ARTICLE

Reactive oxygen species are required for spore-wall formation in *Physcomitrella patens*

Fazle Rabbi, Karen S. Renzaglia, Neil W. Ashton, and Dae-Yeon Suh

Abstract: A robust spore wall was a key requirement for terrestrialization by early plants. Sporopollenin in spore and pollen grain walls is thought to be polymerized and cross-linked to other macromolecular components, partly through oxidative processes involving H_2O_2. Therefore, we investigated effects of scavengers of reactive oxygen species (ROS) on the formation of spore walls in the moss *Physcomitrella patens* (Hedw.) Bruch, Schimp & W. Gümbel. Exposure of sporophytes, containing spores in the process of forming walls, to ascorbate, dimethylthiourea, or 4-hydroxy-TEMPO prevented normal wall development in a dose, chemical, and stage-dependent manner. Mature spores, exposed while developing to a ROS scavenger, burst when mounted in water on a flat slide under a coverslip (a phenomenon we named "augmented osmolysis" because they did not burst in phosphate-buffered saline or in water on a depression slide). Additionally, the walls of exposed spores were more susceptible to alkaline hydrolysis than those of the control spores, and some were characterized by discontinuities in the exine, anomalies in perine spine structure, abnormal intine and aperture, and occasionally, wall shedding. Our data support the involvement of oxidative cross-linking in spore-wall development, including sporopollenin polymerization or deposition, as well as a role for ROS in intine/aperture development.

Key words: augmented osmolysis, exine, perine, intine, oxidative cross-linking, sporopollenin polymerization, microscopy.

Résumé : Une paroi des spores robuste a constitué une exigence clé de la terrestrialisation des premières plantes. On croit que la sporopollénine des parois des spores et des grains de pollen se polymérise et forme des pontages avec d'autres macromolécules, en partie par des processus oxydatifs impliquant le H_2O_2. C'est pourquoi les auteurs ont examiné les effets de piégeurs des dérivés réactifs de l'oxygène (DRO) sur la formation des parois des spores de la mousse *Physcomitrella patens* (Hedw.) Bruch, Schimp & W. Gümbel. L'exposition des sporophytes, contenant des spores dont la paroi est en cours de formation, à l'ascorbate, à la diméthylthiourée ou au 4-hydroxy-TEMPO, empêchait le développement normal de la paroi de manière dépendante de la concentration, du produit et du stade. Les spores matures, exposées au cours de leur développement à un piégeur de DRO, éclataient lorsqu'elles étaient montées dans l'eau sur une lame plate et sous une lamelle (un phénomène que les auteurs ont appelé « osmolyse augmentée » puisqu'elles n'éclatent pas dans une solution saline tamponnée au phosphate ou dans l'eau sur une lamelle creuse). En outre, les parois de spores exposées étaient plus sensibles à l'hydrolyse alcaline que les spores témoins, et certaines se caractérisaient par des discontinuités de l'exine, des anomalies de la structure des épines de la périne, une intine et une aperture anormales et occasionnellement, une perte de la paroi. Ces données soutiennent l'implication de pontages oxydatifs dans le développement de la paroi des spores, y compris la polymérisation ou le dépôt de sporopollénine, ainsi qu'un rôle des DRO dans le développement de l'intine/aperture. [Traduit par la Rédaction]

Mots-clés : osmolyse augmentée, exine, périne, intine, pontage oxydatif, sporopollénine, polymérisation, microscopie.

Introduction

The evolution of land plants ~500 million years ago (Morris et al. 2018) required innovations to counter challenges posed by dehydration, UV radiation, and gravity. One such innovation was a durable spore wall, which provided protection from dehydration and UV radiation. The bryophyte spore wall is composed of a minimum of two layers: an inner intine, and an outer exine. An additional outermost perine layer is found in moss and fern spores (Brown and Lemmon 1990; Wallace et al. 2011). Sporopollenin is the main polymeric constituent of the walls of spores (exine and perine) and pollen grains (exine). It is chemically resistant and composed of polyhydroxylated fatty acid derivatives and oxygenated aro-

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matic compounds linked together by extensive ester and ether bonds (Dominguez et al. 1999). A recent study provides chemical and spectroscopic evidence that acetal linkages are also prevalent in pine sporopollenin (Li et al. 2019).

Several enzymes involved in the biosynthesis of sporopollenin monomers and transcription factors regulating their expression are known (Ariizumi and Toriyama 2011; Quilichini et al. 2015). In *Arabidopsis*, a fatty acyl reductase (MS2), two cytochrome P450-dependent fatty acid hydroxylases (CYP703A2, CYP704B1), an acyl-CoA synthetase (ACOS5), two type III polyketide synthases (PKSA, PKSB), and two tetraketide α-pyrone reductases (TKPR1, TKPR2) participate in the biosynthesis of sporopollenin monomers (Morant et al. 2007; de Azevedo Souza et al. 2009; Dobritsa et al. 2009; Grienenberger et al. 2010; Kim et al. 2010; Chen et al. 2011). Also in *Arabidopsis*, two transcription factors, ABORTED MICROSPORES and MALE STERILE 188, form a regulatory feed-forward loop in which the former induces the latter, and they co-operatively induce the expression of *MS2*, *CYP703A2*, *PKSA*, and *PKSB* (Wang et al. 2018). Data from phylogenetic and gene expression studies of *Physcomitrella* orthologs imply that the sporopollenin biosynthetic pathway is widely conserved in land plants (Colpitts et al. 2011). This conclusion is strengthened by the finding that *Physcomitrella* strains possessing deletion mutations of *MS2* and *PKSA/B* orthologs produce spores with defective walls (Wallace et al. 2015; Daku et al. 2016). However, the enzymes that catalyze sporopollenin polymerization and deposition are yet to be discovered.

The chemical stability of lignin, suberin, and the polymeric matrices of the plant primary cell wall is largely due to C–C and C–O (ether) bonds formed by oxidative coupling reactions (Bernards et al. 1999; Burr and Fry 2009; Shigeto and Tsutsumi 2016). Class III peroxidases catalyze the oxidative coupling reactions of lignin polymerization using H_2O_2 as oxidant. It is reasonable to expect similar processes to occur in sporopollenin polymerization and its deposition in the walls of spores and pollen grains. Matveyeva et al. (2012) reported evidence, using a fluorescent dye, that the superoxide anion radical ($O_2^{\bullet-}$) and probably other reactive oxygen species (ROS) are present in the exine of tobacco microspores at the tetrad stage when sporopollenin polymerization and deposition are occurring.

In culture, *Physcomitrella* sporophytic development, including sporogenesis, occurs when the sporophytes are submerged in water, thus enabling both chemical rescue and inhibitor studies. To obtain evidence of the involvement of ROS in sporopollenin polymerization and spore-wall formation, we investigated the effects of ROS scavengers on the formation of spore walls by exposing sporophytes containing developing spores to ascorbate (AsA), *N,N′*-dimethylthiourea (DMTU), and 4-hydroxy-Tempo (Tempol). AsA is a natural antioxidant, and DMTU and Tempol are representative thiourea and nitroxide antioxidants, respectively. These scavengers are highly soluble in water, less toxic, and have already been studied in-planta within the context of plant developmental processes (Soule et al. 2007; Akram et al. 2017; Mei et al. 2017).

