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Dynamics of intracellular mannan and cell wall folding in the drought responses of succulent Aloe species

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Abstract
Plants have evolved a multitude of adaptations to survive extreme conditions. Succulent plants have the capacity to tolerate periodically dry environments, due to their ability to retain water in a specialized tissue, termed hydrenchyma. Cell wall polysaccharides are important components of water storage in hydrenchyma cells. However, the role of the cell wall and its polysaccharide composition in relation to drought resistance of succulent plants are unknown. We investigate the drought response of leaf-succulent Aloe (Asphodelaceae) species using a combination of histological microscopy, quantification of water content, and comprehensive microarray polymer profiling. We observed a previously unreported mode of polysaccharide and cell wall structural dynamics triggered by water shortage. Microscopical analysis of the hydrenchyma cell walls revealed highly regular folding patterns indicative of predetermined cell wall mechanics in the remobilization of stored water and the possible role of homogalacturonan in this process. The in situ distribution of mannans in distinct intracellular compartments during drought, for storage, and apparent upregulation of pectins, imparting flexibility to the cell wall, facilitate elaborate cell wall folding during drought stress. We conclude that cell wall polysaccharide composition plays an important role in water storage and drought response in Aloe.

KEYWORDS
adaptation, Aloe, CoMPP, drought, hydrenchyma, leaf anatomy, morphology, plant cell walls, polysaccharides, succulence

1 INTRODUCTION
The remarkable diversification success of land plants has been attributed to the evolution of adaptations to cope with potential abiotic stressors such as drought, temperature, and light (Raven, Evert, & Eichhorn, 2005). In desert environments, plants must be able to cope with all three factors (Moore, Vicré-Gibouin, Farrant, & Driouich, 2008). The ability to store and use water in a regulated manner is a remarkable and crucial adaptation in the ecological success of succulent plants (Grace, 2019), which are estimated to include ca. 12,000 species in diverse lineages of the angiosperm tree of life (Moore et al., 2008; Nyffeler & Eggl, 2009).

Succulent plants typically possess thick and fleshy leaves and/or stems as an adaptation to periodically dry environments (Landrum, 2008). This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

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2.1 | Growth conditions and sampling

Plant material of *A. helenae* Denguy (a tree Aloe, voucher number Ahl 96 deposited at C) and *A. vera* L. (a stemless Aloe, voucher number Ahl 94 deposited at C) was obtained from the living collections at the Botanic Garden of the Natural History Museum of Denmark, University of Copenhagen, Denmark. *A. helenae* was selected for its known visible reactions to drought stress (colour change and thinning of leaves), whereas *A. vera* was chosen for its adaptability to different growing conditions. An additional four species were included in the initial CoMPP screening (Figure S1).
Mature (>20 years old) plants of the two species were grown under glass in conditions mimicking the daylight changes and water availability of their natural habitat. Accordingly, irrigation is changed depending on the seasons and natural light of the Northern hemisphere, with more watering (2–3 times per week) in the summer (June–August) and less in the spring, autumn, and winter (0–2 times per week). Plants are regularly given Nitrogen Phosphor Potassium (NPK) fertilizer, and artificial light is supplied during the autumn and winter to resemble the light availability of the southern hemisphere, where most Aloe species originate. Finally, temperature is kept at a minimum of 13–17°C. Three plants were sampled for each species before and after exposure to drought (no watering), on August 01, 2017, and September 11, 2017, respectively. At the same time, three control plants watered according to the normal watering regime were sampled for each species. The drought experiment was repeated on the same plants between May 08, 2018, and June 19, 2018.

2.2 Relative and SWC

Leaf cores were taken from the drought stressed and control species of A. vera and A. helenae in the greenhouse. Samples were taken between 12:00 p.m. and 1:00 p.m. in accordance with the original recommendations of Smart and Bingham (1974), as a standardized approach (Smart & Bingham, 1974). Two leaf core samples from each species were placed in preweighed tubes. Samples were weighed, and 1 ml of distilled water (dH₂O) was added to each tube, covering the samples completely. All samples were refrigerated at 5°C for 3–4 hr to all full rehydration. Excess water was removed before samples (and tubes) were weighed to obtain full turgid weight (TW). Tubes and samples were then incubated in an oven at 80°C for 62 hr with the lids off before they were weighed to obtain the dry weight (DW). All measurements were made in milligrams (mg) with two decimal places. For each species and treatment, calculations were made using the following formulas:

Calculation of relative water content (RWC) in %:

\[
\text{RWC (\%) = \left(\frac{W - DW}{TW - DW}\right) \times 100,}
\]

Calculation of saturated water content (SWC) in %:

\[
\text{SWC (\%) = \left(\frac{TW - DW}{DW}\right) \times 100,}
\]

where W is the fresh weight (mg), TW is the turgid weight (mg), and DW is the dry weight (mg).

