UNDERSTANDING DEVELOPMENTAL PROCESSES

IN EARLY-DIVERGING PLANT MODEL SYSTEMS

by

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Abstract

The study of evolutionary developmental biology relies on a detailed understanding of model systems. Whilst the flowering plants are the most successful and valuable plant group today, they do not tell us much about the change and progression that was initiated by an ancestral aquatic photosynthetic unicell millions of years ago. The expansion of bryophyte and algal model systems was developed as part of this research

The moss *Physcomitrella patens* is descended from the ancestral bryophytes that first colonised land. As such it is well-placed, as a model organism, to provide insight into terrestrialisation. The germination of spores or seeds is one of the key stages in the land plant life cycle. Comparison of the influences on spore and seed germination provides insight into the conservation of functions spanning 450 million years of evolution.

The role of phytohormones in the control of spore germination was assessed by analysing the response of *P. patens* spores to different exogenously applied hormones. Endogenous roles were explored using hormone biosynthesis mutants and semi-quantitative analysis of signalling genes.

This research shows that *P. patens* spore germination is regulated by some of the same hormones that regulate seed germination. The extent of regulation varies between hormone types but this has demonstrated previously unknown characteristics of the *P. patens* hormone signalling network.

This work also highlights the importance of establishing tractable model systems with robust methodological procedures.

This thesis is dedicated to my daughter Edith Rose Thomas for all the things she has taught me that I could never have learned in the lab.

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CHAPTER I:

INTRODUCTION

1.1 Project Introduction

This thesis investigates developmental processes in early-diverging plant model systems. The model Bryophyte *Physcomitrella patens* is used to study the essential process of spore germination and analyse the roles of identified phytohormones. The green macroalga *Ulva intestinalis* is investigated as a new model system for the study of plant evolutionary developmental biology.

The importance of plants, of model systems and an understanding of their biological processes is the driving theme behind this research and will enable the conclusions to be applied to future research in these fields.

1.2 The importance of plants

Plants control a multitude of processes that form the backbone of life on Earth. They are not only the basis of all food chains but also regulate our climate through carbon storage, release of oxygen and control of the water cycle. They provide over a quarter of all prescription medicine either directly or through derivatives and provide shelter for wildlife in an array of habitats (Wong, 2001; Butler, 2008)

Other than the obvious climatic, nutritional and ecological importance of plants they have a multitude of other day-to-day uses. As well as direct consumption by humans, plants also form the base of all food chains in nature and agriculture. 24% of the global crop production by mass is used as animal feed, equating to over 36% of global calorie production and as much as 53% of global plant protein production (Emily *et al.*, 2013).

In addition to their familiar uses as clothing, building materials and beauty products, the coupling of plant capabilities with technological processes and advances has resulted in an array of exploitative and essential plant-based biotech innovations.

Since the introduction of morphine to the commercial market in 1826, plant derived pharmaceuticals have contributed enormously to the prevention and treatment of human disease. The use of plant-based remedies for health began thousands of years ago and is still important today, with over 80% of the population still using them as primary medicine (Wootton, 2006). Moreover, pharmocognostic studies have isolated, refined and even synthesised the active components, resulting in a multi-billion pound drugs market. The approximate 120 plant-derived drugs in use today were originally obtained from just 90 plant species (Rates, 2001). With a conservative estimate of over 400,000 plant species currently on Earth, the potential for discovery of more plant-based pharmaceuticals is vast.

The negative associations of burning fossil fuels and their finite nature has increased the popularity and focus on alternative energy including biofuels. Biodiesel and bioalcohol both offer alternatives to fossil fuels and the use of plants such as soybean and rapeseed account for a large percentage of production. There are also significant advances being made in the generation and refinement of algal biofuels (Allen *et al.*, 2013). With a finite amount of fossil fuels available, the option of plant-based alternative energy is both desirable and achievable (GenomeWeb, 2010).

Relatively recent biotechnological advances such as plant-based pharmaceutical production (pharming), bioremediation, iron seeding and artificial photosynthesis demonstrate the physical and informative importance of all plant groups (Yao *et al.*, 2015; Santiago *et al.*, 2004; Listorti *et al.*, 2009; Barber and Tran, 2013).

1.3 The importance of plant science

With such a heavy dependence on plants, it is essential to understand how they work. From an anthropocentric point of view, in order to manipulate and exploit plant form and function, we must first understand why that form exists and how it performs those functions.

The study of plants has provided some of the most important discoveries in the history of science: Gregor Mendel's study of heredity; isolation of the first virus from tobacco; discovery of transposable elements; and the linking of microorganisms as the source of disease to name a few (Bos, 1999; McClintock, 1950). Plant biology also has a profound societal, economic and political impact on our nations. Over 1 million people emigrated from Ireland during the potato famine (Ross, 2006). Over 670,000 children under the age of 5 die from vitamin A deficiency every year and as many 190 million are recognised as deficient by the World Health Organisation (World Health Organization 2009). Food security through GM crops and biofortification are all options available to us due to years of research into plant science (Nestel *et al.*, 2006; Qaim and Kouser, 2013; Zhu *et al.*, 2007). Humanity has a critical and growing reliance on plants and a better

understanding of how they work enables us to improve their usefulness to benefit society.

The impact of historical plant research is often underappreciated. Most of the vegetation we consume today is vastly different to the ancestral species first manipulated by early botanists. The development of crop plants has made it possible to feed the current population of 7.5 billion people. Complex breeding programmes and more recently, targeted genetic manipulation has provided us with highly nutritious and more economically produced crop plants (Varshney *et al.*, 2005; Burger *et al.*, 2008). The development of genetic manipulation as a tool in plant research has many current and future applications (Qaim and Kouser, 2013; Zhu *et al.*, 2007; Gosal and Kang, 2012). Disease is a major threat to crop plants in particular and in the case of bananas, where minimal genetic diversity exists, resistance would only be possible through insertion of genetic material from wild species. The development of such techniques has been and will continue to be the saviour of many plant species (Strange and Scott, 2005; Ghag and Ganapathi, 2017).

We now understand how delicately balanced our ecosystems can be and the impact that plants have on the stability and longevity of habitats. Research on conservation and resource management has provided a wealth of useful information for scientists and policy makers, the importance of which is constantly increasing in light of global climate change.

Concerns about the impact of climate change on plants have also resulted in multiple safety nets being established. Centres such as Kew's Millennium Seed Bank (Millenium Seed Bank Partnership, 2016), Svalbard Global Seed Vault and BGCI's Safety Nets Project have the capacity to store millions of seed samples. But it is only through decades of plant research that we know *how* to store them and more importantly, how to successfully germinate them when required. With over 400,000 known plant species, the variety in seed type is enormous. Research into areas such as seed production, dormancy and germination are essential to ensure the success of these centres.

1.4 The study of plants

In order to understand plants, one must demonstrate where they have come from (their evolution), study representative examples (model systems) and explore and elucidate the key processes that make them what they are. This research aims to tackle these key points by looking at recently developed model systems, their evolutionary standing in plant developmental biology and using that information to expand our knowledge of essential processes such as germination.

1.5 The Evolution of Plants

1.5.1 Green plant systematics

The Viridiplantae or plants are distinct from other kingdoms due to the presence of chlorophyll, cellulose in the cell wall and the use of starch as polysaccharide storage (Leliaert *et al.*, 2012; Raven *et al.*, 2005). The Viridiplantae are split into the chlorophytes and streptophytes (figure 1.1) (Leliaert *et al.*, 2011). The streptophytes are

made up of the embryophytes (land plants) and charophytes - a group of multicellular freshwater algae. The chlorophytes contain all other green algae, freshwater and marine. Embryophytes and charophytes are distinguished from chlorophytes by the presence of a phragmoplast which serves as a scaffold for assembly of the cell plate during cell division (Leliaert *et al.*, 2011). Research on characteristics such as glycolate metabolism and cellulose synthesis suggests that charophyte algae and land plants shared a more recent common ancestor than charophyte and chlorophyte algae (Frederick *et al.*, 1973; Jacobshagen and Schnarrenberger, 1990; Graham *et al.*, 1991). It is therefore assumed that land plants evolved from a charophyte ancestor similar to the Charales (stoneworts) that then made the permanent transition to land (Lewis and McCourt, 2004; Bowman, 2013; Delwiche and Cooper, 2015).

Due to the freshwater habitat of the charophyte algae, physiological differences may have made them more adaptable than their predominantly marine sister group. Bodies of fresh water tend to be smaller and more susceptible to periods of drying. They are generally shallower and as such will face greater fluctuations in pressures such as temperature and UV radiation. The natural selection of plants that were able to adapt to these environmental conditions would have led to characteristics and features suited to a terrestrial existence.

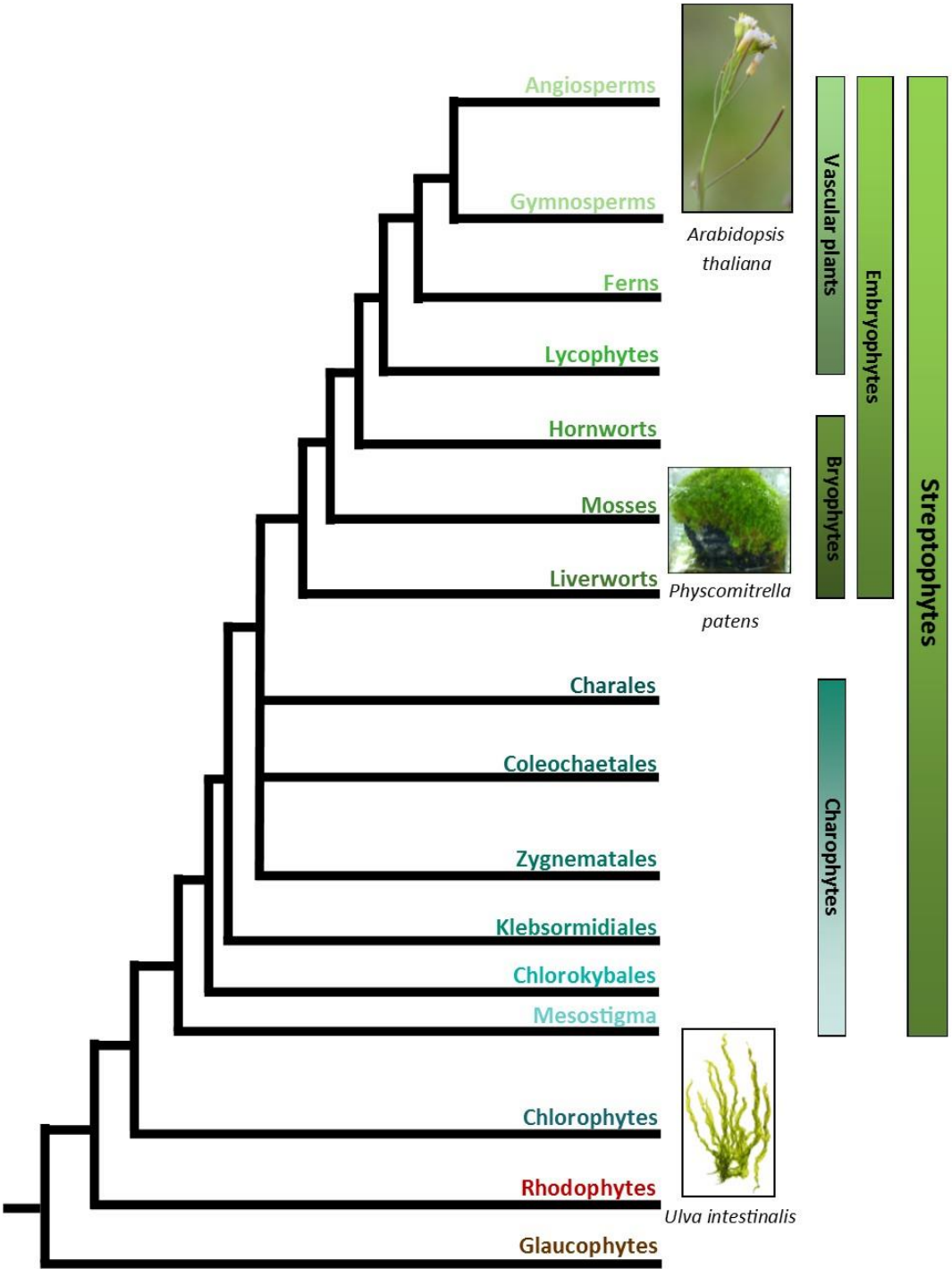


Figure 1.1 The Viridiplantae consists of the chlorophytes and streptophytes. The presence of chlorophyll b as the main photosynthetic pigment distinguishes them from other kingdoms. The vascular plants and non-vascular bryophytes make up the land-dwelling embryophytes and the charophytes are a group of multicellular freshwater algae containing the ancestor of all land plants.

1.5.2 Aquatic origins of plants

The origin of photosynthesis was the first key step in plant evolution and fossil evidence places this around 3000 million years ago (mya) in an aquatic prokaryotic cyanobacteria (Douglas, 1998; Adrian Reyes-Prieto *et al.*, 2007). The establishment of an endosymbiotic relationship between cyanobacteria and other prokaryotic organisms produced the first recognisable photosynthesising eukaryote.

During evolution, plants went through a further series of key steps that resulted in the plethora of forms we see today (Graham, Cook *et al*. 2000; De Smet, Beeckman 2011; Price, Chan *et al*. 2012; Spiegel 2012; Pires and Dolan, 2012) . Movement onto land, the establishment of a vascular system and the development of seeds and flowers all shaped the evolutionary tree (figure 1.2).

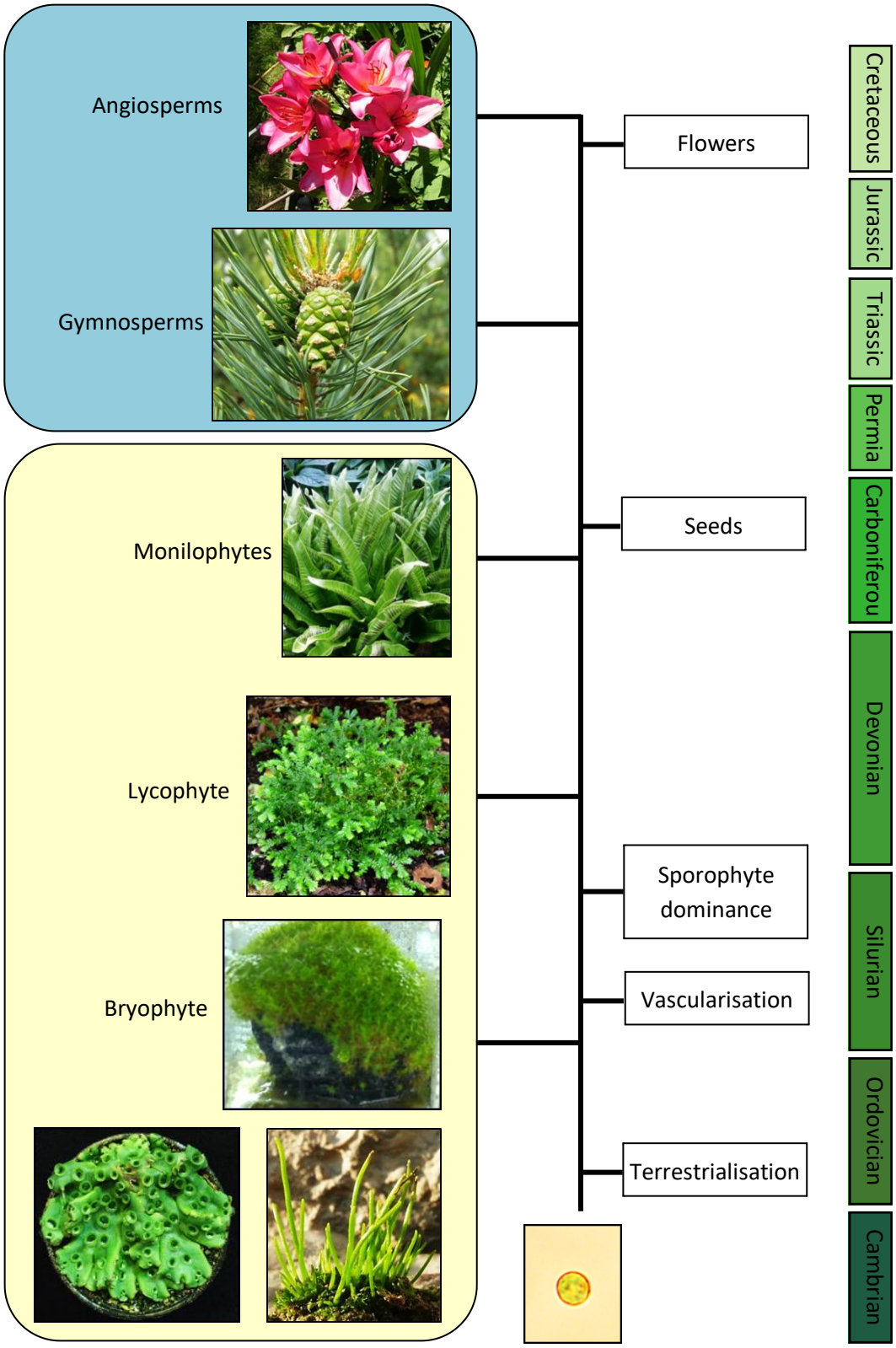


Figure 1.2 Major transitions in plant evolutionary developmental biology

1.5.3 Terrestrialisation of plants

Like all life, plants were solely aquatic for most of their evolutionary history. Evidence of plant tissues in the Cambrian period (485-541 mya) is minimal due to the soft-bodied nature of early plants. However by the Ordovician period (443-485 mya) the presence of desiccation resistant spores in the fossil record provided definitive evidence of true land plants (figure 1.2) (Wellman *et al.*, 2003; Gensel, 2008; Rubinstein *et al.*, 2010; Lenton *et al.*, 2012; Edwards and Kenrick, 2015)

The leap from an aquatic to a terrestrial environment presented many challenges. As sessile organisms, plants could not escape these challenges and had to deal with them head on. Major body plan changes were needed to adjust to the new and intense stresses that were being placed on them (Graham *et al.*, 2000a; Jill Harrison, 2017). One of the greatest threats was desiccation. Unlike in an aquatic environment, water supply was neither reliable nor plentiful. Plants also needed to find ways to extend their reach in terms of sourcing nutrients as well as water. In an aquatic system essential minerals such as nitrates and phosphates could be found dissolved in the water. On land these were not as accessible, often buried in the soil. Similarly the oxygen source was permanently altered; plants had to develop a way to absorb it from the air. Living in air also meant plants had to be able to support themselves. In water they are naturally buoyant and therefore have no need to develop any specialisations for support. The final great challenge was reproduction. Aquatic species have aquatic germ cells, flagellated gametes and spores, which when coupled with the natural movement of water make it relatively easy for them to reproduce by simply releasing hundreds of

germ cells and letting the currents do the rest. Land plants had a multitude of problems to face when it came to reproducing. Without water to move through, flagellated gametes were not fit for purpose and plants had to find new ways to disperse their sex cells. Once gametes had managed to meet, they were not necessarily in a hospitable enough environment to germinate and grow. Adult plants had to find a way to protect the next generation and provide suitable conditions for it to germinate and develop. So the emergence from water, whilst providing a huge increase in the number of available habitats, presented some extreme and combinatory pressures which land plants had to cope with. In the end they not only coped but flourished, radiating out to colonise all continents and produce the thousands of extant species we see today (Graham *et al.*, 2000a Kenrick and Crane, 1997).

1.5.4 Roots, shoots and leaves

The body plan of land plants changed significantly to cope with the pressures outlined above (Graham, 1993; Graham, 1996; Graham *et al.*, 2000a). Tissues became three dimensional and more robust. Apical cell proliferation allowed complex branching, which along with widespread cell specialisation allowed development of structures such as roots, vascular tissue and stomata (Langdale and Harrison, 2008). Early vascular plants are evidenced in the Silurian period (419-443 mya) (Gensel, 2008) but it is the Devonian period (358-419 mya) that saw a rapid colonisation and diversification of truly vascular plants (Kenrick and Crane, 1997; Bateman *et al.*, 1998). During the Carboniferous and Permian periods (252-359 mya) many new plant groups appeared including the

lycophytes, ferns and eventually gymnosperms (cycads, conifers and gnetophytes) which then dominated the Triassic and Jurassic periods (145-252 mya) (figure 1.2).

In terms of reproduction, the alternation of generations reversed once plants moved onto land (figure 1.3) and the two generations became morphologically distinct and highly specialised (Graham *et al.*, 2000b). The gametophyte generation is considered to be evolutionarily older as evidenced by the requirement of water for sperm motility (Bennici, 2008). Over time the sporophyte generation became more dominant (Blackwell, 2003) and the male and female gametophytes reduced to the pollen grains and eggs that we see in the most recently evolved land plants, the angiosperms.

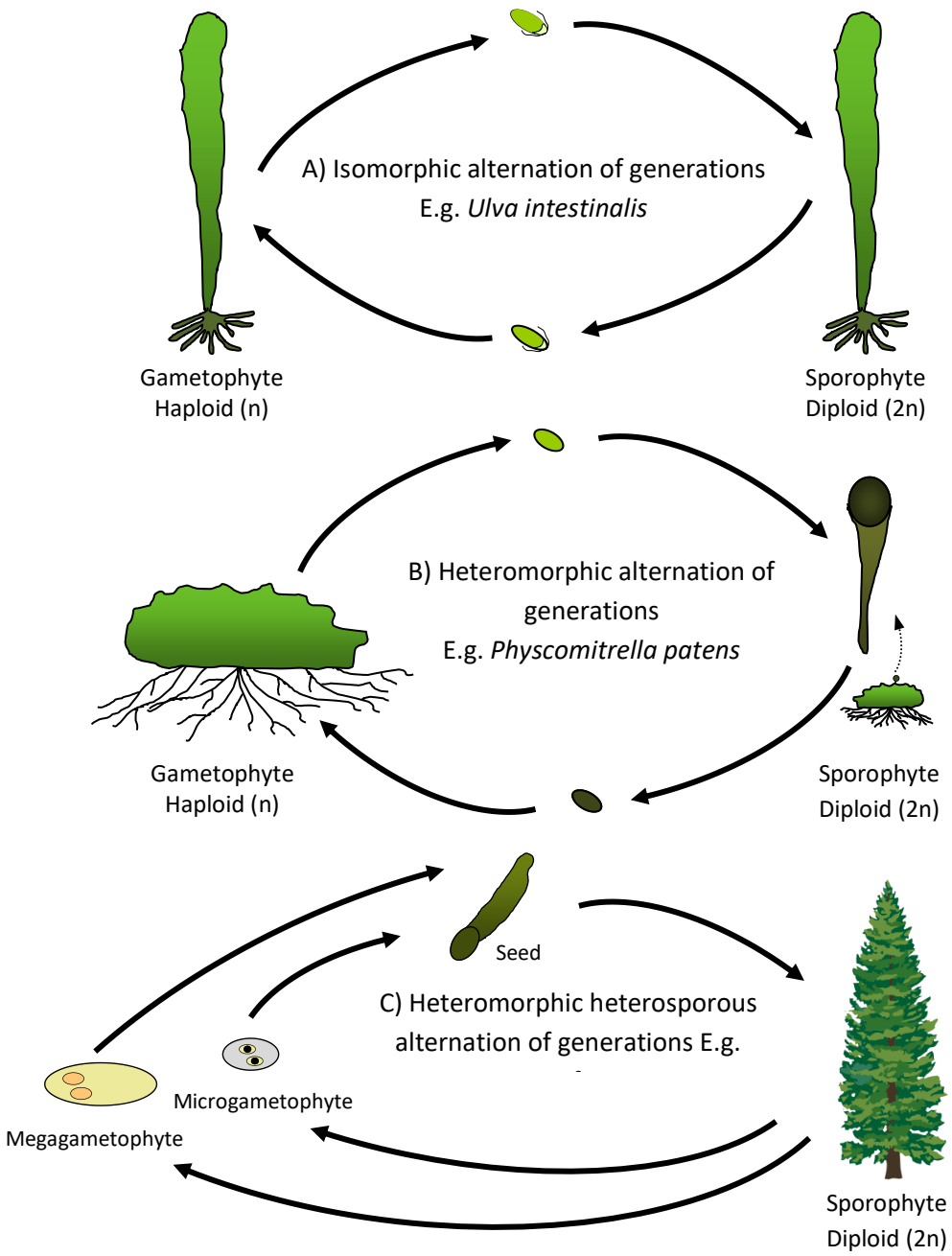


Figure 1.3 Life cycle of organisms with isomorphic and heteromorphic alternation of generations. A) Gametophyte and sporophyte of an aquatic multicellular species such as *U. intestinalis* are very similar in appearance and can only be distinguished by chromosome number or during reproduction. B) Early terrestrial plants such as *P. patens* have a dominant gametophyte generation with a retained sporophyte. C) Seed plants such as conifers produce two types of spores (megaspores and microspores) which produce the highly reduced megagametophyte and microgametophyte respectively. Fertilisation results in an embryo enclosed in a seed ready for dispersal

1.5.5 The angiosperms

Angiosperms appeared during the Cretaceous period (66-145 mya) (Friis *et al.*, 2006) but did not flourish until the end of the period and into the Cenozoic (66 mya to present). They now represent 96% of all vascular plants and are the most diverse extant land plant group. Their success was a result of the evolution of flowers (Friis *et al.*, 2006) and an enclosed seed - angio is from the Greek for vessel (*angeion*) and sperm from the Greek for seed. The monocots (e.g. grasses, orchids and palms) separated from the other angiosperms (eudicots) approximately 35 mya so providing the final step in the establishment of all extant plant groups.

The movement of plants onto land caused major biogeochemical changes to Earth and its atmosphere (Berner, 1997; Algeo, 1998; Kenrick *et al.*, 2012). Vegetation shaped terrestrial ecosystems, creating new and diverse habitats. Atmospheric carbon dioxide levels were dramatically reduced through carbon burial and chemical weathering (Mora *et al.*, 1996; Berner, 1997; Lenton *et al.*, 2012) and oxygen levels increased due to photosynthesis (Berner *et al.*, 2007). All of these changes set the stage for colonisation by early animal life and this primary production is still the basis of all food chains supporting organisms from all taxonomic groups.

1.6 Plant evolutionary developmental biology

Theophrastus presented the first clear principles of botany in 320BC. One of the themes he stressed the importance of was an understanding of the mode of generation and life cycle. Understanding of the developmental stages in plants was increased by the

observations of Nehemiah Grew (1671) and Marcello Malpighi (1675) on the transitions from seed to mature plant. The sexual nature of plant reproduction was confirmed by Rudolf Camerarius and when furthered by Wilhelm Hofmeister's elucidation of the alternation of generations enabled the field of plant developmental biology to take shape.

The study of developmental processes tells us a lot about how that organism functions, but when observed from an evolutionary perspective it also tells us how that organism relates to its ancestors and how those developmental processes have changed over time. The precise classification system developed by Linnaeus in the eighteenth century and the emergence of Darwinism in the nineteenth enabled an evolutionary slant on developmental biology which was enhanced by the rediscovery of Gregor Mendel's Laws of Inheritance. Evolutionary developmental biology (evo-devo) incorporates developmental morphology, systematics, developmental genetics, phylogenetics, fossil studies and molecular genetics and has implications in multiple scientific areas. It observes the stages of plant development, compares those stages with other plants it is related to and, more recently, identifies the genetic changes that have taken place to allow the modifications of developmental processes that led to the establishment of a new species.

With an ever-increasing number of plant genomes being sequenced (Bowman *et al.*, 2007), comparative genomics is coming to the forefront as a way of answering major questions in plant evolutionary developmental biology. A combination of these molecular biology techniques with traditional plant physiology, cell biology, biochemistry and

ecology provides a comprehensive understanding of the value of plants and their essential role in the maintenance of our planet.

1.7 Plant model systems

1.7.1 The importance of model systems

Our understanding of the key stages in plant evolution creates an appropriate list of relevant plant groups that can inform evolutionary developmental biologists and other related fields (Coates, 2016).

Model organisms allow the detailed study of life cycles, processes and behaviour of a system with the view to applying knowledge gained to other related organisms. Model organisms are often selected due to their suitability to a laboratory-based environment, their ease of maintenance and culture and their relevance and importance in comparison with other similar systems.

It is not feasible to study every extant biological system, so biologists select key examples that can provide insight into a range of similar systems. In animal systems for example, mice, pigs and chimpanzees are used as models for mammal systems. Their possession of defining mammalian features and the reaction of those features to experimental conditions provides insight into the functioning of the group as a whole and the applicability of conditions to other organisms such as humans.

In plant systems, movement onto land, development of vasculature and evolution of seed and flowers represent key innovations and defining features that facilitate the grouping of plant species and the identification of relevant plant model organisms.

1.7.2 Angiosperm model systems

Angiosperms can be further subdivided into monocots and dicots. One of the most economically important monocot orders is the Poaceae or grasses. The grasses *Brachypodium distachyon*, *Oryza sativa* and *Zea mays* are excellent models for cereal crops. Some published genome sequences, short life cycles and ease of culture make them fairly straightforward systems as models for the multiple crop plants that are so essential for humans.

Arabidopsis thaliana is a dicot and historically the most studied angiosperm system at a molecular level. It is a member of the economically important Brassicaceae family and was the first plant to have its genome sequenced (Initiative, 2000). It has a small genome, can complete its entire life cycle in six weeks and can be cultured in a variety of laboratory methods. As a representative flowering plant it can tell us a lot about the process of flowering and the environmental and genetic influences that drive it. The presence of roots, vascular tissues and leaves with stomata allows evolutionary developmental biologists to compare it with all other land plants as the most recently evolved group. The production and subsequent germination of seeds also allows comparison with the preceding evolutionary group, the gymnosperms.

1.7.3 The gymnosperms

Gymnosperms are characterised by the situation of their seeds, exposed on modified leaves or stalks, in comparison to the enclosed seeds in the angiosperm ovary. The group is dominated by the conifers which includes the extensively used pines. However, their large size, long life cycle and large genome do not make them ideal model organisms. Despite this, the genome of *Picea abies* or Norway spruce was sequenced in 2013 (Nystedt *et al.*, 2013) and multiple members of the family are intensely studied due their economic and medicinal value.

The spermatophytes encompass all plants that produce seeds to disperse the next generation, the evolution of which can be dated to approximately 365 mya. Prior to that, dispersal was facilitated by spores. This method is a feature of all other land plants including the vascular lycophytes and monilophytes and the non-vascular bryophytes.

1.7.4 Vascular plant models

Monilophytes (ferns and horsetails) are distinguished from lycophytes by their possession of megaphyll leaves with branched veins. The most extensively studied fern *Ceratopteris richardii,* is relatively easy to culture in a laboratory environment and is being established as a tractable genetic model by recent advances such as efficient transformation (Plackett *et al.*, 2014; Bui *et al.*, 2015). However a large genome size has hindered sequencing attempts, limiting its capacity as a model system. As a representative of the closest extant sister group to seed plants however, it can tell us a

lot about key features such as production of vascular tissue and life cycle processes such as sex determination.

The lycophytes are distinct from the monilophytes by having simpler microphyll leaves with a single unbranched vein. The genome sequence of the model species *Selaginella moellendorffii* (spikemoss) was published in 2011 (Banks *et al.*, 2011) and when coupled with a short life cycle and ease of culturing this provides an ideal system for the study of this key evolutionarily ancient lineage. It represents the oldest extant vascular plant lineage and as such provides a wealth of comparative genomic data on the transition from a gametophyte to sporophyte dominated life cycle and the development of vasculature.

The presence of vascular tissue in both monilophytes and lycophytes distinguishes them from the non-vascular bryophytes.

1.7.5 The bryophytes

The bryophytes (mosses, liverworts and hornworts) are all non-vascular land plants that reproduce using spores and possess no true roots, leaves or lignified vascular tissue. They are normally relatively small ephemeral plants that, as the first group of land plants, still retain a dependence on water to facilitate reproduction and resist desiccation. Within this group, model organisms such as the moss *Physcomitrella patens*, the liverwort *Marchantia polymorpha* and the newly emerging hornwort model *Anthoceros agrestis* enable exploration of the key features of bryophytes, comparisons within it and with all other land plants. All three species can be cultured axenically in the

laboratory and manipulated to reproduce both sexually and vegetatively. The availability of the *P. patens* and *M. polymorpha* genomes (Rensing *et al.*, 2008a) and protocols for establishment of stable transformation of somatic cells (Schaefer *et al.*, 1991; Takenaka *et al.*, 2000; Kubota *et al.*, 2013; Alam and Pandey, 2016) has enabled extensive comparison of molecular, genetic, physiological and life cycle processes between this basal plant group and the more complex seed and flower-bearing plant groups.

1.7.6 Algal models

The aquatic ancestry of all land plants has drawn evolutionary biologists further back in time to try to establish when key features such as multicellularity, sexual reproduction and heteromorphic alternation of generations evolved.

The establishment of tractable algal model systems has been slower than in terrestrial lineages but species from key economically and systematically valuable groups are changing this and allowing elucidation of the major evolutionary steps that occurred before plants made the transition onto land. The Volvocacean algae such as *Chlamydomonas reinhardtii* and *Volvox carteri* provide a model for the molecular genetic basis of multicellularity (Miller, 2010; Nishii and Miller, 2010; Prochnik *et al.*, 2010). Brown and red algae such as *Ectocarpus* and *Porphyra* enable insights into broad phylogenetic questions as well as providing biological and genetic back-up for economically valuable algae-based industries. Multicellular green algae such as *Ulva spp.* provide many points of comparison with multicellular green land plants. The current *Ulva* genome sequencing project along with establishment of multiple species that can

complete the life cycle in laboratory culture will further plant biologist's abilities to answer fundamental evolutionary questions using model systems from key plant lineages (Coates, 2016).

1.8 Plant developmental processes

1.8.1 The evolution of life cycle strategies in plants

The availability of model organisms allows the investigation of a biological process from an evolutionary perspective. The plant life cycle has gone through vast changes in terms of relative complexity of the two generations. Figure 1.3 shows the progression from an isomorphic to a heteromorphic gametophyte then sporophyte dominated life cycle and the variation in the relative size and complexity of each generation.

1.8.1.1 Aquatic life cycle strategies

Land plants are characterised by an alternation of morphologically distinct generations between the haploid gametophyte and the diploid sporophyte. In aquatic ecosystems there are a range of life cycle strategies displayed by the many marine and freshwater species including isomorphic and heteromorphic and multicellular and unicellular gametophyte and sporophyte phases. Most green algae exhibit isomorphic gametophyte and sporophyte forms that release the next generation, gametes and spores respectively, directly into the aquatic environment to facilitate fertilisation and dispersal. For example the multicellular alga *Ulva intestinalis* (figure 1.3) has virtually indistinguishable generations that can only be identified by observation of chromosome number or during release of gametes or spores.

During the evolutionary process, mutations in one generation could lead to differential gene expression in the two generations which, over time, resulted in alternation of heteromorphic generations that were often distinct in both size and complexity. This variation in life cycle strategy can be observed across the green, red and brown algal groups. The green algal Charales (class *Charophyceae*) display many of the features of land plants such as oogamy, retention and encasement of the ovum, particular sperm morphology, apical growth and differentiated nodal and intermodal regions (Umen, 2014 Graham *et al.*, 2000a). In addition to this *Chara vulgaris* displays a heteromorphic alternation of generations similar to early land plants with dominance by the haploid gametophyte generation. In combination with DNA sequence data this has led to Charales being considered the closest living relatives of the first land plants (Brodie and Lewis, 2007).

1.8.1.2 Gametophyte dominant land plant life cycle

Plants with a gametophyte dominant life cycle exhibit a larger haploid gametophyte as the main 'plant' with the sporophyte being reduced in size and cell number in comparison. Reproductive organs are produced on mature gametophytes. Male antheridia and female archegonia develop on one plant in monoecious species (e.g. *P. patens*) or on separate plants in dioecious species (e.g. *Ceratodon purpureus*). Gametes are produced by mitosis in the gametangia and fertilisation is facilitated by water as the biflagellate sperm swims down the archegonial neck to fuse with the ovum. The resulting zygote develops into the diploid sporophyte through apoplastic matrotrophy, resulting in a mature sporangium containing spores at the end of the seta.

Spores are produced by meiosis from spore mother cells inside the sporangium. Spores are released by dehiscence of the calyptra as the sporangium dries. A mature sporophyte contains up to 4,000 haploid spores which can be spread by various methods including wind and water.

P. patens is the typical model system displaying the above alternation of generations and represents the dominant lifecycle option for the earliest land plants (figure 1.4). During the course of plant evolution the gametophyte generation was suppressed and the diploid sporophyte became the dominant recognisable plant (figure 1.3). An increase in the number of mitotic divisions in the sporophyte before meiosis occurs increases the number of spores that can be produced inside the sporangium. The lack of water for fertilisation for early land plants meant it would have been an advantage to produce more spores per fertilisation event. Consequently, the sporophyte generation evolved to be bigger, more complex and more long lasting at the expense of the gametophyte.

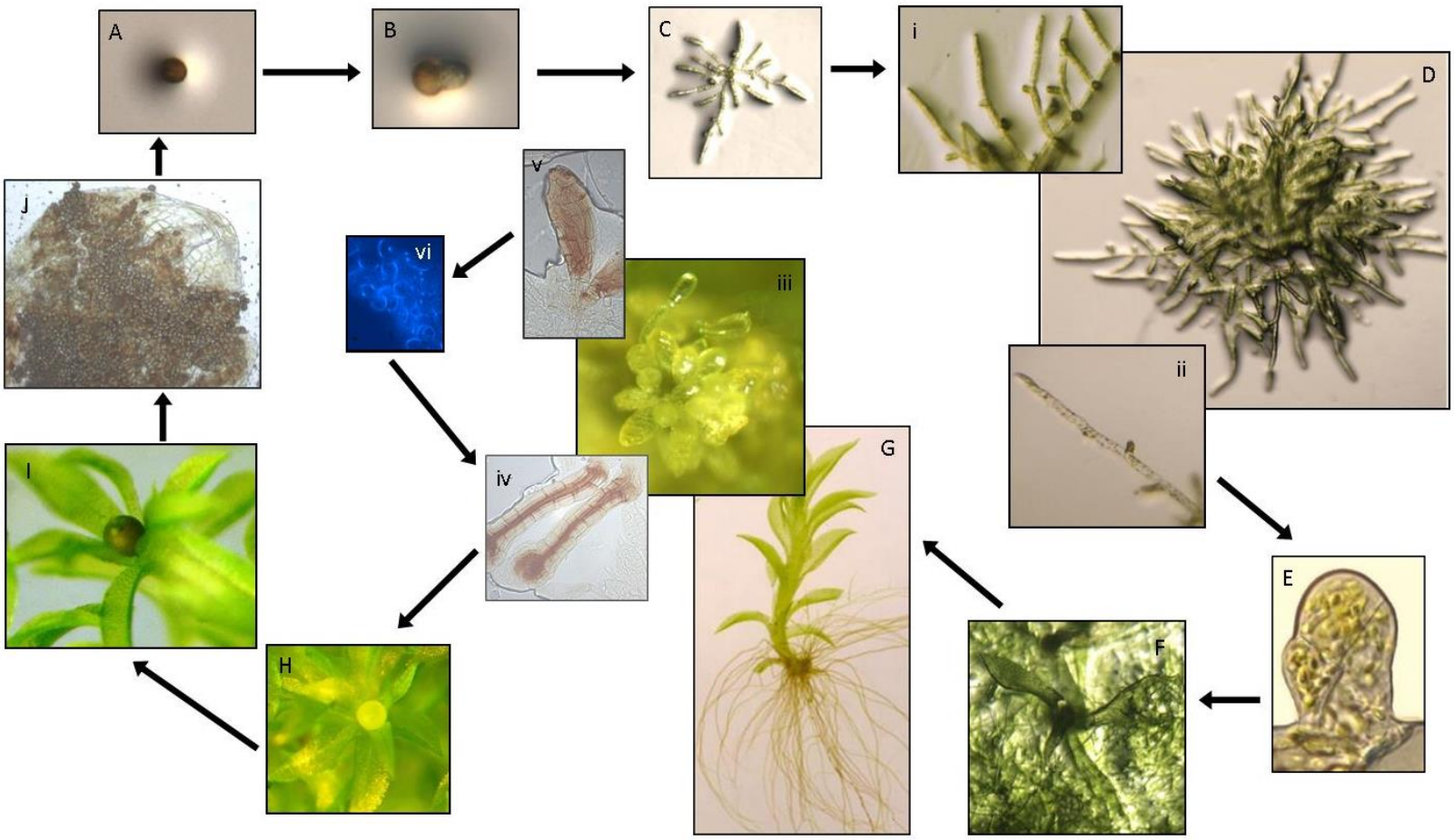


Figure 1.4 Lifecycle of *Physcomitrella patens*. A - ungerminated spore; B - germinating spore; C - early protonemal plant; D - established protonemal plant containing chloronemal (i) and caulonemal (ii) filaments; E - bud develops on caulonemal filament; F - leafy gametophore develops from bud; G - mature gametophore which will develop reproductive organs (iii) under the right conditions that develop into female archegonia (iv) and male antheridia (v) which produce flagellated sperm (vi) that swim down the archegonia and fertilise the egg to produce a zygote; H - immature green diploid sporophyte grows from zygote; I - sporophyte turns dark brown as it matures; J - sporangium ruptures to release 4,000+ haploid spores.

1.8.1.3 The evolution of sporophyte dominance in land plants

In early vascular plants, such as the extinct *Cooksonia*, features such as dichotomous branching of the stem and synthesis of lignin enabled the sporophyte generation to grow larger, produce multiple sporangia and become free living away from a permanent water source. Further specialisation of different parts of the plant led to the overall structure of the typical modern plant: roots, stems and leaves.

The progressive shrinking and eventual enclosure of the gametophyte within the sporophyte was accompanied by the switch from homospory to heterospory (Raven *et al.*, 2005; Taylor *et al.*, 2009). Bryophytes and some early vascular plants such as pteridophytes produce one type of spore through meiosis. Heterospory evolved in parallel in some lycophytes, ferns and all seed plants (Chaloner, 1967). The differentiation of sporangia into the highly reduced male microsporangia producing microspores and female megasporangia producing megaspores meant the resultant gametophytes developed inside the spore wall and, in a reversal of roles, became dependent on the mature sporophyte plant (Raven *et al.*, 2005). The pinnacle of this process of reduction, specialisation and enclosure was the complete retention of the gametophyte generation and dominance of the free living sporophyte. In angiosperms, the role and size of the gametophyte is reduced even further and encompassed into the multiple organs of the complex flower. The evolution of flowers allowed the angiosperms to increase the frequency of successful fertilisation events and expand genetic diversity through increased spread of microspores (pollen) (Raven *et al.*, 2005; Taylor *et al.*,

2009). This diversity enabled the subsequent radiation of flowering plants to colonise and dominate all continents on the planet.

1.8.2 Dispersal of the next generation

As the spore was the main method of dispersal in gametophyte dominant plants, an alternative strategy was needed to ensure the next generation grew at a distance from the parent in sporophyte-dominant plants. Seeds allowed dispersal of the next generation away from the parent, reducing competition for valuable nutrients, water and light and also facilitating colonisation of new environments leading to increased diversity and ultimately evolution of new species (Baskin and Baskin, 2014).

Seeds are the product of fertilisation, being composed of the embryo, a food store (endosperm) and a tough seed coat (Raven *et al.*, 2005). Their multicellular nature is in contrast to the unicellular spores found in early evolving plants such as the bryophytes. Despite this, the sporopollenin-walled spores and the multi-layered seeds are comparable in terms of their role as desiccation resistant dispersal structures.

Dispersal of spores and seeds is achieved by wind, water and animals such as mammals and insects (Dieter and Bouman, 1995; Howe and Miriti, 2004). The multicellular nature of seeds has facilitated the evolution of an array of morphological adaptations to aid dispersal (Linkies *et al.*, 2010; Yamaguchi and Nambara, 2007). Once a spore or seed had been dispersed it needs to germinate to release the next generation. In the case of both spores and seeds this involves multiple environmental and hormonal cues to ensure successful emergence and growth of the new plant

(Koornneef *et al.*, 2002; Kucera *et al.*, 2005; Bentsink, 2008; Footitt *et al.*, 2011; Gazzarrini and Tsai, 2015).

1.8.3 Germination

The array of plant forms and the availability of model systems provides multiple points of comparison throughout plant evolution. *P. patens* and *A. thaliana* represent plants at either end of the land plant evolutionary timescale - a basal, non-vascular, gametophyte-dominant, seedless bryophyte and a highly specialised, recently evolved, vascular, seed and flower bearing eudicot. For the purpose and scope of this research, these two systems provided ideal models. With the overwhelming majority of research in plant biology focusing on angiosperms such as *A. thaliana* the potential to compare knowledge of germination, a key stage in the land plant life cycle, in recently evolved plants with representatives of their distant ancestors was appealing.

Germination is the key point at which a new generation is established and begins its own life cycle for the purposes of further reproduction and ultimately survival and spread of its species. The timing and speed of germination is optimised for the species and its environment but varies widely. Germination takes place by cell division and cell enlargement in a pattern that is specific to the plant species. Key points include imbibition and rupture of the seed or spore coat.

1.8.3.1 *P. patens* spore germination

Figure 1.5 shows the stages of germination of *P. patens* spores under normal conditions. After imbibition, bulges in the spore coat are often visible before the rupture of the coat and protrusion of the first protonemal (chloronemal) filament. Germination is often bipolar with two protonemal filaments emerging in quick succession. Serial divisions of apical chloronemal cells and occasional division of subapical cells produces a branching protonemal plant. Caulonemal filaments emerge from a limited number of apical chloronemal cells and these extend and divide much more rapidly and ultimately go on to produce buds which develop into the leafy gametophores.

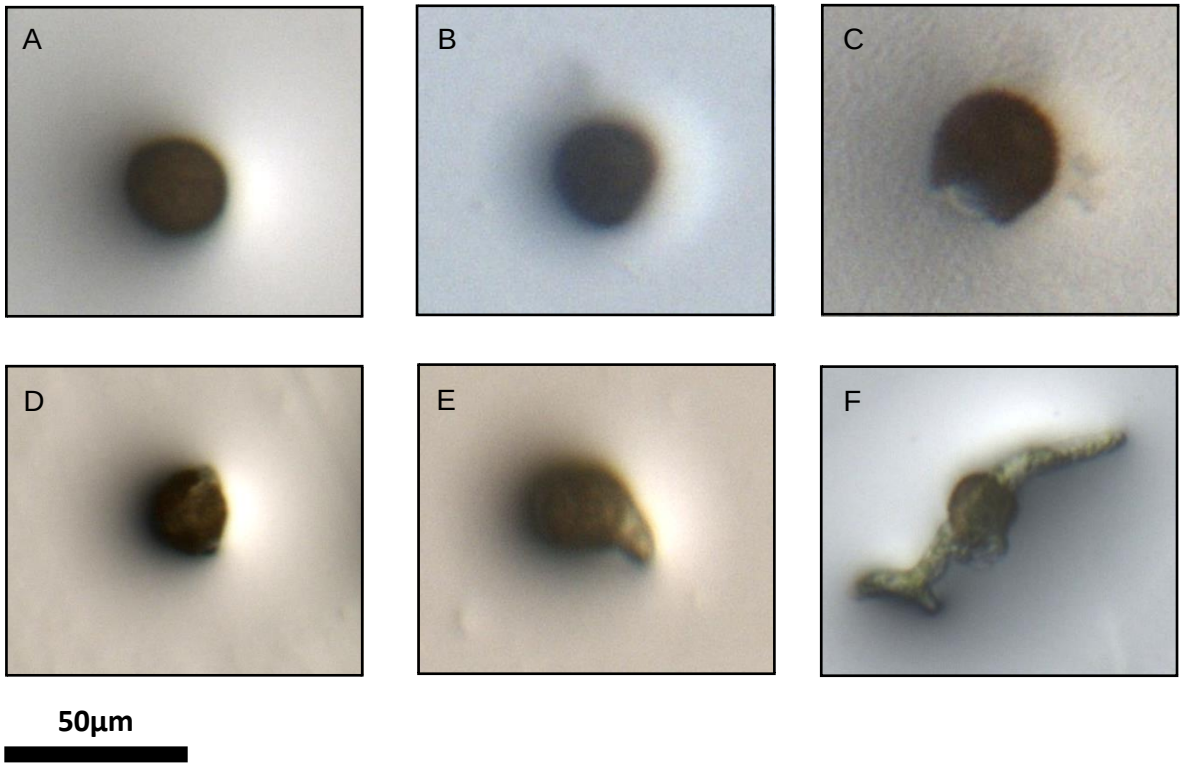


Figure 1.5 Early stages of germination of *P. patens* spores. Imbibed ungerminated spores (A) will start to bulge (B) before cracking of the spore coat (C) allows emergence of single (E) or multiple (D) protonemal filaments which will then branch and develop into caulonemal and chloronemal tissue (F).

1.8.3.2 *A. thaliana* seed germination

Germination of *A. thaliana* and most other angiosperm seeds is a carefully timed interaction between the multiple tissues within the seed (Koornneef *et al.*, 2002; Finch-Savage and Leubner-Metzger, 2006; Finch-Savage and Footitt, 2012). The seed coat (testa) ruptures first to reveal a small portion of the endosperm which itself then ruptures as the emerging radicle extends. Elongation of the hypocotyl region pulls the cotyledons up through the soil to reveal the typical early seedling stage of all eudicots. The cotyledons provide the energy for early growth until the first foliage leaves develop which take over the production of energy by photosynthesis. In laboratory conditions the progression from germination, through foliar growth to opening of the first flower takes approximately 32 days (Boyes *et al.*, 2001).

1.8.3.3 Germination is influenced by internal and external cues

The environment surrounding a spore or seed will vary in many factors such as temperature, the amount and wavelength of light, humidity and oxygen levels. These can all influence when germination occurs (Baskin and Baskin, 2001; Finch-Savage and Leubner-Metzger, 2006; Bentsink, 2008). A suite of metabolic processes are required for germination and the enzymes involved require water, oxygen and often a temperature range specific to that species. So the presence or absence of these external factors must be noted by the spore or seed so it can react when conditions are favourable.

Internally germination is regulated by plant hormones (Gazzarrini and Tsai, 2015). These mobile chemical signals allow communication between different plant tissues and also between different individuals, species and even kingdoms. The complex antagonistic relationships between different plant hormones and the fine balance of synthesis and degradation integrates the external environmental factors and the internal plant system.

1.9 Plant hormones

Hormone signalling is the most essential communication mechanism in the plant world (Davies, 2010). With limited mobility, plants must be able to respond to their environment by modifying their growth and behaviour. Hormones control fundamental processes such as germination, morphogenesis, reproduction and adaptation to environmental change, and also have more far-reaching roles within ecosystems, such as plant to plant communication and interaction with pollinators (Santner and Estelle, 2009; Davies, 2010; Durbak *et al.*, 2012a).

There are five major phytohormones on which most historic research has been centred: abscisic acid; gibberellins; auxins; cytokinins and ethylene. Early studies often identified an effect before the corresponding compound was isolated. For example, observations of the effects of auxin in tropic responses (Went, 1926), preceded the identification and isolation of the then unknown molecule. Their importance as growth regulators has led to extensive research and biotechnological application of phytohormones in areas such as agriculture.

Plant hormones vary widely in their chemical structure and biosynthesis and the interactions between different hormones and the sensitivity of different plant tissues all affects the impact they have. Abscisic acid (ABA) and gibberellins (GAs) are key to multiple plant processes (Finkelstein *et al.*, 2002; Swain and Singh, 2005) and interest in their range of roles has increased rapidly in the last few decades.

1.9.1 Abscisic acid (ABA)

Abscisic acid (ABA) is an isoprenoid hormone. It is an evolutionarily ancient plant hormone that has been found in multiple basal land plants such as bryophytes and all subsequent plant groups (Hauser *et al.*, 2011; Sakata *et al.*, 2014). It was first identified in the 1960s from studies of abscission of cotton flowers (Ohkuma *et al.*, 1963; Addicott *et al.*, 1968). Despite its name, ABA has a role in many other plant processes including stress responses; stomatal control; seed dormancy; germination and fruit ripening (reviewed in Wasilewska *et al.*, 2008; Takezawa *et al.*, 2011; Nakashima and Yamaguchi-Shinozaki, 2013; Sakata *et al.*, 2014). It is ubiquitous in plants but can also be found in fungi, bacteria and metazoans, including humans (reviewed in Wasilewska *et al.*, 2008). The wide distribution of ABA across multiple groups suggests it is an evolutionarily ancient molecule, most likely with a conserved function.

In angiosperms the main functions of ABA are as a biotic and abiotic stress response, regulation of germination and control of growth (Fujii and Zhu, 2009; Nakashima and Yamaguchi-Shinozaki, 2013). It regulates the genes involved in dehydration tolerance (Khandelwal *et al.*, 2010) and closure of stomata (Pantin *et al.*, 2013; Chater *et al.*,

2014), the maturation and germination of seeds (Koornneef *et al.*, 2002; Finch-Savage and Footitt, 2012) and the inhibition of lateral root growth (Ding and De Smet, 2013; Harris, 2015) and development of inflorescences (Verslues and Zhu, 2007). ABA has also been identified in the other major vascular plant groups such as gymnosperms (Feurtado *et al.*, 2004), lycophytes (Brodribb and McAdam, 2011) and ferns, with similar roles in stress tolerance, seed dormancy and control of growth. Despite the specificity of ABA's role in vascular plants, the functions evolved prior to the appearance of stomata and seeds (Hauser *et al.*, 2011; Sakata *et al.*, 2014). Non-vascular plants such as bryophytes also display an ABA response (Minami *et al.*, 2003; Takezawa and Minami, 2004; Cuming *et al.*, 2007; Bhyan *et al.*, 2012). For example, the plant-specific transcription factor ABA INSENSITIVE 3 (ABI3) has been shown to control desiccation tolerance in *P. patens* through gene knockout studies (Sakata *et al.*, 2010; Yotsui *et al.*, 2016). The role of ABI3 in seed and vegetative tissue desiccation tolerance in angiosperms suggests that this function has been evolutionarily conserved (Sakata *et al.*, 2010). Thousands of other genes are regulated by ABA and over 200 loci have been identified so far as regulators of the ABA response (Finkelstein, 2013).

1.9.1.1 ABA in bryophytes

Endogenous ABA has been detected in bryophytes by enzyme-linked immunosorbent assays (ELISA) and gas chromatography-mass spectrometry (GC-MS). Evidence for roles in desiccation and freezing tolerance, inhibition of gametophores and gametangia and inhibition of protonemal growth and differentiation have been shown in the moss *P .patens* (Minami *et al.*, 2005) as well as in liverworts and hornworts (reviewed in

Sakata *et al.*, 2014). Unlike in angiosperms, the full array of ABA functions as well as its biosynthesis and signalling pathways are not as well understood in non-vascular land plants. A more detailed investigation of its roles would aid the understanding of the evolution of the essential desiccation tolerance mechanisms that enabled the movement of plants onto land.

1.9.1.2 ABA signalling

A suite of ABA biosynthesis, signalling and response genes have been identified (Hanada *et al.*, 2011). The majority have come from angiosperm models such as *A. thaliana* and rice. The presence of some in early evolving models such as *P. patens* provides targets for comparison (Takezawa *et al.*, 2011). Functional conservation of ABA signalling molecules between seed plants and mosses has been revealed by molecular analyses which are continuously being updated and expanded. The presence of so many homologous genes in *P. patens* provides a smörgåsbord of potential opportunities for comparison across many plant processes. The biosynthesis, perception and transduction of ABA and the functional response is well characterised in seed plants and increasingly in other model systems (Wasilewska *et al.*, 2008; Sakata *et al.*, 2014), with over 200 loci identified so far.

1.9.1.3 ABA biosynthesis

ABA metabolism is coordinated by a feedback mechanism with certain regulatory genes being regulated by ABA levels. Environmental signals also play a key role by controlling certain enzymes involved in biosynthesis (Xiong and Zhu, 2003; Cutler *et al.*, 2010;

Hauser *et al.*, 2011).

In plants, ABA synthesis begins by production of isoprenoids through the methylerythritol 4-phosphate (MEP) pathway in chloroplasts (figure 1.6). Conversion to a series of intermediate carotenoids follows with the conversion of zeaxanthin to violaxanthin being the first step identified through analysis of ABA deficient mutants. The *A. thaliana* ABA-deficient mutant *aba1* shows increased zeaxanthin levels (Rock and Zeevaart, 1991) suggesting that *ABA1* encodes a key conversion enzyme in the process. Isolation of other ABA-deficient mutants led to the identification of other key enzymes such as ABA2, responsible for converting xanthoxin to abscisic aldehyde (Schwartz *et al.*, 1997; Gonzalez-Guzman *et al.*, 2002) and AAO3 (Seo *et al.*, 2000; Gonzalez-Guzman *et al.*, 2004; Seo *et al.*, 2004), responsible for converting abscisic aldehyde to abscisic acid (figure 1.6). Homologues for many of the ABA-deficient mutant alleles have been identified and the identification of homologs of principle genes such as *ABA2* and *AAO3* in *P. patens* (Hanada *et al*., 2011) enables investigation of evolutionary conservation across millions of years at a transcriptional level. Sequencing of the *P. patens* genome has provided an essential starting point for investigation of such homologues in basal land plants as many have now been uncovered in *P. patens*. Multiple cases of functional redundancy have been highlighted across many plant groups and there also appear to be additional minor pathways of ABA biosynthesis (Cowan, 2000; Seo and Koshiba, 2002; Nambara and Marion-Poll, 2005), the role and importance of which is still to be elucidated.

