viabilidad de las células vegetales aún necesitaría una explicación adicional. Explorar la apogamia en especies de helechos podría representar la posibilidad de aumentar nuestra comprensión de aquellos procesos que eluden la reproducción sexual.

Planteamiento y Objetivos

Los procesos de crecimiento y desarrollo del gametofito y esporófito de helechos, así como los representantes de otros taxones, están controlados por un sistema hormonal multicomponente. Así, por ejemplo, se ha observado que auxinas como AIA indujeron el desarrollo apogámico de esporófitos en gametofitos estériles del helecho *Platycerium*, y el etileno endógeno con la adición de AIA, se observó una depresión en el desarrollo apogámico en gametofitos de *P. coronarium*, que podría ser causado por un deterioro en el transporte de AIA en células gametofitas.

Para una mejor comprensión de las funciones de las fitohormonas y las interacciones entre ellas, la determinación exhaustiva del contenido hormonal es por lo tanto de gran importancia. El hecho de que las diferentes hormonas tienen diversas

propiedades químicas y estructurales dificulta su cuantificación conjunta y también sus bajas concentraciones tisulares, por lo general en partes por mil millones (*ppb*) o nivel nanomolar. Con el advenimiento en los últimos años, de métodos rápidos, sensibles, precisos y eficientes para el análisis de fitohormonas a partir de muestras biológicas, resulta factible analizar un abanico amplio de las fitohormonas. En la actualidad, el HPLC-MS es el método más extendido para realizar estos análisis en plantas, detectando cantidades más pequeñas que las que pudimos detectar hace diez años.

En cuanto a un posible enfoque molecular, cabe decir que los helechos tienen un mayor número de cromosomas y genomas más grandes que los musgos y las plantas de semillas, lo que complica la estabilización de los datos genómicos y de secuencias de transcripción. Sin embargo, el desarrollo de las tecnologías de secuenciación de próxima generación (NGS), así como, la aparición de los secuenciadores Roche'454 GS-FLX *Titanium* e *Illumina HiSeq*. Gracias a estos aparatos es posible caracterizar el transcriptoma de las plantas. Aunque, representa un objetivo de menor alcance en comparación con el genoma de una especie completa, pero es un comienzo.

La variación en la expresión génica inducida por cualquier condición ambiental o interna puede ser examinada en organismos no modelo porque estas técnicas se han vuelto más factibles a medida que la automatización y la eficiencia han reducido el costo. Recientemente, algunos conjuntos de datos se han publicado en helechos como la especie *Pteridium aquilinum, Ceratopteris richardii, Lygodium japonicum* y *Dryopteris affinis ssp. affinis* y algunos otros resultantes del proyecto OneKP.

Recientemente, se realizaron análisis transcriptómicos y proteómicos para aumentar nuestro conocimiento sobre la base molecular de la apogamia en *D. affinis* ssp. *affinis* y que representan los primeros intentos en esta especie. Entre otras, se han detectado algunas proteínas de helecho homólogas a ARGONAUTE10/PINHEAD/ ZWILLE. También se hallaron homólogos de la proteína efectora de ARN *A. thaliana* SERRATE (SE) que podría participar en la actividad meristemática del incipiente embrión apogámico o roles desconocidos en el cambio entre la reproducción sexual y asexual y tal vez en la regulación de la apogamia en helechos. Además, también se identificaron proteínas implicadas en el silenciamiento de genes o enzimas en modificaciones de la pared celular, como las pectinesterasas, que podrían tener algún papel en la apogamia. Además, se realizó un ensayo de ARN-Seq para comparar perfiles de expresión génica de gametofitos unidimensionales y bidimensionales de esta especie,

encontrando varios miles de genes expresados diferencialmente, y relacionados con diferentes aspectos del comportamiento vegetativo o reproductivo del gametofito.

En conclusión, el **objetivo principal** del presente estudio es obtener información sobre algunas claves fisiológicas y moleculares que operan en este este proceso. Para lograr este objetivo principal, se definieron los siguientes **objetivos parciales**:

1. Para probar el posible papel de los reguladores del crecimiento de la planta (PGRs), se propone añadir estas compuestos o inhibidores de síntesis o transporte (IBTH) al medio de cultivo donde el gametofito está creciendo, (Capítulo 2), y medir los niveles endógenos de las fitohormonas utilizando UHPLC, (Capítulo 3).

