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Early events leading to water deficit responses in the liverwort *Marchantia polymorpha*

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ABSTRACT

To understand the genetic and evolutionary basis of the responses to water deficit in plants, we turned to the basal liverwort *Marchantia polymorpha*, representing one of the most ancient plant lineages that first colonized land. Thalli exposed to water deficit showed a decreased growth rate and morphological modifications. In addition, water deficit led to relocation of cell organelles, mostly due to changes in volume of the central vacuole, which correlated with adjustments of osmotic potential. Absciscic acid (ABA) is a phytohormone that mediate responses to environmental stress in vascular plants. Consistently, we observed an increase in endogenous ABA levels upon water deficit in *Marchantia*, which correlated with the increased expression of genes encoding for LATE-EMBRYOGENESIS-ABUNDANT proteins, which are thought to protect cells from damage caused by water deficit. Transcriptome-level changes upon ABA and water deficit treatments revealed that several genes modify their expression under these conditions. Consistent with our cellular level observations, differentially expressed GO categories related to ion binding and transport may explain the changes in vacuole volume. Induction of stress-related genes, including several LEA genes, also points towards a possible conservation of ABA-dependent signaling pathways in *M. polymorpha* and defines a framework to understand the underlying molecular events.

1. Introduction

Water limitation is one of the most restricting factors for plant growth, inducing detrimental effects on crop yields (Araus et al., 2002; Moradi et al., 2014). The main detonators of water deficit are drought and salinity soils, which may cause irreversible damage at the physiological and cellular levels (Madhava, 2006), consequently vascular plants have developed several mechanisms to contend with such conditions. Among these, plants deploy various strategies to cope with drought and high salinity present in the soil, including stomatal adjustment, osmotic balance, protection against damage to cellular components, exclusion of Na⁺ ions from photosynthetic tissues, and tolerance of tissues to ion accumulation (Munns and Tester, 2008). One of the most general response mechanisms that plants mount to mitigate osmotic stress is reducing water loss while maximizing water uptake (Deinlein et al., 2014). Thus, a common response is the accumulation of organic and inorganic osmolytes such as KCl, proline, glycine betaine, sugar alcohols or polyamines, which consequently promotes water influx, increasing cell osmotic potential. Compatible osmolytes along with

antioxidant enzymes and proteins from the late embryogenesis abundant (LEA) superfamily, among others, play an important role in preventing the harmful effects of drought or high salinity (Buitink et al., 2002; Verslues et al., 2006; Battaglia and Covarrubias, 2013). In addition, through the regulation of the activity of different genes and proteins, plants adjust their metabolism, growth and reproduction to cope with these adverse conditions, and some species are even able to resprout vegetatively after an extensive loss of biomass caused by drought (Zeppel et al., 2015).

Absciscic acid (ABA) is a phytohormone known to initiate some of the signaling pathways that lead to many cellular and molecular stress responses, and whose synthesis is triggered by different conditions leading to low water availability in higher plants. ABA plays an essential role in seed maturation, germination, stomatal closure, and in stress responses in vascular plants (Lee and Luan, 2012). ABA induces a rapid response in guard cells by controlling a network of ion channels in the plasma and the vacuolar membranes, promoting stomatal closure. Moreover, ABA induces changes in gene expression leading, among other processes, to accumulation of compatible solutes, which enhances

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the plant capability to cope with low water availability (Wasilewska et al., 2008; Hauser et al., 2011). Tissue cultured cells of angiosperms, gymnosperms and ferns, develop desiccation and freezing tolerance upon exogenous ABA treatments, indicating that ABA has a key role in stress tolerance at the cellular level (Reynolds and Bewley, 1993; Gusta et al., 2005; Tougane et al., 2010). Also, recent reports have revealed that ABA induces stress tolerance in some non-vascular plant species, including bryophytes, indicating a conservation of ABA roles in the stress response throughout the plant kingdom (Takezawa et al., 2011). In mosses, ABA promotes formation of brood cells for vegetative reproduction, stomatal closure in sporophytes, inhibits growth and development of gametophores and induces tolerance to environmental stresses in protonemata (Menon and Lal, 1974; Cuming, 2009). ABA treatments in *Physcomitrella patens* lead to an increase in freezing tolerance while hyperosmotic stress treatments of protonemata induce an increment in endogenous ABA levels (Minami et al., 2005, 2006). Furthermore, in *Atrichum androgynum* exogenous application of ABA followed by storage for three days can fully substitute for partial dehydration (Mayaba et al., 2001). In nature, presumably, partial dehydration will often precede a more severe water loss, resulting in induction of ABA synthesis, which in turn activates signal transduction pathways that increase desiccation tolerance. These data highlight the importance of ABA as a critical factor during evolution for plant adaptation to low water availability.

Marchantia polymorpha is included within the Bryophyte division in the Marchantiophyta (liverworts) group, it is a representative of the most ancient plant lineages that colonized land and offers the opportunity to understand the physiological and molecular basis of key features that allowed green plants to evolve and adapt to a terrestrial environment (Bowman et al., 2007; Ishizaki et al., 2008). A few studies have hinted at the activity of ABA in the context of stress responses in liverworts; for example, in the xerophilic liverwort *Exormothesa holstii*, ABA induces desiccation tolerance (Hellwege et al., 1994), and in *Riccia fluitans* and *Pallavicinia lyellii* an enhanced desiccation tolerance was associated with an increased accumulation of total soluble carbohydrates upon ABA addition (Pence et al., 2005). Desiccation tolerance of *M. polymorpha* callus cells was induced by preculture with sucrose and ABA (Hatanaka and Sugawara, 2010). Akter et al. (2014) reported that ABA treatment of gemmalings of *M. polymorpha* caused accumulation of soluble sugars and it was further increased by addition of sucrose to the culture medium. Furthermore, ABA affects growth rates in both gemmalings and adult thalli and induces expression of known ABA-responsive genes (Tougane et al., 2010; Eklund et al., 2018; Jahan et al., 2019). The machinery responsible for ABA signaling has begun to be unraveled as well; MpoPYL1 has been shown to function as a ABA receptor similar to the PYL family of proteins described in Arabidopsis (Jahan et al., 2019) and moreover, the recent annotation of the *M. polymorpha* genome revealed the presence of a complete ABA signaling pathway (Bowman et al., 2017). In this work, we characterized physiological, cellular and molecular responses to water limitation and ABA treatment of thalli of the liverwort *M. polymorpha*, allowing us to establish an integrative framework to understand the genetic and evolutionary basis of plant responses to water limitation and salinity.

2. Material and methods

2.1. Plant materials

Male gametophyte of *M. polymorpha*, accession Takaragaike-1 (Tak1), was asexually maintained and propagated via gemmae and thalli. To maintain a continuous source of gemmae, plants were grown on trays with metromix (Sunshine, Sungro Horticulture, US) and turf as substrate (Athletics MVP, IL, USA), and incubated in a growth chamber at $22\text{ }^{\circ}\text{C} \pm 1$ under long day conditions (16 h light/ 8 h dark) at $50-80\text{ }\mu\text{mol m}^{-2}\text{s}^{-1}$ of light. To generate young thalli of seven days to perform experiments, gemmae were cultured on half-strength

Gamborg's B5 solid medium (Tougane et al., 2010) at $22\text{ }^{\circ}\text{C} \pm 1$ under long day conditions (16 h light/ 8 h dark) at $50-80\text{ }\mu\text{mol m}^{-2}\text{s}^{-1}$ of light.

2.2. Water deficit treatments

Gemmae were pooled from different conceptacles, washed with bleach (0.3 % sodium hypochlorite in H_2O) and rinsed with sterile distilled water. For each replicate, fifty gemmae were cultured on polyester meshes placed on top of half-strength Gamborg's B5 solid medium in optimal conditions. After seven days, resulting thalli were used for water deficit treatments. Petri dishes containing cotton pads moistened with sterile distilled water were placed inside plastic desiccators. Incubation of thalli in cotton pads moistened with distilled water or with Gamborg's B5 liquid media showed no apparent differences during the water deficit treatments at the time intervals tested here. The water deficit treatments began when polyester meshes with thalli were transferred to the moist pads and subjected to controlled water deficit conditions: 60 % relative humidity (RH), $22\text{ }^{\circ}\text{C} \pm 1$ and long day conditions. To maintain 60 % relative humidity (RH), a defined amount of desiccant (Easy dry, Mexico) was introduced inside the desiccators in order to reduce the relative humidity. Plastic desiccators without desiccant remained close to 100 % RH and were considered as control conditions. The growth and development of the thalli were evaluated after 0, 6, 12, 24, 48 and 72 h. Each experiment consisted of three plates, each containing fifty plants, and it was independently repeated three times.

2.3. Salt and ABA treatments

For salt treatments, seven-day old thalli were transferred to half-strength Gamborg's B5 medium supplemented with different NaCl concentrations (150, 250 or 500 mM). Thalli development was evaluated after 12, 24, 48 and 72 h. For ABA treatments, thalli of seven days were transferred to half-strength Gamborg's B5 medium supplemented with different ABA concentrations (1 or 5 μM), and thalli development was evaluated at 12, 24, 48 and 72 h. Experiments consisted of three separate plates, each with forty plants and each experiment was repeated three times.