1.9.1.4 ABA perception and signal response

The RCAR (Regulatory Component of ABA Receptor) family of proteins, also known as the PYRs (PYrabactin Resistant) or PYLs (PYR-like) are the most well-characterised ABA receptors (Park *et al.*, 2009; Klingler *et al.*, 2010). Binding of ABA to a PYR/PYL/RCAR protein induces a conformational change that stabilises its interaction with a protein phosphatase 2C (PP2C). Multiple PP2C-encoding genes have been identified in vascular models such as *A. thaliana* including *ABI 1, 2, 3, 4* and *5* (Gosti *et al.*, 1999; Chak *et al.*, 2000; Lopez-Molina *et al.*, 2001; Merlot *et al.*, 2001; Khandelwal *et al.*, 2010; Liu *et al.*, 2013; Shu *et al.*, 2013; Shu *et al.*, 2015). Inactivation of the PP2C through binding of ABA is key in the subsequent transduction of the ABA signal as it removes the repression of SnRK (SNF1-related protein kinase). SnRKs such as SnRK2 activate multiple mediators of the ABA response by carrying out roles such as activation of transcription factors (Nakashima *et al.*, 2009; Feng *et al.*, 2014). The discovery of *ABI 1* and *3,* RCAR and *SnRK2* homologs in *P. patens* provided targets for investigation of these signalling pathways in non-vascular models and further elucidation of their ancestry (Table 1.1)

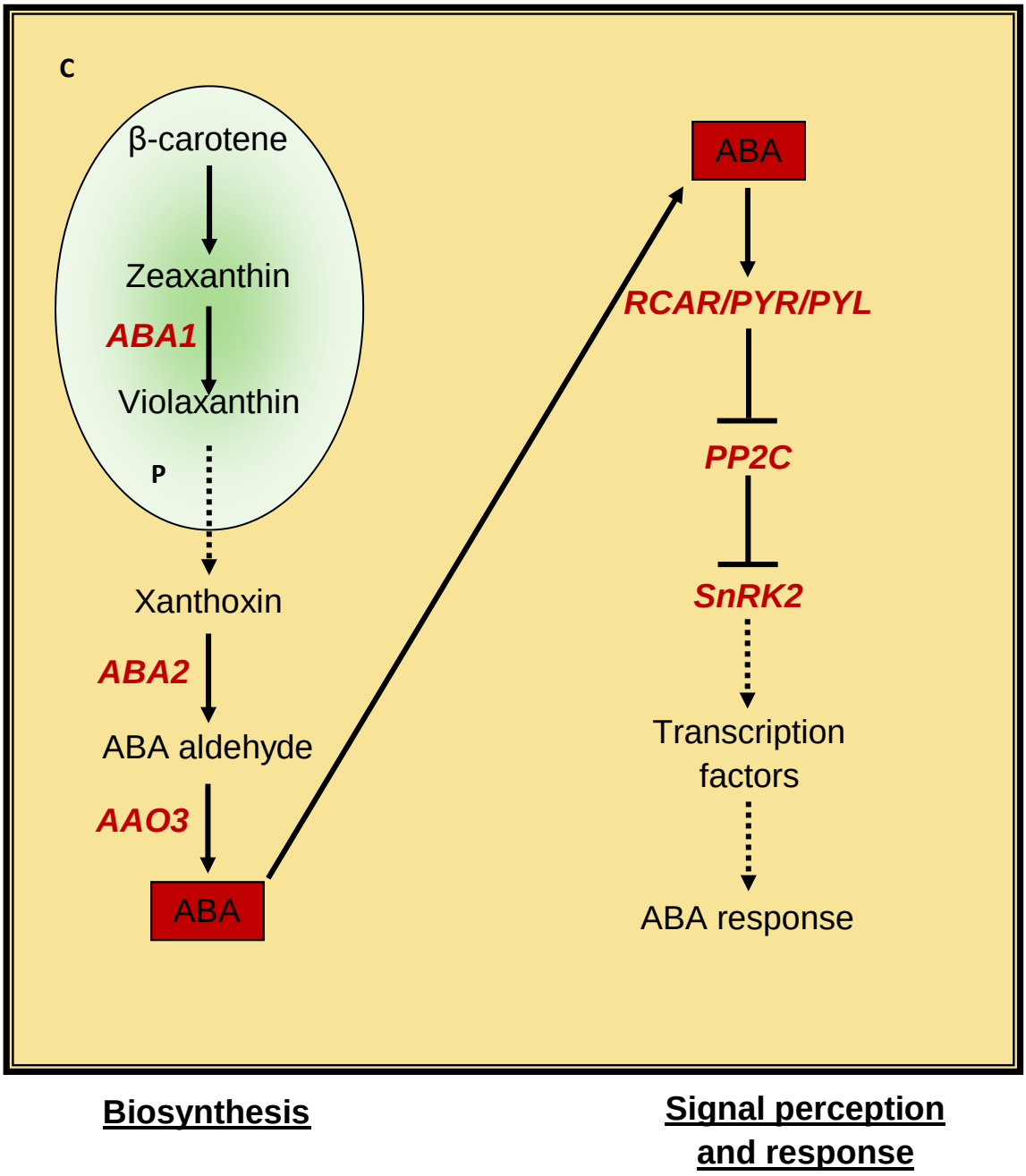


Figure 1.6 Outline of the ABA biosynthesis and signalling pathways. Xanthoxin is produced in the plastid by oxidative cleavage of carotenoids such as β-carotene. ABA2 and AAO3 convert xanthoxin to ABA in the cytosol. ABA is perceived by the RCAR/PYR/PYL receptors which interact with multiple PP2Cs (e.g. ABI1 and ABI3). This removes repression of SnRK2 which mediates the ABA response through the action of transcription factors. Putative homologs of ABA2, AAO3, RCAR, ABI1, ABI3 and SnRK2 have been identified in *P. patens*. Characterised genes are indicated in red. **P** = plastid, **C** = cytosol

Function	Gene	*C. reinhardtii* Green alga	*P. patens* Bryophyte	*S. moellendorffi* Lycophyte	*O. sativa* Angiosperm	*A. thaliana* Angiosperm
ABA metabolism	ABA1	1	1	1	1	1
	ABA2	0	0	0	1	1
	ABA3	1	1	1	0	1
	AAO3	0	0	0	0	1
ABA receptors	PYR/PYL/RCARs	0	4	5	11	14
ABA signalling	Group A PP2Cs	0	2	3	10	9
	SnRK2s	0	4	2	3	3
	ABI3	0	3	3	1	1
	ABI4	0	0	0	1	1
	ABI5	0	2	4	5	7

Table 1.1 Putative ABA signalling orthologues in *C. reinhardtii, P. patens, S. moellendorffi* and *O. sativa* were identified from BLASTP search of *A. thaliana* genes ABA DEFICIENT 1 (ABA1), 2 (ABA2) and 3 (ABA3); ABSCISIC ALDEHYDE OXIDASE 3 (AAO3); REGULATORY COMPONENT OF ABA RECEPTOR / PYRABACTIN RESISTANT / PYR-LIKE (RCAR/PYR/PYL); Protein Phosphatase 2C (PP2C); SNF1-RELATED PROTEIN KINASE (SnRK2); ABA-INSENSITIVE 3 (ABI3), 4 (ABI4) and 5 (ABI5). This data suggests that *P. patens* does not contain homologs for ABA2, AAO3 and ABI4. Adapted from Sakata *et al.*, 2014.

1.9.1.5 ABA in germination

The impact of ABA has been shown in land plants other than angiosperms, such as ferns, mosses, liverworts and hornworts (Swami and Raghavan, 1980; CHIA and Raghavan, 1982; Hickok and Kiriluk, 1984; Werner *et al.*, 1991; Pence *et al.*, 2005; Hartung, 2010). However the effect of ABA on germination has been studied most extensively in *A. thaliana* and other seed plants (Finkelstein *et al.*, 2002; Nambara and Marion-Poll, 2003; Finch-Savage and Leubner-Metzger, 2006; Gianinetti and Vernieri, 2007; Goggin *et al.*, 2009; Khandelwal *et al.*, 2010).

The period of seed dormancy observed in most seed plants is maintained by high levels of ABA present mainly in the endosperm (Lefebvre *et al.*, 2006). The subsequent catabolism of ABA and the synthesis of the mutually antagonistic gibberellins (GAs) releases dormancy and promotes germination of the seed (Finch-Savage and Leubner-Metzger, 2006; Finkelstein *et al.*, 2008; Finch-Savage and Footitt, 2012).

ABA is also a key integrator of environmental signals during germination (Hauser *et al.*, 2011). Conditions such as dark and cold soil temperatures both promote expression of ABA biosynthesis and *SnRK2* genes, serving to increase production and signal transduction of ABA respectively (Soitamo *et al.*, 2008; Finkelstein, 2013). Sensitivity to the availability of nutrients such as nitrate is also relayed by changes in ABA signalling due to upregulation of the ABA-catabolising *CYP707A2* gene (Matakiadis *et al.*, 2009).

There are many examples of conserved functions of ABA biosynthesis and signalling genes in bryophytes and higher plants (Marella *et al.*, 2006; Sakata *et al.*, 2010; Wang *et al.*, 2010). However evidence of conservation of ABA function in germination is lacking.

1.9.1.6 Interaction with other hormones

The complex nature of hormone signalling pathways creates multiple points of intersection allowing varying levels of control. The elucidation of the interplay between ABA and gibberellins (GAs) provides a target for further comparison of the conserved and divergent roles of ABA across evolutionarily distinct plant systems. GA signalling evolved after ABA signalling (Wang *et al.*, 2015) and the requirement of both for seed dormancy and germination (Finch-Savage and Leubner-Metzger, 2006; Finkelstein *et al.*, 2008; Finch-Savage and Footitt, 2012) in more recently evolved plants raises the question of their relative importance in basal land plants.

1.9.2 Gibberellins

Gibberellins (GAs) are diterpenoid acids that were first isolated from the fungus *Gibberella fujikuroi* in 1935. The production of GAs by *G. fujikuroi* as a metabolic by-product caused excessive stem elongation in rice seedlings which led to its identification as an endogenous plant growth regulator. GAs are found in multiple bacterial and fungal groups and also throughout the vascular land plants (MacMillan, 2001; Bottini *et al.*, 2004; Tudzynski, 2005; Bomke and Tudzynski, 2009). There are many different

types identified which control multiple plant processes (Richards *et al.*, 2001) including germination, growth and morphogenesis, root growth and reproduction. The main gibberellins identified as bioactive in plants are GA_1, GA_3, GA_4 and GA_7. Over 130 other GAs have been identified that are mainly precursors for the bioactive forms.

1.9.2.1 Gibberellins in Bryophytes

Not all GAs are bioactive in all plants. It was thought that mosses did not produce any GAs (Hirano *et al.*, 2007; Vandenbussche *et al.*, 2007; Yasumura *et al.*, 2007), however recent work has suggested that GA_9 methyl ester may be the bioactive gibberellin in this ancient group (Hayashi *et al.*, 2010). *Ent*-kaurene (figure 1.7) has been identified in moss, suggesting a possible evolutionary starting point for the complex biosynthesis pathway that exists in higher species (Anterola *et al.*, 2009; Hayashi *et al.*, 2010; Miyazaki *et al.*, 2011; Sun, 2011).

1.9.2.2 Gibberellin signalling

Like most historical phytohormone research, the majority of knowledge comes from investigation of angiosperm models. Genetic screening and characterisation of mutant *A. thaliana* and rice plants has revealed a suite of gibberellin signalling components (figure 1.7). The essential role of gibberellin signalling in plant growth has been investigated in multiple guises as its control of growth responses have been revealed. Agricultural applications such as the increase in wheat and rice yield in the 1960s (Peng *et al.*, 1999; Ueguchi-Tanaka *et al.*, 2007) were possible due to characterisation of gibberellin biosynthesis and signalling mutants. Its tightly controlled antagonistic

relationship with ABA and downstream response to auxin signalling suggests it is an essential signalling pathway for control of plant processes and provides a vast potential for investigation of processes in evolutionary developmental biology in green multicellular plants.

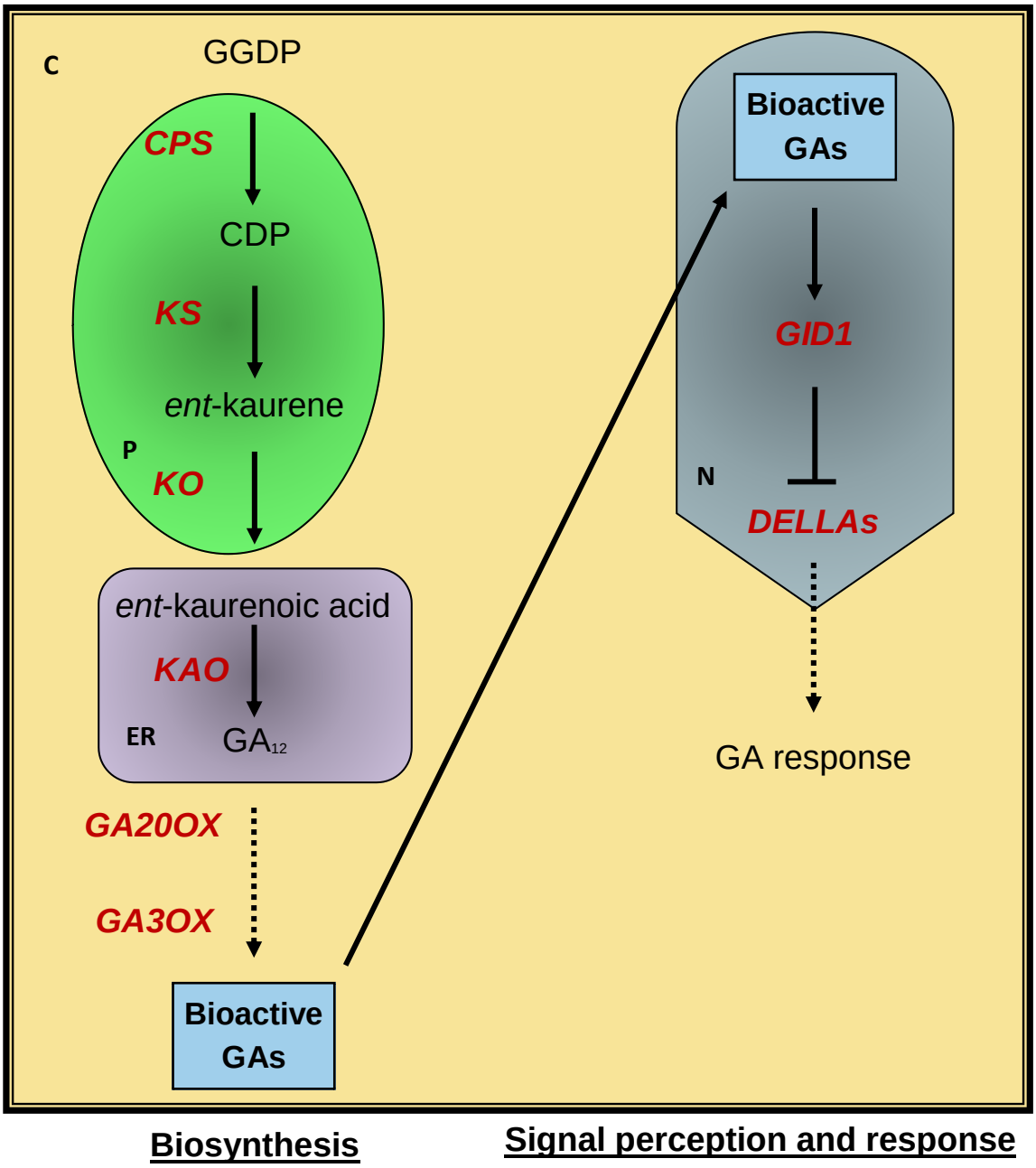


Figure 1.7 Outline of the GA biosynthesis and signalling pathways. Geranylgeranyl diphosphate (GGDP) is converted to GA_{12} by enzymes copalyl diphosphate synthase (CPS), *ent*-kaurene synthase (KS), *ent*-kaurene oxidase (KO) and *ent*-kaurenoic acid oxidase (KAO). GA 20 and GA 3-oxidases catalyse sequential oxidation steps (dashed line) to yield bioactive GAs such as GA_4 and GA_1. These are then perceived by a GID1-mediated mechanism leading to the degradation of DELLA—the repressor of the GA response. Identified genes are indicated in red. **P** = plastid, **ER** = endoplasmic reticulum, **N** = nucleus, **C** = cytosol

1.9.2.3 Gibberellin biosynthesis

Gibberellin biosynthesis (figure 1.8) is a complex series of enzymatic steps that occurs in the plastid via the methylerythritol phosphate (MEP) pathway. The first committed step is cyclization of geranylgeranyl diphosphate to *ent*-kaurene. In angiosperms this is catalysed by two separate diterpene cyclase enzymes via the intermediate *ent*-copalyl diphosphate (*ent*-CDP). Following conversion of *ent*-kaurene by two P450 monooxygenases to GA_{12}, a further 120 different GAs can be synthesised by the actions of GA 2 and 20-oxidases (GA2ox and GA20ox) and finally GA 3-oxidases (GA3ox) to yield bioactive gibberellins such as GA_1, GA_3 and GA_4.

The formation of *ent*-kaurenoic acid from *ent*-kaurene is a key step in the study of GA biosynthesis as some plants can produce one or the other even if a specific bioactive GA has not been identified (Miyazaki *et al.*, 2011).

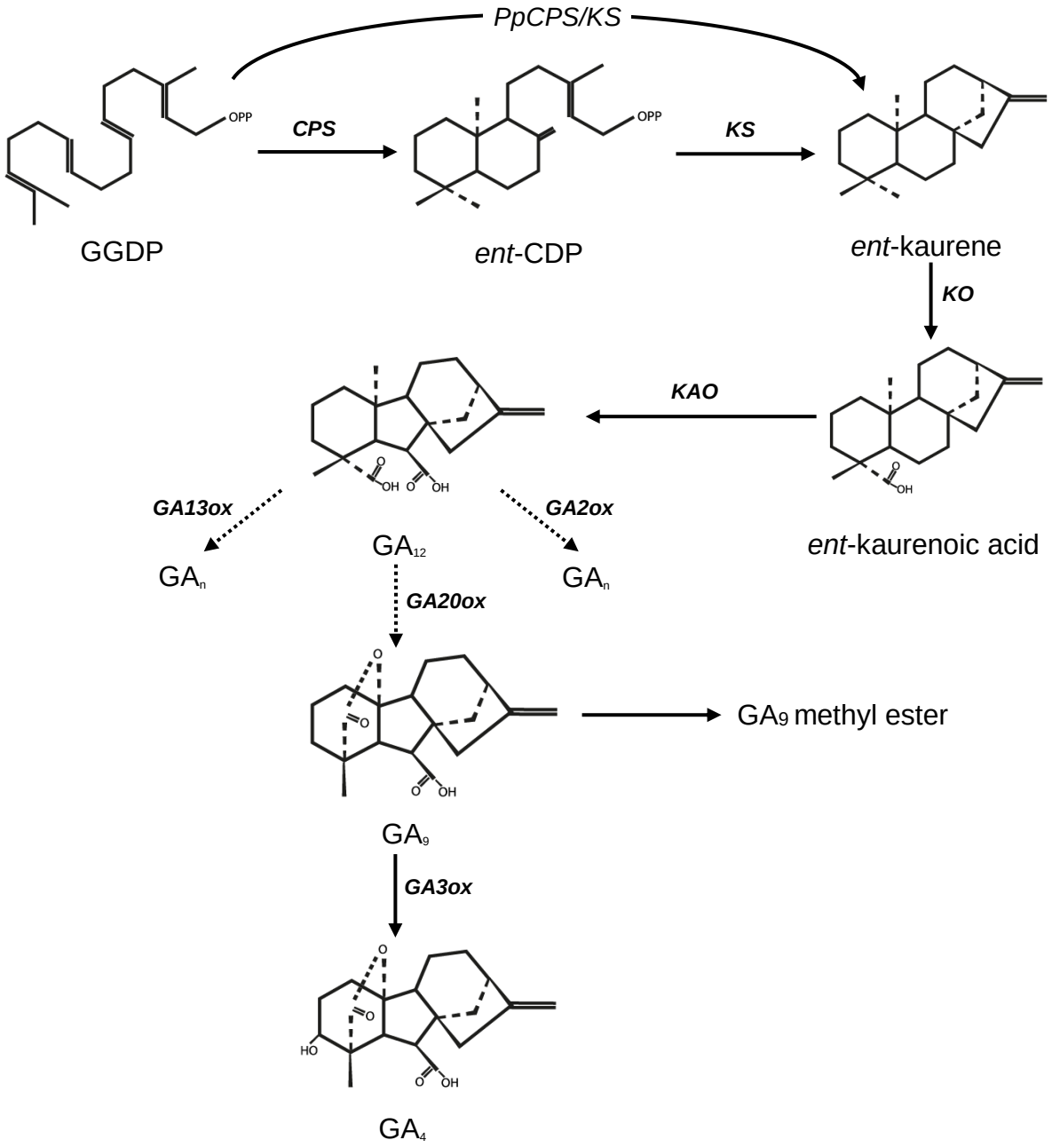


Figure 1.8 Initial stages of the GA biosynthesis pathway identified in angiosperms. Steps for conversion of geranylgeranyl diphosphate to GA_{12} have been identified in basal land plants such as *P. patens* in which the cyclization of GGDP to *ent*-kaurene is carried out by a single bifunctional CPS/KS enzyme. Dashed lines represent multiple oxidation steps yielding a range of gibberellins, gibberellin precursors and degradation products. Different hydroxylation pathways exist through the action of GA2, 13 and 20-oxidases. Production of bioactive GAs such as GA_4 is catalysed by a GA3-oxidase. Gene products are in bold/italics.

1.9.2.4 Gibberellin perception and signal transduction

Bioactive GAs are perceived by the soluble nuclear receptor GIBBERELLIN INSENSITIVE DWARF1 (GID1) (Griffiths *et al.*, 2006). Research on the crystal structure of GID1 (Shimada *et al.*, 2008) shows a GA-binding pocket that is covered by an N-terminal extension that closes due to conformational changes brought about by the binding of GA to the pocket (figure 1.9). The closed lid of this pocket can then interact with two regions (DELLA and TVYYNP) of the DELLA proteins, a subset of the plant-specific GRAS transcriptional regulators. This induces a conformational change in the GRAS domain allowing enhanced recognition between two DELLA-specific motifs and the SLY1/GID2 F-box proteins of an E3 ubiquitin ligase complex. This catalyses the attachment of polyubiquitin chains which targets the DELLA protein for degradation by the 26S proteasome (Ueguchi-Tanaka *et al.*, 2007; Hirano *et al.*, 2008; Murase *et al.*, 2008; Harberd *et al.*, 2009). DELLA proteins repress all GA growth responses (e.g. germination and floral induction) by interaction with multiple transcription factor targets to control gene expression (Locascio *et al.*, 2013). Yeast two-hybrid assays have revealed that DELLA proteins interact with DNA-binding proteins and interfere with their activation or repression of a promoter (de Lucas *et al.*, 2008; Feng *et al.*, 2008). So the induction of the GA response is through repression of a repressor (DELLA).

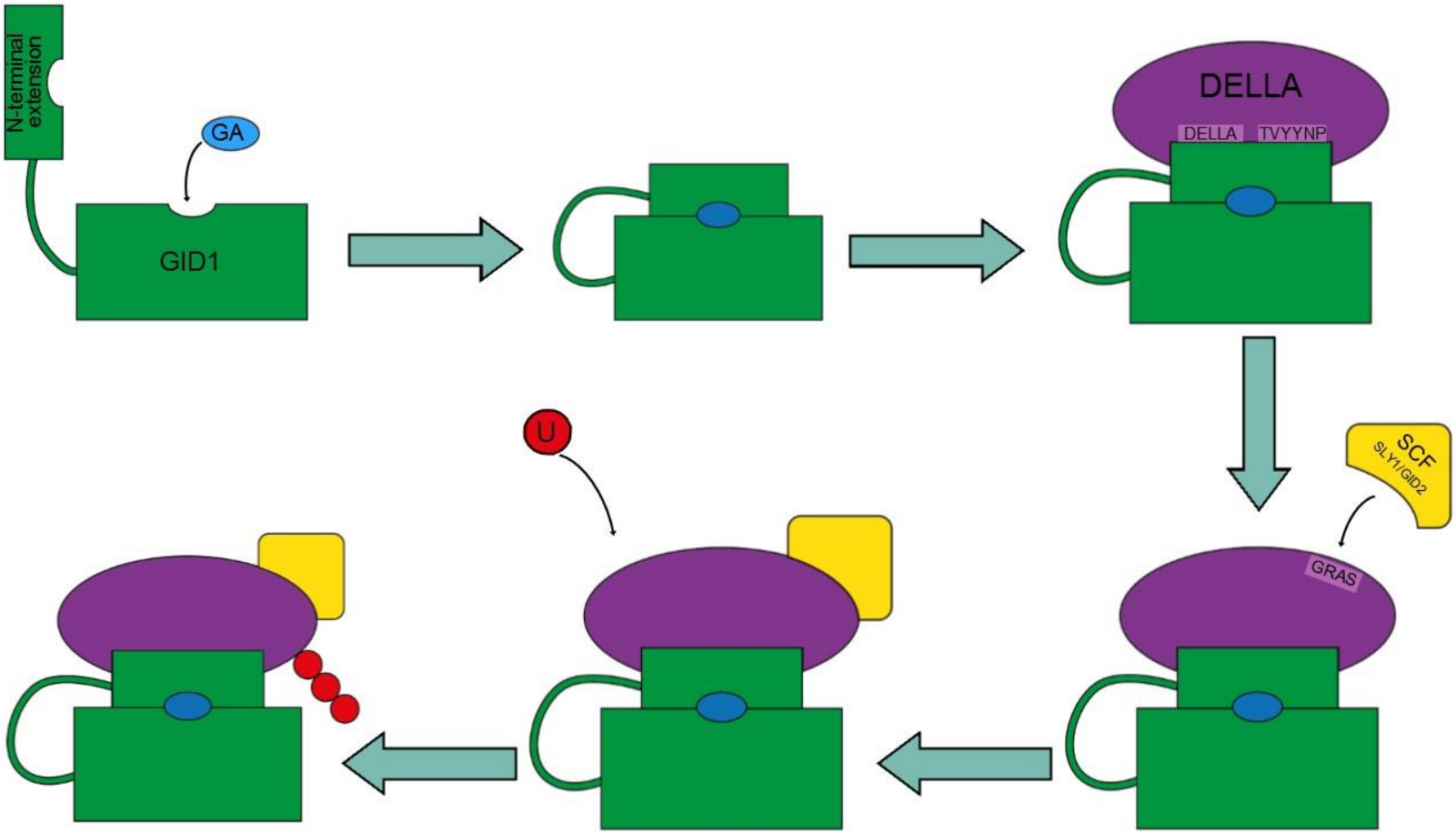


Figure 1.9 Mechanism for perception of GA by the GID1 mediated degradation of DELLA. Binding of GA to GID1 induces a conformational change that closes the N-terminal extension over the GA-binding pocket. The closed lid can then interact with the DELLA and TVYYNP regions of the DELLA protein. Binding alters the GRAS domain of DELLA enhancing recognition of an E3 ubiquitin ligase complex containing the F-box proteins SLY1/GID2. Binding catalyses polyubiquitination of DELLA, targeting it for destruction by the 26S proteasome (not shown).

1.9.2.5 Gibberellin signal response

The main observed effects of GA signalling are in the de-repression of DELLA target proteins. Resultant gene expression facilitates expansion and division of cells, hence GA's determination as a positive growth regulator. Transcription of hydrolytic enzymes involved with the loosening of cell walls, rearrangement of microtubules, stimulation of cell proliferation and promotion of downstream auxin transport have all been attributed to the degradation of DELLA (Cao *et al.*, 2006; Zentella *et al.*, 2007; Achard and Genschik, 2009; Hou *et al.*, 2013). The carefully controlled timing and localisation of GA growth responses results in the multitude of morphogenic and developmental features.

1.9.2.6 The role of Gibberellins in germination

The breaking of seed dormancy is essential for germination in most seed plants and periods of cold and exposure to light can both enable this. These environmental signals promote the accumulation of GAs by increasing transcription of the GA biosynthesis enzyme GA 3-oxidase (Yamaguchi and Kamiya, 2000; Oh *et al.*, 2006; Oh *et al.*, 2007; Yamaguchi, 2008). GA3ox produces bioactive gibberellins such as GA_4 from inactive gibberellin precursors (figure 1.8). They carry out the essential final step in the GA biosynthesis pathway allowing accumulation and dispersal of GAs as signalling molecules in response to the environment. GA-mediated degradation of DELLAs also allows integration of environmental signals into the process of germination by removing repression of proteins such as PIFs (Phytochrome Interacting Factors) which control etiolated growth in response to light (de Lucas and Prat, 2014).

1.9.2.7 The evolution of gibberellin signalling

Despite the fact that bioactive gibberellins have not been identified in the early evolving mosses, some of the components of the signalling pathway have (Hayashi *et al.*, 2006). Homologs for genes involved in GA biosynthesis (*PpCPS/KS*), perception (*PpGLP1*) and transcriptional regulation (*PpDELLA*) suggest that whilst the components of the elucidated angiosperm GID1-DELLA signalling mechanism are present in basal land plants, their interactions may not reflect what is seen in vascular plants (Gao *et al.*, 2008). The increase in diversity of plant tissues was largely a consequence of the evolution of vascular tissue and diverse reproductive strategies. The lycophyte *Selaginella* represents one of the earliest vascular land plant groups. Yeast two-hybrid assays demonstrate that *Selaginella* GA signalling homologs do behave like those found in later evolving plants by using GAs as mediators for the interaction of GID1 and DELLA (Hirano *et al.*, 2007). This is not apparent in *P. patens* though, where *GID1* and *DELLA* homologs are unable to complement vascular plant activity (Hirano *et al.*, 2007). The establishment of a GID1-DELLA mechanism for perception of gibberellin appears to be a vascular plant specific feature. However the lack of identifiable bioactive gibberellins in *P. patens* complicates this conclusion.

Bioactive GAs such as GA_1 and GA_4 have not been identified in mosses. However the genes involved with the initial stages of the GA biosynthesis pathway (*PpCPS/KS*) and the relevant products (*ent*-kaurene, *ent*-kaurenoic acid) are present. Moreover, their conserved nature, as exemplified by the essential isoleucine residue in the kaurene synthase enzyme (Jia and Peters, 2016) (figure 1.10), suggests that this is an

evolutionarily ancient component of the pathway. The end product, which subsequently interacts with GID1/DELLA, has been identified as gibberellin in vascular plant groups. As we do not know the end product in bryophytes, only that is it not an identified gibberellin, it is unsurprising to find no conserved interaction between GAs and GID1/DELLA proteins in this basal land plant representative.

The characterisation of GA biosynthesis and signalling mutants across multiple land plant groups has allowed detailed analysis of the later stages in the functional evolution of this phytohormone. The gaps in our knowledge of non-vascular groups such as mosses presents an opportunity to uncover the origin of this system in land plants and may provide a key evolutionary stage for comparison with ancestral aquatic plant groups.

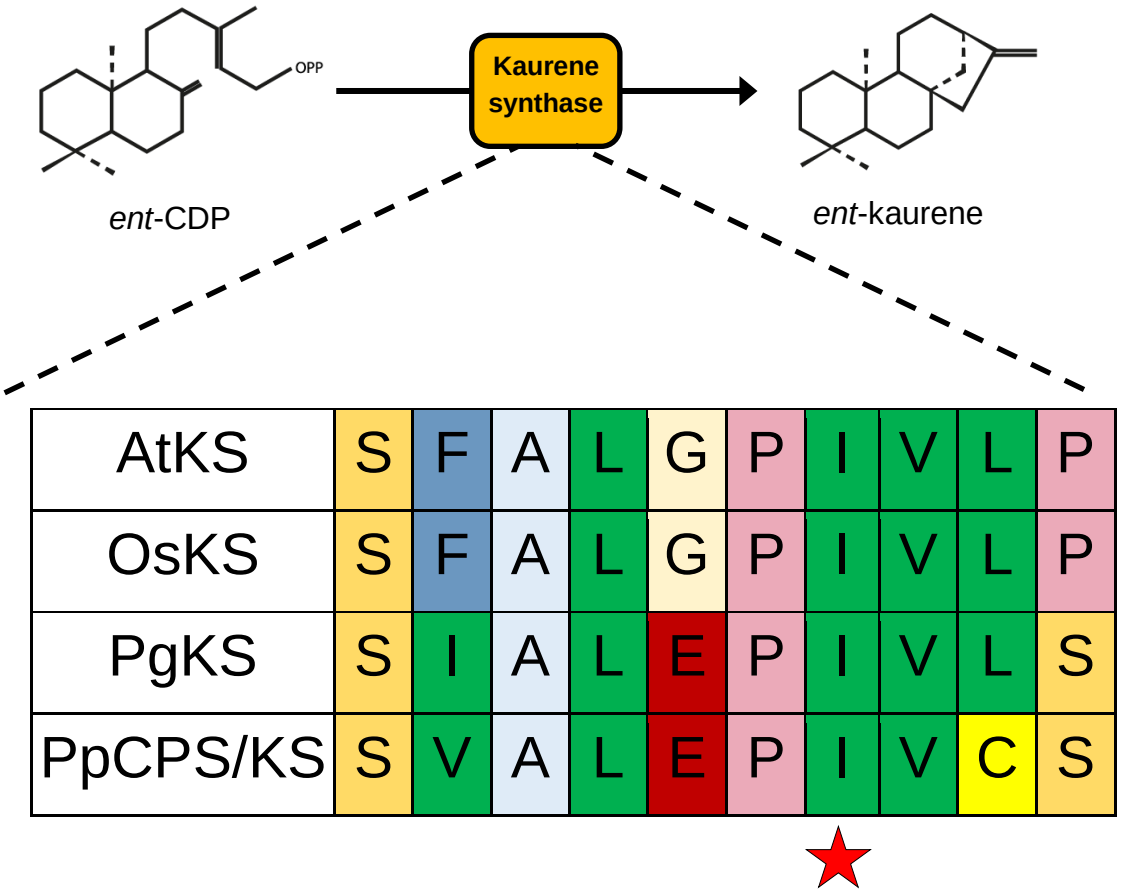


Figure 1.10 Conserved isoleucine residue in kaurene synthase

Conversion of *ent*-CDP to *ent*-kaurene is catalysed by kaurene synthase (KS). An alignment of *A. thaliana* (At), *O. sativa* (Os), *P. glauca* (Pg) and *P. patens* (Pp) kaurene synthase showing the conserved isoleucine (I) residue ★ Swapping the Ile residue results in the abortive production of *ent*-pimaradiene.

1.9.3 Other phytohormones

1.9.3.1 Strigolactones

Strigolactones (SL) were first identified as signalling molecules between organisms (Xie *et al.*, 2010; Delaux *et al.*, 2012). They were shown to control the germination of parasitic weeds in response to exogenous strigolactones from root exudate of neighbouring plants (Yoneyama *et al.*, 2008; Zwanenburg *et al.*, 2009). There is now an increasing amount of research suggesting that strigolactones also have endogenous hormonal roles (Gomez-Roldan *et al.*, 2008; Dun *et al.*, 2009; Koltai *et al.*, 2010; Foo and Davies, 2011; Kapulnik *et al.*, 2011a; Kapulnik *et al.*, 2011b; Proust *et al.*, 2011; Toh *et al.*, 2012; Brewer *et al.*, 2013; Koltai, 2013; Seto and Yamaguchi, 2014). Research has focused on its effects on shoot and root architecture in angiosperms, in particular its interactions with auxin transport mechanisms (Kapulnik *et al.*, 2011a; Kapulnik *et al.*, 2011b; Ruyter-Spira *et al.*, 2011; Kapulnik and Koltai, 2014) and establishment of symbioses with arbuscular mycorrhizae (Akiyama *et al.*, 2005; Besserer *et al.*, 2006; Garcia-Garrido *et al.*, 2009). More recently, its role in control of seed germination, particularly in secondary dormancy through thermoinhibition (Toh *et al.*, 2012) has been highlighted, providing a starting point for comparisons with spore germination in models such as *P. patens*.

The influence of SL on early-evolving plants such as moss has been relatively understudied with Delaux (Delaux *et al.*, 2012) and Proust (Proust *et al.*, 2011) the main contributors. In their guise as signalling molecules between plants they have been

shown to regulate protonemal growth by inhibiting extension and division of *P. patens* caulonemal cells. This appears to be a way of interacting with neighbouring plants and regulating growth to ensure optimal use of available resources in the manner of bacterial quorum sensing (Proust *et al.*, 2011). The characterisation of the SL biosynthesis mutant *Ppccd8* confirmed this role through a lack of colony arrest which was rescuable by application of exogenous SL in the form of the synthetic SL GR24 (Proust *et al.*, 2011). Interestingly Proust *et al* also provided key evidence of the ancientness of SL-controlled regulation of branching through the rescue of *Ppccd8* by seed plant CCD8.

In light of previous investigations, an understanding of the role of SL in *P. patens* spore germination will provide a comparative time point in the understanding of this evolutionarily ancient system. The activity of SL in seed germination and confirmation of its bioactivity in *P. patens* combines to necessitate an understanding of the role of SL in spore germination.

1.9.3.2 Auxin

Auxin was the first plant major plant hormone to be discovered and has been identified in all land plant lineages and some multicellular Charophycean algae (Ross and Reid, 2010). Indoleacetic acid (IAA) is the most abundant naturally occurring auxin in plants and is an essential regulator of plant growth. It is synthesised primarily in the leaf primordia and developing seeds and fruits and has roles in a variety of plant processes including apical dominance, differentiation of vascular tissue and tropic responses

(Friml, 2003; Mockaitis and Estelle, 2008). Research suggests that auxins have a role in germination in seed plants in particular through crosstalk with other plant hormone signalling pathways such as ABA (Liu *et al.*, 2007). In moss, auxin has been shown to control processes such as rhizoid development (Sakakibara *et al.*, 2003) and differentiation of caulonema (Jang and Dolan, 2011). Whilst the roles of auxin in basal land plants may differ, the mechanisms behind its action do show molecular similarities to more recently evolved plants (Prigge *et al.*, 2010).

1.9.3.3 Cytokinins

The main role of cytokinins is the promotion of cell division primarily for growth and differentiation (Sakakibara, 2006). The highest concentrations are therefore found in areas of continuous growth such as roots, young leaves and developing fruits. The ratio of auxin to cytokinin is very carefully regulated as variations can alter a multitude of morphological and physiological characteristics (Coenen and Lomax, 1997). Cytokinins have been shown to have a role in seed germination, mainly through interactions with auxin and ABA (Coenen and Lomax, 1997; Y. Wang *et al.*, 2011). A preliminary investigation of cytokinin action on *P. patens* spores was fairly inconclusive with no obvious effects demonstrated (J. Coates unpublished data).

1.9.3.4 Brassinosteroids

Brassinosteroids are a class of 40 sterol derivatives structurally similar to animal steroid hormones (Clouse, 2002). They regulate the expression of multiple genes and appear to have extensive control over developmental patterns and morphogenesis (Clouse and

Sasse, 1998; Haubrick and Assmann, 2006; Clouse, 2011). Example areas of action include cell division, differentiation and elongation, fertility and germination (Clouse and Sasse, 1998; Haubrick and Assmann, 2006). The occurrence of enzymes and products of the sterol biosynthetic pathway in *P. patens* suggests conservation throughout the evolution of plants, but the exact role(s) of brassinosteroids in *P. patens* is significantly less well known than in more recently evolved angiosperm models.

1.9.3.5 Ethylene

Ethylene is a gaseous hormone that is most often associated with fruit ripening but has a wide range of physiological roles including flower opening, root hair growth, leaf abscission and stress responses (Lin *et al.*, 2009). Ethylene receptors have been found in charophyte algae as well as all land plant groups including *P. patens* (Gallie, 2015) and may have been key in the ability of early fresh-water plants to tolerate desiccation during the transition to a terrestrial environment (Gallie, 2015).

Ethylene has been shown to promote germination in seed plants alongside its other roles. Biosynthesis is upregulated upon imbibition and increases as germination proceeds (Corbineau *et al.*, 2014). It also displays multiple interactions with GA and ABA, the other main regulators of germination.

1.9.3.6 Jasmonates

Jasmonates (JAs) and their precursors generally act as growth inhibitors and regulators of stress responses (Wasternack and Hause, 2013; Ahmad *et al.*, 2016). In angiosperms, JA content is high in dry seeds in a non-dormant state and decreases rapidly upon imbibition (Vigliocco *et al.*, 2007; Bai *et al.*, 2012). The expression pattern in dormant seeds is in need of further investigation but appears to be expressed in low levels when seeds are in a dry dormant state (Linkies and Leubner-Metzger, 2012). The lack of primary dormancy in *P. patens* spores (Vesty *et al.*, 2016) provides a comparable process that would aid understanding of the evolutionary ancientness of this particular phytohormone signalling pathway.

1.9.3.7 Homoserine lactones are an example of an external biotic factor

Acyl homoserine lactones (AHLs) are quorum-sensing molecules that regulate gene expression in Gram-negative bacteria to control population density (Parsek and Greenberg, 2000; Fuqua *et al.*, 2001). In bacterial colonies, AHLs are synthesised and distributed into the environment. At a certain threshold concentration AHLs bind to receptors to form complexes with promoter sequences causing transcription of specific quorum-sensing genes (Reading and Sperandio, 2006; Fast and Tipton, 2012). These genes can have a variety of different roles from virulence to swarming (Parsek and Greenberg, 2000; Bassler *et al.*, 2001). Recent research has shown that AHLs can also interact with eukaryotic organisms including plants (Joint *et al.*, 2002; Marshall *et al.*, 2006; Schuhegger *et al.*, 2006; Ortiz-Castro *et al.*, 2008; von Rad *et al.*, 2008; Schenk

et al., 2012) and that plants themselves can produce AHL mimics that affect bacterial quorum-sensing (Gao *et al.*, 2003; Bauer and Mathesius, 2004; Perez-Montano *et al.*, 2013 Corral-Lugo *et al.*, 2016).

The most prominent effects of AHLs on plants is through systemic resistance to plant pathogens (Zarkani *et al.*, 2013; Cheng *et al.*, 2016), regulation of root growth (Ortiz-Castro *et al.*, 2008; Jin *et al.*, 2012) and formation of root nodules (Gray *et al.*, 1996). In this way AHLs mainly act as promoters of growth and regulators of plant responses.

AHLs are categorised by the length of the acyl chain (i.e. the number of carbons (CN)) and the substitution at the C3 position (H, O or OH). The types and quantities of AHLs produced by Gram-negative bacteria varies dependent on species and environmental conditions. Eukaryotic interactions provide more insight into the mechanism of this essential signalling pathway. Its effects are far-reaching in terms of the current environment and the evolutionarily ancient terrestrial systems that nurtured early plant life. Interactions between bacterial signals and eukaryotes have even been observed in aquatic plants (Joint *et al.*, 2007) hinting at an intercommunication that pre-dates terrestrialisation.

1.9.3.7 Conclusions on plant hormones

The essential role of phytohormones in the control of plant processes makes them a key target to expand our knowledge of plants. They also provide a point of comparison in evolutionary developmental biology due to variations in the presence, synthesis, signalling and relative importance of each across different plant groups.

1.10 Aims of this research

1. Understanding a new developmental process

Compared to seed germination, spore germination is not well studied and is little understood. Previous observations on the germination of *P. patens* spores have not always followed a detailed enough methodology. This has allowed subtle variations in germination to go unnoticed and unquantified. The subtle nature of phytohormone action and sensitivity means that an in depth assessment of its effects on germination is the only way to begin to draw conclusions. My thesis will address this question by developing, for the first time, a robust assay for analysing the effects of genes, hormones and signals on spore germination in *P. patens*.

The development of a detailed germination assay will allow quantification of the response observed during the investigation of the role of phytohormones in spore germination. The assay will provide robust, replicable data that can be used in future comparative studies with other model plant systems.

2. Further understanding of bryophyte diterpene signalling

The current dogma suggests a lack of gibberellin signalling in bryophytes (Hirano *et al.*, 2007). However, the presence of putative homologues of gibberellin receptor- and DELLA-like proteins in *P. patens* (Yasumura *et al.*, 2007) along with a role for diterpenes in *P. patens* development (Hayashi *et al.*, 2010) presents an opportunity to challenge this dogma by discovering potential new roles for diterpene-like signalling in

mosses. My thesis will address this by investigating the roles of diterpene signalling in spore germination on a physiological and molecular level.

The effect of exogenous diterpenes on spore germination will be analysed by application of varying concentrations of identified bioactive diterpenes to the growth media of *P. patens* spores. The endogenous control of germination will be investigated through the use of gene knockout mutants. The disruption of hormone encoding genes will allow observation and quantification of the role of that gene in spore germination. Complementation with exogenous hormones of interest will then allow confirmation of the genes and hormones involved and their relative importance in the germination process. As the significance of hormones such as GAs in *P. patens* germination is currently up for debate, the results from this investigation will also yield poignant further avenues of investigation.

3. Comparative studies of spore and seed germination regulation to further understand early land plant hormone signalling

The review above has identified some of the gaps in our knowledge of hormone signalling in *P. patens* as a model system. Its position as a basal land plant model system has allowed evolutionary biologists to make great strides in the understanding of the colonisation of land and the early adaptations plants had to develop. The successful germination of dispersed spores was the crucial step that allowed the spread of newly terrestrial plants. This research will deepen our understanding of this process and the influences on it. The multitude of stresses that early land plants had to contend with has

left their ancestors such as *P. patens* with some very desirable characteristics. Tolerance to salt and drought are fortes of basal bryophytes, and when combined with knowledge of the role of ABA in stress responses, provides a wealth of physiological and molecular starting points for application of such traits to agriculturally important species.

This investigation seeks to demonstrate the role(s) of specific hormones in the germination and early development of *P. patens* spores. This will be done by direct observation of germination under the influence of exogenously applied known phytohormones at varying concentrations.

The molecular basis of phytohormone action provides another level of understanding and this will be investigated by recording expression levels of key ABA biosynthesis and signalling genes in multiple life cycle stages. This will allow basic quantification of the transcription of genes that are involved in phytohormone action and the variations across key life cycle progressions such as germination.

4. The extension and development of *P. patens* and *U. intestinalis* as model systems

The relevance and suitability of *P. patens* as a tractable model system is already established. The more detailed analysis of the influence of phytohormones on germination not only provides a relevant basal land plant for comparison with angiosperms but also creates a point in evolutionary time from which one can work backwards. The abundance of green multicellular plants in aquatic environments

provides testable systems that can represent the aquatic origins of land plants. The emerging green algal model system *Ulva* presents an opportunity to apply knowledge gained in land plants, both basal and higher, to an observable aquatic species. *Ulva* sp. such as *U. intestinalis* produces spores as a means of distribution and establishment of the next generation. Like moss spores and spermatophyte seeds these have to germinate and will also experience a multitude of environmental and internal cues. One facet of this research has been the establishment of a reproducible culture method to allow completion of the *U. intestinalis* life cycle and generation of spores in a laboratory environment.

1.11 Concluding remarks

My thesis aims will allow comparison of the essential process of germination to be analysed on a cellular and molecular level to truly understand the role of phytohormones in evolutionarily distinct plant species.

Our reliance on plants for food, fuel and medicine and their ecological importance highlights how essential a better understanding of their functioning is. This research contributes to key areas of research in plant biology including germination, environmental responses and development of model systems. The rapidly changing nature of our planet and the often underappreciated role that plants have in it means that a better understanding of plant systems will be key to enabling a sustainable future.

Chapter II:

Materials and methods

2.1 *Physcomitrella patens* tissue culture

2.1.1 Preparation of BCD growth media

BCD minimal medium contained 10ml each of stock solutions B, C and D and 1ml of trace element solution (TES), made up to 1 litre with distilled water (dH_2O). This was supplemented with filter-sterilised $CaCl_2$, ammonium tartrate and agar (7.2g/litre) depending on use and sterilised by autoclaving at 121°C.

BCD+AT was prepared by adding 10ml/litre ammonium tartrate and 1ml/litre 1M $CaCl_2$ to minimal medium. This was used for the production of chloronema-rich tissue e.g. liquid cultures and protonemal plates. Spore germination BCD was prepared by adding 10ml/litre ammonium tartrate and 5ml/litre 1M $CaCl_2$. For germination assays and protonemal tissue culture autoclaved cellophane discs (A.A. Packaging limited) were placed on each plate before storage at 4°C.

Hormone treatment plates were prepared by cooling spore germination BCD + agar to 50°C then adding required hormone or solvent before pouring into 90mm petri dishes. Gibberellic acids (GAs) GA_3, GA_4 (Sigma Aldrich) GA_9 and GA_9-methyl ester (GA_9-ME) (Peter Hedden), ent-kaurenoic acid, ent-kaurene (both Peter Hedden), abscisic acid (ABA) (Sigma Aldrich) homoserine lactones (AHLs) (Miguel Camara) and paclobutrazol (PAC) (Sigma Aldrich) were all dissolved in methanol and concentrated stock solutions stored at -20°C. Rac-GR24 (Stichting Chemiefonds Paddepoel) was dissolved in acetone and stored at -80°C. Working solutions were made in dH_2O and added to 1ml of dH_2O to give desired final concentration when added to medium. Appropriate solvent

controls were included in all assays and additional solvent was added to plates with a lower concentration of hormone to ensure all plates had an equal amount. Agar was overlaid with sterile cellophanes (AA packaging) which had been autoclaved between moist filter papers and applied in a flow hood with sterile forceps. Prepared plates were stored inverted at 4°C until required, up to a maximum of one week.

Stock solutions

Solution B

$MgSO_4.7H_2O$	25g
distilled H_2O	to 1 litre

Solution C

KH_2PO_4	25g
distilled H_2O	to 1 litre

Solution D

KNO_3	101g
$FeSO_4.7H_2O$	1.25g
distilled H_2O	to 1 litre

Trace element solution

H_3BO_3	614 mg	$MnCl_2.4H_2O$	389 mg
$AlK(SO_4)_2.12H_2O$	110 mg	$CoCl_2.6H_2O$	55 mg
$CuSO_4.5H_2O$	55 mg	$ZnSO_4.7H_2O$	55 mg
KBr	28 mg	KI	28 mg
LiCl	28 mg	$SnCl_2.2H_2O$	28 mg
$Na_2MoO_4.2H_2O$	25 mg	$NiCl_2.6H_2O$	59 mg
distilled H_2O	to 1 litre		

2.1.2 Tissue culture

The ‘Gransden’ wild type laboratory strain of *P. patens* was provided by Andy Cuming from the University of Leeds, UK and was used for all experiments unless otherwise stated. Mutant strains were provided by Catherine Rameau at the French National Institute for Agricultural Research, Paris (*ccd8* and *ccd8* background wt), Henrik Toft Simonsen at the University of Copenhagen, Denmark (*cps/ks* KO line #29 and pBK3-e, *cps/ks* YFP pBK3-1 and *cps/ks* background wt) and Nick Harberd at the University of Oxford, UK (*Ppdella A, B* and *AB).*

Liquid cultures of *P. patens* were prepared by adding 3-4ml of homogenised protonemal tissue to 200ml of liquid BCD+AT media in a 500ml conical flask. Cultures were kept at 22°C on an orbital shaker at 90 rpm under 24 hour light to generate fresh biomass (figure 2.1 A). Fresh cultures were established as needed by collecting tissue from flasks, homogenising in sterile water for 1 minute at 19,000rpm using a polytron tissue tearer (IKA® T25 digital Ultra-Turrax) then adding to fresh media. Samples from every culture were stored in dH_2O at 4°C for long term and back-up storage.

Homogenised tissue was used to inoculate peat plugs for gametophyte and subsequent sporophyte production (figure 2.1 B). Sterile peat plugs were inoculated with 5-6ml of homogenised tissue and grown at 22°C for 6-8 weeks to produce colonies of mature gametophytes. Plugs were then placed at 15°C for 2-3 weeks under short day conditions (8 hours light and 16 hours dark) to induce formation of sexual organs. Fertilisation was achieved by returning plugs to standard conditions and periodically

spraying with sterile water to allow movement of sperm cells. After 3-4 weeks mature sporophytes were collected and any gametophyte tissue removed using fine forceps under a dissecting microscope (Nikon SMZ645). Tubes of sporophytes were left with caps off for one week to dry then stored in the dark at room temperature.

Protonemal tissue was cultured on solid cellophane-overlaid BCD+AT plates by inoculating with 500µl of homogenised tissue, sealing with Micropore tape (3M Healthcare, Germany) and growing at 22°C under 24hr light for 10 days (figure 2.1 C).

Gametophyte tissue was propagated by removing individual leafy blades or gametophytes from mature plants and placing on fresh solid minimal BCD plates (figure 2.1E). Under standard conditions (22°C, 24h light) protonemal tissue would regenerate from the blade and new gametophytes would start to form after 2 weeks.

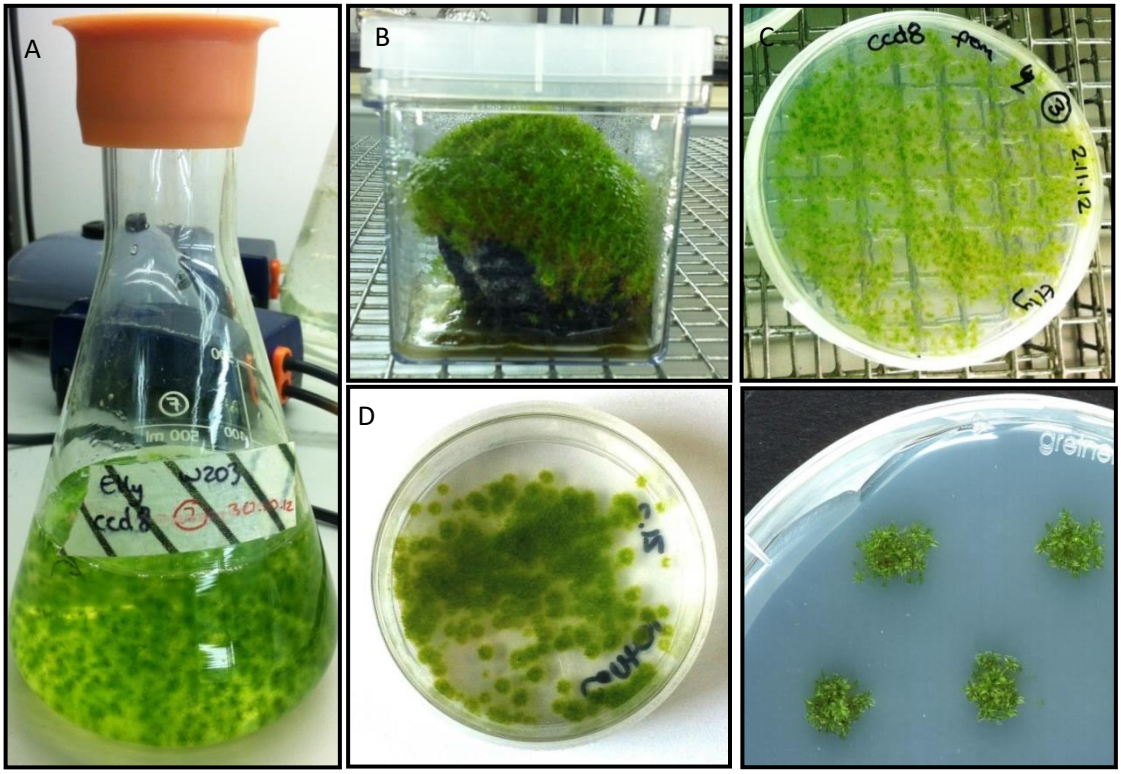


Figure 2.1 Culture conditions used during routine *P. patens* tissue culture. Different strategies were used depending on the desired tissue type. Liquid cultures (A) enabled large scale production of tissue for subsequent homogenisation for inoculation of peat plugs (B); protonemal plates (C) were produced by spreading homogenised tissue onto cellophane overlaid agar plates and were used for the production of tissue for RNA isolation. Germination assays were carried out on agar plates (D). For long term maintenance and storage of different lines, individual gametophyte blades were transferred to thick agar plates (E) allowed to regenerate and then stored at 4°C.

2.1.3 Microscopy

P. patens images were captured using a Nikon Digital Sight DS-Fil camera on a Nikon SMZ 1000 stereomicroscope. Measurements of colony length and area were carried out using the NIS-Elements BR software (version BR3.0)

2.1.4 Germination assays

Sporangia were sterilised in 25% Parazone™ solution for 10 minutes on a tissue culture rotator at room temperature. They were then washed 3-4 times using sterile distilled water. Spores were released from sporangia by perforating them with a sterile pipette tip into a set volume of sterile water. Spores were plated such that 3 sporophytes per 10 plates were distributed, in a volume of 500µl per plate of spore germination medium. Each plate was sealed with Micropore tape (3M Healthcare), chilled for 3 days at 4°C then transferred to a growth cabinet and kept in 24 hour light at 22°C. Plates were checked daily and counts of germinated and ungerminated spores were taken. Spores with at least one filament emerging were counted as germinated (figure 2.2). Most treatments were counted every other day over a period of 7-10 days. After approximately 15 days individual plants were often large enough to cover up any ungerminated spores. At this point no more counting was carried out.

Various statistical analysis methods were trialled to best represent that data from germination assays (figures 3.10). Calculation of the z-ratio for the significance of the difference between two independent proportions was found to best fit the data gathered.

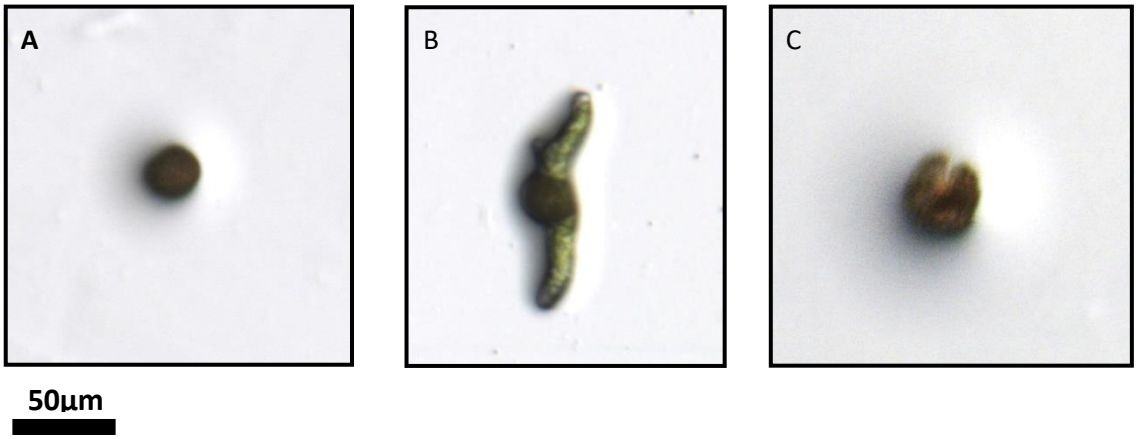


Figure 2.2 Images of ungerminated and germinated *P. patens* spores showing what three spore types were looked for when counting. 'Dud' spores (C) were those with rough edges, discolouration and/or reduced size and were discounted as they would not have the potential to germinate. The ratio of ungerminated (A) to germinated (B) spores gave overall % germination across 2-3 independent counts.

2.2 Nucleic acid isolation and analysis

2.2.1 RNA isolation

Dry sporophytes were pooled in batches of approximately 250 into ribonuclease (RNase)-free tubes, frozen in liquid nitrogen and ground using micropestles (Sigma-Aldrich). Imbibed spores were prepared prior to RNA extraction by perforating 250 sporophytes with a sterile pipette tip in liquid spore germination BCD media and placing on a platform rocker for 18 hours under standard conditions. Spores were spun down and excess liquid removed before freezing in liquid nitrogen and grinding with micropestles (Sigma-Aldrich). Germinating spores were prepared as per germination assay and RNA extraction was carried out once the level of germination reached approximately 65% (usually 7 days). Protonemal and germinating tissue was scraped from plates using an RNase-free microspatula, placed in RNase-free tubes, frozen in liquid nitrogen and ground using micropestles. Gametophyte tissue was collected from mature peat plugs, placed in a pre-chilled RNase-free mortar, frozen by covering with liquid nitrogen and ground to a fine powder. Frozen tissue was transferred to RNase-free tubes using microspatulas. RNA was extracted using a Bioline Isolate II RNA plant kit (Bioline) according to manufacturer's instructions. Briefly, homogenised tissue was lysed and filtered through a column. Binding conditions were then adjusted using 70% ethanol and RNA bound to a column membrane and desalted. On-column DNA digestion was followed by three washings before elution in warm RNase-free water. RNA samples were quantified using NanoDrop spectrophotometer (Thermo Scientific)

and stored at -20°C prior to RT-PCR. For long term storage samples were stored at -80°C.

2.2.2 DNA isolation

Protonemal samples were prepared as above and DNA extracted using a Bioline Isolate II DNA plant kit (Bioline) according to manufacturer's instructions. Briefly, homogenised tissue was lysed and filtered through a column. Binding conditions were then adjusted with binding buffer and DNA bound to a column membrane. Membrane was washed three times before adding warmed (65°C) elution buffer, incubating at 65°C and centrifuging to elute DNA. Samples were quantified using NanoDrop spectrophotometer (Thermo Scientific) and stored at -20°C prior to PCR. For long term storage samples were stored at -80°C.

2.2.3 Primer design

Primers were designed by hand from sequence data of putative ABA and GA signalling genes (see table 2.1 and 2.2 for sequences). Efficacy of primers was determined using OligoCalc.

(Northwestern University. http://www.basic.northwestern.edu/biotools/oligocalc.html).

Gene	Forward primer sequence	Reverse primer sequence
PpDELLAa	AACAAGAGTTGCACATGGCGTA	GGTGGCAGACTAAGCAGCTC
PpDELLAb	CTGGAGAACAACGCGATGGC	CCCTCGCTGGATAGATTCCG
GLP1 GID1-like	CTCTATTACCATGGAGGCGGA	CCGAATATCTCATCTCCCAATC
GLP2 GID1-like	TTATTACTACCACGGAGGCGGG	CTCCGCAAATCGCATCTCCCAA
GLP3 GID1-like	GGATGTATGGGTGCGTCTTTTC	TGCGTATCTCGTCTCCCAGTC
GLP4 GID1-like	CTGCCCAGGCGAGCTCCGG	GAACATATGGACGCCATCCTCG
GLP5 GID1-like	CCCAATCACTCCCGGGCCGT	AGCTCCGCGACTACGACCAGA
GLP6 GID1-like	AATGCAGGCGGTGAGAGTCCC	GGGATCTTTGCCACCTACAA
PpGAMYB1	CATGGCTGCCCAACTTCCCG	GAGCGGACTAGGATTGGTAATC
PpGAMYB2	TTGATGCCTTAATGCAGGATGC	GCGGAGCACACGGAACAGG
CPS/KS	CACAGACTTCCGATACCCATGG	GCCTTGGCATCTTCCATCATCG
ent-k oxidase	CCTTCGCCTTGCAGCAGGTG	AGCGCACATCCTCTTTCCAGC

Table 2.1 Primer list for putative *P. patens* GA biosynthesis and signalling genes.