Materials and methods

Plant material, culture conditions, and chemicals

The *pabB4* strain of *Physcomitrella patens* (Hedw.) Bruch, Schimp & W. Gümbel (Ashton and Cove 1977) was used in this study. *PabB4* was obtained originally by *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine mutagenesis and shown by conventional genetic analysis through sexual crossing to possess a single *pab* biochemical mutation (Ashton and Cove 1977; Courtice et al. 1978). When grown on medium containing adequate *p*-aminobenzoic acid (PABA) (1.8 and 18 μmol/L for gametophytes and sporophytes respectively), the *pabB4* control strain is phenotypically (i.e., morphologically and developmentally) indistinguishable from the original Gransden wild type strain from which it was derived. Like the original Gransden wild type, obtained from a single spore isolated from nature by H.L.K. Whitehouse in 1962, it produces abundant sporophytes containing numerous viable spores with normal spore coat ornamentation and typical stratification of the spore wall into outer perine, middle exine, and inner intine layers (Ashton and Cove 1977; Courtice et al. 1978; Singer et al. 2007; Singer and Ashton 2007; Daku et al. 2016). Gametophytic plants were grown axenically in 30 mL glass tubes containing ABC medium (Knight et al. 1988) solidified with 1.5% agar and supplemented with 1.8 μmol/L PABA. Cultures were maintained under continuous white light (25–40 μmol·cm^{-2}·s^{-1}) at 20–22 °C and 30%–50% relative humidity. To produce sporophytes, 2–3-month-old gametophytes were subjected to cold stress at 16 °C for 3 weeks and then irrigated with 5 mM HEPES buffer (pH 8.0) containing 18 μmol/L PABA to facilitate fertilization and support growth and development of the PABA-requiring sporophytes.

AsA, DMTU, and Tempol were purchased from Sigma–Aldrich (Oakville, Ontario, Canada).

Effects of ROS scavengers on the development of sporophytes and spores

At 0, 10, or 20 days post-irrigation (dpi), ROS scavengers were added at 0.1, 1, and 10 mmol/L to the irrigation solution in which plants had been submerged. By 35 dpi, sporophytes and the spores within them had reached maturity (late orange stage) (Daku et al. 2016). For each treatment, three culture tubes were used. Under the standard conditions, ~80 sporophytes were produced in each tube. Two or more sporophytes from each of three culture tubes were collected, and the spores were gently released into 200 μL of water or PBS (phosphate buffered saline, pH

7.4). The spores were immediately mixed by gentle pipetting, and then 10 μL aliquots, each containing ~1000 spores, were mounted on slides. For each observation, at least three slides were prepared and representative images recorded. All of the experiments involving 10 mmol/L of scavengers added at 20 dpi were duplicated.

Swelling and bursting of spores

Aliquots (10 μL) of spore suspension in water or in PBS were mounted on a flat glass slide or on a depression slide. The slide was overlaid with a coverslip and its edges were sealed with nail polish to prevent evaporation. Images of spores were taken as soon as possible after mounting (within 5 min), and the spores were monitored for up to 2 h to detect swelling and bursting. The times taken for bursting to occur were recorded. To quantify swelling in water, spore diameters were measured from spore images taken within 5 min of mounting and 30 min after mounting. Spore diameter was taken as the mean of the largest diameter and that perpendicular to it.

For Sudan IV-staining of oil droplets, spores from sporophytes treated with 10 mmol/L AsA in 20 μL water were placed between a slide and a cover slip. After bursting, aliquots (5 μL) were withdrawn and mixed with an equal volume of Sudan IV solution (0.1% in ethylene glycol) for staining. Images were obtained with a Nikon Eclipse 80i compound light microscope equipped with a DS-Ri1 digital camera.

Transmission electron microscopy

Capsules of mature sporophytes were punctured with a razor blade or fine needle to facilitate infiltration of solutions and resin. The specimens were fixed in 2% glutaraldehyde in 50 mmol/L sodium phosphate buffer (pH 7.2) for 1 h at room temperature, then overnight at 20 °C. Following three rinses in buffer for 30 min each, the sporophytes were post-fixed for 1 h in buffered 2.5% OsO_4 followed by three 10-min rinses in distilled water. The plants were dehydrated in a graded ethanol series, rinsed three times in 100% ethanol, and slowly infiltrated over 4–7 days by increasing the ratio of Spurr's resin to ethanol. The plants were placed in molds with fresh resin, and cured for 16 h at 65 °C. Thick sections (500–1000 nm) were mounted on glass slides and stained with 1.5% toluidine blue in distilled water to monitor spore stage and integrity using light microscopy. Thin sections (60–90 nm) were collected on nickel grids and observed unstained and post-stained with methanolic uranyl acetate and basic lead citrate. The specimens were observed on a Hitachi H7650 transmission electron microscope at 60 kV and images were captured digitally. Three to ten capsules of each treatment were used to observe the ultrastructural features of mature spores.

Scanning electron microscopy

Three to six capsules were harvested from each treatment, air-dried, and opened on stubs to disperse the spores. The specimens were sputter-coated with 50 nm of Au/Pd using a Denton Desk II Vacuum Sputter Coater, and imaged using a scanning electron microscope (QUANTA FEG 450; FEI) with 5 kV acceleration voltage.

Alkaline hydrolysis

For alkaline hydrolysis, spores from sporophytes that had been treated with 10 mmol/L ROS scavengers at 20 dpi were heated at 70 °C in 2 mol/L KOH for various periods of time. After treatment, the spores were washed with water (3×) by centrifugation, and their images recorded. For each treatment, six sporophytes from three culture tubes were used, and two slides were prepared for each observation.
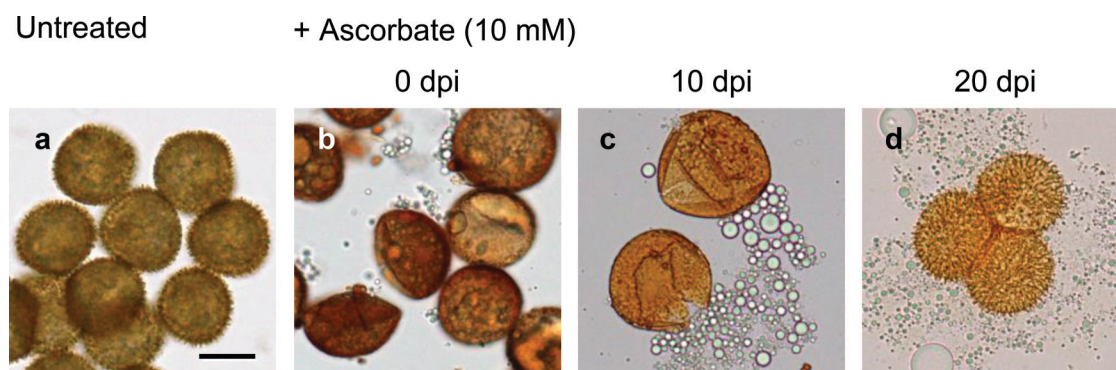
Statistical analysis

The significance of a difference between a given pair of means was assessed with the Student t test.

