Xyloglucan and cellulose form molecular cross-bridges connecting root border cells in pea (Pisum sativum)

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Short paper

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Abstract

Pea (*Pisum sativum*) root cap releases a large number of living border cells that secrete abundant mucilage into the extracellular medium. Mucilage contains a complex mixture of polysaccharides, proteins and secondary metabolites important for its structure and function in defense. Unlike xyloglucan and cellulose, pectin and arabinogalactan proteins have been investigated in pea root and shown to be major components of border cell walls and mucilage. In this study, we investigated the occurrence of xyloglucan and cellulose in pea border cells and mucilage using cytochemical staining, immunocytochemistry and laser scanning confocal microscopy. Our data show that i) unlike cellulose, xyloglucan is highly present in the released mucilage as a dense fibrillary network enclosing border cells and ii) that xyloglucan and cellulose form molecular cross-bridges that tether cells and maintain them attached together. These findings suggest that secreted xyloglucan is essential for mucilage strengthening and border cell attachment and functioning.

Keywords: border cells; cellulose; mucilage; polysaccharides; root; xyloglucan.

1. Introduction

The root cap plays a central role in root protection and development. It consists of several layers of cells including the gravity-sensing columella cells and the lateral root cap cells (Kumpf and Nowack, 2015). It is well established that cells in the last layer of the root cap are released into the external environment as border cells or border-like cells (Hawes *et al.,* 2003; Vicré *et al.,* 2005). While border-like cells are shed as organized layers in which cells remain attached to each other even after their separation from the root, border cells are often observed as single cells non-attached to each other (Driouich *et al.,* 2007; Hawes *et al.,* 2016). Most of these cells remain intact and viable for a long period of time after their separation from the root (Hawes *et al.,* 2003; Vicré *et al.,* 2005; Koroney *et al.,* 2016; Hawes *et al.,* 2016). Their secretory activity also continues after their release (Cannesan *et al.,* 2011; 2012; Wang *et al.,* 2017).

Border cells and border-like cells both secrete mucilage that influences the microenvironment of the root system and its interaction with soil-borne microorganisms (Driouich *et al.,* 2013; Hawes *et al.,* 2016). Mucilage is also thought to lubricate the root tip to facilitate its growth as it moves through the soil. Mucilage production varies in density and composition depending on the species, the developmental stage of the plant and the surrounding environment; and it embeds the cells themselves (Walker *et al.,* 2003; Driouich *et al.,* 2012). Remarkably, the mucilage consists of diverse classes of molecules including cell wall polysaccharides and proteoglycans (Knee *et al.,* 2001; Wen *et al.,* 2007a; Ma et *al.,* 2010; Mravec *et al.,* 2017), secondary metabolites (Bais *et al.,* 2006; Cannesan *et al.,* 2011; Barilli *et al.,* 2015), anti-microbial proteins and peptides (Wen *et al.,* 2007b; Weiller *et al.,* 2017) and extracellular DNA (Wen *et al.,* 2009; Tran *et al.,* 2016; Wen *et al.,* 2017). Association of mucilage with these cells form a protective complex also known as RET (stands for Root Extracellular Trap) believed to play a major role in root immunity (Driouich *et al.,* 2013).

The release of border cells and border-like cells from the root depends on the action of cell wall-degrading enzymes, including pectinases and cellulases. In pea for instance, separation of border cells from the root cap and from each other was shown to rely on the activity of two pectin-modifying enzymes, polygalacturonase and pectin-methylesterase (Wen *et al.,* 1999). In Arabidopsis, mutants deficient in the pectic polysaccharide homogalacturonan were found to release individual cells rather than layers of border-like cells supporting the role of pectin hydrolysis in cell detachment (Durand *et al.,* 2009). Furthermore, release of Arabidopsis border-like cells was also shown to require the transcription factor NLP7 (NIN-

LIKE PROTEIN 7) that controls the expression of genes encoding enzymes responsible for pectin and cellulose hydrolysis, including cellulase 5 (CEL5) (del Campillo *et al.* 2004; Karve *et al.,* 2016). Thus, although the pattern of cell detachment is different, alteration of cell wall components is necessary for the release of border cells and border-like cells.

