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Molecular and physiological responses to desiccation indicate the abscisic acid pathway is conserved in the peat moss, *Sphagnum*

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Abstract

Mosses of the genus *Sphagnum* are the main components of peatlands, a major carbon-storing ecosystem. Changes in precipitation patterns are predicted to affect water relations in this ecosystem, but the effect of desiccation on the physiological and molecular processes in *Sphagnum* is still largely unexplored. Here we show that different *Sphagnum* species have differential physiological and molecular responses to desiccation but, surprisingly, this is not directly correlated with their position in relation to the water table. In addition, the expression of drought responsive genes is increased upon water withdrawal in all species. This increase in gene expression is accompanied by an increase in abscisic acid (ABA), supporting a role for ABA during desiccation responses in *Sphagnum*. Not only do ABA levels increase upon desiccation, but *Sphagnum* plants pre-treated with ABA display increased tolerance to desiccation, suggesting that ABA levels play a functional role in the response. In addition, many of the ABA signalling components are present in *Sphagnum* and we demonstrate, by complementation in *Physcomitrium patens*, that *Sphagnum* ABI3 is functionally conserved. The data presented here, therefore, support a conserved role for ABA in desiccation responses in *Sphagnum*.

Keywords: ABA, chlorophyll fluorescence, desiccation, drought, moss, peat, relative water content, *Sphagnum*

Introduction

One of the biggest challenges facing primordial plants as they moved to terrestrial environments was how to cope with water stress. Arguably, the most important development of the water-to-land transition was the development of systems that allowed the plant to maintain an internal water potential independently from the water potential of the environment. These include the development of vascular systems, physical barriers to water loss in the form of cuticles, and complex drought-responsive cellular signalling pathways, all features of vascular plants. However, some plants developed strategies to avoid water stress...
by living in water-saturated terrestrial environments, increasing plant density or reducing plant size to reduce evaporation. Others evolved physiological mechanisms to tolerate short periods of drought and a few can survive nearly full desiccation. Despite this, a degree of tolerance to water loss is necessary in all the habitats that are not constantly water saturated. Even short periods of mild drought have deleterious effects in terms of plant growth and development, and plants need to develop strategies to mitigate these. Understanding these strategies is even more important given climate change scenarios in which temperature and rainfall are predicted to become more stochastic.

Traditionally, plant responses to drought have been defined as escape (accelerating reproduction), avoidance (developing strategies to maintain high internal water content), and tolerance (maintaining growth under low internal water content). Drought tolerance has been extensively studied in vascular plants and involves the activation of mechanisms to prevent water loss and increase water uptake, and pathways leading to the induction of drought responsive genes (Chaves et al., 2003; Yao et al., 2021). Central to these tolerance processes is the stress hormone abscisic acid (ABA). ABA rapidly increases at the onset of drought and triggers a complex and multi-layered signalling cascade leading to the transcription of drought responsive genes that enable the plant to maintain and adapt physiological processes under drought (Ali et al., 2020).

Although most attention has focused on the effect of drought on agricultural systems, drought also poses a significant threat to wetland environments such as peatlands (Stirling et al., 2020). Peatlands are important carbon-storing ecosystems in temperate and boreal regions and they are now seen as crucial to climate change mitigation strategies (Leifld and Menichetti, 2018). Reversing human impact on peatland degradation often involves restoring the water table and vegetational cover on bare peat surfaces. However, response of key plants in these peatland ecosystems to drought is poorly understood.

Mosses of the genus Sphagnum are major components of acidic peatlands where they regulate water relations and carbon sequestration through peat accumulation. In common with all bryophytes, Sphagnum mosses are poikilohydric meaning that they are unable to maintain internal water potential different from their environment (Raven, 1995). Although typical mosses lack many of the adaptations of vascular plants to reduce water loss such as cuticle and stomata, they have developed alternative specialized features to store and regulate water distribution across the plant (Proctor et al., 2007; Charron and Quatrano, 2009). These include morphological adaptations such as long and densely packed branches, growing in tightly packed cushions to prevent evaporation, and the development of specialized water storage cells that use water tension to keep the plant hydrated while minimally affecting gas exchange (Dilks and Proctor, 1979; Proctor, 2000; Proctor et al., 2007). Physiological adaptations include the development of desiccation tolerance that enables mosses to recover after completely drying out (Oliver et al., 2005). This is achieved through the presence of constitutive mechanisms of cellular protection and a rehydration-induced repair and recovery process (Proctor et al., 2007). These mechanisms are well studied in the moss Tortula ruralis, where the constitutive expression of Late Embryogenic Abundant (LEA) proteins is essential for cellular protection and maintaining membrane integrity during drying (Oliver et al., 2005). The development of desiccation avoidance and tolerance mechanisms may have allowed mosses to colonize a wide range of habitats across all continents, making the group very successful and resilient (Charron and Quatrano, 2009).

Unlike most bryophytes, Sphagnum species have been considered to be drought and desiccation-intolerant (Clymo, 1973; Clymo and Hayward, 1982). More recently, this view has been challenged, and while some authors report low recovery even after mild desiccation, other studies suggest that Sphagnum shoots can tolerate strong desiccation (reviewed in Hájek and Vicherová, 2014). The ability (or otherwise) of Sphagnum to survive water-limiting conditions would have implications for the restoration and management of peatland ecosystems. With predicted changes in climatic patterns, peatlands are considered to be key to mitigation strategies. Therefore, understanding how Sphagnum mosses respond to drought and desiccation is necessary and timely (Leifld and Menichetti, 2018).

