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1 **Short paper**

2

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

5

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19

20 **Abstract**

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

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

34

35 **1. Introduction**

36

37 The root cap plays a central role in root protection and development. It consists of
38 several layers of cells including the gravity-sensing columella cells and the lateral root cap cells
39 (Kumpf and Nowack, 2015). It is well established that cells in the last layer of the root cap are
40 released into the external environment as border cells or border-like cells (Hawes *et al.*, 2003;
41 Vicré *et al.*, 2005). While border-like cells are shed as organized layers in which cells remain
42 attached to each other even after their separation from the root, border cells are often observed
43 as single cells non-attached to each other (Driouich *et al.*, 2007; Hawes *et al.*, 2016). Most of
44 these cells remain intact and viable for a long period of time after their separation from the root
45 (Hawes *et al.*, 2003; Vicré *et al.*, 2005; Koroney *et al.*, 2016; Hawes *et al.*, 2016). Their
46 secretory activity also continues after their release (Cannesan *et al.*, 2011; 2012; Wang *et al.*,
47 2017).

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

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

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

73 Pea is currently the most studied species for border cell release and function in root
74 protection (Wen *et al.*, 2007b; Wen *et al.*, 2009; Cannesan *et al.*, 2012; Mravec *et al.*, 2017). A
75 growing root tip of pea releases a large number of border cells (~3500-4500 cells per day per
76 root) (Hawes *et al.*, 2003), far more than the number of border-like cells released by *Arabidopsis*
77 root tip (~130 cells per day per root) (Driouich *et al.*, 2012). Once a set of cells detaches from
78 the root tip and disperses into the surrounding environment, the root cap generates another set
79 of cells; thus maintaining a continuous production of border cells and release within the
80 rhizosphere. A number of studies have clearly shown that pea border cells function in the
81 protection of root tip from infection (Hawes *et al.*, 2000; Wen *et al.*, 2009; Cannesan *et al.*,
82 2011; Driouich *et al.*, 2013; Tran *et al.*, 2016). For instance, pea border cells and their secretions
83 were shown to inhibit germination of zoospores of the oomycete *Aphanomyces euteiches*,
84 growth of the fungus *Nectria hemtococca* and proliferation of the bacteria *Ralstonia*
85 *solanacerum*, thus limiting invasion of root tips by these pathogens (Gunawardena *et al.*, 2005;
86 Wen *et al.*, 2007b; Cannesan *et al.*, 2011; 2012; Tran *et al.*, 2016). Generally, border cells were
87 found to exhibit different responses to microorganism infection; by either attracting, trapping,
88 immobilizing or repelling bacteria, nematodes, oomycetes and fungi (Gunawardena *et al.*, 2005;
89 Wen *et al.*, 2009; Cannesan *et al.*, 2012; Driouich *et al.*, 2013; Koroney *et al.*, 2016; Tran *et al.*,
90 2016).

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

97

98 **2. Methods**

99

100 *2.1. Biological material*

101

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

104

105 *2.2. Light microscopy, mucilage and cellulose staining*

106

107 Mucilage surrounding border cells was visualized using India ink (Salis International
108 Inc., Dr. Ph. Martin's black india ink hicarb) as described by Miyasaka and Hawes, (2001).
109 Roots were gently removed and mounted on glass microscope slides in a droplet of sterile water.
110 Then, India ink (0.05%) was added between the slide and the coverslip. Roots were then
111 observed using a Leica DMI6000B bright-field microscope. Staining of β -glucans with
112 calcofluor white M2R (Sigma-Aldrich) was performed as described previously (Durand *et al.*
113 2009). Roots were observed using an Epifluorescence microscope equipped with UV
114 fluorescence (Leica DM6000 B; Excitation filter: 359 nm; barrier filter: 461 nm). Staining of
115 cellulose with Direct Red 23 probe (Sigma-Aldrich) was performed as described by Ezquer *et*
116 *al.*, (2016). Fresh roots were incubated with the probe (0.1 mg ml^{-1}) for 30 min in dark
117 conditions. After three washes with distilled water, roots were observed using a confocal
118 microscope (Leica TCS SP5; Excitation: 560 nm; Emission: 570-655 nm).