Results

Effects of ROS scavengers on spore development

Although in culture it is impossible to achieve complete synchronization of sporophytic development because fertilization may occur prior to and at different times following irrigation, most *Physcomitrella* sporophytes are in the late green stage at 20 dpi. During this stage, the formation of sporopollenin-containing walls is initiated around developing spores (Daku et al. 2016). During the subsequent yellow stage, which lasts ~5 days, spines of the perine begin to appear, and both the exine and perine become pigmented. Spore maturation and wall development continue for approximately another 10 days through the orange stage, when the perine spines develop further and pigmentation intensifies. The effects on developing spores of exposure to ROS scavengers in comparison to untreated spores (Fig. 1a) are stage-dependent: the earlier and longer the exposure, the greater the severity. Thus, for example, when 10 mmol/L AsA was present from 0 dpi, some spores became malformed and oblong-shaped (5 of 25 spores), and all lacked prominent perine spines (Fig. 1b). When the same scavenger was added at 10 dpi, spherical spores without prominent spines were formed. When mounted on a slide for observation, it was immediately apparent that there were fissures in the spore wall and the spores were already flattened, having lost most of their contents (Fig. 1c). By contrast, when 10 mmol/L AsA was added at 20 dpi, the spores appeared to be normal and possessed prominent perine spines and dark pigmentation by 35 dpi (Fig. 1d). However, when mounted in water on a flat slide with a coverslip and left for >15 min, the spores swelled and burst, releasing Sudan IV-positive oil droplets. Similar stage-dependent effects on spore morphologies were also obtained with DMTU and Tempol (data not shown). We concluded that swelling and bursting, which did not occur unless developing spores had been exposed to a ROS scavenger, are indicative of a partially compromised

Fig. 1. Bright-field images of *Physcomitrella* spores that developed in the absence (*a*) or presence (*b–d*) of 10 mmol/L ascorbate from 0, 10, or 20 days post-irrigation (dpi) until 35 dpi. All images are to the same scale. Scale bar in (*a*) = 20 μm.

spore wall. Additional experiments were performed to elucidate the nature of this phenomenon.

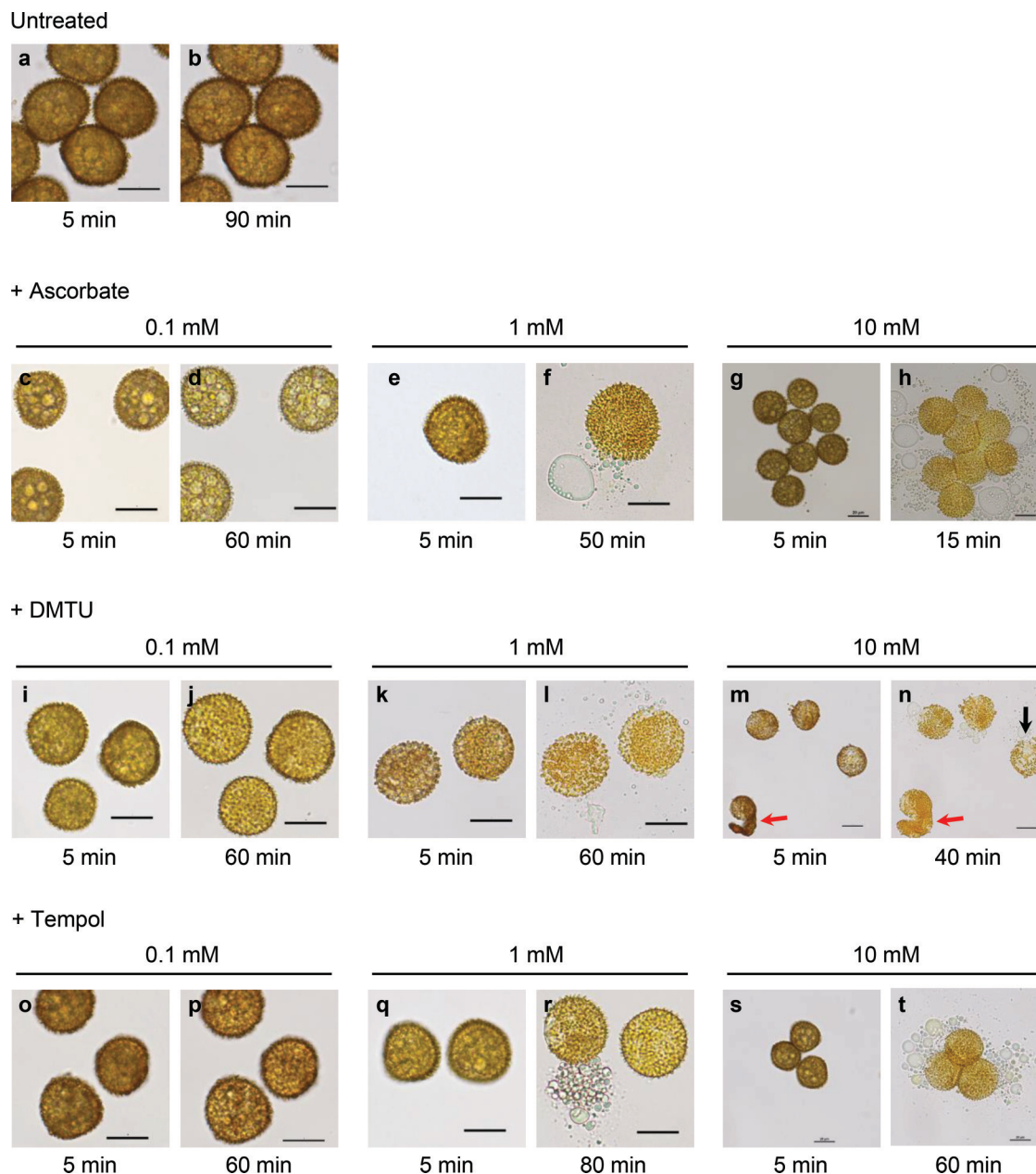### Effects of ROS scavengers on spore wall integrity: swelling and bursting