There may be intrinsic variation in desiccation tolerance within the genus. A typical peatland consists of a variety of microhabitats ranging from permanently flooded pools, wet depressions (hollows), elevated hummocks, and everything in between. Access to the water table in these microhabitats is different and it has been proposed that the Sphagnum species that inhabit each habitat have evolved different mechanisms to cope with changes in water availability (Johnson et al., 2015). Thus, hummock species that grow further away from the water table are expected to be better at maintaining colony water content and thus avoiding desiccation, while hollow species with constant access to the water table would be less desiccation tolerant. Experimental evidence seems to show that this is not always the case, with some studies finding no differences between position along the water table and desiccation tolerance (Wagner and Titus, 1984) while others find that hollow species actually show better recovery after desiccation (Schipperges and Rydin, 1998).

While some of the physiological mechanisms for Sphagnum response to water limiting conditions have been investigated, including the loss and recovery of photosynthetic function, not much is known about the molecular responses to desiccation (Wagner and Titus, 1984; Schipperges and Rydin, 1998; Hájek and Vicherová, 2014; Winnicka and Melosik, 2019). It has previously been shown that drought responsive gene expression is up-regulated in Sphagnum when subjected to drought and that ABA increases desiccation tolerance in Sphagnum (Marshall and Borbely, 2011; Hájek and Vicherová, 2014; Winnicka and Melosik, 2019). Despite this, a systematic and comparative analysis including both physiological and molecular responses.
of different Sphagnum species from different microhabitats to controlled drought and rehydration is lacking.

Here we show that desiccation responses differ in different Sphagnum species from the same blanket bog. Photosynthetic function and water loss are different between the species and this translates into differential activation of drought responsive gene expression. Our data confirm that ABA pre-treatment improves desiccation tolerance in Sphagnum and, significantly, that ABA levels increase upon desiccation in Sphagnum. Finally, we show that components of the ABA signalling pathway in Sphagnum are functional when expressed in Physcomitrium (Physcomitrella) patens. Our results therefore support a functional role for ABA and ABA responsive genes in desiccation responses in Sphagnum.

Materials and methods

Site collection and plant material

Sphagnum species were collected from a minerotrophic mire in Pen y Garn (SN791758) situated in the Cambrian Mountains, Wales, UK. The mire is located at the base of a mountain and contains dense carpets of Sphagnum as well as several pool areas that exhibit mild flow after heavy rain. Four Sphagnum species, Sphagnum fallax (Cuspidata), Sphagnum papillosum (Sphagnum), Sphagnum capillifolium (Acutifolia), and Sphagnum inundatum (Subsecunda), were selected to cover the four most abundant species from the same blanket bog. Photosynthetic active radiation, after which Fv/Fm was calculated using the formula $F_{m} - F_{o}/F_{m}$. In order to determine how chlorophyll fluorescence values change in the different parts of the Sphagnum stem as it dries, areas containing the different zones (capitula, live stem, senescing stem) were manually selected and $F_{v}/F_{m}$ calculated for these areas.

Relative water content

RWC was calculated for each plate by weighing fresh four capitula and then allowing them to completely dry in an oven at 80 °C for at least 24 h before weighing them again (dry weight). RWC was calculated as a difference between fresh and dry weight divided by dry weight and expressed as a percentage relative to the non-droughted material.

RNA extraction and qPCR

Capitula from the different species were harvested at the different time points as described above and ground to a fine powder in liquid nitrogen; 500 mg was used for ABA analysis and the rest for RNA extraction using the Spectrum Total RNA kit (Sigma) with in-column DNase treatment (Quagen) following the manufacturer’s instructions. RNA integrity was determined by gel electrophoresis and 500 ng of total RNA was used to prepare cDNA using the SuperScript III First Strand Synthesis System (Thermo Fisher Scientific). Quantitative polymerase chain reactions (qPCRs) were performed using a LightCycler 480 (Roche). Drought responsive genes were selected based on a study (Cuming et al., 2007) in P. patens and Sphagnum homologues identified using Phytozone (https://phytozone.jgi.doe.gov/). The gene identifiers for both P. patens and Sphagnum are included in Supplementary Table S1. Specific primers able to amplify the genes in the four different species were designed using Primer 3 (Supplementary Table S1). Typically, 10 ng of cDNA was used in a 20 μl reaction containing 0.25 μM of each primer and 10 μl LightCycler 480 SYBR Green I Master (Roche). Three biological samples per species per time point were used and each reaction was carried out in triplicate. Sphagnum EF1α (Sphfad03G00870000) and GAPDH (Sphfad16G0760000) transcripts were used as references (Winnicka and Melosik, 2019; see Supplementary Table S1 for primer sequences). Data were analysed using the LightCycler 480 Software (Roche). The data shown are means ± SD of three biological replicates.

ABA measurements

Capitula from the different species were harvested at the different time points as described above and ground in liquid nitrogen to a fine powder. For each sample 50–500 mg of tissue was weighed and extraction performed in 70% (v/v) HPLC grade MeOH (Fisher Scientific) with ultra-pure H2O (18.2 MΩ). The samples were analysed with flow infusion–electro-spray ionization–high resolution mass spectrometry (FI-ESI-MS). The mass spectra were acquired on an Accela (ThermoFinnigan, San Jose, CA, USA) ultra-performance liquid chromatography system coupled to an Exactive Orbitrap (ThermoFinnigan).
ABA-mediated desiccation responses in *Sphagnum*