2.4. Determination of osmotic potential and ion leakage

To determine osmotic potential, pooled thalli were frozen in liquid nitrogen for 1 min and thawed at room temperature for 10 min. Five cycles of freezing/thawing were performed and tissue was then centrifuged for 20 min at 16,000 g. The upper phase was transferred to a new tube and osmotic pressure was determined according to Money (1989), using a Vapro 5600 Vapor Pressure Osmometer (Wescor, Inc.). The osmolality was obtained from three technical replicates for each sample, performed in three independent experiments. The mean value of osmolality was obtained and the solvent contribution (correction factor = osmolality of distilled water) was subtracted from the mean. Osmolality measurements were converted to MPa using the formula $H = RTc$; where c = osmolality in moles.kg^{-1} and $RT = 2.446\text{ kg.MPa.mol}^{-1}$ at $21\text{ }^{\circ}\text{C}$. To measure ion leakage, thalli were first harvested, rinsed with distilled water, and then maintained in 25 ml of sterile distilled water for 24 h at room temperature. Each 12 h, water was changed and collected for subsequent measurement. Determination of ion leakage was carried out from three technical replicates for each sample, and performed in two independent experiments, using a S230 Seven Compact conductivity meter (Mettler-Toledo, Columbus, OH). Ion leakage was expressed in microsiemens/centimeter ($\mu\text{S/cm}$).

2.5. Measurement of endogenous ABA

The preparation of cell-free total extracts and determination of ABA

content in thalli were performed according to the antibody-based assay Plant Hormone Absciscic Acid (ABA) ELISA Kit (CUSABIO, Wuhan, China) following manufacturer's instructions. For all samples, the dry weight of an equivalent number of plants was determined and used to express ABA contents as ng/g dry weight. These experiments consisted of two separate plates with fifty plants each, and it was performed in two independent experiments.

2.6. Image analysis by confocal microscopy and transmission electron microscopy

Images in bright field of thalli were obtained using a Nikon Eclipse E600 Microscope, objective 20X/0.5 Plan Fluor/DICM. Fluorescence images were obtained using a Nikon Eclipse 80i Microscope, objective 40X/0.75 PH₂ DLL, and inverted confocal Zeiss LSM510 META Microscope (Zeiss Axiovert 200 M), objective C-Apochromat 63X/1.2 W Korr. For electron microscopy analyses, thalli were washed with distilled water, and fixed with 4 % paraformaldehyde and 2.5 % glutaraldehyde in sodium cacodylate buffer (pH 7.4) for 2 h at 4 °C. After fixation, thalli were treated with 1 % osmium tetroxide for 90 min at 4 °C, and then rinsed twice in chilled buffer and cold bidistilled water. Then, thalli were dehydrated by ethanol series and embedded in Epon/Araldite (Mollenhauer, 1964). Ultrathin sections of 55–65 nm in diameter, cut with a diamond knife (Micro Star Technologies, Inc, Huntsville, USA) equipped ultramicrotome, were stained with 3 % uranyl acetate, incubated in darkness at room temperature for 5 min and observed with a ZEISS Libra 120 plus transmission electron microscope. A total of 200 ultrathin sections, an average of 105 cells per treatment, were observed, analyzed and used to estimate the vacuolar volume.

2.7. Image analysis

Images of eighty individual thalli were collected from water deficit and images of fifty thalli from NaCl treatments. For water deficit treatments, twenty images corresponded to initial time (T0) and ten images were taken for each time point evaluated (12, 24 and 48 h), per treatment (control or water deficit treatment). For NaCl treatments, two images corresponded to T0 and three images were taken for each time point evaluated (12, 24, 48 and 72 h), per treatment (control or 150, 250 or 500 mM NaCl). The collection of images was obtained using a Nikon SMZ 1500 Stereoscopic Microscope (Objectives Plan Apo 1x WD 70), with a Nikon DS Camera Head DS-Fi1 (5.24 megapixels CCD/Color/ non-cooled). We used Nikon NIS Elements Imaging Software to process images. All images were obtained using the same magnification, resolution (Fast focus 1280 × 960) and lighting conditions and stored as RGB/TIFF files. For image analysis, the Fiji-ImageJ v1.46 program was used (Schindelin et al., 2012). The Trainable Weka Segmentation (TWS) plugin, combining a collection of learning algorithms to produce segmentations (https://github.com/fiji/Trainable_Segmentation/) (Arganda-Carreras et al., 2014), was used to produce pixel-based segmentation from 2D images. In the graphic interface of TWS the image “typo” was used as reference to perform segmentation of all images. To determine the area, the images were converted to 8-bit and analyzed by threshold color considering the total area as reference.

2.8. RNA isolation and quantitative real time RT-PCR

Total RNA was isolated from thalli using Trizol reagent (Invitrogen, Piscataway, NJ, USA) according to manufacturer's instructions. For cDNA synthesis, we used 1 µg of total RNA treated with DNase I (Fermentas), oligo-dT primer and Superscript III Reverse transcriptase (Invitrogen). Quantitative PCR was performed using SYBR-Green qPCR Master mix kit (Thermo Scientific) in a Step One real-time thermocycler (Applied Biosystems). Actin gene (Mapoly0016s0137, *M. polymorpha* genome v3.1 at Phytozome <https://phytozome.jgi.doe.gov/>) was used as a reference gene, using the 2^{-ddCT} method to determine relative

transcript accumulation. The stress gene markers and other primers used in this work are listed in Supplemental Table S1. Results were obtained from three independent experiments where three replicates for each time point were analyzed.

2.9. RNA-Seq analyses

Total RNA (1 µg) from thalli exposed to water deficit for 6 and 9 h or exposed to 1 µM ABA for 6 and 9 h was assessed for quality and integrity using a Bioanalyzer (Agilent Technologies). Total RNA from two independent experiments was processed to obtain cDNA libraries using TrueSeq Stranded mRNA Library Prep kit (Illumina, San Diego, CA), and sequenced using a HiSeq 2000 platform (Illumina) with a configuration of 150 cycles for 2 × 100 paired-end sequences (Unidad Universitaria de Secuenciación Masiva y Bioinformática, Instituto de Biotecnología-UNAM). Low-quality reads were removed from 273.3 million total reads of which 9–23 million reads per library mapped to the *M. polymorpha* genome v3.1 available at Phytozome (<https://phytozome.jgi.doe.gov/>) (Bowman et al., 2017). Overall, mapped reads identified 13,248 annotated genes (Supporting Information Dataset 1). Differential expression analysis was carried out using EdgeR (Robinson et al., 2010), DESeq, DESeq2 (Anders and Huber, 2010) and NOISeq (Tarazona et al., 2011), only genes that showed a significant change as determined by the four tools (corresponding to p-value < 0.001, logFC > 0.5, and FDR < 0.01, values from EdgeR) were retained for downstream analysis (Supporting Information Dataset 2). We carried out a Gene Ontology (GO) enrichment analysis as follows: first a GO annotation was conducted via InterProScan version 5 using the genes from *M. polymorpha* genome (Bowman et al., 2017), resulting in a 1:1.5 GO annotation ratio (66.6 % proteins with at least one GO category). Then a GO enrichment analysis was performed using the R-package TopGO [<https://bioconductor.org/packages/release/bioc/html/topGO.html>], over the differentially expressed genes from comparisons (WD 6 h vs WD 9 h, and ABA 6 h vs ABA 9 h). This analysis was conducted at the three GO levels: Molecular Function (MF), Biological process (BP) and Cellular Component (CC). To evaluate the results and in order to account for multiple testing, we used the classic fisher statistic with a cut-off value of 0.05.

2.10. LATE EMBRYOGENESIS ABUNDANT protein family classification

To identify LATE EMBRYOGENESIS ABUNDANT genes in the *M. polymorpha* genome, we performed a classification by gene families using the nomenclature introduced by Battaglia and Covarrubias (2013) with the corresponding PFAM number. Gene classification was performed using InterPro software and KAAS (<https://www.genome.jp/kegg/kaas/>) for members of each family using the *M. polymorpha* genome v3.1 annotation provided by Phytozome (<https://phytozome.jgi.doe.gov/>) (Bowman et al., 2017). For the resulting LEA gene list, the most similar genes in the *Arabidopsis thaliana* genome were obtained by a BLASTP search against the TAIR10 protein database, and we obtained their differential expression based on the RNA-seq results described in this work and classified by treatment (water deficit or ABA). These data are available in Supplemental Table S2. See text for details.

2.11. Statistical analysis

Each experiment was performed with three separate growth plates, and all experiments repeated three independent times. In each case, values represent the mean and error bars represent the confidence interval, which considers standard deviation, sample size and a Student's t distribution of the data ($\alpha = 0.05$). We used Microsoft Excel (Version 16.32) to concentrate all data and Prism8 (Version 8.4.2) for ANOVA analyses.