When mounted in water on a flat slide with a coverslip, spores that had developed in the absence of scavenger (untreated) remained unchanged during the observation period (90 min) (Figs. 2*a* and 2*b*). However, spores exposed to a ROS scavenger during the maturation period from 20 to 35 dpi swelled and eventually burst, releasing oil droplets and other spore contents (Figs. 2*c*–2*t*; Supplementary data files cjb-2020-0012suppla.docx and supplb.avi.[1] The Supplementary video shows the bursting of mature spores that had developed in the presence of 10 mmol/L DMTU from 20 to 35 days post-irrigation (dpi). At 35 dpi, spores were released from sporophytic capsules, placed in water, and mounted for observation on a flat slide with a coverslip. Time elapsed from the beginning of observation is shown on the screen.). The rapid outflow of spore contents through an opening typically lasted 20–30 s. Most bursting spores released their contents through a single opening, but simultaneous release through two openings was occasionally observed. The extent of swelling and the time it took for spores to burst were scavenger and dose-dependent. Swelling without bursting was observed when spores had been exposed during development to a ROS scavenger at a low concentration (0.1 mml/L). Oil droplets were visible through the translucent walls of the swollen spores as seen in Figures 2*d* and 2*p*. Previous exposure to higher concentrations (1 and 10 mmol/L) of ROS scavengers resulted in the mature spores swelling and ultimately bursting. AsA was the most effective in causing bursting, and all of the spores exposed while developing burst within 50 min at 1 mmol/L AsA (Fig. 2*f*) and 15 min at 10 mM AsA (Fig. 2*h*). Tempol was the least effective, requiring 80 min (Fig. 2*r*) and 60 min (Fig. 2*t*) at 1 and 10 mmol/L, respectively. Bursting was observed in all of

the treated (10 mmol/L) spores that were examined. When mounted in PBS (instead of water) on a flat slide under a coverslip, spores previously treated with DMTU at 10 mmol/L swelled slightly (0.69 ± 0.06 μm, *n* = 8) but did not burst during the observation period (60–90 min) (Figs. 3*c* and 3*d*), similar to spores from untreated sporophytes (Figs. 3*a* and 3*b*). Also, bursting depended on the type of slide employed. Thus, spores exposed to 10 mmol/L DMTU while developing burst within 40 min when mounted in water on a flat slide under a coverslip (Figs. 2*n* and 3*g*). However, despite becoming swollen, they did not burst during the observation period (120 min) when mounted in water on a depression slide with a coverslip (Fig. 3*h*). The mean diameter of the DMTU-exposed spores increased after 30 min in water, from 22.88 to 24.80 μm when mounted on a flat slide under a cover slip, while that of control spores changed from 22.72 to 22.82 μm measured under the same conditions. Thus, the magnitude of swelling, expressed as increase in spore diameter, of DMTU-exposed spores in water (1.92 ± 0.10 μm) was significantly larger than that of control spores (0.10 ± 0.01 μm) (*P* < 0.01) (Fig. 3*i*). Presoaking in water had little effect on the time taken to burst. When spores previously treated with 10 mmol/L DMTU were soaked in water for 2 h before being mounted on a flat slide under a coverslip, they took approximately the same time (40–45 min) to burst as in the absence of pre-soaking. Only when the spores were presoaked in water for 24 h did a small percentage (5%) of them burst within 10 min, whereas the rest (95%) still took 40–45 min to burst. By contrast, spores that had not been exposed to a ROS scavenger while they were developing failed to burst under any of the experimental circumstances described above even after 2 h (Figs. 3*e* and 3*f*).

Except for swelling and bursting in water, most spores exposed from 20 dpi to AsA or Tempol appeared, under light microscopy, to be similar morphologically to con-
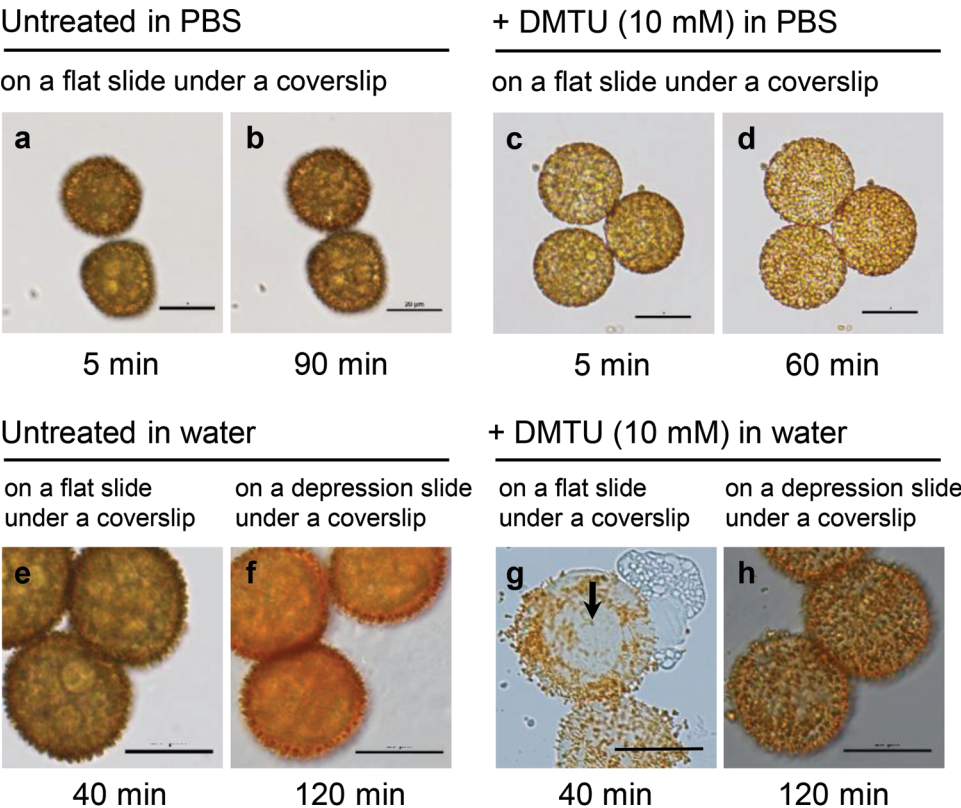

Fig. 2. Bright-field images of spores, which developed in the absence (*a* and *b*) or presence (*c–t*) of a ROS scavenger during the period from 20 to 35 days post-irrigation (dpi). At 35 dpi, the spores were harvested in water and immediately mounted on a flat slide with a coverslip. Spores were monitored for a minimum of 60 min to detect swelling and bursting. Shown are representative images of spores, which had been exposed to a ROS scavenger at 0.1, 1, or 10 mmol/L, taken at the beginning of the observation period (5 min post-harvest) and at the time when most spores had burst (e.g., 50 min for 1 mmol/L ascorbate, *f*). While unexposed spores neither swelled nor burst within 90 min (*a* and *b*), scavenger-treated spores swelled and burst dose-dependently. Spores that had been exposed to a ROS scavenger at 0.1 mmol/L swelled but did not burst within 60 min (*d*, *j*, and *p*). Spores exposed to a scavenger at 1 or 10 mmol/L burst after 15–80 min, depending on the scavenger and dose applied (*f*, *h*, *l*, *n*, *r*, and *t*). Some spores exposed to 10 mmol/L DMTU exhibited wall shedding (red arrows, *m* and *n*) or under-developed spines (black arrow, *n*). Scale bars = 20 μm.