2. Realizar comparaciones de los perfiles proteómicos entre dos etapas de desarrollo de los gametofitos uni- y bidimensionales, (Capítulo 4).

Resultados y discusión

El primer cometido ha sido definir un sistema experimental adecuado. Dada la gran capacidad de regeneración exhibida por el gametofito de helechos, el cultivo de gametofitos homogeneizados, se consideró una buena opción para nuestros experimentos. Cada fragmento de tejido alberga alguna célula capaz de reconstruir un nuevo gametofito, siendo un recurso útil de material vegetal para llevar a cabo, de una manera fácil, los ensayos planteados. En realidad, los gametofitos homogeneizados eran más susceptibles de responder a la presencia de PGR y IBTH añadidos al medio de cultivo, que el uso de esporas o gametofitos enteros como sistema experimental, quizás porque la espora o el gametofito completo representan una estructura más diferenciada y organizada que sólo una pieza de células alteradas y desconectadas.

Antes de empezar a discutir los resultados en su conjunto, sería necesario rescatar una vez más, la idea sobre lo que representa el gametofito de helecho desde un punto de vista estructural y tartar de encajar los resultados en el marco del desarrollo de gametofitos, centrándonos en esos episodios cruciales que ocurren desde la germinación de esporas hasta que se alcanza la morfología acorazonada o cordiforme.

El cambio en la morfología del gametofito de helecho durante el crecimiento ha atraído la atención de las investigaciones durante décadas. Aunque es un individuo que posee una estructura muy simple, de una capa de células, el gametofito posee cierto grado

de complejidad, como una polaridad apical-basal, una simetría dorso-ventral, rizoides diferenciados directamente de las células verdes basales (como consecuencia de una división celular inusual, altamente asimétrica, y destinados a la absorción de iones y agua y fijación al sustrato), órganos sexuales masculinos (anteridios) y femeninos (arquegonios), un meristemo responsable de la expansión de los lóbulos o la diferenciación de numerosos tricomas por toda la superficie, en el caso de nuestra especie. La estructura y la función del meristemo en los esporófitos de las principales plantas vasculares, son relativamente bien conocidos. Sin embargo, no se sabe mucho sobre el de gametofitos de plantas vasculares, que por otra parte sólo tienen razón de ser en helechos, dado que, en plantas con semillas están fuertemente reducidos (grano de polen y saco embrionario) y dependen por completo del esporófito.

A continuación, sería conveniente tomar una aproximación transversal a los análisis realizados, reclutando la información más relevante obtenida sobre cada evento crucial antes mencionado, del desarrollo vegetativo y reproductivo del gametofito. Vamos a dividirla discusión en base a los dos enfoques planteados: fisiológico y transcriptómico.

Ciertamente, a partir de los datos recopilados mediante la adición de reguladores de crecimiento de plantas o sus inhibidores de la biosíntesis y el transporte, y también los derivados de la cuantificación de los niveles endógenos de fitohormonas. Se constata que el crecimiento del gametofito de helecho está influenciado por las fitohormonas

Esta estructura aparentemente insignificante, se asemeja a una planta en miniatura, que es como un panel solar fotosintético, análogo a una hoja, y sus rizoides con una alta relación superficie/volumen, como pelos de raíz, presentando comportamientos básicamente similares al de individuos más complejos, regulados por fitohormonas. En este sentido, se observa una conexión entre la citoquina BA y la morfología filamentosa, En trabajos anteriores, se observó una fuerte correlación entre la adición de citoquinas al medio de cultivo y la morfología filamentosa. Al igual que ocurre en otras especies de helechos, como *B. spicant* y *Osmunda regalis*. Durante el comienzo del desarrollo de gametofitos, la división celular representa un evento crucial, en el que las citoquinas deben desempeñar un papel importante; sin embargo, la adición de estos compuestos podría superar el umbral de tolerancia y causar el efecto contrario: inhibiendo la transición al crecimiento bidimensional.