Pea is currently the most studied species for border cell release and function in root protection (Wen *et al.,* 2007b; Wen *et al.,* 2009; Cannesan *et al.,* 2012; Mravec *et al.,* 2017). A growing root tip of pea releases a large number of border cells (~3500-4500 cells per day per root) (Hawes *et al.,* 2003), far more than the number of border-like cells released by Arabidopsis root tip (~130 cells per day per root) (Driouich *et al.,* 2012). Once a set of cells detaches from the root tip and disperses into the surrounding environment, the root cap generates another set of cells; thus maintaining a continuous production of border cells and release within the rhizosphere. A number of studies have clearly shown that pea border cells function in the protection of root tip from infection (Hawes *et al.,* 2000; Wen *et al.,* 2009; Cannesan *et al.,* 2011; Driouich *et al.,* 2013; Tran *et al.,* 2016). For instance, pea border cells and their secretions were shown to inhibit germination of zoospores of the oomycete *Aphanomyces euteiches*, growth of the fungus *Nectria hemtococca* and proliferation of the bacteria *Ralstonia solanacerum*, thus limiting invasion of root tips by these pathogens (Gunawardena *et al.,* 2005; Wen *et al.,* 2007b; Cannesan *et al.,* 2011; 2012; Tran *et al.,* 2016). Generally, border cells were found to exhibit different responses to microorganism infection; by either attracting, trapping, immobilizing or repelling bacteria, nematodes, oomycetes and fungi (Gunawardena *et al.,* 2005; Wen *et al.,* 2009; Cannesan *et al.,* 2012; Driouich *et al.,* 2013; Koroney *et al.,* 2016; Tran *et al.,* 2016).

As indicated above, pea border cells are defined as populations of single cells fully separated from each other. Here, we show these cells can remain attached to each other through short molecular "cross-bridges" made up of xyloglucan and cellulose as revealed by cytochemical staining, immuno-cytochemistry and confocal microscopy. In addition, we show that the released mucilage enclosing border cells is enriched in xyloglucan and that this polysaccharide forms a web-like network likely to maintain mucilage integrity and function.

2. Methods

2.1. Biological material

Pea seeds (*Pisum sativum* cv Le Normand - Mangetout) were prepared and seedlings were grown as described by Cannesan *et al.,* (2012).

2.2. Light microscopy, mucilage and cellulose staining

Mucilage surrounding border cells was visualized using India ink (Salis International Inc., Dr. Ph. Martin's black india ink hicarb) as described by Miyasaka and Hawes, (2001). Roots were gently removed and mounted on glass microscope slides in a droplet of sterile water. Then, India ink (0.05%) was added between the slide and the coverslip. Roots were then observed using a Leica DMI6000B bright-field microscope. Staining of β-glucans with calcofluor white M2R (Sigma-Aldrich) was performed as described previously (Durand *et al.* 2009). Roots were observed using an Epifluorescence microscope equipped with UV fluorescence (Leica DM6000 B; Excitation filter: 359 nm; barrier filter: 461 nm). Staining of cellulose with Direct Red 23 probe (Sigma-Aldrich) was performed as described by Ezquer *et al.,* (2016). Fresh roots were incubated with the probe (0.1 mg ml^{-1}) for 30 min in dark conditions. After three washes with distilled water, roots were observed using a confocal microscope (Leica TCS SP5; Excitation: 560 nm; Emission: 570-655 nm).

2.3. Immunofluorescence labeling of xyloglucan

The anti-xyloglucan mAb used in this study was LM15 (PlantProbes). The secondary antibody used was Tetramethylrhodamine isothiocyanate (TRITC)-conjugated to goat anti-rat (Sigma-Aldrich). Immunolabeling of root with LM15 was performed as described by Durand et *al.,* (2009) and root observed using a confocal microscope (Leica TCS SP5; Excitation: 550; Emission: 560-600).