Untreated

a | b

5 min | 90 min

+ Ascorbate

0.1 mM | 1 mM | 10 mM

c | d | e | f | g | h

5 min | 60 min | 5 min | 50 min | 5 min | 15 min

+ DMTU

0.1 mM | 1 mM | 10 mM

i | j | k | l | m | n

5 min | 60 min | 5 min | 60 min | 5 min | 40 min

+ Tempol

0.1 mM | 1 mM | 10 mM

o | p | q | r | s | t

5 min | 60 min | 5 min | 80 min | 5 min | 60 min

trol spores. By contrast, some spores previously exposed to 10 mmol/L DMTU exhibited surface regions with poorly developed perine spines that became more noticeable after spore swelling (black arrows, Figs. 2*n* and 3*g*); occasionally, wall (presumably perine) shedding was also observed (red arrow, Figs. 2*m* and 2*n*). Spores ex-

posed to 10 mmol/L DMTU were also examined using SEM and compared with the untreated spores (Fig. 4). Following air-drying and no chemical processing, untreated spores maintained their rounded shape with slight compression along the proximal spore face (Figs. 4*a* and 4*c*). In contrast, most DMTU-exposed spores completely col-

Fig. 3. Bright-field images of spores that developed in the absence (*a*, *b*, *e*, and *f*) or presence (*c*, *d*, *g*, and *h*) of 10 mmol/L DMTU from 20 to 35 days post-irrigation (dpi). At 35 dpi, the spores were harvested in water or phosphate-buffered saline (PBS) and immediately mounted on a flat slide with a coverslip, or on a depression slide with a coverslip. The spores from untreated sporophytes exhibited little swelling, and did not burst after 90 min in PBS (*a* and *b*), whereas the spores from the DMTU-treated sporophytes did not burst after 60 min in PBS (*c* and *d*). In water on a flat slide under a coverslip, the spores from DMTU-treated sporophytes swelled and burst within 40 min (*g*), whereas in water on a depression slide (where there was no direct contact between the coverslip and the spores) they swelled but had not burst after 120 min (*h*). The spores from untreated sporophytes also did not burst in water on a flat (*e*) or a depression slide (*f*). An area with under-developed spines, also seen in Figure 2*n*, is clearly recognizable in *g* (black arrow). Scale bars = 20 μm. (*i*) Swelling of spores from untreated and DMTU-treated sporophytes in water. Spore diameter was taken as the mean of the largest diameter and that perpendicular to it.

Untreated in PBS — on a flat slide under a coverslip

a — 5 min b — 90 min

+ DMTU (10 mM) in PBS — on a flat slide under a coverslip

c — 5 min d — 60 min

Untreated in water

on a flat slide under a coverslip — e — 40 min

on a depression slide under a coverslip — f — 120 min

+ DMTU (10 mM) in water

on a flat slide under a coverslip — g — 40 min

on a depression slide under a coverslip — h — 120 min

i Spore swelling in water

	Spore diameter at 5 min (μm)	Spore diameter at 30 min (μm)	Swelling (μm)
Control	22.72 ± 0.26	22.82 ± 0.26	0.10 ± 0.01*
+ DMTU (10 mM)	22.88 ± 0.46	24.80 ± 0.45	1.92 ± 0.10*

All data are mean ± SE, *n* = 15. * *P* < 0.01 (*t*-test).

lapsed along the proximal surface at the aperture during drying (Figs. 4*b* and 4*d*). Disrupted perine deposition in many DMTU-treated spores resulted in abnormal aggregations of sporopollenin globules terminating perine spines (Fig. 4*f*), differing markedly from the layered spines with gradually tapering ends on untreated spores (Fig. 4*e*). Small rosettes of aggregated sporopollenin were very abundant on tapetal cell walls, especially along radial walls, in DMTU-treated (Fig. 4*h*) and less so in un-

treated plants (Fig. 4*g*). The rosettes are composed of globules of sporopollenin and small spines with layers similar to those of spore wall spines (Fig. 4*i*).

The distal spore wall of untreated plants consists of an electron-lucent intine, thin continuous exine and spiny perine (Fig. 5*a*). The aperture extends across the proximal surface and includes a swollen intine and thin exine subtended by a fibrillar layer (Fig. 5*b*). Spore wall anomalies are evident in the TEM in all three treatments.