En este sentido, la transición hacia un crecimiento bidimensional implica un aumento en la tasa de divisiones anticlinales (es decir, perpendicular a la superficie apical), y la formación de un meristemo en la zona apical. Este evento fue favorecido por

dosis auxina-citoquina que incluían como auxina al ANA, pero no cuando la auxina era el AIB, que tuvo un efecto en la embriogénesis. Altas dosis de ANA resultaron tóxicas para el gametofito. Como se ha comentado en el capítulo 2, las diferencias observadas entre estas dos auxinas podrían deberse al hecho de que la AIB es un mejor sustrato para los transportadores de entrada de auxina en la célula (AUX1). En presencia de la auxina AIB la frecuencia de la apogamia aumentó y también el tamaño de los embriones. Por otra parte, el hecho de que el inhibidor del transporte de auxina NPA, promovió gametofitos más anchos que largos y embriones más grandes y cónicos, apoya un papel de auxinas en el desarrollo vegetativo y embrionario del gametofito. De hecho, en un experimento anterior utilizando el inhibidor del transporte de auxina TIBA, los gametofitos regenerados permanecieron principalmente como filamentos.

A partir de estos resultados, se sugiere la existencia de un posible transporte de auxina (polar?) que podría contribuir a generar gradientes de auxinas en el gametofito. En análisis proteómicos realizados recientemente en las especies *D. affinis y D. oreades* se han anotado proteínas de transporte de auxinas como, la familia de transportadores tipo ABC B 19 o *Clathrin* (clatrinas) que media la endocitosis y es necesaria para una correcta distribución polar de los transportadores de auxina PIN. El transporte de auxina mediado por PIN ha sido comentado en ambas etapas del ciclo de vida, gametofito y esporófito, en musgos, pero la existencia de estos portadores no se ha demostrado en helechos hasta ahora. En el helecho *Ceratopteris*, la auxina regula la función del meristema lateral, y se contempla que el transporte de auxina pueda ser necesario para la comunicación entre el meristemo lateral y otras regiones del gametofito en desarrollo.

En el gametofito, podemos distinguir una disposición polar que implica una muesca apical en la parte superior y los rizoides en la parte inferior, con los lóbulos expandiéndose hacia la derecha y la izquierda, y en consecuencia una polaridad a medida que el gametofito se desarrolla. El transporte intercelular a lo largo del gametofito es extremadamente importante ya que el desarrollo normal del gametofito maduro depende de la localización de la célula apical; con la supresión simultánea de esta condición en todas las demás células. El gametofito de helecho puede servir como modelo para el dominio apical en plantas superiores, y el papel de transporte intercelular podría jugar esto.

El aumento de GA_3, observado en los gametofitos en forma de espátula, podría estar relacionado con la expansión celular que se produce en esta etapa, y que se ha atribuido a las giberelinas, en especies de helechos sexuales como *B. spicant*, *Anemia*

phillitidis y *P. aculeatum*. Los datos obtenidos comparando los efectos de la fitohormona y un inhibidor de su biosíntesis: el flurprimidol, refuerzan la idea de la influencia de las giberelinas en la transición unidimensional a bidimensional del protalo. Este hecho, está contrastado en plantas con semilla. El efecto del inhibidor disminuyendo el tamaño del embrión, podría hacer pensar en alguna conexión entre las giberelinas y la apogamia, sin embargo, el que la adición exógena de GA_3 al medio de cultivo no afecte al proceso reproductivo, podría significar que otra giberelina pueda tener una función más decisiva, como GA_4 y GA_7, cuyos niveles sí aumentaban cuando el centro apogamous comienza a desarrollarse, como así se evidenciaba en trabajos precedentes, y que estaría también respaldado por los resultados obtenidos en el capítulo 3 de la presente memoria.

La poliamina (espermidina) añadida al medio de cultivo, favoreció la transición y la expansión lateral de los lóbulos, siendo los gametofitos más anchos que más largos. No obstante, estos resultados no fueron neutralizados por la adición de su inhibidor al medio, no quedando claro el papel de estos compuestos en el desarrollo vegetativo del gametofito. En cuanto a la apogamia, la espermidina parece influir en el desarrollo embrionario, junto con su inhibidor, la CHA, en la inducción de la apogamia.