**Fig. 1.** Details of the species used in this study and experimental set-up. (A) Four *Sphagnum* species were used, *S. fallax*, *S. papillosum*, *S. capillifolium*, and *S. inundatum*, belonging to the most abundant subgenera and spanning a range of microhabitats as indicated. Mean distances to the water table, as measured over 3 years at the Pen y Garn site, are also shown. Top images show the species in the field (scale bar: 10 cm) and bottom images after they have been assembled into square Petri dishes opened at the top to allow evaporation (cosms) at a density comparable to that observed in the field (scale bar: 1 cm). (B) Desiccation treatment set-up. The cosms were placed upright in a tray and kept in the greenhouse under natural light conditions. (C) Morphology of the different *Sphagnum* species as indicated. Top row, whole plants; bottom row detached capitula detail (scale bar: 1 cm).

| Species   | Subgenus  | Habitat       | Distance to water table |
|-----------|-----------|---------------|-------------------------|
| *S. fallax* | Cuspidata | Hollow/Lawn   | 12.3 cm (n=55) (min 2–max 23) |
| *S. papillosum* | Sphagnum | Hummock       | 11.3 cm (n=43) (min 1–max 30) |
| *S. capillifolium* | Acutifolia | Hummock      | 22.4 cm (n=7) (min 12–max 30) |
| *S. inundatum* | Subsecunda | Hollow/Pool  | 5.6 cm (n=11) (min 0–max 7) |

**ABA and desiccation treatments**

*Sphagnum fallax* plants were placed in cosms as described above at a density of 33 plants per plate, four plates per each of the three ABA treatments and two desiccation regimes, 24 plates in total. The plates were then placed in tubs filled with water, 10 μM ABA, or 50 μM ABA solution so that about two-thirds of the cosm was submerged and left for 24 h. After this time, the liquid was drained out and excess allowed to drain from the plates for 1 h. All the cosms were weighed (starting weight) and chlorophyll fluorescence measured (see above), and this corresponded to time point 0. Plates were again weighed, and imaged every day until each plate...
reached a value of \( F_1/F_0 \) of 0.2. At that point the plate was immediately rehydrated in water overnight (1 d water withdrawal) or left for 4 d before rehydration (4 d water withdrawal) according to what treatment it was part of. Plates were then weighed and chlorophyll fluorescence measured 1 and 2 d after rehydration. Chlorophyll fluorescence recovery was calculated as the ratio of the \( F_1/F_0 \) measurement at day 2 after re-watering and the \( F_1/F_0 \) measurement on day 0.

**Phylogenetic analysis of ABA signalling genes in Sphagnum**

For the construction of the phylogenetic trees, we used a dataset of homologues from Arabidopsis (Arabidopsis thaliana Araport11, Cheng et al., 2017), Oryza sativa (Oryza sativa v7.0, Ouyang et al., 2007), Selaginella moellendorffii (Selaginella moellendorffii v1.0, Banks et al., 2011), Marchantia polymorpha (Marchantia polymorpha v3.1, Bowman et al., 2017), Physcomitrium patens (Physcomitrium patens v3.3, Lang et al., 2018), Sphagnum fallax (Sphagnum fallax v1.1, DOE-JGI, http://phytozome.jgi.doe.gov/), Sphagnum magellanicum (Sphagnum magellanicum v1.1, DOE-JGI, http://phytozome.jgi.doe.gov/), and Ceratopteris richardii (Ceratopteris richardii v2.1, DOE-JGI, http://phytozome.jgi.doe.gov/). For ABI3 only. The homologues were obtained with a BLASTP search against the proteome database from Phytozome 13 (http://phytozome-next.jgi.doe.gov; Goodstein et al., 2012). Deduced amino acid sequences were aligned using MAFFT (Katoh et al., 2019). After elimination of all positions of gaps and short sequences manually, conserved amino acid residues were used in calculations for each gene using the maximum likelihood (ML) method and JTT model (Jones et al., 1992) to construct a ML tree in MEGA-X (Kumar et al., 2018). Statistical support for internal branches by bootstrap analyses (Felsenstein, 1985) was calculated using 1000 replications. All sequences were obtained using the Arabidopsis homologues as query. To build the tree for ABI3 proteins, a significant similarity E-value <1e−10 was used, amino acid sequences lacking conserved B1, B2, and B3 regions (Marella et al., 2006) were deleted from the alignment, and 191 amino acid residues were used to calculate evolution distances for 18 genes. For SnK2 an E-value <1e−63 was used and 300 amino acid residues were used to calculate evolution distances for 35 genes. For PYR1 the E-value was <1e−11 and 129 amino acid residues were used to calculate evolution distances for 48 genes. For ABI1 an E-value <1e−35 was used and 234 amino acid residues were used to calculate evolution distances for 53 genes. For ABI5, an E-value <1e−8 was used, the amino acid sequences lacking a conserved domain (bZIP_plant_BZIP46: cd14707) were deleted from the alignment, and 53 amino acid residues were used to calculate evolution distances for 47 genes.

**Sphagnum ABI3 gene identification and cloning**

The ABI3 homologues were identified using the P. patens ABI3C protein sequence (Pp3c4_7328V3.1) to interrogate the translated Sphagnum fallax database in Phytozome (https://phytozome.jgi.doe.gov/; Goodstein et al., 2012). Specific primers were designed for the selected candidate genes and used to amplify the corresponding transcripts from Sphagnum cDNA (Supplementary Table S1). The transcript from Sphafx01G102500 (which we called SphABI3-16) was the most abundant under our conditions and the full length cDNA cloned into pDONR207 using Gateway technology (Thermo Fisher Scientific). PpABI3A coding region was amplified with specific primers (Supplementary Table S1) from P. patens cDNA and then cloned into pENTR/D-TOPO (Thermo Fisher Scientific) according to the manufacturer’s instructions. SphABI3-16, and PpABI3A full length cDNA sequences were transferred to pMN601 expression vector (Yoshida et al., 2019) under the control of the EF1α promoter by LR reaction (Gateway, Thermo Fisher Scientific).

