Localization and Quantification of Callose in the Streptophyte Green Algae Zygnema and Klebsormidium: Correlation with Desiccation Tolerance

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Freshwater green algae started to colonize terrestrial habitats about 460 million years ago, giving rise to the evolution of land plants. Today, several streptophyte green algae occur in aero-terrestrial habitats with unpredictable fluctuations of land plants. Today, several streptophyte green algae occur worldwide (Lewis and McCourt 2004, Leliaert et al. 2012), numerous studies investigated the physiological adaptations of aero-terrestrial streptophyte green algae to their habitats (Hawes 1990, Elster and Benson 2004, Gray et al. 2007, Karsten and Rindi 2010, Karsten et al. 2011, Kaplan et al. 2012, Aigner et al. 2013, Karsten et al. 2013, Kaplan et al. 2013, Karsten and Holzinger 2014, Pichrtova et al. 2014, Pichrtova et al. 2014b, Vilumbrales et al. 2014, Herburger et al. 2015). Despite detailed knowledge on the composition of the cell walls of streptophyte algae being available (Domozycz et al. 2012), the contribution of these walls to coping with harsh environmental conditions is very limited so far. This is surprising, as the extracellular matrix is the only barrier between the algal protoplasts and the environment.

In the past decade, research has focused on the architecture and chemical composition of cell walls in unicellular Zygnematophyceae, a late diverged streptophyte green algae lineage which founded the line of embryophytes after colonizing terrestrial habitats about 460 million years ago (Wickett et al. 2014), by using immunocytochemistry and focusing on pectins (Domozycz et al. 2007, Eder and Lütz-Meindl 2008, Domozycz et al. 2009, Eder and Lütz-Meindl 2010, Domozycz et al. 2011, Domozycz et al. 2014) or hemicelluloses (Eder et al. 2008, Domozycz et al. 2009), or by the use of general polysaccharide staining (Brosch-Salomon et al. 1998). High-throughput techniques such as glycan microarrays gave semi-quantitative insights into the occurrence of cell wall components in several green algae (Sørensen et al. 2011). These findings were set into an evolutionary context to show significant differences between early- (e.g. Klebsormidiophyceae) and late-branching (e.g. Zygnematophyceae) streptophyte green algae and land plants (Popper and Tuohy 2010, Domozycz et al. 2012). Recently, transcriptome and genome analysis of species belonging to the Klebsormidiophyceae (Wodniok et al. 2011, Timme et al. 2012, Holzinger et al. 2014, Hori et al. 2014) and Zygnematophyceae (Timme et al. 2012) also became available. These data sets were mined for enzymes involved in cell wall biosynthesis and compared with land plants, confirming their epidermal layers, periderm or bark (Graham et al. 2009). Nevertheless, some of these green algae occur in aero-terrestrial habitats, where they are frequently exposed to desiccating conditions causing drastic mechanical deformations of the cell wall and protoplast (Holzinger and Karsten 2013). As many of these green algae occur worldwide (Lewis and McCourt 2004, Leliaert et al. 2012), numerous studies investigated the physiological adaptations of aero-terrestrial streptophyte green algae to their habitats (Hawes 1990, Elster and Benson 2004, Gray et al. 2007, Karsten and Rindi 2010, Karsten et al. 2011, Kaplan et al. 2012, Aigner et al. 2013, Karsten et al. 2013, Kaplan et al. 2013, Karsten and Holzinger 2014, Pichrtova et al. 2014a, Pichrtova et al. 2014b, Vilumbrales et al. 2014, Herburger et al. 2015). Despite detailed knowledge on the composition of the cell walls of streptophyte algae being available (Domozycz et al. 2012), the contribution of these walls to coping with harsh environmental conditions is very limited so far. This is surprising, as the extracellular matrix is the only barrier between the algal protoplasts and the environment.

Introduction

In contrast to land plants, thalli of streptophyte green algae are not protected by watertight covering tissues such as cutinized
most important core wall polysaccharides to be already present in some streptophyte green algae (Mikkelsen et al. 2014, Yin et al. 2014).