Gene	Forward primer sequence	Reverse primer sequence
PpABI1a	AGACCGTCCGGAGGTGACTG	GCGGACTCAACTTCCTCTGCT
PpABI1b	TATGCCTGGTGACTTATACCAG	CTCGCTCTGGCTTGTGATCC
PpABI3A	CGGTTGATGGTTGAGGGCGA	GGCCAAAACCTGTATCGAATGT
PpABI3b	GAAATGAGAAAAGTCCCTGCCC	TCGGCCAGAACCTGTATCTGAT
PpABI3C	CAGCAGCAGAGGCAGAGGCG	TGCGCCCCTTCTGTTCAGCA
ABA2 homologue Phypa_125575	TCAGAACTTGTTTGAGGGGATC	GCTGGCACAGTATGTGTGGG
ABA2 homologue Phypa_202254	CTACAGCATGTGGCATCTCCG	ACTCGAATACCGTAACCCGCAT
AAO3 homologue Phypa_106708	TCCTTTCCGGAGAGCCAACC	TGTCGTCACTATGCCCTCAGA
AAO3 homologue Phypa_140802	GGGCACGCACAATGTAACGTCA	GCACCTTCAACCTGTCCGACG
Putative ABA receptor Phypa_209242	GAGAAACAGGGGCGGCCGGA	GCCGCTGGTTTCTCGTTGGAG
Putative ABA receptor Phypa_222359	GATGCTACCCACCCCGCCA	CCCCTCTCCATATCTAAGCATT
Putative ABA receptor Phypa_132509	CGTCACAGGGCAGCGGCG	CAGGTGCAAATTACAGTACTGG
Putative ABA receptor Phypa_213389	AGGAGGAGCACGCGTACGCA	GGACGCTGTGAGCACGCAAG
Putative SnRK2 Phypa_195464	GTGAAGGACATTGGGTCGGG	CGGGATCCTCAAACGGATATG
Putative SnRK2 Phypa_194508	TTCGAAGTCCTCCTTGTTGCAC	CCCACATCGGGATCCGCATC
Putative SnRK2 Phypa_106968	TCCTCCTGTACTTGCTTCTGG	CGCTCAAGATACGTCCAATGG
Putative SnRK2 Phypa_215231	ACTCGGGAGCTTGTTGCGGTG	GGACCCCAACCATCCCCCTC

Table 2.2 Primer list for putative *P. patens* ABA biosynthesis and signalling genes.

2.2.4 Reverse-transcription PCR (RT-PCR)

RNA samples were diluted to a final concentration of 4ng/µl to give a total of 20ng in each PCR reaction for use in Bioline MyTaq™ One-Step RT-PCR kit (Bioline) as per manufacturer's instructions. Briefly, first-strand cDNA synthesis and subsequent PCR are carried out in a single tube through the use of a one-step mix combined with gene-specific primers at a final concentration of 400nM.

PCR one-step mix

MyTaq™ One-Step mix	12.5µl
Reverse transcriptase	0.25µl
RiboSafe RNase inhibitor	0.5µl
Forward primer (10µM)	1µl
Reverse primer (10µM)	1µl
DEPC-H_2O	4.75µl
RNA template (4ng/µl)	5µl

In most cases the recommended PCR conditions were followed - one reverse transcription cycle of 20 minutes at 45°C, one polymerase activation cycle of 1 minute at 95°C and 40 cycles of denaturation (10 seconds at 95°C), annealing (10 seconds at 60°C) and extension (30 seconds at 72°C)

2.2.5 Agarose gel electrophoresis

1% agarose gels were made using TBE (90mM Tris, 90mM boric acid, 2.5mM EDTA) supplemented with 1.5µl/100ml ethidium bromide. Nucleic acid samples were mixed with 6x loading buffer (New England BioLabs® Inc.) and run alongside a 100bp marker ladder (New England BioLabs® Inc.). Expression was visualised with Molecular Imager® Gel Doc™ XR+ system with Image Lab™ software (Bio-Rad).

2.3 *Ulva intestinalis* tissue culture

A variety of tissue culture methods were trialled as part of the method development section of chapter V.

2.3.1 Preparation of growth media

Artificial seawater (ASW) was made by diluting 34g/litre Marin sea salt (Tropic Marin) in distilled H_2O. Provasoli media was made by adding 20ml/litre enrichment solution (see table 2.3 and 2.4) to ASW and sterilised by steaming to 100°C.

Ulva culture media (UCM) was made by adding 10ml of solutions II, III and IV to 1 litre of solution I then adding 2ml of filter sterilised vitamin solution V (see tables 2.5 and 2.6). Solutions II, III and IV were autoclaved to sterilise and stored in UV resistant amber bottles (Duran®) at 4°C to limit degradation of solutions. Solution V was filter sterilised into 50ml Falcon™ tubes (Fisher Scientific) and stored at -20°C.

Enrichment solution	
Chemical	Quantity
TRIS base	5g
$NaNO_3$	3.5g
Na_2 β-glycerophosphate H_2O	0.5g
Iron EDTA solution (see below)	250ml
PII trace metal solution (see below)	25ml
Thiamine	0.5mg
Biotin (5mg/litre dH_2O)	1ml
Cyanocobalomin (10mg/litre dH_2O)	1ml
distilled H_2O	to 1 litre

Table 2.3 Components of the enrichment solution for addition to ASW to make Provasoli media

Iron EDTA solution	
Chemical	Quantity
$Na_2EDTA \cdot 2H_2O$	841mg
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	702mg
distilled H_2O	to 1 litre

PII trace metal solution	
Chemical	Quantity
$Na_2EDTA \cdot 2H_2O$	12.74g
$FeCl_3 \cdot 6H_2O$	484mg
H_3BO_3	11.44g
$MnSO_4 \cdot 4H_2O$	1.62g
$ZnSO_4 \cdot 7H_2O$	220mg
$CoSO_4 \cdot 7H_2O$	48mg
distilled H_2O	1 litre

Table 2.4 Components of the two solutions (Iron EDTA and PII trace metal) needed to prepare the enrichment solution for addition to ASW to make Provasoli media

Solution I		Solution II (100x stock)	
Chemical	Quantity	Chemical	Quantity
NaCl	19.14g	NaH_2PO_4	0.7g
Na_2SO_4	3.21g	$NaHCO_3$	8.8g
$MgCl_2$	8.39g	$C_4H_{11}NO_3$	10g
$CaCl_2$	0.83g	distilled H_2O	1 litre
$NaNO_3$	8.5g		
$(NH_4)_2SO_4$	1.1g		
distilled H_2O	1 litre		

Solution III	
Chemical	Quantity
KBr	7.84g
KCl	54.2g
$SrCl_2$	1.95g
distilled H_2O	1 litre

Table 2.5 Solutions I, II and III for assembly of Ulva Culture Media (UCM)

Solution IV (100x stock)		Vitamin solution V	
Chemical	Quantity	Chemical	Quantity
$C_{10}H_{16}N_2O_8$ (50x)	20ml	Vitamin B_{12} (2.3mM)	1.6µl
H_3BO_3 (100x)	10ml	Thiamin (16.62mM)	4ml
$FeSO_4$ (1000x)	1ml	Nicotinic acid (4.06mM)	20ml
$CuSO_4$ (10,000x)	100µl	Pantothenic acid (11.4mM)	4ml
$NaMoO_4$ (10,000x)	100µl	Pyridoxine (59.11mM)	400µl
$MnCl_2$ (1000x)	1ml	ρ-aminobenzoic acid 18.23mM)	400µl
$ZnSO_4$ (1000x)	1ml	Biotin (1.02mM)	2ml
$CoCl_2$ (10,000x)	100µl	Thymine (15.86mM)	40ml
NH_4VO_3 (10,000x)	100µl	Inositol (55.51mM)	10ml
KI (10,000x)	100µl	Orotic acid (41.64mM)	4ml
Na_2SeO_3 (5000x)	50µl	Folic acid (45.34mM)	1µl
Na_2WO_4 (20,000x)	50µl	Putrescine (113.44mM)	400µl
distilled H_2O	to 1 litre	Riboflavin (13.29mM)	100µl
		Pyridoxamine (14.86mM)	800µl
		Choline (345.6mM)	1ml
		distilled H_2O	to 100ml

Table 2.6 Solutions IV and V for assembly of Ulva Culture Media (UCM)

2.3.2 Artificial induction of gametogenesis and sporogenesis

Mature *U. intestinalis* blades were induced to produce gametes and spores by chopping (Zyliss® food chopper) individual blades into small pieces approximately 30x30 cells. Tissue was washed three times by leaving in 100ml ASW for 15 minutes, filtering through gauze and placing in fresh ASW. This process removes the naturally occurring sporulation inhibitor, allowing induction to take place (see chapter V). After three repeats pieces were placed in petri dishes containing approximately 75ml UCM or Provasoli media and cultured at 18°C for 3 days. To release gametes/spores from gametangia/sporangia the swarming inhibitor had to be diluted (Wichard and Oertel, 2010). This was done by filtering tissue, washing with ASW and returning to petri dishes containing fresh media. These were then placed in front of a bright lamp. Biflagellate gametes are positively phototactic and, once released, will swim towards the light and pool together at the closest point. Quadriflagellate spores are negatively phototactic and will swim to the furthest point away from the light in the petri dish (figure 2.4). Once gametes/spores had collected in high enough densities they were transferred to microcentrifuge tubes and returned to the light to facilitate further concentration and purification.

2.3.3 Gamete purification

The positive phototaxis displayed by *U. intestinalis* gametes made it possible to purify samples to give axenic cultures. After swarming, gametes are able to swim faster than any flagellated cells, bacteria or diatoms that may be contaminating the culture. All stages of purification were carried out in a sterile flow hood with autoclaved equipment and media. Gametes were pipetted into the wide end of a Pasteur pipette filled with ASW (figure 2.3). A bright light was placed at the narrow end and gametes moved from one end to the other within 7-10 minutes. As soon as the majority of gametes had collected at the narrow end of the pipette they were removed by tipping the pipette slightly to produce one or two drops of concentrated gametes. A drop of the liquid directly behind the collected gametes was dropped onto an LB agar plate to check for bacterial contamination. If the concentration of gametes was high, purification was repeated to get the cleanest sample possible. The LB plate provided a good indication of how clean a sample was. The clean samples were then used for culturing new tissue and RNA and DNA extraction.

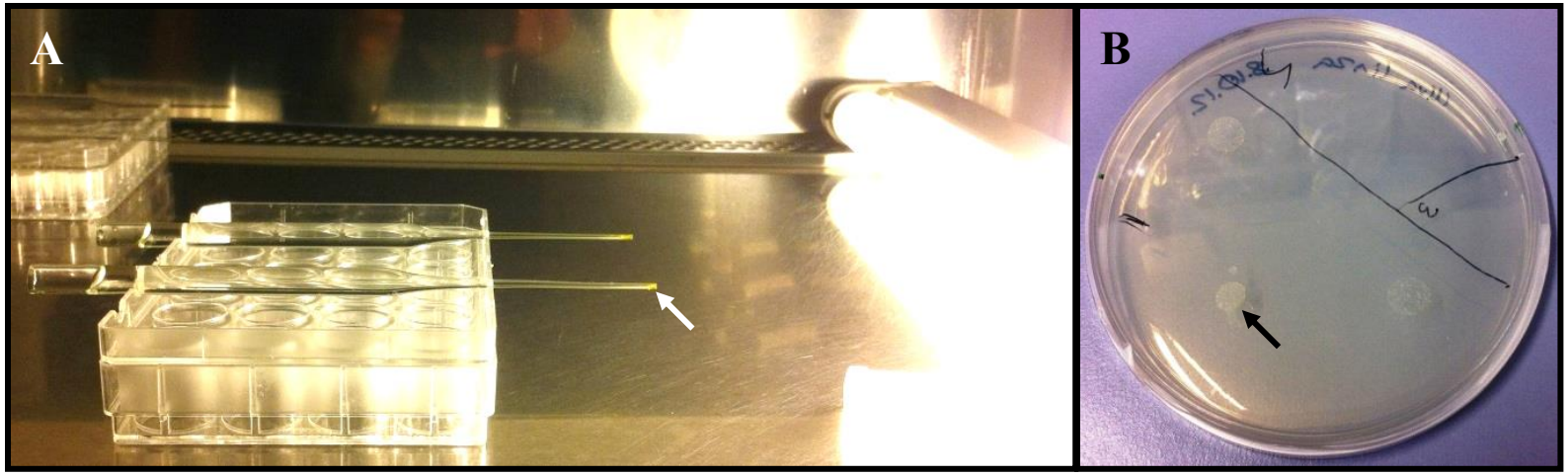


Figure 2.3 Purification of gametes by positively phototactic movement along a Pasteur pipette. Gametes swam towards light at narrow end of the pipette (A) and collected at the tip (⇦). Contamination by bacteria was checked by putting a single drop of the liquid directly behind the collected gametes onto an LB agar plate (B). Salt residue shows where water droplet was placed on plate (⬅).

2.3.4 Nucleic acid isolation

Spores and gametes were spun down and excess liquid removed before freezing in liquid nitrogen and grinding with micropestles (Sigma-Aldrich). Mature gametophyte and sporophyte blades were dried with tissue paper, placed in a pre-chilled RNase-free mortar, frozen by covering with liquid nitrogen and ground to a fine powder. Frozen tissue was transferred to RNase-free tubes using microspatulas. RNA was extracted using a variety of methods as detailed in the method development section of chapter V. RNA samples were quantified using NanoDrop spectrophotometer (Thermo Scientific) and stored at -20°C or -80°C.

Chapter III:

The role of terpenoids as plant hormones

3.1 Introduction

Terpenoids are a large group of organic chemicals found in all living organisms. They are composed of a carbon skeleton and multiple functional units, the number and arrangement of which defines their classification. Diterpenoids are a class of terpenoids with 20 carbons and are classified by the number of structural rings. In plants, diterpenoids are produced in the plastid via the methyl-erythritol 4-phosphate pathway (MEP) yielding the primary intermediate geranylgeranyl pyrophosphate (GGPP).

In plants, the biologically active diterpenoid gibberellins mainly act as promoters of growth (Richards *et al.*, 2001; Claeys *et al.*, 2014). This includes breaking seed dormancy to induce germination (Cao *et al.*, 2006), an increase in cell division and elongation to promote growth and development (Chhun *et al.*, 2007; Achard *et al.*, 2009; Ubeda-Tomas *et al.*, 2009; Claeys *et al.*, 2012; Nelissen *et al.*, 2012) and as a stress response (Colebrook *et al.*, 2014). They can also work positively to delay senescence in leaves (Schippers *et al.*, 2007; Jyothsna and Murthy, 2016) and establish parthenocarpic fruit development (Garcia-Hurtado *et al.*, 2012; Mesejo *et al.*, 2016) - traits which have been seized upon by the agricultural sector.

The gibberellin biosynthesis pathway in flowering plants produces bioactive gibberellins such as GA_1 and GA_4 from GGPP (figure 1.7). Whilst no bioactive gibberellins have been identified in *P. patens*, the early diterpenoid precursors *ent*-kaurene (*ent*-k) and *ent*-kaurenoic acid (*ent*-KA) have (Anterola *et al.*, 2009; Hayashi *et al.*, 2010) (figure 1.8). The bifunctional enzyme copalyl diphosphate/kaurene synthase (CPS/KS) carries

out the conversion of GGPP to *ent*-kaurene and the cytochrome p450 enzyme kaurene oxidase catalyses the oxidation to produce *ent*-kaurenoic acid. However, the enzymes responsible for subsequent production of GA_{12} (kaurenoic acid oxidase) and further GAs (GA 20-oxidase and GA 3-oxidase) are not encoded by the *P. patens* genome and consequently no gibberellins are produced. Intriguingly, the gibberellin GA_9 methyl ester (GA_9-ME) (figure 1.8), an identified antheridiogen in ferns (Tanaka *et al.*, 2014) was found to be bioactive in *P. patens* in regulation of protonema (Hayashi *et al.*, 2010).

Whilst there are over 130 identified GAs, only a small number are biologically active phytohormones with the others acting as precursors or degradation products of bioactive GAs. The role of GAs in seed germination, in particular the antagonistic relationship with ABA has led them to be a prominent area of phytohormone research.

The work in this chapter is based on the hypothesis that moss spore germination is regulated by similar hormones to those with roles in seed germination. If the germination-specific function of diterpenes has been conserved then:

- Exogenous application of bioactive diterpenoids *ent*-kaurene and GA_9-ME will promote germination of *P. patens* spores
- Spores of the diterpene biosynthesis mutant *Ppcps/ks* will have a reduced germination phenotype when compared to wild-type (wt)
- The expression profile of diterpenoid biosynthesis and GA signalling genes will corroborate previous literature from seed gene expression
- Gene expression will coincide with identified bioactivity germination assays

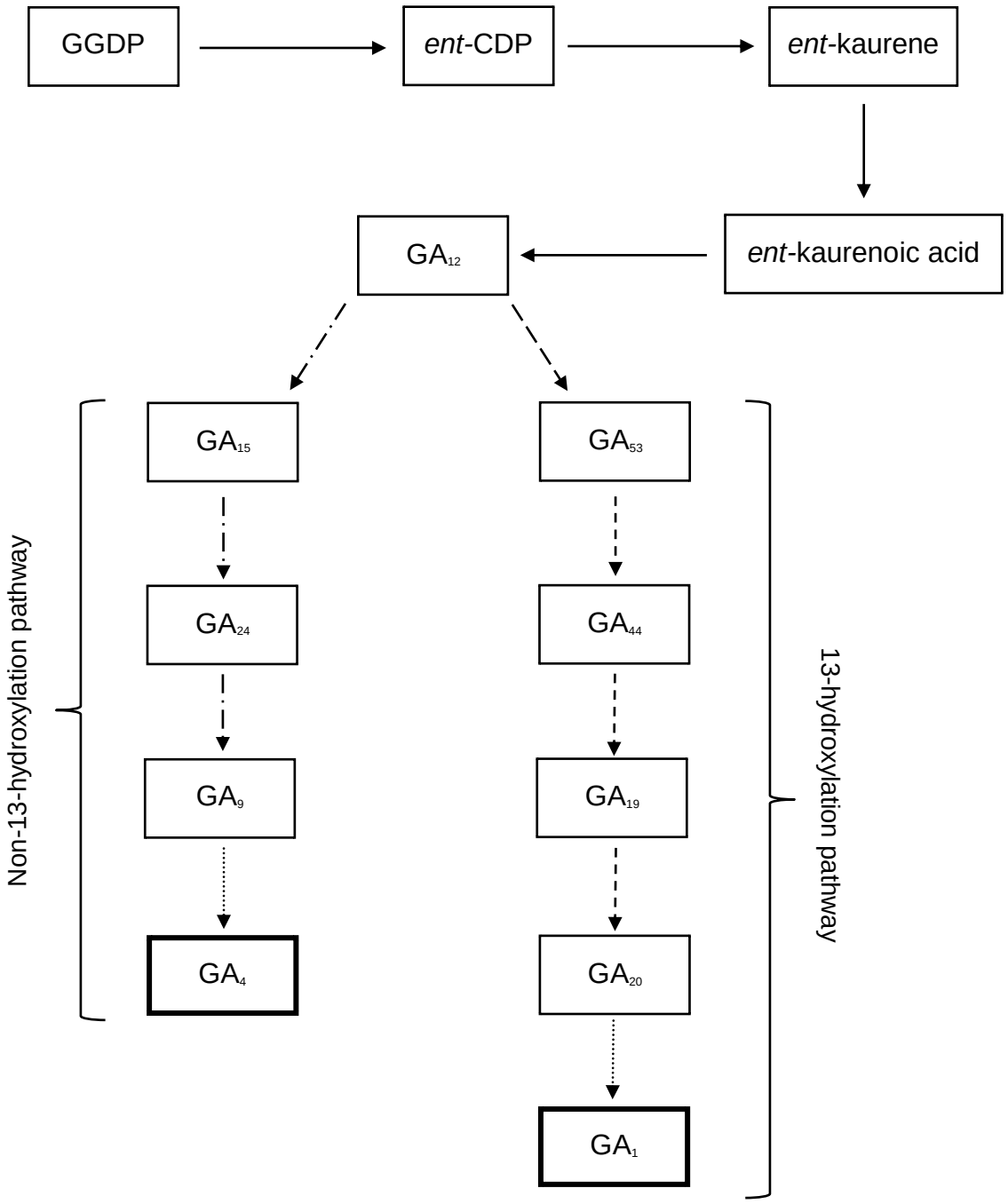


Figure 3.1 Major angiosperm gibberellin biosynthetic pathways. Conversion of geranylgeranyl diphosphate (GGDP) to GA_{12} is carried out by a series of enzymes (see figure 1.8). GA 20-oxidases (◄·—·—) and GA 13-oxidases (◄———) produce a series of GA precursor molecules which are then converted to bioactive gibberellins such as GA_1 and GA_4 by GA 3-oxidases (◄·····) via the 13-hydroxylation pathway or non-13-hydroxylation pathway.

3.2 The diterpenoids GA_9 methyl ester and *ent*-kaurene promote *P. patens* wild type spore germination

In order to first determine the effect of bioactive moss diterpenoids on spore germination, wt *P. patens* spores were assayed for germination on two concentrations of GA_9-me and *ent*-kaurene previously shown to be fully bioactive (Hayashi *et al.*, 2006; Hayashi *et al.*, 2010). Exogenous application of GA_9-me and *ent*-kaurene caused a significant acceleration of germination in three or more biological replicates of each assay (Figures 3.2 and 3.3). Wild-type spores germinated earlier and at a faster rate than the untreated controls. All treatments achieved just under 100% germination efficiency, suggesting that these compounds act as positive regulators of germination. In light of this confirmation of bioactivity, the analysis of a mutant that cannot synthesise diterpenes would provide additional insight into their endogenous roles.

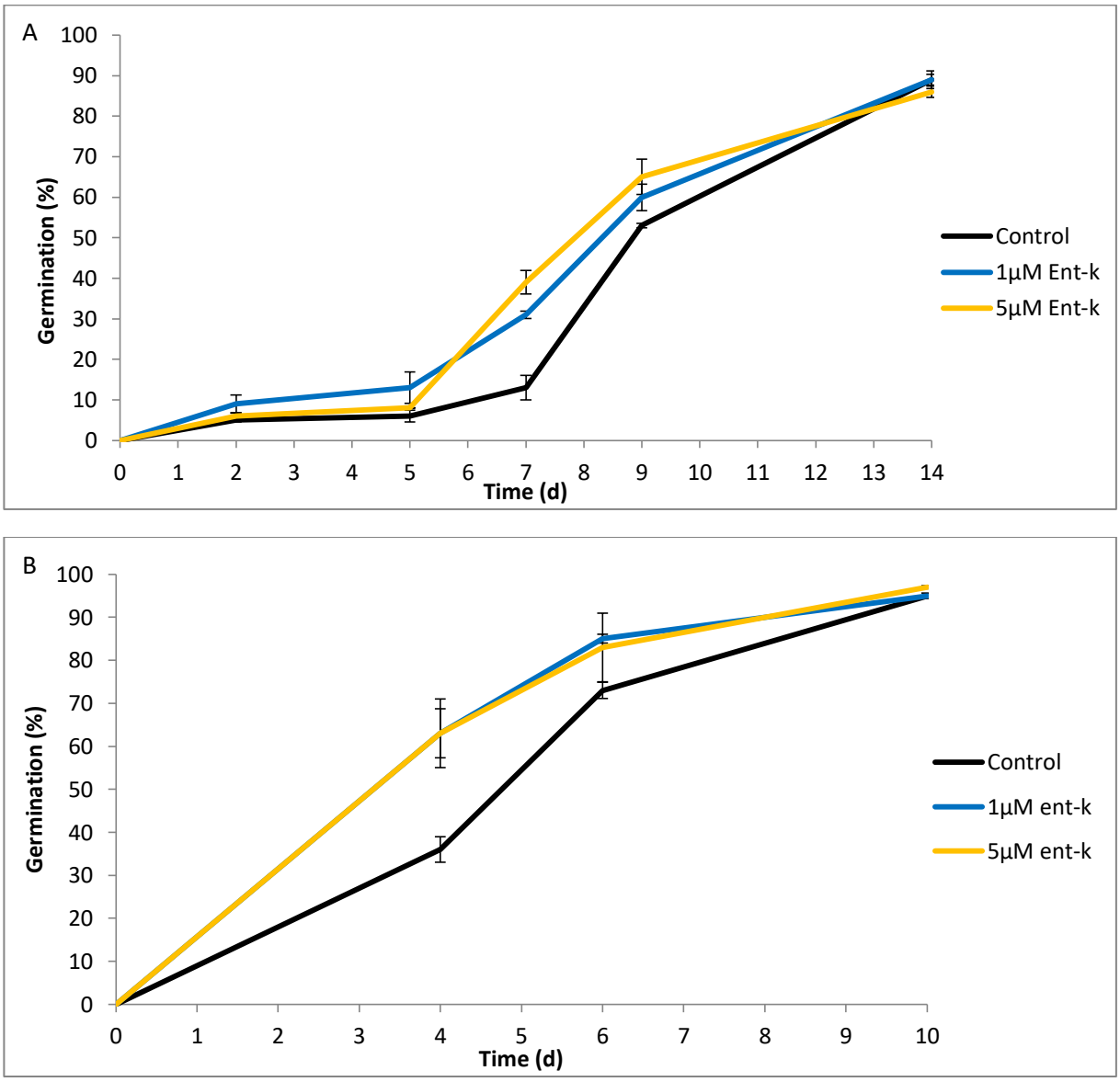


Figure 3.2 Promotion of germination by *ent*-kaurene

A *P.patens* spores were germinated on media containing 0, 1 or 5µM *ent*-kaurene. The number of spores germinated was counted as a % of total spores on the plate. Approximately 300 spores were counted per plate and three plates were counted per treatment to provide replicates. Counts were done every two to three days. Error bars represent ± SEM. Z test indicated significant differences between untreated and treated spores on days 2, 5, 7 and 9 with 1µM *ent*-k and days 7, 9 and 14 with 5µM *ent*-k P >|t| 0.05

B Representative second biological replicate of effects of *ent*-k on *P. patens* spore germination. Z test indicated significant differences between untreated and treated spores on days 4 and 6 with 1 and 5µM. Two tailed P-value is less than 0.05 (>|t| 0.05)

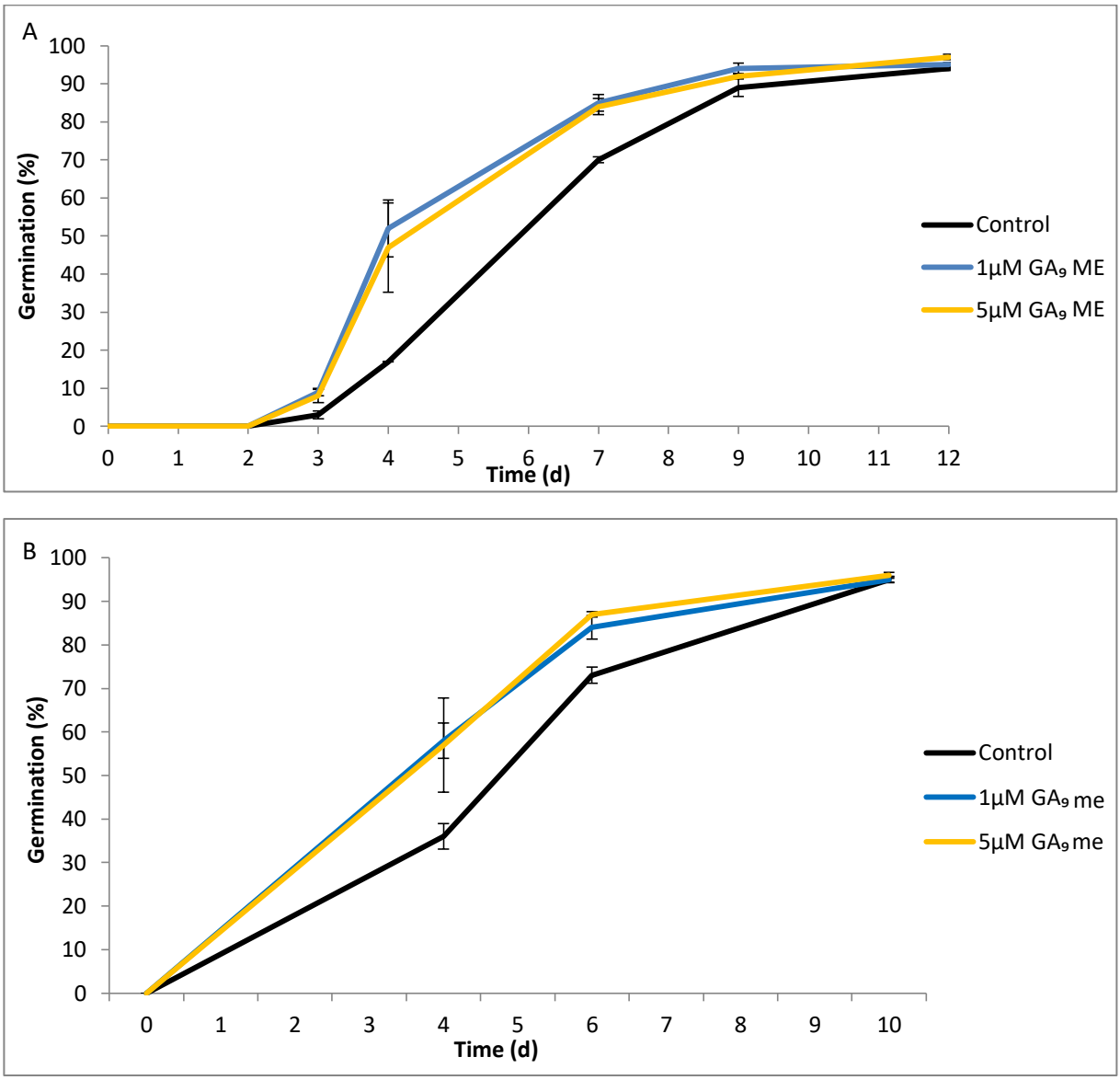


Figure 3.3 Promotion of germination by GA_9 methyl ester

A *P. patens* spores were germinated on media containing 0, 1 or 5µM GA_9-me. The number of spores germinated was counted as a % of total spores on the plate. Approximately 300 spores were counted per plate and three plates were counted per treatment to provide replicates. Counts were done every two to three days. Error bars represent ± SEM. Z test indicated significant differences between untreated and treated spores on days 3, 4 and 7 with 1µM and 5µM *ent*-k P >|t| 0.0002

B Representative second biological replicate of effects GA_9-me on *P. patens* spore germination. Z test indicated significant differences between untreated and treated spores on days 4 and 6 with 1 and 5µM P >|t| 0.0002

3.3 The diterpenoid biosynthesis mutant *Ppcps/ks*

3.3.1 *Ppcps/ks* displays reduced spore germination

The gibberellin biosynthesis pathway is a complex multi-route pathway. The first two steps, converting trans-geranylgeranyl diphosphate (GGDP) to *ent*-kaurene (*ent*-k), are catalysed by the dual function enzyme *ent*-copalyl diphosphate synthase (CPS) / *ent*-kaurene synthase (KS) (Figure 1.4) in *P. patens*. The production of these terpenoids by *P. patens* has been investigated from a biotechnological perspective by Simonsen (unpublished data). The generation and provision of a *Ppcps/ks* disruption mutant by this group (Zhan *et al.*, 2015) allowed analysis of the role of diterpenoid GA-precursors in *P. patens* spore germination. Two independent knock-out lines were germinated alongside corresponding wt spores. In both lines, the *Ppcps/ks* mutant spores germinated at a slower rate than wt (figures 3.4 and 3.5). This suggests that inhibition of this early step in the gibberellin biosynthesis pathway impacts on gibberellin-mediated functions in spore germination. However, the spores were able to germinate successfully and displayed a high final germination efficiency (91% +) (data not shown). This suggests that, unlike in flowering plants, gibberellins are not absolutely required for germination to occur.

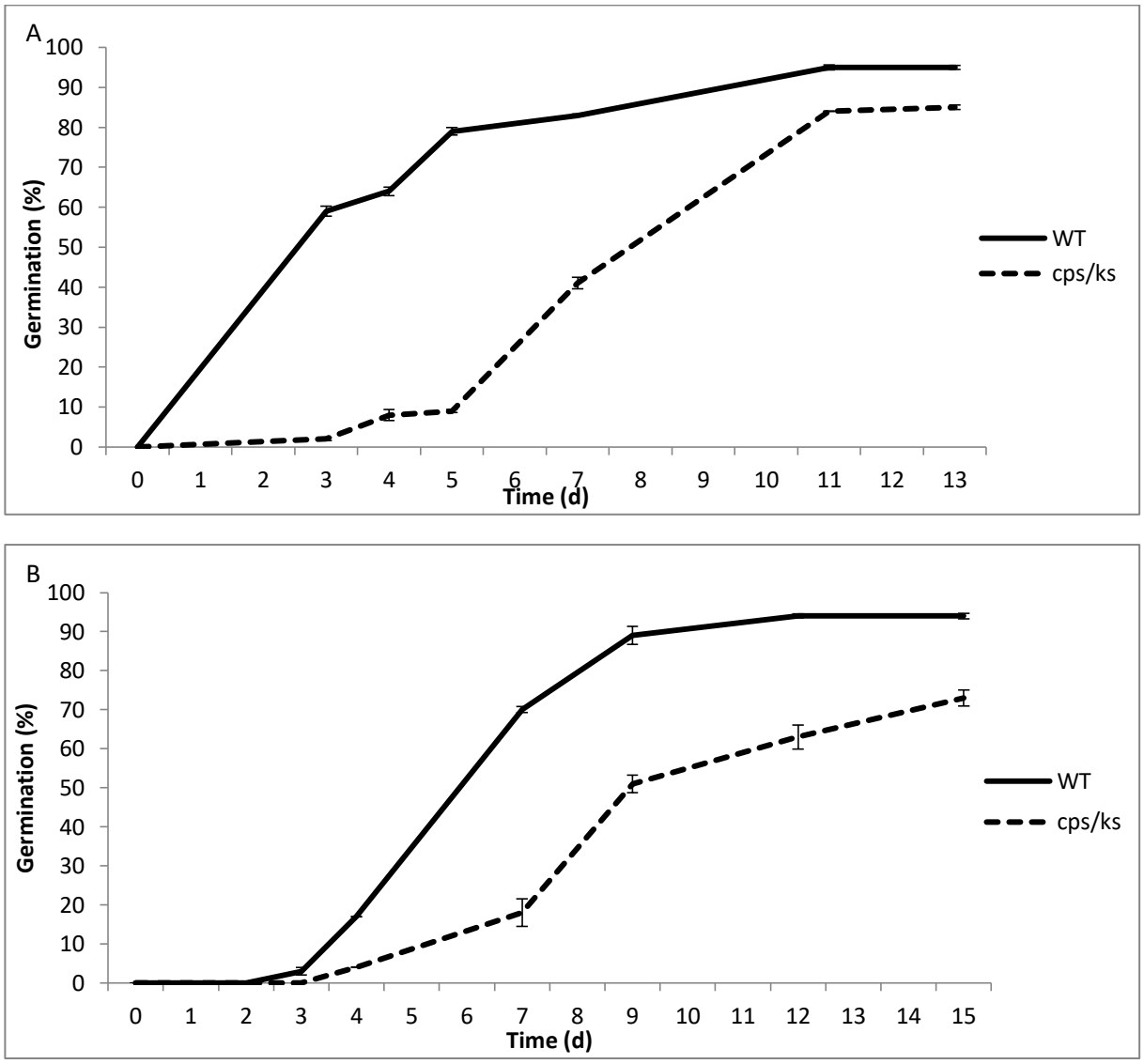


Figure 3.4 Germination is slower in the diterpene mutant *Ppcps/ks* (line pCL755#29)

A *P. patens* wt and *cps/ks* (line pCL755#29) spores were germinated on BCD media. The number of spores germinated was counted as a % of total spores on the plate. Approximately 300 spores were counted per plate and three plates were counted per treatment to provide replicates. Counts were done every two to three days. Error bars represent ± SEM. Z test indicated significant differences between wt and *cps/ks* spores on days 3 (P >|t| 0.0007), 4, 5, 8, 10 and 17 P >|t| 0.0002

B Representative second biological replicate showing slower spore germination in *Ppcps/ks* spores. Z test indicated significant differences between phenotypes on days 4 to 15 P >|t| 0.0002

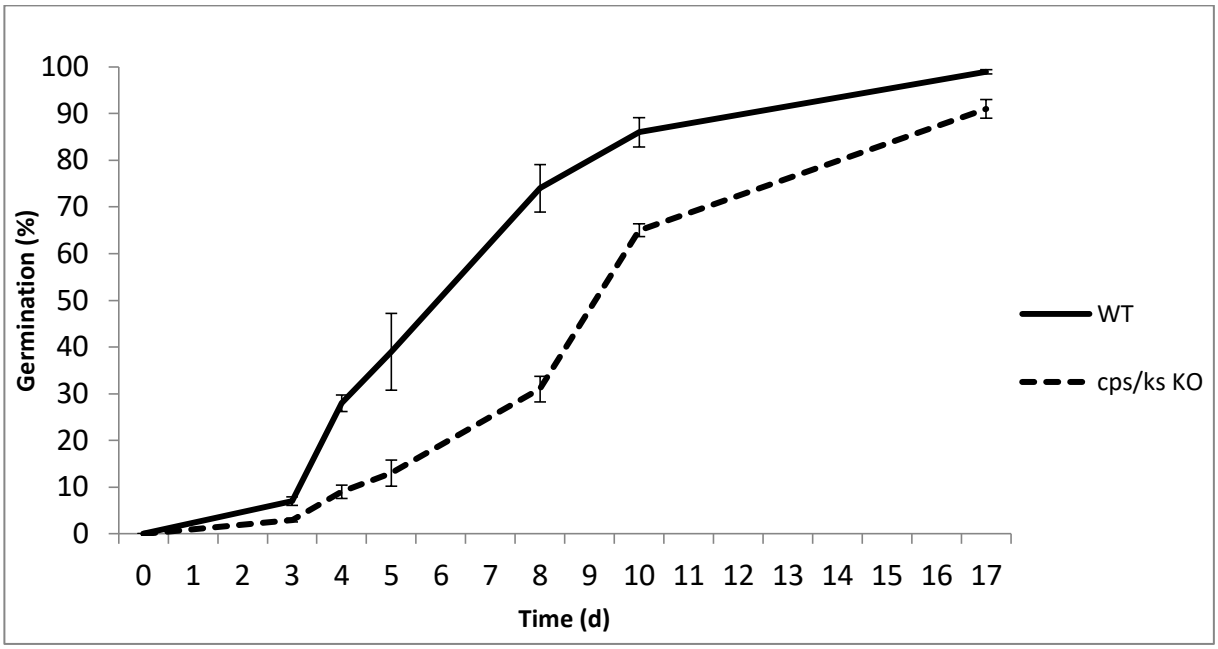


Figure 3.5 Germination is slower in the diterpene mutant *Ppcps/ks* line (pBK3)

P. patens wt and *cps/ks* (line pBK3) spores were germinated on BCD media. The number of spores germinated was counted as a % of total spores on the plate. Approximately 300 spores were counted per plate and three plates were counted per treatment to provide replicates. Counts were done every two to three days. Error bars represent ± SEM. Z test indicated significant differences between wt and *cps/ks* spores on days 3 (P >|t| 0.0007), 4, 5, 8, 10 and 17 P >|t| 0.0002

3.3.2 *Ppcps/ks* displays reduced colony size

The *Ppcps/ks* mutant also shows a smaller protonemal colony size than wt after 8-10 days growth (figure 3.6). The maximum diameter of individual protonemal plants was significantly reduced when compared with wt. This may be attributed to the additional roles of gibberellin-like compounds in plants that have been defined in more depth in angiosperms (Claeys *et al.*, 2014). Further investigation of this at later developmental time-points would allow additional comparison of the roles of GAs in basal and higher land plants.

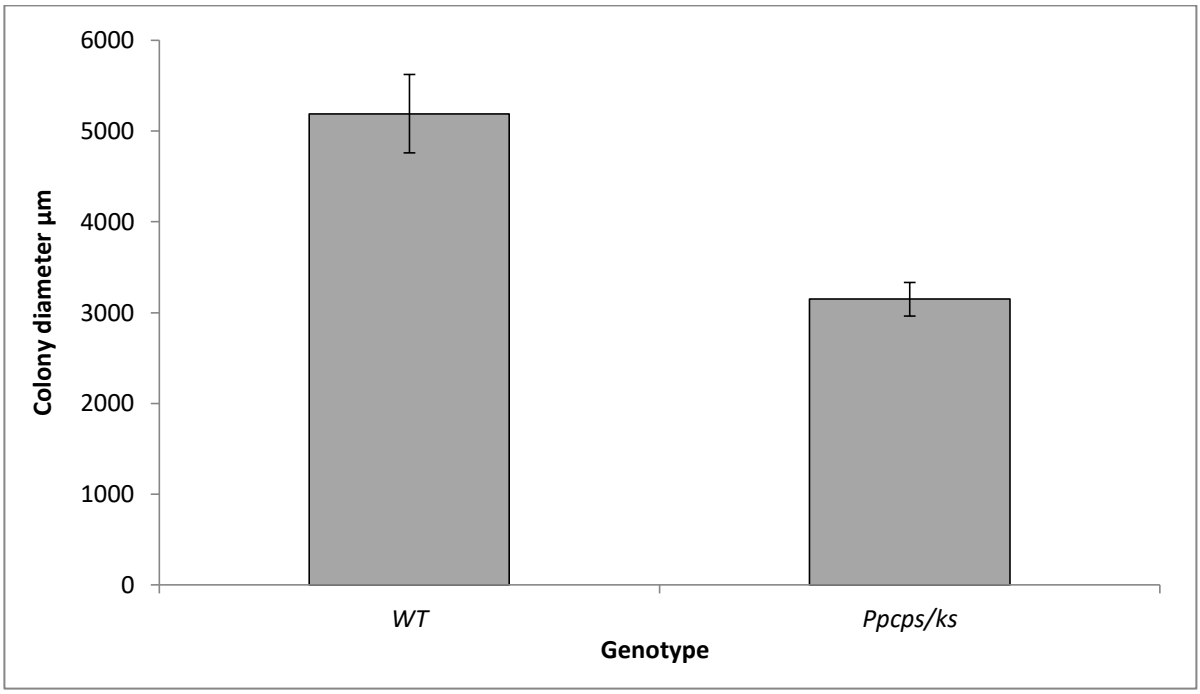


Figure 3.6 Colony size is reduced in the *Ppcps/ks* mutant

10 independent measurements were taken of wt and *Ppcps/ks* protonemal plants. Error bars represent ± SEM. T-test indicates significant difference in size between wild-type and mutant (P = 0.001)

3.3.3 The *Ppcps/ks* germination phenotype can be rescued by GA_9 methyl ester and *ent*-kaurene

The slower rate of germination in the *Ppcps/ks* mutant was attributed to its inability to manufacture a functional CPS/KS enzyme. In wt this enzyme catalyses the production of *ent*-kaurene from GGPP so my hypothesis was that exogenous addition of *ent*-kaurene into the growth media of *Ppcps/ks* spores would rescue this phenotypic reduction. The previously demonstrated bioactivity of GA_9-me in *P. patens* warranted its inclusion as another potential source of rescue.

Both *ent*-k and GA_9-me were able to rescue the germination phenotype of the *Ppcps/ks* mutant (figures 3.7 and 3.8). Spores treated with *ent*-k or GA_9-me germinated at a faster initial rate than untreated mutant spores and this effect was most pronounced early on in culture. However in most cases the germination rate did not return to that observed in untreated wt control spores.

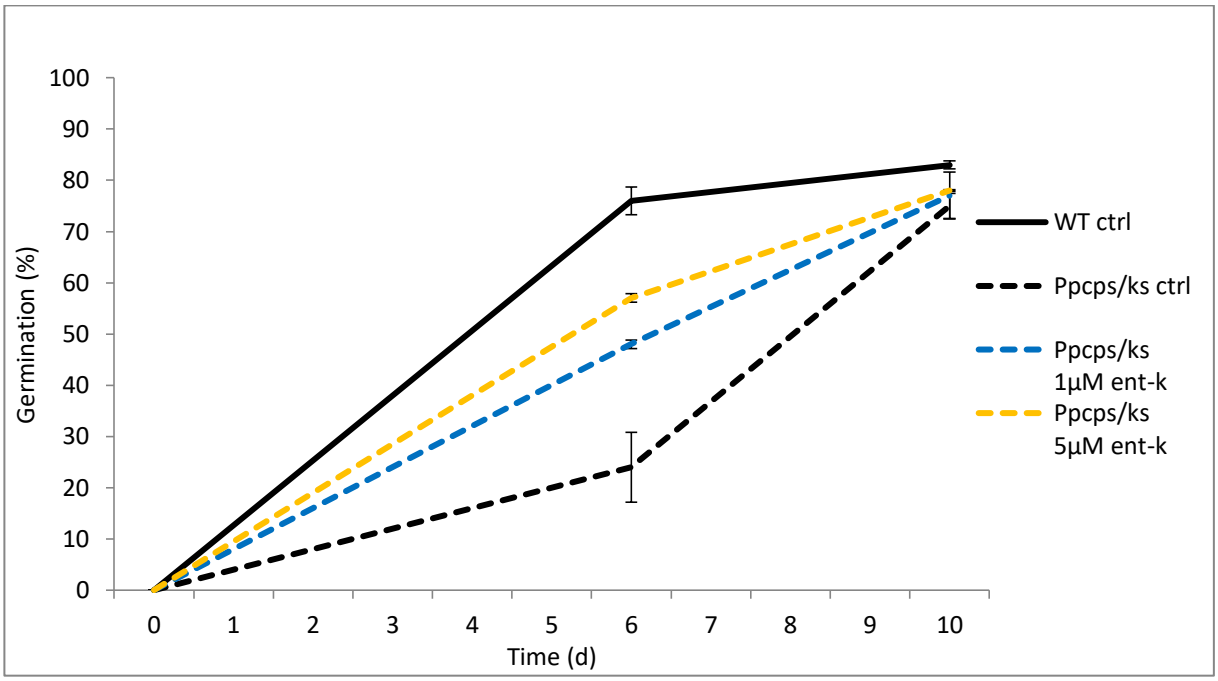


Figure 3.7 The *Ppcps/ks* germination phenotype can be rescued by ent-kaurene

Ppcps/ks mutant spores were germinated on media containing 1 or 5µM *ent*-K to see if exogenous application of a diterpenoid was able to rescue the biosynthesis mutant. Untreated mutant spores displayed the typical delayed germination phenotype with an overall slower rate of germination. Germination rate of untreated wt spores was also included to demonstrate the extent to which exogenous diterpenoids could rescue the *Ppcps/ks* mutant phenotype. Both concentrations of *ent*-K resulted in a statistically significant increase in germination rate against untreated *Ppcps/ks* spores on day 6, Z test (P >|t| 0.0002). Error bars represent ± SEM. Despite promotion of germination by *ent*-K, germination rate of treated *Ppcps/ks* spores was still significantly slower than untreated wt spores (P >|t| 0.0002).

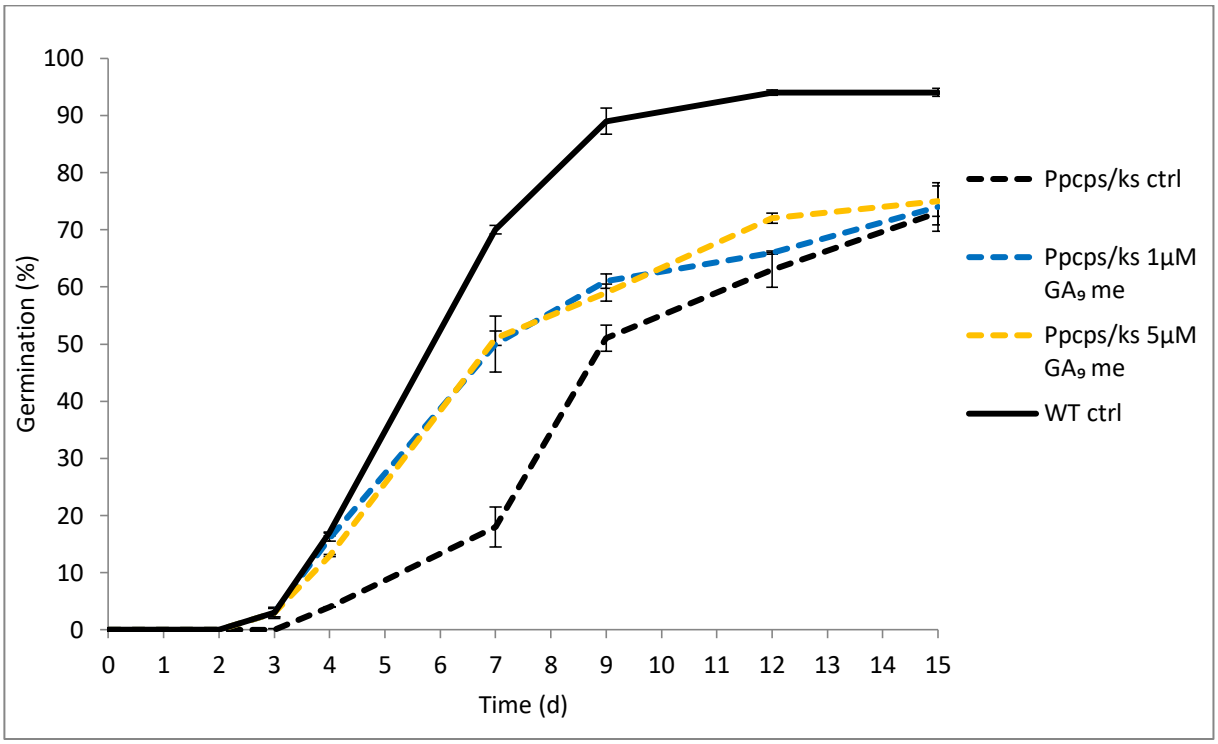


Figure 3.8 The *Ppcps/ks* germination phenotype can be rescued by GA_9-me

Ppcps/ks mutant spores were germinated on media containing 1 or 5µM GA_9-me to see if exogenous application of a diterpenoid was able to rescue the biosynthesis mutant. Untreated mutant spores displayed the typical delayed germination phenotype with an overall slower rate of germination. Germination rate of untreated wt spores was also included to demonstrate the extent to which exogenous diterpenoids could rescue the *Ppcps/ks* mutant phenotype. Both concentrations of GA_9-me resulted in a statistically significant increase in germination rate against untreated *Ppcps/ks* spores on days 3 (P >|t| 0.001), 4, 7 (P >|t| 0.0002), and 9 (P >|t| 0.01). Error bars represent ± SEM. Despite promotion of germination by GA_9-me, germination rate of treated *Ppcps/ks* spores was still significantly slower than untreated wt spores (P >|t| 0.0002) on days 7, 9, 12 and 15.

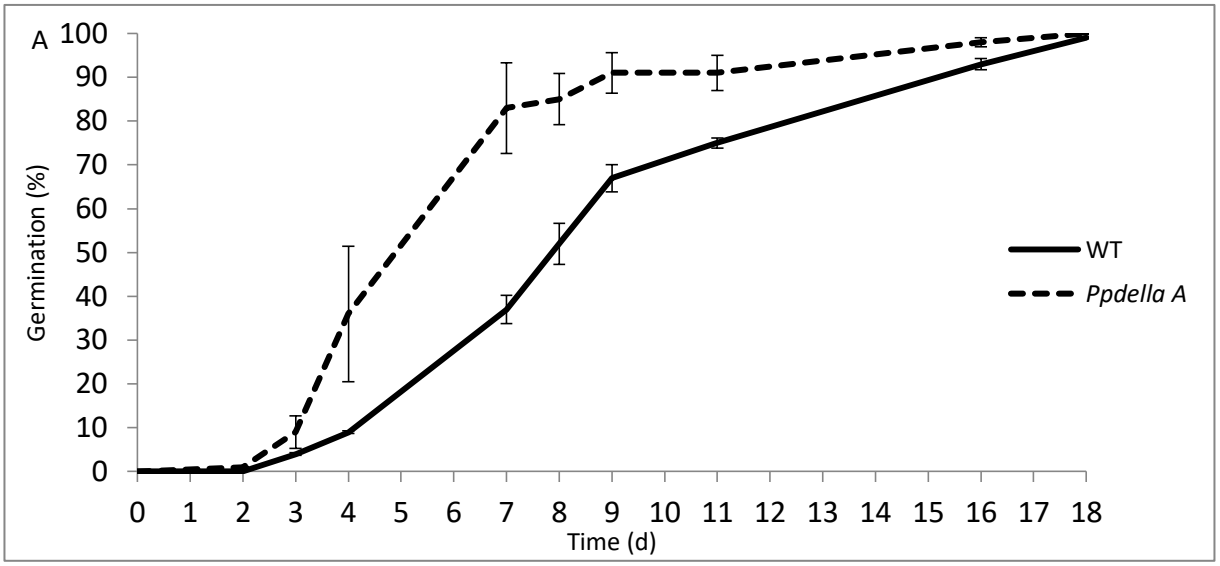
3.4 The germination phenotype of *P. patens* DELLA mutants

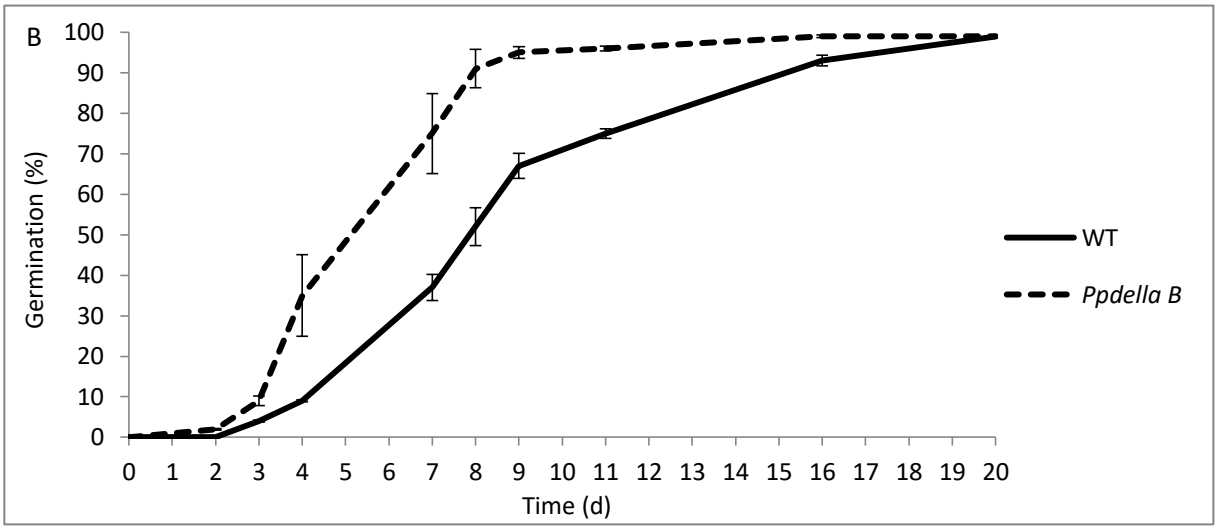
In flowering plants, GAs are perceived by the GID1-DELLA mechanism as discussed in chapter I. This repressor of a repressor action allows GA-mediated processes such as germination to proceed. The central role of the DELLA protein means that *della* mutants do not experience any repression of GA growth responses and subsequent developmental inhibition (Yasumura *et al.*, 2007). Whilst the bioactivity of GAs in *P. patens* spore germination was previously unknown, the confirmation that both *ent*-kaurene and GA_9-me promote germination confirms that *P. patens* does synthesise a GA-type molecule. In seeds, subsequent perception of GAs is facilitated by the GID1-DELLA mechanism. Structural differences between the *P. patens* and *A. thaliana* DELLA and GID1 proteins (Yasumura *et al.*, 2007) renders *Pp*DELLA unable to interact with known bioactive angiosperm gibberellins. This led to the conclusion in previous literature that *P. patens* perceives and regulates GA-type molecules by some other mechanism (Decker *et al.*, 2006; Hirano *et al.*, 2007; Yasumura *et al.*, 2007; Gao *et al.*, 2008). The *P. patens* genome does encode two DELLA genes, A and B; however their role in *P. patens* germination has not previously been investigated.

3.4.1 Germination rate is increased in the DELLA mutants *Ppdella A, B and AB*

The two single and the double *Ppdella* mutants all displayed the same germination phenotype in all assays conducted as part of my research (figure 3.9 A-C). As early as day three of the assay, statistically significant differences in germination % were observed. The overall rate of germination was faster in all three mutant phenotypes with

wt spores taking almost twice as long to reach a germination percentage of 90%+. This early and fast response in the mutant spores suggests that the degradation of DELLA proteins could be one of the controlling factors in the ability of spores to germinate.


A
Germination (%)
Time (d)
WT
Ppdella A


B
Germination (%)
Time (d)
WT
Ppdella B

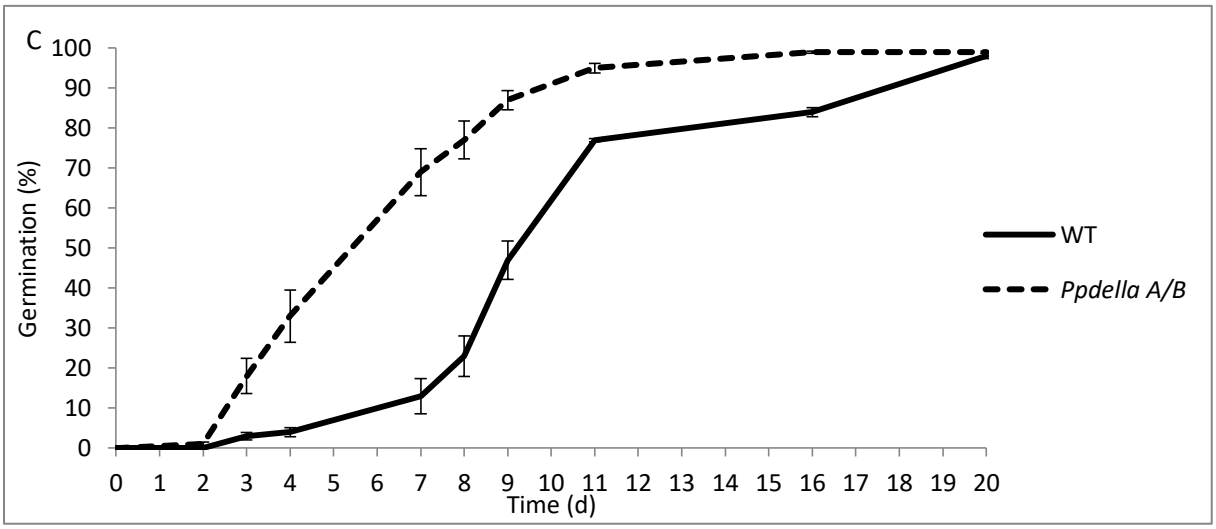

C
Germination (%)
Time (d)
WT
Ppdella A/B

Figure 3.9 The GA signalling mutant *Ppdella* displays an increased rate of *P. patens* spore germination

P. patens wt and *DELLA* spores were germinated on BCD media. The number of spores germinated was counted as a % of total spores on the plate. Approximately 300 spores were counted per plate and three plates were counted per treatment to provide replicates. Counts were done every two to three days. Error bars represent ± SEM. Statistically significant promotion of germination was displayed in all three independent *Ppdella* lines.

A - Z test indicated significant differences in germination % between wt and *Ppdella A* spores on days 3 to 16 (P >|t| 0.0002). Germination rate is much more rapid in mutant spores than wt spores. Germination efficiency is unaffected as both wt and mutant spores achieve 100% germination by day 18.