Fig. 4. Scanning electron micrographs of spores and tapetum from air-dried capsules that developed in the absence (left column) or presence of 10 mmol/L DMTU (right column). (*a*) Cluster of untreated spores that are compressed on the proximal surface and rounded distally. (*b*) DMTU-treated spores are irregularly shrunken with collapsed proximal faces. (*c*) A tetrad of untreated spores showing distal perine spines and slightly compressed proximal surface. (*d*) Single DMTU-treated spore showing abnormal perine spines and collapsed proximal surface that marks the site of the aperture. (*e*) Higher magnification reveals details of the distal spore surface perine with striated spines and surface papillae (arrow) on an untreated spore. (*f*) DMTU-treated spore surface with papillae (arrow) and aberrantly constructed spines that are striated at their bases and terminated by abnormal aggregates of perine globules. (*g*) Scattered rosettes of sporopollenin globules on the inner walls of tapetal cells in untreated capsules. (*h*) Abundant rosettes of sporopollenin globules on the inner walls of tapetal cells, especially along radial walls (arrow), in DMTU-treated capsules. (*i*) Higher magnification of a rosette in (*g*) showing striated nature of sporopollenin globules similar to that in spore spines. Scale bars: *a* and *b* = 25 μm; *c*, *d*, *g*, and *h* = 10 μm; *e* and *f* = 2 μm; *i* = 1 μm.
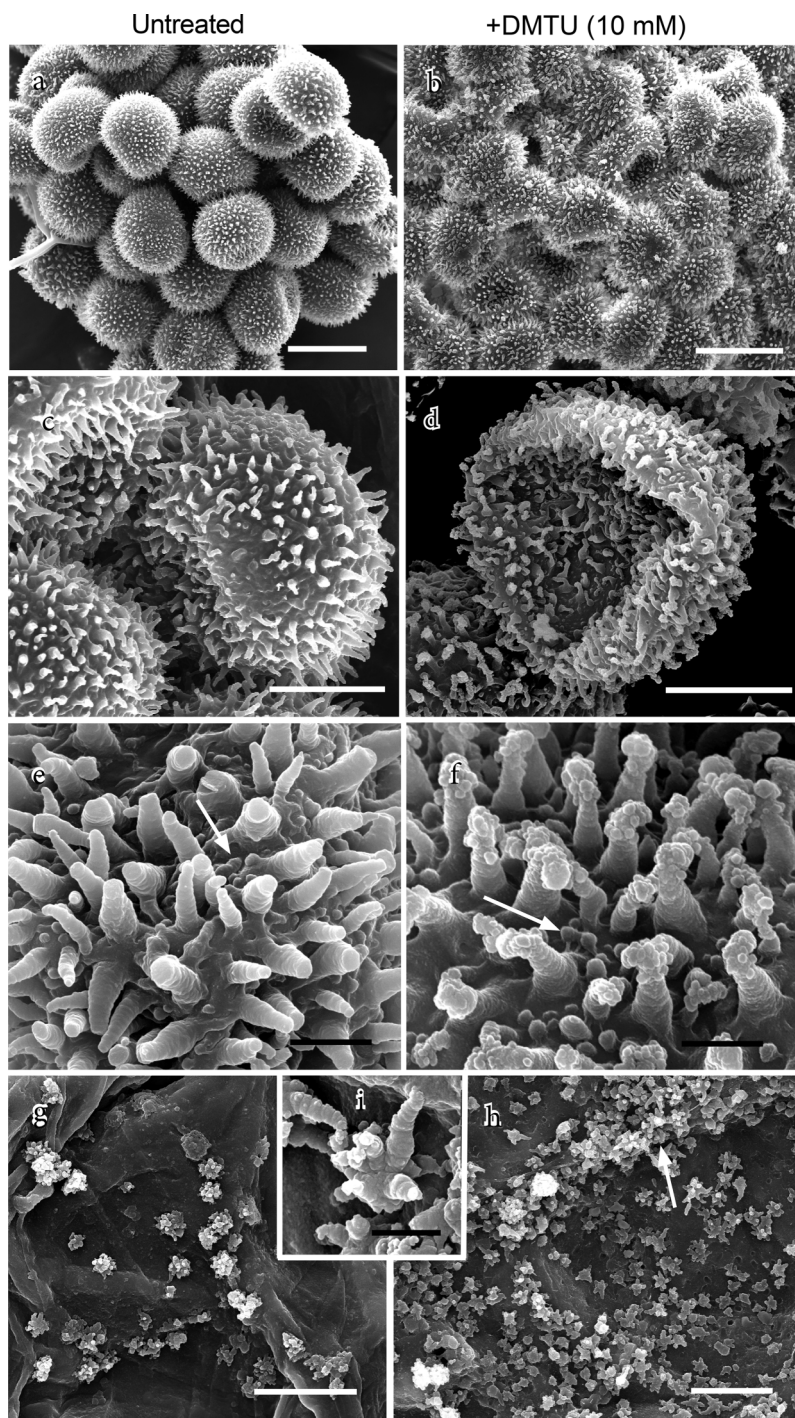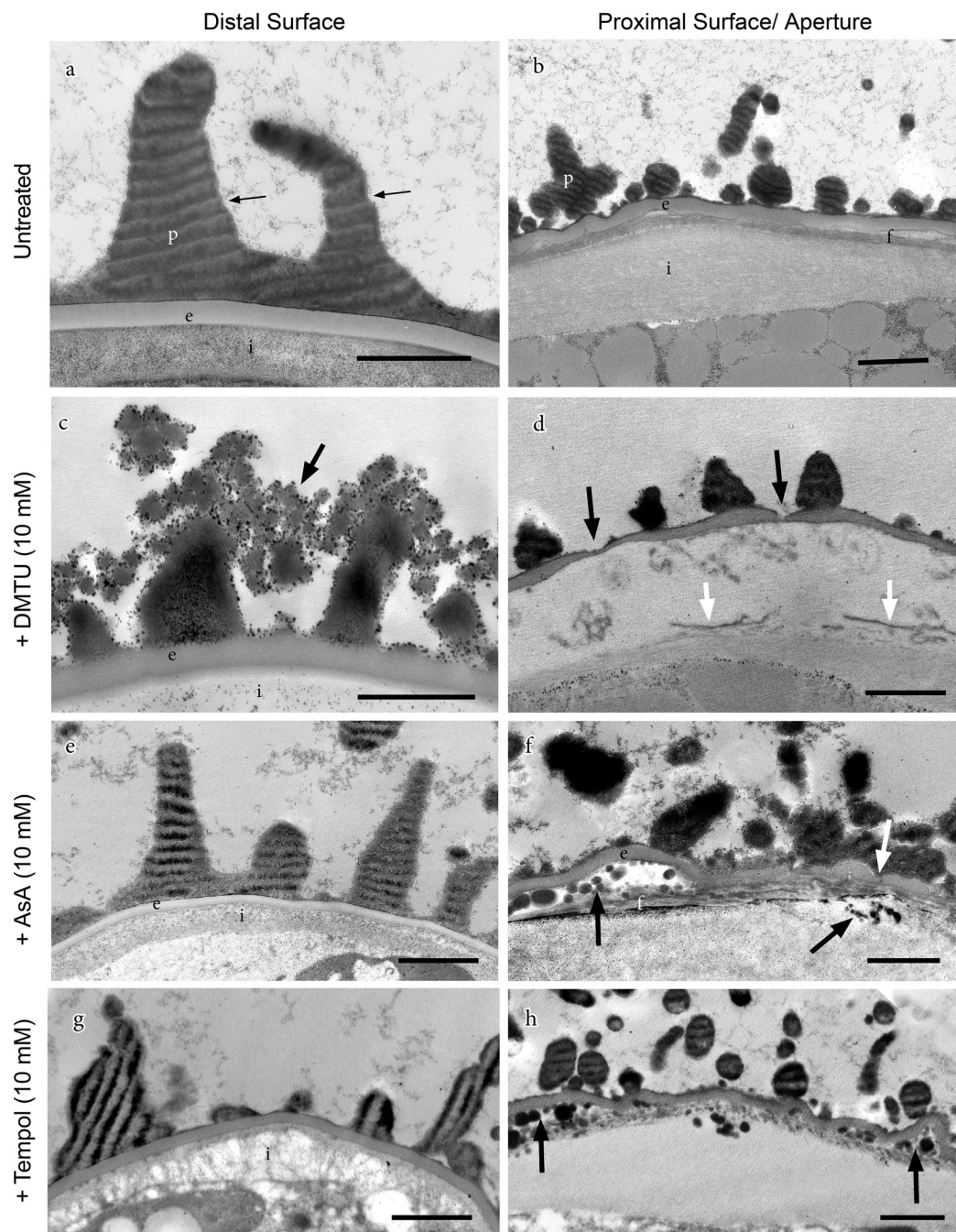

Untreated   +DMTU (10 mM)

Fig. 5. Transmission electron micrographs showing the effects of treatments with ROS scavengers, introduced at 20 days post-irrigation, on distal and proximal spore wall construction. Light-dark stripes in the perine are artifacts that result from the resistance created by dense sporopollenin during the sectioning process. (*a*) Untreated spore with perine (p), including spines, over a homogeneous layer of exine (e) and electron-lucent intine (i). (*b*) Untreated spore showing proximal aperture with irregular perine spines (p) and wavy exine (e) subtended by a fibrillar layer (f) and thickened intine (i). (*c*) DMTU-treated spore showing defective perine spines terminated by aggregations of sporopollenin globules (arrow). (*d*) Aperture of DMTU-treated spore has exine with thin to broken areas (black arrows) and disrupted fibrillar layer (white arrows). (*e*) Perine spines (p), exine (e), and intine (i) of ascorbate (AsA)-treated spores resemble those of untreated spores. (*f*) Aperture of AsA-treated spore exhibits abnormalities in the exine thickness (white arrow) and accumulation of lipid droplets below the exine and below the fibrillar (f) layer (black arrows). (*g*) The disrupted intine (i) in Tempol-treated spores has an abnormal fibrillar substructure. (*h*) Aggregations of lipid droplets (arrows) in Tempol-treated spores resemble those in AsA treatments (*f*). Scale bars = 1 μm.