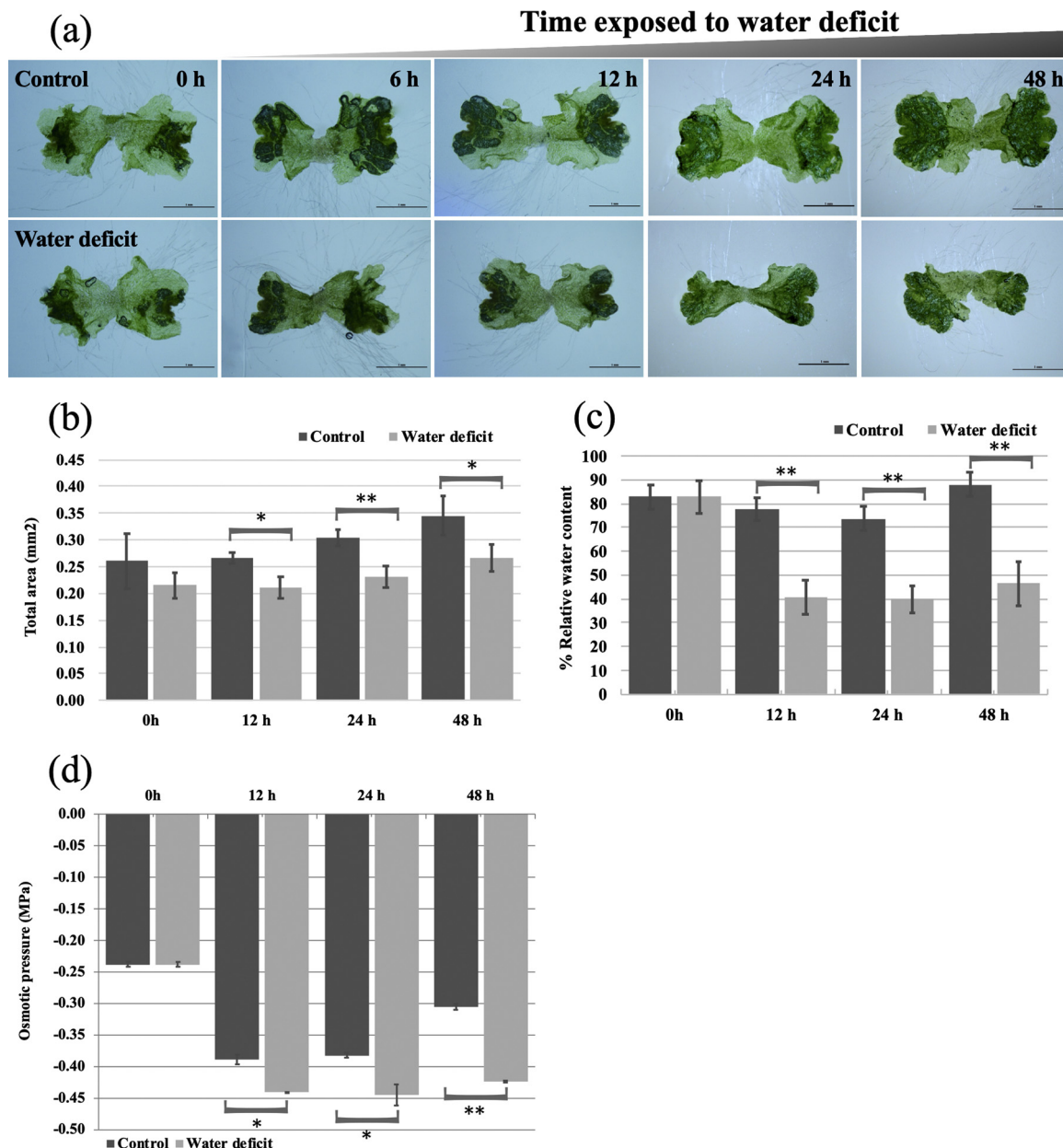


Fig. 1. Water deficit arrests thalli growth and affects water content status. (a) Thalli grown in optimal conditions for seven days and then exposed to water deficit, were photographed at 0, 6, 12, 24 and 48 h of water deficit (dark grey bars and light grey bars, respectively). (b) Total area (in mm²) was determined for thalli under control conditions or exposed to WD for 0, 6, 12, 24 and 48 h (dark grey bars and light grey bars, respectively). Thalli grown under the same conditions were used to determine relative water content (c) and their osmotic potential (d). Each experiment consisted of three separate plates each with fifty thalli, performed in three independent experiments (n = 3). ** Represents significant differences according to Student's t distribution of the data ($\alpha = 0.05$).

3. Results

3.1. Water deficit modifies water status and affects the development of *Marchantia*

To investigate the responses of the basal plant *M. polymorpha* to water deficit conditions and salt treatments, we assessed the effects of these treatments on thalli, the dominant gametophytic stage during *Marchantia* development. Seven-day old thalli, grown in Gamborg's B5 culture medium in optimal conditions, were exposed to water deficit conditions by transferring them to a moist pad at 60 % RH (see Materials and methods for details) and their growth was monitored for different time lengths, while thalli maintained in control conditions (moist pad at 100 % RH) were used for comparison. After 12 h, the growth of thalli exposed to water deficit was visibly affected (Fig. 1a).

To quantify this effect, the growth of eighty individual thalli was followed to determine the plant total area using image analysis with the TWS plugin (Supporting Information Fig. S1). Importantly, image analysis to monitor plant growth has the advantage that the same individuals can be followed throughout the experiment to document the change in growth. We observed a decrease of 21 % in the total area growth of thalli exposed to water deficit for 12 h, in contrast to thalli in control conditions (corresponding to 100 %, Fig. 1b). After 24 and 48 h, a significant decrease in area growth of 24 % and 23 %, respectively, was observed. In addition, this reduction was associated with a significant decrease in relative water content (RWC) of 48 % (Fig. 1c) and with a decrement of 12 % in the osmotic potential, which was most evident at 48 h (Fig. 1d). These findings indicate that water deficit affects several physiological parameters in *Marchantia*, which in turn generates changes in its growth, relative water content and the internal

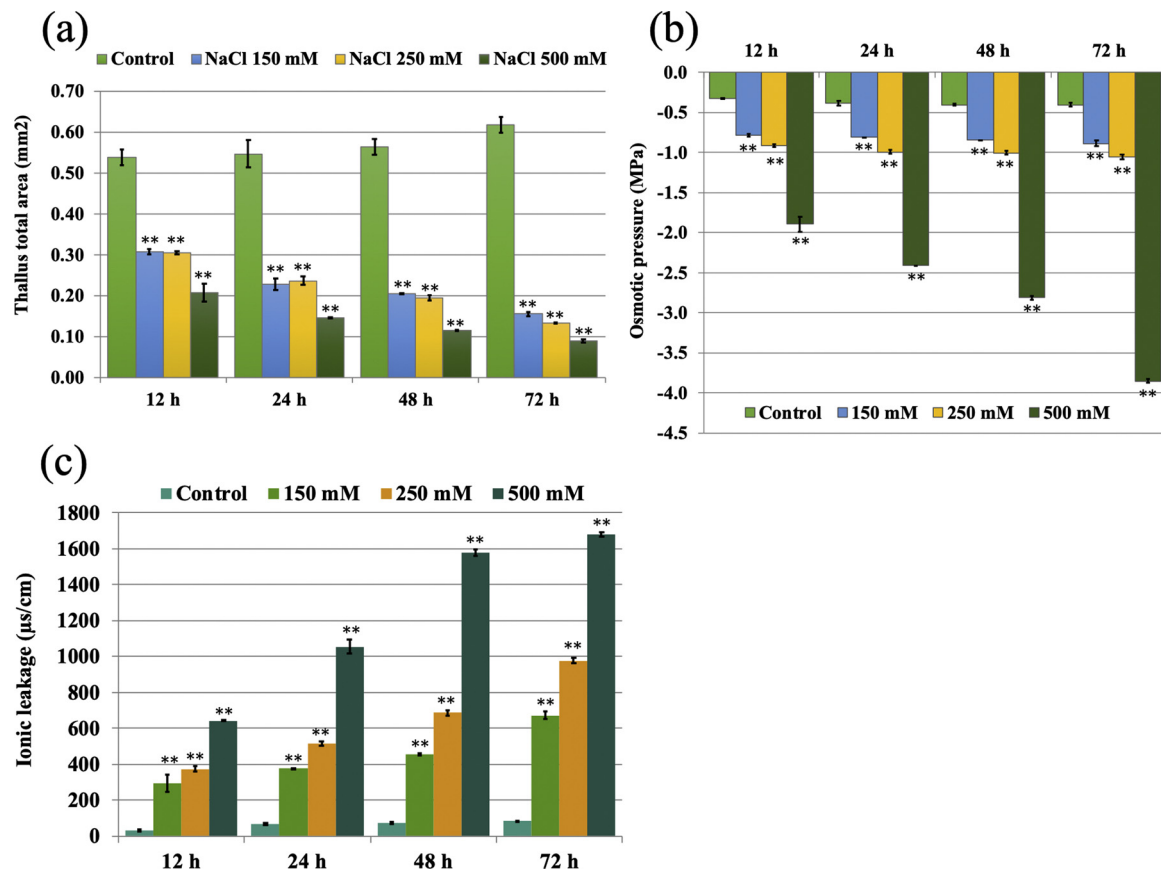


Fig. 2. NaCl treatments affect thalli growth and modify osmotic potential. (a) Thalli grown in optimal conditions for seven days and then exposed to different NaCl concentrations (150, 250 or 500 mM). (b) Osmotic potential was determined at 12, 24, 48 and 72 h of treatment and (c) ion leakage by cell death was determined at the same timepoints. Each experiment consisted of three separate plates each with fifty thalli and was performed in three independent experiments ($n = 3$). ** Represents significant differences according to Student's t distribution of the data ($\alpha = 0.05$).

osmotic potential to cope with this stress.

