1   Immunolocalization of some arabinogalactan protein epitopes during indirect somatic

2   embryogenesis and shoot organogenesis in leaf culture of centaury (*Centaurium erythraea*

3   Rafn)

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22 **Abstract**

23 Common centaury (*Centaurium erythraea* Rafn) is a medicinal plant of great importance for

24 both pharmaceutical and food industries. Distribution of arabinogalactan proteins (AGPs)

25 with specific epitopes recognized by seven monoclonal antibodies (mAbs) was investigated

26 during indirect somatic embryogenesis (ISE) and shoot organogenesis (ISO) in centaury leaf

27 culture. Dynamic changes were observed in localization of JIM4, JIM8, JIM13, JIM15, LM2,

28 LM14 and MAC207 epitopes during somatic embryo (SE) and adventitious bud (AB)

29 development. AGPs responded to all tested mAbs and expressed in numerous meristematic

30 centers that were formed on leaf explants. In globular SEs, the distribution pattern of JIM4,

31 JIM13, JIM15, LM2 and MAC207 epitopes was observed, while during the progression of

32 somatic embryo development, the number of detected AGPs decreased. During formation of

33 ABs, the number of detected AGPs also decreased. In fully formed ABs only JIM4 and

34 MAC207 were detected. The present study suggests JIM13 antibody as a marker for ISE in

35 centaury leaf culture. These results implicated that AGPs were developmentally regulated

36 during centaury ISE and ISO.

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Introduction

Arabinogalactan proteins (AGPs) are a highly diverse group of hydroxyproline-rich proteoglycans that are implicated in numerous aspects of plant growth and development including cell differentiation and morphogenesis *in vitro* (Rumyantseva 2005; Ma *et al.* 2018). AGPs are located at the plant plasma membrane, cell wall, extracellular space and intracellular compartments. Structurally, AGPs consist of a relatively small highly variable protein core which is covalently linked through hydroxyproline to various carbohydrate side chains (AG sugar chains), consisting mainly of β-1,3-galactan with β-1,6-galactan substitutions decorated with arabinosyl, glucuronosyl, rhamnosyl, fucosyl, xylosyl and other sugars (Ellis *et al.* 2010).

Modifications of cell wall components, including precise tempo-spatial regulation of AGPs appearance at the cell surface, are important for many aspects of plant growth and development (Ma *et al.* 2018). AGPs are highly glycosylated proteins and more than 90% of their total molecular mass comes from glycan moieties, which can undergo tissue-specific degradation during differentiation and morphogenesis. Monoclonal antibodies that recognize specific carbohydrate epitopes of the AGP molecules are powerful tools widely used to monitor their dynamic modifications, visualize tempo-spatial patterns of expression and investigate possible biological roles of AGP glycan epitopes in plant tissues or cells during various developmental stages (Knox *et al.* 1991; Moller *et al.* 2008). Most studies of AGP functions regarding *in vitro* morphogenesis were focused on somatic embryogenesis (Saare-Surminski *et al.* 2000; Ellis *et al.* 2010; Namasivayam *et al.* 2010; Pan *et al.* 2011; Steinmacher *et al.* 2012; Pilarska *et al.* 2013; Potocka *et al.* 2018; Betekhtin *et al.* 2016, 2019; Pérez-Pérez *et al.* 2019). One of the first reports on AGPs role during somatic embryogenesis

71 appeared three decades ago and was based on localization of the JIM4 monoclonal antibody

72 on the surface of proembryogenic carrot cell cultures (Stacey *et al.* 1990). Extensive studies

73 have revealed that AGPs are developmentally regulated during somatic embryogenesis, and

74 act either as positional markers (Stacey *et al.* 1990), messengers during cell fate selection

75 (McCabe *et al.* 1997) or early molecular markers for embryogenic competence (Šamaj *et al.*

76 1999). However there were a few studies on AGPs functions in shoot organogenesis

77 (Konieczny *et al.* 2007; Wiśniewska and Majewska-Sawka 2007; Johnson *et al.* 2011;

78 Orbović *et al.* 2013; Trifunović *et al.* 2015; Simonović *et al.* 2015).

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80 Common centaury (*Centaurium erythraea* Rafn), a member of the Gentianaceae family, is an

81 important medicinal plant which aerial organs contain numerous pharmacologically valuable

82 metabolites with therapeutic properties, such as secoiridoid glucosides and xanthones