**Physcomitrium patens transformation and ABA sensitivity assays**

SphABI3-16 and PpABI3A in pMN601 and pMN601 empty vector were used to transform the P. patens Δabi3 strain (Khandelwal et al., 2010) by the polyethylene glycol-mediated method as previously described (Nishiyama et al., 2000). Colonies carrying a successful insert were selected on BCDAT plates containing 75 μg ml−1 nourseothricin (NTC, Jena Bioscience). The presence of the insert, the targeting to the PTA-1 locus and the presence of single and multiple insertions were detected by PCR, using specific primers as detailed in Supplementary Fig. S1 and Supplementary Table S1. Lines carrying the transgene were selected and transgene expression confirmed by qPCR using SphABI3-16 specific primers (Supplementary Table S1). For the colony growth in ABA medium, 10 single leaves of each of the different genotypes were removed from young gametophytes and transferred to both BCD medium and BCD medium supplemented with 25 μM ABA and incubated at 25 °C for 32 d. After this time, plates were scanned and colony diameter measured in ImageJ (https://imagej.nih.gov/). Percentage of growth in the ABA medium was calculated in relation to colonies grown in BCD medium alone. For the ABA protonemal growth assays, an equal amount of material for each genotype was homogenized in sterile water and used to inoculate BCDAT plates overlaid with cellophane disks (3 ml per plate) and allowed to grow for 7 d. After this time, cellophane disks were then transferred to BCD plates and BCD plates supplemented with 25 μM ABA (three replicates per genotype, per treatment) and grown for a further 14 d. After this time, plates where imaged in colour using a flatbed scanner at a resolution of 300 dpi using a white background. For each image, the red, green, and blue channels were separated and binary masks were created in R (version 3.5.2) from the RGB tiff image. The binary masks were reapplied to the original image to create a pair of images with green-dominant-pixels and red-dominant pixels respectively. Green pixels were counted using the filters green:blue>1.5 and green:red>1.05 and red pixels were counted using the filters green:blue>1.5 and red:green>1.05. The percentage of green-dominant pixels in the treatment compared with the control was calculated for each line. The presence of red-dominant pixels was used as a proxy for stress in the treatment plates.

**Results**

**Differential responses of Sphagnum species to controlled desiccation**

To establish if Sphagnum species show differential desiccation responses related to their position within the habitat, we chose four local species, representing the four major subgenera and with different distributions across the water table. Sphagnum fallax (subgenus Caspidata) is found in lawns and hummocks, S. papillosum (subgenus Sphagnum) and S. capillosum (subgenus Acutifolia) are predominantly hummock species, while S. inundatum (subgenus Subsecunda) is a typical hollow/pool species (Fig. 1A). All four species are present in the bog at Pen y Garn (van de Koot et al., 2021) at different distances from the water table. Sphagnum capillosum grows further away from the water table while S. inundatum is found primarily in ponds. Sphagnum fallax and S. papillosum occur at similar distances from the water table (Fig. 1A). These four Sphagnum species were collected from the site (Fig. 1A top row images) and subjected to a desiccation treatment in

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cosms, under greenhouse conditions (Fig. 1A bottom row, B). Besides occupying different microhabitats we noted marked morphological differences between the species, including colony density, which may affect responses to desiccation treatments (Fig. 1C). To take this into consideration, the average stem density in the field was determined for each species and used when planting the cosms. Cosms were weighed and imaged regularly over the water withdrawal period. Fresh weight loss and relative water content was calculated for each time point and the stress caused by water withdrawal assessed by measuring the photosynthetic activity recovery of PSII by calculating the $F_v/F_m$ parameter in dark adapted samples (Maxwell and Johnson, 2000). Sphagnum fallax, S. capillifolium, and S. inundatum showed similar rates of fresh weight loss and, 9 d after water withdrawal, plant weight was down to less than 10% of the original weight (Supplementary Fig. S2). Sphagnum papillosum showed much slower fresh weight loss and did not reach the same stage of desiccation until 13 d after water withdrawal (Supplementary Fig. S1). Sphagnum’s ability to store water has been attributed to anatomical features such as the size of capitula and the shape of the branches as well as the shape and number of specialized water-storage hyaline cells (Bengsson et al., 2020). To take this into account, we calculated the RWC for each sample. As seen with the fresh weight loss, S. fallax and S. inundatum showed a rapid decrease in RWC and by 7 d after water withdrawal the RWC of the capitula was near zero, while S. capillifolium showed a slightly slower decrease (Fig. 2A). Sphagnum papillosum again showed a slow decrease in RWC across time and only reaching a similar point to the other species at day 13 (Fig. 2A). While the slower decrease in S. papillosum and to a lesser extent in S. capillifolium can be correlated with their position in relation to the water table, the same is not true for S. fallax and S. inundatum as they show similar rates of fresh weight loss despite being collected at different water table distances (Fig. 2A). We next looked at the effect of desiccation on the photosynthetic activity of the different species by calculating the $F_v/F_m$ ratio (see ‘Materials and methods’). Within the first 4 d after water withdrawal, the $F_v/F_m$ ratio stayed constant or even showed a small increase in some of the species (Fig. 2B). This increase in photosynthetic activity might be due to the reduction of the excess surface water allowing improved gas exchange. From day 4 onwards, the $F_v/F_m$ ratio started to decrease, and as observed with the RWC, this decrease was more pronounced in S. fallax and S. inundatum and slightly slower in S. capillifolium (Fig. 2B). By day 9, these three species showed $F_v/F_m$ values between 0.2 and 0.4. In S. papillosum, the ratio $F_v/F_m$ decreased more slowly and only reached a value of 0.3 at day 13 (Fig. 2B). At $F_v/F_m$ values between 0.2 and 0.4, full recovery was still possible when plates were re-watered (Fig. 2B). We also measured $F_v/F_m$ recovery after re-watering when compared with the starting $F_v/F_m$ ratio (Supplementary Fig. S3). Sphagnum capillifolium, present at higher water table distances, fully recovered while S. inundatum and S. papillosum had recovery rates of around 90%. Sphagnum fallax showed high variation in recovery between replicates with three out of four replicates making full recovery and one not recovering (Supplementary Fig. S3).

