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Original Research

How does the cell wall 'stick' in the mucilage? A detailed microstructural analysis of the seed coat mucilaginous cell wall

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ABSTRACT

The seed mucilage envelope of myxospermatic diaspores is considered as a modified cell wall. Its chemical constitutes are mainly polysaccharide groups typical of the cell wall, however, pectins are very often the main component. A detailed analysis of the mucilaginous cell wall spatial architecture was demonstrated for the first time using a less-invasive CPD + SEM technique, which allowed the preservation of the mucilaginous cell wall structure. The examined pectic (*Linum usitatissimum*) and cellulose (*Neopallasia pectinata*) seed mucilage showed the fibrillary character of the components. The second type of mucilage indicated a much more arranged structure than that of the pectic one due to the presence of cellulose microfibrils. We showed cellulose organization in a net-like scaffold on which other mucilage components were spread and shoved how the mucilage was anchored to the seed surface through the cellulose skeleton, preventing it from being lost. Our detailed analysis gave an insight into how the mucilage is spatially arranged and also provided direct microstructural evidence of cell wall polysaccharides structure, distribution and interactions especially for widely-postulated xylan-cellulose linking. We demonstrated that xylan might be represented by long chains covering the surface of cellulose fibrils. The main advantage of the applied technique is its less-invasive character which retains the 3D structure of the components within the intact mucilaginous cell wall. As utilized in our studies, preparation and visualization methods with seed mucilage as a model system can give us new possibilities in structural studies of the (mucilaginous) cell wall.

1. Introduction

The plant cell wall (CW) is involved in different processes which maintain the proper functioning of cell and plant survival. Such processes range from the control of cell expansion, transport regulation, enhancing mechanical strength, maintaining cell shape and defending the cell against pathogens to signal transduction and cell adhesion. Cell wall structure and composition differ between the primary (PCW) and secondary cell wall (SCW) and can continually be modified as a result of diverse environmental conditions (Bidlack et al., 1992; Caffall and Mohnen, 2009; Banasiak, 2014).

The typical plant cell wall of dicotyledonous plants is a complex structure composed of three main groups of polysaccharides: cellulose, pectins and hemicellulose linked together by different bonds in the form of a spatial network (Somerville et al., 2004; Zykwinska et al., 2005; Zykwinska et al., 2007; Donaldson, 2007; Jarvis, 2009). However, such a composition is more typical of the PCW, whereas the SCW mainly consists of cellulose and hemicelluloses and appears to be more structurally organized than the PCW in this instance (Bidlack et al., 1992). The size of cellulose microfibrils can differ between diverse plants and cell wall types (PCW, SCW). The width of a microfibril was assessed at 8–15 nm (McCann et al., 1990; Fujino et al., 2000). Microfibrils can aggregate forming bundles (macrofibrils) of different size e.g. 12–24 nm or even from 50 to 250 nm (Ding and Himmel, 2006; Donaldson, 2007; Terashima et al., 2009; Ding et al., 2014).

The difference between the PCW and SCW also involves hemicellulose constitution. One of the most important hemicelluloses in the primary cell wall is xyloglucan (XG), whereas xylan (XY) and arabinoxylan are less substituted polymers, characteristic of the secondary cell walls in dicots (Zykwinska et al., 2007; Scheller and Ulvskov, 2010; Banasiak, 2014; Busse-Wicher et al., 2014; Cosgrove, 2014). Pectins are linear or branched (with attached side chains) polymers of galacturonic acid residues, they are characteristic of the primary cell wall in all land plants and play diverse

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roles in wall structure and function (Willats et al., 2001; Pelloux et al., 2007; Mohnen, 2008; Caffall and Mohnen, 2009; Banasiak, 2014). The most abundant pectic polysaccharide is homogalacturonan (HG) which represents a linear, unbranched homopolymer of galacturonic acid. Other important pectins of the cell wall are rhamnogalacturonan II and/or rhamnogalacturonan I (RG I) the backbone of which is composed of repeat disaccharides i.e. rhamnose and galacturonic acid (Mohnen, 2008).

Mucilage secreting cells (MSCs) are characteristic of many types of fruits and seeds able to produce a mucilage envelope after hydration. This phenomenon, known as myxodiaspory, is a common adaptation to drought habitats in many families of angiosperms (Grubert, 1974; Kreitschitz, 2009; Kreitschitz, 2012). Soon after hydration, the mucilage secreting cell walls burst, loosening their material in the form of an easily accessible, gel-like envelope (Arsovski et al., 2010; Haughn and Western, 2012; Kreitschitz, 2012). The mucilage envelope is characterized by a complex composition and structure and could be considered as a specialized pectin-rich secondary cell wall (Haughn and Western, 2012). Mucilage composed mainly of pectins and hemicelluloses is known as pectic ('true') mucilage typical of *Linum* sp. The second type has additional cellulose fibrils and was described as cellulose mucilage characteristic of many diverse families such as Asteraceae, Brassicaceae, Lamiaceae or Plantaginaceae (Western, 2012; Kreitschitz, 2009; Kreitschitz, 2012). As the seed coat of *Arabidopsis thaliana*, a model plant, is amenable to genetic manipulation and due to the special character of its mucilage, it has been adapted to diverse studies of the functional aspects of the cell wall (Haughn and Western, 2012; North et al., 2014; Voiniciuc et al., 2015a). Although our knowledge of seed coat mucilage composition, function and development is quite extensive, the detailed structural, spatial organization of the mucilage components still remains unclear (Macquet et al., 2007; Mendu et al., 2011; Sullivan et al., 2011; Haughn and Western, 2012).