83 (Subotić *et al.* 2006; Šiler and Mišić 2016). This plant is also used as a natural flavor additive

84 in both food and beverage industries (Newall *et al.* 1996). Due to its commercial value and

85 over-harvesting from natural populations, this species is considered to be endangered (Subotić

86 *et al.* 2009). Therefore, centaury is the subject of biotechnological interest in order to protect

87 this species in nature and provide new resources to obtain desired secondary metabolites

88 (Subotić *et al.* 2006; Trifunović-Momčilov *et al.* 2016a; Matekalo *et al.* 2018). Many reports

89 describing *in vitro* regeneration studies in centaury revealed that this plant is characterized

90 with enormous regeneration potential and can be successfully propagated via two different

91 developmental pathways, somatic embryogenesis and shoot organogenesis (Filipović *et al.*

92 2015).

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94   Although the involvement of AGPs in centaury morphogenesis *in vitro* has been already

95   shown in our previous studies, information on the distribution of AGP epitopes during

96   indirect regeneration in leaf culture is still unknown. Trifunović *et al.* (2014, 2015), using

97   antibody-based approach and histochemical localization with β-D-glucosyl Yariv (βGlcY)

98   reagent, which can also visualized the distribution of AGPs, demonstrated that AGPs played a

99   significant role during the induction of direct somatic embryogenesis and shoot organogenesis

100 in centaury root culture. Simonović *et al.* (2015) investigated centaury morphogenesis in leaf

101 culture at the molecular level and showed that among four identified centaury AGPs genes,

102 only *CeAGP1* and *CeAGP3,* an membrane-anchored AG peptide (Simonović *et al.* 2016), are

103 involved in indirect morphogenesis. Therefore, the objective of this research was to

104 investigate changes in localization of selected AGP epitopes during indirect somatic embryo

105 (SE) and adventitious bud (AB) development in leaf culture of centaury using

106 immunolabeling.

107 **Materials and Methods**

108 **Plant material and culture conditions**

109 Indirect somatic embryogenesis (ISE) and shoot organogenesis (ISO) were induced from leaf

110 culture of *C. erythraea*, as previously described (Filipović *et al.* 2015). For induction of ISE,

111 leaf explants were kept both in the light, under 16 h photoperiod, and in darkness on culture

112 medium composed of Murashige and Skoog (MS; Murashige and Skoog 1962) full-strength

113 macro and micro mineral salts (Lachner, Brno, Czech Republic), MS vitamins (Sigma-

114 Aldrich, Saint Louis, MO), 0.2 mg L^{-1} 2,4–dichlorophenoxyacetic acid (2,4-D; (Sigma-

115 Aldrich, Saint Louis, MO), 0.5 mg L^{-1} *N*-(2-chloro-4-pyridyl)-*N'*-phenylurea (CPPU; (Sigma-

116 Aldrich, Saint Louis, MO), 30 g L^{-1} sucrose (Sigma-Aldrich, Saint Louis, MO), 100 mg L^{-1}

117 myo-inositol (Sigma-Aldrich, Saint Louis, MO) and 7 g L^{-1} agar (Torlak, Belgrade, Serbia).

118 In accordance with our previous findings, for induction of ISO, leaf explants were kept in the

119 light on the same medium, since light was required for induction of adventitious buds in our

120 model system (Filipović *et al.* 2015).

Sample preparation, monoclonal antibodies and immuno-fluorescence labeling

122 For immuno-fluorescence labeling of AGPs, leaf explants were collected during induction of

123 ISE and ISO every wk for four wk. Tissue pieces were processed as described by Trifunović

124 *et al.* (2015). Briefly, excised leaf explants were fixed in FAA (5.4% *v/v* formalin, 65.5%

125 ethanol and 5% glacial acetic acid, Zorka Pharma, Šabac, Serbia), dehydrated in an ascending

126 ethanol series (30, 50, 70, 96 and 100%) and embedded in Histowax (Histolab, Gotëborg,

127 Sweden). Embedded tissues were sectioned (5μm thick) with microtome blades (Leica,

128 Wetzlar, Germany, Type 819) in a rotary microtome (Reichert, Vienna, Austria) and collected

129 on SuperFrost®Plus slides (VWR International, Strasbourg, France).