Similar to land plants, the cell walls of Zygnematophyceae comprise load-bearing compounds such as cellulose and hemicelluloses (Scheller and Ulvskov 2010, Sørensen et al. 2011), matrix carboxylic polysaccharides (i.e. pectins; Popper et al. 2011) and glycoproteins [arabino-galactan proteins (AGPs)], extensins and expansins (Vannerum et al. 2011, Domozych et al. 2012). In contrast, most of the cell wall polymers usually found in the Zygnematophyceae and land plants are absent in the Klebsormidiophyceae (Sørensen et al. 2011). However, both classes contain callose, a β-1,3-glucan (Bacic et al. 2009). Biosynthesis of callose is catalyzed by family 48 glycosyltransferases (Cantarel et al. 2009), which is part of a very ancient eukaryotic pathway (Michel et al. 2010). It occurs in many land plants as a regular component of developing cell plates during cytokinesis (Verma 2001), in pollen walls and tubes (Dong et al. 2005, Nishikawa et al. 2005, Shi et al. 2014) and in sieve elements (Chen and Kim 2009), and it acts as a regulator of plasmodesmata for controlling the movement of molecules through the symplasmic continuum (Chen and Kim 2009). Callose is involved in many stress responses, such as plugging plasmodesmata or to prevent pathogen spread throughout the plant (Iglesias et al. 2000, Eggert et al. 2014). It is incorporated within minutes after cellular damage due to mechanical strain, heavy metal treatment or stress caused by plasmolysis and temperature fluctuations (Bhuja et al. 2004, Bacic et al. 2009). In several green algae, callose was found in newly formed septae during cytokinesis (Scherp et al. 2001), the chamber walls of developing meiospores in Coleochaete (Graham and Taylor 1986), in rhizoids and conjugation tubes of Spirogyra (Yamada et al. 2003), and it is incorporated after ultrasonic treatment (Scherp et al. 2001). However, knowledge of the contribution of callose to the aero-terrestrial lifestyle of green algae is lacking.

In the present study, we employed immunocytochemistry in live cells as well as in high-pressure frozen cells. Additionally, standard staining procedures and spectrofluorimetry were used to visualize and quantify the change of callose content after experimental desiccation stress. The physiological status of the desiccated and recovered individual algal filaments was monitored by Imaging-PAM measurements. As desiccation stress causes drastic cell wall deformations and callose is involved in many wound responses related to mechanical strain, we hypothesize that it is incorporated in deformed areas of the cell wall. This would imply an important contribution to desiccation tolerance. Flexible cell walls are crucial for surviving cellular water loss by allowing regulated shrinkage of the protoplast (Holzinger et al. 2011), as shown for desiccation-tolerant ‘resurrection plants’, lichens, seeds and the intertidal macroalga Ulva (Webb and Arnott 1982, Brown et al. 1987, Moore et al. 2013, Holzinger et al. 2015). We have chosen two species of filamentous green algae from the class Klebsormidiophyceae [Klebsormidium crenulatum (Karsten et al. 2010, Kaplan et al. 2012) and Klebsormidium nitens (Kaplan et al. 2012)] and two species from the class Zygnematophyceae [Zygnema sp. 'Saalach' (S) and Zygnema sp. 'Elmau-Alm' (E-A) (Herburger et al. 2015)]. As each of these species is assigned to different subclades within the respective genus (Kaplan et al. 2012, Herburger et al. 2015), both genera investigated are represented on a broad phylogenetic base. This enabled us to put the findings on the role of callose for an aero-terrestrial lifestyle in an evolutionary context, by comparing early-branching Klebsormidiophyceae with the later branching Zygnematophyceae.

**Results**

**Spectrofluorimetric quantification of callose**

Control samples of Zygnema contained between half and a third of the amount of callose when compared with Klebsormidium (Fig. 1). Desiccation for up to 210 min did not change the callose content in Zygnema (Fig. 1; Supplementary Table S1). In contrast, 30 min of desiccation increased the callose content in K. crenulatum and K. nitens significantly by 109.8 ± 9.5% and 70.0 ± 8.6%, respectively (Fig. 1). Desiccation for 210 min led to an even stronger increase compared with the initial value: 207.2 ± 21.6% and 122.9 ± 11.8% in K. crenulatum and K. nitens, respectively (Fig. 1).