We also explored NaCl exposure, a condition inducing water deficit but also ion toxicity. To investigate the responses of thalli to salt treatments, seven-day old thalli grown in Gamborg's B5 culture medium in optimal conditions were transferred to medium supplemented with different NaCl concentrations (150, 250 or 500 mM), and their growth for different time lengths was followed. First, upon NaCl exposure thalli showed different morphological changes, such as differential growth (some thallus regions larger than others or sideways curvatures) and raised thalli with rhizoids excluded from the stressful medium, which increased with time (Supporting Information Fig. S2). Importantly, we observed a marked decrease in their growth and the appearance of tissue bleaching at higher NaCl concentrations and longer time periods, which we quantified through image analysis (Supporting Information Fig. S3). The total area of thalli under standard growth conditions was larger than that of thalli exposed to NaCl in all concentrations tested (Fig. 2a); thus, we observed a significant reduction in growth at 150 mM NaCl, even at 12 h. These changes prompted us to determine the osmotic potential, which as expected decreased as the NaCl concentration was raised but it was more pronounced at 500 mM NaCl (Fig. 2b). In addition, we also observed an increase in ion leakage upon salt addition (Fig. 2c), likely to be the result of damage to cells and subsequent cell death, as confirmed by Evans blue staining (Supporting Information Fig. S4).

Finally, we also found that, even though exposure of plants to severe stressful conditions or during time to a mild stress caused extensive detrimental phenotypical effects, it did not result in the plant's death. Thalli exposed to water deficit or to different NaCl concentrations for 48 and 72 h, respectively, were then transferred to medium without

stress. After three days under optimal conditions, thalli exposed to water deficit and from all NaCl concentrations tested regenerated new green tissue (data not shown).

We also analyzed the effect of osmotic stress induced by mannitol addition. Seven-day old thalli were exposed to increasing mannitol concentrations for 12, 24, 48, and 72 h. However, we observed a plethora of effects like tissue malformation, growth arrest, dehydration, and loss of chlorophyll even with a lower mannitol concentration after 24 h of exposure. Tissue damage was too severe to allow us to reproducibly estimate water deficit effects upon mannitol exposure, and thus it was not further explored in this study.

3.2. Water deficit induces cell vacuole expansion in *Marchantia*

To investigate the effects of water deficit at the cellular level, we followed the growth of thalli of seven days of age exposed to water deficit for different periods of time. We obtained confocal micrograph images from the dorsal epidermal cells located next to one of the meristematic regions of thalli grown under control conditions, these cells showed small vacuoles and homogeneous chloroplast distribution (Fig. 3a). In contrast, in thalli exposed for 24 h to water deficit, cells showed a large central vacuole surrounded by chloroplasts that showed a periclinal placement along the cell wall (Fig. 3b; additional images showing independent results are provided in Supporting Information Fig. S5). Similarly, thalli exposed to 150 mM NaCl for 24 h showed cells with a larger central vacuole surrounded by chloroplasts with anticlinal and periclinal placement along the cell wall (Fig. 3c). These results were confirmed by electron micrograph images of ultrathin sections. Thalli grown under control conditions showed cells with small vacuoles

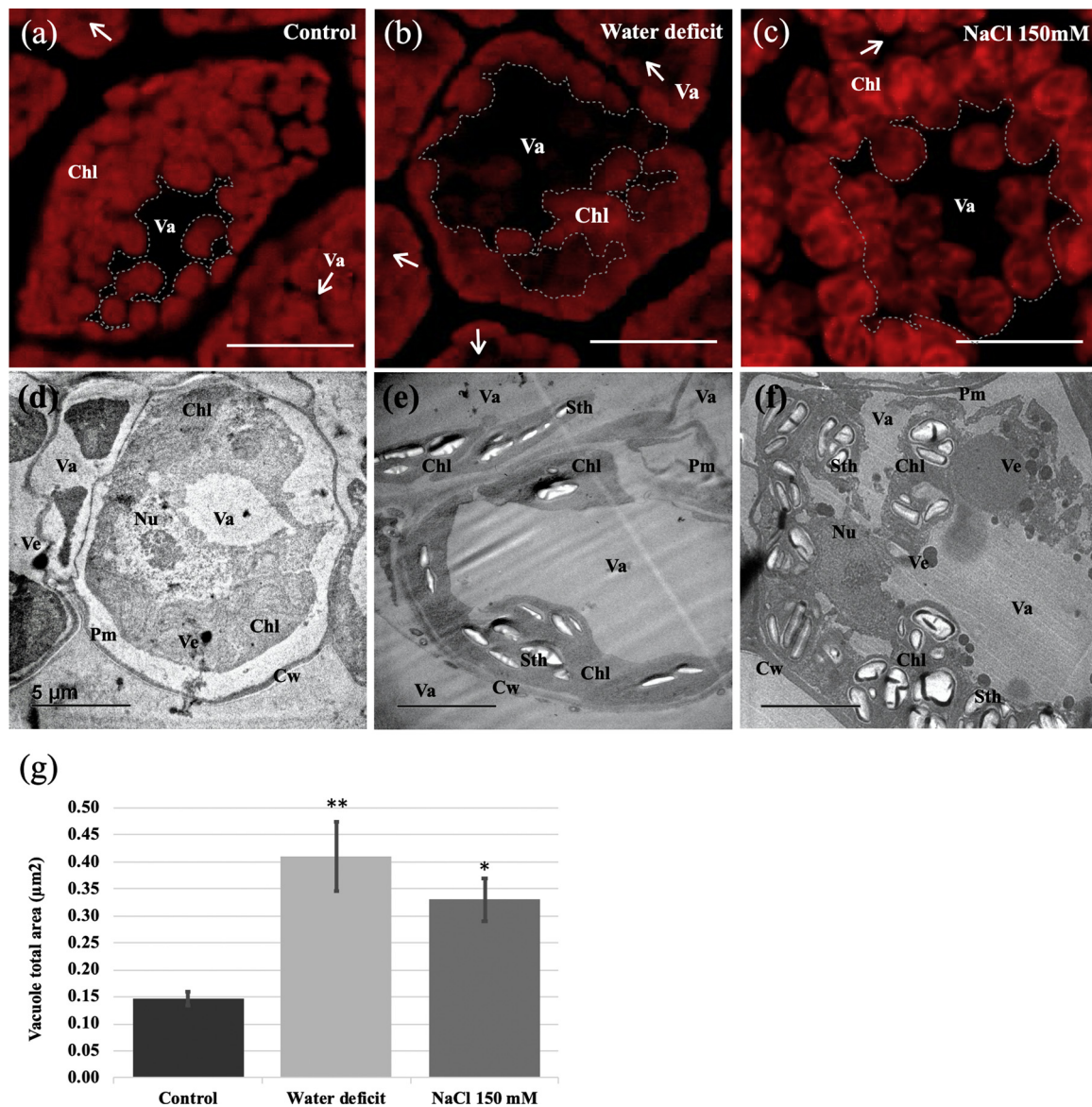


Fig. 3. Water deficit induces cell vacuole expansion and organelle redistribution in *Marchantia*. (a, d) Confocal and electron micrograph images of thalli grown under control conditions; (b, e) thalli exposed for 24 h to water deficit or (c, f) 150 mM NaCl. Confocal image bars = 50 μm , electron micrograph bars = 5 μm . Chl = Chloroplast, Cw = Cell wall, Nu = Nucleus, Pm = Plasma membrane, Sth = Starch, Va = Vacuole, Ve = Vesicle. The dotted line and arrowheads indicate the vacuole position. (g) Quantification of vacuole total area in thalli exposed to water deficit and salt treatments. Each experiment consisted of three separate plates and was performed in three independent experiments ($n = 3$). ** Represents significant differences according to Student's t distribution of the data ($\alpha = 0.05$).

and homogeneous organelle distribution (Fig. 3d; Supporting Information Fig. S5); while in thalli exposed to water deficit or NaCl, cells showed a large central vacuole surrounded by elongated periclinal chloroplasts containing small portions of starch granules (Fig. 3e and f). The presence of starch granules has been previously observed in *M. polymorpha* cells first cultured in growth media supplemented with sucrose and then exposed to desiccation. Under these conditions, chloroplasts with small grana and accumulation of starch grains were detected (Hatanaka and Sugawara, 2010). The presence of starch grains observed here could suggest that changes in carbohydrate metabolism are occurring during water deficit and salt stress, possibly to contend with cell damage; however, further studies are necessary to clarify this issue.