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131 A method described in Knox *et al.* (1989) was used for indirect immunohistochemical

132 localization. In brief, the sections were de-waxed and rehydrated in an ethanol series (100, 96

133 and 70%). After rinsing in phosphate-buffered saline (PBS, pH 7.2; 136.9 mM NaCl, 2.7 mM

134 KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, Centrohem, Stara Pazova, Serbia), the sections

135 were treated with a blocking reagent (PBS (pH 7.2) supplemented with 3% milk protein

136 (Nestle, Vevey, Switzerland) and 0.05% Tween 20 (Serva, Heidelberg, Germany) for 30 min

137 at room temperature (22 ± 1 ºC) and then incubated in a five-fold dilution of primary anti-

138 AGP mAbs in a blocking reagent at 4 ºC for 24 h in order to detect presence and distribution

139 of different arabinogalactan epitopes. Immunofluorescence labeling of leaf sections during the

140 first four wk of culture was performed with a set of mAbs (JIM4, JIM8, JIM13, JIM15, LM2,

141 LM14 and MAC207), listed in Table 1. All of the primary mAbs used in this study were

142   purchased from Plant Probes (Leeds, UK). Negative controls were performed by incubation in

143   PBS instead of the primary antibodies.

144

145   After several rinses with PBS (each for five min), secondary Alexa Fluor 488 conjugated goat

146   anti-rat IgG antibody (Thermo Fisher Scientific, Rockford, IL), diluted 1:1000 in a blocking

147   reagent, was applied at room temperature (22 ± 1 ºC) for 30 min in darkness. After labeling,

148   the slides were rinsed in PBS (three times, each for five min). Finally, the sections were

149   rinsed with PBS again and mounted in a 1:1 mixture of PBS and glycerol (Zorka Pharma,

150   Šabac, Serbia). All images were obtained using a camera associated with Zeiss Axiovert

151   fluorescent microscope (Zeiss, Jena, Germany).

152   **Results**

153   **Indirect somatic embryogenesis and shoot organogenesis in *C. erythraea* leaf culture**

154   The leaf explants cultured in darkness developed embryogenic callus cultures during the

155   second wk of incubation and, after 28 d, SEs of different developmental stage on the surface

156   of the calluses were observed (Figs 1a-d). Centaury ABs (Figs 1e, g-i) and SEs (Fig 1f)

157   developed simultaneously on leaf explants cultured under light conditions. This provided the

158   material to investigate the localization of diverse AGP epitopes (JIM4, JIM8, JIM13, JIM15,

159   LM2, LM14 and MAC207) during formation of SEs and ABs in centaury leaf culture. AGPs

160   were recognized by all tested mAbs and also expressed in numerous meristematic centers that

161   were formed on leaf explants after 7 and 14 d in culture (Fig 2). Immunolocalization using

162   JIM4 showed strong immunofluorescence in numerous meristematic cells formed after 14 d in

163   culture under light conditions (Fig 2a). This epitope was detected with high abundance in cell

164   walls and cytoplasm of small isodiametric cells with high cytoplasmic content. After seven-d

165 in darkness, JIM4 epitope showed strong expression in cell walls of dividing epidermal and

166 subepidermal cells which formed meristematic centers as well as in embryogenic cells in

167 meristematic calluses (Fig 2a inset). JIM8 epitope showed moderate signal in meristematic

168 callus cells formed after seven-d under light conditions (Fig 2b). The strong fluorescence of

169 JIM8 was visible in extracellular matrix covering dividing epidermal cells (Fig 2b).

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171 After 14 d in darkness, JIM13 epitope was distributed moderately in the cell walls and

172 intracellularly in meristematic cells and was also localized in the cell walls of some

173 parenchyma cells of regenerating leaf explants (Figs 2c, d). In the vascular bundle cells of the

174 explant, JIM13 epitope was abundantly present (Fig 2c). The strong fluorescence due to

175 JIM15 binding was identified in meristematic cells of leaf explant formed after seven-d under

176 light conditions (Fig 2e). Stronger activity of LM2 and LM14 epitopes was observed in

177 meristematic cells formed in epidermal and subepidermal layers of regenerating leaf explants

178 after seven-d in the light (Figs 2f, g). The LM2 epitope was detected in meristematic centers

179 formed in epidermis, subepidermis and mesophyll of regenerating leaf explants after seven-d

180 in the light (Fig 2f).

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182 As shown in Fig 2g, the LM14 epitope was present in meristematic cells with stronger signal

183 on the surface of regenerating leaf explants while no signal was detected in vascular elements.