B - Z test indicated significant differences in germination % between wt and *Ppdella B* spores on days 3 to 16 (P >|t| 0.0002). Germination rate is much more rapid in mutant spores than wt spores. Germination efficiency is unaffected as both wt and mutant spores achieve 100% germination by day 20

C - Z test indicated significant differences in germination % between wt and *Ppdella A/B* spores on days 3 to 16 (P >|t| 0.0002). Germination rate is much more rapid in mutant spores than wt spores. Germination efficiency is unaffected as both wt and mutant spores achieve 100% germination by day 20

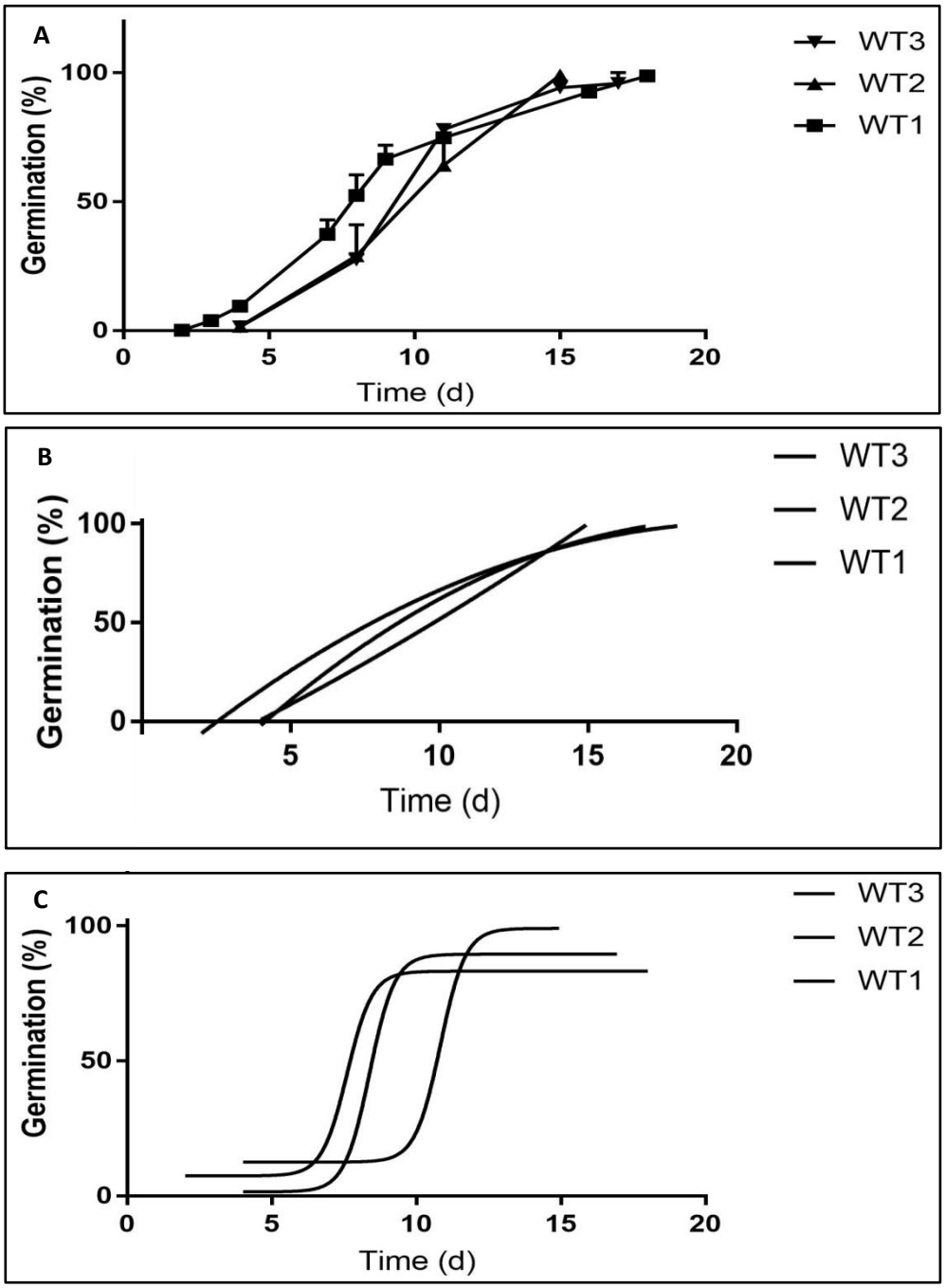


Figure 3.10 Additional data analysis methods trialled

Z-ratio (A) for the significance of the difference between two independent populations was used for analysis of data in chapters III and IV. Nonlinear regression analysis of data to a sigmoidal dose response (B) or second order polynomial (C) fit did not improve scope for analysis. Kruskal-Wallis and Mann Whitney were also considered. The above graphs are a comparison of methods between three biological replicates of wt (control).

3.5 Transcription of GA biosynthesis and signalling genes demonstrates evolutionary conservation

The degradation of RGA (DELLA) by the GID1-mediated perception of GA in angiosperms is well characterised. The divergence of the GID1-DELLA mechanism during plant evolution means it was unlikely functional conservation between *P. patens* and a later evolving land plant such as *A. thaliana* (Yasumura *et al.*, 2007) would be observed. Previous research using yeast two hybrid assays corroborated this, leading to the assumption that basal land plants such as *P. patens* used some other method to detect and relay the GA signal (Yasumura *et al.*, 2007). The presence of homologues of *DELLA* and *GID1* (*PpGLP1*) in *P. patens* provided the opportunity to explore the ancestry of this mechanism through RT-PCR analysis of gene transcription.

3.5.1 Transcription of *P. patens DELLA A* decreases during spore imbibition and germination

In order to clarify the importance of *Pp*DELLAs in germination, transcriptional analysis of DELLAs during the process of germination was carried out. Germination assays led to the hypothesis that *P. patens DELLA* homologues act as inhibitors of germination which must subsequently be degraded, by a pathway involving an as yet unknown GA-type molecule, before germination is observed in culture. Gene expression analysis through RT-PCR of tissue from different life cycle stages supports this hypothesis by showing a marked decrease in *PpDELLA A* transcript levels during imbibition and germination when compared with dry spores (figure 3.11). Transcript levels only began to increase

again as more complex protonemal and gametophyte tissue stages developed. This reinforces the above hypothesis and suggests that removal of DELLA-mediated repression of gene expression is required in order for germination to occur and provides a point of comparison with the GID1-DELLA mediated control of germination observed in angiosperms. Levels of *PpDELLAb* transcript were extremely low in all RT-PCRs performed (data not shown) which did not allow observation of significant variation across life cycle stages. The possibility of functional redundancy would require further investigation.

3.5.2 Transcription of *P. patens* GID1-like *GLP1* gene is highest in dry spores

In flowering plants GID1 acts as a receptor of GA, in conjunction with which it degrades DELLA to relieve repression of GA-mediated growth responses. During *A. thaliana* seed germination expression of *GID1a* and *GID1c* is high in dry seeds and decreases during imbibition (Griffiths *et al.*, 2006). Sensitivity to GA is essential for breaking primary dormancy in seeds. The immediate response to changes in GA levels due to environmental cues or degradation of ABA is only possible through perception by receptors such as GID1 and this is facilitated by transcription of *GID1* in dry seeds. Once the decision to germinate is made and DELLA is degraded during the transition from dry to imbibed and then germinating seeds, GA perception and transduction is no longer necessary and the receptor is not as readily transcribed. Transcription of the GID1-like *GLP1* in *P. patens* suggests a similar mode of action. Despite previous conclusions that *P. patens* does not use a GID1-DELLA mechanism for GA perception, the levels of *GLP1* are highest in dry spores and decrease upon imbibition (figure 3.11),

in a similar pattern to DELLA A. The transcript data from my research challenges the current dogma by suggesting that *P. patens GID1* and *DELLA* homologues are transcribed in a similar pattern to seeds in the key stages of germination. This suggests that GID1-DELLA signalling is an evolutionarily ancient mechanism that may have diverged in conjunction with the expansion of the gibberellin family of diterpenoids.

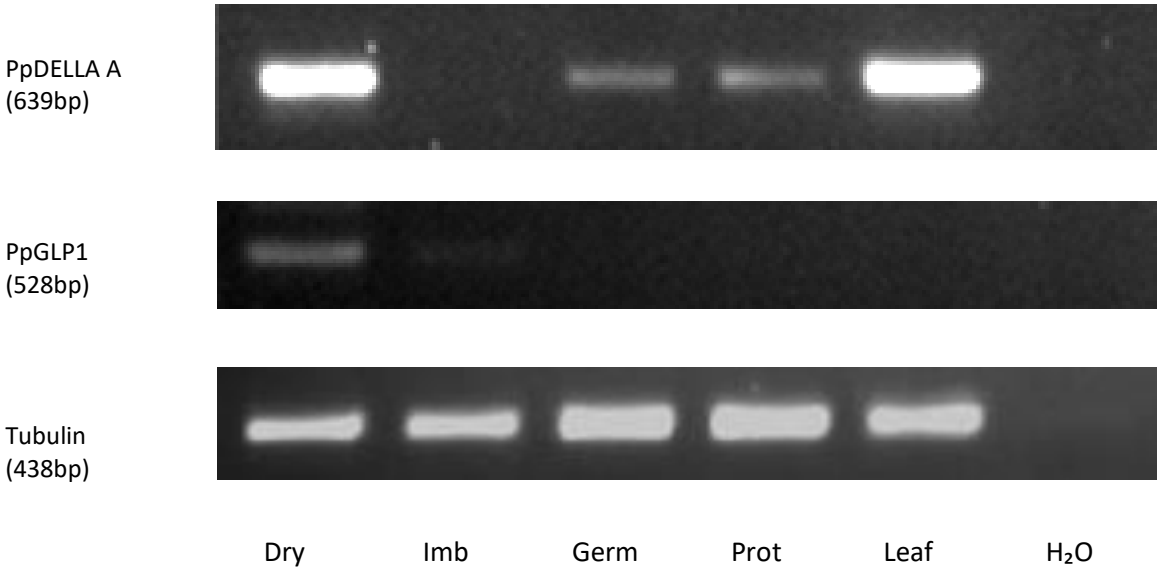


Figure 3.11 Expression of putative GA signalling genes in different tissue types

RNA was extracted from 250 dry spores (dry); 250 imbibed spores (Imb); germinating spores (Germ) and protonemal tissue (Prot) were cultured on plates for 7 days before 100mg of tissue was scraped from plates; 100mg of whole leafy gametophyte tissue (Leaf) was removed from mature peat plug cultures; a water control (H_2O) was included as an experimental negative control. PCR product was visualised by ethidium bromide (EtBr) staining on agarose gel. Brightness of bands demonstrates a semi-quantitative measure of transcript level in total cellular RNA. Tubulin is used as a control as expression should be constitutive across all tissue types. All gels were run alongside a 100bp molecular weight marker to confirm product size against predicted transcript length.

PpDELLA A is expressed in all tissue types but is highest in dry spores and mature leafy tissue. There is a marked reduction in transcription upon imbibition with only a very small amount of transcript detected.

PpGLP1 is only detected in dry and imbibed spores. Transcript levels are low in both tissues but there is a clear decrease in expression upon imbibition.

3.5.3 Transcription of the *ent*-kaurene synthesis gene *PpCPS/KS* is highest in imbibed and germinating spores

In *P. patens* the gibberellin precursor *ent*-kaurene promotes spore germination (figure 3.2). Biosynthesis and perception of subsequent GA-type molecules from *ent*-kaurene triggers germination by degrading DELLA. Conversion of GGPP to *ent*-kaurene is the first step in the GA biosynthesis pathway (figure 3.1) so transcription of the CPS/KS enzyme responsible is key to the induction of germination. RT-PCR analysis indicates that transcription of *PpCPS/KS* occurs upon imbibition (figure 3.12). Transcription is also evident in early germinating spores as different spores in the population used for RNA extraction would be at different stages in the germination process. Transcription is never observed in dry spores.

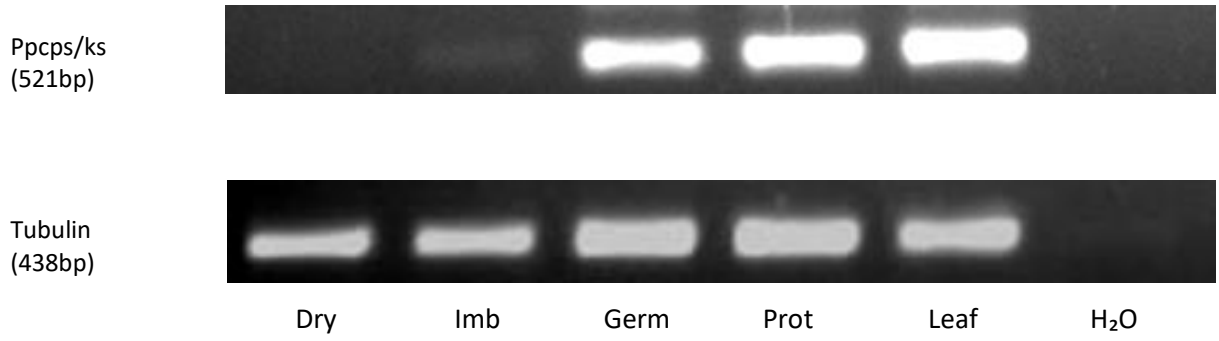


Figure 3.12 Expression of putative GA biosynthesis gene *PpCPS/KS* in different tissue types

RNA was extracted from 250 dry spores (dry); 250 imbibed spores (Imb); germinating spores (Germ) and protonemal tissue (Prot) were cultured on plates for 7 days before 100mg of tissue was scraped from plates; 100mg of whole leafy gametophytes tissue (Leaf) was removed from mature peat plug cultures; a water control (H_2O) was included as an experimental negative control. PCR product was visualised by ethidium bromide (EtBr) staining on agarose gel. Brightness of bands demonstrates a semi-quantitative measure of transcript level in total cellular RNA. Tubulin is used as a control as expression should be constitutive across all tissue types. All gels were run alongside a 100bp molecular weight marker to confirm product size against predicted transcript length.

PpCPS/KS is detected in all tissue types except dry spores. Transcription appears to be upregulated upon imbibition and increases dramatically in germinated spores.

Gene	Dry	Imbibed	Germinating
GLP1 GID1-like	+	-	-
GLP3 GID1-like	+	-	-
Ent-K oxidase	+	-	-
PpDELLAa	+	+	-
GAMYB2	+	+	-
GLP4 GID1-like	+	+	+
GLP5 GID1-like	+	+	+
GAMYB1	+	+	+
CPS/KS	-	+	+
GLP2 GID1-like	+	-	+
GLP6 GID1-like	-	-	-
PpDELLAb	-	-	-

Figure 3.13 Expression of putative GA biosynthesis and signalling genes in dry, imbibed and germinating *P. patens* spores.

Genes are grouped by expression pattern to demonstrate which are transcribed in the same life cycle stage. **+** Indicates gene was expressed and **-** indicates gene was not expressed. Symbol represents a consensus from multiple biological replicates. At least one of the six GID1-like gene was expressed in all three tissue types. GA biosynthesis genes *ent*-kaurene oxidase and CPS/KS were expressed in dry and imbibed and germinating tissues respectively.

3.6 Discussion

3.6.1 Development of a spore germination assay was necessary to generate accurate data

In *P. patens,* many environmental and hormonal factors have been shown to affect spore germination (chapter I). However, discrepancies and failings in previously published methodology has led to a lack of reliable and consistent evidence on the extent of their impact.

Many spore assays have not recorded germination on multiple days, and as such cannot be considered a measure of germination rate, rather a final measure of germination efficiency (Anterola *et al.*, 2009; Hayashi *et al.*, 2010). Additionally, consistently low (less than 60%) levels of germination in control conditions even after 21 days suggests a methodological reduction in efficiency that makes comparisons with treatments less valid. A final germination % of over 90% was obtained in all assays carried out in my research, in most cases within 14 days of culture. In any assays where overall germination rate appeared slower (i.e. wt/control spores were taking more than 10 days to reach 50%), germination counts were carried out for an extended period of up to 24 days to ensure all observable patterns were included. This allowed more subtle and reliable observations of the effects of hormone treatments and phenotype on spore germination to be made. In contrast with previous strategies that either did not record spore number often enough, for a long enough period, or had methodological failings such as not including solvent controls, my research has generated a consistent, reliable

and repeatable spore germination assay (chapter II – figure 2.2). This was necessary in order to provide a more clear point of comparison with the wealth of seed germination data that is available. Recent publication of this assay (Vesty *et al.*, 2016) will also provide a key reference for any future work that intends to record the true effects of factors affecting germination of *P. patens* spores.

3.6.2 The germination-specific function of diterpenes is non-essential in *P. patens*

The hypothesis behind this section of research was that diterpenoids are evolutionarily ancient phytohormones that will have a comparable mode of action to later evolving vascular plant gibberellins in the process of germination. Exogenous application of diterpenes and phenotypic analysis of biosynthesis mutants suggests that whilst diterpenes do contribute to the successful germination of *P. patens* spores, they are not an absolute requirement for the process.

3.6.2.1 Bioactive diterpenes have a role in *P. patens* spore germination

Endogenous *ent*-kaurene has been isolated in *P. patens (*Anterola *et al.*, 2009; Hayashi *et al.*, 2010) and provides evidence for the presence of the first stage of the GA biosynthesis pathway that is so well characterised in seed plants. The role of GA biosynthesis genes has been well characterised in seed plants (chapter I) and inhibition of any through gene disruption or the use of growth retardants such as paclobutrazol (PAC) severely reduces germination. The promotion of germination through exogenous application of *ent*-kaurene (figure 3.2) reinforces the idea that GA-precursors are key to the process of germination and this importance has been maintained throughout land

plant evolution. The proposal of GA_9-me as a bioactive gibberellin in *P. patens (*Hayashi *et al.*, 2010) is reinforced by its action on spore germination (figure 3.3). Whilst none of the identified angiosperm GAs have been isolated from moss, the bioactivity of GA_9-me hints at the likelihood of *P. patens* synthesising other GA-type molecules to fulfil the roles of GA_1 and GA_4 identified in angiosperms.

3.6.2.2 Synthesis of *ent*-kaurene is not essential for *P. patens* spore germination

The specialisation and refinement of the roles of an expanding number of GAs has increased as the gymnosperms and then angiosperms evolved. Homologues for the first enzymatic steps in diterpenoid synthesis have been found in all tested land plant groups. This provided a source of genetic potential, with which later evolving groups could expand their GA repertoire through duplication of individual genes or whole genomes. The vast number and structural diversity of GAs found in flowering plants suggests an increase in the relative importance of this large family during evolution. This is evident in the severe mutant phenotype of seed plants which are unable to germinate. My research demonstrates that the initial stage of the GA biosynthesis pathway i.e. the conversion of GGPP to *ent*-kaurene, does have a role in the germination of *P. patens* spores, but does not appear to be the only controlling factor in the spore's ability to germinate (figure 3.4). Whilst bioactive angiosperm GAs have not been identified in *P.* patens, other GA-type molecules may be synthesised by an alternative pathway or a previously unidentified branching of the known GA biosynthesis pathway (figure 3.14). The incomplete rescue of the *Ppcps/ks* germination phenotype by *ent*-k or GA_9-me (figure 3.5) supports this hypothesis and suggests that other, as yet unidentified, GA-like

molecules may be involved. Until such hypothetical proteins can be found, characterised and used to generate mutant spores, the now apparent role of the bifunctional CPS/KS enzyme provides a clear starting point for investigation in *P. patens*.

3.6.3 The expression profile of diterpenoid biosynthesis and GA signalling genes corroborates previous literature from seed gene expression

The identification of *P. patens DELLA* and *GID1*-like genes and analysis of the GID1-DELLA mediated perception of GA-type compounds in later evolving plants led to the hypothesis that *P. patens* may demonstrate a previously unknown ability to perceive a GA-type signalling molecule through this vascular-plant specific mechanism. Whilst spores do not exhibit primary dormancy (Vesty *et al.*, 2016), environmental cues and antagonistic phytohormone interactions may control the synthesis of GAs to the extent that biosynthesis only occurs when conditions are optimal. Inhibitory cues such as exposure to far red light or high temperatures can induce secondary dormancy in *P. patens* spores (Vesty *et al.*, 2016). The pattern of gene transcript levels (figures 3.11 and 3.12) from dry spores, through imbibition and germination suggests that despite previous conclusions *P. patens* may use a GID1-DELLA-like mechanism for perception of GA-type molecules synthesised initially by the dual function CPS/KS enzyme. Germination assays in conjunction with transcriptional analysis provides salient evidence for the ancestry of this essential mechanism. The conclusions from this section of my research therefore challenges the current dogma on the evolution of GA-signalling in land plants and provide new avenues of investigation.

3.6.4 The evolution of GA signalling

The combination of germination assays and transcriptional analysis carried out as part of this thesis provides clear evidence of a start and end point in the GA signalling network in *P. patens* (figure 3.14). The presumptive conclusions from previous comparisons with seed networks have left a significant gap in our knowledge of this process in a basal land plant. As one of the first land plant groups, the bryophytes would have had to adjust to radical changes in environmental cues which would have had to be communicated to tissues such as spores by a changing phytohormone network. Consequently, elucidation of these networks in basal land plants provides evidence for conservation of ancestral characteristics across 450 million years of evolution. The presence of *ent*-kaurene in *P. patens* and the confirmation of its action in my research suggests it is an evolutionarily ancient hormone that arose before the divergence of vascular plants from bryophytes. The subsequent diversification of GA signalling components in vascular plants supports the observed differences between bryophytes and angiosperms and consolidates my research as a key step in uncovering what happened in between. The expansion of available genome sequences for groups such as lycophytes, liverworts gymnosperms and even charophyte algae will facilitate this and, one would expect, provide further confirmation of the evolutionary ancientness of this network.

Phytohormone networks generally consist of the stages of biosynthesis, signal perception, transduction and response. The action of *CPS/KS, GID1* and *DELLA* genes in control of germination in *P. patens* provides evidence for the presence of these

networks despite assumptions of their vascular plant-specific roles. Figure 3.14 presents a proposed pathway of the GA signalling network in *P. patens* with evidence provided by the data above. Whilst there is a large section missing in the middle, the evidence for the roles of the genes and processes identified as part of my research provides a new and interesting starting point for future work to join the dots.

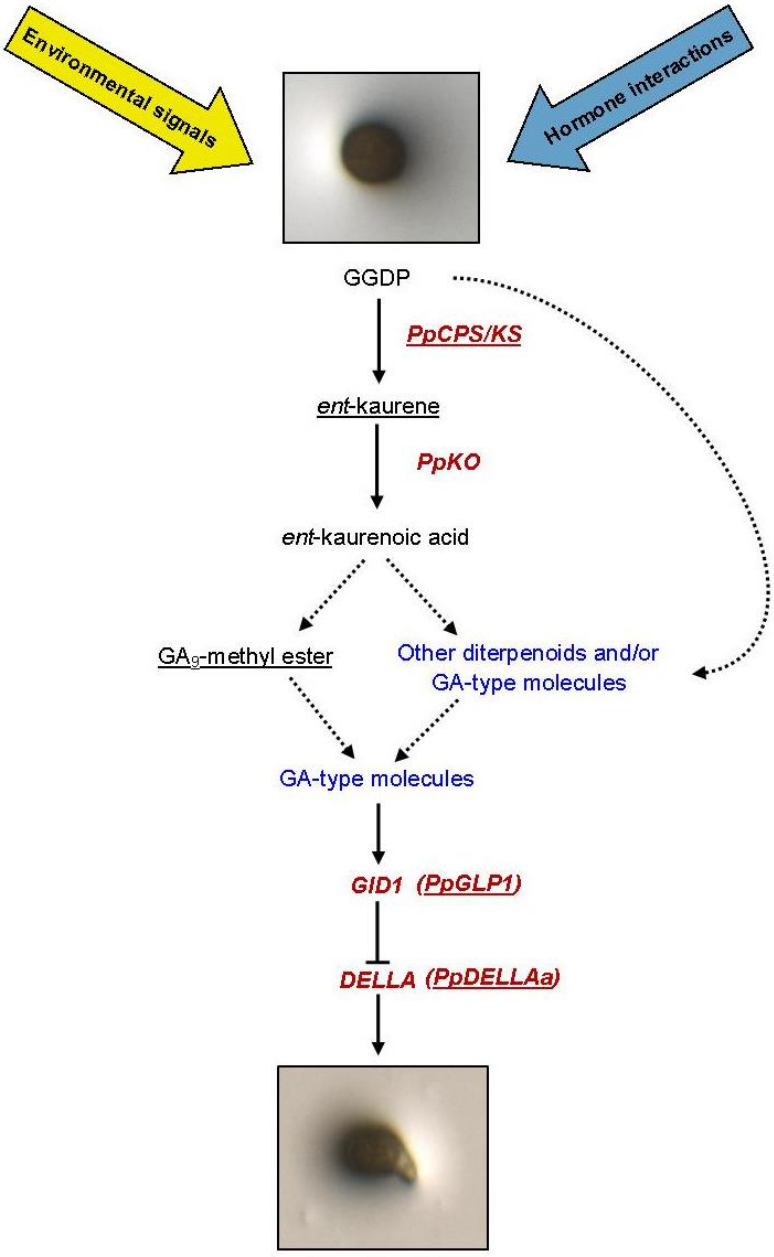


Figure 3.14 Proposed GA signalling network in *P. patens* from thesis data

P. patens spores integrate environmental signals into internal interacting hormone networks. Germination assay and gene expression analysis suggests the above network. Elucidation of intermediate steps may be possible through characterisation of signalling mutants. Confirmed bioactive diterpenoids are in black, theoretical/proposed components of the pathway in blue. Identified genes are in red. Underlined products/genes indicate those analysed as part of this thesis. Solid lines indicate direct reaction/interaction. Dashed lines indicate multiple unknown/theoretical steps.

3.7 Future work

3.7.1 A role for *ent*-kaurenoic acid

The isolation of *ent*-kaurenoic acid (ent-KA) from *P. patens* raised further avenues of investigation in the scope of this research. Preliminary data and conversations with key personnel in the field (E Vesty unpublished, J Coates personal communication, H Toft-Simonsen personal communication) suggests that *ent*-KA is not as essential in *P. patens* as in later evolving land plants. In angiosperms, the clear progression along the biosynthesis pathway (figure 3.1) from *ent*- K to *ent*-KA is a key step in GA production. Preliminary spore germination assays provide no evidence of the necessity of this compound in its isolated form. Suggestions (H Toft-Simonsen personal communication) are that the excessive production of this compound by *P. patens* begs the question of what role it is playing and in what form. One possibility is that it is the subsequent degradation products of *ent*-KA that play a role in GA-mediated growth responses. In angiosperms, an array of GAs and other diterpenoids are produced with only a few playing identified essential roles. The multitude of other compounds are precursors or degradation products. The production of *ent*-KA may represent the starting point of a bryophyte-specific pathway of GA synthesis that diverges from the known vascular plant pathway (figure 3.1). The ineffectual and inconsistent effect of *ent*-KA on *P. patens* spore germination (data not shown) does not necessarily indicate a dead end in the understanding of this pathway, more a fork in the road at which bryophytes took a different turn.

5. 3.7.2 Generation of *P. patens* GA signalling mutants will further our understanding of the evolutionary origins of this network

The consistently obvious germination phenotype of *Ppdella* spores (figure 3.9) and corroborating gene expression patterns (figure 3.11) casts doubt on the previous assumption of the vascular plant-specific nature of the GID1-DELLA mechanism. The expression patterns of other related genes such as *GLP1, GLP2* and *GAMYBs* justifies further investigation of this pathway through generation of additional mutant knockout lines. The *P. patens GID1* mutant *Ppglp1/glp2* provides the next step in the unravelling of this network and its phenotypic characterisation will be a future facet of my work.

3.8 Conclusion

The detailed analysis of GA-signalling in angiosperms highlights the lack of understanding and gaps in the corresponding basal land plant systems. My research has begun to fill some of these and lends its hand to a step-by-step uncovering of this essential feature of the moss germination network and highlights the importance of evolutionarily ancient plant model systems.

CHAPTER IV:

MOSS GERMINATION NETWORKS

4.1 Introduction

Germination is the process by which a new generation emerges from a desiccation-resistant structure. All plants produce the next generation via this mechanism and in non-seed plants this structure is a spore. Spore and seed germination can require a very complex and extensive array of hormone signalling pathways. The quantities and timings of these compounds must be balanced in order for every stage of the process to proceed.

Plant hormone signalling is the most essential communication mechanism in nature (Davies, 2010). Plants have limited mobility and must be able to respond to their environment by modifying their behaviour. The roles of plant hormones in moss spore germination have not been fully determined and any insight into the origin of these pathways will provide new information about the evolution of this essential process in all plants. Along with gibberellins (discussed in chapter III) the role of ABA in germination has been researched extensively in seeds (Nambara and Marion-Poll, 2003; Rodriguez-Gacio Mdel *et al.*, 2009; Nambara *et al.*, 2010). The development of a consistent moss spore germination assay as part of my research (chapter III) has enabled quantifiable assessment of the effect of GAs, ABA and other phytohormones on this key evolutionarily distinct model system.

P. patens is a well-established model bryophyte system in plant biology (Cove, 2005). Features such as highly efficient homologous recombination, relatively quick development and growth, a body plan containing only a few tissue types and a fully

sequenced genome (Rensing *et al.*, 2008b) make it a very amenable model for comparative and functional genomics.

In order to provide comparative insights with other evolutionarily important plant groups, the specific role(s) of a range of plant hormones were identified and characterised. These could then be contrasted with angiosperm models such as *A. thaliana* to begin to map the changes that have taken place during the course of evolution.

4.1.1 Chapter aims

The role of known phytohormones in germination was assessed by:

- Analysing the effects of exogenously applied hormones on germination rate
- Analysing the effects of exogenously applied hormones on plant growth
- Analysing the germination phenotype of hormone biosynthesis mutants
- Transcriptional analysis of hormone signalling genes during germination and early growth

Exogenous application of hormones provides insight into their endogenous role. Confirmation of bioactivity in *P. patens* also provides scope for further investigation of biosynthesis mutants as they become available.

4.2 The role of abscisic acid in germination

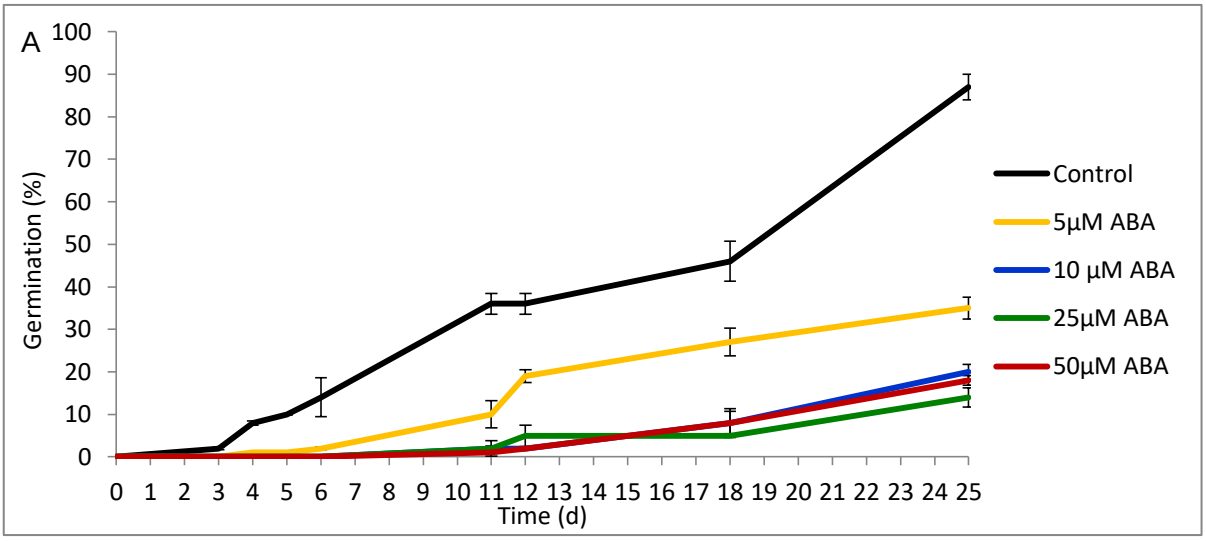
Abscisic acid (ABA) is an isoprenoid phytohormone involved in a variety of plant processes (reviewed in Wasilewska *et al.*, 2008; Takezawa *et al.*, 2011; Nakashima and Yamaguchi-Shinozaki, 2013; Sakata *et al.*, 2014). In *A. thaliana* it has been shown to

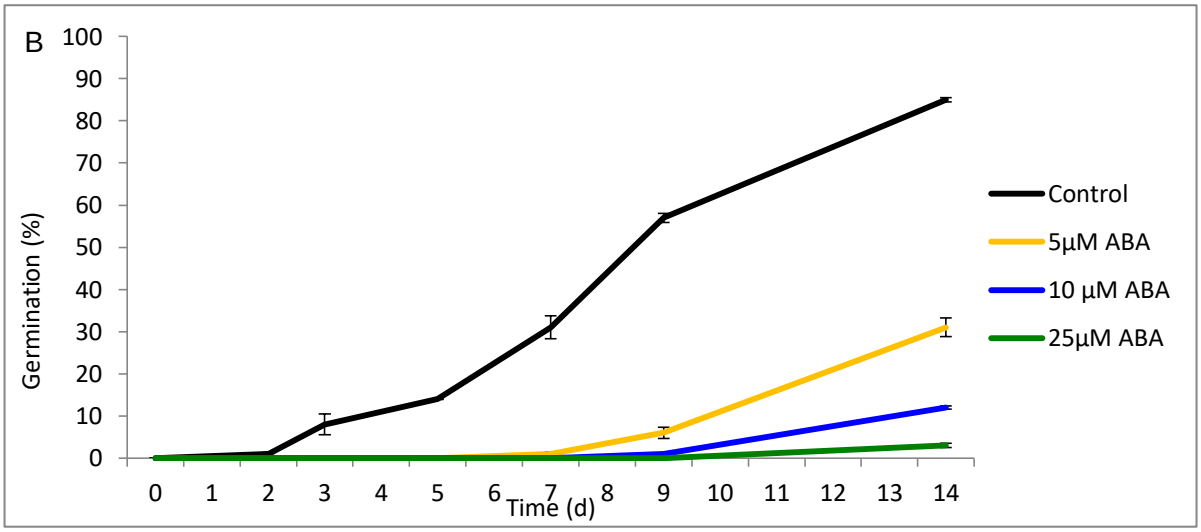
induce primary dormancy and inhibit precocious germination through gene regulation and the pathways discussed in chapter I.

In *P. patens,* ABA affects the regulation of over sixty-five proteins, including those involved in cell division and growth (4%) and transcriptional regulation (11%) (Wang *et al.*, 2010). This suggests a role in the control of spore germination, which is initiated by a suite of genes whose transcription is regulated by environmental and hormonal factors. The process of spore germination involves extension of individual cells and cell division, similar to in seeds. However a key difference is the single-celled nature of a spore when compared to the multiple tissues and cells of a seed.

4.2.1 Abscisic acid inhibits *P. patens* spore germination

In order to identify the role of ABA in *P. patens* spore germination, the effect of exogenous application on germination rate was observed over a period of approximately two weeks. Concentrations as low as 2µM ABA significantly reduced the germination efficiency of spores (Figure 4.1 C), with over 50% of spores unable to germinate. When ABA concentration was increased to 25µM, germination was almost completely inhibited with only 3% successfully germinated (figure 4.1 B). Figure 4.1 shows two independent repeats of the germination assay with A and B demonstrating the effect of ABA by both delaying germination and reducing the overall potential of spores to germinate. C demonstrates the effect of increasing ABA concentration and therefore the dose-dependent nature of this inhibitory effect.


A
Germination (%)
Time (d)
Control
5µM ABA
10 µM ABA
25µM ABA
50µM ABA


B
Germination (%)
Time (d)
Control
5µM ABA
10 µM ABA
25µM ABA

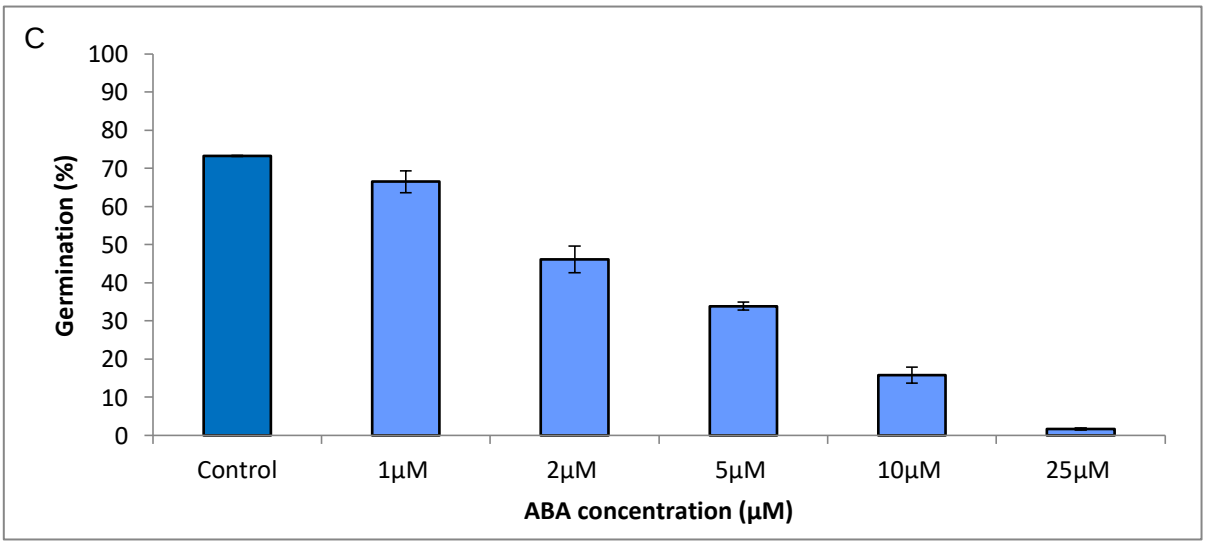

C
Germination (%)
Control
1µM
2µM
5µM
10µM
25µM
ABA concentration (µM)

Figure 4.1 Abscisic acid inhibits *P. patens* spore germination

A - *P. patens* spores were germinated on media containing 0, 5, 10, 25 or 50µM abscisic acid (ABA). The number of spores germinated was counted as a % of total spores on the plate. Error bars represent ± SEM. Z test indicated significant differences between untreated and treated spores on all days counted with all concentrations of ABA P >|t| 0.0002. Germination is both delayed and inhibited in terms of the total number of spores able to germinate, with treated spores never exceeding 40% germination (data not shown)

B - Representative second biological replicate of inhibition of *P. patens* spore germination by ABA. Z test indicated significant differences between untreated and treated spores on all days counted with all concentrations of ABA P >|t| 0.0002

C - % of spores germinated by day 7 after plating on different concentrations of ABA. The number of spores germinated was counted as a % of total spores on the plate. Error bars represent ± SEM. Z test indicated significant differences between untreated and treated spores at all concentrations. 1µM P |t| 0.0038 2-25µM P >|t| 0.0002.

4.2.2 Abscisic acid inhibits *P. patens* colony growth

ABA also affects the growth and morphology of developing *P. patens* colonies. Increasing concentrations of exogenously applied ABA correspond to smaller colony size (Figure 4.2). Even at a low dose of 10µM, colony size is reduced by over 50% and at 100µM this increases to 85%.

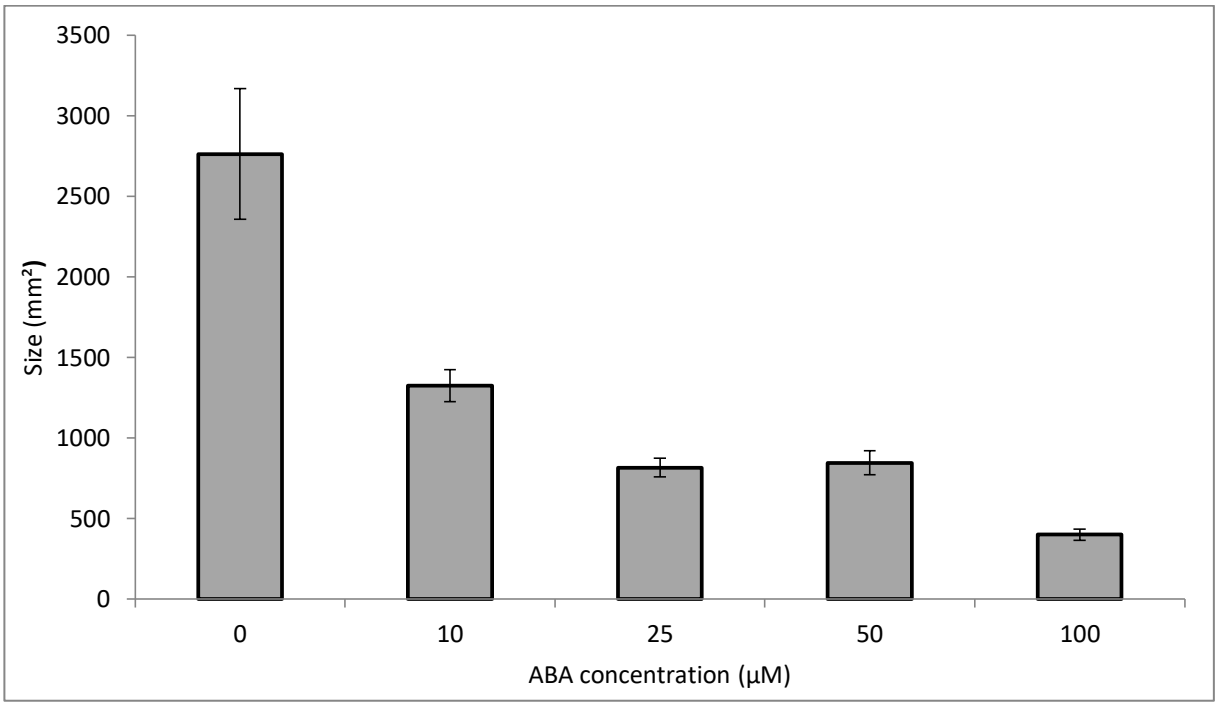


Figure 4.2 Abscisic acid inhibits *P. patens* colony growth.

Spores were germinated at a low density on media containing ABA. Measurements of size were made by measuring the maximum diameter of 20 individual protonemal plants from three different culture plates after 10 days of culture. Error bars represent ± SEM. t test indicated significant difference in colony diameter between untreated and treated cultures of all concentrations. $p > 0.01$

4.2.3 ABA biosynthesis genes are expressed in dry spores

The above data corroborates previous investigation of the effect of ABA on spore germination (Moody *et al.*, 2016). Numerous studies have identified the endogenous role of ABA and highlighted the many genes that are involved (reviewed in Sakata *et al.*, 2014). This available data was used to analyse the expression of different ABA-regulated genes across different stages in the life cycle.

Figure 4.3 displays the expression pattern of identified ABA biosynthesis homologs (Takezawa *et al.*, 2011) in *P. patens*. Putative *ABA2* and *AAO3* genes are expressed in all tissue types however there is some clear variation throughout the life cycle. *ABA2* 125575 is more highly expressed in dry spores than any other tissue type, with a marked decrease in transcript level upon imbibition. The two *AAO3* genes (106708 and 140802) are fairly ubiquitously expressed but do show slight increases in transcription in dry and imbibed (106708) and germinating spores (140802)

4.2.4 ABA signalling genes are expressed ubiquitously in *P. patens* tissues

The putative *P. patens RCAR* ABA receptor gene homologues are generally expressed in all tissue types but at consistently lower levels in imbibed spores when compared with dry (figure 4.4). All putative ABA receptors tested (213389, 209242, 222359 and 132509) were expressed in multiple biological repeats in all tissue types. All show a marked decrease in transcript levels upon imbibition but there is a consistent low level of transcription in that stage of the life cycle in all except putative receptor 213389.

Only two of the six putative *SnRK2* ABA signalling genes were detectable in these assays, 195464 and 194508 (figure 4.5). Both are highly transcribed across all tissue types but show slight decrease in transcript levels upon imbibition.

All of the ABA response genes were expressed highly in dry spores with most being expressed ubiquitously across all tissue types (figures 4.6 and 4.7). The protein phosphatase *ABI1a* showed consistent decrease in transcript levels upon imbibition when compared with dry spores. This was also evident in *ABI1b*, in which transcript levels decreased dramatically upon imbibition and were not obvious again until the growth of mature leafy tissue (figure 4.6). The ABA-regulated transcription factors *ABI3a, ABI3b* and *ABI3c* all showed a marked decrease or absence of transcription upon imbibition (figure 4.7) but a generally high level of transcription across all tissue types.

In the majority of cases the putative ABA biosynthesis and signalling genes were expressed in all tissue types representing key life cycle stages. The key trend emerging is of a decrease in the transcription of these genes upon imbibition. There is also a trend for amplification of multiple different sized sequences that are evidently also transcribed.

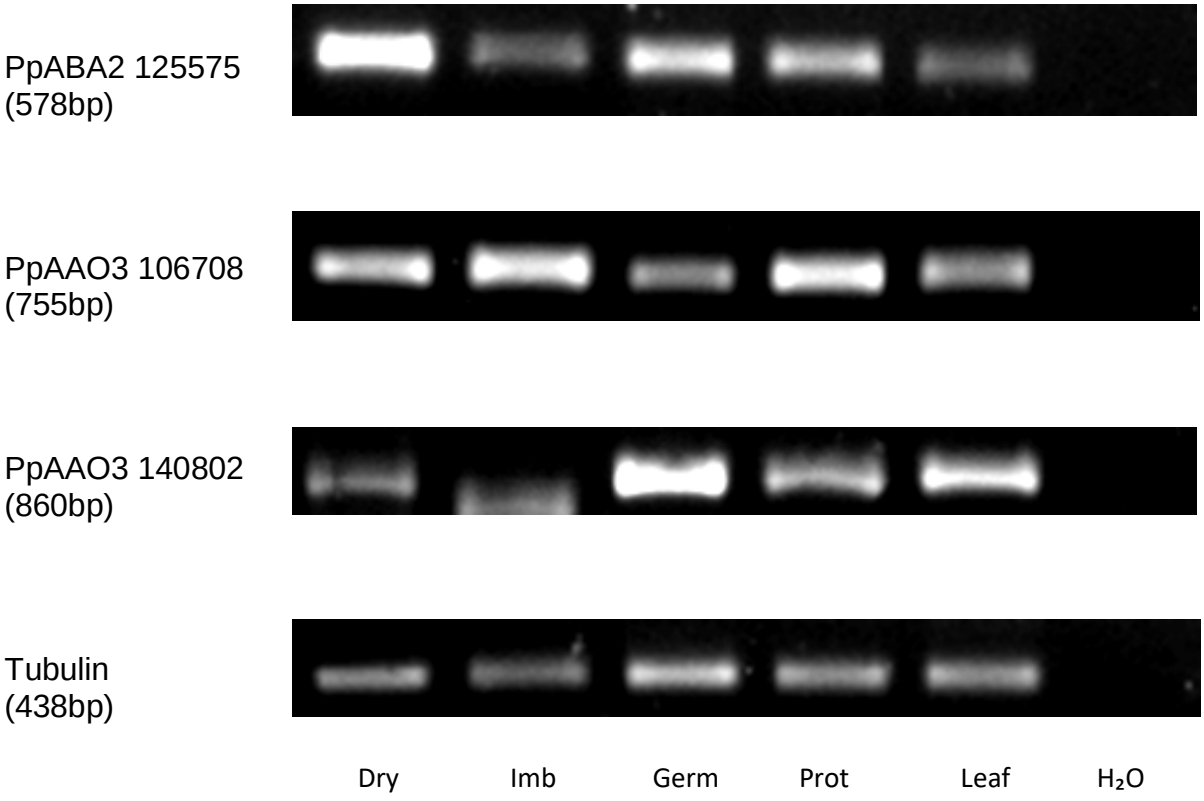


Figure 4.3 Expression of putative ABA biosynthesis genes in different tissue types

All three genes are expressed in all tissue types. *PpABA2* is more highly expressed in dry spores and decreases upon imbibition. *PpAAO3* 106708 shows higher transcription in dry and imbibed spores and in protonemal tissue. *PpAAO3* is highest in germinating spores with relatively lower levels of expression in dry and imbibed spores.

RNA was extracted from 250 dry spores (dry); 250 imbibed spores (Imb); germinating spores (Germ); and protonemal tissue (Prot) cultured on plates for 7 days before 100mg of tissue was scraped from plates; 100mg of whole leafy gametophytes tissue (Leaf) was removed from mature peat plug cultures; a water control (H_2O) was included as an experimental negative control. PCR product was visualised by ethidium bromide (EtBr) staining on agarose gel. Brightness of bands demonstrates a semi-quantitative measure of transcript level in total cellular RNA. Tubulin is used as a control as expression should be constitutive across all tissue types. All gels were run alongside a 100bp molecular weight marker to confirm product size against predicted transcript length.

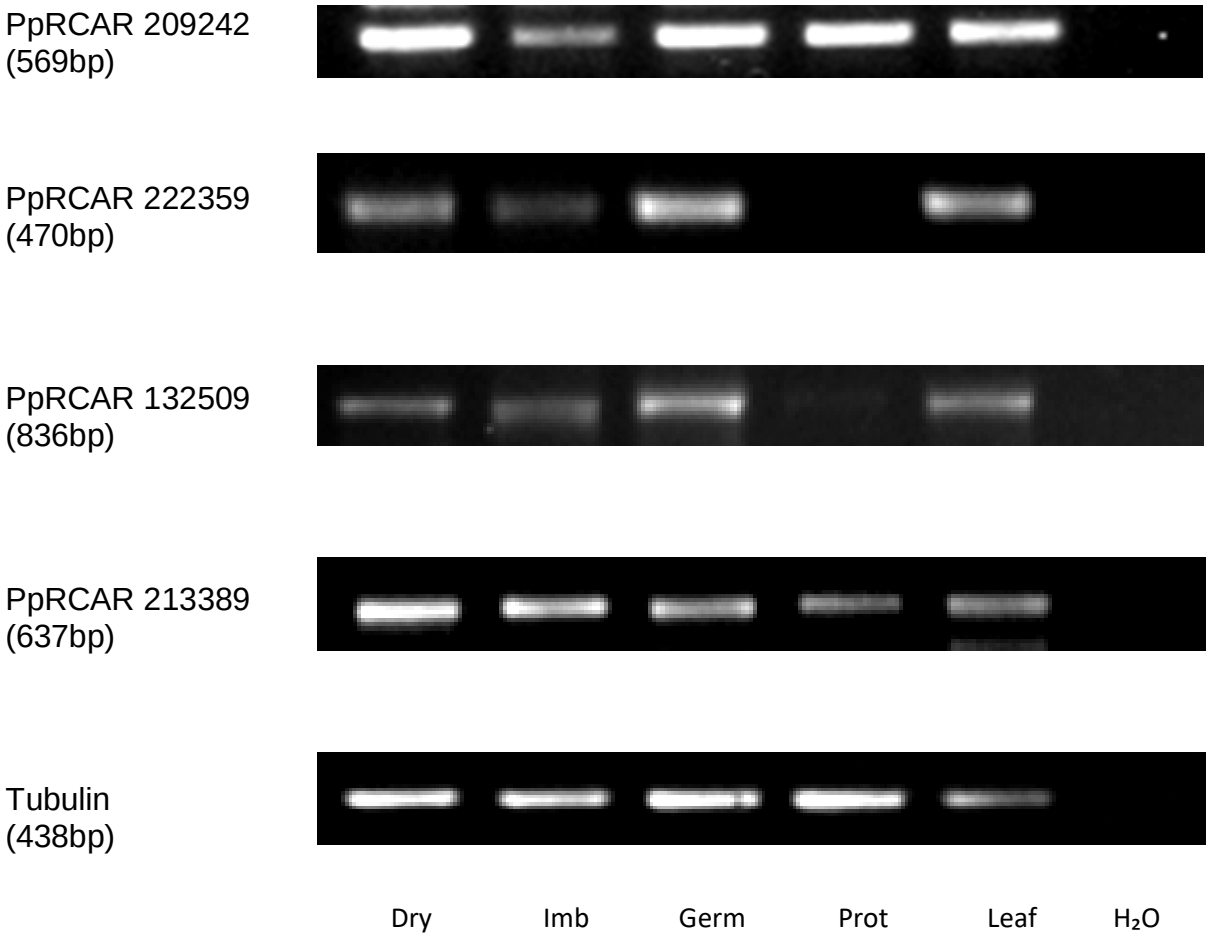


Figure 4.4 Expression of putative ABA receptor genes in different tissue types

All four genes are expressed in all tissue types and showed consistently higher levels of expression in dry spores in comparison to imbibed spores. This is most evident in *PpRCAR* 209242, 222359 and 213389. Presence of *PpRCAR* 222359 and 132509 transcript was often low in protonemal tissue but was detected on more than one occasion (above is a representative image to show difference between dry and imbibed spores).

RNA was extracted from 250 dry spores (dry); 250 imbibed spores (Imb); germinating spores (Germ); and protonemal tissue (Prot) cultured on plates for 7 days before 100mg of tissue was scraped from plates; 100mg of whole leafy gametophytes tissue (Leaf) was removed from mature peat plug cultures; a water control (H_2O) was included as an experimental negative control. PCR product was visualised by ethidium bromide (EtBr) staining on agarose gel. Brightness of bands demonstrates a semi-quantitative measure of transcript level in total cellular RNA. Tubulin is used as a control as expression should be constitutive across all tissue types. All gels were run alongside a 100bp molecular weight marker to confirm product size against predicted transcript length.

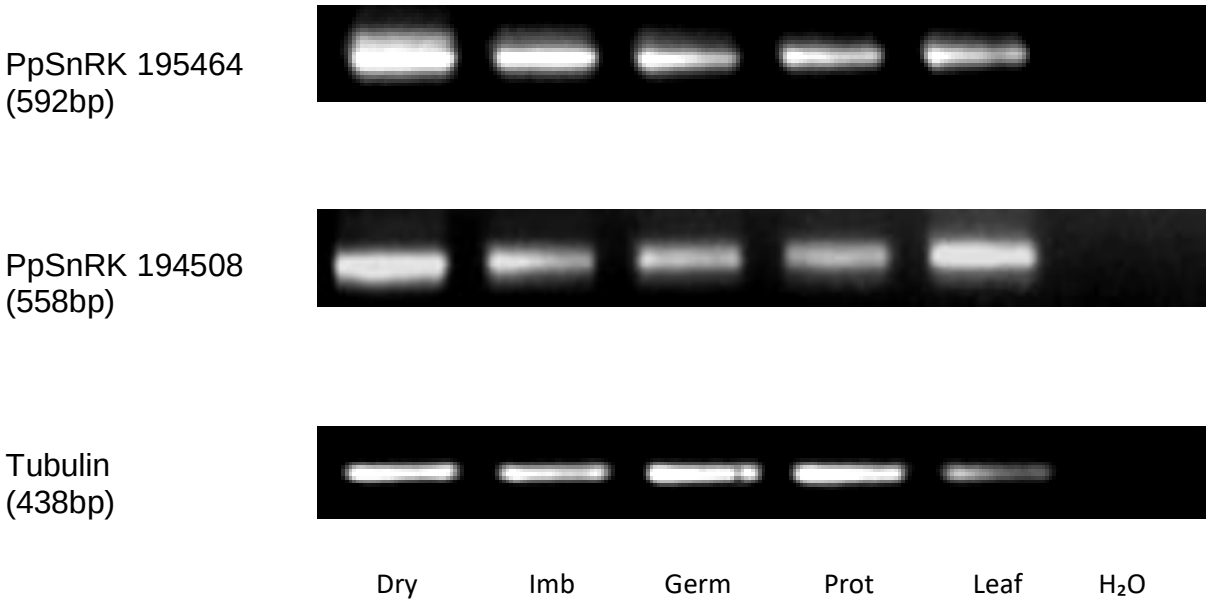


Figure 4.5 Expression of putative ABA signalling kinase genes in different tissue types

Both genes are expressed in all tissue types but show higher transcript levels in dry spores when compared to imbibed.

RNA was extracted from 250 dry spores (dry); 250 imbibed spores (Imb); germinating spores (Germ); and protonemal tissue (Prot) cultured on plates for 7 days before 100mg of tissue was scraped from plates; 100mg of whole leafy gametophytes tissue (Leaf) was removed from mature peat plug cultures; a water control (H_2O) was included as an experimental negative control. PCR product was visualised by ethidium bromide (EtBr) staining on agarose gel. Brightness of bands demonstrates a semi-quantitative measure of transcript level in total cellular RNA. Tubulin is used as a control as expression should be constitutive across all tissue types. All gels were run alongside a 100bp molecular weight marker to confirm product size against predicted transcript length.

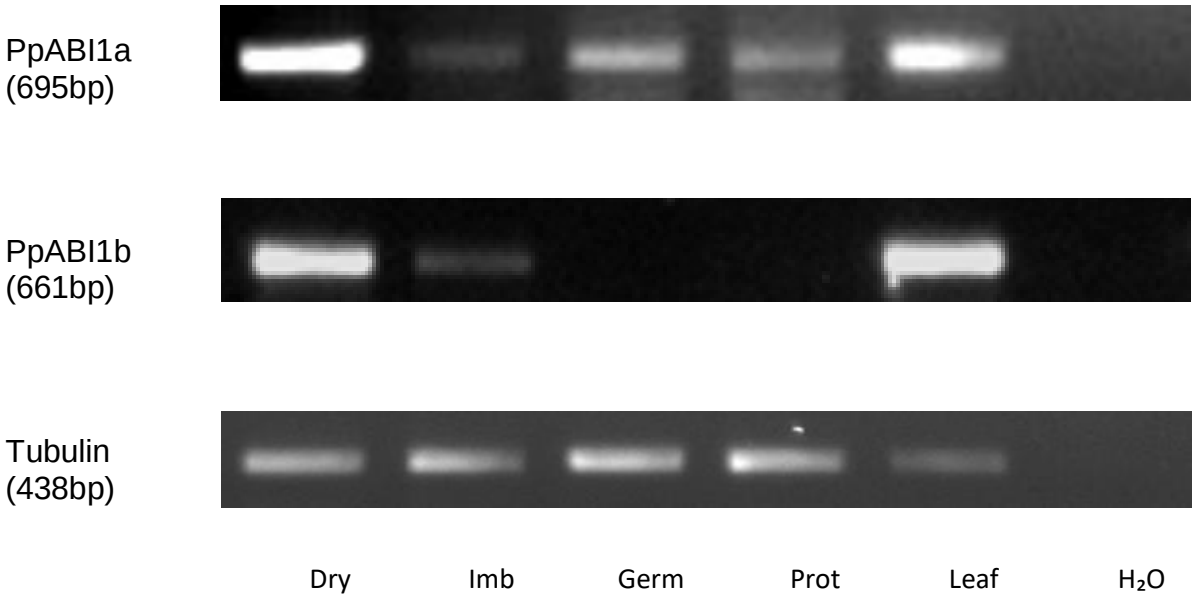


Figure 4.6 Expression of putative ABI1 ABA response genes in different tissue types

Both genes were highly expressed in dry spores and leafy tissue with a marked decrease in expression upon imbibition. *PpABI1a* was expressed ubiquitously across all tissues whereas *PpABI1b* was never detected in germinating or protonemal tissue.

RNA was extracted from 250 dry spores (dry); 250 imbibed spores (Imb); germinating spores (Germ); and protonemal tissue (Prot) were cultured on plates for 7 days before 100mg of tissue was scraped from plates; 100mg of whole leafy gametophytes tissue (Leaf) was removed from mature peat plug cultures; a water control (H_2O) was included as an experimental negative control. PCR product was visualised by ethidium bromide (EtBr) staining on agarose gel. Brightness of bands demonstrates a semi-quantitative measure of transcript level in total cellular RNA. Tubulin is used as a control as expression should be constitutive across all tissue types. All gels were run alongside a 100bp molecular weight marker to confirm product size against predicted transcript length.

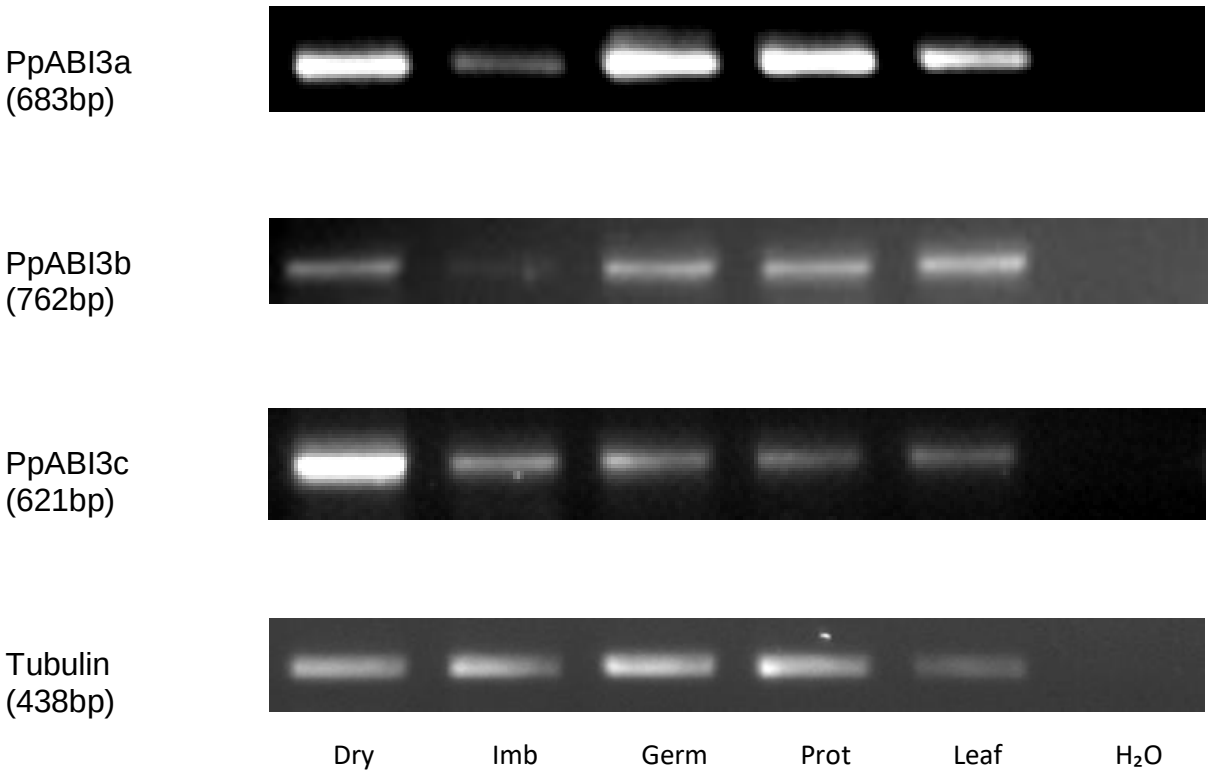


Figure 4.7 Expression of putative ABI3 ABA response genes in different tissue types

The three putative ABA regulated transcription factors (ABI3s) were expressed in all tissue types. All showed high levels of expression in dry spores which decreased upon imbibition. Transcription of *PpABI3a* and *PpABI3b* then increased in subsequent developing tissue types whereas levels of *PpABI3c* stayed constant.