**Desiccation effects, Calcofluor white and Aniline blue staining**

To visualize the effect of cellular water loss on algal cell morphology already after 30 min of desiccation, we used confocal laser scanning microscopy (CLSM) and Chl autofluorescence (Fig. 2). In both Zygnema (Fig. 2A–D) and Klebsormidium (Fig. 2E–J), desiccation resulted in a drastic deformation of the cell walls and protoplasts (Fig. 2). In Zygnema, the longitudinal cell walls...
**Immunocytochemistry**

Live cell labeling with the monoclonal antibody (AB) 400-2 allowed a specific detection of callose in *Zygnema* (*Fig. 4*) and *Klebsormidium* (*Fig. 5*). Hydrated filaments of both *Zygnema* S and *Zygnema* E-A showed a similar pattern of callose distribution: callose was abundant in terminal cross cell walls (*Fig. 4A, B*) and in detaching or deformed parts of the walls (*Fig. 4C, D*). Despite long incubation times, AB penetration into unexposed cross cell walls was not satisfactory. Therefore, we labeled semi-thin sections of high-pressure-fixed algae (*Fig. 4E–H*). There, the callose signal usually was restricted to cell corners between the cells of control *Zygnema* filaments (*Fig. 4E, F*). In shorter, i.e. younger, cells, the cross cell walls also contained callose (*Fig. 4E, F*). Similarly, desiccated *Zygnema* filaments exhibited callose-rich cell corners and terminal cell walls, while the unexposed cross and longitudinal cell walls lacked callose (*Fig. 4G, F*). Occasionally, desiccated cells of *Zygnema* E-A with protoplasts closely attached to the wall contained abundant callose in the longitudinal cell walls (*Supplementary Fig. S2*). In *K. crenulatum*, live cell labeling
showed callose to be abundant in cell corners and terminal cell walls (Fig. 5A), while in *K. nitens* it was mainly restricted to terminal walls (Fig. 5B), and after cell detachment in newly exposed cross cell walls (Fig. 5C) and deformed cells (Fig. 5D). Labeling of semi-thin sections of hydrated *Klebsormidium* filaments revealed callose occurring in cell corners and in most unexposed cross cell walls (Fig. 5E, F), while after desiccation the cell corners and undulated cross cell walls were rich in callose (Fig. 5G, H). Moreover, desiccated filaments of *K. nitens* showed a dotted callose distribution in the longitudinal cell walls (Fig. 5H). Negative control samples after omitting incubation with the primary AB showed no staining due to a lack of binding of the secondary AB (Supplementary Fig. S3A–E).

**Immunogold labeling**

Labeling of callose epitopes with 10 nm gold particles on ultra-thin sections showed callose throughout the cross cell walls of *K. crenulatum* and also in the centrally located protuberances (Fig. 6A) and particularly in the terminal cell walls of *K. nitens* (Fig. 6B).

**Imaging-PAM**

To exclude that the drastic deformation of the cells and protoplasts after desiccation is lethal, we used the microscopic version of an Imaging-PAM to monitor the effective quantum yield of PSII (Y(II)) prior to and after desiccation and upon rehydration (Figs. 7, 8). In *Zygnema* S and *Zygnema* E-A, 30 min of desiccation caused a strong reduction of the Y(II) in all cells from approximately 0.65 to approximately 0.25 and from approximately 0.60 to 0.15, respectively (Fig. 7A, B). Subsequent rehydration allowed the cells of both *Zygnema* strains to restore their initial shape and the Y(II) to recover homogeneously to about half of the initial value (Fig. 7A, B), while *Zygnema* S cells with thicker cross cell walls showed the highest Y(II) after 180 min of recovery (Fig. 7A). Furthermore, after 180 min, the characteristic stellate shape of the two chloroplasts was remodeled in several cells (Fig. 7A, B). The cells of *K. crenulatum* within one filament exhibited a similar Y(II), which declined after desiccation from approximately 0.65 to approximately 0.35 (Fig. 8A). In contrast, the Y(II) in *K. nitens* varied between the cells of one filament (~0.45 to ~0.60; Fig. 8B). Thirty minutes of desiccation reduced the Y(II) uniformly to approximately 0.35 (Fig. 8B). Rehydration allowed both *Klebsormidium* strains to recover their Y(II) almost fully, and the filaments expanded to the initial value (Fig. 8A, B). In both *Klebsormidium* strains, the shape of the parietal chloroplast was restored after rehydration for 10 min (Fig. 7A, B). The Y(II) in terminal cells of fragmented *Zygnema* and *Klebsormidium* filaments and small fragments (2–3 cells) of...
Fig. 4 Micrographs of *Zygnema* S (A, B, E, G) and *Zygnema* E-A (C, D, F, H); hydrated cells (A–F), 210 min desiccated cells (G, H). (A–D) Live cell labeling (red, Chl autofluorescence); (E–H) the corresponding bright field image and labeling of semi-thin sections with the monoclonal antibody 400-2 (green). (A) Convex terminal cross wall stained abundantly. (B) Recently fragmented cells with massive staining in the new terminal wall. (C) Detaching filament; a strong signal in the terminal cells (asterisks) and weak labeling in longitudinal walls (arrow). (D) Filament with a deformed longitudinal cell wall abundantly stained (arrow). (E) Filament with clearly visible starch grains in pyrenoids (arrowheads); cell corners show strong labeling; only cross cell walls of young cells are stained (arrow). (F) Filament with a deformed cell (asterisk) and strong callose labeling in the area of deformation and in cell corners (arrowheads). (G) Desiccated filament with labeling restricted to the terminal cell wall and cell corners. (H) Desiccated filament with labeling in cell corners. Scale bar = 10 μm.