3. Results

3.1. Mucilage staining and localization of cellulose

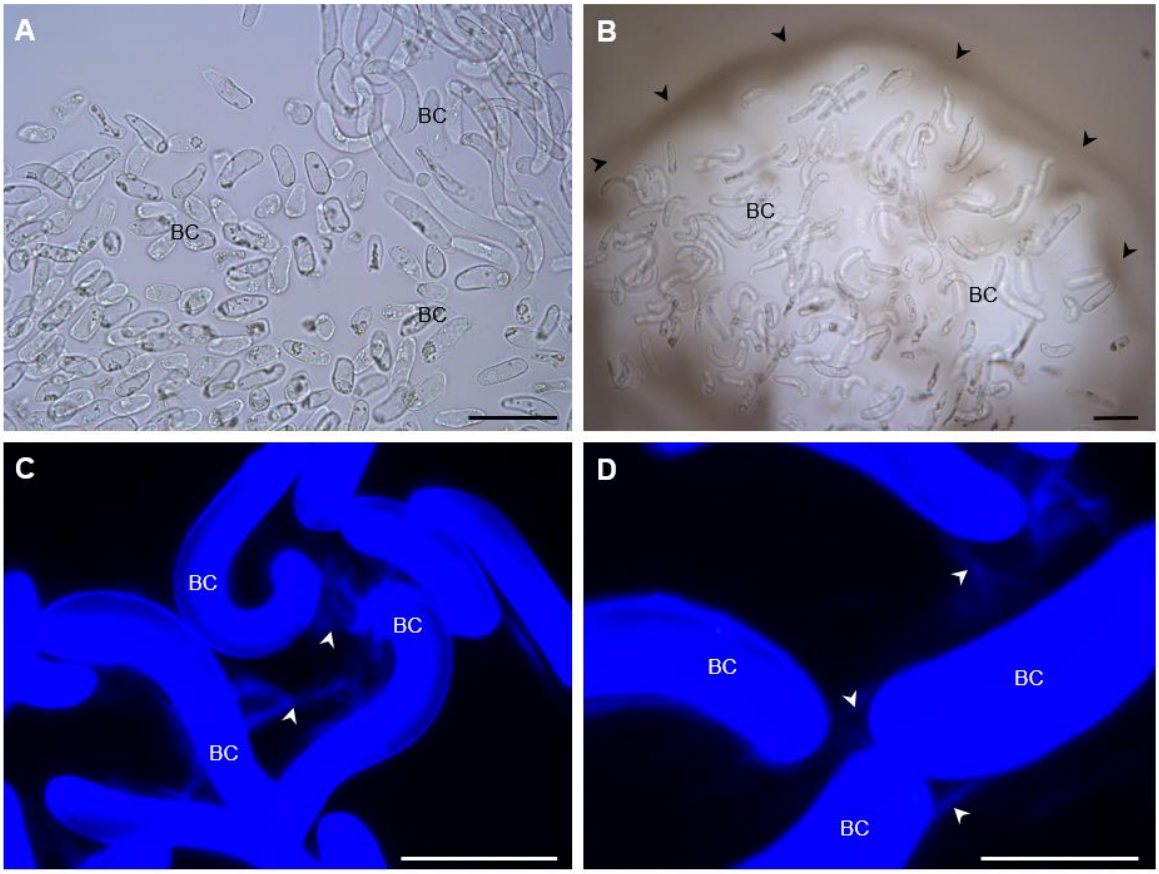

Figure 1: Microscopical observation and cytochemical staining of β-Glucans in pea root border cells and mucilage.
Light microscopical image showing border cells released from root tip as (A) individual cells very close to each other. (B) Mucilage is stained with India ink. Note the presence of a thick mucilage (outlined by black arrowheads in B) enclosing cells. (C and D) border cells are stained with Calcofluor white M2R and visualized by epifluorescence microscopy. Note the presence of fibrillary-like structures still attached to border cells in the surrounding area (white arrowheads). Some of these seem to link two cells together under the form of molecular "short bridges". BC: Border cell. Scale bars: 100 µm (A-B), 50 µm (C) or 25 µm (D).

Cellulose is a major component of plant cell walls that is also commonly observed in seed mucilage (Cosgrove, 2005; Voiniciuc *et al.*, 2015; Ezquer *et al.*, 2016). However, it has not been described in root mucilage so far. Herein, using two cellulose-recognizing probes and fluorescence microscopy, we checked for the occurrence of cellulose in pea root mucilage. Mucilage was also stained using India ink.

Figure 1a shows border cells that are released by a pea root tip after immersion in a drop of water. Border cells are seen as individual cells of different sizes and shapes (*i.e.*, spherical,

intermediate or elongated) and sometimes very close, nearly adhering to each other. Further microscopical examination of root tip after negative staining with India ink, revealed the presence of a thick mucilage embedding border cells (Fig. 1B). In addition, cells were strongly stained with the histochemical dye Calcofluor white M2R (blue fluorescence) that is specific for β-glucans (Durand *et al.*, 2009). In contrast, mucilage surrounding the cells was not -or very weakly- stained with Calcofluor, suggesting that it is devoid of β-glucans. However, stained fibrillary structures were observed in the mucilage area surrounding the cells forming aggregates very close to the cells (Fig. 1C). Careful examination of the staining showed that some of these structures seemed to connect adjacent cells, sometimes forming "cross-bridges" between two border cells as illustrated in Figure 1D. Calcofluor is a specific dye that stains β-glucans and chitin, and, in higher plants, it primarily binds to cell wall cellulose. Therefore, the observed Calcofluor-stained molecular "bridges" are most likely to be made up of cellulose.