2.3 Microscopy

Thick transverse sections (0.5 cm²) were carefully excised from a mature leaf from both drought-stressed and control plants of A. helenae and A. vera before and after the drought period and placed in 2-ml Eppendorf tubes in 100 μl 99.8% methanol (CH₂OH, 322415 Sigma-Aldrich, Merck, USA). Samples were frozen and stored at 20°C prior to investigation.

Transverse sections of approximately 3 mm in diameter were excised from the collected material and fixed for 30 min in 4% formaldehyde prepared from paraformaldehyde in phosphate-buffered saline (PBS). After two washes in PBS, sections were dehydrated in series of methanol/water solutions until reaching a final concentration of 100% methanol. The methanol was substituted with methanol: London Resin (LR) white resin mixture (1:1) for 8–10 hr and, finally, the sections were transferred to pure LR resin overnight. The specimens were placed and oriented in gelatin capsules filled with pure LR resin. The polymerization was performed in an oven at 60°C overnight. An ultramicrotome (Leica EM-UC7) was used to produce 1 μm-thick sections. These were adhered on Superfrost Slides (Thermo Scientific, Roskilde, Denmark) in a drop of water at 60°C.

Immunolocalization was performed as described by Mravec et al. (2017) using the antibodies LM21 and LM22 binding galactomannan or glucomannan (Marcus et al., 2010), BS-400-4 binding mannan (Pettolino et al., 2001), and CCRC-M170 binding acetylated mannan (Zhang et al., 2014) to understand the mannan distribution. In brief, leaf sections were placed on glass microscope slides, surrounded by a hydrophobic circle drawn with a PAP pen (Merck Life Science, Darmstadt, Germany), and blocked with 5% milk powder in PBS for 15 min. The leaf tissue was probed with antiglycan monoclonal antibodies for 1 hr at 1:10 dilution in 5% milk powder in PBS, washed two times with 5% milk powder in PBS, then probed with a secondary antibody. The secondary antibodies used were antirat or antimouse conjugated to Alexa Fluor 555 or Alexa Fluor 488 (Invitrogen) at 0.1 mg/ml concentration for 10 min. Finally, the leaf tissue was washed once more and mounted in Citifluor, an antifading reagent (Agar Scientific, Essex, United Kingdom).

The COS488 staining binding to deesterified homogalacturonan was performed as described by Mravec et al. (2014). "COS" stands for "chitosan oligosaccharides" in which the interaction between COS and homogalacturonan (HG) is governed by multiple polar interactions of protonated NH2 on COS and carboxyl groups on demethyl esterified HG. COS recognizes HG with degree of esterification from 0 to approximately 38% (Mravec et al., 2014). Leaf sections were incubated with 1:1,000 COS488 (Mravec et al., 2014) and 0.1 mg/ml of Calcofluor White (Sigma) in 25-m 2-(N-morpholino)ethanesulfonic acid (MES) buffer for 30 min at room temperature. The leaf sections were then washed twice with MES buffer and mounted in MES:glycerol (1:1) solution.

Sections of A. helenae and A. vera were stained with 1% toluidine blue for 10 min to highlight all cell walls in the section, washed twice with distilled water, and mounted on glass slides under a coverslip. Images were taken on Olympus BX41 microscope with mounted Olympus ColorView I camera.

The fluorescently labelled samples were scanned using a Leica SP5 confocal laser scanning microscope equipped with UV diode (405 nm),
Ar (488 nm), and HeNe (543 nm) lasers at either 20× or 63× water objectives. The pictures were processed with GIIMP2 software for colour enhancement and contrast. Control samples were treated equally.

For general comparative observations of leaf anatomy in a range of Aloe species, we used the extensive collection of permanent microscope slides located at the Royal Botanic Gardens, Kew. A Leica (Wetzlar, Germany) DM LB 100T light microscope was used to examine the prepared slides of leaf surfaces, transverse, and longitudinal sections; photomicrographs were taken using a Zeiss (Jena, Germany) AxioCam HRc mounted camera.