184 Moderate MAC207 signal was observed in the cytoplasm and cell walls of dividing cells in

185 meristematic tissue and also in parenchyma cells after seven-d in darkness (Fig 2h). The

186 negative control for JIM4 (performed by omitting the primary antibodies) did not show any

187 antibody signal (Fig 2i).

Immunolocalization of AGPs epitopes during somatic embryos development

Epitopes recognized by JIM4 were found during formation of early four-cell proembryo at the periphery of the callus after 14-d in darkness (Fig 3a). Strong signal of JIM4 was observed in thick outer cell walls of the proembryos (Fig 3b). During further proembryo development, strong expression was detected only in extracellular matrix surrounding proembryogenic nodule (Figs 3c, c inset). At the globular stage of development AGP epitopes recognized by JIM4 were detected in cell walls of protodermal cells (Fig 3d). Somatic embryos at globular stages showed a very weak (or none) signal of the JIM8 epitope (Fig 3e). Similarly, very weak (or none) signal was detected in globular somatic embryo in the case of the LM14 binding AGP (data not shown). In contrast, high intensity labeling pattern in the whole centaury globular somatic embryo was observed for JIM13 and JIM 15 epitopes (Figs 3f, g). Strong expression of LM2 epitope was detected in cell walls during somatic embryos formation in embryogenic callus and in the whole globular embryo (Figs 3h, i, j). During formation of somatic embryos in embryogenic callus, specific distribution pattern of MAC207 epitope was also observed, since strong signal of this epitope was present in the extracellular matrix (Figs 3k, k inset). The labeling pattern of MAC207 in globular somatic embryos was similar to that of JIM4 epitope (Fig 3l). During further SE development, a moderate fluorescence of JIM4 epitope was observed in early cotyledonary stage (Fig 4a). Expression of JIM13 epitope decreased in cotyledonary embryos compared with globular embryos (Figs 4b, c). No obvious signal could be detected in later developmental stage of SEs for all the rest of mAbs (JIM8, JIM15 and LM2). The result obtained with JIM15 was selected as the representative one (Fig 4d). Strong fluorescence of LM14 epitope was evenly distributed throughout the longitudinal sections of heart embryo (Fig 4e). The MAC207 epitope was the most strongly expressed AGP epitope mainly localized in developing cotyledons (Fig 4f).

Immunolocalization of AGPs epitopes during adventitious bud development

JIM8 epitope was moderately expressed in meristematic bud primordium with stronger signal on the outer cell wall of surface cells of meristem (Fig 5a). At this stage of AB development, LM2 epitope showed strong and even distribution (Fig 5b). The presence of MAC207 epitope was more abundant in the initials of ABs than in the surrounding callus tissue (Figs 5c, c inset). Strong immunofluorescence of the AGPs epitopes recognized with JIM15 (Fig 5d) and LM14 (data not shown) mAbs were detected in meristematic bud primordium, while JIM4 showed weak signal (data not shown). LM2 and JIM4 were observed in the cells of *de novo* formed adventitious buds (Figs 5e, f). During *in vitro* culture the signal of JIM8 and JIM15 epitopes was poor (Figs 5g, h). MAC207 epitope showed strong expression in regenerated adventitious bud with a well-developed first leaf and second leaf which was developed later (Figs 5i, i inset). In fully formed ABs with well-defined shoot apex and leaf primordia, only JIM4 and MAC207 were detected with stronger signal compared to callus cells (Figs 5j, k, l). During AB development, the presence of JIM13 epitope was not detected (data not shown).

Discussion

Indirect morphogenesis via two developmental pathways, ISE and ISO, was achieved from centaury leaf explants incubated on medium containing 2,4-D and CPPU, in light and in darkness, which is in accordance with our previous report (Filipović *et al*. 2015). SEs and ABs at different stages of development were observed on the same leaf explant cultured under light conditions, while in darkness only SEs were formed. Histological characterization of events that lead to the indirect formation of somatic embryos and adventitious buds in leaf culture of *C. erythraea* has been described previously (Filipović *et al*. 2015). This analysis showed that SEs originated indirectly from subepidermal cells of cultured leaf segments, which involved dedifferentiation of these cells and formation of meristematic centers and then

236 differentiation into proembryogenic structures. During this period of culture, dedifferentiation

237 within mesophyll cells of the leaf segments led to formation of meristematic centers from

238 which ABs were produced, by differentiation of meristematic callus cells (Filipović *et al.*

239 2015).