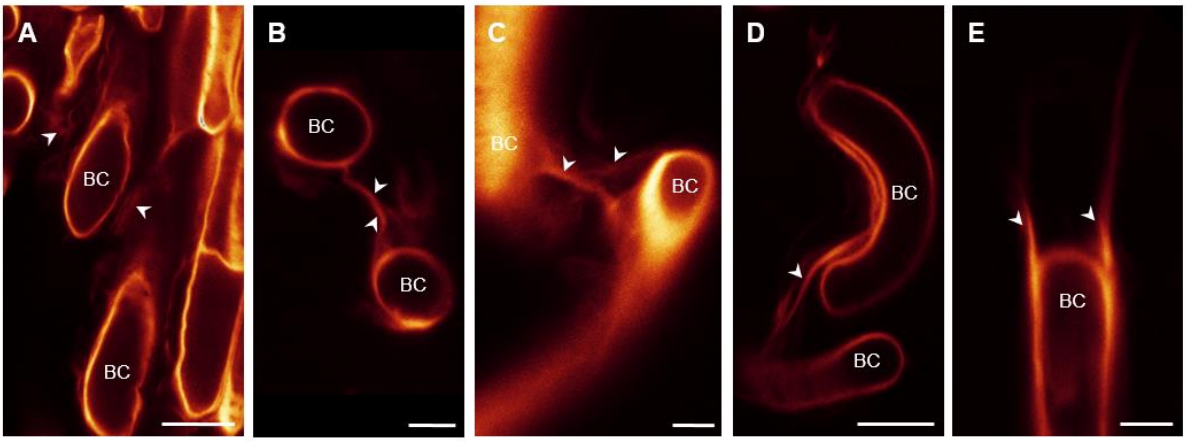

Figure 2: Cytochemical staining of crystalline Cellulose in root border cells and mucilage.
Mucilage and border cells are stained with Direct Red 23 and visualized using laser scanning confocal microscopy. Note the strong fluorescent labeling of the cell wall and the presence of stained fibrillary-like structures still attached to cells (white arrowhead in A). Note also that some of these structures seem to form molecular "short bridges" connecting cells together (white arrowheads in A, C and D). Remnants of these bridges are observed at the tip of a boder cells (white arrowheads in E). BC: Border cell. Scale bars: 25 µm (A, B and D), 5 µm (C) or 7.5 µm (E).

To confirm these observations, we probed border cells with Direct Red 23, a fluorescent dye that is highly specific for crystalline cellulose microfibrils (Ezquer *et al.*, 2016). As shown in Figure 2, the dye stained strongly the cell wall of border cells, but rarely the secreted mucilage. Close examination of the staining pattern confirmed the presence of fibrillary structures that can be seen peeling-off from the surface of border cells (Fig. 2A). Furthermore, the "bridges" stained with Calcofluor (Fig. 1D) were also confirmed with Direct Red 23 staining (see Fig. 2B, C, D). These "bridges" appear to link two cells together as clearly seen in Figure

2A-D. Finally, remnants of these structures were also observed sometimes at the tip of isolated border cells (Fig. 2E). Together these observations indicate that the “bridge”-like structures contain the cell wall polysaccharide, cellulose.