2.4 Comprehensive microarray polymer profiling

The CoMPP analysis followed the protocol described by Ahl et al. (2018), optimized for succulent tissues from that of Möller et al. (2007). Hydrenchyma was carefully excised from mature harvested leaves of A. helenae and A. vera and immediately placed in labelled Falcon tubes (Corning, New York, USA) and snap frozen in liquid nitrogen. For each species, three samples were taken from three plants grown in individual pots. Samples were kept at −20°C for 24 hr before they were freeze dried. Three samples of approximately 5 mg were weighed to 1 decimal accuracy, and each placed in the well of an 8 ‐ strip tube (CLS4413 SIGMA, Merck Life Science, Darmstadt, Germany). The samples were homogenized in a Tissuelyser II (Gentec Biosciences, Columbia) using glass beads prior to extraction.

A three-step sequential extraction series was employed. For each sample, the extractant volume was adjusted to the exact weight of each sample. Glass beads were kept in the tubes to enhance mixing of tissues and solvents. For the first extraction step, dH2O was added at a concentration of 250 mg mL−1 for 2 hr. After extraction, the samples were centrifuged at 4,000 RPM (Thermo Fisher Scientific, Waltham, MA USA 02451) for 10 min before samples were printed on a 0.45-µm nitrocellulose membrane (Whatman, Maidstone, UK) using an Arrayjet Sprint (Arrayjet Ltd., Edinburgh, UK) piezoelectric robotic printer. The dilution series were printed in duplicate on each microarray. As an earlier study observed problems with detection of mannans (Ahl et al., 2018), sample wait time between extraction and printing was minimized to prevent degradation and optimize quality of print following Ahl et al. (2019).

A total of 19 monoclonal antibodies were used for the microarray analyses as primary antibodies; the secondary antibodies (Merck Life Science, Darmstadt, Germany) were either antirat or antimouse depending on the primary antibody. The printed arrays were developed, quantified, and analysed following the procedures described by Ahl et al. (2018). A total of 62 arrays were used: one for each antibody in triplicates and two for negative controls of the secondary antibodies.

Averages were calculated using the dilution series for each sample and the array triplicates. The highest mean value of the dataset was assigned the value of 100%, and the remainder of the data was normalized with a 5% cut off. The dataset is visualized in a heatmap format.

| Species        | Treatment            | RWC (%) | SWC (%) |
|----------------|----------------------|---------|---------|
| Aloe helenae   | Drought stressed     | 43      | 30      |
| Aloe helenae   | Control              | 88      | 63      |
| Aloe vera      | Drought stressed     | 64      | 33      |
| Aloe vera      | Control              | 84      | 61      |

FIGURE 1 Effect of drought on two Aloe species. (table) The calculated relative and saturated water content for Aloe helenae and Aloe vera and measured before (control) and after 6 weeks of drought. A. helenae showed visible signs of drought stress. Pictures (a) and (b) show A. helenae (a) before and (b) after the drought period.
3 | RESULTS

3.1 | RWC and SWC in A. vera and A. helenae

RWC and SWC were calculated for A. helenae and A. vera in both drought-stressed plants and well-watered control plants. Pronounced differences were seen in both species between the drought-stressed species and control plants (Figure 1). For both species, RWC of the control plants were consistently above 80%, whereas the drought-stressed plants were significantly reduced to below 65% for A. vera and as low as 43% for A. helenae. Similar differences were observed for the SWC, control species reaching above 60%, and the drought-stressed species being 33% for A. vera and 30% for A. helenae.

3.2 | In situ microscopy analysis of hydrenchyma cell walls in A. vera and A. helenae

Cell wall morphology of the hydrenchyma cells in watered and drought-stressed A. helenae and A. vera was investigated using microscopy (Figure 2). The leaves of Aloe helenae were longer, thinner, and

FIGURE 2  Comparative morphology and anatomy of Aloe helenae (a, b, d, e, and f), Aloe vera (a, c, g, h, and i), and Aloe somaliensis (j and k). Transverse sections (marked by dashed squares) of hydrenchyma tissue stained with toluidine blue showing typical leaf tissue arrangement in Aloe species with hydrenchyma, (h) surrounded by an outer photosynthetic chlorenchyma, (c) epidermis and cuticle (d, e, g and h). Convoluted folding of hydrenchyma cell walls in drought-stressed plants (f and i) indicated by arrows. Aloe somaliensis (j and k) from the Kew slide collection also showing folds in the hydrenchyma cell walls.
the margins rolled inwards than those of A. vera. The epidermis of A. helenae was thicker than that of A. vera, whereas the hydrenchyma layer was thicker in A. vera than in A. helenae (Figure 2b, c). Based on the toluidine blue-stained micrographs, both species have chlorenchyma region composed of approximately 15 cell layer, whereas the thickness of hydrenchyma varied in both the watered and drought-stressed species (Figure 2d–i).