Fig. 5 Micrographs of *Klebsormidium crenulatum* (A, E, G) and *Klebsormidium nitens* (B, C, D, F, H). (A–D) Live cell labeling (red, Chl autofluorescence), and labeling of semi-thin sections (E–H) of hydrated (E, F) and desiccated filaments (G, H) with the monoclonal AB 400-2 (green). Bright field image and corresponding labeling is shown. (A) Callose labeling in the longitudinal cell walls between individual cells (arrowheads). (B) Filament fragment with callose labeling in the terminal cross cell walls. (C) Detached cells (asterisks) show labeling in the terminal walls (arrow). (D) 3D projection of a deformed cell filament with strong callose labeling in cross cell walls, with maximal labeling in the center of the cross cell walls and protuberances (arrows). (F) Callose in the center of cross cell walls (arrows). (G) Undulated cross cell walls with abundant callose staining. (H) Callose labeling in cross cell walls and longitudinal walls (arrows). Scale bar = 5 μm.
K. nitens was similar to that of adjacent cells in long filaments (Supplementary Fig. S4A–D).

Discussion

Desiccation usually leads to a clearly visible deformation of plant cell walls (Moore et al. 2006, Holzinger et al. 2011). While the walls of some species exhibit a random wrinkling leading to irreversible damage, others show highly regular and reversible shrinking patterns (Brown et al. 1987, Moore et al. 2008), which is important for surviving cellular water loss (Webb and Arnott 1982). As information on deformable cell walls in algae is scarce, we used spectrofluorometry and immunocytochemistry to estimate the role of callose in coping with mechanical stress of the algal cell wall and protoplast during desiccation. Recently, in a transcriptomic analysis, callose synthase complex (UNO 39686, KEGG orthology: K 11000, EC 2.4.1.-) was found to be up-regulated by approximately 1.5-fold upon severe desiccation stress in *K. crenulatum* (Holzinger et al. 2014). This key enzyme has also been detected in the genome of *K. flaccidum* (gene family number OG00420; biological process category GO: 0052544—defence response by callose deposition in cell wall; Hori et al. 2014). It has to be emphasized that in our study, callose was not only detected as a stress response, but is already present in mechanically undisturbed algal filaments. Callose allows a regulated shrinking process in *Klebsormidium*

We found a high abundance of callose in the cross walls of *Klebsormidium* filaments which allow a regulated reduction...
of the cell filament diameter during cellular water loss. The longitudinal cell walls stayed closely attached to the protoplasts. This is crucial for preserving the structural integrity of the basic cell organelles (Holzinger et al. 2011), maintaining the turgor pressure and a high photosynthetic performance during short-term desiccation, as shown by microscopic Imaging-PAM. Furthermore, as the plasma membrane did not retract from the cell wall, additional callose can be incorporated during water loss to repair local injuries of the walls. This is indicated by higher callose contents and stronger AB labeling throughout the cross cell walls in both Klebsormidium species after desiccation. Furthermore, in K. nitens, callose was incorporated in the longitudinal cell walls after desiccation, pointing to local repairs of strained areas. In contrast to land plants, algae do not form wound-induced regenerative tissues, and therefore maintaining the integrity of individual cells is particularly important (Menzel 1988). In Desmidiaceae, wound plug formation after experimental microinjection has been shown to occur within 15 min (Holzinger et al. 1995), and even when cell development is supressed, e.g. by an G-actin-binding protein, the wound plug is formed undisturbed (Holzinger et al. 1997). Sometimes, cross cell walls in older or field-collected filaments of K. crenulatum become thick and multilayered due to the mode of cell division (Mikhailyuk et al. 2014). We demonstrate marked callose staining by Aniline blue as well as AB labeling in the corners between individual Klebsormidium cells. This finding is in agreement with observations of Mikhailyuk et al. (2014), who demonstrated triangular spaces of unidentified content in transmission electron microscopy samples. As shown moreover by immunogold labeling in 1-month-old cell filaments, callose occurs throughout the central part of cross cell walls and in the central protuberances of K. crenulatum and the terminal walls of K. nitens. The function of these protuberances remains largely unknown; they have also previously been shown in the Klebsormidiophyceae Entransia (Cook 2004). These structures could contribute to the fragmentation of the filaments, but are clearly distinct from the cylinders of cell wall material found during the division process in Desmidiaceae (Hall et al. 2008), which have been immunologically shown to contain highly methylsterified homogalacturonans in Desmidium swartzii (Andosch et al. 2015).