In addition to whole plant responses, we also looked at how different parts of the Sphagnum plant responded to desiccation. For this, we divided the chlorophyll fluorescence images into three regions, one encompassing the capitulum (top), the other the middle section of the stem (middle), and the last the basal senescing stem region (base), and calculated the $F_v/F_m$ ratio for these regions across the water withdrawal period. The general changes in $F_v/F_m$ of the three regions for the four species mirrored what was seen for the whole plant but there were differences in the rate of change (Supplementary Fig. S4). While the three regions showed similar starting values for $F_v/F_m$, as expected the top region showed a faster decrease in $F_v/F_m$ suggesting a faster drying rate (Supplementary Fig. 4A) while the base, less exposed to air flow, dried more slowly (Supplementary Fig. S4C).

**Induction of drought-responsive genes in the different Sphagnum species**

After determining that there were indeed differences in physiological responses to desiccation, we next asked if these changes are accompanied by changes in the expression of drought-responsive genes. For this we selected four genes known to be strongly up-regulated upon drought in the moss *P. patens* (Cuming et al., 2007) and identified the *Sphagnum* homologues (Supplementary Table S1). *AWPM19* encodes a drought-induced membrane protein that in rice promotes ABA influx into the cell (Yao et al., 2018); late embryogenesis abundant proteins group 3 (LEAs group 3) are also induced in response to desiccation and are important during seed maturation (Battaglia et al., 2008); synaptotagmins maintain membrane integrity under stress conditions (Schapire et al., 2008) and ABI3 is a central component of the ABA-induced desiccation tolerance in the moss *P. patens* (Khandelwal et al., 2010). We observed increased expression of all genes as desiccation progressed but the magnitude of the change was different for each species (Fig. 3). *Sphagnum fallax* and *S. papillosum* showed larger increases in expression of the genes, with expression peaking at day 7, while in *S. capillifolium* and *S. inundatum* the relative increases were more modest (Fig. 3). Interestingly, these two species showed higher basal levels of expression in untreated material (Supplementary Fig. S5). We observed a marked decrease in drought-responsive gene expression 24 h after re-watering except for *S. papillosum* where expression was maintained at high levels (Fig. 3). It is worth noting the high variability between the three biological samples seen in the large SD observed, especially at later time points, which probably reflect different drying rates for each cosm.
ABA mediates desiccation responses in Sphagnum

It is well established that ABA is a central regulator of drought responses in plants and that ABA production increases upon drought. Despite this, not much is known about the role of ABA in desiccation tolerance in Sphagnum. We measured ABA concentration per gram fresh weight, across the water withdrawal period for all species. As the amount of water held...
ABA-mediated desiccation responses in *Sphagnum* by the plants varies drastically between samples as they dry, we adjusted the values using the RWC for each sample (see ‘Materials and methods’). We detected a low concentration of ABA in well-watered material (day 0) for all four species but ABA levels generally increased as drought progressed, peaking around day 4 (Fig. 4A), preceding the peak of ABA-dependent gene expression at day 7 (Fig. 3). Similar to what was observed with ABA-induced gene expression, the increase was more pronounced in *S. fallax* (Fig. 4A). We also observed significant increases in endogenous ABA for *S. capillifolium* at day 7, while ABA levels increased slightly but not significantly in *S. papillosum* and *S. inundatum* (Fig. 4A). Twenty-four hours after being re-watered, ABA levels had not yet returned to basal levels in the four species (Fig. 4A).

Increases in ABA and ABA-responsive gene expression underpin drought responses, increasing the ability of the plants to tolerate mild drought (Chaves et al., 2003). To determine if this is also the case in *Sphagnum*, we tested the effect of ABA pre-treatment on the desiccation response of *S. fallax* plants in cosms. We used two ABA concentrations, 10 μM and 50 μM, to pre-treat *S. fallax* plants for 24 h before water withdrawal. Desiccation responses were evaluated by measuring photosynthetic performance by calculating the $F_v/F_m$ ratio. *Sphagnum fallax* plants were allowed to dry until $F_v/F_m$ reached 0.2 and
kept at this value (i) for 1 d and then re-watered or (ii) for 4 d and then re-watered. After re-watering, \( F_v/F_m \) was again measured and the percentage recovery \((F_v/F_m \text{ after re-watering} / F_v/F_m \times 100)\) was calculated for all treatments. Pre-treatment with either 10 μM or 50 μM ABA increased \( F_v/F_m \) recovery after 1 d at \( F_v/F_m \) of 0.2, while at the harsher treatment of 4 d at \( F_v/F_m \) of 0.2, only 50μM ABA significantly improved recovery (Fig. 4B).

Taken together, these data suggest that ABA is an important component of desiccation responses in *Sphagnum*.