In A. helenae, the hydrenchyma had shrunk to a very thin layer after the plants had been exposed to drought (Figure 2e). In A. vera, shrinkage was less pronounced, but cell size and shape had changed. The hydrenchyma cells were transformed from uniformly rounded in hydrated samples to elongated and flat with uneven cell walls in drought-stressed samples (Figure 2g,h). In both species, the chlorenchyma was largely unaffected, and cell size was similar before and after drought.

In the drought-stressed plants (Figure 2f,i), the cell walls were pronouncedly convoluted, a type of folding where the cell wall does not collapse, but rather folds in regular patterns (Haberlandt, 1914). In some cells, the entire cell wall was continuously and moderately folded in a wavy or zig-zag pattern; otherwise, it was tightly folded several times in discontinuous regions of the wall (Figure 2f, i). Convoluted cell wall folding was observed in other species of Aloe represented in the permanent microscope slide collection of the Royal Botanic Gardens, Kew and illustrated here by Aloe somaliensis.

| Species    | Extraction | Treatment          | XG | Mannan | Negative |
|------------|------------|--------------------|----|--------|----------|
| Aloe helenae | Water      | Drought            | 0  | 0      | 0        |
|            | Water      | Watered            | 0  | 0      | 0        |
| Aloe helenae | Water      | Control            | 0  | 0      | 0        |
| Aloe vera   | Water      | Drought            | 0  | 0      | 0        |
| Aloe vera   | Water      | Watered            | 0  | 0      | 0        |
| Aloe vera   | Water      | Control            | 0  | 0      | 0        |
| Aloe helenae | ODA        | Drought            | 19| 12| 12      |
| Aloe helenae | ODA        | Watered            | 5 | 5   | 5        |
| Aloe helenae | ODA        | Control            | 0 | 0    | 0        |
| Aloe vera   | ODA        | Drought            | 0 | 0    | 0        |
| Aloe vera   | ODA        | Watered            | 0 | 0    | 0        |
| Aloe vera   | ODA        | Control            | 0 | 0    | 0        |
| Aloe helenae | NaOH       | Drought            | 0 | 0    | 0        |
| Aloe helenae | NaOH       | Watered            | 0 | 0    | 0        |
| Aloe helenae | NaOH       | Control            | 0 | 9    | 0        |
| Aloe vera   | NaOH       | Drought            | 0 | 0    | 0        |
| Aloe vera   | NaOH       | Watered            | 0 | 0    | 0        |
| Aloe vera   | NaOH       | Control            | 0 | 0    | 0        |

**FIGURE 3** Heatmap representation of hydrenchyma polysaccharides of drought stressed, watered, and control treatments of Aloe helenae and A. vera: (a) all data from each extraction; (b) summed data from comprehensive microarray polymer profiling. Sums were made using the measured quantifications and are not the sums of the calculated data shown in (a). XG, xyloglucan; Ab, antibody; R, rat; M, mouse; RGI, rhamnogalacturonan-I. Data not shown here are available in the Supporting information.
(Figure 2j,k). The watering scheme applied to the plants used to prepare permanent mounted slides in the slide collection was not recorded.

3.3 | CoMPP profiling of cell wall polysaccharides in response to drought

Compositional changes of Aloe polysaccharides in response to drought were detected using CoMPP. Of a total of 20 antibodies targeting different epitopes on the most common cell wall polysaccharides, only 10 gave a signal; a selection of these results is shown in Figure 3 (additional data in Figure S1 online). The strongest mannan signals (LM21 and BS-400-4) were observed in the CDTA extraction from both species. Only two of the mannan specific antibodies (LM21 and BS-400-4) detected epitopes in both A. vera and A. helenae in all three extraction steps; both detecting more in A. helenae than A. vera in all extractions.