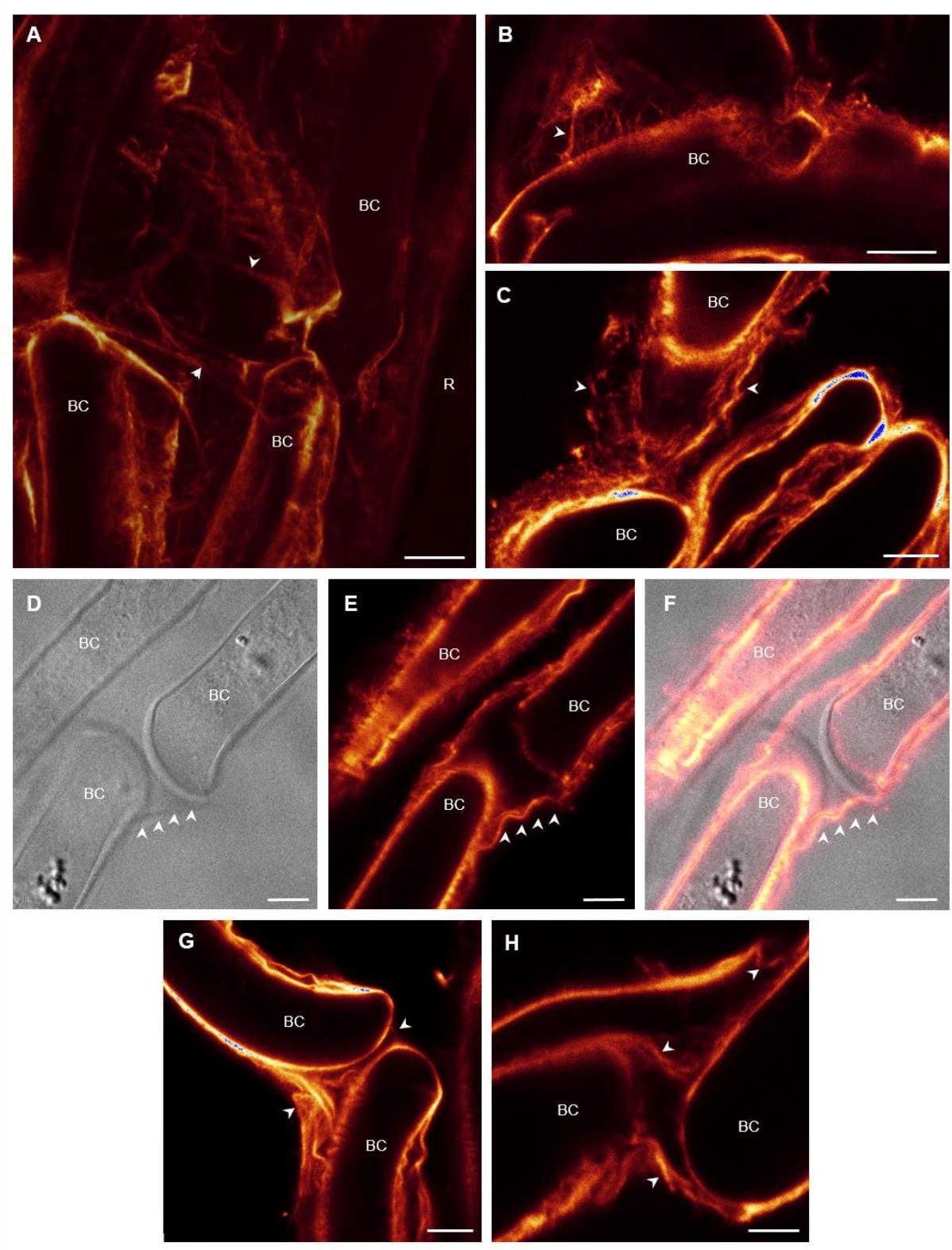

Figure 3: Immunolocalization of xyloglucan in root border cells and mucilage with the mAb LM15. Immunofluorescence labeling is visualized by laser scanning confocal microscopy. (A-C) Cell wall of border cells and the surrounding mucilage are strongly immunostained. Arrowheads in A and B indicate the labeled xyloglucan fibrillary network surrounding border cells. Note that short bridges linking cells together seen in D (arrowheads) are strongly immuno-stained with the anti-xyloglucan antibody (arrowheads in E, and also in G and H). d, e and f are bright-field, fluorescent and merged images, respectively. BC: Border cell; R: Root tip. Scale bars: 7.5 µm (A), 5 µm (B) or 10 µm (C, D and E).