RNA was extracted from 250 dry spores (dry); 250 imbibed spores (Imb); germinating spores (Germ); and protonemal tissue (Prot) were cultured on plates for 7 days before 100mg of tissue was scraped from plates; 100mg of whole leafy gametophytes tissue (Leaf) was removed from mature peat plug cultures; a water control (H_2O) was included as an experimental negative control. PCR product was visualised by ethidium bromide (EtBr) staining on agarose gel. Brightness of bands demonstrates a semi-quantitative measure of transcript level in total cellular RNA. Tubulin is used as a control as expression should be constitutive across all tissue types. All gels were run alongside a 100bp molecular weight marker to confirm product size against predicted transcript length.

Gene	Dry	Imbibed	Germinating
PpAAO3 140802	+	+	+
PpAAO3 106708	+	+	+
PpRCAR 222359	+	+	+
PpRCAR 132509	+	+	+
PpRCAR 209242	+	+	+
PpSnRK2 194508	+	+	+
PpSnRK2 195464	+	+	+
PpABI1a	+	+	+
PpABI3a	+	+	+
PpABI3c	+	+	+

Gene	Dry	Imbibed	Germinating
PpABI1b	+	+	-
PpABA2 125575	+	-	+
PpRCAR 213389	+	-	+
PpABI3b	+	-	+
PpSnRK2 106968	-	-	-
PpSnRK2 215231	-	-	-

Figure 4.8 Expression of putative ABA biosynthesis and signalling genes in dry, imbibed and germinating *P. patens* spores.

Genes are grouped by expression pattern to demonstrate which are transcribed in the same life cycle stage. **+** Indicates gene was expressed and **-** indicates gene was not expressed. Symbol represents a consensus from multiple biological replicates. The majority of genes are expressed in all tissue types. A representative from all tested gene families (PpABI; PpSnRK2; PpRCAR; PpABA2; PpAAO3) was expressed in every tissue type.

4.3. The role of strigolactones as plant hormones

Strigolactones were first identified as signalling molecules between different plant species (e.g. *Striga sp.* and cereal crops) and have since been shown to have endogenous roles in multiple plant processes, in particular the control of root and shoot architecture (Ruyter-Spira *et al.*, 2011; Kapulnik and Koltai, 2014). The availability of synthetic strigolactones has allowed a wider range of plant species and physiological effects to be studied. The effect of synthetic GR24 on *P. patens* spore germination will increase our understanding of the role of strigolactones in basal land plants and provide comparisons with its effects on seeds.

4.3.1 Strigolactone GR24 delays *P. patens* spore germination

Germination was significantly delayed in spores supplemented with GR24 (Figure 4.9). When compared with untreated spores, all biological replicates show a characteristic delay in germination of 3-5 days when treated. In most cases all treatments attain a significantly similar final germination % to wt (data not shown). This suggests that GR24 is having an impact on the initiation of germination as opposed to the germination potential.

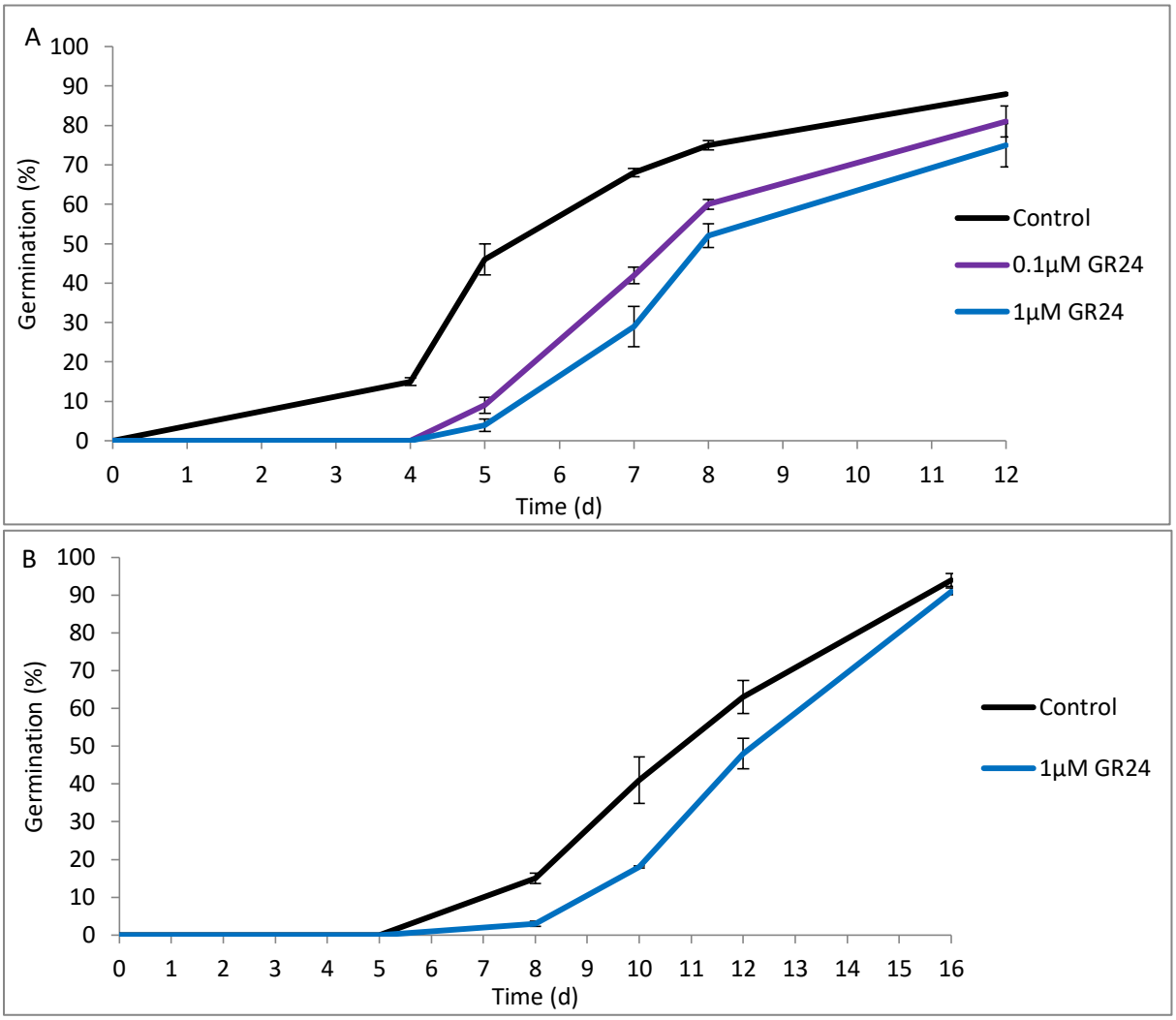


Figure 4.9 Strigolactone GR24 inhibits *P. patens* spore germination

A - Spores were germinated on media containing 0, 0.1 and 1µM GR24. The number of spores germinated was counted as a % of total spores on the plate. Error bars represent ± SEM. Z test indicated significant differences between untreated and treated spores on all days counted with all concentrations of GR24 P >|t| 0.0002. Germination is delayed in treated spores with no observable germination until 5 days after imbibition. Spores on all treatments were able to germinate to close to 100% with no significant differences between treated and untreated spores after 18 days.

B - Biological replicate of inhibition of *P. patens* spore germination by 1µM GR24. Z test indicated significant differences between untreated and treated spores on days 8, 10 and 12 P >|t| 0.0002. Germination was slightly delayed in both treatments but a significantly reduced germination rate was still evident when germination commenced. All treatments were able to achieve a final germination % of over 90% with no significant difference between treatments by day 16.

4.3.2 Strigolactone GR24 inhibits colony growth

Exogenous application of GR24 also inhibits the growth and extension of germinated spores (Figure 4.10). Germinated spores of the same age produce much smaller and less dense filamentous tissue when grown on media containing GR24. *P. patens* naturally produces and releases strigolactones into the surrounding medium as a way of restricting colony growth in response to the proximity of neighbouring colonies (Proust *et al.*, 2011; Hoffmann *et al.*, 2014) by inhibiting branching and elongation of caulonemal cells. Application of additional GR24 to the medium exacerbated this effect resulting in even further restricted colonies.

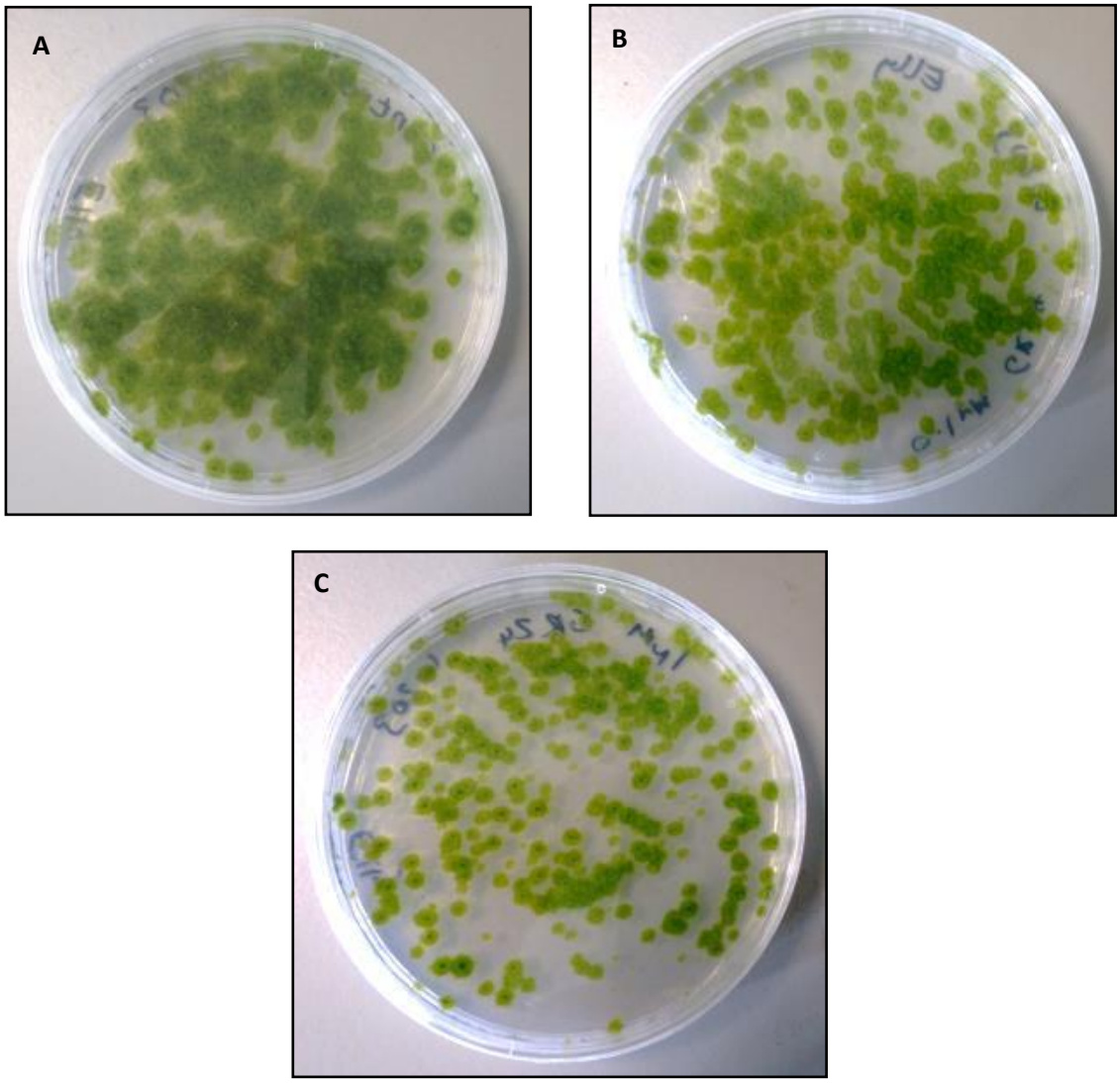


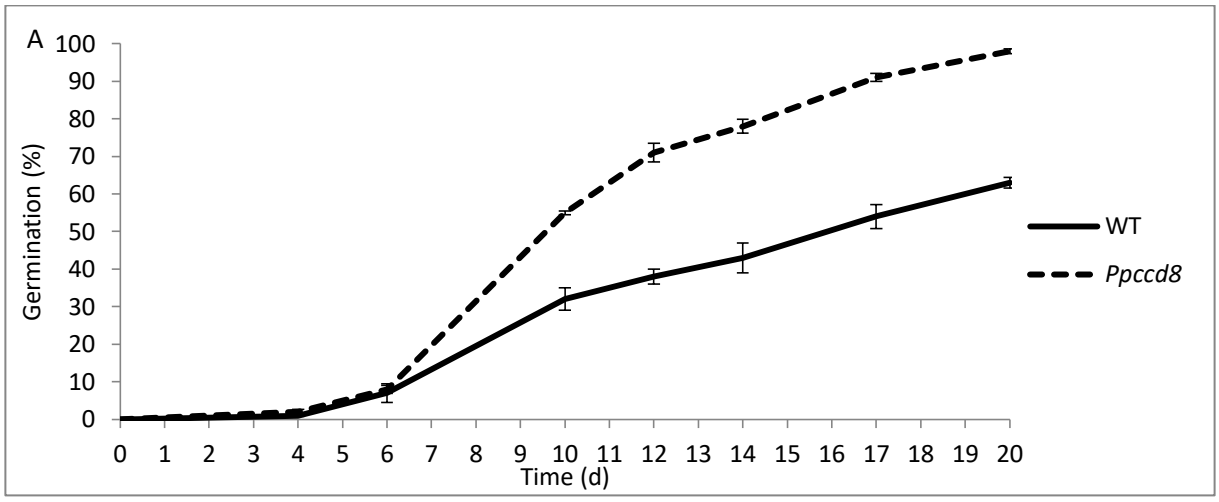
Figure 4.10 GR24 restricts colony extension in *P. patens*

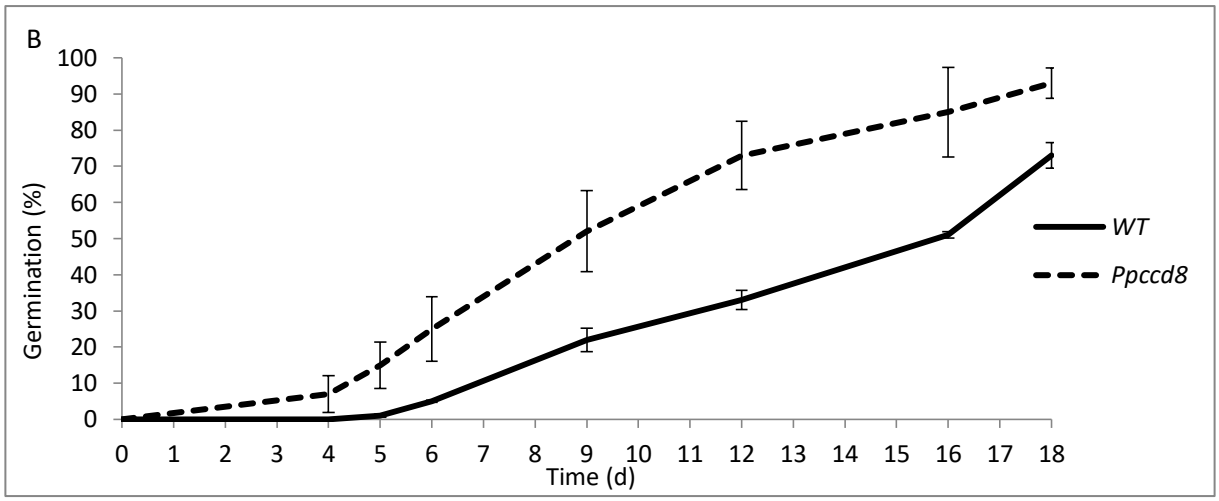
Spores were germinated on media containing 0 (A), 0.1 (B) and 1µM (C) GR24 and cultured until germination % was over 90%. Photographs were taken to demonstrate the restricted colony extension displayed under treatment of GR24. Closer inspection confirmed that decreased coverage under treatment was due to smaller and less dense protonemal colonies as opposed to fewer successfully germinated spores.

4.3.3 The strigolactone mutant *Ppccd8*

The carotenoid cleavage dioxygenases (CCDs) are a family of enzymes that catalyse the first step in the biosynthesis of strigolactones (R. K. Wang *et al.*, 2011). The *Ppccd8* mutant displays an unrestricted colony extension phenotype with elongation and branching of caulonemal cells continuing for significantly longer than in wt (Proust *et al.*, 2011; Hoffmann *et al.*, 2014).

Figure 4.11 shows the accelerated germination phenotype of *Ppccd8* with germination of mutant spores reaching 50% an average of 5 days earlier than wt. Germination potential does not appear to be different as just under 100% of both mutant and wt successfully germinated. This phenotype was observed across multiple biological replicates (figure 4.11 A-C).


A
Germination (%)
Time (d)
WT
Ppccd8


B
Germination (%)
Time (d)
WT
Ppccd8

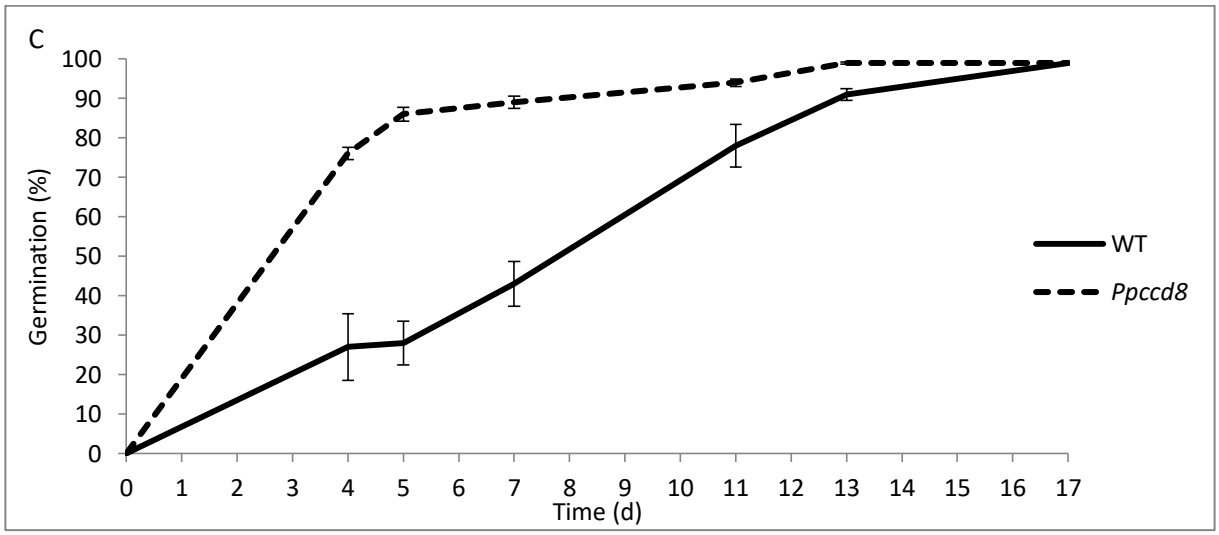

C
Germination (%)
Time (d)
WT
Ppccd8

Figure 4.11 Germination is faster in the strigolactone biosynthesis mutant *Ppccd8*

P. patens wt and *CCD8* spores were germinated on BCD media. The number of spores germinated was counted as a % of total spores on the plate. Approximately 300 spores were counted per plate and three plates were counted per treatment to provide replicates. Counts were done every two to three days. Error bars represent ± SEM. Statistically significant promotion of germination was displayed in all three biological replicates illustrated.

A - Z test indicated significant differences in germination % between wt and *Ppccd8* spores on days 10, 12, 14, 17 and 20 (P >|t| 0.0002). Germination was slightly delayed in both wt and mutant spores but a statistically significant promotion of germination was still evident when germination commenced. Germination efficiency was lower in wt spores but counting was complicated by growth of germinated colonies making it difficult to record spores that may have been germinating underneath.

B - Biological replicate of *Ppccd8* germination phenotype. Z test indicated significant differences in germination % between wt and *Ppccd8* spores on all days counted (P >|t| 0.0002). Germination efficiency was slightly lower in wt spores but did achieve similar levels to *Ppccd8* when cultured for an extended period (data not shown)

C - Biological replicate of *Ppccd8* germination phenotype. Z test indicated significant differences in germination % between wt and *Ppccd8* spores on days 4, 5, 7, 11 and 13 (P >|t| 0.0002).

4.4 The role of *N*-acyl-homoserine lactones in plant signalling

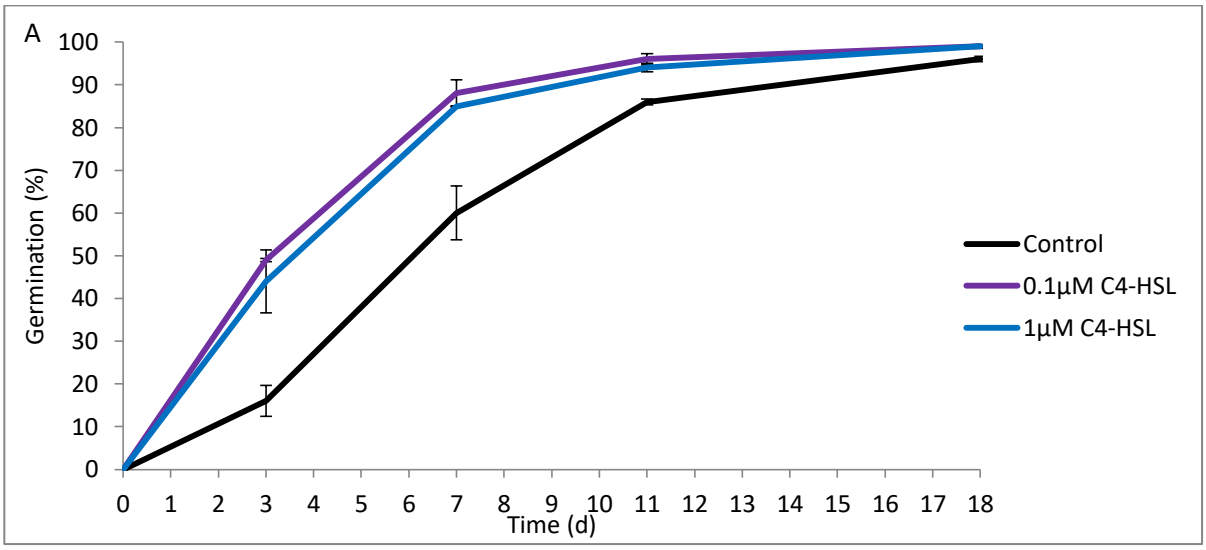
N-acyl-homoserine lactones (AHLs) are produced by Gram-negative bacteria as quorum-sensing molecules (Williams *et al.*, 2007). Their presence in the environment can also be detected by eukaryotes in a form of cross-kingdom signalling (Hartmann and Schikora, 2012). In plants, this interaction often occurs in the rhizosphere where roots come into contact with AHLs in varying concentrations due to bacterial growth (Ortiz-Castro *et al.*, 2008; Klein *et al.*, 2009; Tsuchiya and McCourt, 2009; Foo and Davies, 2011; Kapulnik *et al.*, 2011a; Ruyter-Spira *et al.*, 2011; Rasmussen *et al.*, 2012; Brewer *et al.*, 2013; Koltai, 2013; Zarkani *et al.*, 2013). Studies have shown that plants are able to respond to these bacterial compounds and even absorb them from the surrounding environment (Sieper *et al.*, 2014). The AHLs can then affect the activity of endogenous hormones causing a downstream effect on plant growth. The response is often dependent on the structure and concentration of the AHL encountered and can be positive or negative in terms of growth (Palmer *et al.*, 2014). Certain plant species have also been shown to produce AHL mimics which induce a premature quorum-sensing response in bacteria that serves to protect the plant or aid establishment of symbiotic relationships (Gao *et al.*, 2003; Bauer and Mathesius, 2004; Perez-Montano *et al.*, 2013).

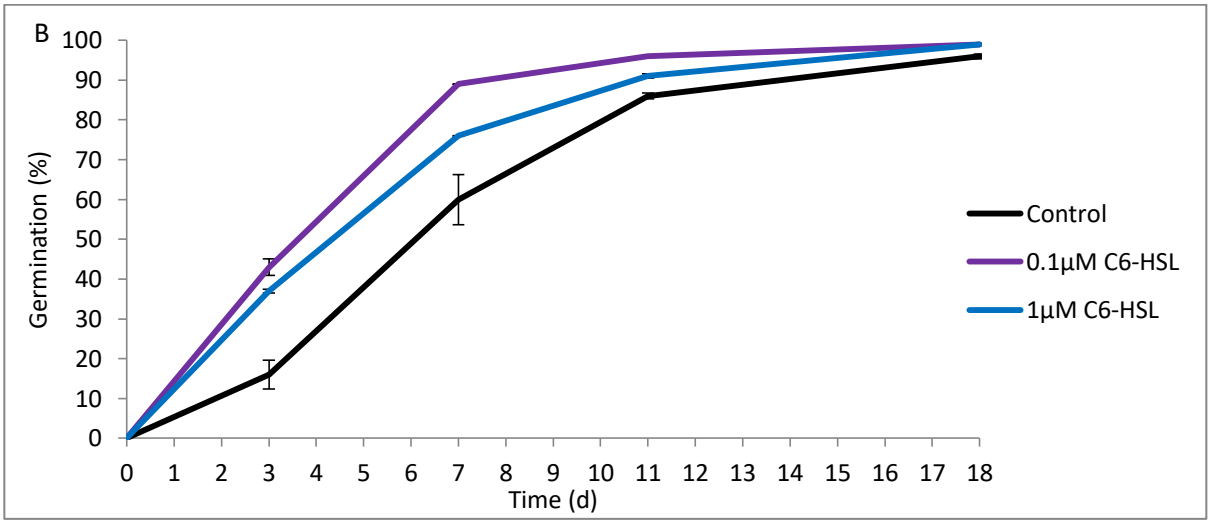
Spores and seeds will also encounter multiple bacterial species in the soil. Despite the protective seed or spore coat limiting the influence of AHLs directly, its role as an environmental signal to be subsequently integrated into the endogenous hormone network could be investigated by exogenous addition and observations of changes in

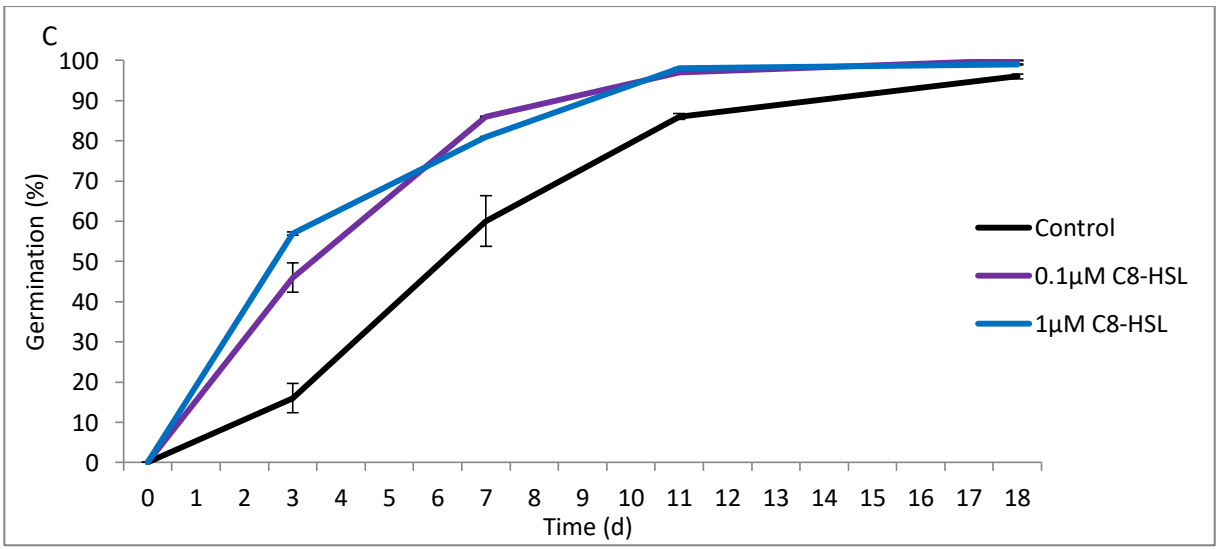
plant processes. Early land plants such as mosses did not possess true roots, so their interaction with soil microflora might have been during earlier life cycle stages such as spore germination.

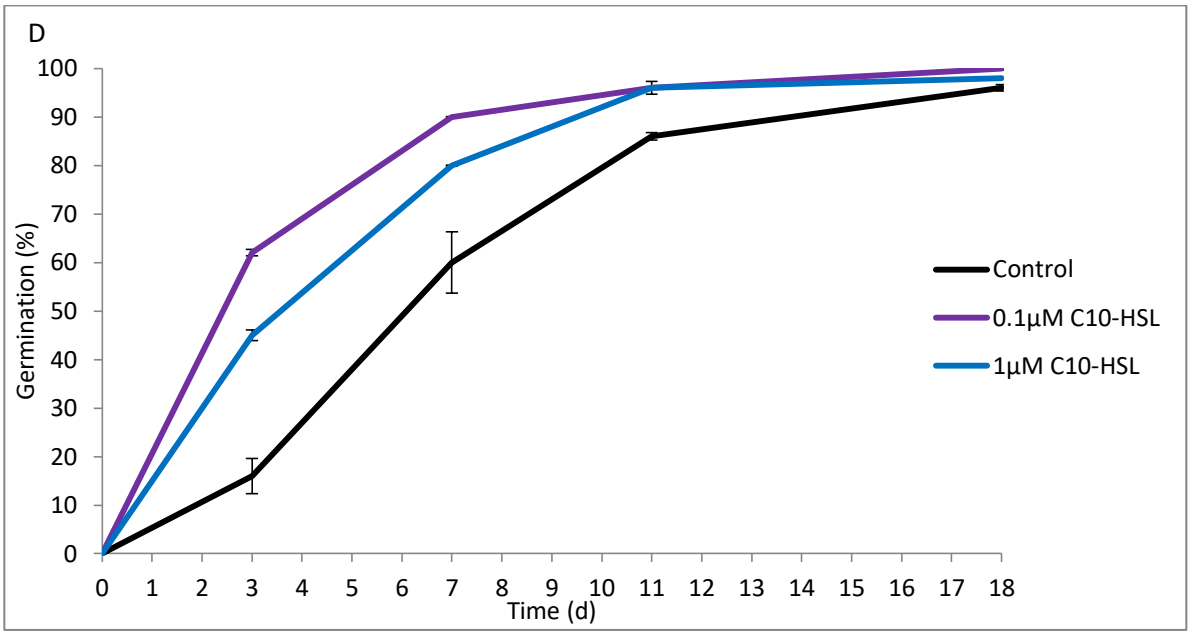
4.4.1 *N*-acyl homoserine lactones promote *P. patens* spore germination

Germination of *P. patens* spores was significantly faster when treated with *N*-acyl homoserine lactones (AHLs) (figure 4.12 A-E). Preliminary investigation of the effects of AHLs on *P. patens* spore germination showed that although both short and long chain AHLs increased germination rate (figure 4.12) the long chains C10 and C12 showed the most consistent increase across biological replicates (figure 4.13). It also showed that lower concentrations were more effective at promotion (figure 4.13) leading to a focus on the effects of 0.1 and 1µM C10 and C12 AHL.


A
Germination (%)
Time (d)
Control
0.1µM C4-HSL
1µM C4-HSL


B
Germination (%)
Time (d)
Control
0.1µM C6-HSL
1µM C6-HSL


C
Germination (%)
Time (d)
Control
0.1µM C8-HSL
1µM C8-HSL


D
Germination (%)
Time (d)
Control
0.1µM C10-HSL
1µM C10-HSL

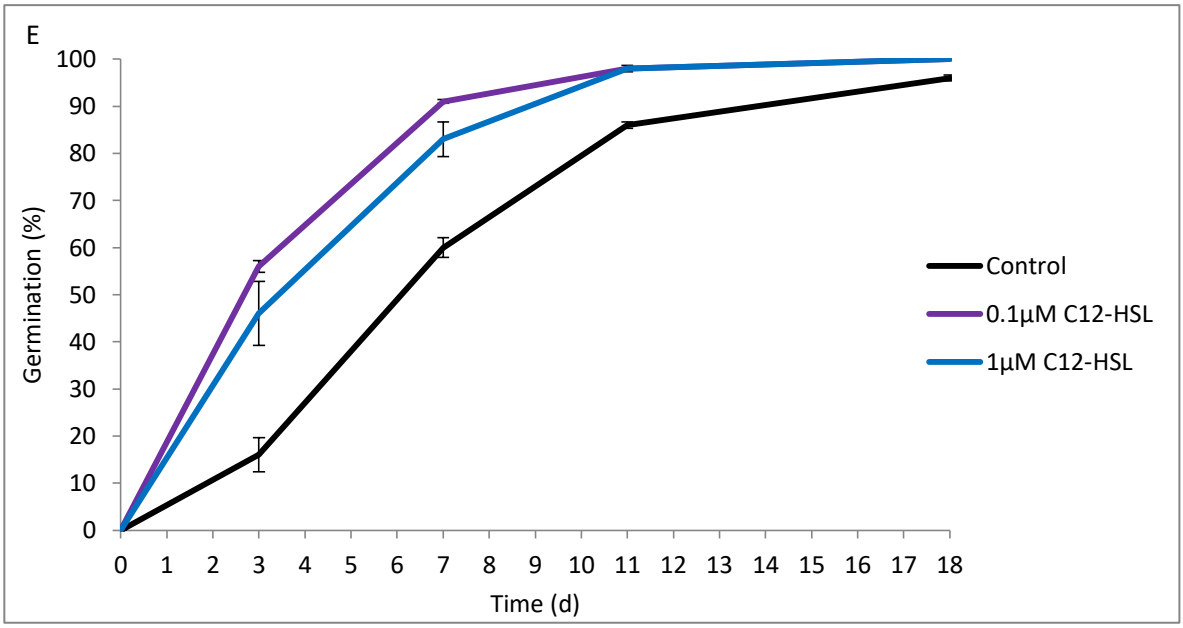

E
Germination (%)
Time (d)
Control
0.1µM C12-HSL
1µM C12-HSL

Figure 4.12 *N*-acyl homoserine lactones (AHLs) promote *P. patens* spore germination

P. patens spores were germinated on media containing 0, 0.1 and 1µM AHLs of varying chain lengths (C4-C12). The number of spores germinated was counted as a % of total spores on the plate. Error bars represent ± SEM. Both concentrations resulted in a faster rate of germination when compared with untreated spores.

A - C4-HSL with a 4 carbon R-group side chain length showed statistically significant promotion of germination against untreated *P. patens spores*. Z test indicated significant differences in germination % between treated and untreated spores on days 3, 7 and 11 (P >|t| 0.0002). Overall germination efficiency was not affected with all treatments achieving a final germination % of over 95%

B - C6-HSL with a 6 carbon R-group side chain length showed statistically significant promotion of germination against untreated *P. patens spores*. Z test indicated significant differences in germination % between treated and untreated spores on days 3, 7 and 11 (P >|t| 0.0002). Treatment with the lower concentration of 0.1µM was significantly more effective in promoting germination on days 3, 7 and 11 when compared to 1µM. Overall germination efficiency was not affected with all treatments achieving a final germination % of over 95%

C - C8-HSL with an 8 carbon R-group side chain length showed statistically significant promotion of germination against untreated *P. patens spores*. Z test indicated significant differences in germination % between treated and untreated spores on days 3, 7 and 11 (P >|t| 0.0002). Overall germination efficiency was not affected with all treatments achieving a final germination % of over 95%

D - C10-HSL with a 10 carbon R-group side chain length showed statistically significant promotion of germination against untreated *P. patens spores*. Z test indicated significant differences in germination % between treated and untreated spores on days 3, 7 and 11 (P >|t| 0.0002). Treatment with the lower concentration of 0.1µM was significantly more effective in promoting germination on days 3 and 7 when compared to 1µM. Overall germination efficiency was not affected with all treatments achieving a final germination % of over 95%

E - C12-HSL with a 12 carbon R-group side chain length showed statistically significant promotion of germination against untreated *P. patens spores*. Z test indicated significant differences in germination % between treated and untreated spores on days 3, 7 and 11 (P >|t| 0.0002). Treatment with the lower concentration of 0.1µM was significantly more effective in promoting germination on days 3 and 7 when compared to 1µM. Overall germination efficiency was not affected with all treatments achieving a final germination % of over 95%

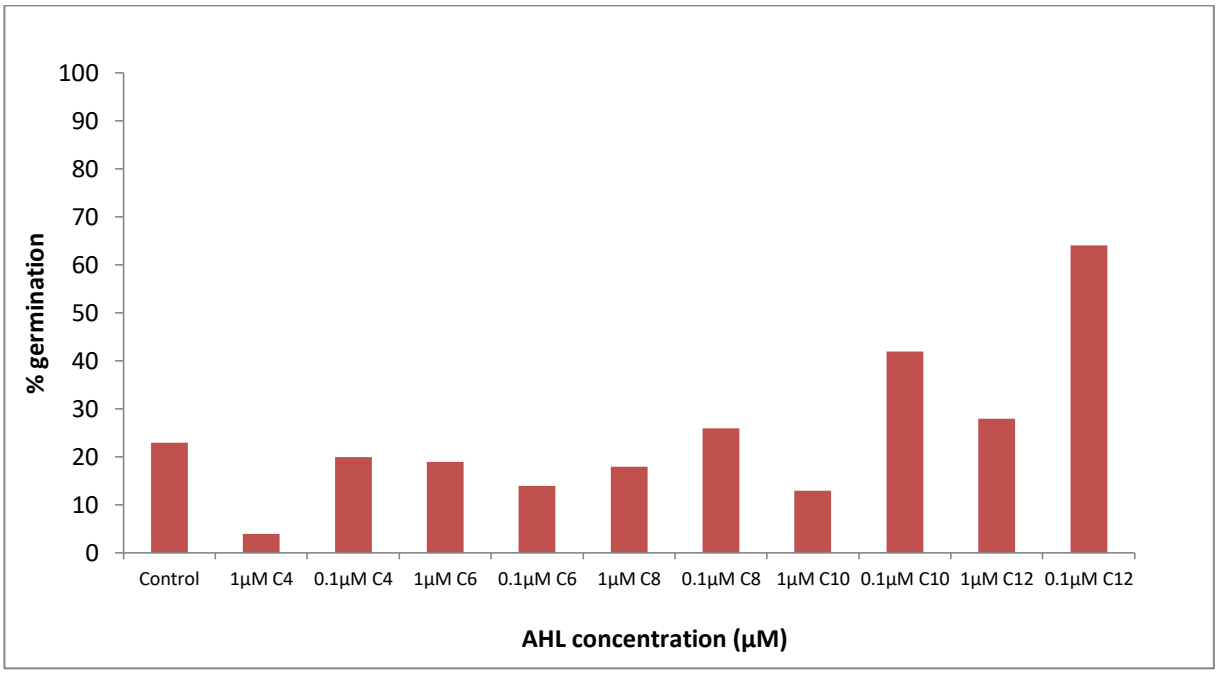


Figure 4.13 Promotion of *P. patens* spore germination by AHLs is affected by chain length and concentration

Spores were germinated on media containing 0, 0.1 and 1µM AHL of varying R-group side chain lengths (4 -12 carbons). Figure shows % of spores germinated 5 days after imbibition and plating onto AHLs. Significant promotion of germination is only observed under treatment of 0.1µM C10 and C12 AHLs ($P < 0.05$).

4.5 Discussion

Germination is one of the key stages in a plant's life cycle. The decision to germinate is tightly regulated by an integrated network of environmental and hormonal cues. Phytohormones such as ABA provide a link between external signals and internal control of plant processes and as such have become key areas of research. The catalogue of plant hormones is constantly expanding as endogenous roles for an increasing number of compounds are being discovered. More recently, signalling molecules from outside the plant kingdom have begun to feature in the understanding of the complex regulatory networks of plants.

4.5.1 The role of ABA in *P. patens* germination

Abscisic acid is one of the most ancient hormones and is found across multiple kingdoms (Wasilewska *et al.*, 2008). Its ubiquitous nature and physiological versatility means it is involved in many essential processes, not just in plants. Its role in later evolving vascular plants is well documented on a physiological and genetic level. The purpose of this chapter was to begin to reveal its role and relative importance in a basal land plant model. The focus of ABA-related research in *P. patens* has been its role in stress tolerance (Sakata *et al.*, 2009; Khandelwal *et al.*, 2010; Takezawa *et al.*, 2011; Sakata *et al.*, 2014) which shows a high level of conservation across the 450 million year evolutionary gap between bryophytes and angiosperms. The ability to tolerate stresses such as desiccation and UV radiation would have been key for early colonisers of the land and this essential signalling network has been retained and refined in

multiple tissues of later-evolving plants. Experimentation with exogenous application of ABA to different plant tissues has demonstrated its importance as a negative growth regulator as well as a regulator of stress tolerance. My research has revealed that ABA also has a role in the negative regulation of the germination process. Exogenous application of ABA significantly inhibited *P. patens* spore germination to the extent that large numbers of spores were unable to germinate. This suggests that tight metabolic regulation of endogenous levels of ABA is crucial for initiation of the germination process.

In seed plants, germination is controlled by the antagonistic relationship between ABA and gibberellins (GAs). ABA acts as negative regulator and GAs as a positive, with the balance between synthesis and degradation of both being the pivotal point. Interruption of the synthesis or signalling of GA in angiosperms renders seeds unable to germinate - the equilibrium between ABA and GA is lost. Exogenous application or overexpression of ABA also results in an imbalance resulting in an inability to germinate. The previous chapter demonstrated the subtle role of the biosynthesis and signalling of a GA-type molecule in *P. patens* spore germination. It does not have the same 'all or nothing' level of control as GA in seed plants and as such led me to question if the role of the GA antagonist, ABA, also has a more subtle mode of action. However, the consistent effect of ABA on spore germination suggests its relative level of importance is high and has been conserved during the evolution of all plant groups. Its ancient nature and broad range of roles has cemented ABA as a major factor in the hormonal regulation of plant processes such as germination.

4.5.1.1 ABA biosynthesis is controlled by multiple genes

The biosynthesis of ABA involves multiple genes, yielding bioactive ABA from carotenoid precursors (figure 1.6). The final two steps are catalysed by a reductase enzyme encoded by *ABA2* and an oxidase encoded by *AAO3*. Characterisation of *ABA2/SDR1* and *AAO3* mutants in angiosperms has confirmed their essential role and allowed identification of homologs in other plant groups (Schwartz *et al.*, 1997; Seo *et al.*, 2000; Gonzalez-Guzman *et al.*, 2002; Gonzalez-Guzman *et al.*, 2004). The presence of putative homologs in *P. patens* provided a point of comparison between basal and later evolving plants. In *P. patens,* transcription of *ABA2* (125575) was highest in dry spores. This suggests that a relatively large amount of ABA is being synthesised, which concurs with the pattern in seed plants (Ali-Rachedi *et al.*, 2004; Nambara *et al.*, 2010; Okamoto *et al.*, 2010). The accumulation of ABA suppresses GA biosynthesis in seeds (Weiss and Ori, 2007; Zentella *et al.*, 2007; Ye *et al.*, 2011; Liu *et al.*, 2016). The expression pattern of *ABA2* in conjunction with GA biosynthesis gene expression data from chapter III suggests that a similar pattern of interaction and control may occur in *P. patens* spores.

There is a marked decrease in the level of transcript upon imbibition of spores. Due to a lack of primary dormancy in *P. patens* spores (Vesty *et al.*, 2016), the availability of water and key environmental signals such as light and temperature could trigger biosynthesis of germination-promoting genes such as those involved in diterpenoid synthesis (see chapter III). This in turn could negatively regulate the transcription of ABA-related genes leading to lower transcript levels in the total cellular RNA extracted

during this assay. Further analysis of transcription levels in a diterpenoid synthesis mutant such as *Ppcps/ks* or in spores treated with bioactive diterpenoids would enable a more definitive assessment of this relationship and suggest a molecular network warranting further investigation.

The pattern is less clear in the expression of *AAO3* homologs. The putative homolog *AAO3* 106708 shows high transcript levels in dry spores and upon imbibition but a clear decrease when spores are actively germinating. *AAO3* 140802 is highest in germinating spores and relatively weakly expressed in dry and imbibed spores. There are two salient points to consider when analysing these results in light of current knowledge of ABA signalling: 1 - ABA is everywhere in plant tissues and 2 - differential expression enables regulation of growth response.

ABA has roles in many plant processes across multiple tissue types and life cycle stages. Its ubiquitous nature means that biosynthesis and degradation will constantly vary in a temporal and spatial manner. As spores germinate, multiple tissue types are produced all requiring their own unique suite of regulatory signals. The differentiation of caulonema and chloronema and initiation of rhizoids can all be controlled by the metabolism of phytohormones such as ABA (Decker *et al.*, 2006). The potentially complex biosynthesis pathway and negative feedback mechanisms of ABA metabolism in conjunction with the dearth of characterised *P. patens* ABA signalling mutants limits the ability to draw conclusions on the specific role of individual ABA biosynthesis genes in spore germination. If ABA is everywhere then observing germination-specific functions of ABA signalling genes is more difficult.

When the expression pattern of the two identified *AAO3* homologs are considered in conjunction then consistently high levels of expression are seen across all tissue types. It may be that *AAO3* 106708 has a spore-specific role in ABA biosynthesis whereas *AAO3* 140802 is transcribed more in later multicellular tissues. The differential expression of hormone signalling genes is often the controlling factor in a plant's developmental response. The expression pattern of *AAO3*s in *P. patens* provides insight into one facet of this complex network in a basal land plant.

4.5.1.2 ABA perception and signalling in *P. patens*

The RCAR (regulatory component of ABA receptor) proteins bind ABA and relay the ABA signal through inactivation of protein phosphatase 2Cs (PP2Cs) and subsequent removal of SnRK (SNF1-related protein kinase) repression. This pathway and the genes involved have been well characterised in seed plants with generation of multiple signalling mutants and reporter expression analysis (Seo and Koshiba, 2002; Schwartz *et al.*, 2003; Xiong and Zhu, 2003; Nambara and Marion-Poll, 2005; Marion-Poll and Leung, 2007).

Transcription of all four *RCAR* genes was observed in all tissues assayed. There was a consistent decrease in transcript levels upon imbibition but this generally increased again as spores germinated and developed into multicellular protonemal and then leafy tissues. This pattern was also observed in the signal transduction genes, the class II SnRKs. The initial high level of transcription in dry spores was reduced upon imbibition and this level was then maintained throughout further life cycle stages.

This pattern suggests that the multitude of processes in which ABA is involved requires a consistently high level of production and the ability to perceive and relay that signal across multiple tissue types and life cycle stages. There are multiple functionally redundant groups within the identified ABA signalling gene families (Klingler *et al.*, 2010; Umezawa *et al.*, 2010). Generation of mutant lines in seed plants has enabled more detailed characterisation of the specific roles of different genes. The expression data from my research highlights the necessity of such characterisation in basal land plants and provides a starting point for selection of genes of interest.

4.5.1.3 Relaying the ABA signal response in *P. patens*

ABI1 and ABI3 act as ABA signal response factors. In seeds, *ABI1* encodes a protein phosphatase which acts as a negative regulator of the ABA signalling pathway. *ABI3* genes encode a suite of transcription factors that act as positive regulators of ABA-mediated control of seed germination (Nambara *et al.*, 2000). Putative *P. patens ABI1* homologues *PpABI1a* and *PpABI1b* are both highly expressed in dry spores. This could suggest they are acting in a regulatory manner to control the transduction of the consistently high ABA signal being perceived as shown by transcript data in figures 4.3 to 4.6. The negative feedback loop they are part of enables temporal and spatial control of the ABA signal as a way to control different plant growth responses such as germination. *PpABI1* expression decreases when spores are imbibed and in the case of *PpABI1b* disappears completely in actively germinating spores. My data suggests that the decision to germinate is controlled by both a decrease in ABA signalling and an increase in GA signalling. Upon imbibition, as this decision is made, the negative

regulation of ABA by ABI1 may no longer be required. The negative regulation of ABA by GA that occurs during germination may take over this role and exert enough control through its own ABA degradation pathways. The data from figures 4.3 to 4.6 supports the more detailed analysis of this process in seeds and also provides a potential target for germination-specific mutant analysis in *P. patens* in the form of *PpABI1b*

Putative *P. patens ABI3* homologues *PpABI3a* and *PpABI3c* also show high expression in dry spores which decreases markedly upon imbibition. As a positive regulator of the ABA response the transcription factors encoded by *ABI3* control the expression of genes that inhibit seed germination through maintenance of primary dormancy (Bentsink, 2008). A decrease in the expression of this transcript will down-regulate ABA-related gene expression and relieve the ABA signal pressure, allowing GA-mediated promotion of germination to occur. This balance between ABA and GA is key for control of seed germination. Transcriptional analysis in *P. patens* shown in figures 4.3 to 4.6 suggests that many of the components of this system may have their evolutionary origins in basal land plants.

4.5.2 The role of strigolactones in *P. patens* germination

Proust et al. identified the role of strigolactones in *P. patens* in controlling colony extension as a response to proximity of neighbouring colonies (Proust *et al.*, 2011), reminiscent of bacterial quorum-sensing pathways. The strigolactone biosynthesis mutant *Ppccd8* continues to extend colony size after wt plants have arrested growth approximately 20 days after germination. The diameter of wt colonies plateaus whilst

continued elongation and branching of caulonema in *Ppccd8* colonies increased colony size for a further 25 days. Figure 4.9 corroborates this previous data by showing the inhibitory effect of the synthetic strigolactone GR24 on colony extension. When investigated further Hoffmann et al. showed that strigolactones reduce caulonemal cell elongation and cell division rate (Hoffmann *et al.*, 2014).

The process of germination in both seeds and spores involves continual cell expansion before cell division takes place. The inhibition of germination by the exogenous application of the synthetic strigolactone GR24 (figure 4.8) suggests that its ability to reduce cell elongation has a negative effect on the germination rate of *P. patens* spores. The most consistent pattern was of a delay in germination caused by application of GR24. The overall germination efficiency of treated spores was not affected as all treatments achieved a final germination % comparable to untreated spores. This hints at the specificity of the role of strigolactones as quorum-sensing type signalling molecules. Under natural conditions the release of strigolactones appears to be a response to increasing plant size and proximity to neighbours (Proust *et al.*, 2011; Hoffmann *et al.*, 2014). In this situation, endogenous levels of GR24 in addition to that produced by neighbouring plants are sufficient to arrest growth. The immaturity and small size of the germinating spore may not warrant strigolactone biosynthesis in a similar manner. However it would still be of benefit to detect and respond to a strigolactone signal from a mature plant as a way of evaluating the external environment before deciding to germinate. Exogenous application of GR24 clearly has an inhibitory effect (figure 4.8) but this is not sustained and spores appear to overcome this after a few days and

germinate rapidly to achieve similar levels of germination to untreated spores. The mechanism by which they do this is an intriguing area for future study. Upregulation of genes involved in GR24 catabolism, or downregulation of genes involved in GR24 perception may decrease the impact of the GR24 signal. The typical germination-promoting signals (i.e. GAs) may need to accumulate to higher levels to outweigh the GR24 signal before germination can proceed. Whilst this is purely conjecture, the inhibitory role of GR24 demonstrated in figures 4.8 and 4.9 may provide further insight into the complexity of the regulatory network that controls spore germination. The availability of the *P. patens* strigolactone biosynthesis mutant *Ppccd8* provided additional evidence for the conclusions made.

4.5.2.1 Strigolactones are an evolutionarily ancient signalling molecule

The role of strigolactones in control of colony extension in *P. patens* has been studied in detail through characterisation of the biosynthesis mutant *Ppccd8* (Proust *et al.*, 2011). The similarity of its role in *P patens* to that in seed plants suggests a level of evolutionary conservation. Early land plants would have needed to develop novel ways of interacting with their new environment. The perception of diffusible signals in an aquatic environment is much easier when compared to the bare rocks and shallow soils of early terrestrial landscapes. The discovery and demonstrable importance of strigolactone signalling in basal land plants makes sense in light of early land plant colonisation. One of the crucial requirements for the subsequent diversification and radiation of terrestrial plant groups was their ability to interact with the rhizosphere. The role of strigolactones here and subsequent diversification into control of branching in

roots and shoots indicates that strigolactones are evolutionarily ancient molecules. They may have played a key role in the colonisation of the terrestrial environment through adaptation of hormonal growth responses.

The endogenous production of strigolactones was confirmed much later than its identification as an external signalling molecule. The importance of additional ecophysiological communication pathways is considered below through analysis of the effects of known bacterial quorum-sensing molecules on *P. patens*.

4.5.3 External signalling molecules - a role for *N*-acyl homoserine lactones

When plants first colonised the terrestrial environment they would have been bombarded with signalling molecules from their new microbial neighbours. Quorum sensing molecules as autoinducers regulate growth and metabolism of bacteria and this is well studied in the case of *N*-acyl-homoserine lactones (AHLs) synthesised by Gram-negative bacteria (Fuqua *et al.*, 2001). The terrestrial rhizosphere contains a diverse microbial fauna so the ability to listen in on the bacterial conversation would have been an evolutionary advantage for plants. Indeed, recent research has shown that many vascular plants are able to perceive these diffusible signals and use them to get a head start on protecting themselves from a potential pathogenic attack (Zarkani *et al.*, 2013; Cheng *et al.*, 2016). Tissue-specific changes in detoxification enzyme activity (Götz-Rösch *et al.*, 2015) and expression of salicylic acid-dependent genes (Schuhegger *et al.*, 2006) allow a more rapid response to pathogenic attack and can limit damage caused. But the ability to eavesdrop on the rhizosphere can also be used positively by

plants. Interaction with rhizobacteria can promote growth in plants, particularly in terms of root growth and promoting formation of root nodules (Veliz-Vallejos *et al.*, 2014). This has been observed in detail in *A. thaliana* roots, where two G-protein coupled receptors (GPCRs) were shown to be responsible for perceiving the AHL signal which then caused transcriptional changes in roots leading to alterations in primary root growth, lateral root formation and root hair development (Ortiz-Castro *et al.*, 2008).

Whilst seeds and spores also find themselves amongst this bacterial community in the soil, the resistant nature of their outer coat raises the question of whether they are able to respond to signals such as bacterial AHLs and also if they need to. The unicellular spores of *P. patens* provide an ideal model system in this respect as they are simpler than multicellular seeds so may be more accessible to the diffusible bacterial signals. At low concentrations, the AHLs C4 to C12-HSL appear to have a promotional effect on the germination of *P. patens* spores (figure 4.11). Across all biological replicates this was more often observed with the long chain C10 and C12-HSL. There was also, more often than not, a significantly greater effect when using a lower concentration of AHL in the growth media (figure 4.11). Preliminary assays using 5, 50 and 100µM AHL concentrations were fairly inconclusive and most often resulted in inhibition of germination (data not shown). In nature, these molecules are present in very low concentrations in comparison to endogenous hormone levels. As a germinating spore the detection of bacterial signals could indicate that it is a suitable time and place to germinate. The presence of beneficial rhizobacteria could stimulate the germination of seeds as a way of exploiting the environment. At very high concentrations, AHLs may

be perceived as a negative environmental characteristic, nullifying any benefits of early germination. The promotion of *P. patens* spore germination by exogenous application of AHLs was demonstrated in multiple biologically replicated assays and by other members of Coates labs research team. The simple structure of its spores and the key evolutionary position *P. patens* occupies make it an ideal system for studying this cross-kingdom signalling mechanism. The recent identification of an AHL-producing bacterial species on the sporophyte of wild grown *P. patens* (Coates unpublished data) supports the idea of a role for AHLs in *P. patens* growth and development. The lack of true roots in *P. patens* could also help confirm what the 'pre-root' function of AHL perception was.

In aquatic systems AHLs act as attractants to zoospores of *Ulva* spp. as they will preferentially settle on biofilms containing AHL-producing bacterial species (Tait *et al.*, 2005; Wheeler *et al.*, 2006; Joint *et al.*, 2007; Twigg *et al.*, 2014). However, it has recently been shown that zoospore germination and germling growth is negatively regulated by the long chain C12-HSL (Twigg *et al.*, 2014). This is in contrast to the observed land plant response as demonstrated in angiosperm models and now bryophytes as discussed above.

The majority of research on AHL signalling has focussed on the later-evolving vascular plant groups and a key point arising from this, carried out largely in the past decade, is that the type of response is often dependent on the structure of the AHL perceived and that the response to a specific AHL structure may be different in different plants (Ortiz-Castro *et al.*, 2008; von Rad *et al.*, 2008; Hartmann and Schikora, 2012; Schenk *et al.*, 2012; Palmer *et al.*, 2014). This area is of increasing interest in the agricultural sector,

demonstrated by a patent application recently submitted for the utilisation of AHLs as growth promoters in agriculture (Bassler *et al.*, 2001).

4.5.4 Concluding remarks

As with most areas of biotechnology, the process needs to be understood before it can be applied to a different system. This is only possible through the study of natural plant processes in multiple model systems. When combined with answering questions on the evolutionary origins of those processes, the option of representative model systems is crucial. As a basal land plant, *P. patens* represents a key step in the evolution of plants. However, as with all life, the origins were aquatic.

CHAPTER V:

ULVA AS AN EMERGING MODEL SYSTEM FOR PLANT EVOLUTIONARY DEVELOPMENTAL BIOLOGY

5.1 Introduction

Macroalgae (seaweeds) are one of the most environmentally and economically important algal groups. In conjunction with microalgae they play a major role in the maintenance of global oxygen levels and carbon sequestration through photosynthesis. They are a food source for many nationalities and countries and are also an emerging system for biotechnological areas such as bioremediation and production of biofuels (Vandermeulen and Gordin, 1990; Abd El-Baky *et al.*, 2009; Baweja *et al.*, 2009; Bolton *et al.*, 2009; Godard *et al.*, 2009; Toskas *et al.*, 2011; Lawton *et al.*, 2013; Sode *et al.*, 2013).

As multicellular photosynthetic organisms they inhabit an evolutionarily relevant time-point for increasing our understanding of the development of multicellularity and colonisation of the terrestrial environment (Leliaert *et al.*, 2011; Leliaert *et al.*, 2012).

The green chlorophyte macroalgae are the seaweed group most recently diverged from the land plant lineage (Leliaert *et al.*, 2011) (figure 5.1). The importance of this group and the requirement for an understanding of their functioning necessitates investigation of processes in a variety of species through characterisation of new model systems.