To quantify the change in the central vacuole detected, we determined the vacuole area as observed in the confocal microscopy images (dotted areas in Fig. 3a–c). Thalli exposed to water deficit or salt treatment showed an increase in the vacuole area corresponding to 2.8-

fold, and 2.2-fold respectively, when compared to thalli grown under control conditions (Fig. 3g). These results indicate that adjustments in the volume of the vacuole occur in response to water deficit and NaCl, likely to be involved in adjusting cellular osmotic potential to contend with water deficit effects and ionic detoxification.

3.3. Endogenous ABA levels increase upon water deficit exposure

Responses to water deficit have been extensively studied in vascular plants (Raghavendra et al., 2010), where the phytohormone ABA plays an essential regulatory role. To investigate the water deficit effect on ABA levels in *Marchantia*, we determined the endogenous levels of this phytohormone in thalli grown under control conditions and after exposure to water deficit for 6, 12, 24 and 48 h, employing a widely used antibody-based assay (Faralli et al., 2016; Luo et al., 2016). At 6 h, thalli in control conditions presented an ABA content of 33.9 ng/g dry weight; these levels increased after 12 h to 75.6 ng/g dry weight, likely

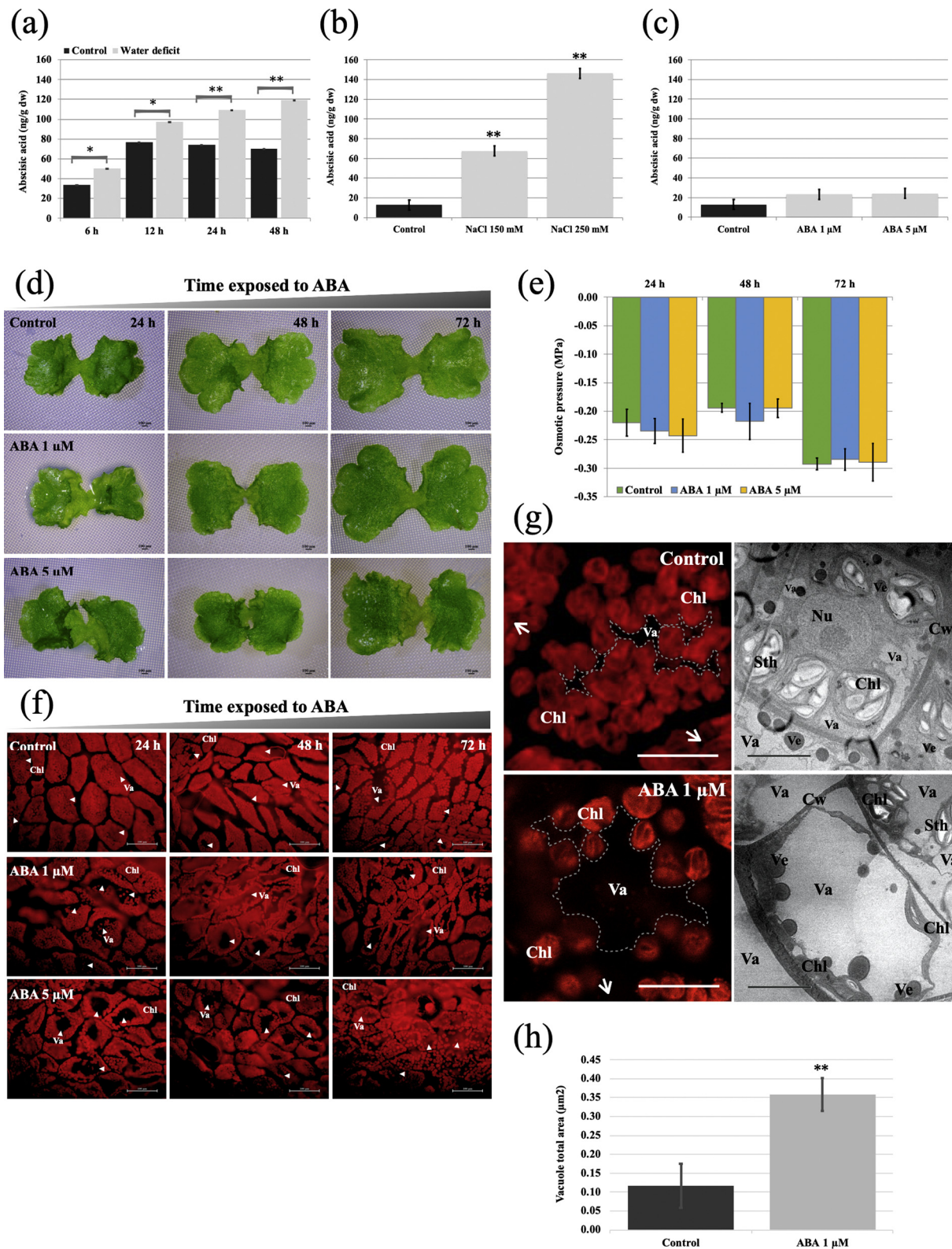


Fig. 4. Water deficit increases endogenous ABA levels and ABA treatment induces increased vacuole volume. Endogenous ABA levels were determined for thalli of seven days of age exposed to (a) water deficit for 6, 12, 24 and 48 h, (b) 150 or 250 mM NaCl at 24 h, and (c) thalli exposed to 1 or 5 μ M ABA concentrations for 24 h. (d) Thalli exposed to 1 or 5 μ M ABA for 24, 48 or 72 h were photographed, or (e) used to determine their osmotic potential and (f) auto-fluorescence as observed by microscopy. (g) Confocal and electron micrograph images showing cells with central vacuole and chloroplasts. Microscopy image bar = 100 μ m, confocal image bar = 50 μ m, electron micrograph bar = 5 μ m. Chl = Chloroplast, Cw = Cell wall, Nu = Nucleus, Pm = Plasma membrane, Sth = Starch, Va = Vacuole, Ve = Vesicle. The dotted line and arrowheads indicate the vacuole position. (h) Quantification of vacuole total area in thalli exposed to 1 μ M ABA at 24 h. Each experiment consisted of three separate plates each with fifty thalli, performed in three independent experiments ($n = 3$). ** Represents significant differences according to Student's t distribution of the data ($\alpha = 0.05$).