Many genes involved in ABA signalling pathways are conserved in *Sphagnum*

Many of the ABA signalling components from perception to signal transduction have been identified in the model plant Arabidopsis and found to be conserved across many other species (Cai et al., 2017; Sun et al., 2020). Querying of the available *Sphagnum* genome sequences (*S. fallax* and *S. magellanicum*) showed that these components are also present in *Sphagnum* (Supplementary Figs S6–S8). All the core ABA signalling
proteins, PYR/RYL (ABA receptors; Supplementary Fig. S6), SnRK2 (ABA-signalling regulators, Supplementary Fig. S7), and ABI5s (transcription factors phosphorylated by activated SnRK2, Supplementary Fig. S8) were found in the Sphagnum genome. In particular, the phylogenetic trees of PYR/RYC and SnRK2 indicate that bryophyte species have fewer PYR/RYC and SnRK2 genes than vascular plants (Supplementary Fig. S6, S7). Surprisingly, while homologues of the clade F of protein phosphatases (PP2Cs) are present in Sphagnum, we were not able to find any homologues for clade A (Supplementary Fig. S9). This clade of PP2Cs is found in other bryophytes and includes Arabidopsis ABI1/2, HAB1/2, and AHG1/3 that together with PYR act as co-receptors for ABA (Nishimura et al., 2010).

The plant-specific ABI3 family of transcription factors plays a critical role during seed development desiccation tolerance and is an important regulator of ABA-mediated responses to abiotic stresses (Sakata et al., 2010). In P. patens, mutants (Δabi3) lacking three of the ABI3 genes (PpABI3A/B/C) have increased drought and desiccation tolerance and reduced sensitivity to exogenously applied ABA (Khandelwal et al., 2010; Tan et al., 2017; Zhao et al., 2018).

In order to determine if these functions are conserved in ABI3 proteins from Sphagnum moss, we cloned one of the five Sphagnum ABI3 homologues (Fig. 5; SphABI3-16 (SphFalx01G102500)) and asked if it could rescue the Δabi3 phenotype. When grown on medium supplemented with 25 μM ABA for 32 d, wild type P. patens showed around 20% of the growth in the ABA-free medium while the Δabi3 mutant showed a growth of around 50% (Fig. 6A, B). This increased growth was maintained when the Δabi3 mutant was transformed with an empty vector construct, but growth returned to wild type levels when the Δabi3 mutant was transformed with the endogenous PpABI3A gene (Fig. 6A, B). When the Δabi3 mutant was transformed with SphABI3-16, growth in ABA medium was also inhibited, returning to wild type levels in lines #31 and #37 (Fig. 6A, B). Interestingly, in line #7, growth was indistinguishable from the untransformed Δabi3 mutant (Fig. 6A, B) but, despite carrying the SphABI3-16 construct, we could not detect expression in this line (Fig. 6C). As the transgene in this line is not found at the PTA-1 locus (Supplementary Fig. S1), the lack of expression might be due to where the transgene was inserted within the genome.

Exogenous ABA application is also known to affect protonemal growth and choronemal cell morphology in P. patens. Protonemal growth was inhibited when wild type P. patens was grown on medium containing 25 μM ABA (Fig. 6D, E). Consistent with having decreased sensitivity to ABA, this growth inhibition was not observed in the Δabi3 mutant. Expression of PpABI3A in the Δabi3 mutant background recovered the wild type phenotype (Fig. 6D, E). Expression of SphABI3-16 in the Δabi3 mutant background not only restored growth sensitivity to ABA, but also resulted in the protonemal growth becoming oversensitive to exogenous ABA application (Fig. 6D, E). Interestingly, the Δabi3 lines expressing either PpABI3A or SphABI3-16 when grown on ABA medium also produced

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**Fig. 5.** A phylogenetic tree of ABI3 proteins. The tree was constructed with the maximum likelihood method using the JTT-based matrix. The tree with the highest log likelihood is shown. Bootstrap values of >50% are shown on the branches. Horizontal branch length is proportional to the estimated evolutionary distance. Schematic representations of the corresponding protein structure, including the conserved B1–B3 domains indicated by the green boxes, are also shown.
Fig. 6. *Sphagnum* ABI3 rescues the phenotype of the Δabi3 mutant in *P. patens*. (A) Colony growth in wild type *P. patens* (Gransden), Δabi3 mutant, and Δabi3 mutant transformed with all the different potential rescue constructs as indicated. Single leaves were grown in BCD medium (top row) or BCD medium supplemented with 25 μM ABA (middle row) for 32 d. Scale bar: 500 μm. Bottom row shows detail of the colony structure of the plants grown in the ABA medium (scale bar: 100 μm). (B) Relative colony growth in ABA medium for wild type (Gransden), Δabi3 mutant, and the rescue transformants as shown above. After 32 d growth, colony diameter was measured and the percentage of growth in the ABA-containing medium was calculated in relation to the media without ABA. Data show means ±SD of 10 colonies. *Significantly different from wild type, P<0.01.* (C) Increased ABI3 expression in the transformed Δabi3 mutant was confirmed by qPCR. Data show means ±SD of three replicates. Expression of *P. patens EF1α* was used as a reference. (D, E). Protonemal growth of wild type (Gransden), Δabi3 mutant and the rescue transformants in BCD medium or BCD medium supplemented with 25 μM ABA as indicated. (D) Representative images of each genotype after 14 d growth. (E) Quantification of protonemal growth. After 14 d growth, plates were scanned and growth (total green pixels in the image) was calculated for each plate. The percentage of growth in the ABA containing medium was calculated in relation to the medium without ABA. Data show means ±SE of three plates per genotype. *Significantly different from wild type, P<0.01.*
increased amount of red pigments suggesting increased stress levels in these plants (Supplementary Fig. S10). Another effect of ABA application is the increased branching of chloronemal cells. As expected from the reduced sensitivity of the Δabi3 mutant, we observed reduced chloronemal branching when this line was grown on ABA-containing medium (Fig. 6A, bottom row). Consistent with the results described above, the Δabi3 mutant transformed with either the endogenous PpABI3A or SphABI3-16 showed wild-type like phenotype of increased branching (Fig. 6A, bottom row).