In natural habitats, green algae occurring in soil crusts strongly depend on rainwater as a source of available moisture. Rainwater is hypotonic compared with the cytoplasm of Klebsormidium, which exhibits a very negative osmotic potential (K. crenulatum = −2.09 MPa, K. nitens = −1.67 MPa; Kaplan et al. 2012) compared with obligatory submersed living green algae. These osmotic values are an adaptation to habitats with low water availability (Holzinger and Karsten 2013). A sharp osmotic gradient causes a rapid water uptake of desiccated protoplasts, while the closely attached cell walls act as an antagonist to the increasing turgor and prevent harmful expansion rates causing rupture of the plasma membrane. In desiccation-tolerant resurrection plants and the intertidal green macroalga Ulva compressa, maintaining flexible cell walls is also important for efficient rehydration (Moore et al. 2013, Holzinger et al. 2015). The processes in U. compressa are different, as inner pectic layers are responsible for the flexibility of the extremely thick cell walls; however, the effect, i.e. avoidance of mechanical damage, is similar. As shown by microscopic Imaging-PAM, rehydration of single algal filaments allowed photosynthesis to recover almost fully after 1 h (K. nitens) or 3 h (K. crenulatum), respectively. In part, this might be related to the morphology of the parietal chloroplast in Klebsormidium, which we consider as another adaption to changing filament diameters during desiccation and rehydration cycles: the chloroplast is shaped like an incompletely closed tube. Thus reducing the cell diameter during desiccation allows the margins of the chloroplast to become closely attached to each other (Supplementary Fig. S5). Near infrared (NIR) remission images of rehydrated Klebsormidium filaments revealed a similar chloroplast morphology to that prior to desiccation. This supports that the chloroplast ultrastructure in K. crenulatum remains intact even after desiccation for 4 d at 5% relative humidity (Holzinger et al. 2011).

Rapid incorporation of callose in response to deformation (e.g. perturbation) also occurs in Embryophytes (Bacic et al. 2009). In resurrection plants, arabinose-rich cell wall components in particular contribute to the cell wall’s flexibility (Moore et al. 2013). However, as shown by glycan microarray analysis (Sørensen et al. 2011), cell walls of Klebsormidium lack these polymers. Furthermore, pectic substances related to cell wall flexibility in desmidiaecean green algae [methylsterified homogalacturonan (HG); Eder and Lütz-Meindl 2008] and wall loosening in land plants (demethylsterified homogalacturonans; Peaucelle et al. 2011) are absent in Klebsormidium. Klebsormidiophyceae lack most of the components (e.g. extensin) that are typical for cell walls of Embryophytes and late-branching streptophytes green algae (Domozych et al. 2012). However, the walls of Klebsormidium contain cellulose, which was visualized in the present study by Calcofluor white staining, where fluorescence was found in the cross cell walls while the longitudinal walls exhibit rather weak fluorescence. However, due to the non-specifity of calcofluor staining, callose-rich cell wall areas (e.g. centers of cross cell walls) are also likely to be stained (Krishnamurthy 1999). The low amount of load-bearing cell wall components in Klebsormidium (i.e. cellulose and hemicelluloses), which was also reported previously (Domozych et al. 1980, Sørensen et al. 2011), probably reduces the cell wall’s rigidity. Another cell wall-related factor supporting desiccation tolerance is mucilage layers (Shephard 1987). As discussed for Interflium strains, these layers increase the water-holding capacity of the cells and contribute to a higher photosynthetic performance during desiccation stress (Karsten et al. 2014). Species of Klebsormidium belonging to clades E (K. nitens), B and G also form a mucilage envelope by gelatinization of the parietal wall, while members of the clades D, F (K. crenulatum) are not coated by such layers (Rindi et al. 2011, Mikhailyuk et al. 2014). As on the filament level both K. crenulatum and K. nitens show similar responses to desiccation stress followed by rehydration, the presence or absence of a mucilage layer is probably not involved in surviving short-term desiccation stress in Klebsormidium. However, the contribution of callose to survive water scarcity by restricting desiccation-induced
mechanical damage is indicated by three major findings: (i) the callose content in the cell walls increased strongly during desiccation stress; (ii) cellular water loss caused cell wall undulations allowing regulated shrinkage and expansion, with callose particularly abundant in the strained areas; and (iii) the cell wall of *Klebsormidium* lacks other cell wall components which allow cell wall flexibility (Sørensen et al. 2011).