119

120 *2.3. Immunofluorescence labeling of xyloglucan*

121

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

127

128 **3. Results**

129

130 *3.1. Mucilage staining and localization of cellulose*

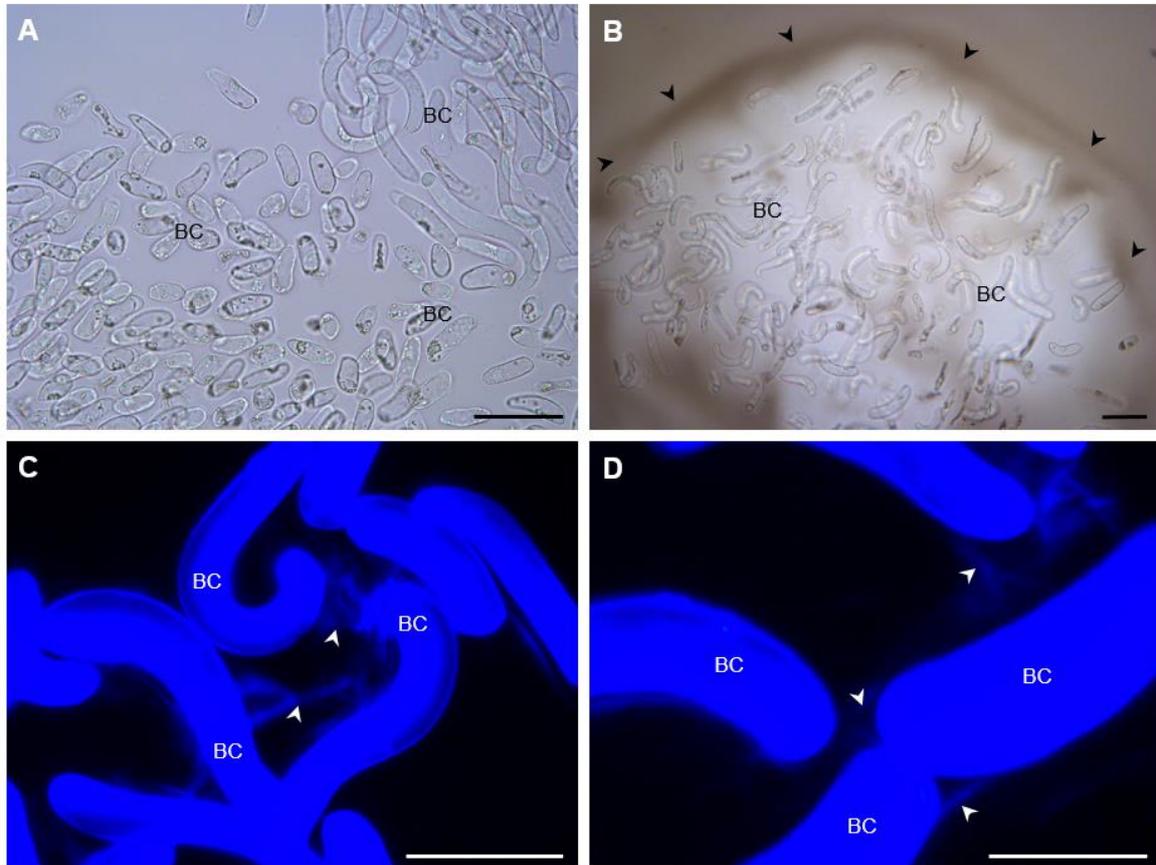


Figure 1: Microscopical observation and cytochemical staining of β -Glucans in pea root border cells and mucilage.

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 μ m (A-B), 50 μ m (C) or 25 μ m (D).

131

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

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

139 intermediate or elongated) and sometimes very close, nearly adhering to each other. Further
140 microscopical examination of root tip after negative staining with India ink, revealed the
141 presence of a thick mucilage embedding border cells (Fig. 1B). In addition, cells were strongly
142 stained with the histochemical dye Calcofluor white M2R (blue fluorescence) that is specific
143 for β -glucans (Durand *et al.*, 2009). In contrast, mucilage surrounding the cells was not -or very
144 weakly- stained with Calcofluor, suggesting that it is devoid of β -glucans. However, stained
145 fibrillary structures were observed in the mucilage area surrounding the cells forming
146 aggregates very close to the cells (Fig. 1C). Careful examination of the staining showed that
147 some of these structures seemed to connect adjacent cells, sometimes forming “cross-bridges”
148 between two border cells as illustrated in Figure 1D. Calcofluor is a specific dye that stains β -
149 glucans and chitin, and, in higher plants, it primarily binds to cell wall cellulose. Therefore, the
150 observed Calcofluor-stained molecular “bridges” are most likely to be made up of cellulose.
151