These data indicate that Sphagnum contains an ABA signalling system functionally equivalent to that of other mosses and higher plants, further supporting a role for ABA during desiccation responses in Sphagnum.

Discussion

By using a comprehensive physiological and molecular approach, we show that different Sphagnum species respond differently to a controlled induced desiccation, but, surprisingly, this is not always correlated with their position relative to the water table.

Sphagnum mosses have been considered to be desiccation-avoiding rather than tolerant (Clymo, 1973; Clymo and Hayward, 1982). In this scenario, they have evolved systems to store large amounts of water with 90% of all water stored in extracellular spaces and 10% in hyaline cells (Clymo and Hayward, 1982). Detached Sphagnum capitula have been shown to have a water content of 700–1200% relative to plant weight for optimal photosynthesis (Bengtsson et al., 2016), high values as compared with other bryophytes and most other land plants.

Of the four species that we used for our studies, S. inundatum showed the highest capitula water content (2498 ± 181%), followed by S. papillosum (2121 ± 205%) and S. fallax and S. capillifolium having similar water content values (1458 ± 107% and 1441 ± 217%). Unexpectedly these values do not correlate with their position along the water table (Fig. 2A). For S. fallax, S. capillifolium, and S. inundatum, the F/v ratio was stable for the first 4–5 d suggesting that the plant was not stressed at RWC of around 50%. The first physiological signs of stress appeared between day 5 and day 7 after water withdrawal. During this period, the RWC fell to less than 20% and there was a sharp decrease in the F/v ratio to values between 0.4 and 0.6, accompanied by the induction of the expression of drought-related genes (Fig. 3). At day 9, the RWC neared zero and the F/v ratio decreased to around 0.2. For S. papillosum on the other hand, this was not attained until day 13, again underscoring the high water holding capacity of this species. As early as 24 h after re-watering, both the RWC and the F/v ratio recovered to levels close to the starting point, suggesting that Sphagnum is able to tolerate mild desiccation without significant plant damage. While some studies describe similar levels of recovery, others report very poor recovery after desiccation (Wagner and Titus, 1984; Gerdol et al., 1996; Hajek and Beckett, 2008; Hajek and Vicheronová, 2014). One possible factor in these differences is the levels of hardening that the plants were subjected to before the experiment. The plants we used had been naturally exposed to varied degrees of desiccation and rehydration in the field and we did not allow for any greenhouse adaptation time. They may have developed a degree of hardening that was impacting on the observed desiccation tolerance. In agreement with some recent studies, we did not observe significant differences in desiccation tolerance between hummock and hollow species (Wagner and Titus, 1984; Schipperges and Rydin, 1998; Oliver et al., 2005; Proctor et al., 2007; Hajek and Beckett, 2008; Hajek and Vicheronová, 2014; Bengtsson et al., 2020).

The molecular signatures of drought responses seem to be conserved in Sphagnum as many of the genes found to be up-regulated by drought in other species are also up-regulated in drying Sphagnum (Fig. 3), and together with the observed increases in ABA, this suggests the existence of an inducible desiccation response pathway in Sphagnum.

ABA as an important component of desiccation responses in Sphagnum

Evolutionary analysis of ABA signalling pathways suggest that ABA has been used ancestrally as a stress hormone in land plants...
and that it might have evolved to enable drought and desiccation tolerance in the new land environment (Wang et al., 2015; Cuming, 2019; Sun et al., 2020). Many of the ABA synthesis, perception, and signalling components have been identified in a range of land plants and their functions seem to be broadly maintained (Guillory and Bonhomme, 2021). In the mosses P. patens and Funaria, drought has been shown to increase endogenous ABA concentrations (Werner et al., 1991; Knight et al., 1995). ABA pre-treatment has been shown to increase drought tolerance in several crop species (Lu et al., 2009; An et al., 2014; Changning et al., 2014; Liting et al., 2015; Brito et al., 2020; Skowron and Trojak, 2021) and also in mosses, including one study in Sphagnum (Wagner and Titus, 1984; Beckett et al., 1999, 2000; Hájek and Beckett, 2008; Khandelwal et al., 2010). We also observed an increase of endogenous ABA during desiccation in Sphagnum. ABA levels were at the highest at day 4 (Fig. 4), preceding the peak of ABA-induced gene expression observed at day 7 (Fig. 3). ABA levels did not increase after day 4, in line with what was observed in other species (Scholz et al., 2015). Notably, 24 h after the plants were re-watered, we observed decreased gene expression but levels of ABA were still high, suggesting that it takes longer for ABA levels to return to normal in re-watered Sphagnum plants. In S. fallax, the most desiccation sensitive of the species used, the increases in ABA levels were the highest while more modest increases were observed in S. capillifolium. In the most desiccation resistant species, S. papillosum, levels of ABA were lowest, and possibly sampling at later time points would be necessary to detect larger increases in ABA. In S. inundatum, ABA levels stayed constant at low levels and this was also the case for drought responsive gene expression (Figs 3, 4). Being a hollow species, one would expect that S. inundatum would need to quickly respond to the lack of water, and it has been proposed that it does not possess the same desiccation avoidance mechanisms as hummock species (Mazziotta et al., 2019), which may have adapted by evolving the faster ABA-dependent pathways. Our data suggest that this may not be the case and S. inundatum relies on other mechanisms for desiccation tolerance such as the proposed resource acquisition (Mazziotta et al., 2019). Alternatively, as the basal level of drought-responsive gene expression is high in this species when compared with the others (Supplementary Fig. S5), it is possible that some aspects of the desiccation response may be constitutively activated in this species.