**Callose acts as a flexible supporter between individual cells of a Zygnema filament**

We found that the cell walls of *Zygnema* contained less callose compared with *Klebsormidium*, and desiccation did not change their content. In contrast to *Klebsormidium*, desiccation forced the proplasts of *Zygnema* to retract completely from the strongly expanded longitudinal cell walls, which prevents incorporation of callose into the walls as callose synthase is located in the plasma membrane. As *Zygnema* is closely related to land plants, the cell walls contain other flexibilizing components, such as AGPs, pectins and extensin (Sørensen et al. 2011). Cross cell walls, where the proplast remains attached centrally after desiccation, only contain callose in cells with shorter longitudinal walls (i.e. recently divided cells), possibly indicating remnants of a newly formed septum after cytokinesis (Scherp et al. 2001). This callose accumulation has been found in numerous filament-forming or multicellular plants (Scherp et al. 2011); however, this has not been further investigated in the present study.

Most interestingly in *Zygnema*, AB labeling of semi-thin sections revealed callose to be restricted to cell corners between individual cells. In this area, biomechanical forces are greatest during desiccation, when the longitudinal walls expand convexly but the cross cell walls do not change their shape. The flexible callose-rich areas between the cells probably act as supporters and help in dissipating shearing forces to avoid rupture of the load-bearing cell wall scaffold. The conspicuous deformation of the callose-rich longitudinal cell walls was already clearly visible after 30 min of desiccation, but, surprisingly, not lethal, as shown by microscopic Imaging-PAM by monitoring increasing photosynthetic performance after rehydration. Lacking full recovery of photosynthesis is in good agreement with previous findings showing young vegetative filaments of Zygnematophyceae to be more sensitive to water loss compared with Klebsormidiophyceae, which explains their preference for moister habitats (Holzinger and Karsten 2013). However, as a response to long-term desiccation or nutrient starvation, vegetative cells of *Zygnema* can be transformed into specialized resistant cells (‘pre-akinetes’ or akinetes), to increase the resistance against desiccation stress (Pichrtová et al. 2014a, Pichrtová et al. 2014b, Herberger et al. 2015). However, it is not clear whether *Klebsormidium* also forms such resistant cells (Mikhailyuk et al. 2014), and therefore the focus of this study was on comparing young vegetative filaments of *Zygnema* and *Klebsormidium* that were fully metabolically active. Mucilage layers may be more or less abundant in different *Zygnema* species; however, culture age is a critical factor, where older cultures produce more mucilage (Herberger et al. 2015). Moreover, filaments obtained from polar field populations of *Zygnema* often produce fibrilllose mucilage layers, which, in combination with the formation of extensive mats on the soil, protect against cellular water loss (Pichrtová et al. 2014b). The diameter of these pectic-rich layers increases when *Zygnema* is exposed to mild long-term desiccation stress (several months), which probably increases the water-holding capacity of the filaments during prolonged dry periods (Pichrtová et al. 2014b, and references therein). In contrast, the present study investigates the effects of severe short-term desiccation (30–210 min) on individual young filaments, resulting in retraction of the proplast from the cell wall.