Seguidamente, al comparar gametofitos apogámicos frente a gametofitos sexuales, hasta siete de catorce fitohormonas analizadas, se acumularon más en el gametofito sexual respecto al apogámico, incluyendo la auxina AIA, las citoquinas *trans*-Z, RZ, IPA, la giberelina GA_4, y los brasinosteroides: epibrasinolido y su precursor la castosterona, aunque sólo una muestra fue analizada en el primer caso. Las citoquinas isoprenoideas son características de musgos y helechos, y su presencia parece ser relevante también en helechos, siendo interesante echar un vistazo a estas vías biosintéticas en la evolución de las plantas. En *D. oreades*, GA_4 parece tener un mayor protagonismo en el desarrollo del gametofito sexual y asexual, siendo especialmente mayor en las especies sexuales. Ello podría estar relacionado con un comportamiento reproductivo dependiente de anteridiógenos, como se discutió en el capítulo 2. En cuanto al ácido abscísico, su papel resulta más incierto.

Sin duda, uno de los datos más sorprendentes fueron los altos niveles detectados de brasinoesteroides, que ponen sobre la pista de algún papel en el desarrollo sexual de gametofitos en *D. oreades* por parte de estas fitohormonas. Se deben realizar más investigaciones para dilucidar en detalle la relación entre los brasinoesteroides y la reproducción del gametofito, así como una posible relación con auxinas y giberelinas, entre otras posibles fitohormonas.

En los cultivos homogeneizados de gametofitos, algunas células fueron capaces de dividirse y formar masas celulares desorganizadas, tipo callo. La inducción del callo en esta subespecie apogámica representa un buen resultado en sí mismo, pues representa una herramienta biotecnológica adecuada para lograr la transformación estable por bombardeo de partículas como se ha visto recientemente en el helecho *Ceratopteris richardii*, y podría ser útil para validar genes candidatos de la apogamia.

Por último, en el capítulo 4 se realizó un transcriptoma de gametofito de alta calidad en helecho apogámico *D. affinis* ssp. *affinis*, y con él se porta el primer análisis de expresión diferencial entre gametofitos uni y bidimensionales, con el objetivo de alcanzar una mejor comprensión de la regulación génica subyacente al desarrollo gametofito y la apogamia, en esta especie. Sin duda *Dryopteris affinis* ssp. *affinis* puede ser considerada como una especie modelo para estudiar la apogamia a pesar de su complejidad genética, y gracias a las nuevas tecnologías.

Los genes expresados diferencialmente en gametofitos unidimensionales estaban enriquecidos en GO´s relacionados con la "respuesta al estímulo ambiental" y los procesos de "defensa". Además, también se encontraron relacionados con biosíntesis de poliaminas y el catabolismo del precursor de la arginina, sugiriendo una necesidad de protección pero también una posible relación con un evento apogámico, como se comenta en el capítulo 4. Aparentemente, las poliaminas parecen tener un papel importante en el desarrollo de gametofitos en general, pero se deben llevar a cabo más estudios para determinar con mayor precisión su función biológica.

Tras el crecimiento unidimensional, el gametofito debe ganar en complejidad, gracias a la expresión de genes implicados en aquellos procesos de importancia fundamental para definir el aspecto acorazonado, basado en planos de divisiones orientados y patrones de diferenciación. Entre ellos, se encontraron genes relacionados con división celular asimétrica, la determinación de la identidad dorsal y reguladores transcripcionales del desarrollo de meristemo como SMT, y otros pertenecientes a la familia NAC (NAM) involucrados en el desarrollo de plantas, incluyendo la formación del meristemo apical de brote o SAM. Otro estímulo importante, relacionado con la apogamia sería el azúcar, y que se ha visto fuertemente enriquecido en términos GO en gametofitos bidimensionales entre las categorías de procesos biológicos, fortaleciendo su papel en este proceso.

Finalmente, en esta especie se contaron anotaciones de dominio proteico que implicaban la regulación epigenética en la apogamia. En este estudio, identificamos

algunos ortólogos de proteínas MULTICOPY SUPPRESSOR OF IRA1 (MSI1) y *Enhancer of zeste* [E(z)], tanto codificando subunidades PCR2, como *Enhancer Polycomb-like* (proteínas Epl1); un miembro de un complejo de histona acetiltransferasa involucrado en la activación transcripcional, que se expresa más en gametofitos bidimensionales frente a unidimensionales. Otros, incluyendo componentes de la vía de degradación de la histona mediada por ubiquitina. Además, nuestro análisis identificó cuatro genes relacionados con la regulación epigenética a través de ARN-pequeños, reguladas en gametofitos bidimensionales, que codifican las proteínas de dominio Paz, Piwi y Dufi785 que están asociadas con las proteínas ARGONAUTE y DICER-LIKE en *A. thaliana*.