The most noticeable differences between the polysaccharide composition of drought-stressed and watered control samples were observed in A. helenae in the CDTA extraction, which contained very low pectin levels as seen by the homogalacturonan detected by the antibodies JIM5, JIM7, LM18, and LM19 (Clausen, Willats, & Knox, 2003; Vandenbosch et al., 1989; Verhertbruggen, Marcus, Haeger, Ordaz-Ortiz, & Knox, 2009; Willats et al., 2000). However, in the drought-stressed samples, the signals were strong even for antibodies that showed no detection in the watered and control samples. A similar pattern was observed for the mannan-specific antibodies LM21 and BS-400-4, for which signal strengths were almost double in the CDTA extraction of A. helenae. Drought also increased pectin signal in A. helenae, although the pectin specific antibody LM20 gave a weak response. No pectin signals were seen for A. vera in the water and CDTA extractions, yet a very vague LM20 signal appeared in drought-stressed A. vera in the NaOH extraction. A. vera also showed differences in the mannan signals between the watered and drought-stressed plants in all extractions but were relatively weakly compared with A. helenae.

No signals were detected for the antibodies targeting xylan (LM10 and LM11) and only very little AGP (JIM8, JIM13, and Jim14; Knox, Linstead, Cooper, & Roberts, 1991; McCabe, Valentine, Forsberg, & Pennell, 1997; McCartney, Marcus, & Knox, 2005; Pedersen et al., 2012; Yates et al., 1996). Likewise, only sporadic signals were

![Figure 4](image-url)
detected from the RG-I specific antibodies (LM5, LM6, LM13, and LM16; Lee et al., 2005; Moller et al., 2008; Willats, Marcus, & Knox, 1998). Data shown in the Supporting information online.

3.4 | Distribution of structural polysaccharides in hydrenchyma cell walls and intracellular compartments

To further understand the mechanism behind the cell wall folding, we investigated the composition of the hydrenchyma cell walls in situ (Figure 4). We analysed the presence of homogalacturonan as the main pectic component and one of the crucial determinants of cell wall mechanical properties. Both immunological and nonimmunological probes were used: The oligosaccharide probe COS488, staining longer stretches of deesterified homogalacturonan (Mravec et al., 2014), and JIM5 and JIM7, recognizing homogalacturonan with a low or high level of deesterification, respectively (Clausen et al., 2003; Vandenbosch et al., 1989; Willats et al., 2000; Figure 4). In A. vera (Figure 4a–f), the JIM5 signal was restricted to the chlorenchyma cell walls and gave only a weak and sporadic signal in the hydrenchyma cell walls, especially at the triangular junctions. In contrast, JIM7 gave a strong signal throughout the leaf section and was unaffected by drought. In the drought-stressed specimens, the signal for JIM5 disappeared from the shrunken hydrenchyma. COS488 gave a strong signal in all cell walls in the control sample. However, in the drought-stressed specimen, the signals for JIM5 and COS488 were noticeably reduced in hydrenchyma. This effect was also clearly seen in A. helenae (Figure S2). When examined in higher magnification (Figure 4g–j), none of the immunological probes gave middle lamellar and cell corner staining typical of other parenchymatic tissues. The JIM7 signal also appeared inside the cells as distinct compartments different to those stained with Calcofluor White (Figure 4i). Instances of middle lamella staining were sporadically observed only for COS488 (Figure 4g,h).

We also investigated the distribution of other pectin types. Immunolocalization showed no appreciable labelling with either LM5 (binding (1,4)-β-D-galactan) or LM6 (binding (1,5)-α-L-arabinan) monoclonal antibodies (Figure S3), suggesting cell walls in hydrenchyma are low in RG-I side chains (Jones, Seymour, & Knox, 1997; Lee et al., 2005; Verhertbruggen et al., 2009; Willats et al., 1998). We also tested the presence of other cell wall components for which antibodies were available: Relatively small amounts of xyloglucan (using LM15) were detected, but other polysaccharides and proteoglycans (extensins, AGPs) were almost completely absent when probed on sections (data not shown). These observations would be in line with CoMPP results indicating that the cell walls of hydrenchyma are formed mainly with mannans, xyloglucan, and homogalacturonan out of the polysaccharides that we measured for. The hydrenchyma cell walls are thus lacking some of the compositional complexity seen in other primary cell wall types found in other organs (Fangel et al., 2012).