240

241 In the present study, we found broad presence of all tested AGP epitopes in meristematic

242 centers that formed on culture leaf explants. Our results are consistent with the literature

243 where monoclonal antibody JIM4 could serve as an early marker of embryogenic competence

244 in maize callus culture (Šamaj *et al.* 1999). The authors showed the presence of AGP epitope

245 recognized with this antibody in the extracellular matrix on the surface of embryogenic maize

246 cells, while non-embryogenic callus cells are devoid of this epitope. McCabe *et al.* (1997)

247 showed that JIM8 epitope was necessary for formation of SEs, since this epitope labels the

248 surface of embryogenic cells which give rise to SEs of carrot. The JIM8 and JIM13 epitope

249 were localized both in embryogenic callus cells and in non-embryogenic callus cells of

250 *Fagopyrum tataricum* (Betekhtin *et al.* 2019) and *Brassica napus* (Namasivayam *et al.* 2010).

251 JIM13 epitope is often found in provascular cells, xylem or phloem of different species

252 (Rumyantseva 2005) and in cell walls of parenchyma cells (Betekhtin *et al.* 2016), which is in

253 accordance with our results. JIM15 epitopes were also present in the embryogenic cells of

254 carrot (McCabe *et al.* 1997). In the developing ovary of a facultative apomict *Fragaria x*

255 *ananassa,* epitopes recognized by the JIM15 epitope were located in the cell walls of

256 reproductive cells (Leszczuk and Szczuka 2018). Strong detection of LM2 epitope was

257 observed in the cell walls of meristematic cells from which SEs develop and in cells of

258 embryogenic swellings in *Trifolium nigrescens* (Pilarska *et al.* 2013). In *F. tataricum* the LM2

259   antibody can be used as a marker of the embryogenic determined cells in morphogenic callus

260   (Betekhtin *et al.* 2019).

261

262   In contrast to above mentioned reports, LM2 is a negative marker of embryogenic cells in

263   *Arabidopsis* explants (Potocka *et al.* 2018). MAC207 antibody seems to be a marker of the

264   morphogenic callus of *F. tataricum* which consists of proembryogenic cell complexes, since

265   lack of its signal in the non-embryogenic callus (Betekhtin *et al.* 2019). In embryogenic callus

266   of *Brachypodium distachyon,* LM2 and MAC207 were detected intracellularly as well as in

267   the cell walls (Betekhtin *et al.* 2016). In banana (*Musa* spp. AAA cv. 'Yueyoukang 1'),

268   among 16 mAbs used, LM14 and JIM16 epitopes were strongly expressed in embryogenic

269   cells, MAC204, MAC207, CCRCM7 and JIM13 epitopes were moderately present, while

270   very weak or no signal was detected in the case of JIM4, JIM8, JIM14, JIM15, JIM101, LM2,

271   MAC265, MAC266, PN16.4B4 and JIM17 epitopes (Pan *et al.* 2011).

272

273   Studies concerning the somatic embryogenesis of different species have revealed that the

274   spatiotemporal occurrences of AGPs are developmentally regulated, for example in *Daucus*

275   *carota* (Stacey *et al.* 1990), *Zea mays* (Šamaj *et al*. 1999), *Cichorium intibys* (Chapman *et al.*

276   2000) and *T. nigrescens* (Pilarska *et al*. 2013). Our results showed that the same

277   developmental stage of somatic embryogenesis, globular somatic embryo, was characterized

278   with different spatial distribution pattern of analyzed AGP epitopes. In the present study,

279   expression pattern of JIM4 and MAC207 epitopes was restricted to protodermal cells of

280   globular SEs. Our results are in agreement with distribution of MAC207 epitope and its

281   strong expression in protodermal cells of the globular SEs during induction of direct somatic

282   embryogenesis in centaury root culture (Trifunović *et al.* 2015). During somatic

283 embryogenesis of *D. carota*, the JIM4 epitope was enriched at the surface of peripheral cells

284 of proembryogenic masses and in the protoderm of globular embryos (Stacey *et al.* 1990).

285 Similar labeling pattern in protodermal tissue of globular somatic embryo was shown with

286 other monoclonal antibodies in many other species, for example with LM2 in *Euphorbia*

287 *pulcherrima* (Saare-Surminski *et al.* 2000) and JIM13 in peach palm (Steinmacher *et al.*

288 2013). In chicory, AGPs recognized by monoclonal antibodies LM2, JIM13 and JIM16 were

289 localized at the surface of proembryo and at the outer cell walls of epidermal cells in globular

290 embryos (Chapman *et al.* 2000).