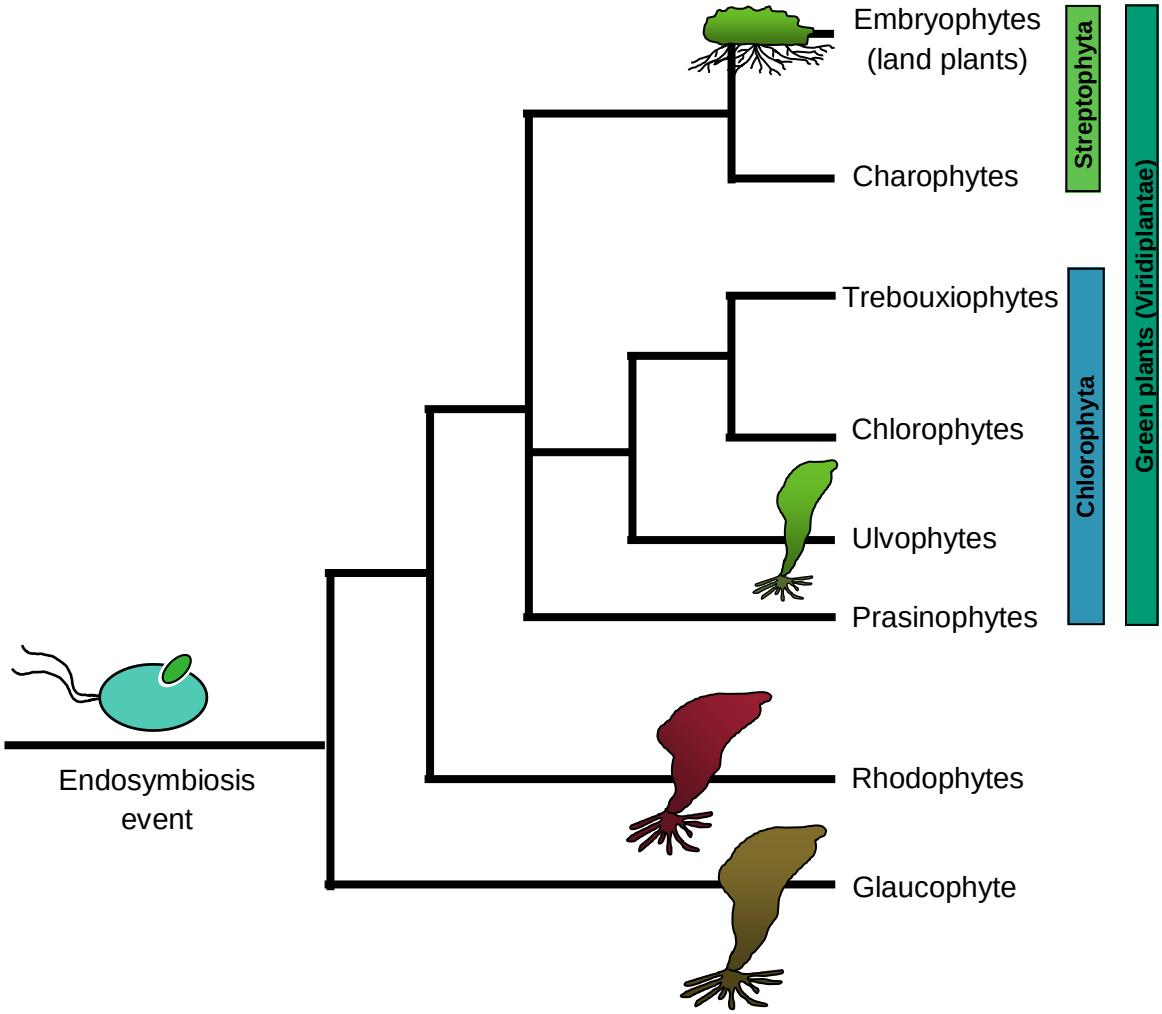


Figure 5.1 Phylogeny of plant life

After the primary endosymbiosis event approximately 1.6 billion years ago the other algal groups (Rhodophytes and Glaucophytes) diverged from the green plants (Viridiplantae). The Streptophyta contains all land plants (e.g. *Physcomitrella patens)* and the freshwater Charophytes. The Chlorophyta contain all other marine and freshwater algae including multicellular green macroalgae such as *U. intestinalis.*

5.2 *Ulva* as a new genus for understanding the evolution of developmental mechanisms

The Ulvophyceae contains a vast array of morphologically and cytologically diverse groups that can provide insight into the morphological evolution of green plants (Wichard *et al.*, 2015). Species from the genus *Ulva* are mostly known as sea lettuces and form a large group of mainly coastal phenotypically plastic multicellular green seaweeds.

As an aquatic multicellular green plant, species such as *Ulva intestinalis* are also a source of information for the understanding of the green plant lineage and the evolution of features such as multicellularity and desiccation tolerance. Moreover, unlike land plants, green algae of the genus *Ulva* exhibit clear alternation between distinct unicellular and multicellular stages twice in their life cycle (Figure 1.4)

5.3 *Ulva mutabilis* as an emerging molecular genetic green non-land plant model system

Previous research on two *Ulva* species, *U. mutabilis* and *U. lactuca* has provided a wealth of information on the physiology, cell biology and life cycle of this algal group (Løvlie and Bråten, 1970; Bråten, 1971; Hoxmark, 1975; Wichard and Oertel, 2010; Spoerner *et al.*, 2012; Wichard *et al.*, 2015). *U. mutabilis* is found mainly off the coast of Portugal in Europe. Historic research on the species revealed many key characteristics of the genus particularly its life cycle progression and alternation of multicellular and

unicellular isomorphic generations (Løvlie and Bråten, 1970; Bråten, 1971; Hoxmark, 1975; Wichard and Oertel, 2010; Spoerner *et al.*, 2012). More recently the majority of research is carried out on the *U. mutabilis* 'slender' mutant (Løvlie and Bråten, 1970) which displays a much shorter life cycle and is amenable to laboratory culture.

The recent sequencing of the *U. mutabilis* slender genome has led to this being considered the type species for *Ulva* research, with implications for phylogenetic and molecular biology fields. As an emerging model system it provides a key point of comparison when investigating the evolution of green plant life. Comparisons with terrestrial models such as *P. patens* and *A. thaliana* could begin to answer some of the big questions in plant evolutionary developmental biology, particularly regarding multicellularity, terrestrialisation and life cycle progression.

The availability of genetic data will support the in-depth knowledge we currently have on the cell biology, life cycle progression and physiology of this particular species. It also provides grounds for development of other *Ulva* model systems that may offer additional information.

5.3.1 Features of the *Ulva mutabilis* life cycle

The life cycle of *U. mutabilis* is consistent with most other *Ulva* species with mature isomorphic sporophytes and gametophytes releasing spores and gametes respectively, that germinate and develop into new individuals. One of the advantageous features of *U. mutabilis* slender is that the induction of gametogenesis and sporogenesis can be carried out in a laboratory environment, something that has proved difficult in other

commonly studied species such as *U. lactuca* and *U. prolifera.* This is due to the detailed characterisation of the controlling signalling molecules in this particular species (Wichard and Oertel, 2010; Spoerner *et al.*, 2012).

5.3.2 Induction of gametogenesis and sporogenesis

Gametogenesis and sporogenesis are induced naturally upon maturation of *U. mutabilis* blades in concurrence with optimal environmental conditions such as high tides. Two key internal signals have been identified that control the formation of spores and gametes and the release of them into the environment (Wichard and Oertel, 2010). Sporulation inhibitor 1 (SI-1) is a glycoprotein that inhibits the differentiation of cells into gametangia or sporangia. It is produced consistently throughout the development of both sporophyte and gametophyte blades and as they mature the SI-1 levels decrease, allowing induction of gametogenesis or sporogenesis. Sporulation inhibitor 2 (SI-2) is a low molecular weight molecule that inhibits differentiation of basal sections of blades ensuring that gametogenesis and sporogenesis only occur in the apical region of the blade through maintenance of a vertical concentration gradient (Wichard and Oertel, 2010). Once gametes or spores have been produced a second inhibitor, swarming inhibitor 1 (SW1), stops them from being released into the surrounding media until it too is diluted below inhibitory levels. At this point gametes or spores exit through a pore in the gametangial or sporangial cell and begin to move about in the media by beating of flagella. The removal of these inhibitors through dilution or a decrease in synthesis is essential for these processes and overrules signals such as diurnal light patterns (Wichard and Oertel, 2010).

In the natural aquatic environment, the removal of inhibitors occurs by a natural decrease in the levels of each. This may be due to decreased synthesis or increased degradation. However the critical drop in levels is what controls the timing of sporogenesis and gametogenesis and release of their respective reproductive cells. The timing of gamete and spore release with high tides consolidates this.

5.4 Other *Ulva* species and their economic importance

One of the characteristics of *Ulva* species is the large volume of spores and gametes produced on a regular basis (Reddy *et al.*, 2007; Gao *et al.*, 2010; Mantri *et al.*, 2011; Lawton *et al.*, 2013). This makes it particularly amenable to studies of reproductive output and growth and development. As a source of food and also potential biotechnological impact an understanding of its individual traits and features would expand and consolidate previous research.

Ulva species are also important models for the study of invasive macroalgal blooms. The green tides such as those caused by *U. prolifera* during the Beijing Olympics (Leliaert *et al.*, 2009) can generate over 1,000,000 tons of wet mass in as little as two months. The immediate and long lasting effects of such an event can cause significant ecological and economic damage to a region, like in Qingdao in 2008. Subsequent studies have shown that it is *Ulva's* ability to rapidly respond to variations in salinity, nutrient availability and temperature and to form floating mats that facilitate such invasive capabilities (Teichberg *et al.*, 2010; Xu *et al.*, 2012; Kang *et al.*, 2016). A better understanding of the physiology and developmental patterns of *Ulva* species will allow a

more detailed understanding of the causes and consequences of these events that appear to be increasing in frequency.

More recently, this exploitative feature has been harnessed in a beneficial way. The use of *Ulva* species as bioremediation agents for waste water treatment and removal of contaminants (via their ability to form a large amount of biomass) has been investigated by multiple groups and in multiple locations (Zakhama *et al.*, 2011; Sode *et al.*, 2013)

Ulva species are also a common biofouling group due to the strong adhesive nature of their zoospores when settled (Callow *et al.*, 1998). This has been investigated by analysis of influences on settlement and adhesion of zoospores (Callow *et al.*, 1998; Tait *et al.*, 2005; Wheeler *et al.*, 2006; Finlay *et al.*, 2008; Rosenhahn *et al.*, 2009; Wendt *et al.*, 2013). The subsequent germination of spores and also the behaviour of reproductive cells (gametes, zygotes) have not been as thoroughly assessed. The availability of *Ulva* tissue from Jim and Maureen Callow's research group at the University of Birmingham allowed me to begin investigation of an alternative *Ulva* system. Recent Coates lab sequencing data (F. Ghaderi, unpublished) suggests that the species previously identified as *Ulva linza* by the Callow lab is actually *Ulva intestinalis.* All samples taken from the collection site in Wales have been analysed in this way to confirm this and therefore the experimental species referred to in the rest of this chapter will be *U. intestinalis*. Both *U. linza* and *U. intestinalis* have a broad range and are extensively phenotypically plastic species. They are intertidal epilithic species found along most UK coastlines and thrive in both marine and brackish conditions. Whilst the implications of this mix up are yet to be confirmed the development of an

alternative and more widespread *Ulva* model system is the salient achievement of this thesis.

5.5 Chapter objectives

The main objective of this chapter was to develop methods for the routine culture of an alternative *Ulva* species to enable comparison with the newly emerging *U. mutabilis* model system. The information already available from research on *U. mutabilis* enabled several key characteristics to be investigated:

1. Characterisation of the life cycle for comparison with *U. mutabilis*

2. Cross-species comparison of the control of morphology by certain bacterial species

3. Development of culture methods and nucleic acid isolation

The achievement of these three objectives led to the publication of the paper included in the appendix (Vesty *et al.*, 2015). The establishment of routine culture methods and characterisation of the *U. intestinalis* life cycle will enable further comparative studies and establishment of robust green algal model systems.

5.6 Characterisation of the *U. intestinalis* life cycle

Like many *Ulva* species *U. intestinalis* displays a certain amount of morphogenic plasticity, but it is generally longer than both *U. mutabilis* and *U. lactuca* with a narrower thallus. It displays an isomorphic alternation of generations (figure 5.2) with both sporophyte and gametophyte forming multicellular blades with a network of rhizoids at the base. Blades are two cells thick often with a gap in between the layers such that they form a flattened tube when mature. Figure 5.6 provides images of the key stages in the *U. intestinalis* life cycle and provides points of comparison with the other *Ulva* species. Detailed characterisation such as this will enable investigation of the influence of factors such as phytohormones and environmental signals on developmental processes, including my particular interest of spore germination.

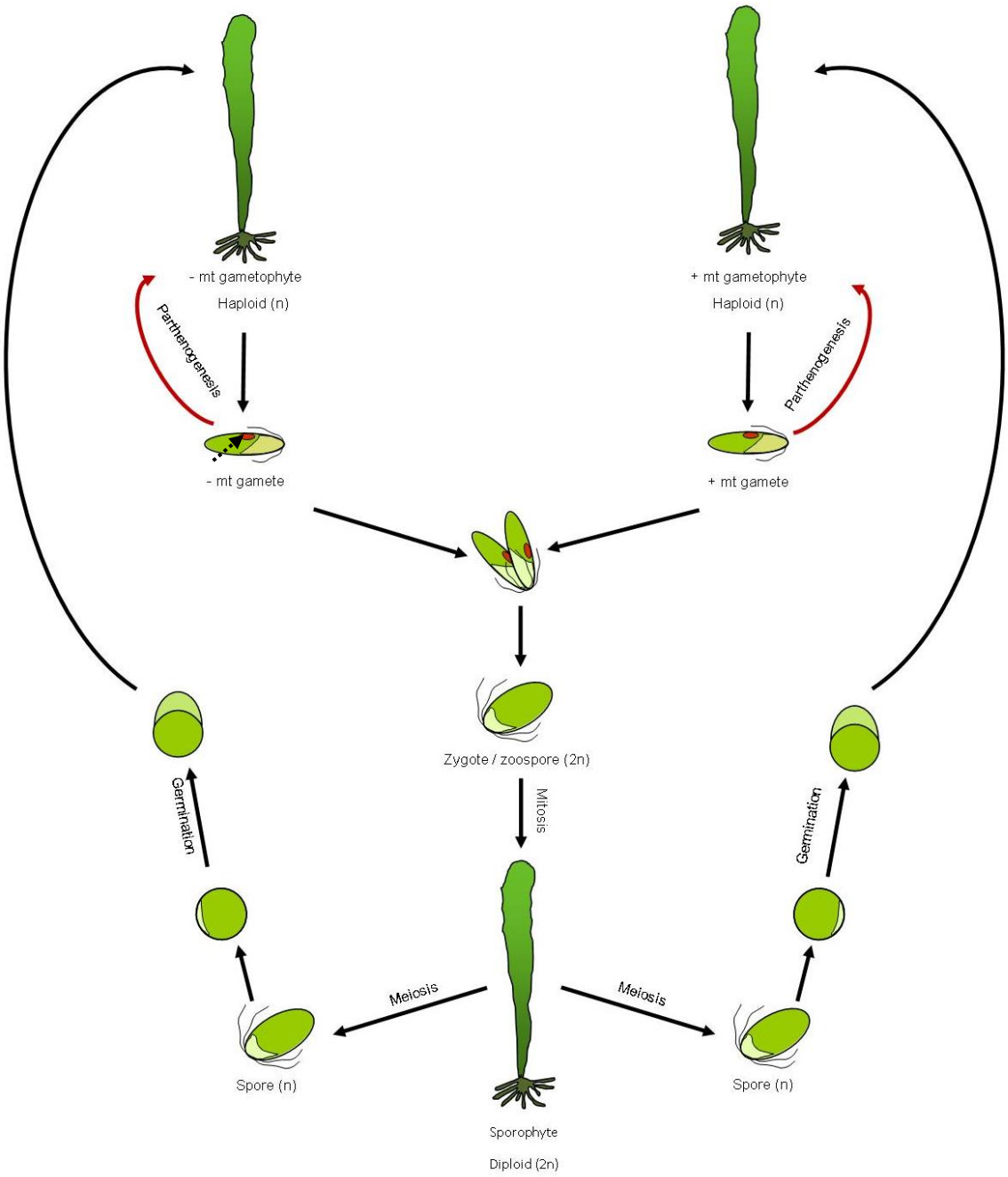


Figure 5.2 Life cycle of *U. intestinalis*

Mature haploid gametophyte blades produce either + or - biflagellate gametes with a visible eyespot (◂···). Gametes can fuse to produce a quadriflagellate zoospore or germinate to form another gametophyte through parthenogenesis (red line). Zygote develops into mature sporophyte which produces haploid spores through meiosis. These germinate and develop into haploid gametophytes.

5.6.1 Induction of gametogenesis and sporogenesis

As a mainly tidal species, *U. intestinalis* experiences variations in water level that would affect the dilution of any secreted signals analogous to *U. mutabilis* SW1. It is therefore reasonable to assume that as in *U. mutabilis*, the natural maturation of *U. intestinalis* blades causes a decrease in sporulation inhibitor concentrations leading to induction of gametes and spores. These reproductive cells are then held inside gametangia and sporangia by high levels of swarming inhibitors that are then sufficiently diluted by the increasing water volume of a high tide to allow their release into the environment to begin the next stage in the life cycle.

5.6.2 Artificial induction

The importance of SIs and SWs in *U. mutabilis* 'slender' meant that an efficient method for the removal of both at specific times had to be developed in order to establish laboratory cultures of *U. intestinalis*. This was based mainly on the protocol developed by Wichard *et al*. for use with *U. mutabilis* slender (Wichard and Oertel, 2010) and the aim was to see if it could be successfully applied to an alternative and more widespread wild-type species such as *U. intestinalis*.

5.6.2.1 Sporulation

Sporulation inhibitors were removed by washing and fragmenting mature *U. intestinalis* blades (see materials and methods and figure 5.3). Fragments of approximately 900 (30 x 30) cells were found to be optimal to ensure induction of the majority of cells. These

were washed multiple times to ensure sufficient dilution of inhibitors and then suspended in UCM growth media for induction to take place.

Differentiation of cells was observed through light microscopy of multiple technical and biological experimental replicates. Immediately after fragmentation cells appeared normal with a conspicuous vacuole. Images taken on day two show an obvious change with the appearance of multiple granular bodies (figure 5.3). By day three, spores or gametes were visible and this was generally the day on which release was induced. Occasionally fragments had to be left for a further day to achieve the maximum release of reproductive cells.

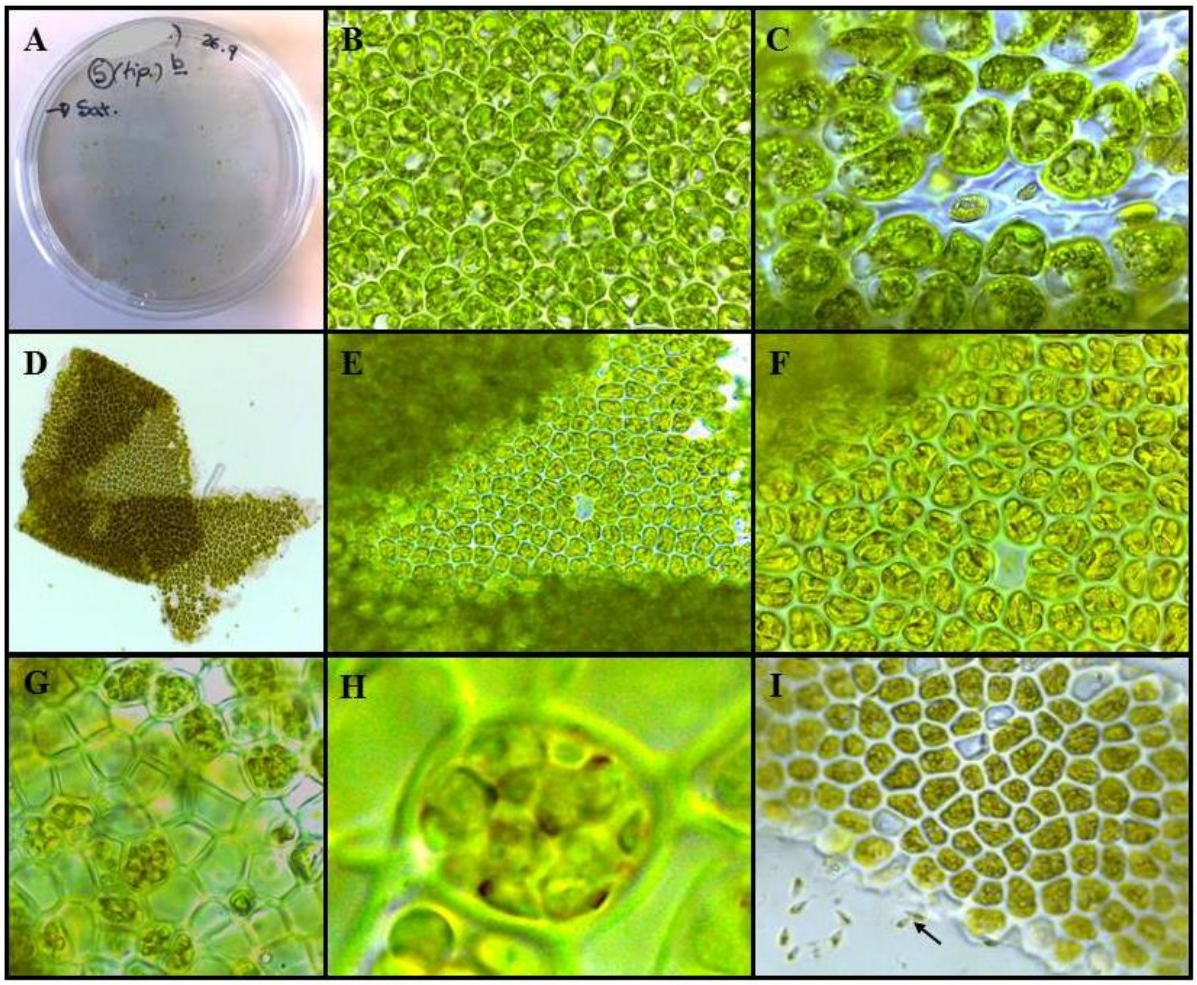


Figure 5.3 Artificial induction of sporogenesis and gametogenesis

Thallus was fragmented, washed and re-suspended in UCM media in petri dishes (A). Cells appeared normal immediately after fragmentation (B) but by day two multiple granular bodies were visible (C). Three days after induction (D-I) tissue was much darker in colour (D) and closer inspection revealed gametes (E and F) and spores (G and H) tightly packed into each cell. In some cases there was premature release of gametes or spores before swarming was induced so some were visible in the media (I) (←)

5.6.2.2 Swarming

The removal of swarming inhibitors was carried out by washing induced fragments multiple times and re-suspending in fresh UCM in petri dishes. These were placed in front of a lamp in a dark area to encourage phototactic movement towards (gametes) or away (spores) from the light source (figure 5.4 B and C). Swarming generally occurred within half an hour at which point enough gametes or spores had collected to be visible to the naked eye (figure 5.4 C). At this point they were collected and transferred to microcentrifuge tubes. Those containing gametes were then further concentrated by placing them back in front of the lamp and subsequent removal of excess media (figure 5.4 D). The positively phototactic nature of *U. intestinalis* gametes enabled application of the purification technique developed by Wichard *et al*., (Wichard and Oertel, 2010) for purification of *U. mutabilis* gametes.

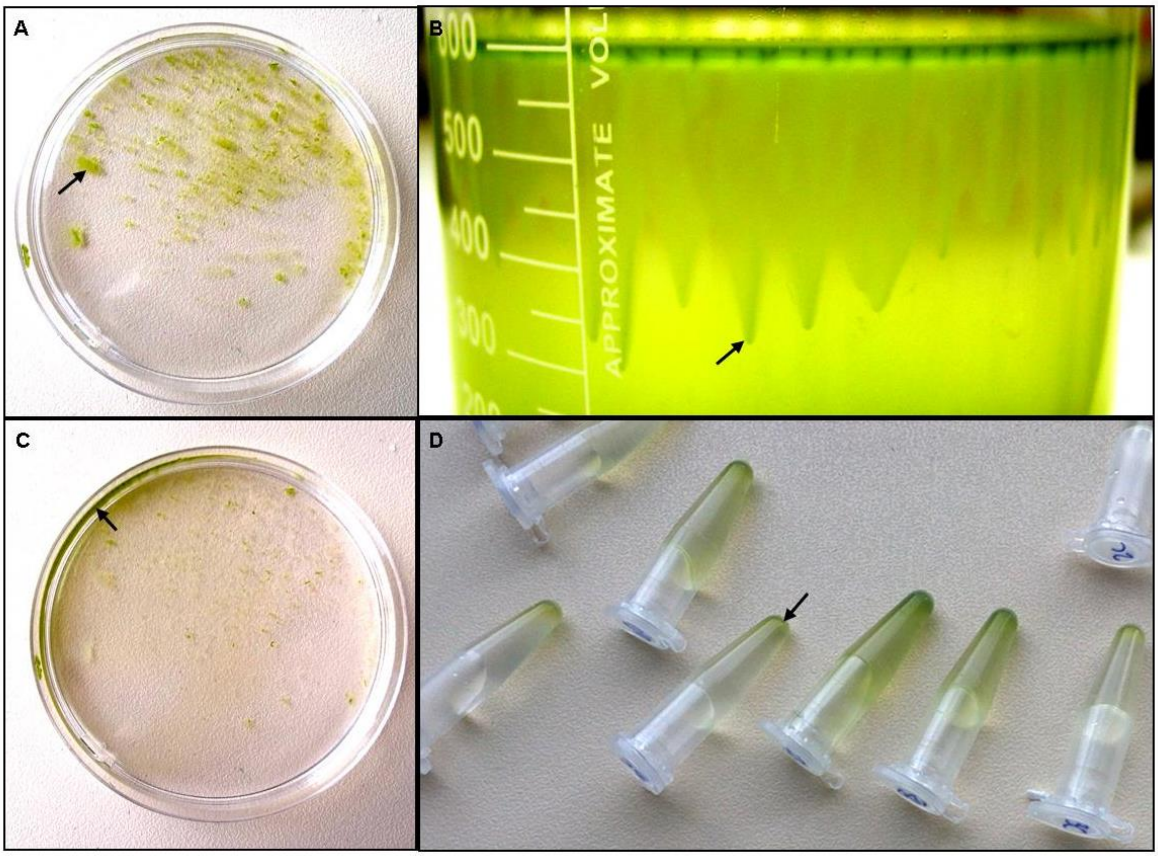


Figure 5.4 Release and swarming of spores and gametes

Induction of fragments was confirmed by microscopy before washing and re-suspending in fresh media. Gamete/spore release was visible after approximately 10 minutes (A). Spores are negatively phototactic and will move away from light (B) whereas gametes are positively phototactic and aggregated at the side of the petri dish closest to the light (C). Gametes were collected and transferred to microcentrifuge tubes for further concentration (D). Arrows indicate spore or gamete location.

5.6.2.3 Purification

The generation of axenic cultures is essential for molecular biology techniques such as genome sequencing. Contamination by any unicellular green algae or bacteria could inhibit analysis of sequence data and may also lead to tissue culturing problems through competition and rapid growth. The speed at which *U. intestinalis* gametes swim in response to light is much faster than any epiphytic contaminants commonly found on mature blades. This was exploited by getting them to swim along a Pasteur pipette towards a lamp (figure 5.5). As soon as a dense concentration of gametes had collected at the end they were removed before the slower moving contaminants could contaminate the sample (see materials and methods and figure 5.5).

This method was repeated and refined to facilitate efficient and consistent generation of material for further use in setting up of cultures and extraction of nucleic acids. Experimentation with the timings of each purification step, light source and distance and control of external light levels were combined with constant checking of aseptic technique (figure 5.5 D) to establish a suitable method for future applications.

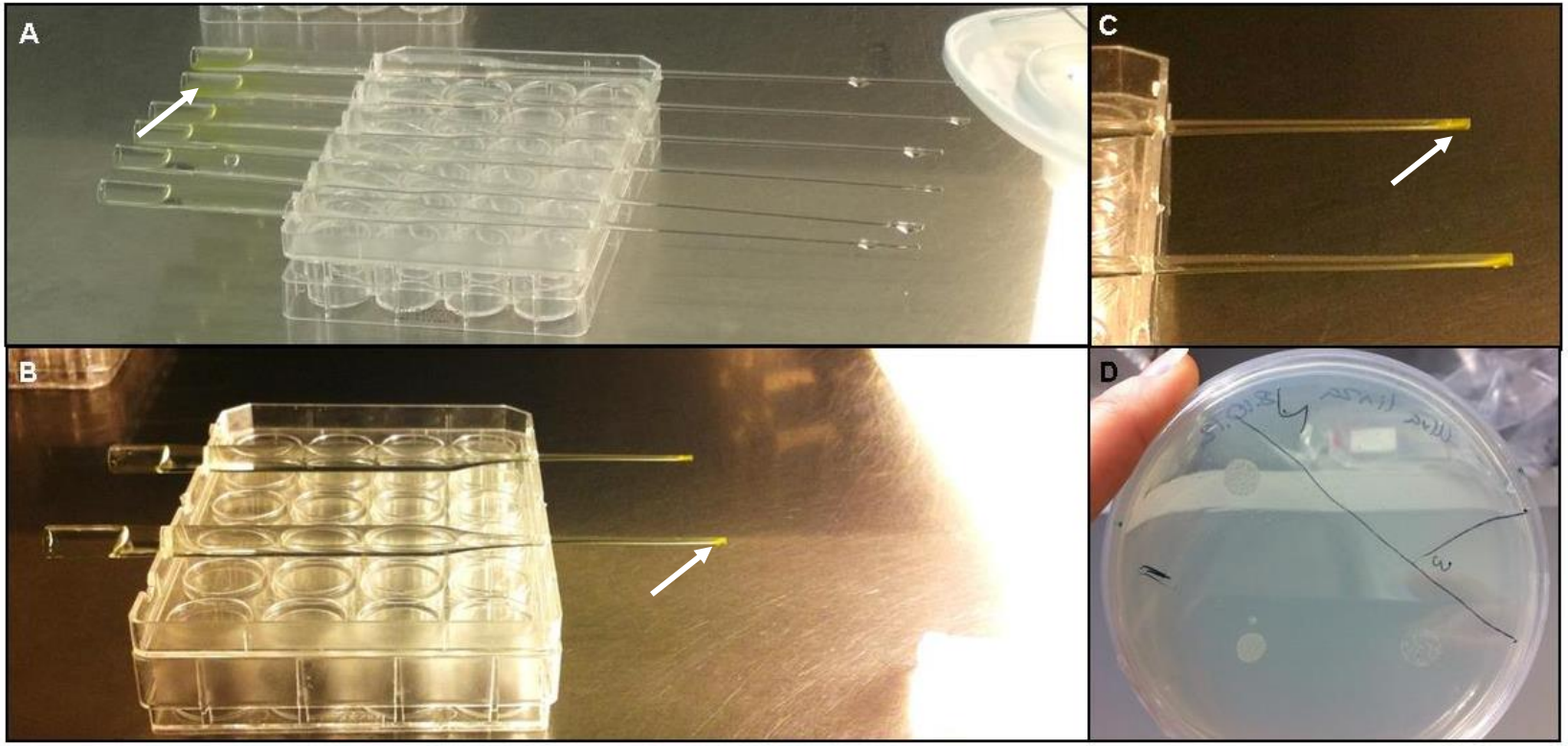


Figure 5.5 Purification of gametes

Gametes were transferred to the wide end of a sterile glass Pasteur pipette (A) and placed in front of a light in a sterile hood. After approximately 15 minutes gametes had reached the other end and concentrated at the narrow tip (B and C). Gametes were then removed by tipping the droplet of liquid into a microcentrifuge tube. The next three droplets were placed onto a marine agar plate to check for presence of contaminating bacteria (D). White spots on (D) are salt crystals from dried droplets. Arrows indicate gamete location.

5.6.3 Reproductive strategies of *U. intestinalis*

U. intestinalis gametophytes produce only one gamete mating type (+ or -) (figure 5.2). These biflagellate cells can parthenogenetically produce new gametophytes without the need for fertilisation (Wichard *et al.*, 2015). This was carried out on multiple occasions to generate new tissue for further gamete induction. Combining + and - gametes in culture produced zygotes that grew into the diploid sporophyte. These would produce haploid spores by meiosis once mature. Observations of mating in *U. mutabilis* (Bråten, 1971; Hoxmark, 1975; Wichard and Oertel, 2010; Wichard *et al.*, 2015) suggests that suspensions of a single spore mating type can also form parthenosporophytes by fusing and undergoing meiosis to produce haploid zoospores. Due to the parthenogenetic capabilities of *U. intestinalis* and the isomorphic nature of its multicellular stages there was no way of ensuring that all plants in a culture flask were sporophytes and therefore that all reproductive cells were spores. For this reason spores for culture, observations and nucleic acid extraction were collected from sporophytes sampled from the beach at Llantwit Major, South Wales (51°40′N; 3°48′W). Laboratory cultures for future use were established using parthenogenetic gametophytes.

5.6.4 Germination and development of blades and rhizoids

Figure 5.6 shows some of the early stages in the germination and development of a spore. The initial polar division forms a primary rhizoid cell that extends and divides further. These cells have fewer chloroplasts and divide on multiple planes to produce a mass of filaments to secure the developing thallus blade to a surface. The blade

develops by multiple divisions in a single plane and then subsequent widening by perpendicular divisions (figure 5.6 F). The unbranched thallus can grow up to 45cm in length and is often wider in the middle with a frilled margin. It can grow as tubes or flat blades but is always just two cells thick. In the UK *U. intestinalis* grows mainly in littoral and sublittoral zones attached to rocks or human structures. It can also live free-floating but this fragmentation from holdfast often leads to early maturation of tissue leading to tissue death or induction of reproductive cells.

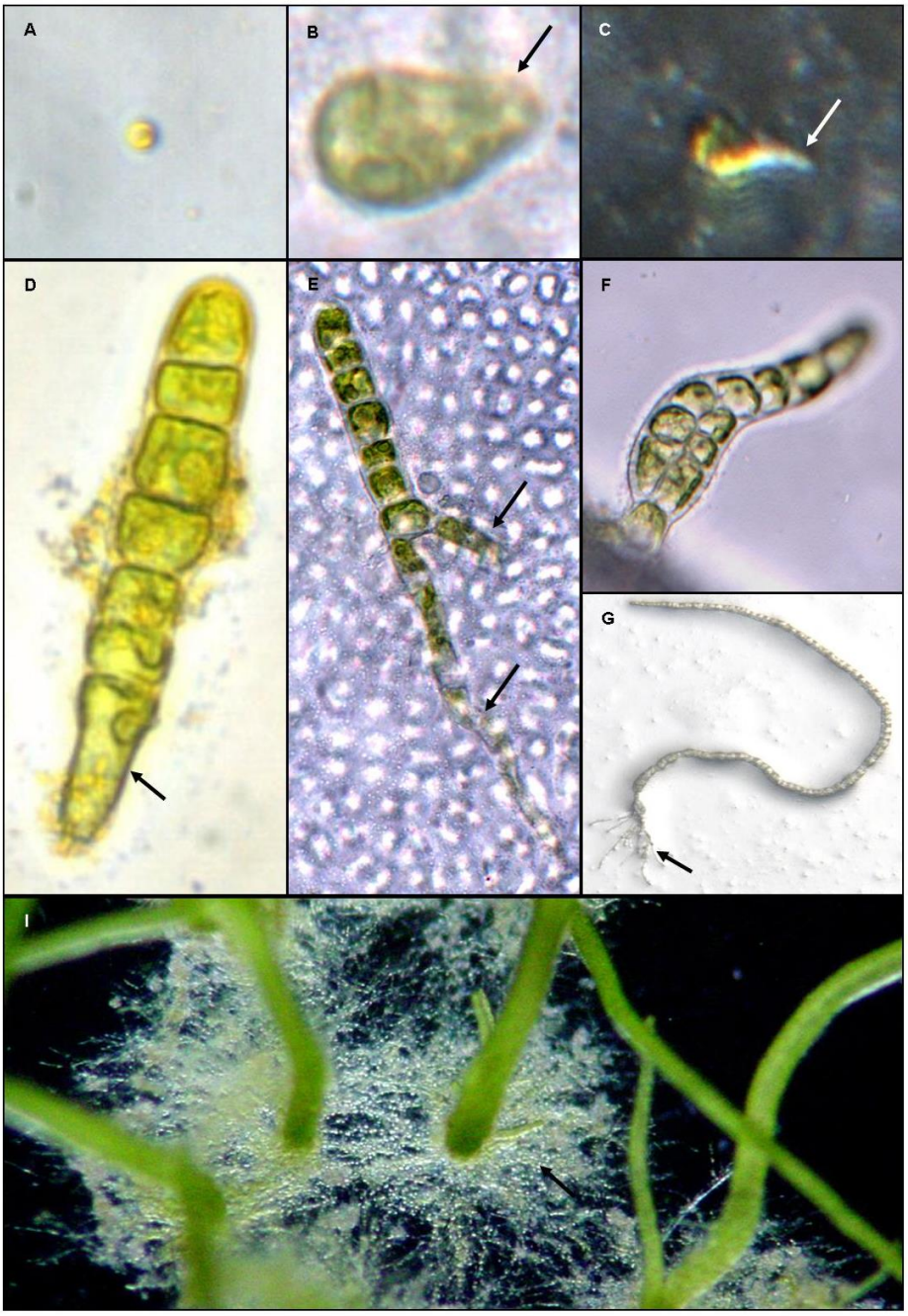


Figure 5.6 Germination and early developmental stages of *U. intestinalis*

Spores were settled in culture vessels (A). First cell division forms a primary rhizoid cell and a cell that will develop into the thallus (B and C). Additional polar divisions extend thallus while primary rhizoid cell extends, divides and branches (D and E). Perpendicular division widens blade (F) and forms tubular filament (G) with rhizoid mass at the base (H). Arrows indicate rhizoid cells.

5.6.5 Maturation of blades and progression to natural induction of gametogenesis and sporogenesis

The importance of inhibitory signals has been discussed above in terms of induction of gametogenesis and sporogenesis. In nature, the thallus takes 8-10 weeks to mature, depending on seasonal variations in temperature and day length. The closely related *U. mutabilis* displays a clear synchronised daily division of blade cells to increase in size. This detailed level of cell cytology has not been observed as yet in *U. intestinalis* but preliminary observations suggest a similar rate of growth that would support any theoretical consistency between the two species.

The induction of gametogenesis/sporogenesis results in a colour change in the apical region of mature blades. In *U. intestinalis* this can be used to distinguish between sporophyte (white) and gametophyte (orange) blades. The release of reproductive cells leaves the apical region transparent and degradation of tissue occurs rapidly.

In laboratory culture, induction often occurs prematurely in blades that are only 2-3 weeks old (figure 5.7 D and 5.9 C). This may be due to induction of reproductive structures as a response to unnatural conditions during the refinement of culturing techniques.

5.7 Development of culture conditions and molecular biology techniques

The development of routine culture conditions for *U. mutabilis* (Stratmann *et al.*, 1996; Wichard and Oertel, 2010) enabled rapid establishment of functioning cultures of *U. intestinalis*. Training received at the Institute for Inorganic and Analytical Chemistry at

the Friedrich Schiller University allowed me to apply known techniques to a new Ulvophyte system and enabled cross species comparison of culture requirements and life cycle progression (Vesty *et al.*, 2015).

5.7.1 Culture conditions

The Ulva Culture Media (UCM) developed for *U. mutabilis* was capable of supporting *U. intestinalis* in laboratory conditions. The specific combination of vitamins, minerals and salts found in UCM was prepared (see chapter II) and used for all culturing methods.

A variety of different culture vessels were tested during the establishment of the laboratory culture (figures 5.7 - 5.9). These included polystyrene tissue culture flasks with vented caps (figure 5.7), submerged glass slides in multiwell plates, submerged agar plates and conical flasks on an orbital shaker (figure 5.8). All strategies enabled new tissue growth but each also presented individual obstacles.

By far the most successful culturing strategy was inoculation of vented culture flasks with gametes or spores. These settled and attached to the base of flasks allowing observation of growth and development on an inverted microscope (figure 5.7 A). It was a simple process to routinely change media and also remove germlings for sub-culturing as required (figure 5.7 B). However blades did not grow very large before natural induction of gametogenesis or sporogenesis (figure 5.7 D). It was difficult to stop this process occurring naturally and this was the major challenge faced in most culturing strategies used. Whilst the ability to culture large thallus blades would have been useful to maximise tissue culture, the speed at which thalli could be grown, matured and

induced enabled a constant supply of reproductive cells for analysis, nucleic acid extraction and further culturing. The well characterised *U. mutabilis* ‘slender’ mutant is currently preferred mainly due to the short duration of the life cycle from germination to maturation. The rapid turnover of *U. intestinalis* therefore makes it an ideal candidate for future investigation of a naturally occurring widespread Ulvophyte species.

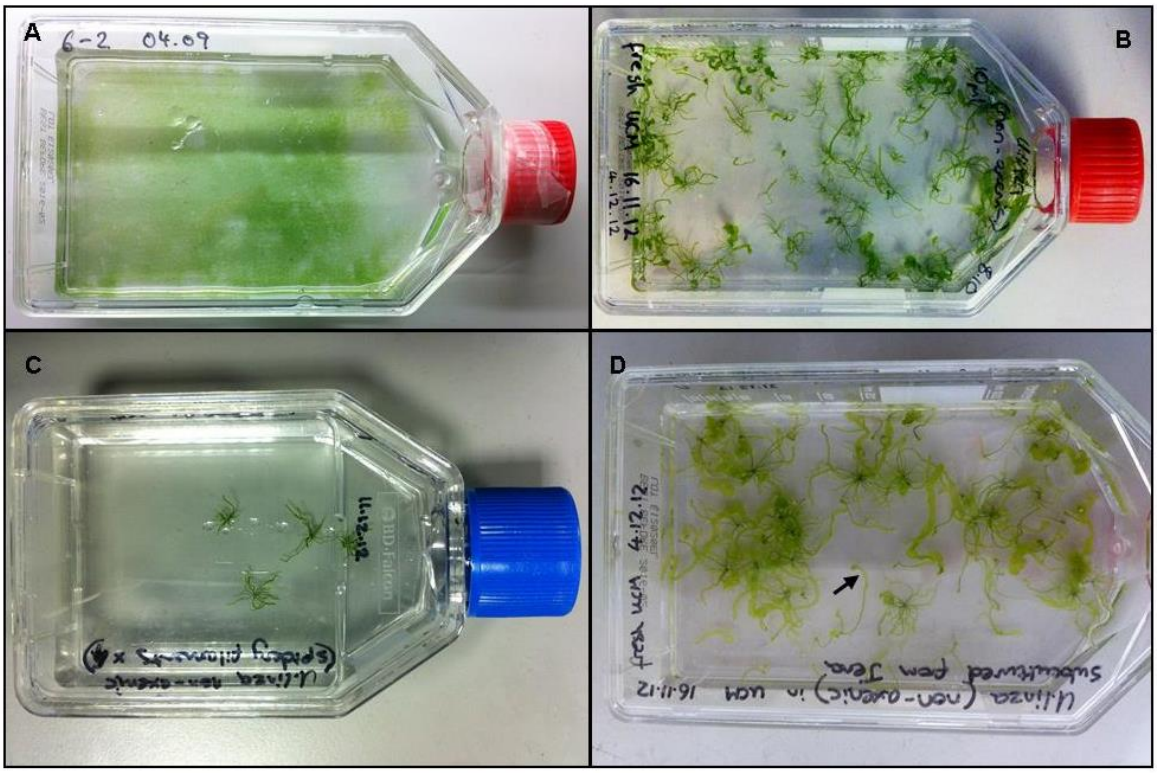


Figure 5.7 Culture of *U. intestinalis* in vented culture flasks

Flasks were seeded with gametes or spores while still motile. Within five to seven days a carpet of developing germlings was visible on the bottom of the flask (A). Sterile cell scrapers were used to remove a small number of developing germlings and transfer them to a fresh culture vessel at a lower density for further growth (B). When individual blades were large enough to be handled with forceps, they could be sub-cultured even further to facilitate larger growth (C). In some cases this would trigger premature induction of gametogenesis/sporogenesis and blades would change colour as tissue differentiated and eventually released gametes/spores (D). Note flasks are labelled *U. linza* as cultures were prepared before sequencing established that experimental species was *U. intestinalis.*

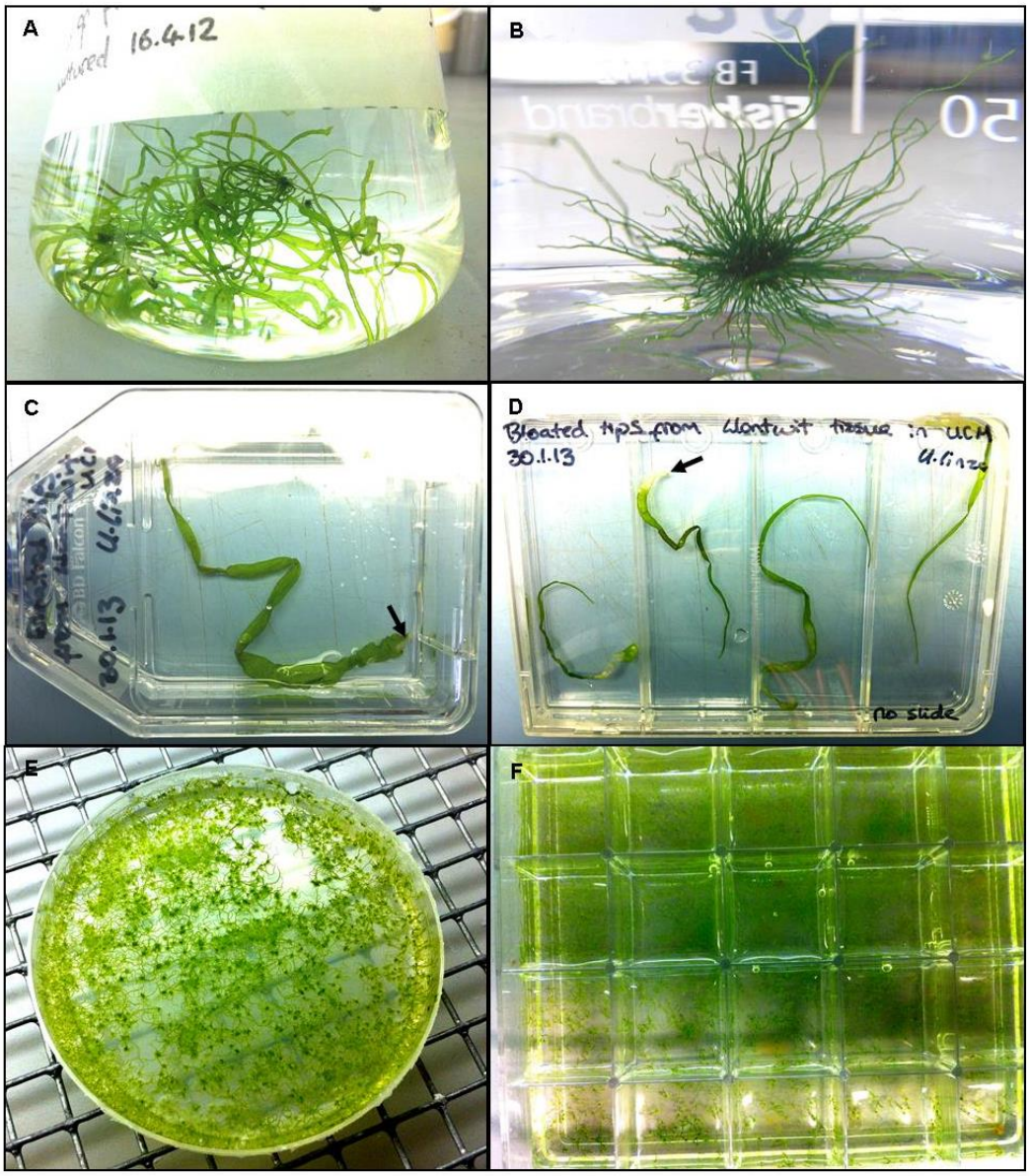


Figure 5.8 Culture techniques for *U. intestinalis*

Germlings from settled gametes were scraped from vented flasks and placed into conical flasks on an orbital shaker. These cultures typically formed large balls of filaments from multiple individuals (A and B). Naturally induced sporophyte blades collected from Llantwit Major were placed in culture vessels (C), multiwell plates with slides (D) and submerged agar plates (E) (arrow indicates differentiated tip). Once spores had been released from the white tips the tissue was removed, leaving a flask, slide or plate with settled spores that would then germinate and develop (E). Multiwell plates were used for preliminary observations of germination at different spore densities (F). Note flasks are labelled *U. linza* as cultures were prepared before sequencing established that experimental species was *U. intestinalis.*

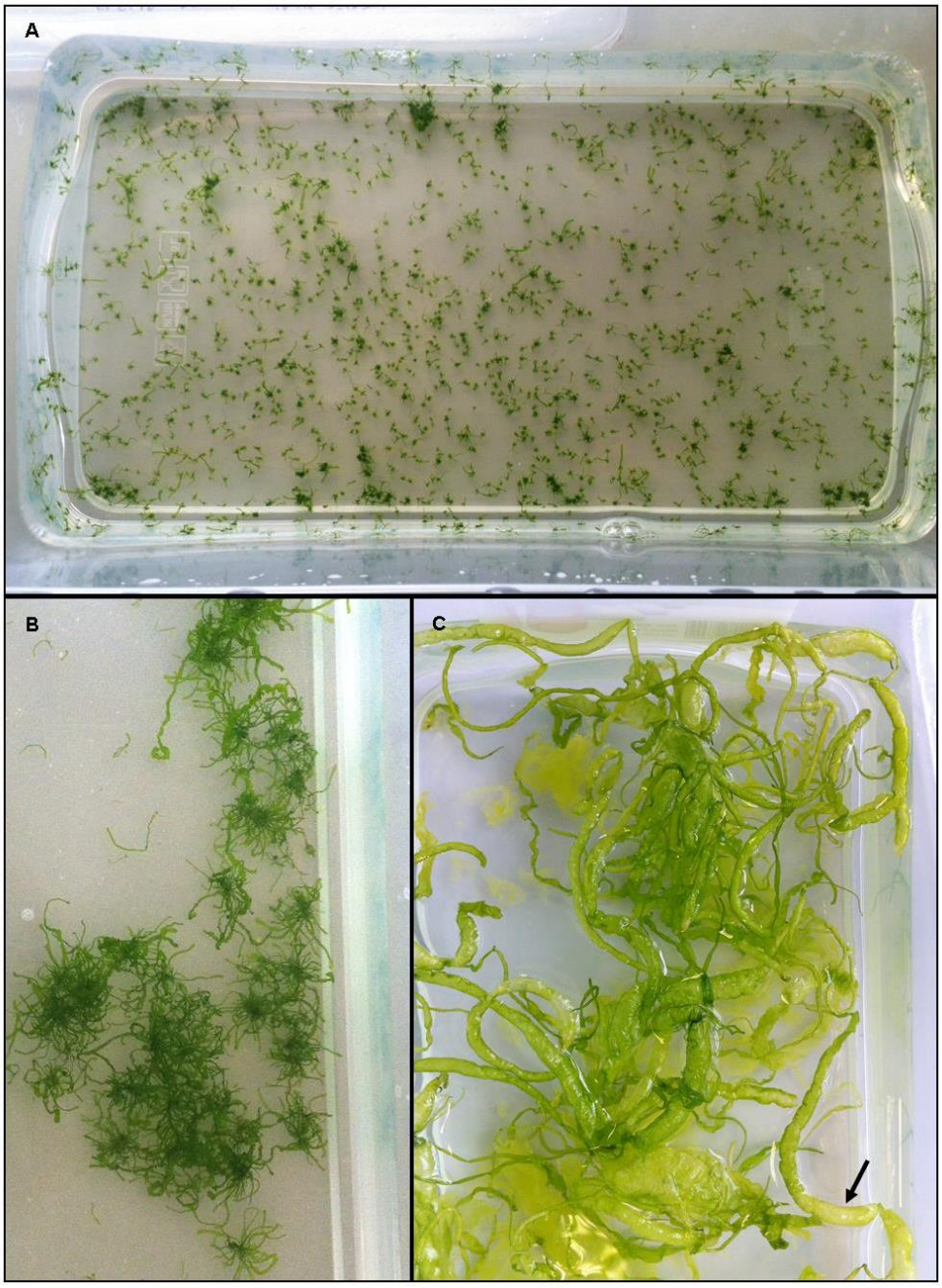


Figure 5.9 Additional culture techniques for *U. intestinalis*

Cultures were also established in large polypropylene boxes for bulking of tissue and long term storage at 4°C. Spores or gametes were seeded directly into boxes where they developed into small blades (A). These could then be sub-cultured as required (B) but would sometimes undergo premature induction of gametogenesis/sporogenesis and blades would change colour as tissue differentiated and eventually released gametes/spores (C) (arrow indicates differentiated tip)

5.7.2 Nucleic acid isolation

Whilst the focus for *U. mutabilis* culture was for analysis of biochemical processes, the protocols used for routine nucleic acid extraction during my research on *P. patens* provided the opportunity to establish molecular biology techniques in the *U. intestinalis* system.

Green multicellular algae present significant challenges when it comes to nucleic acid extraction. *Ulva* spp. have a high polysaccharide and glycoprotein content in cell walls as well as multiple secondary metabolites such as polyphenols (Percival, 1979). Incomplete removal of cell debris during nucleic acid isolation will be apparent when analysing quality (i.e. 260/280 and 260/230) as many can co-purify with nucleic acid and interfere with downstream enzymatic applications. This was tackled by carrying out multiple extraction techniques (see appendix) and comparing total yield as well as purity.

Hexadecyltrimethylammonium bromide (CTAB) selectively precipitates nucleic acids allowing separation from contaminating polysaccharides and some polyphenols. Utilisation of a high salt concentration solubilises the CTAB-nucleic acid complex whilst precipitating polysaccharides. This was used in combination with chloroform:isoamyl alcohol extraction and LiCl for precipitation of RNA. Experimentation with LiCl concentration and overnight incubation temperature enabled a final yield of ≈80ng/µl. A LiCl concentration of 2M and incubation temperature of -20°C produced the highest RNA yield (~80ng/µl) but consistently low ratios on both 260/280 absorbance (≈1.7) and

particularly 260/230 (≈0.8) indicated the presence of proteins, phenols and other contaminants. For this reason, other methods were investigated.

Guanidinium thiocyanate-phenol-chloroform (TRIzol) extraction solubilises cell contents and denatures proteins. Addition of chloroform separates RNA from DNA and proteins allowing removal and subsequent precipitation of cellular RNA. RNA yield was better (≈100ng/µl) when compared to the CTAB method and the protocol was shorter and easier to carry out with multiple samples. However an average 260/230 ratio of 0.31 suggested significant levels of contaminants remained in the sample.

Both methods involved the use of extremely hazardous reagents such as β-mercaptoethanol, phenol and guanidium thiocyanate. They were also quite long protocols with lengthy incubation steps and very precise timings. The success with Bioline Isolate II plant RNA kits during extraction of *P. patens* RNA for semi-quantitative RT-PCR justified trying them with *U. intestinalis* despite previous reservations about their ability to efficiently remove the large polyphenols and other macromolecules that are found abundantly in macroalgae. The extraction is based on the preferential binding of nucleic acid to a silica membrane allowing the removal of contaminants by simple washing steps. DNA is removed by on-column digestion and total RNA is then eluted.

Extraction from *U. intestinalis* thallus and gametes using the Bioline Isolate II plant RNA kit (Bioline) yielded between 300 and 800 ng of RNA per microlitre. This was much higher than any extractions using either CTAB or TRIzol methods. The purity was also much better with an average 260/280 and 260/230 of 2.2. An absorbance ratio of

between 2.0 and 2.2 is considered pure when analysing RNA. Multiple repeats of this extraction protocol yielded consistent results that would easily enable downstream applications as required. The only modification that was made was to warm the RNase-free water to be used in the elution step to 37°C. This was found to consistently yield higher RNA concentrations.

5.8 Comparisons with *U. mutabilis*

Vesty *et al.* showed that *U. intestinalis* controls gametogenesis and sporogenesis in much the same way as *U. mutabilis (*Vesty *et al.*, 2015). The presence of inhibitors and the cross species capabilities of both consolidates the importance of this process in *Ulva* species and provides a point of comparison with other green algae.

5.8.1 Bacterial control of morphology

A feature of *Ulva* spp. is the requirement for epiphytic bacteria to ensure correct morphology (Provasoli, 1958; Nakanishi *et al.*, 1996; Joint *et al.*, 2002; Marshall *et al.*, 2006; Joint *et al.*, 2007; Spoerner *et al.*, 2012). Under axenic conditions many species display aberrant growth and development, with incomplete cell division resulting in an undifferentiated ‘pin cushion’ appearance. This has been well characterised in *U. mutabilis* with the identification of two essential bacterial species able to rescue morphology (Spoerner *et al.*, 2012). Morphogenetic compounds produced by *Cytophaga* and *Rosebacter* sp. restore the appearance of both blades and rhizoids in a manner similar to cytokinin and auxin-induced morphogenesis (Grueneberg *et al.*, 2016).

The establishment of axenic *U. intestinalis* cultures allowed cross-species comparisons of bacterial interactions with green macroalgae. The two bacterial species identified by Wichard were not able to fully rescue the morphology of *U. intestinalis* (Vesty *et al.*, 2015). Blade development was fairly typical but rhizoid growth showed consistent morphological defects despite the presence of bacteria in close proximity. This suggests a species-specific interaction between different *Ulva* species and the microbiome. The isolation of 38 unique bacterial species from *U. intestinalis (*Marshall *et al.*, 2006) provides a starting point for identification of these specific interactions. My development of culturing methods for *U. intestinalis* has allowed subsequent investigation of these bacterial species by colleagues. By identifying the species enabling correct morphology in *U. intestinalis* it may be possible to isolate and characterise the molecule responsible and its mode of action. It may be that the bacterial species is specific but the type of molecule it is producing is not.

5.9 Observations on germination

Earlier chapters in this thesis have focussed on the key developmental process of spore germination. The expansion of this theme into the aquatic ancestors of land plants is now possible through the establishment of reproducible culture methods for *U. intestinalis*. Whilst the initial cell division of the meiotically-derived single cells show some similarities, there are many important differences in the strategies of and influences on the spores of *P. patens* and *U. intestinalis*.

The very first division of an *U. intestinalis* spore establishes the two main tissue types, thallus and rhizoid (figure 5.6). This contrasts with the prolonged period of protonemal extension in *P. patens* that precedes establishment of rhizoid cells. These progressions are quite different, but how they relate to the process of that initial decision to divide is unknown.

Ulva spores do not have a comparable spore coat like that of *P. patens* spores or the functionally-equivalent angiosperm seed coat. The perception of external signals through this coat controls many germination-specific processes so an absence may represent a different strategy for sensing and responding to the environment by *Ulva* sp. As motile cells, zoospores have the ability to identify and respond to environmental signals well in advance of actually committing to germination. The decision to germinate is preceded by a decision to settle. It is also worth noting that the length of time between spore release and initial cell division can be as little as an hour. The presence of a barrier, in the form of a spore coat, would surely be a hindrance in this rapid progression of the life cycle.

The impact of hormone signals on germination does provide a point of comparison despite the developmental differences. Molecules such as nitric oxide and homoserine lactones have been shown to affect seed and spore germination (Bethke *et al.*, 2006; Arc *et al.*, 2013; Vesty *et al.*, 2016) and also influence settlement and germination of *Ulva* spores (Egan *et al.*, 2001; Wheeler *et al.*, 2006; Twigg *et al.*, 2014). The establishment of *U. intestinalis* as a tractable model system provides avenues of further

investigation into the effects of signalling molecules on developmental processes such as germination.

5.10 Implications of results and future work

The establishment of *U. mutabilis* as the first green multicellular algal model system will facilitate significant advances in the characterisation of this phylogenetically important plant group. The evolutionary time point of *Ulva* species and their ecological and economical influences reinforce the need for detailed knowledge of a model system. As with all research of this type, the ability to compare *U. mutabilis* with other *Ulva* species is essential for drawing comparisons and providing robust evidence of characteristics and experimental divergence from the norm. Previous attempts to establish axenic laboratory cultures of other species have proved difficult due to the involvement of specific bacterial compounds and unidentified developmental inhibitors.

The successful establishment of axenic laboratory cultures of *U. intestinalis* and subsequent development of molecular techniques evidenced in this thesis provides a significant step in the establishment of comparative model systems within this algal group. The recent progression of biological and phylogenetic characterisation of *U. intestinalis* undertaken by other members of the Coates research group is dependent on the knowledge and methodology developed as part of this research. *U. intestinalis* is a more widespread species than *U. mutabilis* and consequently experiences a vast range of environmental influences. An understanding of a species such as this can provide

clues as to the factors that enable similarly widespread species such as *U. prolifera* to inflict such environmental damage.

From a thematic point of view, the establishment of a reliable culture method enabling production of spores and gametes in a laboratory environment will allow future analysis of the features of germination in an aquatic multicellular green plant system. The expansion of knowledge of the ancestral origins of hormonal control of germination will benefit greatly from the ability to investigate a comparable aquatic system. Whilst the terrestrial functions of hormones such as ABA and GA are being investigated in detail in species such as *A. thaliana* and *P. patens*, comparable investigation in green multicellular aquatic groups is hindered by the lack of suitable model systems. The recent sequencing of the *U. mutabilis* 'slender' genome and the methodological advances demonstrated in this thesis will greatly aid this area of research.

CHAPTER VI:

GENERAL DISCUSSION

6.1 Introduction

Well characterized model systems are essential for the understanding of evolutionary developmental biology (Flavell, 2009; Coates, 2016). The ability to compare and contrast species from key time points in evolution allows us to identify when particular traits arose, the relative importance of such traits and how they may be exploited in areas such as agriculture and biotechnology. One of the key stages in the evolution of green plants was colonization of the terrestrial environment (Graham *et al.*, 1991; Graham, 1993; Graham, 1996; Bateman *et al.*, 1998; McCourt *et al.*, 2004; Delwiche and Cooper, 2015). The ability to source water and nutrients, survive vast fluctuations in temperature and disperse the next generation were all tackled in a variety of ways that led to the progressive increase in size and complexity of land plants. The plethora of forms that exist today is due to a relatively recent extensive radiation of the angiosperms (Graham *et al.*, 2000a). However in the 300 million years prior to their appearance the other major plant groups tackled these land-specific problems and worked out the multitude of kinks that enabled the evolution of such a perfectly adapted and successful division.