3.2. Localization of xyloglucan in border cells and mucilage

Xyloglucan is the major hemicellulosic polysaccharide in cell walls of pea root cells (Hayashi, 1989) and it is known to bind cellulose microfibrils (Cosgrove, 2005). We investigated the occurrence of xyloglucan in border cells and mucilage using the monoclonal antibody LM15 specific for the XXXG motif of this polysaccharide. As shown in Figure 3, the antibody bound strongly to cell wall and mucilage. Fibrillary-like structures were also observed peeling off the surface of the cells and present in the mucilage network embedding border cells (Fig. 3A-C). Again, close examination of the pattern of xyloglucan labeling revealed the presence of "bridges" linking cells together, as clearly illustrated in figure 3D-H. Thus, these data indicate that, in addition to cellulose, the molecular "bridges" seen between cells also contain the hemicellulosic polysaccharide xyloglucan. These structures are most likely to serve as tethers to maintain cells attached together within the mucilage network.

4. Discussion

In this study, we have been able to show that xyloglucan is released into mucilage secretions and forms a fibrous network that holds and links cells together. The links can sometimes occur through short molecular cross-bridges that also contain cellulose. Such molecular tethers connecting root border cells together have never been revealed earlier. Our findings clearly establish that cell-to-cell contact is maintained by border cells of pea after their separation from the root cap. Therefore, these cells do not seem to be fully separated from each other (*i.e.*, single cells) as has been previously described in several studies (Hawes *et al.*, 2003; Driouich *et al.*, 2007; Cannesan *et al.*, 2012; Mravec *et al.*, 2017). We suggest that xyloglucan may serve as a scaffold maintaining structural integrity of mucilage and cell attachment required for proper functioning of root border cells and secreted components.

4.1. Xyloglucan and cellulose form short molecular bridges tethering border cells

Xyloglucan is a major polysaccharide of primary cells walls in eudicotyledonous plants (Hayashi, 1989; Cosgrove, 2005). It consists of a β-D-(1→4)-glucan backbone and side chain structures containing different sugar residues including xylose, galactose and fucose. In the cell wall, xyloglucan binds to cellulose microfibrils to form a major load-bearing network that contributes to the control of cell expansion (Cosgrove, 2005). Xyloglucan has also been

implicated in cell adhesion in tomato fruit pericarp (Ordaz-Ortiz *et al.,* 2009). Xyloglucan and cellulose are thus closely linked within the primary cell wall. Both polysaccharides are revealed as molecular constituents of the cross-links observed between border cells in the present study (Fig. 1-3) and most likely interact closely within these structures. In addition, crystalline cellulose, which is detected in border cell cross-bridges by the probe Direct Red 23 (Fig. 2), has been shown to have a higher affinity to xyloglucan than amorphous cellulose (Thomas *et al.,* 2013; Cosgrove, 2014). This suggests a strong association between the two polysaccharides forming a rigid structure that stabilizes the "bridges" and maintains border cell attachment after their separation from the root tip. Thus, root border cells organization in pea seems to rely on the complex xyloglucan/cellulose, rather than on pectin as has been shown for border-like cells in Arabidopsis (Durand *et al.,* 2009; Karve *et al.,* 2016). Unlike border cells, attachment of border-like cells is maintained after their release and depends mainly on the pectic polysaccharide, homogalacturonan (Durand *et al.,* 2009). Indeed, defect in this polysaccharide in Arabidopsis mutants or in the activity of the pectin-modifying enzymes, polygalacturonases and pectin-methylesterases, led to separation of cells and their release as isolated cells much like border cells of pea (Wen *et al.,* 1999; Durand *et al.,* 2009). In our observed cells, adhesion in the middle lamellae region is lost but attachment of cells does still occur through specific cross-links containing xyloglucan and cellulose (Fig. 1D, Fig 2B-E and Fig. 3D-H). It is worth noting that pectin epitopes have not been detected in these cross-links (data not shown). During the separation process of pea root border cells, pectin-hydrolyzing enzymes might be highly active, resulting in a complete dissolution of pectin in the middle lamellae (or at least an extended dissolution of pectin) necessary for cell release. This is in contrast to xyloglucan and cellulose, whose hydrolysis and/or remodeling might have not been as extensive as for pectin (or it does occur at a limited extent) leaving these polymers intact enough to form the observed cross-links between cells. Cellulases have been reported to influence detachment of border cells in Arabidopsis (del Campillo *et al.,* 2004) but there has been no previous report on the role of such enzymes or those involved in xyloglucan hydrolysis during pea border cells release (Wen *et al.,* 2007b). The exact mechanisms by which the xyloglucan/cellulose network is remodeled during root border cells formation and release will await further investigations. Interestingly, the separation process of root border cells and border-like cells leads to two different cell phenotypes with regards to cell attachment and organization. Attachment of border-like cells seems to depend on pectic polysaccharides, whereas that of border cells is mediated by the xyloglucan/cellulose network. Both organizations are essential for cell function and survival. Clearly, generation of these phenotypes requires the activity of endogenous cell wall-modifying

enzymes and remodeling of cell wall structure, two related processes that must be tightly controlled during root development and hence deserve further research attention.