Mucilage lacking cellulose fibrils is also regarded as primary-cell-wall-like, whereas the cellulose type is regarded as a specific, modified secondary cell wall (Mühlethaler, 1950; Haughn and Western, 2012). This separation is derived from a number of immuno- and biochemical analyses of mucilage components which showed the presence of hemicelluloses such as arabinoxylan and xyloglucan typical of the PCW (Naran et al., 2008; Young et al., 2008; Arsovski et al., 2010). In the case of cellulose mucilage regarded as a modified SCW, the presence of xylan, typical of this type of cell wall was also shown (Ralet et al., 2016; Voiniciuc et al., 2015b).

Our knowledge of the distribution of mucilage components' within the mucilage envelope is derived mainly from biochemical analysis (Naran et al., 2008; Young et al., 2008; Arsovski et al., 2010; Western, 2012; Voiniciuc et al., 2015a; Voiniciuc et al., 2015b). Another question is the spatial organization of mucilage polysaccharides – does it reflect cell wall arrangement?

Cell wall structure has been examined with diverse microscopy techniques including Atomic Force Microscopy (AFM), Transmission and Scanning Electron Microscopy (TEM, SEM), and Cryo-SEM. They have revealed the fibrillary nature of cell wall components and such analyses have largely been based on the use of invasive sample preparations such as enzymatic or chemical treatments, which can destroy and/or influence cell wall organization and its individual components (McCann et al., 1990; Satiat-Jeunemaitre et al., 1992; McCann and Roberts, 1991; Nakashima et al., 1997; Zykwinska et al., 2007; Sarkar et al., 2009; Terashima et al., 2009; Sarkar et al., 2014). Some general micromorphological studies of seed coat mucilage envelope(s) using SEM analysis were also presented e.g. for nutlets of *Salvia hispanica* L. or *Arabidopsis thaliana* seeds (Windsor et al., 2000; Capitani et al., 2013; Salgado-Cruz et al., 2013; Voiniciuc et al., 2013). However, these studies did not give a detailed insight into the spatial architecture of the mucilage.

Our previous studies on the mucilaginous seeds of selected Asteraceae taxa and *Neopallasia pectinata* allowed us to classify the mucilage according to the cellulose type. Pectins and cellulose were very distinctive components, organized in a smaller or abundant mucilage envelope (Kreitschitz and Vallès, 2007; Kreitschitz, 2012). Taking our results into account, we decided to examine seeds of *Neopallasia pectinata*, which produce abundant cellulose mucilage with very long, complexed cellulose fibrils (Kreitschitz and Vallès, 2007). The second selected plant species is flax *Linum usitatissimum* whose seeds produce pectic mucilage which is a mixture of branched rhamnogalacturonan I and arabinoxylan and lacks cellulose (Naran et al., 2008).

The main aim of this study was to analyze and visualize the spatial structural organization of the seed coat mucilage (seed coat mucilaginous cell wall) of two taxa differing in mucilage composition and morphology i.e. *Linum usitatissimum* with pectic mucilage and *Neopallasa pectinata* with cellulose mucilage. To explore the mucilage spatial organization, we applied less-invasive techniques i.e. critical point drying (CPD) followed by high resolution scanning electron microscopy (SEM). We also analyzed the presence of selected hemicelluloses i.e. xylan/arabinoxylan and xyloglucan whose occurrence was described for the mucilaginous cell wall.

2. Material and methods

Mature seeds of *Neopallasia pectinata* were obtained from Prof. Dr. Joan Vallès (Barcelona University, Catalonia, Spain). The seeds of *Linum usitatissimum* (flax) were obtained from a commercial supplier (SANTE, A. Kowalski Sp. j., Warsaw, Poland). The material for CPD and SEM analysis were not fixed in any special fixative solution and not chemically or enzymatically pretreated.

2.1. Morphology of the mucilage envelope – pectins and cellulose detection

In order to detect the main mucilage components staining with ruthenium red (0.1%, w/v) for pectins, with safranin (0.1%, w/v) for cellulose and pectins and with Direct Red 23 (0,1% w/v) for cellulose (excitation LP 555, emission 560 nm) were done. Images were taken using a DP71 camera connected to an Olympus BX-50 microscope and Cell B imaging software (Olympus BX50, Olympus Optical Co, Poland) and with Zeiss CLSM microscope (LSM 700 AXIO ZEISS; staining with Direct Red 23). Additionally, in order to observe the presence of crystalline cellulose a polarized light microscope was used. The highly ordered cellulose microfibrils present in the mucilage envelope can produce birefringence of polarized light (Sullivan et al., 2011; Yu et al., 2014). A Leica polarized light microscope connected to a Leica DFC 450C camera and Las X software (Leica DM 6000B, Leica Microsystems GmbH, Germany) was used.

2.2. Examination of seed coat mucilage arrangement in dry seeds

Handmade cross-sections of *L. usitatissimum* and *N. pectinata* dry seeds were attached to the SEM stubs using carbon-containing double-sided adhesive conductive tape and coated with gold palladium (film thickness 15 nm) using a Leica EM SCD 500 High Vacuum Sputter Coater (Leica Microsystems GmbH, Wetzlar, Germany). The preparations were visualized in a SEM (Hitachi S-4800, Hitachi High-Tech. Corp., Tokyo, Japan).