**Callose allows terminal walls to expand convexly**

In both *Zygnema* and *Klebsormidium*, high amounts of callose occur in terminal cell walls after fragmentation, which were expanded convexly due to the turgor pressure (Holzinger et al. 2011). Sufficient turgor pressure is also necessary for callose-rich rhizoid formation in detaching terminal cells of Spirogyra filaments (Yamada et al. 2003). Cell detachment was observed in *Zygnema* and *Klebsormidium*, while *K. nitens* showed the strongest tendency to fragment, which explains the highest callose content which probably positively correlates with the more frequent occurrence of terminal cross walls. In *Klebsormidium*, fragmentation into short filaments is commonly seen in liquid cultures (Rindi et al. 2008) and may have an ecological function (Karsten and Holzinger 2014). Fragmentation usually takes place along cross cell walls (Mikhailyuk et al. 2014), and remnants of wall material stay attached to newly exposed callose-rich cross walls. Imaging-PAM revealed that the photosynthetic performance of terminal cells in recently fragmented filaments of *Zygnema* and *Klebsormidium* and small filaments (2–3 cells) of *Klebsormidium* was not affected by cell detachment. Interestingly, in *Klebsormidium*, cell detachment is positively correlated with increasing light intensities or temperature under culture conditions (Dřímalová and Poulíčková 2003). Severe weather conditions entrain fragmented algal colonies into the atmosphere (Sharma et al. 2007). Therefore, we consider fragmentation into small and metabolically active [indicated by high Y(II)] filaments to be a fast and economic way for gaining dispersal units under changing environments (i.e. climate change). In part, this explains the worldwide distribution of *Klebsormidium* (Ryšánek et al. 2015).

**Conclusion**

Under natural conditions, a complex inter-relationship of many responses on the molecular and cellular level affects the ability of green algae to cope with water scarcity. In the present study, we focused on the cell wall and showed for the first time that desiccation stress nearly doubled the amount of callose even after 30 min desiccation in *Klebsormidium*, and callose localization is clearly evident in strained areas (cross walls). This correlates with a higher photosynthetic performance during desiccation and a full recovery after rehydration, while in *Zygnema* desiccation did not change the generally lower callose content and inhibited photosynthesis more strongly. Furthermore, the incorporation of callose in newly formed terminal cell walls may be a
prerequisite to survive the sudden rupture. Short algal filaments were found to be metabolically active [high Y(II)], considered to be important for dispersal and the global success of aero-terrestrial green algae. In future studies, a detailed investigation of AGPs and pectins of the Zygnematophyceae will increase our understanding of the current belief that this class is sister group to land plants (Wickett et al. 2014).

Materials and Methods

Algal material and culture conditions

*Zygnema* S (SAG 2419) and *Zygnema* E-A (SAG 2418) were isolated from aero-terrestrial habitats in different altitudes, and cultures were maintained in BBM (Bischoff and Bold 1963) in 250 ml Erlenmeyer flasks according to Herburger et al. (2015).

*Klebsormidium crenulatum* (SAG 2415) was isolated previously from the Schönwieskopf (Tyrol, Austria; Karsten et al. 2010), and *K. nitens* (SAG 2417; recently determined as *K. dissectum* according to Mikhailyuk et al. 2015) from concrete panels in the Botanical Garden of Innsbruck (Kaplan et al. 2012). Purified unialgal cultures were cultivated in modified BBM (5 NMIBM; Starr and Zeikus 1993) under the same conditions as described in the previous paragraph. Cell filaments of 4-week-old cultures were used in this study.

Desiccation experiments and colorimetric quantification of callose

Desiccation experiments were performed according to Karsten et al. (2014) in desiccation chambers at ambient humidity (AHL: ~65%), monitored by a PCEMSR145 S-TH data logger; PCE Instruments) and room temperature (~21 °C) at 40 μmol photons m⁻² s⁻¹. Reproducibility was guaranteed by using defined starting volumes of culture medium after removing excess moisture. For colorimetric quantification, fresh algae incubated in 200 μl of culture medium were spread evenly onto aluminum foil and desiccated for 0 (control), 30, 60, and 180 min of rehydration. This was repeated at least five times for each strain. Pictures were taken with a modified Axio Scope A.1 epifluorescence microscope (Leica Microsystems Gmbh) and embedding in LR-White (London Resin Company Ltd.). As cell walls in *Klebsormidium* are often multilayered (Mikhailyuk et al. 2014), additionally we used immunogold labeling to estimate whether callose occurs throughout the wall of hydrated filaments. Therefore, ultrathin sections were prepared by using a Leica ultramicrotome. Immunogold labeling and control experiments were performed according to Holzinger et al. (2000) with modifications. Briefly, ultrathin sections were blocked onto droplets of 5% (v/v) hydrogen peroxide (Sigma-Aldrich) and 1 mM MgCl₂ for 1 h. Filaments were washed in 1 ml of phosphate-buffered saline (PBS; 3 × 10 min), blocked in 2% bovine serum albumin (BSA; Sigma-Aldrich) and 0.1% Tween-20 (Sigma-Aldrich) in PBS for 30 min, washed again in 1 ml of PBS (3 × 10 min) and incubated for 2 h at room temperature in the primary AB (AB; 400-2; Biosupplies Australia Pty Ltd; Meikle et al. 1991), 1:10 in PBS containing 0.1% Tween-20. Filaments were washed (1 ml of PBS; 3 × 10 min), incubated in the secondary AB [Alexa Fluor® 488 Goat Anti-Mouse IgG (γ1) (Life Technologies), 1:50 in PBS with 0.1% Tween-20] for 1.5 h at room temperature. Probes were transferred in CitiFluor AF1 (CitiFluor Ltd.) and examined by CLSM. Samples were excited at 488 nm (argon laser) and emission was collected in two separate channels at 505–550 nm (false color green) and long pass 560 nm (false color red). Z-stack projections were generated by overlaying several optical slices through half of the width or the whole cell filament.