3.5 | In situ distribution of Aloe mannans

To analyse the distribution of acetylated mannans and its molecular variants in situ and immunolocalization on LR-resin, sections were made using the same set of antimannan antibodies as for CoMPP with the addition of CCRC-M170. The immunolocalization showed mannans widely distributed in the entire cross-section and not exclusively in hydrenchyma and could be seen as associated with the cell wall and as patchy populations of granular intracellular signals (Figures 5 and 6). The binding of BS-400-4 and to a lesser extent LM21 was

**FIGURE 5** In situ detection of mannan epitopes in Aloe vera hydrenchyma using three monoclonal antibody probes. (a) Colocalization of Calcofluor White (blue signal), LM21 (red signal) recognizing β-(1,4)-mannan/galactomannan/glucomannan, and CCRCM-170 (green signal) recognizing acetylated mannan; (b) colocalization of Calcofluor White (blue signal), LM21 (red signal), and BS-400-4 recognizing β-(1,4)-galactomannan (green signal); cell wall is marked with arrowheads.
enhanced by pretreatment with 200-mM NaOH in all leaf tissues, confirming that the epitopes recognized by LM21 are partially masked by the acetylations (Figure S4). The CCRC-M170 antibody also showed the distribution of mannan throughout the cross-section of a leaf, whereas the pretreatment with 200-mM NaOH diminished the signal, hence confirming the requirement of acetylation for the CCRC-M170 recognition (Figure S4). The close inspection of the sections probed with anti-mannan monoclonal antibodies revealed that all mannan epitopes could be seen as bead-like round compartments 0.1–1 μm in size. These compartments were also observed by in vivo labelling of tissue streaks using Vectabond slides, confirming that they are not an embedding artefact (Figure S5).

The colabelling using CCRC-M170 and LM21 showed only partial overlay (Figure 5a) whereas the colocalization using LM21 and BS-
400-4 showed a near-complete match (Figure 5b). These patterns suggest some intramolecular structural heterogeneity of Aloe mannan polymers and their intracellular distribution in relation to their acetylation (Figure 5). Furthermore, Calcofluor White, a specific dye for β-(1,4)-glucan, stained similar sized compartments yet only partial colocalization was detected, indicating the presence of other compartments possibly filled by nonmannon types of β-glucans (Figure 5). We found that the stressed plants of A. helenae and A. vera gave a lower mannan signal in shrunk hydrenchyma, not only particularly CCRC-M170 but also partially by LM21 (Figures 6 and 7). In watered plants, the mannan compartments were mainly associated with the cell wall or distributed around the borders of the cells, probably pushed toward the cell wall by the central vacuole yet were more dispersed within the cell in drought stressed, which could be an effect of the shrinkage of the central vacuole.

4 | DISCUSSION

4.1 | Convoluted folding of cell walls provides a structured response to drought

Our findings highlight the ecological significance of a somewhat overlooked anatomical feature of hydrenchyma tissue: cell wall convolution as a regulated structural response to drought. Convoluted cell walls were initially reported in Aloe by Pfizer (Haberlandt, 1914), but have since received little attention as a morphological trait associated with succulence (but see an example of Aloe pearsonii in von Willert, Eller, Werger, Brinckmann, and Ihlenfeldt [1992]). The regular folding mechanism is a necessary measure to reduce cell size during dehydration without shrinking or altering the cell wall (Haberlandt, 1914), which could otherwise result in costly cell loss or repair during

**FIGURE 7** Changes in cell wall-associated mannan in watered and drought-stressed leaf hydrenchyma tissue of Aloe helenae. The sections are probed with LM21, CCRC-M170, and BS-400-4 in (a) watered and (b) drought-stressed specimens. Note the disappearance of the CCRCM-170 and partial disappearance of LM21 in hydrenchyma of drought-stressed specimen.
postdrought recovery. Cell wall folding has also been postulated to influence the shape and position of the dehydrated leaf on the plant, minimizing temperature and radiation damage under drought conditions (von Willert et al., 1992).