291

292 Very weak or no labeling was detected for JIM8 epitope in centaury globular somatic embryo

293 which is in agreement with very low abundance of JIM8 epitope, mainly in epidermal cells of

294 the banana embryo (Pan *et al.* 2011). Potocka *et al.* (2018) detected occasionally JIM8

295 epitope in the cell walls of the globular embryos, but in older stage it was abundantly present

296 in the embryo except the protodermis. In contrast, high intensity labeling pattern in the whole

297 centaury globular somatic embryo was observed for JIM13, JIM15 and LM2 epitopes. Similar

298 results were reported for LM2 and LM14 epitopes showing strong signal, while JIM16

299 epitope showed moderate signal in whole globular SEs of banana (Pan *et al.* 2011). JIM15

300 epitope was reactive with AGPs in developed SEs in centaury root culture (Trifunović *et al.*

301 2015). In banana globular somatic embryos lacked JIM15 epitope and only weak signal was

302 detected in cotyledonary embryos (Pan *et al.* 2011). Interestingly, the AGP epitope that is

303 recognized by the LM14 antibody showed specific labeling pattern since it was not present in

304 globular stage of centaury embryo but strong fluorescence of this epitope was detected in

305 heart stage of embryo. AGPs bearing this epitope seem to be important for the differentiation

306 and further development of polar embryogenic stages, from heart to cotyledonary stage. This

307 specific strong labeling pattern could be related with the fact this mAb binds to an epitope of

308 type II arabinogalactan that may occur on both pectins and AGPs (Moller *et al.* 2008). As the

309 embryo further developed, only JIM4, JIM13 and MAC207 epitopes were detected at the

310 cotyledonary stage of somatic embryo.

311

312 The present study showed that all analyzed AGP epitopes were expressed during early

313 embryogenic developmental stages, while during further differentiation of the embryos, the

314 number of detected AGP epitopes and labeling intensity decreased. Our results are consistent

315 with those that have been reported during somatic embryogenesis in banana (Pan *et al.* 2011),

316 where a diversity of immunolabeled AGPs also decreased with SE maturation. No AGP

317 epitopes were detected in cotyledonary-staged SEs of *T. nigrescens* (Pilarska *et al.* 2013). On

318 the contrary, AGP levels increased with the progression of cork oak somatic embryogenesis,

319 since LM2 and LM6 showed an increment in signal intensity at cotyledonary embryo stage

320 (Pérez-Pérez *et al.* 2018). In our study, each of analyzed AGP epitopes showed different

321 spatio-temporal distribution pattern, which appeared to be associated with differentiation

322 during SE development. The expression of JIM4, JIM13 and MAC207 provides an evidence

323 that these epitopes are markers for all stages of the major tissue patterning during centaury

324 ISE. Expression of specific epitopes of AGPs recognized with JIM4, JIM8, JIM13, JIM15,

325 LM2, LM14 and MAC207 was developmentally regulated during ISE. Monoclonal antibodies

326 JIM4, JIM13, JIM15, LM2 and MAC207 were detected in globular SEs, while only JIM4,

327 JIM13 and MAC207 were detected in cotyledonary SEs.

328

329 To the best of our knowledge, the functions and evident involvement of AGPs in shoot

330 organogenesis were described only in a few plant species including *Arabidopsis thaliana*

331 (Johnson *et. al* 2011), two citrus cultivars *Citrus sinensis* and *C. paradisi* (Orbović *et al.*

332 2013), *Beta vulgaris* (Wiśniewska and Majewska-Sawka 2007), *Triticum aestivum*

333 (Konieczny *et al.* 2007) and *C. erythraea* (Simonović *et al.* 2015; Trifunović *et al.* 2015).