El gametofito de helechos es una estructura comprometida con la reproducción, encontrando genes involucrados en el desarrollo del polen, la segregación cromosómica meiótica, y la regulación del ciclo celular entre otros. Otra interesante familia genética que está fuertemente regulada en gametofitos bidimensionales codifica proteínas similares a FRIGIDA similares a FRIGIDA en *A. thaliana*, y algunas más mencionadas en el capítulo 4.

La combinación de diferentes enfoques, fisiológicos y -o*mics*, es una forma prometedora de obtener una imagen completa de los procesos regulatorios. El estudio de los mecanismos moleculares de la reproducción asexual es un tema importante de cara a introducir híbridos autosostenibles en la agricultura. Por lo tanto, la introducción de apomixis tiene un enorme potencial de mejora de cultivos, y extender nuestros análisis a ramas filogenéticas distintas de las de las especies modelo puede ayudar a desentrañar los procesos subyacentes comunes a una amplia gama de organismos.

Conclusiones

1. El sistema experimental utilizado en este trabajo junto con la adición de ciertas fitohormonas e inhibidores de su síntesis o transporte, proporcionaron una mayor respuesta en número de variaciones inducidas sobre el desarrollo vegetativo y la apogamia, en el gametofito de *Dryopteris affinis* ssp. *affinis*, que utilizando la espora o todo el gametofito.
2. Un balance equilibrado de auxina/citoquina favorable a IBA, aumenta el alargamiento del gametofito, la frecuencia de la apogamia y el tamaño de los embriones. GA_3 muestra un papel importante en el desarrollo vegetativo del gametofito, promoviendo la elongación, hecho refrendado por el empleo del

inhibidor de la biosíntesis de GAs, flurprimidol. Aunque la aplicación de flurprimidol inhibe la apogamia, no está clara la posible implicación de los GAs ya que la adición de GA_3 al medio de cultivo, no tuvo ningún efecto. El papel de la espermidina tanto en el desarrollo vegetativo como en la apogamia, es controvertido.

3. Se ha conseguido un protocolo válido para inducir y proliferar el callo de *D. affinis* ssp. *affinis*, que puede conducir a un sistema biológico para profundizar en la apogamia, y también para llevar a cabo más análisis moleculares sobre este tema.
4. El contenido de fitohormona varía entre los tres estadios de desarrollo del gametofito apogámico (filamento, espátula y corazón), y entre el gametofito acorazonado asexual y sexual del progenitor *D. oreades*. En el primero, se observó que la citoquina BA y el ácido salicílico, aumentaban sus niveles en el estadio filamentoso, la giberelina GA_3 en el espatulado, y finalmente se observa una ligera variación de RDHZ en los gametofitos cordiformes. Por su parte, el gametofito sexual femenino, dio mayor espectro de fitohormononas identificadas, incluyendo la auxina AIA, las citoquinas Z, RZ, IPA, la giberelina GA_4, y los brasinosteroides analizados.
5. Los perfiles transcriptómicos de gametofitos unidimensionales y bidimensionales revelaron un total de 10.679 genes que se expresaron diferencialmente, 6.110 en gametofitos unidimensionales y 4.570 en gametofitos bidimensionales. Los genes estaban asociados a procesos de defensa, desarrollo, reproducción, señalización de fitohormonas y regulación epigenética.
6. Se anotaron ortólogos de las proteínas SHOOT MERISTEMLESS y NAC, que apoya la idea de que el meristemo en el protalo de los helechos comparte ciertas características moleculares con SAM, el meristemo apical de angiospermas. Además, se refuerza el papel del metabolismo de la sacarosa como inductor de la apogamia, por el elevado número de genes identificados, asociados con el metabolismo de los carbohidratos en los gametofitos bidimensionales.
7. Se arroja nueva información sobre la transición uni a bidimensional del gametofito del helecho, que podría ayudar a comprender mejor la evolución de las plantas en tierra firme, y también la reproducción asexual en plantas en general, y la apogamia en helechos, en particular.