Our observations indicate that the reduction in the volume of water-storing hydrenchyma in Aloe during drought is not caused by random cell collapse but represents a closely regulated and controlled process in which cell wall folding allows desiccated hydrenchyma cells to retain the potential to unfold subsequently by isotropic expansion of the cell. The folding of hydrenchyma cell walls occurs more frequently in drought-stressed plants but may be a more general mechanism for regulating leaf shape in Aloe in response to less severe fluctuations in water availability. Our results pinpoint homogalacturonan and its esterification in the folding process. The distribution of different types of homogalacturonan in hydrenchyma and chlorenchyma changes under drought and watered conditions with drought-stress-induced loss of homogalacturonan with low degrees of esterification, whereas homogalacturonan with a high degree of esterification was largely unaffected. We postulate that the loss of deesterified homogalacturonan available for Ca$^{2+}$-mediated complexation might release the internal constrains within the cell wall, triggering the folding response. It would be interesting to study the enzymatic mechanisms underlying this homogalacturonan dynamics in the future (Saffer, 2018).

Drought-stressed plants of A. helenae and A. vera differed significantly in RWC and SWC. Despite the resilience imparted by cell walls, drought stress events negatively impact the capacity of hydrenchyma to fully rehydrate following a drought event. Prior to sampling, A. helenae appeared to be more negatively affected by drought than A. vera (Figure 1) with red-coloured leaves, loss of turgidity, and in-rolled margins. Interestingly, A. helenae showed higher RWC and SWC in the control plants, indicating a more rapid cellular response to drought compared with A. vera.

The observed variation in water content storage ability and flexibility could reflect habitat-specific adaptations, and the differences in response time might also be linked to unique polysaccharide composition of the cell walls for each species.

### 4.2 Cell wall polysaccharides with flexibility and storage functions dominate in Aloe

The composition of structural Aloe polysaccharides and the lack of components related to the mechanisms of cell wall extensibility and rigidity (e.g., galactans) are consistent with the requirement for high flexibility to accommodate cell wall folding. Indeed, acetylated mannan and galacto (gluco)mannan were the most frequently detected polysaccharides in both the microscopy and the CoMPP analysis of Aloe hydrenchyma (Figures 3 and 5). It has been suggested that acetylated mannan is involved in water storage in epiphytic orchids (Stancato et al., 2001). In Aloe, the abundance of mannan in the hydrenchyma cell walls of both A. helenae and A. vera would indicate that this polysaccharide is, likewise, implicated in water storage (Figures 5 and 7). The main hemicellulosic components detected in the cell walls of Aloe hydrenchyma were different types of homopolymers of mannan, galactomannans or glucomannans, and in lower amounts xyloglucan (Figure 4). The cellulose content requires an extraction step using cadoxen (Moller et al., 2007), and due to its corrosiveness and toxicity, is usually avoided and cellulose content excluded from CoMPP measurements. However, other studies have already confirmed the presence of cellulose in Aloe parenchymatous tissue (Femenia, Sanchez, Simal, & Rossell, 1999; Ni, Turner, Yates, & Tizard, 2004; Rodríguez-González et al., 2011). This was supported by the Calcofluor White staining in the present study.

Antibody detection of mannan in tissue sections (Figure 5) revealed that different epitopes of mannan are stored separately in distinct intracellular compartments and have a granular appearance as opposed to the more continuous distribution when it is embedded in the cell wall. The granular form of mannan resembles that of starch, a well-known storage polysaccharide. This observation provides further support for the hypothesis that mannan plays a role in energy storage and offers a source of carbohydrates for photosynthesis and maintaining water potential gradients between the chlorenchyma and hydrenchyma during drought conditions (Kluge & Ting, 1978; Nobel, 2006; Surridge, 2019). The precise nature and role of the granules that we identify in this study require further investigation to explore whether this is a common mechanism in succulent plants. That there is a link between polysaccharides and CAM has previously been shown (Kluge & Ting, 1978; Reynolds, 2004), and with the high content of mannan detected in aloe, it seems plausible that the two could be linked and thus enhance the survivability of succulents in extreme conditions. Future studies to resolve the biosynthetic pathway of mannan, the role of the mannan-containing granules and the polysaccharides connection to CAM will improve our understanding of how these two systems interact to improve the resilience of Aloe to drought.

### 4.3 Cell wall polysaccharide composition changes in response to drought

Remobilization of polysaccharides and organized cell wall folds are recognized in the hydrenchyma tissue of drought-stressed Aloe species. Our findings indicate the involvement of mannans in relation to water storage, but the size of the response (increase or decrease) and direction (up or down regulation) may differ between species. As seen for A. helenae, the total mannan and pectin levels increased after drought, whereas the opposite (to a lesser degree) was true for A. vera (Figure 3b). The detection of polysaccharides using CoMPP may be influenced by their extractability (for instance, partial digestion during remobilization), suggesting that further investigations are needed to explore this phenomenon and how it may affect the total level of mannan. This methodological limitation also necessitates micromorphological analysis using monoclonal antibodies.