Ultrastructural details of DMTU-treated spores corroborate the SEM observations relating to the spore wall ornamentation. Sporopollenin globules that are aggregated around perine spines (Fig. 5c) are the same size (~100 nm in diameter) and abundance as those in Figure 4f. Not visible in the SEM but visible in the TEM are the aperture abnormalities found in some DMTU-treated spores (Fig. 5d). Whereas in untreated spores the exine is uniform in thickness, the exine in the DMTU-treated spores is thin and discontinuous in places along the aperture (Fig. 5d). Moreover, the subtending fibrillar wall is disrupted in DMTU-treated spores and the thickened intine contains electron-dense fibers, probably representing the disruption of the fibrillar layer beneath the exine of untreated spores. The distal spore walls of AsA-treated spores (Fig. 5e) are similar ultrastructurally to the untreated spores, whereas the aperture in some spores has thin exine areas and contains aggregates of small electron-dense droplets (Fig. 5f). While many Tempol-treated spores do not display an abnormal ultrastructure, other spores show an anomalous fibrillar network in the intine (Fig. 5g) and dense droplets in the aperture region resembling those in AsA treatments (Fig. 5h).

Alkaline hydrolysis

Spore wall integrity was tested by alkaline hydrolysis, which cleaves ester bonds in wall polymers, including sporopollenin, as well as amide bonds in proteins and glycosidic bonds in polysaccharides. The spore wall pigmentation and perine were very sensitive to alkaline hydrolysis. Control spores and spores exposed to any of the ROS scavengers became colorless and perine-less within 30 min in 2 mol/L KOH at 70 °C. However, all of the spores exposed to a ROS scavenger at 10 mmol/L while developing were more susceptible to alkaline hydrolysis than the untreated spores. Only untreated spores remained intact after 4 h in KOH (Figs. 6a–6d). Spores treated with 10 mmol/L Tempol were the most sensitive, bursting and fragmenting after 2 h and 4 h in KOH, respectively (Figs. 6r and 6s). Among the ROS scavengers, 10 mmol/L DMTU appeared to have the least effect on sensitivity to alkaline hydrolysis, with spores retaining most of their protoplasm after 4 h in KOH (Fig. 6n). After 6 h in KOH, the integrity of spore walls had been breached in all cases, and the spores flattened under the weight of the coverslips.

Discussion

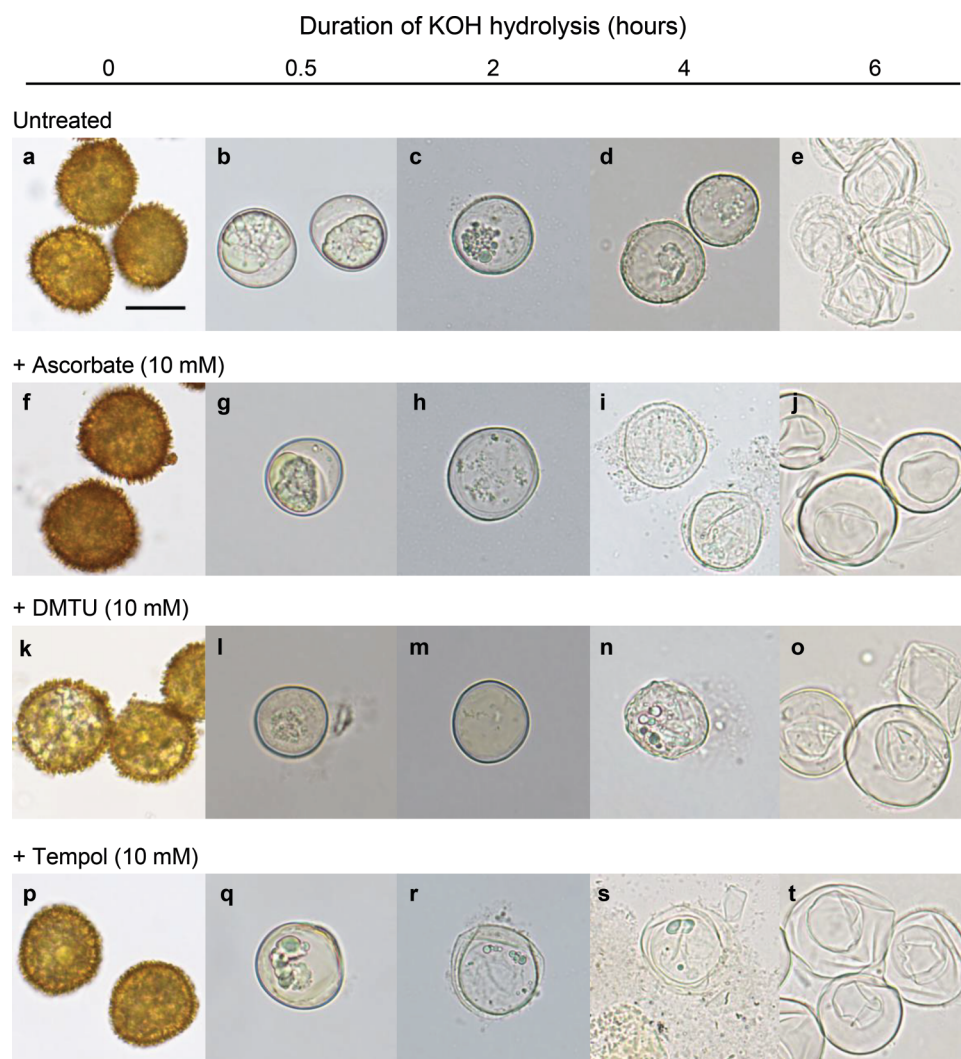### ROS are required for the formation of spore and pollen grain walls

Although the enzymes in sporopollenin polymerization and deposition have yet to be identified, it has been reported that a ROS, the superoxide anion radical, is involved in sporopollenin polymerization in the exine of developing tobacco pollen grains (Matveyeva et al. 2012) and that the class III peroxidases PRX9 and PRX40 may participate in pollen grain development in *Arabidopsis*

(Jacobowitz et al. 2019), perhaps indirectly via cross-linking extensins in tapetal cell walls.

Our findings are the first to demonstrate that ROS scavengers compromise the formation and structural integrity of the moss spore coat, and imply the involvement of ROS and peroxidases in normal spore wall development, including sporopollenin polymerization and (or) deposition and intine/aperture development. Mature spores of *Physcomitrella*, which have been exposed to an ROS scavenger during their development, swell and burst in a scavenger-, dose-, and developmental-stage-dependent manner when placed in water under a coverslip. Their walls are more susceptible to alkaline hydrolysis than those of untreated spores, while some of them are characterized by discontinuities in the exine, anomalies in perine spine structure, abnormal intine and aperture, and occasionally, in DMTU-exposed spores, wall shedding. Some variability in morphology/ultrastructure among spores exposed to a scavenger can be attributed to variability in the stage of sporophytic development at the time of treatment (because the development of sporophytes, and thus also of spores within them, is imperfectly synchronized), and to the plane of section of spores.