4.2. Xyloglucan in mucilage secretions, a structural scaffold

In addition to xyloglucan being found in the short tethers that link border cells (see above), this polysaccharide was also released into the mucilage secretions that embed border cells. Secreted xyloglucan appeared as a dense fibrous network surrounding the cells and linking them to each other. Other studies have described the occurrence of xyloglucan in plant secretions including the extracellular medium of sycamore suspension-cultured cells (Aspinall *et al.,* 1969) and seed coat mucilage of Arabidopsis although in low amounts (Haughn and Western, 2012). Seed xyloglucan was suggested to promote cohesion and structuration of the secreted mucilaginous network (Voiniciuc *et al.,* 2015; Ezquer *et al.,* 2016). Recently, xyloglucan has also been described in root exudates of many plants including wheat, barley, maize, tomato and Arabidopsis (Galloway *et al.,* 2017) and therefore it appears as a common molecular feature of root secretions much like pectin. Furthermore, Galloway *et al.* (2017) have also shown that tamarind seed xyloglucan was able to promote aggregation of soil particles. Although the structure of tamarind xyloglucan might be different from that of xyloglucan released by root cells, this polysaccharide seems to play a significant role in soil cohesion as a particle-stabilizing agent. Xyloglucan found in root exudates may also interact with other components (*e.g.,* polysaccharides) released by soil microbes or by root itself to maintain soil structure, stability and functionality. Xyloglucan was also found in pea root border cell walls and shown to have a polarized distribution *in muro* but its presence in mucilage has not been investigated (Mravec *et al.,* 2017). Based on this observation, the authors also proposed that xyloglucan promotes cell curvature that contributes, in addition to pectin hydrolysis, to cell detachment and release. Thus, xyloglucan may play distinct and significant roles within plant cell walls or outside the cell.

In the present study, we suggest that xyloglucan released by pea root border cells is required for mucilage structural integrity and cohesion. The physical integrity of mucilage is central to its function and we, therefore, propose that xyloglucan serves as a scaffolding structure that i) provides structural support and strength to the whole mucilage network, ii) allows cells to be maintained together within this network for correct functioning and iii) stabilizes functional components contained in the mucilage. Xyloglucan network may also act in conjunction with other mucilage components as a physical barrier against pathogen

penetration. In pea, root mucilage is known to contain a variety of molecules including pectin, arabinogalactan proteins, extracellular DNA, histones, pisatin and other antimicrobial components (Wen *et al.,* 2007b; 2009; Cannesan *et al.,* 2011; 2012). These molecules are secreted by border cells into the mucilage and were shown to play a major role in root defense as part of root extracellular trap (Driouich *et al.,* 2013). Xyloglucan is integrated with all these molecules making up the mucilage network and might possibly interact with some of them to stabilize the whole structure and enables cells to support physical stress during growth through the soil. The structural stabilization of this network (through xyloglucan and potential association with other molecules) would ensure correct organization of cells and secreted molecules including antimicrobial components allowing them to function and act on pathogens. Disruption of xyloglucan may result in mucilage disorganization leading to altered function of root extracellular trap and its physical barrier properties against pathogen penetration. Interestingly, xyloglucan from tamarind seeds was recently shown to function as a protective barrier limiting bacterial adherence and invasion of intestinal mucosal cells (Piqué *et al.,* 2018). Future work will clearly need to define how different molecules are assembled and organized within the mucilage network and how they contribute to proper functioning of root extracellular trap in plants.

Author's contribution

MR carried out the experiments. MR, SB, MLFG, MV, IB and AD analyzed the data. MR, SB, IB and AD wrote the manuscript, with input from MLFG and MV. AD and MR conceived the present idea and designed the study. All authors provided critical feedback.

Acknowledgments

We are grateful to La Région Normandie and FEDER for Financial support (to AD). This work was part of the LIPS-GRRVASI 2015/18 project. We are also grateful to Drs. E. Nguema-Ona and J.C Yvin (Centre Mondial de l'Innovation-Roullier-35400St Malo, France) for their support to our research on "Root Biology and Protection".

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