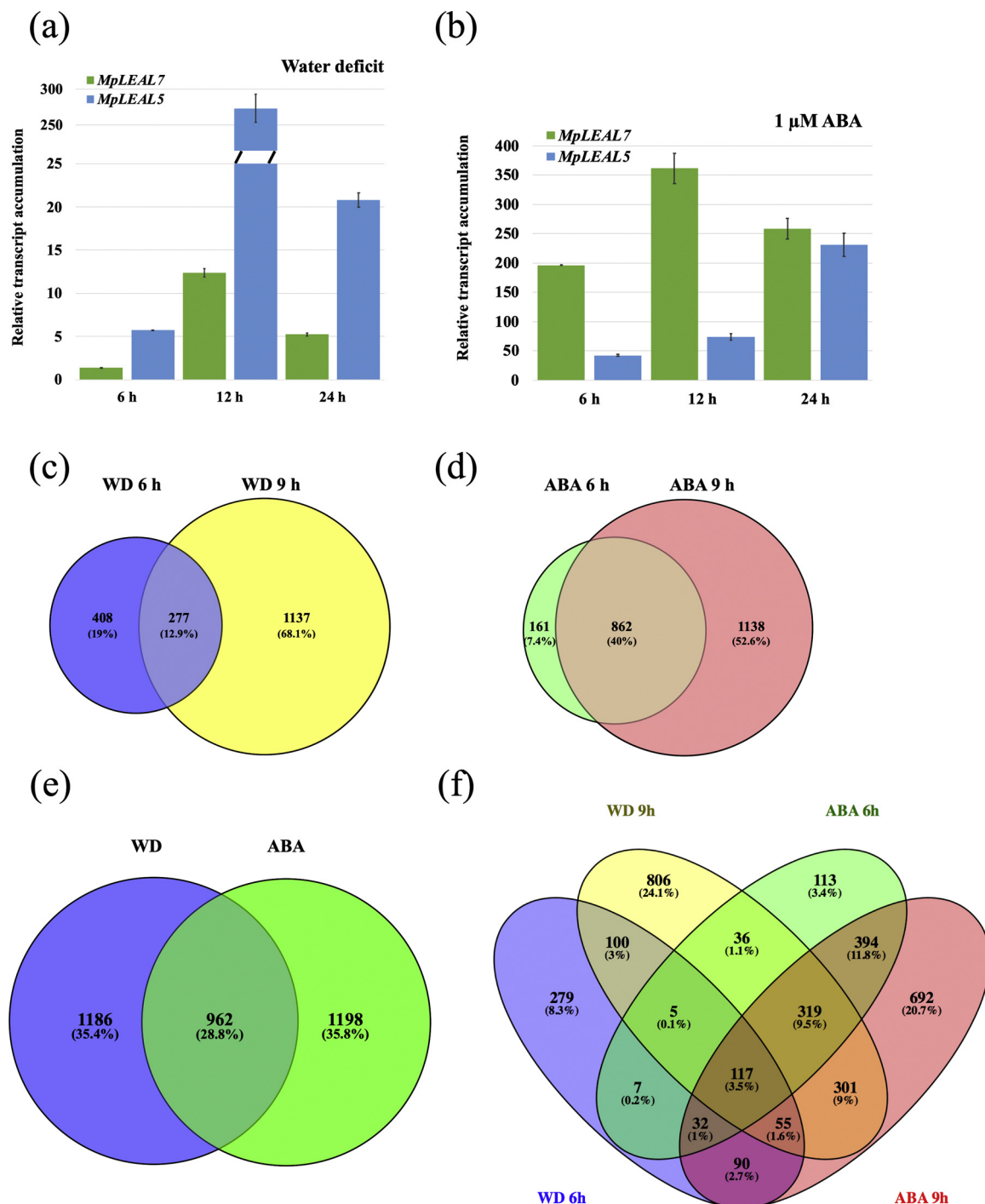


Fig. 5. Water deficit induces expression of stress-related genes in *Marchantia* as in higher plants. Transcript accumulation of *MpLEAL7* and *MpLEAL5* in 7-day old thalli exposed to (a) water deficit or (b) 1 μ M ABA for 6, 12, and 24 h, as determined by RT-qPCR. Gene expression was set at 1 for values obtained at time 0 (not shown in the graph), and Actin (Mapoly0016s0137) was used as the gene of reference. Each WD and ABA experiment consisted of three separate plates and was performed in three independent experiments ($n = 3$). RNA-Seq analysis showed that thalli exposed to (c) water deficit or (d) ABA at 6 and 9 h share differentially expressed genes. (e) Comparison of genes differentially expressed in water deficit and ABA treatments. (f) Venn diagram showing the genes shared by treatment and by time of treatment.

due to transfer of the plants to a moist pad (100 % RH). In contrast, upon water deficit treatments (moist pad, 60 % RH), ABA accumulation increased up to 50.3 ng/g dry weight at six hours (1.5-fold increase); and at 24 and 48 h ABA increased to 109.6 and 119.3 ng/g dry weight, respectively (1.5-fold and 1.7-fold increase with respect to the corresponding control samples, Fig. 4a), indicating that in response to water deficit endogenous ABA levels increase in *Marchantia* as it occurs in vascular plants. Similar to water deficit, NaCl treatments induced the

accumulation of endogenous ABA, reaching an ABA content of 67.5 ng/g dry weight in thalli of seven days of age exposed to 150 mM NaCl for 24 h, a 5.3-fold increase when compared to thalli in standard growing conditions (12.8 ng/g dry; Fig. 4b). Also, we observed that a higher NaCl concentration (250 mM) led to an 11.4-fold increase of ABA levels (146.3 ng/g dry weight), showing a correlation between ABA accumulation and stress severity and supporting the role of ABA as signaling molecule to elicit the required plant defense responses. In addition,

thalli of seven days were exposed to 1 or 5 μM ABA concentrations for 24 h and, as expected, intracellular increases of 1.8 and 1.9-fold in ABA levels were detected when thalli were exposed to 1 or 5 μM ABA concentrations (23.3 and 24.2 ng/g dry weight, respectively), when compared to thalli grown without ABA (12.8 ng/g dry, Fig. 4c); even when a higher ABA concentration was added (10 μM), no further increase in endogenous ABA levels was detected. While endogenous ABA levels increased upon exogenous addition of the hormone, the levels attained are lower than those obtained when water deficit or NaCl were applied to the plants. This finding could be attributed to limited function of ABA transporters mediating cell intake, as free ABA should have a very reduced diffusion rate across cell membranes (Boursiac et al., 2013).

3.4. Exogenous ABA addition induces increase in vacuole volume

Thalli of seven days of age exposed to 1 or 5 μM ABA did not present an apparent effect at the macroscopic level (Fig. 4d), and no significant changes in the osmotic potential were observed (Fig. 4e). However, using confocal microscopy we observed that thalli exposed to 1 or 5 μM ABA for different time periods (24, 48 and 72 h) showed an increase in their vacuole volume, concomitant with the chloroplast relocation and a decrease in their auto-fluorescence (Fig. 4f). Electron microscopy images confirmed these observations, showing that cells of thalli exposed to 1 μM of ABA for 24 h presented a large central vacuole surrounded by chloroplasts which showed a periclinal placement along the cell wall, while these effects were not observed in thalli grown under standard conditions (Fig. 4g, Supporting Information Fig. S6). Furthermore, exposure to 1 μM ABA showed an increase of 3.1-fold in the vacuole area (Fig. 4h), similar to the increase observed in thalli subjected to water deficit or salt treatments (see Fig. 3g), suggesting that ABA acts as a stress signaling molecule modulating osmotic adjustment involving the vacuole. We also explored the effect of ABA addition during gemmae growth, an early stage of *Marchantia* development. In contrast to thalli, gemmae exposed to 0.1 and 0.5 μM ABA presented growth arrest as previously reported (Eklund et al., 2018), showing slight tissue darkening after 24 h of treatment. Moreover, exposure of gemmae to ABA resulted in an increase in the vacuole volume with concomitant relocation of cell organelles and decreased auto-fluorescence (Supporting Information Fig. S6).

3.5. Water deficit induces transcriptome-wide changes in *Marchantia* as in vascular plants

To explore responses of thalli exposed to water deficit and ABA treatments at the molecular level, we started by analyzing the expression of *LEA* protein-encoding genes. *LEA* proteins act as protectors of cellular machinery and are involved in the tolerance of vascular plants to water deficit (Battaglia et al., 2008; Dong-Hong et al., 2012). We determined expression of *MpLEAL7* (Mapoly0025s0039) and *MpLEAL5* (Mapoly0087s0015) encoding for group 3 *LEA* proteins, as previously reported (Aker et al., 2014). In thalli of seven days of age, transcript accumulation of *MpLEAL7* and *MpLEAL5* exposed to water deficit or to 1 μM of ABA showed significant transcript accumulation (Fig. 5a and b, respectively). In contrast, mRNA levels of *MpABI1*, an ortholog of *Arabidopsis ABSICISIC-ACID-SENSITIVE1 (ABI1)*, encoding for a protein phosphatase involved in ABA signaling (Tougan et al., 2010), showed no significant changes upon water deficit or ABA treatments (Supporting Information Fig. S7).

Considering that *MpLEAL7* and *MpLEAL5* transcript accumulation increased as early as 6 h of water deficit and ABA treatments, we sought to identify early transcriptomic responses using an RNA-Seq analysis from thalli exposed to water deficit or 1 μM ABA for 6 and 9 h (see Materials and methods for experimental setup). We obtained between 9 and 23 million reads per library for a total of 273 million raw reads that were mapped to the *M. polymorpha* genome available at phytozome.jgi.doe.gov (Supporting Information Dataset 1). We carried out a

bioinformatic analysis to determine differential expression with four different programs (EdgeR, DESeq, DESeq2 and NOISeq, see Materials and Methods for details) to obtain a final dataset consisting of the differentially expressed (DE) genes shared by all methods employed (Supporting Information Dataset 2). Thus, in thalli exposed to water deficit (WD) we obtained 685 and 1739 DE genes at 6 and 9 h, respectively, and 277 DE genes (13 %) shared between these time points (Fig. 5c). In comparison, for thalli exposed to ABA we found differential expression for 1023 and 2000 genes at 6 and 9 h, respectively, while 862 genes (40 %) were shared between 6 and 9 h (Fig. 5d). In addition, 29 % of DE genes are shared between WD (total of 2148) and ABA treatments (total of 2160). This 29 % represents 962 genes (Fig. 5e), from which 453 up-regulated genes and 243 down-regulated genes were shared between WD and ABA (696 out of 962 genes corresponding to 72 %). The proportion of genes shared with the similar direction of regulation (72 %), suggests that a significant number of stress responses are common to both conditions. However, when DE genes were compared between both conditions and considering each time point evaluated (6 and 9 h), only a limited number of genes were shared (117 genes, corresponding to 3.5 %, Fig. 5f), indicating that as expected, individual treatments trigger changes with different gene expression kinetics.

To predict functional relationships among differentially expressed genes, we carried out a gene ontology (GO) annotation and enrichment analysis of the DE genes for WD and ABA, comparing 6 vs. 9 h. The analysis conducted at the biological process level showed up-regulated genes for transport processes, ion binding and defense response which increased from 6 h to 9 h in thalli exposed to WD (Fig. 6a). As expected, down-regulated genes included photosynthesis and light harvesting terms (Fig. 6b). Similarly, in thalli exposed to ABA up-regulated genes corresponding to metabolite transport, and response to stress increased from 6 h to 9 h (Fig. 6c). As in WD, in thalli exposed to ABA, down-regulated genes were related to photosynthesis, light harvesting and regulation of cyclin-dependent protein (Fig. 6d).