Cryofixation and immunogold labeling

For cryofixation, algae and 200 μl of culture medium were transferred to a Ø 47 mm Whatman GF/F glass microfiber filter and desiccated for 210 min. Fresh and desiccated filaments were cryofixed in a Leica EMPACHT high-pressure freezer (Leica Microsystems) followed by freeze substitution (Leica EM AFS; Lutz-Meindl and Aichinger 2004) and embedding in LR-White (London Resin Company Ltd.). As control and the primary AB was omitted.

Light fluorescence and confocal laser scanning microscopy

Epifluorescence microscopy was performed with a Zeiss Axiocert 200M microscope (Carl Zeiss AG), equipped with a × 631.4 NA objective lens and a Zeiss Filter Set 01 (excitation, 365 nm; emission, 397 nm). Images were captured with an AxioCam MRc5 camera and Zeiss Axiovision software. For CLSM, a Zeiss Pascal CLSM system was used. All images were further processed with Adobe Photoshop (CCS) software version 12.1 (Adobe Systems).

Aniline blue and Calcofluor white staining

Single algal filaments and 1 μl of culture medium were transferred to well plates, desiccated for 30 min and investigated by CLSM. Emission (long pass 560 nm) from a 488 nm excitation laser (argon) and a bright field image were collected in two channels. Callose and cellulose were stained with 1% Aniline blue and 1% Calcofluor white (Sigma-Aldrich), respectively (Krishnamurthy 1999). Both cellulose and callose were visualized by epifluorescence microscopy.

Immunocytchemistry

Live cell labeling was performed according to Domozycz et al. (2011) and Eder and Lütz-Meindl (2010) with modifications. Cell filaments were washed and chemically fixed in PIPES buffer (pH 7.4) with 4% (v/v) paraformaldehyde (Sigma-Aldrich) and 1 mM MgCl₂ for 1 h. Filaments were washed in 1 ml of phosphate-buffered saline (PBS; 3 × 10 min), blocked in 2% bovine serum albumin (BSA; Sigma-Aldrich) and 0.1% Tween-20 (Sigma-Aldrich) in PBS for 30 min, washed again in 1 ml of PBS (3 × 10 min) and incubated for 2 h at room temperature in the primary AB (AB; 400-2; Biosupplies Australia Pty Ltd.; Meikle et al. 1991), 1:10 in PBS containing 0.1% Tween-20. Filaments were washed (1 ml of PBS; 3 × 10 min), incubated in the secondary AB [Alexa Fluor® 488 Goat Anti-Mouse IgG (γ1) (Life Technologies), 1:50 in PBS with 0.1% Tween-20] for 1.5 h at room temperature. Probes were transferred in CitiFluor AF1 (CitiFluor Ltd.) and examined by CLSM. Samples were excited at 488 nm (argon laser) and emission was collected in two separate channels at 505–550 nm (false color green) and long pass 560 nm (false color red). Z-stack projections were generated by overlaying several optical slices through half of the width or the whole cell filament.
Statistical evaluation of the data
Comparison of callose contents (n = 4) of control and desiccated (30 or 210 min) samples by using Origin 8.5 software (OriginLab Corporation) was evaluated by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test (P < 0.001) to find homogeneous subgroups of significantly different means.

Supplementary data
Supplementary data are available at PCP online.

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Disclosures
The authors have no conflicts of interest to declare.

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