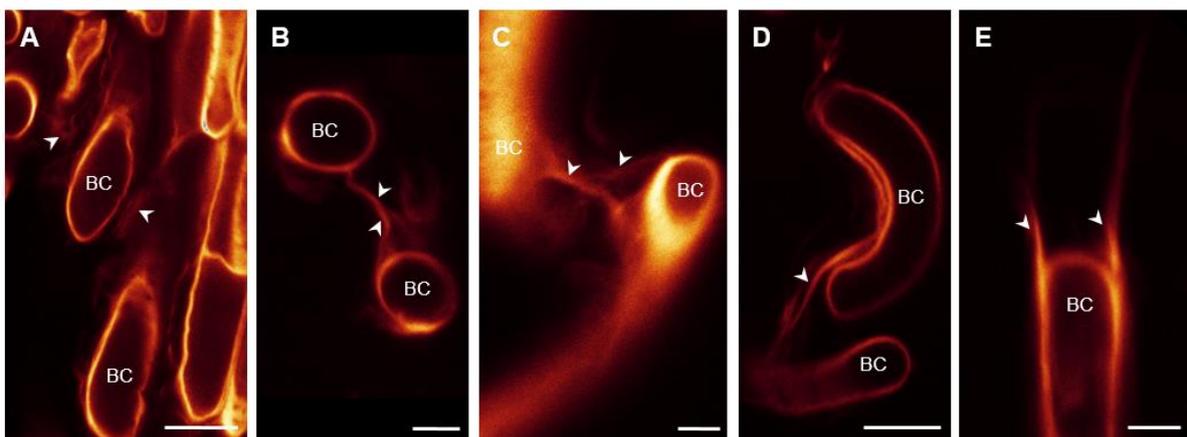


Figure 2: Cytochemical staining of crystalline Cellulose in root border cells and mucilage.

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 border cells (white arrowheads in E). BC: Border cell. Scale bars: 25 μ m (A, B and D), 5 μ m (C) or 7.5 μ m (E).

152

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

160 2A-D. Finally, remnants of these structures were also observed sometimes at the tip of isolated
161 border cells (Fig. 2E). Together these observations indicate that the “bridge”-like structures
162 contain the cell wall polysaccharide, cellulose.