As the first truly terrestrial plant group, the bryophytes and their modern descendants contain a wealth of information on the physiological and genetic adaptations that were necessary for colonisation of the land (Cove, 2005; Cove *et al.*, 2006; Quatrano *et al.*, 2007; Bennici, 2008; Rensing *et al.*, 2008b). The expansion and modification of these traits was the basis for the evolution of subsequent plant groups and as such an

understanding of their roles in bryophytes provides insight into their potential roles in more economically exploited species such as grasses (wheat, maize etc.).

The model moss *P. patens* is a bryophyte that has already provided a vast amount of information on the adaptations required for a terrestrial existence and hinted at the ways in which these may have been modified in the evolution of a vascular system.

The parallel evolution of green multicellular plants in aquatic systems provided an opportunity for comparison of the strategies for becoming multicellular – another key innovation that led to the greening of the planet. The characterisation of the multicellular green algal species *Ulva mutabilis* has provided a model system that, in conjunction with unicellular models, allows evolutionary biologists to work backwards from terrestrialisation and identify the ancestral origins of important traits.

Increasing the understanding of developmental processes across a range of plant model systems can identify similarities and differences between divergent groups and tell us a lot about how plants arrived at the pinnacle of evolutionary adaptation seen in modern angiosperms.

6.2 The development of *Physcomitrella patens* as a model for spore germination

As a model bryophyte system, *P. patens* has provided significant insight into traits such as desiccation tolerance and the role of hormones in developmental processes. The production of spores for the dispersal of the next generation is a characteristic of many plant groups including bryophytes. Their role as a desiccation-resistant dispersal

structures enables functional comparison with seeds, allowing investigation of a process that has undergone 450 million years of evolution – land plant germination.

Germination of spores and seeds is one of the most essential stages in a plant's life cycle. The appearance and growth of the next generation ensures survival of the species and maintains genetic diversity. The single-celled nature of *P. patens* spores makes it particularly amenable to studies of this process; it was therefore a surprise to discover the lack of a robust, reproducible assay for the analysis of this process in the *P. patens* literature. The development of a spore germination assay as part of my research was essential for the subsequent experimental treatments that were the initial focus of my thesis. This provided a robust method for use in my own studies, but may also enable more comprehensive and relatable analysis of the influences on spore germination across multiple research areas. A consistent assay that can be used to produce comparable data across multiple disciplines could greatly aid the study of such an essential process.

6.3 The role of phytohormones in *P. patens* spore germination

This thesis has demonstrated the role of signalling molecules such as diterpenoids and abscisic acid (ABA) in the germination of *P. patens* spores. The control of germination observed mostly concurs with what is known in seeds. However the extent of control does appear to vary in the case of some hormones such as diterpenoids. The inability of seeds of a diterpenoid mutant to germinate is not apparent in the corresponding *P. patens* mutant. This highlights the subtle variations that have arisen over 450 million

years of evolution that are now becoming evident through the detailed analysis of spore germination.

What is clear is that moss spore germination is regulated by similar hormones to those with roles in seed germination. This has begun to fill in some of the gaps in our knowledge of signalling pathways of hormones such as gibberellins and provided targets for future analysis. There are a multitude of phytohormones and as well as their individual roles it is also necessary to investigate their interactions and relative impacts on each other. The task of developing of a novel germination assay has meant that the scope of this research was limited to investigating hormones mainly in isolation. The importance of antagonistic relationships between hormones such as GAs and ABA for example provides a natural progression for this type of research. This investigation has also begun to explore the molecular basis for hormone biosynthesis and signalling

The expression data presented has identified genes of interest whose roles could be further investigated by generation and characterisation of mutant lines. The ability to easily transform *P. patens* cells makes this a very realistic opportunity to further clarify this complex network of control. This too provides scope for further research into the relationships between expression of hormone signalling genes and their spatial and temporal regulation. The roles of plant hormones in angiosperms, particularly in crop species, is a key area of investigation and the expansion of this understanding into early land plants is an obvious bonus.

6.4 Extension of green plant model systems

The expanding investigation of *P. patens* strengthens its role as a model system allowing it to tell us more about the evolutionary developmental biology of land plants. The availability of an aquatic multicellular green plant in the form of *Ulva intestinalis* also enabled considerations of more ancient traits and their relative roles in the evolution of plants. The development of a green macroalgal model system is at an early stage. The emergence of *U. mutabilis* 'slender' as a type species model will greatly advance this field and its application to general evolutionary biology questions. My successful culture and characterisation of the more widespread *U. intestinalis* strengthens the case for *Ulva* spp. as the model system of choice for green macroalgae. The availability of model systems increases the detail with which we can illustrate the evolutionary past and further illuminates our modern plethora of plant groups, hinting at the potential future traits and variations we may discover.

6.5 Final remarks

The dependence of life on plants and the ecosystems they create is an often underappreciated feature of our planet. Our total reliance on them for things like food, fuel and breathable air is magnified when we consider the fact that the evolution of animal life was only possible due to the preceding evolution and terrestrialisation of plant life. The movement of plants onto land boosted atmospheric oxygen, created a multitude of habitats and permanently altered the biogeochemical state of the planet.

The evolutionary position of models such as *P. patens* and *U. intestinalis* provides an insight into how plants did it. How they coped, adapted and ultimately flourished.

This thesis has filled some of the gaps in our knowledge of the key developmental process of germination in an early-diverging plant model system. The role of phytohormones in control of *P. patens* spore germination shows significant similarity to the roles in seed germination. But it is the differences that proved the most thought-provoking outcome of my studies. The differing requirement for diterpenes in *P. patens* and *A. thaliana* suggests that there are fundamental differences in the way these evolutionary divergent species synthesise gibberellins and gibberellin-type molecules. In contrast, the discovery that *P. patens* actively transcribes components of the GA perception network (*GID1* and *DELLA*) hints at a similarity between bryophytes and angiosperms that was previously thought to be non-existent.

Investigation of germination in evolutionarily distinct systems has provided insight into the evolution of this essential process. It has also advanced our understanding of *P. patens* as a model system and established a robust germination assay for future investigations.

The development of methodology for culture of the green macroalga *U. intestinalis* facilitates questions on the aquatic origins of the traits we have become so accustomed to. The evolution of multicellularity, desiccation tolerance, heteromorphic alternation of generations and ultimately terrestrialisation has resulted in the abundance of plant forms

we see today. The more model systems available to study these process the clearer the image becomes.

Our ability to understand and manipulate plants has been the driving force behind many of the technological innovations that support the human population. Whilst the outcome of this research is not going to resolve the food crisis, impact on global warming or provide a novel source of antibiotics, its contribution to the understanding of the fundamental processes occurring in all plant species is something to be proud of.

CHAPTER VII:

REFERENCES

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CHAPTER VIII:

APPENDIX

8.1 CTAB-based extraction of RNA from *Ulva intestinalis*

Day 1

Preparation:

- Preheat extraction buffer to 65°C
- Collect liquid nitrogen from stores
- Select tissue for samples

Reagents:

- Extraction buffer (see recipe)
- β-mercaptoethanol
- chloroform:isoamyl alcohol (24:1)
- 10M lithium chloride (LiCl) solution

Method:

1. Add 100µl of β-mercaptoethanol to 5ml of extraction buffer and heat to 65ºC
2. Half fill a mortar with liquid nitrogen and allow to bubble off
3. Fill mortar again and place metal rack on top (see picture)
4. Place open Eppendorf tubes into rack to chill
5. Cut up tissue and place small amount in each tube
6. Allow tissue to freeze completely then grind to a fine powder using pellet pestle
7. Add 500µl of warm buffer to each tube and vortex to ensure no frozen lumps and full dispersion
8. Leave each tube at room temperature as you do the next samples
9. Vortex all tubes again until the foam at the top
10. Add an equal volume (500µl) of chloroform:isoamyl alcohol and vortex

11. Centrifuge at 11,984g for 10 minutes to separate phases

12. Carefully pipette off the aqueous phase into a fresh RNase-free tube. **Caution:** it is better to leave a small amount of the aqueous phase behind rather than transfer some of the contaminating lower phase

13. Add an equal volume of chloroform:isoamyl alcohol, vortex, centrifuge and pipette off the aqueous phase into a fresh RNase-free tube (steps 10 - 12)

14. Repeat step 13 if organic layer still visible

15. Estimate volume and add LiCl solution to give final concentration of 2M

16. Leave at -20ºC overnight

Day 2

Preparation:

- Preheat SSTE buffer to 65°C
- Place centrifuge in cold room (4°C) to chill for step 23

Reagents:

- SSTE buffer (see recipe)
- chloroform:isoamyl alcohol (24:1)
- Absolute ethanol
- RNase-free water

Method:

1. Centrifuge at 4°C and 11,984g for 20 min

2. Pour off supernatant and invert tubes onto tissue to drain

3. Dissolve pellet in 200µl of preheated SSTE buffer (65°C)

4. Transfer to a fresh 1.5ml RNase -free tube (**NOTE:** if the SDS in the SSTE buffer precipitates to form a white cloudiness, place the Eppendorf in a heating block (37°C) until it dissolves before continuing

5. Vortex then add an equal volume (200µl) of chloroform:isoamyl alcohol to Eppendorf and vortex again immediately to mix

6. Add 2 volumes (800µl) of absolute (100%) ethanol to precipitate RNA

7. Leave for two hours at -20°C or 30 minutes at -70°C

8. Centrifuge at 4°C and maximum speed for 20 minutes

9. Discard supernatant and air-dry pellet

10. Re-suspend pellet in 20µl RNase-free water by gently sucking the liquid up and down with a pipette

11. Quantify and check the purity of the RNA using a nanodrop

17. Store at -20°C if using sample soon or -80°C for long-term storage

Extraction buffer:

- 2% hexadecyl trimethyl-ammonium bromide (CTAB) (2g in 100ml)
- 2% polyvinylpyrrolidone K30 (2g in 100ml)
- 100mM Tris-HCL, pH 8.0
- 25mM EDTA, sodium form, pH 8.0
- 2M NaCl
- 0.5g/L spermidine
- (2% β-mercaptoethanol - to be added during protocol as step 1)

SSTE buffer (sodium dodecyl sulphate-Tris-HCL-EDTA): (heat to 65°C to melt SDS)

- 1M NaCl
- 0.5% sodium dodecyl sulphate (SDS) (0.5g in 100ml)
- 10mM Tris-HCL, pH 8.0
- 1mM EDTA, sodium form, pH 8.0

8.2 TRIzol-based extraction of RNA from *Ulva linza*

Preparation:

- Collect liquid nitrogen from stores
- Select tissue for samples
- Put centrifuge in cold room (4°C) - ALL CENTRIFUGATION STEPS TO BE CARRIED OUT AT 4°C

Reagents:

- Chloroform
- Isopropanol
- 75% ethanol (prepared with RNase-free water)
- 1M potassium acetate (9.8g in 100ml)
- TRIzol reagent
- RNase-free water

Method:

1. Half fill a mortar with liquid nitrogen and allow to bubble off
2. Fill mortar again and place metal rack on top (see picture)
3. Place open RNase-free Eppendorf tubes into rack to chill
4. Cut up tissue and place small amount in each tube
5. Allow tissue to completely freeze then grind to a fine powder using pellet pestle
6. Add 1ml of TRIzol reagent to each chilled tube and continue homogenization (add more liquid nitrogen as required)
7. Leave to thaw then aliquot into four RNase-free tubes (250µl each)
8. Add 50µl of potassium acetate to each sample to give a final concentration of 0.2M
9. Mix and incubate at 20°C for 5 minutes
10. Add 200µl of chloroform and shake for 15 seconds
11. Incubate at 20°C for 10 minutes

12. Centrifuge at 12,000g for 15 minutes to separate phases

13. Carefully pipette off the aqueous phase into a fresh RNase-free tube. **Caution:** it is better to leave a small amount of the aqueous phase behind rather than transfer some of the contaminating lower phase

14. Add 500µl of isopropanol, mix and incubate at -20°C (freezer) for one hour

15. Centrifuge at 12,000g for ten minutes

16. Remove supernatant and wash pellet with 75% ethanol

17. Gently resuspend pellet in solution

18. Centrifuge at 7,500g for five minutes

19. Repeat ethanol wash steps

20. Dry pellet at 50°C for 5-10 minutes (**NOTE:** drying should be terminated when pellet begins to become transparent. Contaminated RNA remains white)

21. Add RNase-free water and incubate at 55-60°C for ten minutes

22. Resuspend pellet completely by pipetting

23. Discard supernatant and air-dry pellet

24. Quantify and check the purity of the RNA using a nanodrop

18. Store at -20°C if using sample soon or -80°C for long-term storage

8.3 Bioline ISOLATE II plant RNA kit

ISOLATE II RNA Plant Kit BENCH-TOP PROTOCOL

PURIFYING TOTAL RNA FROM PLANT TISSUE OR FILAMENTOUS FUNGI

1 **Sample homogenization**
See manual for recommended sample amounts and homogenization methods.

2 **Cell lysis**
Add 350µl Lysis Buffer RLY and 3.5µl ß-ME to a maximum of 100mg ground tissue and vortex vigorously.
If lysate solidifies when adding Lysis Buffer RLY, use 350µl Lysis Buffer RLS instead.

3 **Filter lysate**
Place ISOLATE II Filter (violet) in a 2ml Collection Tube (supplied).
Load lysate and centrifuge 1 min at 11,000 x g.
Transfer filtrate to a new 1.5ml microcentrifuge tube (not supplied).
If visible pellet forms, transfer supernatant avoiding any pellet to a new 1.5ml microcentrifuge tube (not supplied).

4 **Adjust RNA binding conditions**
Discard ISOLATE II Filter and add 350µl ethanol (70%) to homogenized lysate.
Mix by pipetting up and down 5 times.
Alternatively, transfer flow-through into a new 1.5ml microcentrifuge tube (not supplied), add 350µl ethanol (70%) and mix by vortexing (2 x 5s).

5 **Bind RNA**
Place ISOLATE II RNA Plant Column (blue) in a 2ml Collection Tube.
Load lysate onto column and centrifuge 30s at 11,000 x g.
Place column in a new 2ml Collection Tube.

6 **Desalt silica membrane**
Add 350µl Membrane Desalting Buffer (MEM).
Centrifuge at 11,000 x g for 1 min to dry membrane.

7 **Digest DNA**
Add 10µl reconstituted DNase I to 90µl Reaction Buffer for DNase I (RDN).
Mix by gently flicking tube.
Apply 95µl DNase I reaction mixture directly onto center of silica membrane.
Incubate at room temperature for 15 min.

BTP081 2V1

Please consult the ISOLATE II RNA Plant Kit Product Manual before using this protocol for the first time. For technical support please email tech@bioline.com or visit www.bioline.com/isolate.

BIOLINE

ISOLATE II RNA Plant Kit

8 **Wash and dry silica membrane**

1st Wash

- Add 200µl Wash Buffer RW1.
  Centrifuge 30s at 11,000 x g.
  Place column into a new 2ml Collection Tube.

2nd Wash

- Add 600µl Wash Buffer RW2.
  Centrifuge 30s at 11,000 x g.
  Discard flow-through and place column back into Collection Tube.

3rd Wash

- Add 250µl Wash Buffer RW2.
  Centrifuge 2 min at 11,000 x g to dry membrane completely.
  Place column into a nuclease-free 1.5ml Collection Tube (supplied).

9 **Elute RNA**

Add 60µl RNase-free water (supplied) directly onto center of silica membrane.
Centrifuge at 11,000 x g for 1 min.

8.4 First author publications based on thesis

frontiers in
PLANT SCIENCE

ORIGINAL RESEARCH ARTICLE
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Regulation of gametogenesis and zoosporogenesis in *Ulva linza* (Chlorophyta): comparison with *Ulva mutabilis* and potential for laboratory culture

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Green Ulvophyte macroalgae represent attractive model systems for understanding growth, development, and evolution. They are untapped resources for food, fuel, and high-value compounds, but can also form nuisance blooms. To fully analyze green seaweed morphogenesis, controlled laboratory-based culture of these organisms is required. To date, only a single Ulvophyte species, *Ulva mutabilis* Føyn, has been manipulated to complete its whole life cycle in laboratory culture and to grow continuously under axenic conditions. Such cultures are essential to address multiple key questions in *Ulva* development and in algal–bacterial interactions. Here we show that another *Ulva* species, *U. linza*, with a broad geographical distribution, has the potential to be grown in axenic culture similarly to *U. mutabilis*. *U. linza* can be reliably induced to sporulate (form gametes and zoospores) in the laboratory, by cutting the relevant thallus tissue into small pieces and removing extracellular inhibitors (sporulation and swarming inhibitors). The germ cells work as an ideal feed stock for standardized algae cultures. The requirement of *U. linza* for bacterial signals to induce its normal morphology (particularly of the rhizoids) appears to have a species-specific component. The axenic cultures of these two species pave the way for future comparative studies of algal–microbial interactions.

Keywords: green algae, gametogenesis, zoosporogenesis, morphogenesis, life cycle, algal–bacterial interactions, axenic culture, sporulation inhibitor

INTRODUCTION

The growth and development of land plants has been extensively studied and representative model systems have been developed for molecular genetic studies in several major clades, for example, *Arabidopsis* for dicots, *Oryza/Brachypodium* for monocots, *Selaginella* for lycophytes, and *Physcomitrella* for early evolving Bryophytes (The *Arabidopsis* Genome Initiative, 2000; Goff et al., 2002; Rensing et al., 2008; Banks et al., 2011; Girin et al., 2014). This has enabled translation of the understanding of basic biological principles of plant development and evolution from models to crops (Irish and Benfey, 2004; Rensink and Buell, 2004; Coudert et al., 2010; Spannagl et al., 2011; Orman-Ligeza et al., 2014), thus improving the potential of crop plants for food and biofuel, to meet the challenges of population- and climate change. Green macroalgae (seaweeds) represent a new group of organisms with great potential for tackling the challenges of food- and fuel-security (Dibenedetto, 2012), which also cause significant environmental problems in the form of green tides and biofouling (Callow and Callow, 2006a,b; Smetacek and Zingone, 2013). However, unlike land plants, green seaweeds are under-exploited as model organisms, thus the understanding of their mechanisms of growth and development is currently severely limited.

The reason for this under-exploitation is partly due to the extreme challenges faced when growing green seaweeds under sterile laboratory conditions. It has been demonstrated for several species of green algae that the epiphytic bacterial populations with which they naturally associate are absolutely required for correct development and subsequent morphogenesis (Matsuo et al., 2003; Marshall et al., 2006; Spoerner et al., 2012). Thus, the axenic cultures that are normally required for molecular genetic/functional genomic studies in a model organism, such as transformation systems and genome/transcriptome sequencing are not straightforward to develop for green seaweeds. The greatest progress has been made with one species of Chlorophyte macroalga, *Ulva mutabilis*, [which is native to Southern Cost of Portugal and originally collected by Føyn (1958)], with (i) an established laboratory culture protocol (Stratmann et al., 1996; Wichard and Oertel, 2010), (ii) a collection of developmental mutants (Løvlie, 1968), (iii) definition of bacterial species and partially purified substances that are required for proper morphogenesis (Spoerner et al., 2012) and (iv) the isolation of factors that prevent the breakdown of leafy thallus tissue into unicellular spores/gametes (zoosporogenesis and gametogenesis, respectively, collectively "sporulation"; Nilsen and Nordby, 1975; Stratmann et al., 1996). This has paved the way for truly axenic culture of *U. mutabilis*, which will enable sequencing of this species (Spoerner et al., 2012). One general issue with seaweed culture is inducing transition between generations *via* unicellular cell types (gametes or zoospores). *U. mutabilis* produces substances that inhibit the induction of gamete- and spore-formation, 'sporulation inhibitors' (SI; the glycoprotein SI-1 and the low molecular weight SI-2; Stratmann et al., 1996). A third substance, the 'swarming inhibitor' (SWI) prevents gamete

release after induction has occurred (Wichard and Oertel, 2010). Gamete induction and release can be induced in vegetative *U. mutabilis* thallus by washing and tissue fragmentation, and similarly (albeit more slowly) in *U. lactuca* (Wichard and Oertel, 2010). A similar method may also work to induce spores in *U. prolifera*, as tissue fragmentation into disks leads to spore formation (Gao et al., 2010).

Ulva is an economically important genus, and therefore merits a deeper understanding of its growth and developmental mechanisms at the molecular level (Wichard et al., under review). *Ulva* is a food source (Nisizawa et al., 1987; Tabarsa et al., 2012) and a potential source of biomass for fuel production (Bruhn et al., 2011). However, *Ulva* also forms nuisance algal blooms (Blomster et al., 2002; Nelson et al., 2003; Hiraoka et al., 2004; Leliaert et al., 2009; Smetacek and Zingone, 2013) and is a major biofouler (Callow and Callow, 2006a). Although certain worldwide abundant species such as the sea lettuce *U. rigida* (e.g., RFU_77) can be cultured under standardized conditions (Alsufyani et al., 2014), sporulation could not easily be synchronously induced as in tubular *Enteromorpha*-like morphotypes of the genus *Ulva* (Nilsen and Nordby, 1975; Stratmann et al., 1996).

In this paper, we sought to discover whether *Ulva* species other than *U. mutabilis* could be cultured axenically in the laboratory, and whether the signals regulating *Ulva* sporulation, morphogenesis, and development are conserved between species. We chose *U. linza*, a cosmopolitan intertidal alga found, e.g., along the coastlines of the UK and in the Yellow Sea (China; Brodie et al., 2007; Xu et al., 2013), which is a well-established model for biofouling research (Callow and Callow, 2006b) and has a partly characterized microbiome (Marshall et al., 2006). We showed that *U. linza* has the potential for standardized laboratory culture. We also highlight potential species-specific requirements for the bacterial signals required for correct morphogenesis.

MATERIALS AND METHODS

SAMPLING AND CULTIVATION OF *Ulva*

Algal strains

Haploid gametophytes from the fast-growing developmental mutant "slender" (sl) of *U. mutabilis* Føyn (mating type mt+) were used for all comparative studies with *U. linza* (Føyn, 1958; Løvlie, 1964; Fries, 1975). Vegetative and fertile sporophytic *U. linza* plants were collected in March 2013, from Llantwit Major, South Wales (51°40′N; 3°48′W). Gametogenesis and sporogenesis was induced by chopping the harvested tissue using a Zyliss® Smart Clean Food Chopper.

Bacterial strains

Roseobacter sp. MS2 (Genbank EU359909) and *Cytophaga* sp. MS6 (Genbank EU359911) were cultivated in marine broth medium at 20°C on an orbital shaker. They were originally isolated from *U. mutabilis* (Spoerner et al., 2012) and stocks are stored in glycerol at −80°C.

Cultivation conditions

Gametophytes of *U. mutabilis* and *U. linza* were raised parthenogenetically from unmated gametes or from zooids derived from sporophytes under the standard conditions (Stratmann et al., 1996). Small germlings were grown attached to the bottom of sterile culture flasks with gas-permeable screw caps containing 100 mL *Ulva* culture medium (UCM) without antibiotics. The medium for *U. mutabilis* was routinely supplemented with the two bacterial symbionts of the algae, *Roseobacter* sp. MS2 and *Cytophaga* sp. MS6 to secure normal thallus morphogenesis. Until fertility the medium was completely exchanged weekly. Later, the medium was changed only partially (50%) to avoid premature induction of gametogenesis. The medium for experimental *U. linza* was either unsupplemented (axenic), supplemented with MS2 and MS6, or its natural bacterial flora.

Ulva mutabilis and experimental *U. linza* were cultivated in UCM in a 17:7 h light/dark regime at 20°C with an illumination of 60–120 μmol photons m^{-2} s^{-2} (50% GroLux, 50% day-light fluorescent tubes; OSRAM, München, Germany) and no additional aeration. Freshly collected *U. linza* thalli were washed and kept in UCM (Stratmann et al., 1996) in large tanks and boxes (>1 L), in a Sanyo MLR-351 growth cabinet with Osram Lumilux Cool White L36W/840 (36 watt, 4 ft) tubes at an illumination of 50 μmol $m^{-2}s^{-1}$.

BIOASSAYS OF EXTRACTED SPORULATION INHIBITORS

Chemicals

For the extraction of the SI (SI-1, SI-2), tris (hydroxymethyl) aminomethane (Tris) was purchased from VWR (Darmstadt, Germany), HCl (37%), and EDTA (≥99.9%, p.a., ACS) were obtained from Roth (Karlsruhe, Germany). Phenol was purchased from Alfa Aeser (Karlsruhe, Germany), ethanol (99.9%, LiChroSolv) from Merck KGaA (Darmstadt, Germany) and acetone from Fluka (Sigma–Aldrich, Taufkirchen, Germany). Instant Ocean was obtained from Aquarium Systems (Sarrebourg, France). All solutions were prepared with ultrapure water purified by a reverse osmosis system (TKA, Niederelbert, Germany).

Preparation of crude extracts for purification of the sporulation inhibitors (SI-1, SI-2)

The established extraction protocols of the SI-1 and SI-2 by Stratmann et al. (1996) were slightly modified and applied to both *U. mutabilis* and *U. linza*.

For the extraction of the SI-1 from the growth medium, 500 mL of medium from 3 to 4 week old axenic *U. mutabilis* cultures was stirred with 50 mL phenol (saturated with 100 mmol L^{-1} Tris-HCl, 1 mmol L^{-1} EDTA, pH 7.5) in a 1 L two-neck round-bottom flask for 20 min at 20°C. After centrifugation (3800 *g*, 10 min) the phenol phase was transferred into plastic tubes. The extraction was repeated once and the phenol phases were combined. After re-extracting with 100 mL 10 mmol L^{-1} Tris-HCl (pH 8.0), the phenol phase was mixed with three volumes of acetone and subsequently incubated for 30 min at −20°C. The precipitate was collected by centrifugation (3800 *g*, 20 min, 0°C) and washed three times with pre-cooled ethanol (−20°C). After drying in the vacuum, the precipitate was suspended in 100 mmol L^{-1} Tris-HCl (pH 8.0) and stored at −20°C.

For the extraction of the SI-1 from the thallus, 2 g of minced *Ulva* sp. thalli was washed with UCM and frozen with liquid nitrogen. After grinding the thalli with a pestle, the powder was thawed

and resuspended directly in 5 mL of 50 mmol L^{-1} Tris-HCl (pH 8.0). This was repeated once and subsequently thalli were mixed in 2 mL of 10 mmol L^{-1} Tris-HCl (pH 8.0) and 2 mL phenol (saturated with 100 mmol L^{-1} Tris-HCl, 1 mmol L^{-1} EDTA, pH 7.5) at 60°C for 30 min. The extraction was repeated once and the phenol phase was washed with 4 mL 10 mmol L^{-1} Tris-HCl (pH 8.0) and mixed with three volumes of acetone for ≥30 min at −20°C. After drying in a vacuum, the precipitate was suspended in 100 mmol L^{-1} Tris-HCl (pH 8.0) and stored at −20°C.

For the extraction of the SI-2 from the fluid in between the bilayered thallus, *Ulva* thalli were washed for 15 min with ultrapure water and blotted with paper. One gram of thalli was suspended in 4 mL 10 mmol L^{-1} Tris-HCl (pH 8.0) and cut into single-layered fragments with a chopper. After centrifugation (3800 *g*, 10 min), the buffer containing SI-2 was passed through cellulose acetate filters and stored at −20°C (Stratmann et al., 1996).

Bioassay-guided testing of sporulation inhibitors

Fertile *Ulva* sp. thalli were harvested before noon and intensively washed with half-concentrated Instant Ocean for 15 min. According to Stratmann et al. (1996) the induction efficiency (i.e., proportion of cells differentating into gametangia) increases dramatically if sporulation is induced during the G1-cell-cycle phase, which happens before noon in synchronized cultures of *U. mutabilis*. We assumed the same was true for *U. linza* and, indeed, this was the case.

After chopping the thalli, *Ulva* fragments were washed twice in a fine sieve. The fragments ($n = 70 \pm 30$) were transferred into 96 multiwell dishes (Nunc, Roskilde, Denmark) with 100 µl UCM for survey of gametogenesis. The concentration of the SIs was measured *via* dilution series of the partly purified compounds with UCM according to Stratmann et al. (1996). Due to the nature of the discrete dilution series, variance of measurement also depends on the interval of the dilution steps: a dilution series of six steps ranking from 150 units to 1 unit of the respective inhibitor was performed. One unit of the SI-1 and SI-2 is hereby defined as the concentration that inhibits completely the gametogenesis of a mature alga (i.e., fragmented thallus) completely in 1 mL of UCM for 3 days at 20°C upon induction. In parallel, samples with Tris-HCl (negative control) and with defined known amounts of SI (positive control) were tested. After 3 days of incubation the sporulation rates were determined under a Leica DMIL LED microscope equipped with a DFC 280 camera (Leica, Solms, Germany). The one-way Analysis of Variance (ANOVA) and the subsequent Tukey *post hoc* tests were performed by Minitab 16 Statistical Software (2010; State College, PA, USA: Minitab, Inc.).

PREPARATION OF AXENIC CULTURES

For the preparation of axenic cultures of *U. linza*, gametes were purified from accompanying bacteria based on the protocol developed for *U. mutabilis* (Spoerner et al., 2012): purification was performed by phototactic movement of freshly released gametes through a narrow horizontal capillary (see also review by Wichard et al., under review) toward a light source, under strictly sterile conditions in a laminar flow hood. Sterile Pasteur pipettes with 15 cm capillaries were prepared; gametes swim to the top of the pipettes, where they are collected and applied for next run of purification through a further Pasteur pipette (**Figure 3**). In general three runs are necessary to purify the gametes form the bacteria. The final preparations of axenic gametes were routinely tested for axenicity by plating aliquots on marine broth agar (Roth, Karlsruhe, Germany) and checking for absence of bacterial colony formation.

BIOASSAY-GUIDED TESTING OF MORPHOGENESIS INDUCING BACTERIA

Standard bioassays of the activities of the bacterial morphogenetic factors were performed in sterile 50 mL plastic tissue-culture flasks (Nuclon Surface, Nunc Int.) for both *U. linza* and *U. mutabilis* (control strain). 10 mL sterile UCM was inoculated with ∼1000 freshly prepared axenic gametes. After incubation overnight at 20°C in the dark, gametes randomly attached to the bottom of the flask. Axenic gametes of *U. linza* were inoculated with a combination of *Roseobacter* sp. and *Cytophaga* sp. (cell density 10^4 cells mL^{-1}) or with the natural bacterial community. As a negative control, one flask was left without any bacteria for the complete period of the experiment. The flasks were cultured under standard light: dark conditions and analyzed under the inverted microscope during the next 21 days. The observed qualitative features were [as described by Spoerner et al. (2012)]: the presence of bubble-like cell wall protrusions; degenerating blade cells and differentiated rhizoid cells.

RESULTS

INDUCTION OF GAMETOGENESIS AND ZOOSPORANGENESIS

Cutting gametophyte blades of *U. linza* into small pieces using a chopper can induce full gamete formation and release of gametes in the morning of the third day, upon an additional medium change (**Figure 1**). On the day of induction and during the next day, the phenotype of the blade cells does not change visibly, and the orientation of the chloroplasts stays perpendicular to the light for optimal energy uptake (**Figures 1A,B**). During the second day after induction, the cells further differentiate into gametangia containing about 16 progametes, which mature during the following night into fully developed gametes ready for swarming (**Figures 1C,D**). If gametogenesis was induced in a small volume of UCM, the fully developed gametes were not released in the next morning, despite illumination, until the medium was changed again, which implies the accumulation of a SWI as reported in *U. mutabilis* (Wichard and Oertel, 2010; **Figure 1C**). In addition, applying the same protocol to sporophyte blade tissue leads to spore induction and release (**Figures 1E,F**) as observed in *U. mutabilis*. To verify the culture conditions in the laboratory, *Ulva* was grown under quasi-natural conditions, where the medium was turned over on a continuous orbital shaker: spontaneous gametogenesis was not observed until an age of 3–4 weeks or even later as previously reported by Stratmann et al. (1996).

EXTRACTION OF SPORULATION INHIBITORS FROM *U. linza*

The results in Section "Induction of Gametogenesis and Zoosporangenesis" imply that sporulation in *U. linza* has similar regulation to sporulation in *U. mutabilis* and involves the removal of SI (=induction) and SWIs (=release of gametes), although they belong to different clades of *Ulva* (Guidone et al., 2013). To

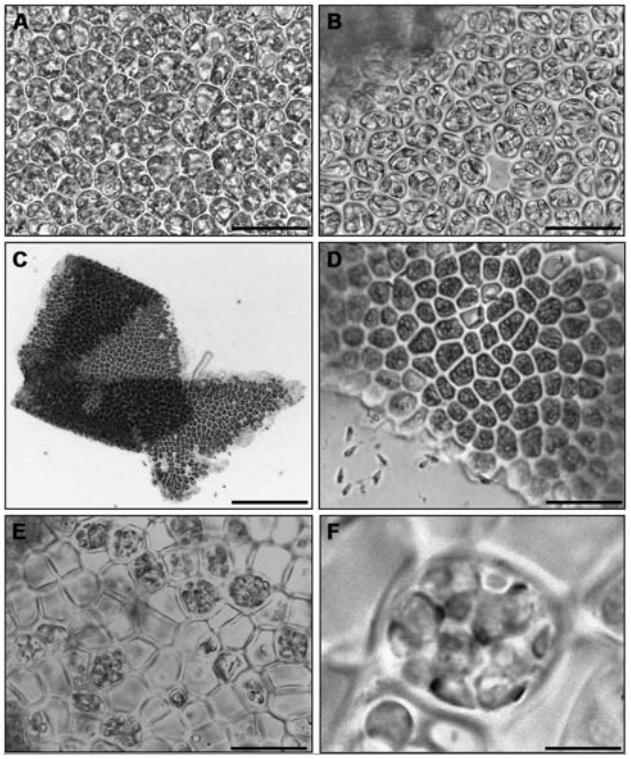

FIGURE 1 | Induction of gametogenesis and zoosporogenesis in *Ulva linza*. Phenotypic changes of blade cells during gametogenesis and gamete release. **(A)** Blade cells 24 h after induction resemble uninduced blade cells: cells are square and often in transverse rows. **(B)** 48 h after induction, blade cells differentiate into gametangia containing finally 16 progametes, which mature during the following night into fully developed gametes ready for swarming. **(C**,**D)** Gametes are discharged in the morning of the third day. **(E)** Discharged sporangia and **(F)** zoospores within a sporangium are shown. Gametophytes **(A–D)** and sporophytes **(E**,**F)** were grown under standard conditions (Scale bars: **A**,**B**,**D** = 25 μm; **C** = 140 μm, **E** = 16 μm, **F** = 4 μm).

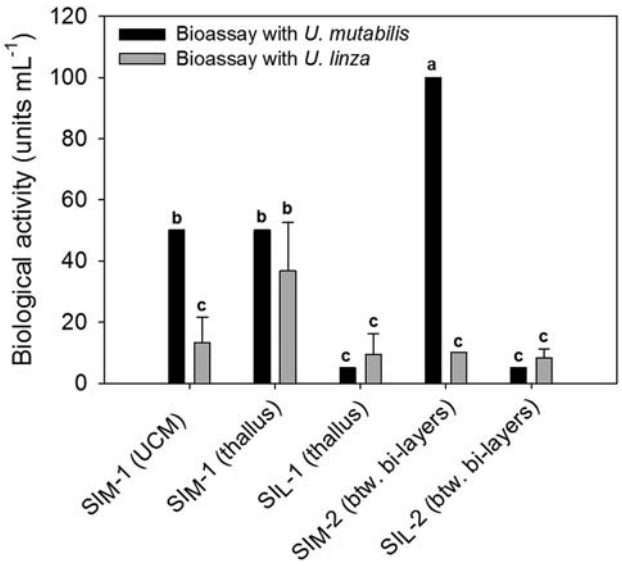

FIGURE 2 | Quantification of the sporulation inhibitors (SI), SI_M and SI_L, extracted from *U. mutabilis* and *U. linza*, respectively. SI_M-1 was extracted from both the *Ulva* culture medium (UCM) and the thallus, whereas SI_L-1 only from the thallus. The inhibitors were then cross-tested on both *Ulva* species (black bars = *U. mutabilis*, gray bars = *U. linza*). Activity of the inhibitors is given in units mL^{-1} (mean ± SD, $n = 3$). One unit of the SI-1 and SI-2 is defined as the concentration that inhibits the gametogenesis/zoosporogenesis of a mature alga completely in 1 mL of UCM for 3 days at 20°C upon induction. One-way ANOVA was performed to determine statistical significance. Tukey's test was used to determine which groups differ (significance level = 5%), indicated by the letters a, b and c.

investigate whether gamete induction in *U. linza* requires the same or similar factors as in *U. mutabilis*, we partially purified SI from both *U. mutabilis* and freshly collected *U. linza* samples using the previously established method (Stratmann et al., 1996) and cross-tested them. We showed that *U. linza* produces SI that work interchangeably with *U. mutabilis* during gametogenesis: both types of *U. mutabilis* SI (SI_M1 and SI_M2) were each able to inhibit gamete production in *U. linza* and *U. mutabilis* (**Figure 2**), albeit to a lesser extent (for medium-derived SI_M1 and between-cell-layers SI_M2) in *U. linza*. Conversely, *U. linza* SI (SI_L1 and SI_L2) were each able to inhibit gamete formation in both *Ulva* species tested (**Figure 2**).

In detail, the determined biologically active concentration depends to some extent on the target species: SI_L1 accounts for 9.4 ± 6.8 units mL^{-1} tested on *U. linza*, which was equal to 5 units mL^{-1} tested on *U. mutabilis* and thus less is less active toward *U. mutabilis*, although the difference is not statistically significant due to the high variance of the biological replicates (one-way ANOVA followed up by Tukey *post hoc* tests with an overall significance level of 5%). Moreover, SI_M2 is significantly (about 10 times) more active when applied to *U. mutabilis* rather than to *U. linza*, but the SI_L2 does not show any species-specific differences in its inhibitory activity (**Figure 2**).

GENERATION OF FEEDING STOCK BY GAMETE PURIFICATION

Understanding the regulation of *U. linza* allows building up of a feedstock for further standardized cultivation, similarly to *U. mutabilis*. *U. linza* gametes from a single blade (i.e., all the same mating type) isolated upon induction of gametogenesis were able to germinate parthenogenetically to form blades. Therefore, we tried to purify *U. linza* gametes for axenic culture and to set up cultures forming thalli parthenogenetically with a controlled microbiome. As *U. mutabilis* can be put into axenic culture by purifying gametes *via* their strong and rapid phototactic response (Spoerner et al., 2012), we investigated whether *U. linza* gametes could behave (and therefore be purified) in the same way. We showed that *U. linza* gametes can be subject to purification in a very similar manner to *U. mutabilis*, over a very similar time frame (**Figure 3**). The gametes were demonstrated to be axenic by inoculation of the medium in which the purified gametes were residing onto Petri dishes: after the third purification run in Pasteur pipettes the gamete containing medium was free of bacteria (**Figure 3C**).

BACTERIA INDUCED MORPHOGENESIS

As epiphytic bacteria are required for correct morphology in both *U. mutabilis* and *U. linza* (Fries, 1975; Marshall et al., 2006;

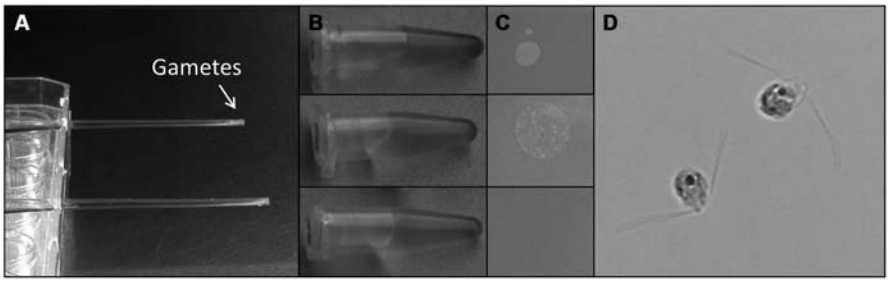

FIGURE 3 | Purification of *U. linza* gametes from accompanying bacteria. Gametes are harvested upon medium change; capitalizing on their movement toward the light. Then collected gametes are purified in capillary pipettes several times **(A)**. The purifications can be quickly tested by placing a drop (10 μL) of the gamete solution **(B)** on marine broth agar plates **(C)**. In general three purification steps are sufficient to separate the bacteria from the gametes. Bacterial colonies forming on marine broth agar plates could be observed after two purification steps but not after the third one **(C)**. Purified bi-flagellated gametes (3 μm) are shown **(D)**.

Spoerner et al., 2012), we tested whether *U. mutabilis* bacteria could drive the correct development of *U. linza*. Gametes were seeded in culture either axenically (purified, no bacteria), with the normal complement of *U. linza* epiphytes (i.e., gametes induced but not purified), or with the two species of bacteria known to restore morphogenesis to axenic *U. mutabilis*, namely *Cytophaga* sp. MS2 and *Roseobacter* sp. MS6 (Spoerner et al., 2012). Axenic *U. linza* formed an undifferentiated mass of cells reminiscent of axenic *U. mutabilis*, with very little cell elongation or longitudinal cell division, compared to non-axenic controls (**Figure 4**). The size of the structure formed was larger than for *U. mutabilis*. However, the callus-like morphology contained the typical colorless protrusions from the exterior cell walls as observed in axenic cultures of *U. mutabilis* (Spoerner et al., 2012), *Enteromorpha compressa* and *E. linza* (Fries, 1975).

Addition of *Roseobacter* sp. and *Cytophaga* sp. to axenic *U. linza* restored blade growth/elongation and rhizoid growth, and the bacteria clustered around the rhizoid as seen in *U. mutabilis*. However, the combination of *Roseobacter* sp. and *Cytophaga* sp. were unable to restore wild type rhizoid morphology to *U. linza*. The rhizoids

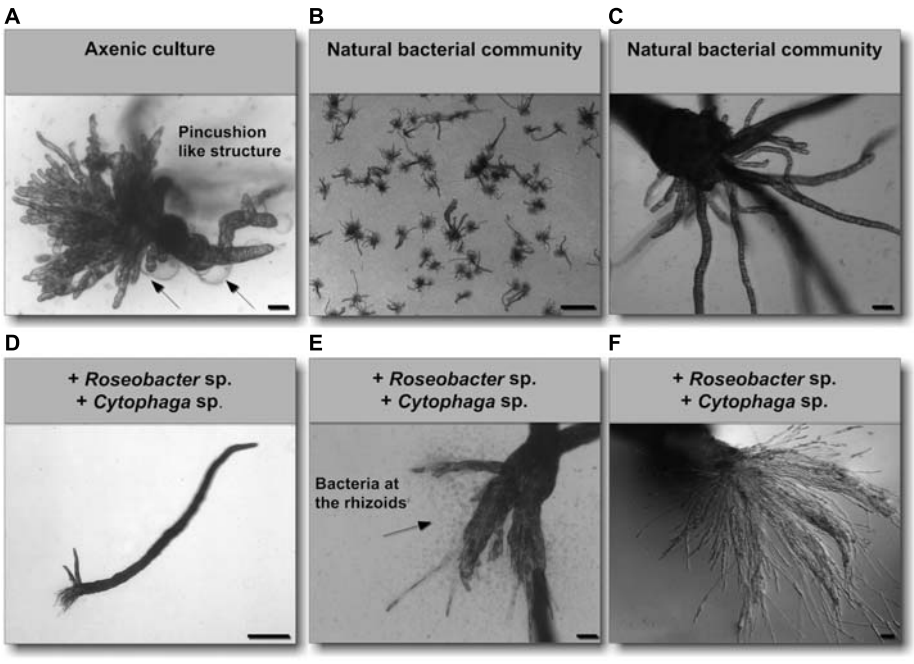

FIGURE 4 | Survey of the effects of know morphogenesis inducing bacteria on *U. linza*. **(A)** Phenotype of an axenic *U. linza* gametophyte after 3 weeks cultivation with few initial thallus stages. Algae grow parthenogenetically from axenic gametes (scale bar = 25 μm). Arrows indicate the typical colorless protrusions from the exterior cell walls of axenic cultures. **(B,C)** Axenic gametes with the natural microbiome develop into a normal plant (scale bar = 1 cm in **(B)** and 50 μm in **(C)**. **(D,E)** Addition of *Roseobacter* sp. and *Cytophaga* sp. induces blade and rhizoid formation and bacterial concentrate at the rhizoids (scale bar **D** = 1 mm, **E** = 50 μm). **(F)** However, rhizoid formation seems to be incomplete in comparison of the *U. linza* grown in natural bacterial community even after 5 weeks compared to **(C)** (scale bar = 50 μm).

formed were extremely numerous and thin, largely only one cell thick (**Figures 4E,F**). In addition, the growth of *U. linza* within this tripartite community was significantly slower than the growth of the "sl" mutant of *U. mutabilis* (3 weeks to achieve maturity; Løvlie, 1964) and the *U. linza* in a natural community.

DISCUSSION

GAMETOGENESIS AND SPOROGENESIS CAN BE INDUCED IN *U. linza*

We have shown that *U. linza* gametes (and zoospores) can now be reproducibly induced by cutting thallus tissue and removing extracellular inhibitors (SI-1, SI-2), as in *U. mutabilis*. This shows that there is potential for laboratory culture of a cosmopolitan *Ulva* species with worldwide distribution including in the UK, which is an established model for biofouling research and algal–bacterial interactions. A lack of gamete release when gametes are induced in a small volume of culture medium implies the existence of a SWI, as in *U. mutabilis*. The presumed release of the SWI has to be further investigated and compared with *U. mutabilis* to see whether the SWIs are exchangeable at the same concentration or whether they are even the same substance.

Ulva linza PRODUCES SPORULATION INHIBITORS

We have partially purified SI (SI_L1 and SI_L2) from *U. linza* and compared their activity to the SI_M previously isolated from *U. mutabilis* (Nilsen and Nordby, 1975; Stratmann et al., 1996). *U. linza* SI_L works interchangeably with *U. mutabilis* during gametogenesis. *U. mutabilis* sporulation inhibitor inhibited gametogenesis in both *U. linza* and *U. mutabilis*, but to a lesser extent in *U. linza*. Conversely, *U. linza* SI inhibited gamete formation in both *Ulva* species. This demonstrates that the tested SIs are not species-specific and indicates that *U. mutabilis* and *U. linza* use similar signals to regulate induction and release of both unicellular life cycle stages. The tendency is that higher amounts of SI are necessary to inhibit the sporulation of the opposite *Ulva* species, indicating that the isolated SIs from both species are probably not identical and may differ slightly in their structure-activity relationship.

There were high variances between biological replicates with *U. linza* and this highlights the advantages of standardized culture conditions with synchronized algae. Whereas the variances were high for the bioassays with the *U. linza* due to its potentially varying age, variances of the inhibitory effects on the gametogenesis of *U. mutabilis* were so small they were not measurable within the resolution of the dilution series for three biological replicates conducted in parallel, (i.e., no SD is seen in **Figure 2**).

Compared to Stratmann et al. (1996), the extracted yield of inhibitor from the UCM (i.e., biological activity) was in general lower than previously reported. This can be explained with the lower cell densities that were used in our study. The SI_L1 was only extracted from the thalli of vegetatively growing *U. linza* cultures and could not be detected in the UCM of *U. linza*, in contrast to *U. mutabilis* laboratory cultures. This is partly due to the fact that bacteria of the undefined microbiome of the collected *U. linza* samples have most likely digested the SI, as was also shown in natural *U. mutabilis* samples (Stratmann et al., 1996).

The observation that *U. linza* and *U. mutabilis* share similar SI and perception systems cannot be generalized within the entire *Ulva* genus, as the SI_M1 was not effective on *U. rigida* (Stratmann et al., 1996). Because the morphology of the distromatic thalli (broad thalli with no hollow parts) of *U. rigida* is very different to the monostromatic thalli of *U. linza* and *U. mutabilis* (broad or ribbon like thalli with hollow parts), further studies need to investigate the underlying evolutionary processes and verify whether, e.g., life-cycle-regulating factors are clade-specific in the genus of *Ulva*, e.g., in the *compressa/pseudocurvata/mutabilis* group versus the *rigida* group (Guidone et al., 2013). Recently, it was suggested that apparently clade-specific biosynthetic pathways are used to transform polyunsaturated fatty acids into oxylipins (Alsufyani et al., 2014). Taking into account that an excess of SI-1, a cell-wall glycoprotein, is released into the environment, inhibitors might regulate the germ cell formation of closely related *Ulva* species in, e.g., tidal ponds or during green tides. This inter-species regulation might become *a fortiori* important, as waterborne breakdown products of the protein can still possess inhibitory activities (Stratmann et al., 1996; Kessler, personal communication).

In nature, the induction of sporulation might be triggered by segmentation as potentially observed by Gao et al. (2010) during green tides. Certainly sporulation events also occur even without fragmentation, whenever the SIs are either not produced or perceived in *Ulva*'s life cycle (Stratmann et al., 1996), which might explain the underlying mechanism of sporulation events reported in a recent study with a tropical *Ulva* species (Carl et al., 2014).

GAMETE PURIFICATION AND AXENIC CULTURE OF *U. linza* AND REQUIREMENT FOR EPIPHYTES

We have shown that *U. linza* gametes can be purified with the same methodology as developed originally for *U. mutabilis* gametes, and can germinate parthenogenetically to form new gametophyte thalli, thus paving the way for axenic culture of a second *Ulva* species. Axenic *U. linza* formed multicellular structures slightly larger than those formed by *U. mutabilis*. This could indicate species-specific differences, or could indicate a small residual (and uncultivable) bacterial load in the culture, that was not detected in the Petri dish test. As 16S PCR was not carried out, we cannot rule out this possibility.

However, when we tested the ability of the two specific bacterial species that rescue morphology in *U. mutabilis* to rescue axenic *U. linza* development, we found that recovery was slow and incomplete, particularly of the rhizoids, despite the clustering of the bacteria around the rhizoids. No filamentous basal system was formed, in contrast to Kapraun's and Flynn's observation with culture studies to *E. linza* (L.; Kapraun and Flynn, 1973). In summary, epiphytic bacteria are required for both growth and differentiation of *U. linza*, but *U. linza* requires different, although probably related, bacteria to *U. mutabilis* for normal morphology, particularly of rhizoid and holdfast formation. In particular, the *Cytophaga* strain releases potentially algae-specific morphogenetic substances inducing rhizoid formation in an auxin-like fashion. It supports Berglund's (1969) studies, which found that growth of *E. linza* can in principle be stimulated by water-soluble organic substances separated from nutrients, although he did not observe changes in morphology at that time.

Marshall et al. (2006) isolated approximately 38 unique bacteria from *U. linza* and categorized them according to their morphogenetic activity within 28 days of incubation. Four categories, based on the number of tubular extensions grown from a central callus, were identified. One category holds for axenic cultures and represents a morphotype very similar to the observed axenic morphotype in this study. However, none of the other categories described the complete recovery of morphogenesis, but a combination of the isolated bacteria was not tested. Therefore, the bacteria should be re-isolated from *U. linza* according to the protocol of Marshall et al. (2006) and tested in combinations of the *Roseobacter* sp. and *Cytophaga* sp. using the newly established laboratory strains of *U. linza*.

SUMMARY AND FUTURE WORK

We have shown that *U. linza* sporulation can be induced using the protocols previously developed for *U. mutabilis* (Stratmann et al., 1996; Wichard and Oertel, 2010; Spoerner et al., 2012), and that *U. linza* likely also produces a SWI, like *U. mutabilis*. Moreover, both species appear to use similar concepts controlling sporulation, as inhibitors purified from *U. mutabilis* and *U. linza* using identical protocols work largely interchangeably in both species. *U. linza* gametes can be purified for axenic culture and can germinate parthenogenetically, similarly to those from *U. mutabilis*. Experiments adding back *U. mutabilis* epiphytic bacteria to axenic *U. linza* gametes suggest the existence of species-specific differences in bacterial signals regulating development, particularly of rhizoids. In future, SI and SWI from *U. linza* should be further characterized, and the *U. linza*-specific bacteria and signals regulating normal development (particularly the *Cytophaga*-equivalent affecting rhizoid development) should be identified. Understanding of sporulation in more than one *Ulva* species will shed light on the formation of green tides (as seen with *U. prolifera*; Gao et al., 2010). Moreover, development of axenic culture for a second *Ulva* species potentially enables future comparative studies, particularly of the bacterial signals regulating green seaweed morphogenesis. However, our results also highlight the usefulness of a standardized model culture system using a single species for a detailed understanding of the principles of seaweed development.

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Research

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The decision to germinate is regulated by divergent molecular networks in spores and seeds

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Summary

• Dispersal is a key step in land plant life cycles, usually via formation of spores or seeds. Regulation of spore- or seed-germination allows control over the timing of transition from one generation to the next, enabling plant dispersal. A combination of environmental and genetic factors determines when seed germination occurs. Endogenous hormones mediate this decision in response to the environment. Less is known about how spore germination is controlled in earlier-evolving nonseed plants.

• Here, we present an in-depth analysis of the environmental and hormonal regulation of spore germination in the model bryophyte *Physcomitrella patens* (*Aphanoregma patens*).

• Our data suggest that the environmental signals regulating germination are conserved, but also that downstream hormone integration pathways mediating these responses in seeds were acquired after the evolution of the bryophyte lineage. Moreover, the role of abscisic acid and diterpenes (gibberellins) in germination assumed much greater importance as land plant evolution progressed.

• We conclude that the endogenous hormone signalling networks mediating germination in response to the environment may have evolved independently in spores and seeds. This paves the way for future research about how the mechanisms of plant dispersal on land evolved.

Introduction

Transition from one generation to the next in land plants is mediated by the formation of desiccation-resistant dispersal units (Finch-Savage & Leubner-Metzger, 2006). Within the spermatophyte lineage, these dispersal units are multicellular seeds, whereas in bryophytes and nonseed vascular plants (lycophytes and ferns) the functionally equivalent dispersal units are unicellular spores (Linkies *et al.*, 2010). How and when germination is initiated in a seed or spore is critical for plant and species reproduction, movement and survival.

Hormonal and environmental factors are both well-established as key players in the regulation of seed germination (Holdsworth *et al.*, 2008a). The regulation of seed germination is highly complex, and involves integration of environmental signals by hormones within the seed and within different seed compartments and cell types (Yamaguchi *et al.*, 2001; Holdsworth *et al.*, 2008b; Linkies *et al.*, 2010; Dekkers *et al.*, 2013). Much is still unknown about exactly how seed germination is controlled at a cellular level (Nonogaki *et al.*, 2010; Bassel *et al.*, 2014).

Spores, unlike seeds, are haploid and are derived from the sporophyte stage of the plant life cycle via meiosis (reviewed in Rubinstein *et al.*, 2010). Despite the different developmental origins of spores and seeds, previous work suggests that at least some aspects of germination regulation may be conserved between the two types of dispersal unit, as outlined later. Because spores are unicellular structures, understanding spore germination provides us with a simplified system for the study of the cellular and hormonal basis of germination regulation, and how this has evolved.

Environmental regulation of seed germination in both monocots and dicots is controlled in part by a phytochrome-mediated reversible system, with red (R) light promoting germination, and even brief exposure to far-red (FR) light inhibiting R light-induced germination (Borthwick *et al.*, 1952; Shinomura *et al.*, 1994; Hennig *et al.*, 2002), although this trait has been bred out of some commercial cereal crops (Barrero *et al.*, 2012). A similar R–FR reversible system regulates spore germination in several ferns (Mohr *et al.*, 1964; Raghavan, 1973; Wayne & Hepler, 1984; Scheuerlein *et al.*, 1989; Tsuboi *et al.*, 2012). In the earliest-evolving land plant lineage, bryophytes, complete inhibition of spore germination by FR light, and reversal of this inhibition by R light via phytochromes, has been demonstrated (Possart & Hiltbrunner, 2013). Phytochrome regulation of spore germination is likely to be extremely ancient, as it appears to exist outside the land plant lineage, also, including in spores of the Charophycean algae *Spirogyra* and *Chara*, and in fungi (Calpouzos &

Chang, 1971; Takatori & Imahori, 1971; Lucas *et al.*, 1975; Mathews, 2006; Agrawal, 2009). The mechanisms downstream of phytochromes that regulate the control of germination throughout the plant lineage are poorly understood.

The onset of germination in seeds is closely regulated by the balance between plant hormone signalling pathways of gibberellin (GA) and abscisic acid (ABA), which interact at multiple levels (Karssen & Lacka, 1986; Holdsworth *et al.*, 2008a). We have recently shown that ABA inhibits spore germination in *Physcomitrella* and that conserved proteins modulate ABA-mediated germination responses in both spores and seeds (Moody *et al.*, 2016). This suggests that downstream signalling components regulating germination may be conserved between spores and angiosperm seeds. In seeds, gibberellins are required for germination: seeds of the *Arabidopsis ga1* mutant, which lacks the first enzyme in the GA biosynthesis pathway, are unable to germinate without exogenously supplied gibberellin (Koornneef & van der Veen, 1980), whereas GA receptor (GID) mutants cannot germinate fully (Voegele *et al.*, 2011). GA overcomes the inhibitory effects of ABA to allow seed germination (Holdsworth *et al.*, 2008a).

Conflicting results relating to the role of GA and ABA in the control of plant spore germination have been reported. Mosses biosynthesize the diterpenes at the start of the GA biosynthesis pathway, *ent*-kaurene and *ent*-kaurenoic acid, but they lack the enzyme that further converts *ent*-kaurenoic acid into bioactive gibberellins (as occurs in seed plants). Thus, the identity of bioactive diterpenes in spore-bearing plants is not yet fully characterized (Von Schwartzenberg *et al.*, 2004; Hayashi *et al.*, 2010; Zhan *et al.*, 2015). Microarray analysis of spore germination in the fern *Ceratopteris* implicated involvement of GA signalling and downregulation of ABA signalling in this process, similarly to seeds (Yao *et al.*, 2008). However, different fern species' spores have different sensitivities to GA and ABA application (Weinberg & Voeller, 1969; Chia & Raghavan, 1982; Singh *et al.*, 1990; Kagawa & Michizo, 1991; Haas *et al.*, 1992). The GA biosynthesis inhibitor AMO-1618, which blocks the first step(s) in the GA biosynthesis pathway (Rademacher, 2000), can inhibit some (but not all) light-induced fern spore germination (Weinberg & Voeller, 1969; Nester & Coolbaugh, 1986; Kagawa & Michizo, 1991). In the best-studied model bryophyte, the moss *Physcomitrella patens* (*Aphanoregma patens*), a *copalyl-diphosphate synthase/kaurene synthase* (*cps/ks*) mutant, which lacks the CYP88A enzyme that catalyses the key step of *ent*-kaurenoic acid oxidation in gibberellin biosynthesis and hence makes no diterpenes, had no reported spore germination phenotype (Hayashi *et al.*, 2010). However, *Physcomitrella* spore germination can be inhibited by AMO-1618, although AMO-1618 may have targets in addition to the CPS enzyme (Anterola *et al.*, 2009). Reports in other bryophytes detail the conflicting effects of exogenously applied gibberellins on spores of different species at different concentrations (Chopra & Kumra, 1988).

ABA is central to dormancy establishment and maintenance in *Arabidopsis* (Finkelstein *et al.*, 2008). Freshly harvested *Arabidopsis* seeds contain high levels of ABA and show primary dormancy. ABA is also implicated in the imposition of secondary dormancy by, for example, high temperatures, via *de novo* synthesis of this hormone (Finch-Savage & Leubner-Metzger, 2006; Toh *et al.*, 2008, 2012). The majority of bryophyte species' spores have not been reported to show primary dormancy (McLetchie, 1999; Glime, 2015). Whether spores can have secondary dormancy imposed on them is currently not well-characterized (Glime, 2015).

ABA also protects plants against abiotic stresses such as desiccation and freezing (Lee & Luan, 2012; Dekkers *et al.*, 2015). Formation of both mature seeds and spores involves desiccation, and aspects of ABA signalling during abiotic stress responses in bryophytes are conserved with angiosperms (Knight *et al.*, 1995; Cuming *et al.*, 2007; Khandelwal *et al.*, 2010). ABA-mediated stress tolerance in bryophytes occurs at least in part via accumulation of soluble sugars including sucrose (Burch & Wilkinson, 2002; Nagao *et al.*, 2006; Oldenhof *et al.*, 2006; Bhyan *et al.*, 2012; Erxleben *et al.*, 2012). Notably, soluble sugars (sucrose, glucose) can inhibit germination in *Arabidopsis* seeds (Dekkers *et al.*, 2004; Li *et al.*, 2012).