Minor differences between the watered and the control measurements in CoMPP are also likely due to natural fluctuations (Ahl et al.,...
2019). The changes between predrought- and drought-stressed specimens were found for both species, although not in the same polysaccharide structures (Figure 3). Follow up studies are likely to illuminate species-specific drought tolerance in Aloe species, given that in this study A. helenae appeared to be more severely drought stressed than A. vera (Figure 1). The role of changing polysaccharide composition in drought recovery and resilience and the water-storing capacity of hydrenchyma tissue also require further consideration.

Considerable diversity in succulence between Aloe species likely reflects adaptations to varied habitats (Grace et al., 2015). The global popularity of A. vera is also attributed to its high polysaccharide content (Ahl et al., 2018; Eberendu et al., 2005; Shi et al., 2017). Therefore, predictable changes in the polysaccharide composition of Aloe species stimulated by drought stress or water availability could have practical ramifications for the management of species farmed for the hydrenchyma tissue, such as Aloe arborescens in Asia, A. vera on the American subcontinents, and A. ferax in South Africa (Grace, 2011).

Cell wall polysaccharides in Aloe are of ecological and economic significance in drought tolerance. We conclude that convoluted cell wall folding allows for controlled shrinking of dehydrated hydrenchyma and subsequent unfolding postdrought, although this process is not straightforward and the tissue may not recover its previous capacity for water storage. The most ubiquitous cell wall polysaccharides in Aloe appear to be different forms of mannan. Drought-induced changes to the polysaccharide composition of Aloe hydrenchyma impact mannan abundance in particular, and these shifts could have implications for the commercial production of A. vera and other species.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

L. I. A. and J. M. conceived and designed the experiments with O. M. G. and P. J. R. L. I. A. conducted the CoMPP experiments. J. M. conducted the microscopy imaging. O. M. G. and P. J. R. contributed micrographs from the permanent slide collection at the Royal Botanic Gardens, Kew. L. I. A. and J. M. wrote the manuscript with the assistance of N. R. and O. M. G. All authors contributed to the analysis and discussion of the results and edited the manuscript.

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**SUPPORTING INFORMATION**

Additional supporting information may be found in the Supporting Information section at the end of the article.

**Figure S1.** CoMPP analysis of hydrenchyma tissue in six Aloe species pre- and post- drought stress, with control treatments. Vouchers are deposited in Herbarium C at the Natural History Museum of Denmark. XG = Xyloglucan. Antibodies are listed in Figure 3 in the main part of the study.

**Figure S2.** COS488 labelling of leaf hydrenchyma in *Aloe helenae* in (a) watered and (b) drought-stressed (b) specimens. Hydrenchyma cell walls are labelled with COS488 but the labelling is significantly reduced in the drought-stressed hydrenchyma, but chlorenchyma is unaffected.

**Figure S3.** Detection of the RG-I side chains in Aloe leaves using LM5 and LM6 monoclonal antibodies (a) Staining with LM5 (green signal) recognizing (1,4)−β-D-galactan shows a discontinued pattern in chlorenchyma tissue; (b) staining with LM6 (green signal) recognizing (1,5)−β-L-arabinan show instances of sporadic binding in chlorenchyma tissue. Calcofluor White was used as counter stain.

**Figure S4.** Specificity analysis of anti-mannan antibodies. Calcofluor White (staining (1,4)−β-glucans, blue signal) is used on all sections. Antibodies used are CCCR-M170 binding acetylated mannan (a-c), BS-400-4 binding (1,4)−β-D-mannan (d-f) and LM21 binding (1,4)−β-D-mannan/galactomannan/glucomannan (g-i). Sections were treated with mannanase (b, e, h) and 200 mM NaOH (c, f, i). Mannanase treatment diminishes staining for all antibodies (b, e, h). De-acetylation partially diminishes binding of CCCR-M170 (c), strongly binding of BS-400-4 (f), and minor changes are seen for LM21 (i).

**Figure S5.** Probing of tissue streaks with CCCR-M170 and LM21 monoclonal antibodies confirms the granular or compartmentalised mannan accumulation in *Aloe* hydrenchyma.

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