163

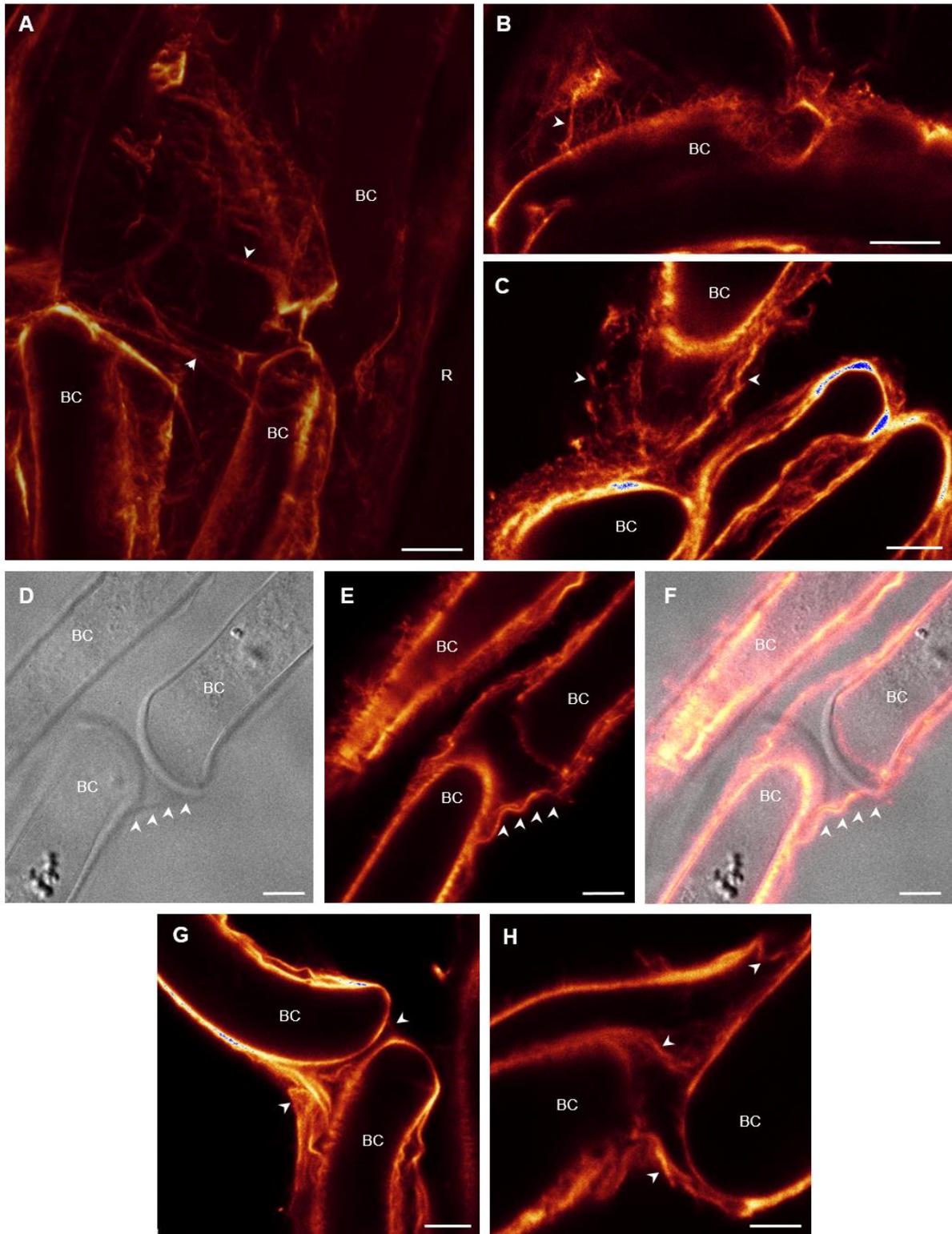


Figure 3: Immunolocalization of xyloglucan in root border cells and mucilage with the mAb LM15. Immunofluorescence labeling is visualized by laser scanning confocal microscopy. (A-C) Cell wall of border cells and the surrounding mucilage are strongly immunostained. Arrowheads in A and B indicate the 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).

165 *3.2. Localization of xyloglucan in border cells and mucilage*

166

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

178

179 **4. Discussion**

180

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

191

192 *4.1. Xyloglucan and cellulose form short molecular bridges tethering border cells*

193

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

199 implicated in cell adhesion in tomato fruit pericarp (Ordaz-Ortiz *et al.*, 2009). Xyloglucan and
200 cellulose are thus closely linked within the primary cell wall. Both polysaccharides are revealed
201 as molecular constituents of the cross-links observed between border cells in the present study
202 (Fig. 1-3) and most likely interact closely within these structures. In addition, crystalline
203 cellulose, which is detected in border cell cross-bridges by the probe Direct Red 23 (Fig. 2), has
204 been shown to have a higher affinity to xyloglucan than amorphous cellulose (Thomas *et al.*,
205 2013; Cosgrove, 2014). This suggests a strong association between the two polysaccharides
206 forming a rigid structure that stabilizes the “bridges” and maintains border cell attachment after
207 their separation from the root tip. Thus, root border cells organization in pea seems to rely on
208 the complex xyloglucan/cellulose, rather than on pectin as has been shown for border-like cells
209 in *Arabidopsis* (Durand *et al.*, 2009; Karve *et al.*, 2016). Unlike border cells, attachment of
210 border-like cells is maintained after their release and depends mainly on the pectic
211 polysaccharide, homogalacturonan (Durand *et al.*, 2009). Indeed, defect in this polysaccharide
212 in *Arabidopsis* mutants or in the activity of the pectin-modifying enzymes, polygalacturonases
213 and pectin-methylesterases, led to separation of cells and their release as isolated cells much
214 like border cells of pea (Wen *et al.*, 1999; Durand *et al.*, 2009). In our observed cells, adhesion
215 in the middle lamellae region is lost but attachment of cells does still occur through specific
216 cross-links containing xyloglucan and cellulose (Fig. 1D, Fig 2B-E and Fig. 3D-H). It is worth
217 noting that pectin epitopes have not been detected in these cross-links (data not shown). During
218 the separation process of pea root border cells, pectin-hydrolyzing enzymes might be highly
219 active, resulting in a complete dissolution of pectin in the middle lamellae (or at least an
220 extended dissolution of pectin) necessary for cell release. This is in contrast to xyloglucan and
221 cellulose, whose hydrolysis and/or remodeling might have not been as extensive as for pectin
222 (or it does occur at a limited extent) leaving these polymers intact enough to form the observed
223 cross-links between cells. Cellulases have been reported to influence detachment of border cells
224 in *Arabidopsis* (del Campillo *et al.*, 2004) but there has been no previous report on the role of
225 such enzymes or those involved in xyloglucan hydrolysis during pea border cells release (Wen
226 *et al.*, 2007b). The exact mechanisms by which the xyloglucan/cellulose network is remodeled
227 during root border cells formation and release will await further investigations. Interestingly,
228 the separation process of root border cells and border-like cells leads to two different cell
229 phenotypes with regards to cell attachment and organization. Attachment of border-like cells
230 seems to depend on pectic polysaccharides, whereas that of border cells is mediated by the
231 xyloglucan/cellulose network. Both organizations are essential for cell function and survival.
232 Clearly, generation of these phenotypes requires the activity of endogenous cell wall-modifying

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

235

236 4.2. Xyloglucan in mucilage secretions, a structural scaffold

237

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

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

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

284

285 **Author's contribution**

286

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

290

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292

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297

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