**ABA-mediated drought signalling is conserved in Sphagnum**

Sphagnum species also merit attention in the context of the plant’s transition from aquatic to terrestrial life. A critical step in the water to land transition was the acquisition of desiccation tolerance and, although sphagna are thought to be mainly desiccation avoidant, our data suggest that they have a basal level of desiccation tolerance and this involves, at least in part, ABA. We found that the main components of ABA responses are conserved in Sphagnum with the notable exception of clade A PPC2As that include the ABA co-receptor ABI1 (Nishimura et al., 2010; Bhaskara et al., 2019). The fact that we were not able to find ABI1 homologues in the Sphagnum genome could be due to the homology thresholds we used, although this is unlikely as we were able to find homologues in P. patens and M. polymorpha. Another possibility is that these genes are not correctly annotated in the Sphagnum genome versions that we used to build our phylogenies (S. fallax v1.1 and S. magellanicum v1.1). Otherwise, the prospect of ABI1 homologues not being present in Sphagnum raises the interesting possibility that Sphagnum has co-opted other proteins, maybe PP2Cs from other clades, for example clade F that seems to have expanded in Sphagnum (Supplementary Fig. S9), as ABA co-receptors.

Our data also show that the function of ABA signalling components is conserved in Sphagnum as demonstrated by the ABI3 protein. In tracheophytes, ABI3 was first identified as a seed specific transcriptional activator (Finkelstein and Somerville, 1990). While its role during seed development remains best documented, ABI3 has been found to be important in other vegetative tissues where it controls developmental transitions and stress responses (Rhode et al., 2000; Bedi et al., 2016). Its functions extend outside seeded plants and may have even diversified in mosses where we find an expansion of the number of ABI3 proteins (five in Sphagnum fallax and four in P. patens compared with one in Arabidopsis and one in rice; Fig. 5). In P. patens, loss of function mutants in three of the ABI3 genes show decreased ABA sensitivity and are more sensitive to desiccation and other stresses (Marella et al., 2006; Takenaka et al., 2007; Khandelwal et al., 2010; Tan et al., 2017; Zhao et al., 2018). We found that heterologous expression of the Sphagnum ABI3 orthologue rescued the phenotype of loss of function Δabi3 mutant in P. patens (Fig. 6). This is the first time that an ABA-pathway gene from Sphagnum has been functionally characterized and has several implications. The first is that ABA-mediated responses to stress are conserved in Sphagnum as was observed for other mosses (Cuming, 2019; Guillory and Bonhomme, 2021). It will be interesting to determine if the role of ABA in other developmental pathways such as gametophyte development and spore germination is also conserved in Sphagnum. In addition, there is evidence that, although there is broader conservation of ABA pathways in other mosses, the molecular and physiological responses as well as the interaction with other hormone pathways are different from the ones observed in vascular plants. Further detailed studies in Sphagnum will give better insight into the evolution of ABA signalling pathways (Cuming, 2019; Guillory and Bonhomme, 2021). Secondly, our studies confirm the usefulness of using P. patens as a heterologous functional expression system for species without an established transformation protocol (Rensing et al., 2020). The ease of transformation and the existence of well-characterized mutants in many of the developmental and stress signalling pathways in Physcomitrium are valuable resources to test the functionality of other Sphagnum genes. Lastly, our data
support earlier observations that *Sphagnum* is able to tolerate significant levels of dehydration without losing photosynthetic capacity, and this should be taken into consideration when modelling the global responses of peatlands to changes in climate patterns especially precipitation (Wagner and Titus, 1984; Schipperges and Rydin, 1998; Proctor, 2000; Oliver et al., 2005; Proctor et al., 2007; Dise, 2009; Hájek and Vícherová, 2014; Cruz de Carvalho et al., 2019; Rastogi et al., 2020).

**Supplementary data**

The following supplementary data are available at *JXB* online.

Fig. S1. Targeting of the *PpABI3A* and *SphABI3-16* expression constructs.

Fig. S2. Percentage fresh weight loss during the desiccation experiment for the four different *Sphagnum* species.

Fig. S3. Chlorophyll fluorescence recovery after desiccation as measured by F₁/Fᵅ.

Fig. S4. Changes of chlorophyll fluorescence upon desiccation in different regions of the *Sphagnum* plants for the four different species.

Fig. S5. Basal expression of drought responsive genes is higher in *S. inunatum*.

Fig. S6. ABA signalling components are present and evolutionarily conserved in *Sphagnum* including the ABA-receptor family PYR1.

Fig. S7. ABA signalling components are present and evolutionarily conserved in *Sphagnum* including SnRK2.

Fig. S8. ABA signalling components are present and evolutionarily conserved in *Sphagnum* including ABI5.

Fig. S9. Clade A PPC2A homologues are absent in *Sphagnum*.

Fig. S10. *Physcomitrium patens* plants with increased expression of ABI3 show signs of stress as measured by the presence of red pixels.

Table S1. List of the primers used in this study.

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**Author contributions**

CN, DS, TK, and YH designed and performed experiments. CN, WvdK, DS, KW, TK, MB, LM, and YH analysed data. CN and JHD wrote the manuscript. JHD and YH obtained funding. All authors read and approved the manuscript.

**Conflict of interest**

The authors declare no conflict of interest.

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**Data availability**

All data supporting the findings of this study are available within the paper and within its supplementary materials published online.

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