The enrichment analysis at the molecular function level complemented the enrichment of biological processes results, showing more specific data in water deficit about the kind of ions and transport, including genes related to metal ion binding, manganese and copper ion binding, transmembrane transporter activity, secondary active transmembrane transporter, carbohydrate transmembrane transporter, oxidoreductase and antioxidant activity among others (Supporting Information Fig. S8). In contrast, in response to ABA we identified genes related to metal ion binding, but mostly related to oxidoreductase activity, to a peptidase regulator, and many others related with carbohydrate kinase activity, hydrolase activity and carbohydrate transmembrane transporter (Supporting Information Fig. S8). These findings are consistent with our previous observations, where we documented changes in vacuole volume in thalli exposed to WD and ABA, where ion binding and metal ion transport activities are likely involved.

As shown above, we determined by RT-qPCR that at least two previously identified *LEA* genes are induced by WD and ABA treatments (Fig. 5). To gain further information about this group of genes, we defined the complete set of *LEA* genes in *M. polymorpha* and their expression patterns under the conditions studied, based on the current annotation of the *M. polymorpha* genome and our RNA-seq data. Gene ontology analysis revealed thirty-six *LEA* genes belonging to six of the seven known families (as defined by Battaglia and Covarrubias, 2013) present in *M. polymorpha* (Supplemental Table S2). In response to the WD and ABA treatments applied, we found several *LEA* genes induced, including two from group 1, two in group 2, all four members in group 3, two in group 4, six in group 5, and three belonging to group 7. In contrast to this induction, 11 *LEA* genes did not show significant changes in expression, and six were even slightly repressed by WD and ABA treatments. Consistently, *MpLEAL5* (Mapoly0087s0015, a group 3 *LEA* protein) was induced by WD and ABA treatments as shown by RT-qPCR and is present among the genes induced in the RNA-seq data

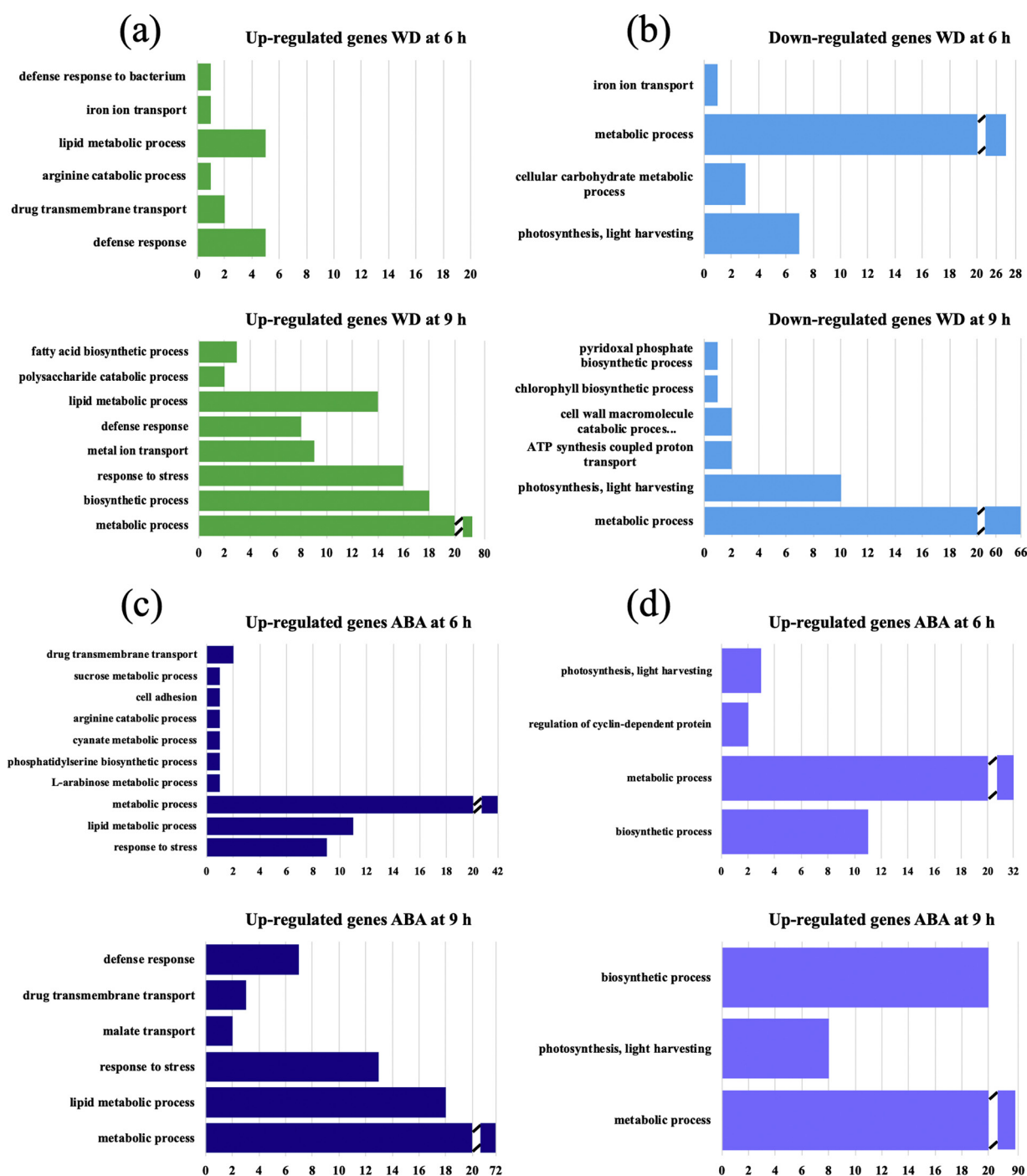


Fig. 6. Gene ontology analysis of differentially expressed genes in Water deficit and ABA treatment. In GO enrichment at the level of biological process of DE genes in thalli exposed to water deficit (a) shows up-regulated genes at 6 and 9 h and (b) shows down-regulated genes at 6 and 9 h. In 1 μ M ABA treatments (c) shows up-regulated genes at 6 and 9 h and (d) shows down-regulated genes at the same time points. X-axis indicates number of genes in each category.

(Supplemental Table S2). In contrast, *MpLEAL7* (Mapoly0025s0039) was not included in our LEA gene dataset, as it is not annotated with a PFAM ID, however it showed increased accumulation in response to WD and ABA treatments. These results confirm that, as in vascular plants (Battaglia and Covarrubias, 2013), LEA proteins participate in the adjustment response to stress in liverworts.

Finally, to corroborate the RNA-seq results we employed RT-qPCR to determine the expression levels of 10 randomly selected genes for high or low expression upon WD or ABA treatments. The selected genes encoded members of KUP system potassium uptake protein (Mapoly0046s0054), carbonate dehydratase (Mapoly0012s0065),

chitinase/Poly-beta-glucosaminidase (Mapoly0147s0004), S-locus glycoprotein domain (Mapoly0061s0017), BLUE COPPER PROTEIN (Mapoly0037s0115), cupin domain (*Cupin2*, Mapoly0180s0030), cupin (*Cupin1*, Mapoly0377s0002), tocopherol cyclase (Mapoly0099s0019), MALIC ENZYME-RELATED (Mapoly0169s0005) and *ABSCISIC-ACID-INSENSITIVE5* (*ABI5*, Mapoly0072s0050). The selected genes included genes with differential expression in WD and/or ABA treatments, showing up-regulation or down-regulation. The RT-qPCR expression profiles obtained were in complete agreement with the RNA-Seq data (Supporting Information Fig. S9).

4. Discussion

In contrast to vascular plants and in particular the model plant *Arabidopsis thaliana*, little is known about water deficit responses in the liverwort *Marchantia polymorpha*. Liverworts belong to the basal plant lineages that first colonized land, and through evolution they must have acquired strategies to contend with the adverse conditions confronted during this transition. The study of the stress responses present in this plant species may reveal fundamental processes that helped in its adaptation to a novel environment. Among bryophytes, *Marchantia* has been described as not tolerant to desiccation; nevertheless, *M. polymorpha* can withstand water potentials of -45 MPa, certainly beyond the range of survival for other bryophytes (-20 to -40 MPa) and some crop species (-1.5 to -3 MPa) (Proctor and Pence, 2002). In this study, we explored *M. polymorpha* responses to water limitation and NaCl stress at different levels. We established a system to achieve gradual water deficit and additionally we tested the effects of different salt concentrations in the growth medium. Even though thalli exposed to water deficit showed reduced growth, and a decrease in RWC and in internal osmotic potential, no further damage was detected. Regarding salinity effects in bryophytes, most have been documented in *P. patens*, a moss able to grow in media containing up to 600 mM NaCl (Oliver et al., 2005; Frank et al., 2005; Benito and Rodríguez-Navarro, 2003). In contrast, here we observed that a concentration of 150 mM NaCl resulted in bleaching and tissue deformation, indicating that *M. polymorpha* is more sensitive to NaCl than *P. patens*. However, when thalli were exposed to severe WD or NaCl treatments and returned to optimal conditions for three days, they were able to regenerate green tissue. This response could be an analogous strategy to that seen in mosses, where the tolerance to severe drought and salt stress is thought to rely on the efficient limitation of stress-induced cell damage and an efficient repair of cell injury upon stress relief (Ruibal et al., 2012; Zeppel et al., 2015).