Several additional hormones regulate seed germination. Strigolactones (SLs) produced by host plants are potent promoters of parasitic plant seed germination (Bouwmeester *et al.*, 2003; Yoneyama *et al.*, 2010). In *Arabidopsis*, strigolactone signalling pathway mutants show reduced seed germination (Stanga *et al.*, 2013) and strigolactone can overcome the secondary dormancy imposed by exposing *Arabidopsis* seeds to high temperatures (Toh *et al.*, 2012). Ethylene also promotes seed germination via multiple routes (Stewart & Freebairn, 1969; Logan & Stewart, 1991; Linkies & Leubner-Metzger, 2012), whereas an inhibitory role for cytokinin in germination is implied in *Arabidopsis* (Riefler *et al.*, 2006). A role for auxin in seed germination under normal conditions has not been demonstrated (Holdsworth *et al.*, 2008a; Park *et al.*, 2011); neither has the effect of these hormones on moss spore germination been investigated systematically (Chopra & Kumra, 1988; Glime, 2015).

Here, we sought for the first time to define comprehensively how hormones and environmental processes regulate spore germination in the bryophyte lineage by using *Physcomitrella* as a model system.

Materials and Methods

Physcomitrella culture and spore generation

Physcomitrella patens (Gransden wild-type (WT) strain, and *Ppcps/ks* and *ccd8* mutants) was cultured and sporulation induced as in Moody *et al.* (2012). Sporophytes were harvested after maturation (dark brown sporophytes with a slightly 'sparkly' appearance) using sterile forceps under a SMZ645 dissecting microscope (Nikon, Tokyo, Japan) and air-dried in sterile tubes for *c.* 1 wk before storage at room temperature.

Spore germination assays

Spores from a minimum of three sporophytes (all of the same age) of a particular genotype were used within each assay. For

larger assays, three sporophytes' worth of spores were used for every 10 Petri dishes (9 cm diameter). For assays comparing WT with a mutant, spores of each genotype were chosen to be of the same age, harvested at the same time.

Sporophytes were bleached in groups of two to three in 1 ml 25% Parozone™ (Jeyes Group, Thetford, UK) for 10 min and then washed three times in 1 ml sterile distilled water (10 min each) in a sterile flow cabinet. The sporophytes were then crushed in 100–200 µl of sterile water to release the spores. Spores were diluted down in sufficient sterile distilled water to allow plating of 500 µl of spore solution per Petri dish. Spores were plated on cellophane-overlaid BCD supplemented with 5 mM $CaCl_2$ and 5 mM ammonium tartrate, or on water agarose for the dormancy experiments in Fig. 1. Cellophane discs (A.A. Packaging Ltd, Preston, UK) were autoclaved for 15 min at 121°C, before use.

For hormone/chemical treatments, the treatment in the relevant solvent (or a solvent-only control) was added to 1 ml of sterile water, which was then added to BCD medium just before plate-pouring to achieve the desired final concentration in the plates. Within each assay, all solvent-containing plates were matched so that all contained exactly the same volume of solvent, even if the hormone concentration varied. The solvents used were methanol for diterpenoids and norflurazon, acetone for GR24 and water for ethylene.

Plated spores were air-dried in a laminar flow hood, sealed with micropore tape, and placed at 22°C in long-day conditions. Spores were counted daily under the ×4 objective of a Leica compound microscope with a ×10 eyepiece; total magnification ×40. A minimum of 200 spores per plate was counted to define the number of germinated and ungerminated spores. Data were expressed as percentage germination, that is: (germinated spores/total spores counted) × 100. A spore was defined as having germinated as soon as the very first deformation of the spore coat by the emerging protonemal filament was observed.

Two to three technical replicates were contained within each assay, and each assay was repeated a minimum of three times on different dates, using different batches of spores and medium, to provide biological replicates. Data from one representative assay is shown in each figure.

RNA preparation

RNA was prepared from dry spores (*c.* 250 harvested sporophytes), imbibed spores (*c.* 250 sporophytes bleached then soaked in liquid BCD for 18 h), germinating spores (spores from *c.* 250 bleached sporophytes plated at high density and left for 7 d until *c.* 50% germination was seen) and 100 mg vegetative (protonemal or leafy) tissue using the Bioline Isolate II Plant RNA preparation kit according to the manufacturer's instructions (London, UK). Average RNA yield was *c.* 300 ng μl^{-1}, with OD260/280 > 2 and average OD 260/230 *c.* 1.8.

RT-PCR

Reverse transcription polymerase chain reaction (RT-PCR) was carried out on 20 ng RNA from each sample using the Bioline MyTaq™ one step RT-PCR kit according to the manufacturer's instructions. Primer sequences are detailed in Supporting Information Table S1.

Generation of *Physcomitrella patens* lines with disrupted *PpCPS/KS* functionality

The moss line pCL755#29 is described in Zhan *et al.* (2015), generated using the methods described in (Bach *et al.*, 2014). Briefly, a cassette containing *p35S-nptII-CaMVter*, expressing *Neomycin Phosphotransferase II* (conferring resistance to G418), was excised from pMBL6 (http://www.biology.wustl.edu/moss/pmbl6.jpg) using *Xho*I and inserted into the *Xho*I site in pDONR201:CPS/KS, generating the knock-out construct pDONR201:CPS/KS-nptII. Following transformation into *P. patens* of this construct, one line – pCL755#29 – with disrupted *PpCPS/KS* functionality was used for further studies.

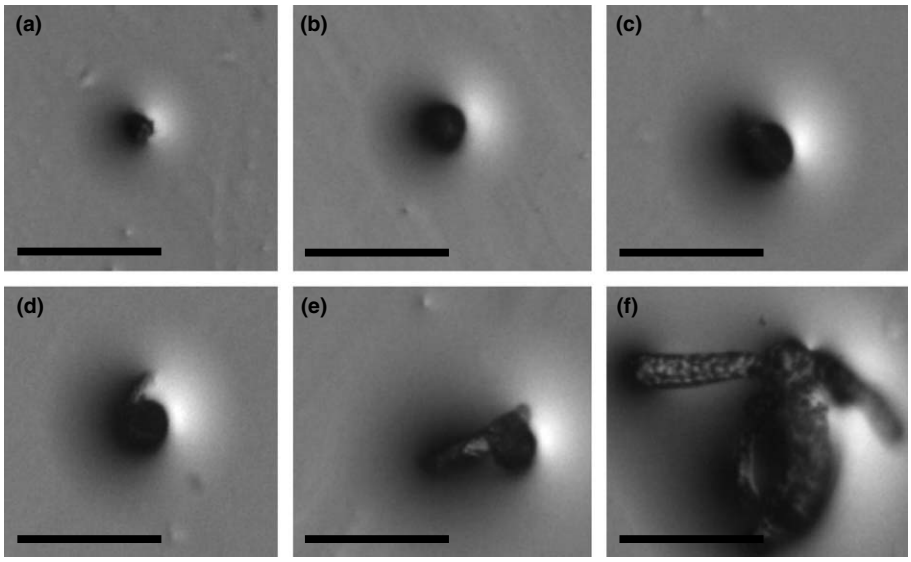

Fig. 1 *Physcomitrella patens* spore germination stages. (a) Dry spore. (b) Imbibed spore. (c) Spore coat cracking. (d) Protrusion of one protonemal (chloronemal) filament. (e) Protrusion of two protonemal filaments. (f) Established protonemal colony. Bars, 100 µm.

A second line, pBK3, was generated by disrupting *PpCPS/KS* via targeted gene replacement using the pBK3 vector first described in Pan *et al.* (2015) and utilizing the method described in King *et al.* (2016), which contains a *p35S-aph4-CaMVter* cassette flanked by 5′- and 3′-genomic sequence of *PpCPS/KS* on its 5′ and 3′ end, respectively. Therefore, genomic sequence of *PpCPS/KS* was replaced with *p35S-aph4-CaMVter*, which gives hygromycin resistance. The two lines were genotyped by PCR with the combination of primers that specifically bind to genomic DNA or selection marker cassette to distinguish knockout mosses from WT (Fig. S1).

GC-MS analysis of diterpenoids

All GC-MS analyses were performed on a Shimadzu GCMS-QP2010 plus (GC-2010) with a CTC auto sampler AOC-5000, with cooled trays, agitation oven, and needle bake-out.

GC-MS analysis utilizing solid-phase microextraction fibers was previously published (Drew *et al.*, 2012; Andersen *et al.*, 2015). Briefly, the injection port temperature was set to 230°C, with a sampling time of 1 min. The flow control mode was pressure control with a total flow of 2.3 ml min^{-1}, with H_2 as carrier gas, and a purge flow of 1.0 ml min^{-1}. The column was a 30 m HP-5MS column. The oven temperature program was 35°C for 3 min, rising by 10°C per min to 230°C and a hold for 3 min. The MS settings were: Ion source temperature 260°C, interface temperature 280°C and the scan range from *m/z* 50 to *m/z* 350 with 70 eV electrical ionization.

All data were analysed using the Shimadzu software Lab Solutions, GCMS Solutions v.2.70, using the libraries provided by NIST (NIST 08) and Wiley (Wiley 8.0). Obtained spectra were compared with the spectra in the mass spectral libraries. Compounds were identified comparing the data with library information of MS and retention indices (*I*). All reference *I*s were taken from Adams (2007).

Results

Physcomitrella spores are nondormant and do not require cold-stratification or after-ripening treatment for germination

We define a *Physcomitrella* imbibed spore as having 'germinated' as soon as the first visible deformation of the spore coat occurs and the spore no longer appears spherical. This marks the first protrusion of protonemal filament(s), which grow with the subsequent formation of further filaments (Fig. 1a–f). Spores typically begin to germinate 2–7 d after imbibition/plating. We found that there was no correlation between spore age (time of dry storage post-harvest) and speed of germination (Fig. 2a), suggesting that

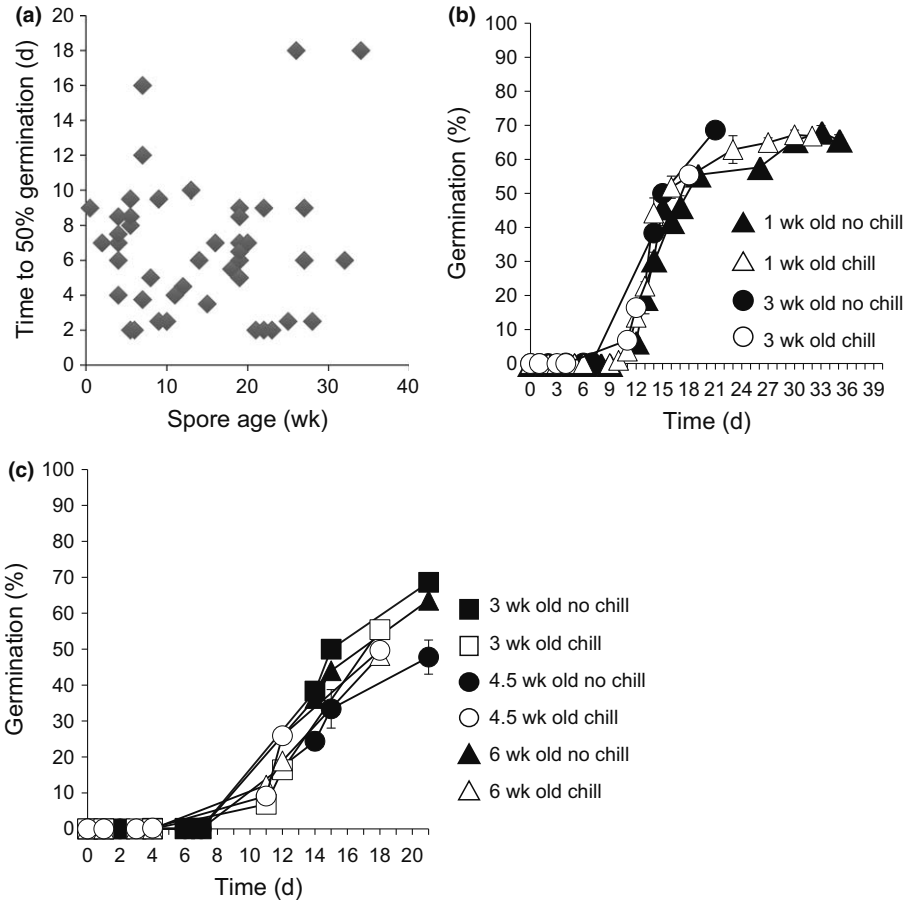

Fig. 2 *Physcomitrella* spores do not appear to show primary dormancy and after-ripening. (a) There is no correlation between dry spore age and time to 50% germination. Pearson–Spearman correlation test, $R^2 = 0.004$. (b) There is no effect of ageing or chilling on a single spore population. Spores from more than one sporophyte harvested from WT plants on the same day were germinated on water agarose with or without 3 d chilling at 4°C, 1 wk after collection and again at 3 wk after collection. Chilling does not accelerate germination. Error bars, ± SEM. (c) There is no effect of chilling on three independent batches of spores. Spores (from more than one sporophyte) from three independent harvests of different ages were germinated on water agarose with or without 3 d chilling at 4°C. Chilling does not accelerate germination. Error bars, ± SEM.

after-ripening does not occur as it does in seeds. Moreover, a period of chilling, which breaks dormancy in seeds, did not affect spore germination (Fig. 2b,c). This suggests an absence of primary dormancy or after-ripening in *Physcomitrella* spores.

Far-red inhibition of *Physcomitrella* spore germination is not rescued by application of diterpenes or inhibition of ABA synthesis

Although *Physcomitrella* spores appear not to have primary dormancy, we investigated whether their germination could be inhibited by environmental signals, as occurs in seeds (imposition of secondary dormancy in otherwise germination-competent seeds). In both seeds and *Physcomitrella* spores, germination can be fully inhibited by a pulse of FR light (Seo *et al.*, 2009; Possart & Hiltbrunner, 2013). In eudicot seeds, FR-inhibition of germination can be fully rescued by treatment with bioactive GAs or by inhibition of ABA biosynthesis (Ikuma & Thimann, 1960; Schopfer *et al.*, 2001; Oh *et al.*, 2006; Seo *et al.*, 2006; Lee *et al.*, 2012).

In order to investigate whether the interface between light- and diterpene/ABA-signalling in *Physcomitrella* spores is conserved with the regulation seen in *Arabidopsis* seeds, we FR-treated spores in the presence of a diterpenoid known to be bioactive in moss (Hayashi *et al.*, 2010), the fern antheridiogen GA_9-methyl ester, and the carotenoid biosynthesis inhibitor norflurazon, which blocks ABA biosynthesis in flowering plants (Chamovitz *et al.*, 1991). We found that neither GA_9-methyl ester nor norflurazon were able to rescue the germination-inhibitory effects of a pulse of FR light on *Physcomitrella* spores, even when control spores had germinated to 100% (Fig. 3a). This suggests that diterpenes and ABA do not facilitate spores' responses to the environmental signal of light at certain wavelengths, and thus the hormonal control of *Physcomitrella* spores and dicot seeds is not conserved.

Physcomitrella spore germination can be inhibited by high temperatures, but this cannot be rescued by ABA-inhibition, or application of diterpenes or strigolactones

In order to further explore the role of diterpenes and ABA in the control of environmentally regulated germination, we inhibited spore germination using another environmental trigger: high temperature (thermoinhibition). Substantial and reversible thermoinhibition of seed germination (imposition of secondary dormancy) is seen at 32°C in *Arabidopsis* (Tamura *et al.*, 2006; Toh *et al.*, 2008), and this can be rescued by GA_3, norflurazon or strigolactone application (Toh *et al.*, 2012).

Incubation of spores at 35°C (but not 32 or 34°C; data not shown) caused complete inhibition of germination that was fully reversible upon return to normal growth conditions (22°C) (Fig. 3b). Thermoinhibition of *Physcomitrella* spores at 35°C could not be alleviated at all by GA_9-methyl ester, norflurazon or the synthetic strigolactone GR24 (Fig. 3c). Although high temperatures can inhibit germination in *Physcomitrella* spores, as in *Arabidopsis* seeds, the hormones mediating this response in *Arabidopsis* are not the same as in *Physcomitrella*, as was also seen with the FR light response.

Diterpenoids can promote *Physcomitrella* spore germination

Our previous work suggested conservation of some hormone function in spore and seed germination, via an ABA-ARABIDILLO/PHYSCODILLO signalling module (Moody *et al.*, 2016). Thus, we further explored the effects of diterpene hormones in *Physcomitrella*, to compare their effects with those of GAs in seeds. We examined *Physcomitrella* mutants in the gene encoding the first enzyme in the putative moss gibberellin biosynthesis pathway, *ent-COPALYL DIPHOSPHATE SYNTHASE/ent-KAURENE SYNTHASE* (*CPS/KS*), which makes no *ent*-kaurene and hence no bioactive diterpenoids/gibberellins (*Ppcps/ks*; Hayashi *et al.*, 2010; Fig. S1), similar to the mutants used by Hayashi *et al.* (2010). Two different *Ppcps/ks* mutant alleles showed a reduced germination speed compared with WT: they attained a lower percentage of germination at any given time on the upwards slope of the graph, although they eventually attained 100% germination (Figs 4a, S1a), and this phenotype could be rescued by application of two diterpenoids known to be bioactive in moss (Hayashi *et al.*, 2010): the fern antheridiogen GA_9-methyl ester or *ent*-kaurene (Fig. 4b,c). This result indicates that bioactive diterpenoid hormones in *Physcomitrella* have a positive effect on spore germination.

In order to further investigate this possibility, we examined the effect of diterpenes on WT *Physcomitrella* spore germination. GA_9-methyl ester and *ent*-kaurene both enhanced spore germination (Figs 4b,c, S1b), whereas GA_3 did not (Fig. S1c). Conversely, the diterpenoid hormones that promote moss spore germination cannot fully rescue the germination defect of the *Arabidopsis ga1-3* mutant (Fig. S2). Together, these data: (i) show that diterpenoid hormones are not absolutely required for spore germination, unlike in seeds, corroborating Hayashi *et al.* (2010); (ii) indicate that diterpenoid hormones increase *Physcomitrella* spore germination speed, thus have a positive effect on germination; and (iii) lend support to the notion that bryophyte bioactive diterpenoid hormones differ from those in seed plants.

ABA reduces *Physcomitrella* spore germination acting synergistically with sucrose

We have shown previously that ABA inhibits *Physcomitrella* spore germination in a dose-dependent manner (Moody *et al.*, 2016). *Physcomitrella* spores require approximately five-fold higher concentrations of ABA for strong inhibition of germination than *Arabidopsis* (Finkelstein, 1994).

In order to examine the effect of inhibiting ABA biosynthesis on spore germination, we treated WT spores with norflurazon. We saw a small but reproducible promotion of germination (Fig. 5a; see also Fig. 3a). To investigate a potential link between diterpenoid hormones and ABA-regulation of spore germination, we first tested whether the *cps/ks* mutant phenotype can be rescued via inhibition of ABA biosynthesis in moss spores.

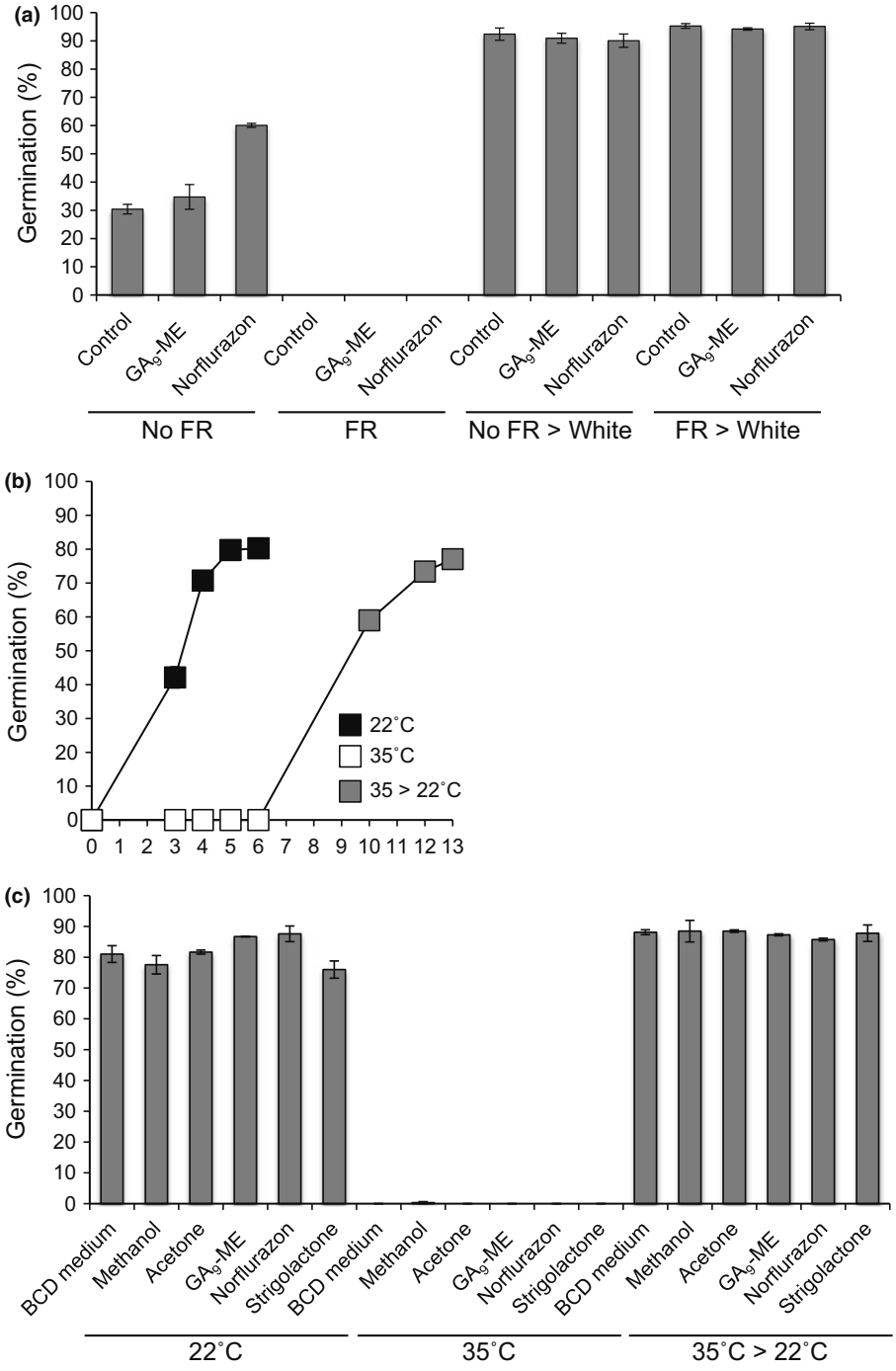

Fig. 3 Effects of environmental signals on *Physcomitrella* spore germination. (a) A pulse of far-red (FR) light inhibits spore germination and this cannot be rescued by norflurazon or GA_9-methyl ester (GA, gibberellin). Spores plated on control medium or GA_9-methyl ester (GA_9-ME) or norflurazon were treated ± FR light ('No FR' and 'FR', respectively) and immediately placed in the dark for 7 d. The spores' germination percentage was measured 7 d after treatment. All spores were then moved to white light for a further 7 d ('No FR > White' and 'FR > White', under which conditions all spores germinated to completion, showing that the effect of FR light is completely reversible. A Kruskal–Wallis test indicates differences between FR and white light-treated samples, $P < 0.05$. Error bars, ± SEM. (b) A temperature of 35°C inhibits spore germination and this is completely reversible when spores are returned to 22°C. (c) High-temperature inhibition of spore germination cannot be rescued by norflurazon, GA_9-methyl ester or the synthetic strigolactone analogue GR24 ('strigolactone' on the graph). Spores were germinated for 7 d at either 22°C or 35°C. The 35°C-treated spores were then transferred to 22°C for another 7 d ('35°C > 22°C'). A Kruskal–Wallis test indicated differences between 35°C and 22°C spores, $P < 0.05$. Error bars, ± SEM.

Norflurazon enhanced the germination of *cps/ks* mutant spores (Fig. 5b). Moreover, exogenously applied diterpenoid hormones could reverse the inhibitory effect of ABA on spore germination (Fig. 5c). This suggests that the balance of ABA and diterpenoid hormone levels may be important for regulating spore germination, but not with the same prominent role that these hormones have assumed in seed germination.

As ABA-mediated stress responses in *Physcomitrella* vegetative tissue involve accumulation of soluble sugars (Burch & Wilkinson, 2002; Nagao *et al.*, 2006; Oldenhof *et al.*, 2006; Bhyan *et al.*, 2012; Erxleben *et al.*, 2012), we tested the effect of low concentrations of sucrose (0.1–1%) on spore germination. Sucrose inhibited spore germination in a dose-dependent manner (Fig. 5d) and acted synergistically with ABA (Fig. 5e). Together these data show that ABA at relatively high concentrations reduces moss spore germination, as it does (although not as strongly as in) in seed germination, and suggest that *Physcomitrella* ABA-mediated inhibition of spore germination could share downstream mechanisms with ABA-mediated desiccation and freezing tolerance responses in the *Physcomitrella* gametophyte.

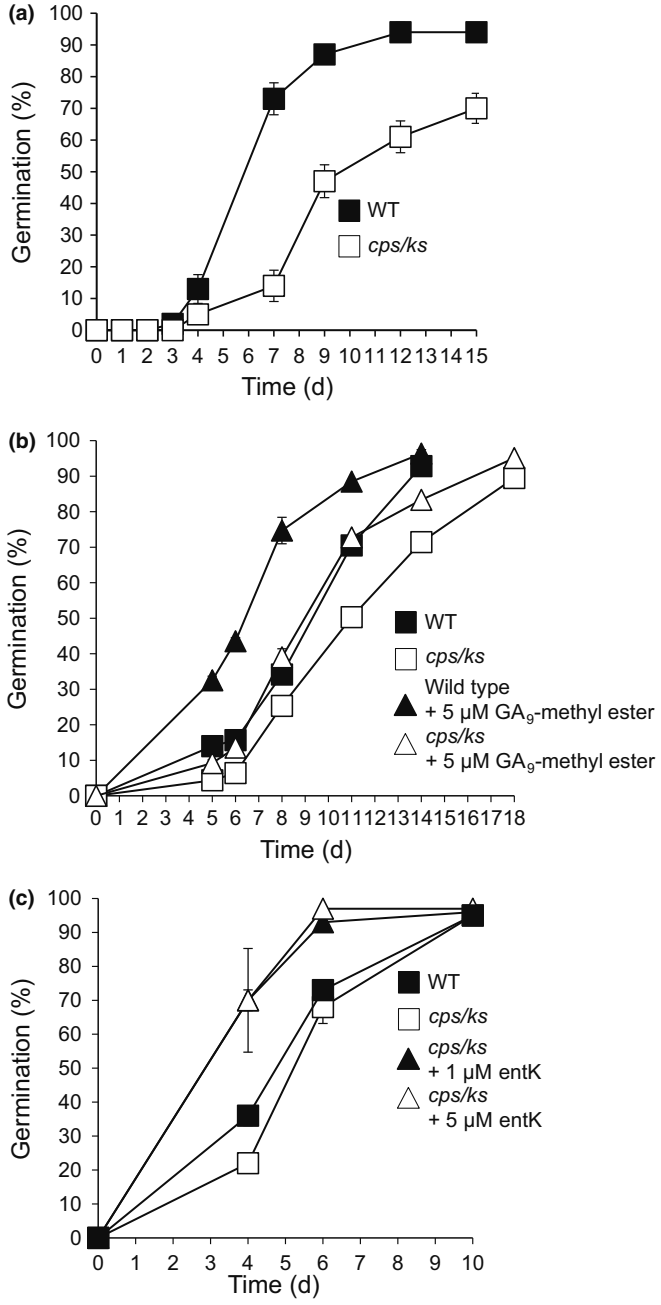

Fig. 4 Diterpenes promote *Physcomitrella* spore germination. (a) Comparison of wild-type (WT) and *copalyl-diphosphate synthase/kaurene synthase* (*cps/ks*) mutant (Zhan *et al.*, 2015) spore germination. (b) Effect of exogenous GA_9-methyl ester on WT and *cps/ks* mutant spores (GA, gibberellin). (c) Effect of exogenous *ent*-kaurene (entK) on *cps/ks* mutant spores. Error bars, ± SEM.

Ent-kaurene and ABA biosynthesis and signalling genes are expressed in spores

Our data suggest that diterpenoid hormones and ABA have subtle effects on spore germination compared with the absolute requirement for these hormones in regulating seed germination. To extend these findings, we asked whether the putative homologues of genes encoding the proteins responsible for biosynthesis and signal transduction of diterpenes and ABA are expressed in spores or during spore germination. We extracted RNA from dry spores, imbibed spores, germinating spores, protonemal filaments and leafy gametophytes. We performed semi-quantitative RT-PCR to detect expression of the *Physcomitrella* homologues of the *ent*-kaurene biosynthesis genes *CPS/KS* and *CYP701A3* (*ent-KO*) (Hayashi *et al.*, 2006; Miyazaki *et al.*, 2011), the first and second enzymes (respectively) in the putative moss diterpenoid hormone biosynthesis pathway (Hayashi *et al.*, 2006). The *CPS/KS* transcript was detectable during spore germination and was absent from dry spores, whereas the *ent-KO* transcript was detectable largely in dry spores (Fig. 6a), suggesting that spatial and temporal regulation of different stages of diterpene biosynthesis occurs during the *Physcomitrella* life cycle.

We also examined the expression of the putative gibberellin receptors *GLP1* and *GLP2* (Yasumura *et al.*, 2007), and the two *PpGAMYB* transcription factors *PpGAMYB1* and *PpGAMYB2* (Aya *et al.*, 2011). The putative gibberellin receptor *PpGLP1*, like *CYP701A3* (*ent-KO*), showed its highest expression in dry spores, decreasing upon imbibition and undetectable once germination occurs (Fig. 6b). The second putative gibberellin receptor, *PpGLP2*, was expressed more strongly than *PpGLP1*, but again showed strong expression in dry spores, decreasing markedly during imbibition (Fig. 6b). *PpGLP2* expression also was detected in germinating spores and, later, in leafy tissue (Fig. 5b). *PpGAMYB1* was expressed in all tissues tested, whereas *PpGAMYB2*, similarly to the *PpGLP*s and *ent-KO*, was detected in dry spores but decreased upon imbibition (Fig. 6b). *PpGAMYB2* was absent from germinating spores but present in protonemal and gametophyte tissues (Fig. 6b), corroborating Aya *et al.* (2011).

We also assessed the expression of the putative genes encoding the final two (cytosolic, ABA-specific) steps in the ABA biosynthesis pathway, namely two putative *Physcomitrella ABA DEFICIENT2* (*ABA2*) homologues and two putative *Physcomitrella ABSCISIC ALDEHYDE OXIDASE3* (*AAO3*) homologues (Hanada *et al.*, 2011). All genes showed expression in dry spores, germinating spores and leafy tissue, with one *PpABA2* and one *PpAAO3* also present in imbibed spores and one *PpAAO3* also present in protonema (Fig. 7a). We also tested the expression of putative ABA signalling genes. The four putative *PYRABACTIN-RESISTANCE 1/PYRABACTIN RESISTANCE 1-RELATED/REGULATORY COMPONENT OF ABA RECEPTOR* (*PYR/PYL/RCAR*) ABA receptors (Takezawa *et al.*, 2011) were expressed in all tissues tested (Fig. 7b), as were the two putative Class II SnRK phosphatases that were detectable in this assay (Fig. 7c) (out of the total six SnRKs in *Physcomitrella*; Takezawa *et al.*, 2011). Out of the two *Physcomitrella ABI-INSENSITIVE1* (*ABI1*) protein phosphatases (Komatsu *et al.*, 2013), *PpABI1a* was expressed ubiquitously (Fig. 7d), whereas *PpABI1b* was highly expressed in dry spores and leafy tissue (Fig. 7d). Out of the three ABA-regulated transcription factors *Physcomitrella ABA INSENSITIVE3A*, *-3B* and *-3C* (*PpABI3A*, *-3B* and *-3C*)(Khandelwal *et al.*, 2010), *PpABI3A* and *PpABI3C* were expressed in all tissues tested, whereas *PpABI3b* was largely absent from imbibed spores but present in other tissues (Fig. 7e).

In summary, all putative ABA biosynthesis genes were expressed in dry spores, with lower levels during imbibition, as is

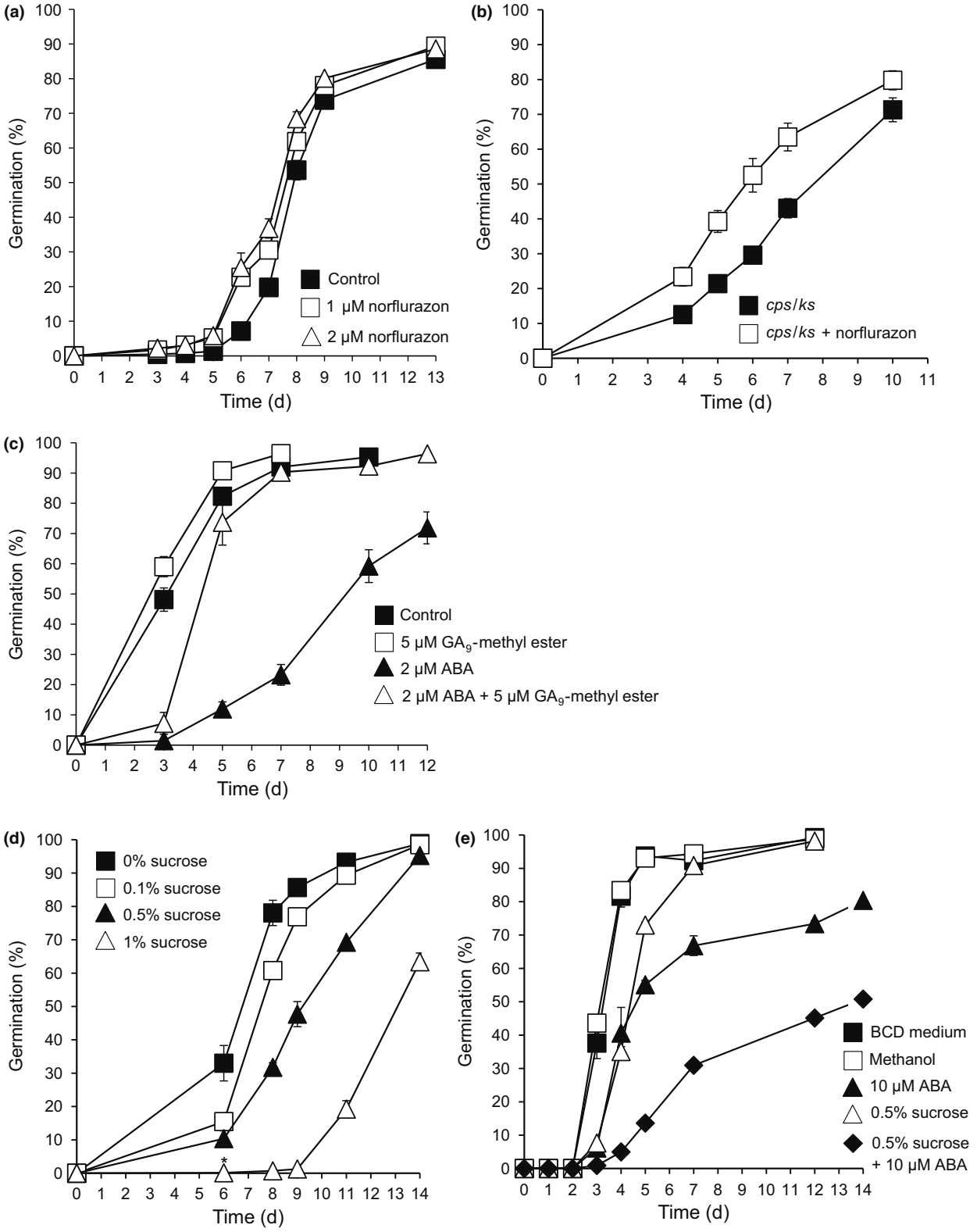

Fig. 5 Abscisic acid (ABA) and sucrose synergistically inhibit *Physcomitrella* spore germination. (a) Effect of norflurazon on wild-type (WT) spore germination. Five micromolar norflurazon has no further effect (data not shown). (b) Effect of 2 μM norflurazon on *cps* spores. (c) GA_9-methyl ester can rescue the inhibition of germination by ABA (GA, gibberellin). (d) Dose-dependent inhibition of germination by sucrose. Sucrose was dissolved in BCD medium. (e) Synergistic inhibitory effect of ABA and sucrose on spore germination: an intermediate concentration of both ABA and sucrose was used; the 0.5% sucrose control also contains matched solvent. Error bars, ± SEM.

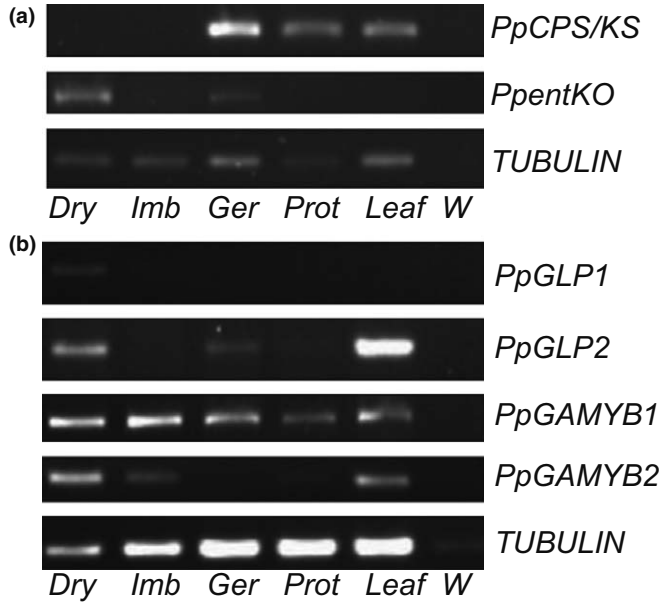

Fig. 6 Expression of diterpene biosynthesis and putative response genes in *Physcomitrella* tissues. (a) Reverse transcription (RT)-PCR of the *Physcomitrella* diterpene biosynthesis gene *PpCPS/KS* and putative diterpene biosynthesis gene *PpentKO* in *Physcomitrella* tissues compared to a *PpTUBULIN* control. (b) RT-PCR of the putative *Physcomitrella* diterpene response genes *PpGLP1* and *PpGLP2* and *PpGAMYB1* and *PpGAMYB2* in *Physcomitrella* tissues compared to a *PpTUBULIN* control. Dry, Dry spores; Imb, imbibed spores; Ger, germinating spores; Prot, protonema; Leaf, Leafy gametophores; W, water control.

the putative second gene in the diterpene biosynthesis pathway. The initial diterpene biosynthesis gene transcript, *PpCPS/KS*, was detected only after imbibition during germination and growth. Putative ABA signalling genes were largely expressed ubiquitously, although many were expressed more highly in dry spores than imbibed spores. Putative GA signalling genes (receptors and GAMYBs) were all expressed in dry spores but largely decreasee in expression level during imbibition.

Strigolactones inhibit *Physcomitrella* spore germination

In order to extend our findings around the hormonal control of spore germination, we examined the effect of other hormones known to affect seed germination. Strigolactones promote seed germination in a variety of plants (Akiyama & Hayashi, 2006) and have been suggested to affect *Physcomitrella* spore germination (Proust *et al.*, 2011). We tested whether strigolactones affected spore germination by comparing the germination of WT spores with those of the *Physcomitrella ccd8* mutant, which cannot synthesize SLs (Proust *et al.*, 2011). The *ccd8* mutant showed increased germination (Fig. 8a), corroborating an unpublished observation by Proust *et al.* (2011). The *ccd8* mutant's increased germination could be reduced to levels closer to that of WT spores by exogenous application of GR24 (Fig. 8b). Moreover, exogenous GR24 reduced the germination speed of WT *Physcomitrella* spores at concentrations of 0.1 μM (Fig. 8b). This indicates that in *Physcomitrella*, unlike in *Arabidopsis* and parasitic plants, strigolactones have an inhibitory role in the germination process.

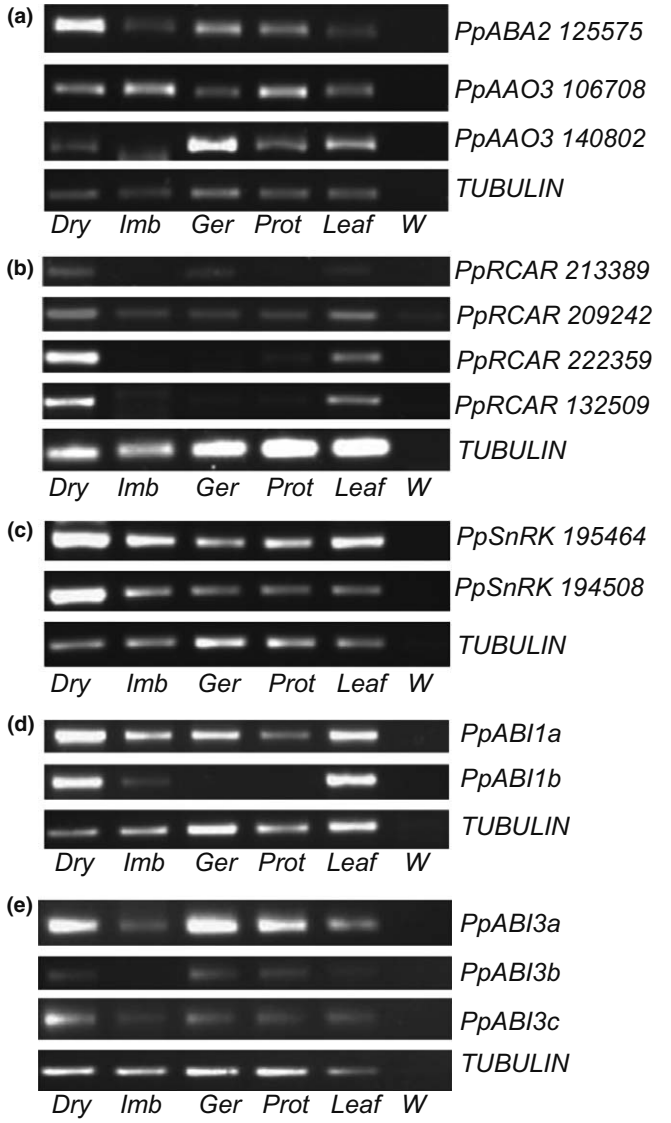

Fig. 7 Expression of abscisic acid (ABA) biosynthesis and response genes in *Physcomitrella* tissues. (a) Reverse transcription (RT)-PCR of the putative *Physcomitrella* ABA biosynthesis genes *PpABA2* and *PpAAO3* (two homologues; Hanada *et al.*, 2011) in *Physcomitrella* tissues. (b) RT-PCR of the four putative *Physcomitrella* ABA receptors (RCARs; Hanada *et al.*, 2011) in *Physcomitrella* tissues. (c) RT-PCR of the two putative *Physcomitrella* ABA signalling kinases (SnRKs; Hanada *et al.*, 2011) in *Physcomitrella* tissues. (d) RT-PCR of the *Physcomitrella ABI1* ABA-response genes (Sakata *et al.*, 2009) in *Physcomitrella* tissues. (e) RT-PCR of the *Physcomitrella ABI3* ABA-response genes (Khandelwal *et al.*, 2010) in *Physcomitrella* tissues. All gene expression was compared to a *PpTUBULIN* control. Dry, dry spores; Imb, imbibed spores; Ger, germinating spores; Prot, protonema; Leaf, Leafy gametophores; W, water control.

The ethylene precursor ACC inhibits *Physcomitrella* spore germination

Like strigolactone, ethylene has been shown to promote seed germination in *Arabidopsis* and other dicots, acting antagonistically to ABA. We showed that application of 50 μM–1 mM 1-aminocyclopropane-1-carboxylic acid (ACC), the ethylene precursor that is cleaved by ACC oxidase to release ethylene, inhibited *Physcomitrella* spore germination in a dose-dependent manner

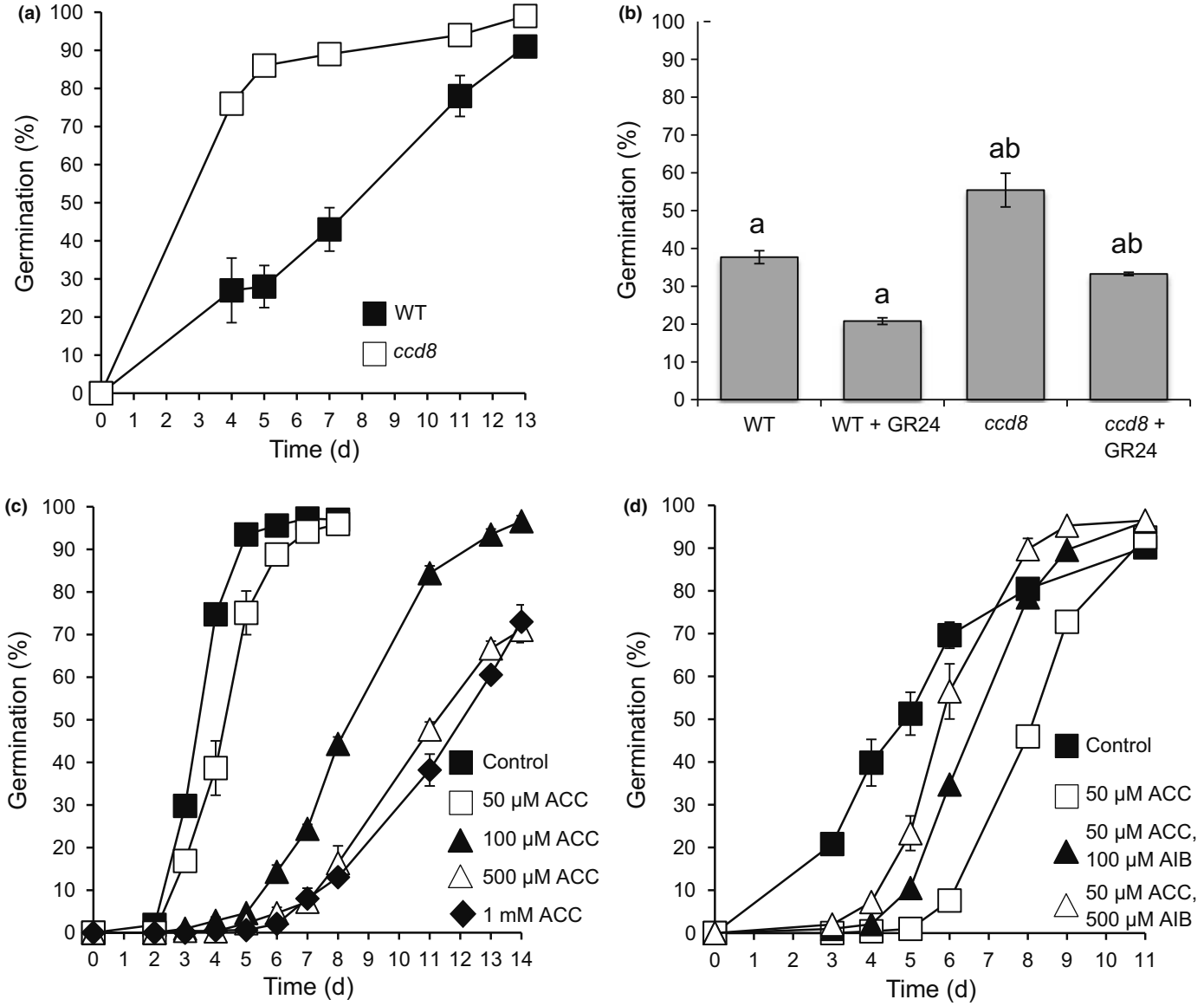

Fig. 8 Strigolactones and an ethylene precursor reduce *Physcomitrella* spore germination rate. (a) *Ppccd8* strigolactone biosynthesis mutants (Proust *et al.*, 2011) germinate faster than their corresponding wild-type (WT). (b) *Ppccd8* mutant germination can be inhibited by exogenously applied synthetic strigolactone GR24, which also inhibits WT spore germination. Day 5 data shown; a Kruskal–Wallis test indicates significant differences between samples, $P < 0.05$ as indicated by the letters on the graph. (c) The ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) inhibits WT spore germination rate. (d) The ethylene inhibitor aminoisobutyric acid (AIB) can rescue the effects of ACC on spore germination. Error bars ± SEM.

(Fig. 8c), which is the converse of its effects in seeds (where experiments commonly use a dose of 1 mM ACC), but similar to strigolactone's effects on spores. Moreover, the ethylene biosynthesis inhibitor aminoisobutyric acid, which competitively inhibits ACC oxidase, can overcome the effects of ACC (Fig. 8d).

Discussion

Environmental regulation of germination by light and high temperature is not integrated by the same hormones in *Physcomitrella* spores compared with seeds

We have shown that unlike most seeds, *Physcomitrella* spores grown in laboratory conditions do not show primary dormancy and after-ripening. A lack of primary dormancy has been reported in several moss and liverwort species (McLetchie, 1999; Glime, 2015).

We have also shown that, in a similar way to seeds, germination-competent *Physcomitrella* spores can be prevented from germinating using environmental cues. This is in line with evidence that conditional (secondary) dormancy exists in *Sphagnum* allowing formation of a spore bank (Sundberg & Rydin, 2000) and in *Physcomitrium sphaericum* (Furness & Hall, 1981). The interface of environmental signals with known hormonal networks is not conserved between *Physcomitrella* spores and flowering plant seeds.

Seed germination and spore germination can both be reversibly inhibited by a pulse of far-red (FR) light (e.g. Seo *et al.*,

2006; Possart & Hiltbrunner, 2013) or elevated temperatures (32°C in *Arabidopsis*, Toh *et al.*, 2008; 35°C in *Physcomitrella*, this work). This shows that both spores and nondormant seeds respond to environmental cues that block germination, and suggests that this is an evolutionarily early adaptation for plants to live, and move around, on land. Our experiments suggest a higher degree of temperature tolerance in *Physcomitrella* spores compared with seeds: *Physcomitrella* vegetative tissue is also more tolerant to abiotic stress than that of seed plants (Frank *et al.*, 2005). Temperatures of 35°C are known to inhibit germination in the moss *Physcomitrium* (Furness & Hall, 1981) and in two species of liverwort (Chopra & Kumra, 1988), although this was not shown to be reversible (Chopra & Kumra, 1988).

Unlike in seeds (Ikuma & Thimann, 1960; Schopfer *et al.*, 2001; Oh *et al.*, 2006; Seo *et al.*, 2006; Nelson *et al.*, 2009), FR-inhibition of *Physcomitrella* spore germination cannot be rescued by addition of diterpenes or inhibition of abscisic acid (ABA) synthesis. Furthermore, unlike in seeds (Toh *et al.*, 2012), addition of diterpenes, norflurazon or strigolactones (SLs) cannot rescue the germination of spores inhibited by high temperature. Thus, a rescue mechanism for FR- and thermo-inhibition in *Physcomitrella* spores is divergent compared with seeds, and remains to be elucidated.

In seeds, environmentally regulated inhibition of germination impinges on ABA and gibberellin (GA) synthesis and metabolism (Seo *et al.*, 2006; Toh *et al.*, 2012). Crosstalk between light and GA/ABA signalling in seeds occurs at multiple levels, including via effects on hormone metabolism (and subsequent hormone levels), via transcriptional changes in signalling genes (reviewed in Piskurewicz *et al.*, 2009), and via interactions between transcription factors from different pathways (e.g. Richter *et al.*, 2010; Casal, 2013; Tang *et al.*, 2013). For example, FR light blocks germination in dicots by repressing the expression of *GA_3-oxidase* (Toyomasu *et al.*, 1998; Yamauchi *et al.*, 2004), which catalyses a step in gibberellin biosynthesis that does not exist in *Physcomitrella* (Stewart & Freebairn, 1969; Hayashi *et al.*, 2010; Zhan *et al.*, 2015). Moreover, *Physcomitrella* seems not to have clear orthologues of FHY3/FAR1 or ABI5 (Rensing *et al.*, 2008) (which integrate ABA and light signalling in *Arabidopsis*; Tang *et al.*, 2013) and has divergent DELLA proteins that have not been shown to transduce gibberellin signalling (Yasumura *et al.*, 2007). Thus, the 'wiring' of the interface between environmental and hormonal regulation of seed germination evolved after the divergence of the bryophyte lineage. Our work suggests that a novel trigger for light-induced germination exists in moss. Furthermore, divergent molecular networks mediate conserved developmental responses to environmental stimuli in spores and seeds to enable plant movement on land.

Diterpenes and ABA affect germination in *Physcomitrella* spores but appear to have a modulatory role, in contrast to the critical role of GA and ABA in seeds

We showed, on the one hand, that certain diterpenoid hormones have a positive effect on germination in *Physcomitrella* spores and, on the other, that ABA has a negative effect on spore germination. Furthermore, *Physcomitrella ent-KO*, ABA synthesis genes and putative diterpene- and ABA-signal transduction pathway genes were expressed in spores. Corroborating previous work (Hayashi *et al.*, 2010; Zhan *et al.*, 2015), we saw that the bioactive diterpenes in *Physcomitrella* are those at an early step in the biosynthesis pathway (*ent*-kaurene), or those that show activity in ferns and also spore-bearing plants (GA_9-methyl ester), rather than those active in seed plants. We found that diterpenoids are not required for *Physcomitrella* spore germination (corroborating Hayashi *et al.*, 2010), but that they do influence germination in a positive way. Although Hayashi *et al.* (2010) did not report a germination phenotype for their *cps* mutant, their mutant spores were compared with wild-type (WT) only at a single time point, so no measure of germination rate was made and thus relatively subtle differences were probably overlooked. *Physcomitrella* GAMYB proteins, homologues of which are regulated by gibberellin signalling in flowering plants, are required for correct spore coat formation (Aya *et al.*, 2011).

Mutant analysis in *Arabidopsis* has demonstrated that bioactive gibberellins and gibberellin signalling are absolutely required for seed germination to occur (Koornneef & van der Veen, 1980), whereas *Arabidopsis* seed germination is completely inhibited by concentrations of ABA as low as 5 μM (Finkelstein, 1994). The effects of gibberellins and ABA in *Physcomitrella* spores were not as extreme: the *cps* mutant has a slower germination rate than WT, but *cps* mutant spores can eventually germinate to the same level as WT controls, demonstrating no loss of germination potential. The concentration of ABA required for strong inhibition of *Physcomitrella* spore germination was also five- to 10-fold higher than for *Arabidopsis* seeds, although different spore batches vary in their ABA sensitivity, as is the case for *Arabidopsis* seeds (Finkelstein, 1994), as might be expected in an ephemeral species (Glime, 2013).

The effect of norflurazon on *Physcomitrella* spores was subtle, which may be due to the lack of primary dormancy in spores, which are essentially 'ready to germinate'. The extent to which norflurazon reduces ABA levels in *Physcomitrella* is not known.

This suggests that diterpenes and ABA perform a modulatory role during *Physcomitrella* spore germination, and gained a more prominent and complex role in multicellular seeds after co-option into the sporophyte during the evolution of seed plants (e.g. Piskurewicz *et al.*, 2009). Our data comparing the behaviour of *physcodillo* mutant spores and *arabidillo* mutant seeds on ABA suggest that these Armadillo-related protein homologues may represent a conserved node in an ancient regulatory network (Moody *et al.*, 2016).

The function of ABA in *Physcomitrella* previously has been studied in gametophytic vegetative tissues, where a role in stress responses (such as drought) has been demonstrated, as in the flowering plant sporophyte (Knight *et al.*, 1995; Cuming *et al.*, 2007; Khandelwal *et al.*, 2010; Takezawa *et al.*, 2011). Several studies have demonstrated a role for sugars (including sucrose) acting synergistically with ABA during moss abiotic stress resistance (Burch & Wilkinson, 2002; Nagao *et al.*, 2006; Oldenhof *et al.*, 2006; Bhyan *et al.*, 2012; Erxleben *et al.*, 2012). We

demonstrated that a similar synergism may exist during spore germination. The level of sucrose that can decrease spore germination rate (0.1–1% in this study) is 10- to 100-fold lower than that used to cause osmotic stress in moss (10%: Garrocho-Villegas & Arredondo-Peter, 2008). Evidence exists for a regulatory role of sugars during seed germination: an inhibitory effect of low levels of sucrose (0.5–3%) on *Arabidopsis* seed germination up to 5 d has been observed (Chen *et al.*, 2006; Li *et al.*, 2012). This effect requires functional ABA biosynthesis, because *aba2* mutant seeds are sucrose-insensitive (Li *et al.*, 2012). However, Finkelstein & Lynch (2000) showed that 1–2% sucrose could counteract the effects of 3 μM ABA on *Arabidopsis* seed germination after 7 d, suggesting that different interactions between sucrose and ABA may occur at different times.

A novel role for SLs and ethylene in inhibiting spore germination in *Physcomitrella*

We showed that SLs have a negative effect on *Physcomitrella* spore germination. The role of SLs in seed plant germination is a positive one: they act as signals to promote seed germination either between plants (via root exudates) or within one plant (e.g. during thermoinhibition) (Bouwmeester *et al.*, 2003; Yoneyama *et al.*, 2010; Toh *et al.*, 2012; Stanga *et al.*, 2013). This occurs via regulation of gibberellin biosynthesis (Nelson *et al.*, 2009), lending weight to the hypothesis that SLs have a different mechanism of action in *Physcomitrella* germination. SLs are well known as regulators of shoot branching in seed plants (Waldie *et al.*, 2014). This branching function is conserved in the *Physcomitrella* gametophyte (Proust *et al.*, 2011). In addition, SLs in moss promote 'self-awareness' and delimit colony spread, acting as quorum-sensing molecules (Proust *et al.*, 2011), suggesting that the 'between-plant' communication function of SLs arose early in land plant evolution. Perhaps in bryophytes the inhibitory role of SLs in spore germination arose as a quorum-sensing function; thus, when spores are released from the plant, the formation of one colony from a spore could prevent the germination of a second colony-forming spore close by, and hence aid colony establishment without competition for resources.

Interestingly, ethylene also has a positive role in seed germination and the ethylene precursor ACC has a negative role in spore germination: as ethylene is also a small, gaseous and easily diffusible hormone, its presence in a developing moss gametophyte could also signal to neighbouring spores and prevent their germination. Ethylene affects seed germination via crosstalk with ABA signalling/synthesis, via synergism with GA signalling and via direct effects on cell separation of the endosperm in a number of plant species (Linkies & Leubner-Metzger, 2012). This suggests that ethylene may have been co-opted separately into spores and seeds to perform different roles.

Comparing the regulation of germination in *Physcomitrella* spores and nondormant seeds

The regulation of equivalent developmental processes between gametophyte and sporophyte seems to show an amazing degree of similarity in comparative studies on key model organisms. For example, *Physcomitrella* rhizoids are developmentally equivalent to *Arabidopsis* root hairs (Menand *et al.*, 2007), and GAMYB functions in spore and reproductive organ development are equivalent (Aya *et al.*, 2011), whereas the liverwort *Marchantia polymorpha* shows circadian regulation of the vegetative-to-reproductive transition, as in the seed plant sporophyte (Kubota *et al.*, 2014). We have shown that spores and seeds respond to the same environmental cues to generate the same developmental output, but via different mechanisms.

Our results suggest evolution of novel hormonal regulation of germination between *Physcomitrella* spores and nondormant/after-ripened seeds, with ABA and GA assuming much greater importance in seed plants, multilevel crosstalk between environmental and hormone pathways evolving in seeds, and some hormones being co-opted into different roles in spores and seeds. Similar network rewiring has been identified between root hairs and rhizoids, between RHD SIX-LIKE transcription factors and auxin signalling (Jang *et al.*, 2011; Pires *et al.*, 2013).

One possibility is that the multicellular nature of seeds may have led to a requirement for complex, coordinated hormonal regulation of the different tissues during germination. Determining the molecular nature of the signalling pathways that regulate spore germination is now a key target for future research. Alternatively, as spores appear not to show dormancy, there may be no requirement for a complex hormonal regime regulating dispersal. It is possible that a 'bet-hedging' strategy controlling germination rates due to life history may exist in *Physcomitrella* spores as in *Arabidopsis* (Springthorpe & Penfield, 2015). The absence of observed dormancy in moss spores also raises the question of how these dispersal units function to colonize new environments.

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Author contributions

E.F.V., Y.S., L.A.M., G.W.B., H.T.S. and J.C.C. planned and designed the research and conceived the experiments. E.F.V., Y.S., L.A.M., D.H., A.W., S.N., A.C., B.B., D.M., S.J.B., H.B., B.C.K. and J.C.C. performed the research. E.F.V., Y.S., L.A.M., D.H., A.W., S.N., H.B., B.C.K., G.W.B., H.T.S. and J.C.C. analysed data. E.F.V., L.A.M., H.B., G.W.B., H.T.S. and J.C.C. wrote the paper.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Moss bioactive gibberellins promote *Physcomitrella* spore germination.

Fig. S2 Gibberellins that are bioactive in *Physcomitrella* cannot rescue the *Arabidopsis ga1-3* mutant seed germination phenotype and substitute for GA_3.

Table S1 Primers used for RT-PCR analysis

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