The three ROS scavengers employed in this investigation elicited different responses from maturing spores. For example, developing spores treated with Tempol were the most sensitive to alkaline hydrolysis, whereas those exposed to DMTU exhibited abnormal perine spines and, on rare occasions, wall shedding. Most striking are the aggregates of electron-dense globules, presumed to be sporopollenin, on the tips of perine spines and the abundance of rosettes composed of sporopollenin aggregates that cover the inner spore sac (tapetal cells) in DMTU-treated capsules. This aberrant deposition of sporopollenin on the perine spines implies inhibition of sporopollenin polymerization and (or) deposition by the ROS scavenger. These differences probably reflect dissimilarities in the bioavailability, chemical stability, and reactivity towards ROS of the scavengers. AsA has a strong affinity for H_2O_2, whereas DMTU is a potent hydroxyl radical scavenger that also reacts with H_2O_2 and the superoxide radical (Curtis et al. 1988; Kelner et al. 1990). Tempol reacts with H_2O_2 and scavenges the superoxide radical (Soule et al. 2007). Further investigation will be needed to elucidate in detail the biochemical mechanisms responsible for some of the effects of ROS scavengers, especially those of DMTU. For example, DMTU-induced wall shedding (presumably of the perine) implies that attachment of perine to the exine is partially compromised. Similar wall shedding has not been reported previously in the literature, and little is known about how the exine and perine are chemically connected. Our results suggest that oxidative coupling of components (proteins, carbohydrates, or sporopollenin) of these layers may be involved in a manner analogous

Fig. 6. Bright-field images of spores subjected to alkaline hydrolysis with 2 mol/L KOH at 70 °C. The spores had developed in the absence (A) or presence (B–D) of a ROS scavenger at 10 mmol/L during the period from 20 to 35 days post-irrigation (dpi). Images of the KOH-treated spores were recorded at regular time intervals from 0 to 6 h. All of the spores exposed to a ROS scavenger during their maturation were more susceptible to alkaline hydrolysis than control spores from untreated sporophytes. All of the images are to the same scale. Scale bar in a = 20 μm.

to, for example, strengthening of the plant primary cell wall matrix by cross-linking of polymer-esterified hydrocinnamic acids and wall polysaccharides or tyrosine-containing glycoproteins (e.g., extensins). In this case, the cross-linking reactions are mediated by peroxidases and H_2O_2 (Lindsay and Fry 2007). Further biochemical studies will be required to confirm this supposition.

Defects in the intine of AsA and Tempol-treated spores are not surprising given that the enzymes involved in cross-linking cell wall constituents require ROS (Ros Barceló and Gómez Ros 2009). The intine is the inner spore wall layer that lacks sporopollenin and has a carbohydrate composition similar to that of the primary cell wall, namely cellulose, pectins, hemicelluloses, and protein (Owen and Makaroff 1995; Huang et al. 2009). These cell wall polymers are cross-linked to form a three-dimensional and insoluble network; a scaffolding that can withstand internal turgor pressure. The intine is the immediate barrier between the spore protoplasm and sporopollenin layers. Disruption of intine construction would have consequences related to porosity, strength, and flexibility of the spore coat, as the spore is subjected to dehydration prior to dispersal and imbibition at the time of germination. Less is known about the molecular composition of the spore aperture. With a thickened intine, this region probably contains the major primary cell wall constituents (Renzaglia et al. 2020). As the site of germination, the aperture is modified to take up water and allow for cell wall expansion that ruptures the spore wall and initiates the development of the filamentous protonema. During normal spore maturation, the aperture is the site of wall shrinking that presumably is critical to successful dehydration and the maintenance of cell wall-plasmalemma integrity as the spore protoplasm

decreases in volume. Anomalous development of the spore aperture could have an impact on a range of spore functions, including dehydration, water uptake, and emergence of the protonema from the spore case. Extracellular ROS have been detected in aperture zones during pollen tube growth, and it is proposed that •OH causes local loosening of the intine in aperture zones (Smirnova et al. 2014). ROS are also implicated in tobacco pollen grain activation, which precedes the formation of the pollen tube (Smirnova et al. 2009).

It is reasonable to conclude that the collective effects of ROS scavengers on spore wall formation in *Physcomitrella* result mainly from the inhibition of oxidative polymerization and (or) cross-linking of sporopollenin and other cell-wall components. Although indirect effects of scavengers on spore wall formation through the synthesis and secretion of sporopollenin monomers appear to be minor, as indicated by the normal morphologies of sporopollenin globules in DMTU-treated capsules (Fig. 4h), the possibility of indirect effects from the inhibition of other functions required for normal spore wall formation, for example, the production or secretion of wall precursors, cannot be excluded.

Composition of the exine is different from that of the perine of the *Physcomitrella* spore

Another notable observation made in this study is that the exine and perine of *Physcomitrella* spores differ with respect to their electron opacity and alkaline resistance. Using TEM, we see that the perine is more electron-dense than the exine and, unlike the exine, perine spines dissolve in hot alkaline solution. Different reactivity to alkaline hydrolysis implies different contributions of hydrolyzable (ester) and non-hydrolyzable (C–C and C–O) linkages. Thus, our findings suggest there are compositional and (or) linkage differences between exine and perine sporopollenin and the associated wall polymers.

Augmented osmolysis, wall shedding, chemical inhibition: *Physcomitrella* is a convenient model for studying spore wall formation

Bursting of pollen grains by osmotic shock has been reported in the literature. About 40% of tobacco pollen grains burst when left for 15 min in a hypotonic medium (Smirnova et al. 2014). When inaperturate pine pollen grains are subjected to osmotic down-shock (e.g., transfer from a saturated salt solution to water), their exines rupture and protoplasts are released (Bohne et al. 2005). In a study of the walls of various pollen grains, Matamoro-Vidal et al. (2016) observed exine rupture and cytoplasmic leakage "through a tiny breakage of the exine", when pollen grains lacking apertures and with thin exines are placed in a hypotonic medium. By contrast, *Physcomitrella* spores do not normally burst in water, indicating the physical robustness of their walls.

We have concluded that the observed swelling in water of mature spores that were exposed to a ROS scavenger during their development is osmotically driven and depends upon their possession of a spore wall whose structural integrity and tensile strength have been compromised, as exemplified by a discontinuous exine (Fig. 5d) and disrupted fibrillar layer (Figs. 5d and 5f). Subsequent bursting when mounted in water on a flat slide under a coverslip is a consequence of additional pressure from the weight of the coverslip being applied directly to the already swollen spores. We have named this phenomenon "augmented osmolysis". Our interpretation is reinforced by the finding that spores, previously exposed to a scavenger, exhibit little or no swelling and do not burst when mounted in PBS under a coverslip. Also, when placed in water on a depression slide, spores are not subjected to the additional pressure resulting from direct contact with the coverslip. In this latter case, the spores swell but do not burst.

In conclusion, the structural integrity of the walls of spores in the process of developing and maturing within *Physcomitrella* sporophytes submerged in aqueous solutions containing ROS scavengers is compromised. The spores with defective walls are susceptible to augmented osmolysis and sometimes display wall (perine) shedding. Spores from sporophytes exposed to DMTU accumulate sporopollenin globules around their perine spines, whereas those subjected to other treatments show structural defects in the intine and (or) aperture. These findings provide support for the proposed oxidative cross-linking during polymerization or deposition of sporopollenin and other wall constituents within maturing spore walls. Augmented osmolysis of *Physcomitrella* spores with defective walls provides a direct and simple test for studying the effects of chemicals on spore wall formation.

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