4.1. *M. polymorpha* preserves several features to outlast water deficit

In vascular plants, unraveling the contribution of individual factors that participate during water limitation is challenging as many events occur concomitantly: turgor is lost, RWC decreases, solute concentrations increase, and cell membranes shrink, altering interactions with the cytoskeleton and cell wall (Haswell and Verslues, 2015; Sack et al., 2018). Similarly, in *M. polymorpha* thalli exposed to WD we observed a reduction in RWC associated with increases in solute concentration, but without an apparent decrease of vacuolar volume. Plants can respond to water limitation through osmotic adjustment via accumulation of compatible osmolytes (Greenway and Munns, 1980; LaRosa et al., 1987; Munns, 2008; Krasensky and Jonak, 2012; Sussmilch et al., 2017). We observed that *M. polymorpha* exposed to water deficit and NaCl stress, showed an increased vacuole volume associated with a gradual decrease in osmotic potential. Recently, it has been suggested that turgor loss provides an endogenous signal to trigger ABA accumulation (Sussmilch et al., 2017). However, in different angiosperms ABA accumulation was not caused by declining turgor pressure but instead was associated with a decline in RWC (Sack et al., 2018). Therefore, it seems likely that in *Marchantia* changes in cell and vacuole volume are a consequence of coordinated regulation of ion concentrations in the vacuole and cytosol through plasma and vacuole membranes, thus triggering ABA accumulation in response to changes in RWC. Our results suggest that *Marchantia* is able to carry out osmotic adjustment via solute accumulation, and that this ability to regulate water status contributes, together with other cellular mechanisms, to mount a defense response to counteract water loss effects.

In *M. polymorpha* we also observed chloroplast relocation upon exposure to water deficit and NaCl. As early as 1908, chloroplast relocation in the moss *Funaria hygrometrica* was observed to be induced by low temperature stress (Senn, 1910). Chloroplasts relocate in response

to high light intensities in *P. patens* (Suetsugu and Wada, 2007) and in response to cold treatments in the fern *Adiantum capillus-veneris* (Kodama et al., 2008) and in *M. polymorpha* (Ogasawara et al., 2013). Ogasawara et al. (2013) suggested that chloroplast relocation induced by cold depends on actin filaments; however, our results indicate that relocation of chloroplasts and other organelles towards the cell periphery under WD and NaCl stress is directly due to the expansion of the central vacuole. Although it is possible that relocation of chloroplasts and other organelles in response to different forms of stress is due to distinct factors, we suggest that regulation of vacuole turgor is an important ancestral component already present in *Marchantia*, likely mediated by yet undefined tonoplast-associated transporters. To shed light in this regard, we explored the responses of thalli to WD and ABA addition at the transcriptomic level. Gene-ontology enrichment analysis provided a broad scenario of cellular activities present in thalli exposed to WD and ABA treatments. *Marchantia* thalli exposed to water deficit, showed enrichment of GO categories related to cation and metal transport, malic and malate enzyme activity, suggesting that ion and water transport are involved in control of vacuolar turgor, directed at preventing cell damage induced by water loss.

4.2. ABA does not modify plant water status but affects cellular ultrastructure

Thalli exposed to different ABA concentrations did not show any apparent change in growth, in agreement with previous reports (Hellwege et al., 1994; Pence et al., 2005; Jahan et al., 2019). In contrast, in this work we found that gemmae exposed to high ABA concentrations presented darkening of tissue and growth arrest. Similar results were reported by Tougane et al. (2010) and Eklund et al. (2018), who showed that addition of exogenous ABA delayed the growth of gemmae. In thalli and gemmae, at the cellular level, we observed chloroplast relocation towards cell boundaries associated to vacuole expansion upon ABA addition, similar to the effect seen in WD and NaCl stress. However, these changes did not correlate with a detectable decrease in osmotic potential, suggesting that ABA treatments promote the movement of pre-existing osmolytes from the cytosol to the vacuole, possibly by the activation of solute transporters, hence favoring water transport to the vacuole. These observations are in agreement with previous reports demonstrating that ABA regulates transporters and ion channels in the tonoplast of *Arabidopsis* cells (Pandey et al., 2007).

ABA-dependent pathways are present in *Marchantia*, and several factors involved have been already identified. Addition of exogenous ABA to cultured *M. polymorpha* cells induces gene expression changes in *MpABI1*, a homolog of *ABA-INSENSITIVE 1* protein phosphatase 2C required for ABA signaling in vascular plants (Tougane et al., 2010; Bowman et al., 2017; Jahan et al., 2019). Also, transient overexpression of *MpABI1* resulted in suppression of ABA signaling, and recently *MpABI1* and *MpABI3* (a homolog of *AtABI3*) were associated to dormancy in gemmae (Eklund et al., 2018; Jahan et al., 2019). In transgenic *P. patens* expressing a mutant version of *MpABI1* (*MpABI1-d2*), reduced osmotic stress tolerance and reduced accumulation of ABA-induced LEA proteins were observed (Tougane et al., 2010; Jahan et al., 2019). In addition, Eklund et al. (2018) have shown that *MpABI3A* activates LEA expression in an ABA-dependent manner, and that it can complement a mutant of *P. patens* *ABI3*. Finally, other essential factors participating in the ABA signaling pathway have been revealed from the analysis of the *M. polymorpha* genome (Bowman et al., 2017).

4.3. Other response mechanisms operate during water deficit in *Marchantia*

In vascular plants, other biochemical pathways contribute to mounting a cellular protection strategy. The accumulation of LEA proteins has been described in orthodox seeds and vegetative tissues of angiosperms, in response to water limitation (Buitink et al., 2002; Oliver et al., 2005). In *Marchantia* thalli exposed to water deficit and

ABA treatments, we found that *MpLEAL7* and *MpLEAL5* showed transcript accumulation. Consistently, our transcriptome-level analysis showed differential expression of several genes belonging to the different groups of LEA protein families present in plants (Close, 1997; Juszczak and Bartels, 2017), in thalli exposed to water deficit and ABA treatments (Supporting Table S2), marking their importance as contributors to plant stress responses.

In addition, in vascular plants water loss also leads to inhibition of photosynthesis thus causing ROS accumulation (Ergen et al., 2009). Consistently, in *Marchantia* exposed to WD and ABA treatments, we detected down-regulation of genes associated to photosynthesis and light harvesting. Moreover, exposure of *M. polymorpha* thalli to water deficit led to a reduction of starch content (Fig. 3), and possibly to the accumulation of soluble sugars to maintain vacuolar turgor, as has been reported in vascular plants (Basu et al., 2007; Kempa et al., 2008; Krasensky and Jonak, 2012). In vascular plants, the response to drought and ABA triggers the production of reactive oxygen species (ROS), which in turn induces an increase in cytosolic Ca^{2+} (Osakabe et al., 2014). Upon exposure to water deficit, the transcriptomic response of thalli showed enrichment of GO categories related to oxidoreductase, antioxidant and peroxidase activities, and calcium ion binding, suggesting that ROS homeostasis is also involved in preventing cell damage induced by water loss.

According to our results, we suggest that the protein synthetic machinery, membrane structure and metabolism, and the need to maintain the integrity and turgor of the vacuole are central to the response in *Marchantia* exposed to water deficit, features that are conserved in this ancestral liverwort.

5. In summary

In this study, we established a system to achieve gradual water deficit in thalli of the basal liverwort *M. polymorpha* in order to understand the basis of the responses to water limitation and NaCl stress in this model plant. Thalli exposed to water deficit and NaCl stress presented increases in volume of the central vacuole and adjustments in the osmotic potential. Also, we showed that water deficit and high NaCl concentrations increased the endogenous ABA levels in thalli, and that upon these treatments several genes modified their expression. In addition, our RNA-seq study revealed that ion binding and transport GO categories could explain the changes in vacuole volume and the adjustments in the osmotic potential. These findings indicate that *M. polymorpha* employs an array of strategies to cope with adverse conditions, including changes in the volume of its central vacuole by osmotic potential adjustments, increased ABA levels and transcriptome-level effects. These results, mirroring the current knowledge gained from vascular plants, strongly indicate that these responses are preserved as an ancestral response system from the first plants that colonized terrestrial environments.

Author contributions

DGV, GL, AAC, JLR designed experimental strategies and analyzed data. DGV performed all experiments. AAC and JLR coordinated work. DGV, AAC and JLR wrote the manuscript, with input from all authors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.envexpbot.2020.104172>.

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