334 Study of expression of *FLA1* gene in callus and shoot developmental processes using a fla1

335 mutant support the role for this *AGP* gene in the early events of shoot development and lateral

336 root development in tissue culture, prior to cell-type specification (Johnson *et al*. 2011). The

337 use of mAbs and Yariv reagent demonstrated the role of carbohydrate moiety of AGPs in

338 shoot organogenesis. The role of AGPs in indirect organogenesis was hindered by βGlcY

339 treatment, which blocks AGPs (Orbović *et al.* 2013; Simonović *et al.* 2015). Previous

340 immunolocalization studies of AGPs during shoot organogenesis demonstrated the role of

341 glycan moietes of AGPs in shoot development. Immunohistological analysis of androgenic

342 wheat callus suggested the involvement of AGPs with LM2 epitope in shoot organogenesis

343 since LM2 antibody labeled the walls and some cytoplasmic regions of actively dividing

344 meristematic cells of regenerated shoot buds and leaves (Konieczny *et al.* 2007). AGP-rich

345 extracts characterized by the widespread occurrence of JIM13, MAC207 and LM2 epitopes

346 stimulate organogenesis in sugar beet callus (Wiśniewska and Majewska-Sawka 2007). In

347 centaury, JIM16 could be a reliable marker for direct shoot organogenesis in root culture

348 since strong expression of JIM16 antibody was detected in *de novo* formed meristematic

349 centers in the root cortex (Trifunović *et al.* 2015).

350

351 In our study, the AGPs recognized by JIM8, JIM15, LM2, LM14 and MAC207 (and JIM4

352 weakly) antibodies were found in meristematic bud primordium, suggesting that these AGPs

353 may participate in their formation. During further development of adventitious buds, the

354 number of detected AGPs decreased. JIM8 and JIM15 epitopes were detected with low

355 abundance, while JIM4, LM2 and MAC207 were abundantly present in ABs. In fully formed

356 adventitious buds with well-defined shoot apex and leaf primordia, only JIM4 and MAC207

357 were detected. Strong expression of MAC207 epitope was detected in regenerated

358 adventitious bud with a well-developed first leaf and second leaf which was developed later.

359 Spontaneous development of this adventitious bud was previously described in centaury root

360 culture (Trifunović-Momčilov *et al.* 2016b). Almost all AGP epitopes (JIM4, JIM8, JIM15,

361 LM2, LM14 and MAC207) were involved in centaury adventitious buds formation and their

362 appearance was developmentally regulated.

363 **Conclusion**

364 Our results revealed dynamic changes in distribution of specific AGP epitopes during SE and

365 AB development in centaury leaf culture. All of the AGP epitopes analyzed in this study were

366 expressed in meristematic centers that were formed on leaf explants, while during

367 development of SEs and ABs the number of detected AGPs decreased. All analyzed AGP

368 epitopes showed different spatio-temporal distribution pattern during ISE, which appeared to

369 be associated with differentiation during SE development. All the antibodies except JIM13

370 were involved in centaury adventitious bud formation. These results implicated that AGPs

371 were developmentally regulated during centaury ISE and ISO. The present study also

372 suggests JIM13 antibody as a marker for ISE in centaury leaf culture. The distribution of

373 AGPs during SE and AB development could suggest that these proteoglycans have

374 fundamental roles in cell division. This immunolocalization study provides new information

375 on the structural changes of cell walls in morphogenesis in leaf culture of this important

376 medicinal plant. Broad presence of all analyzed AGP epitopes in meristematic cells,

377 predetermined to different developmental pathways, indicated great microheterogeneity of

378 glycan part of centaury AGPs and their involvement in two different developmental

379 pathways. Future investigations of cell wall chemical composition are necessary to connect

380 specific AGPs or at least their glycans with cells determined to enter particular developmental

381 pathways.

384 **Author contribution statement BKF** conducted all experimental work, image analysis and

385 manuscript preparation. **MMTM** contributed in image analyses and manuscript preparation.

386 **ADS** revised the manuscript. **SBJ** contributed to image analysis and obtained results

387 interpretation**. SMM** contributed to experimental work considering immunohistochemical

388 analyses. **ARS** supervised the whole study and also contributed in immunohistochemical

389 analyses, fluorescent microscope analyses and preparing the final manuscript. All authors

390 have participated in this research and approved the final manuscript.

391 **Compliance with ethical standards**

392 **Conflict of interest** The authors declare that they have no conflict of interest.

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Figure legends:

Fig. 1 Indirect somatic embryogenesis (ISE) and indirect shoot organogenesis (ISO) in leaf culture of *Centaurium erythraea* Rafn after 28 d. **a-d** ISE in darkness; **e-i** ISE and ISO on light; *ec* embryogenic callus, *se* somatic embryo, *gse* globular somatic embryo, *cse* cotyledonary somatic embryo, *oc* organogenic callus, *og* organogenic globule, *ab* adventitious buds.
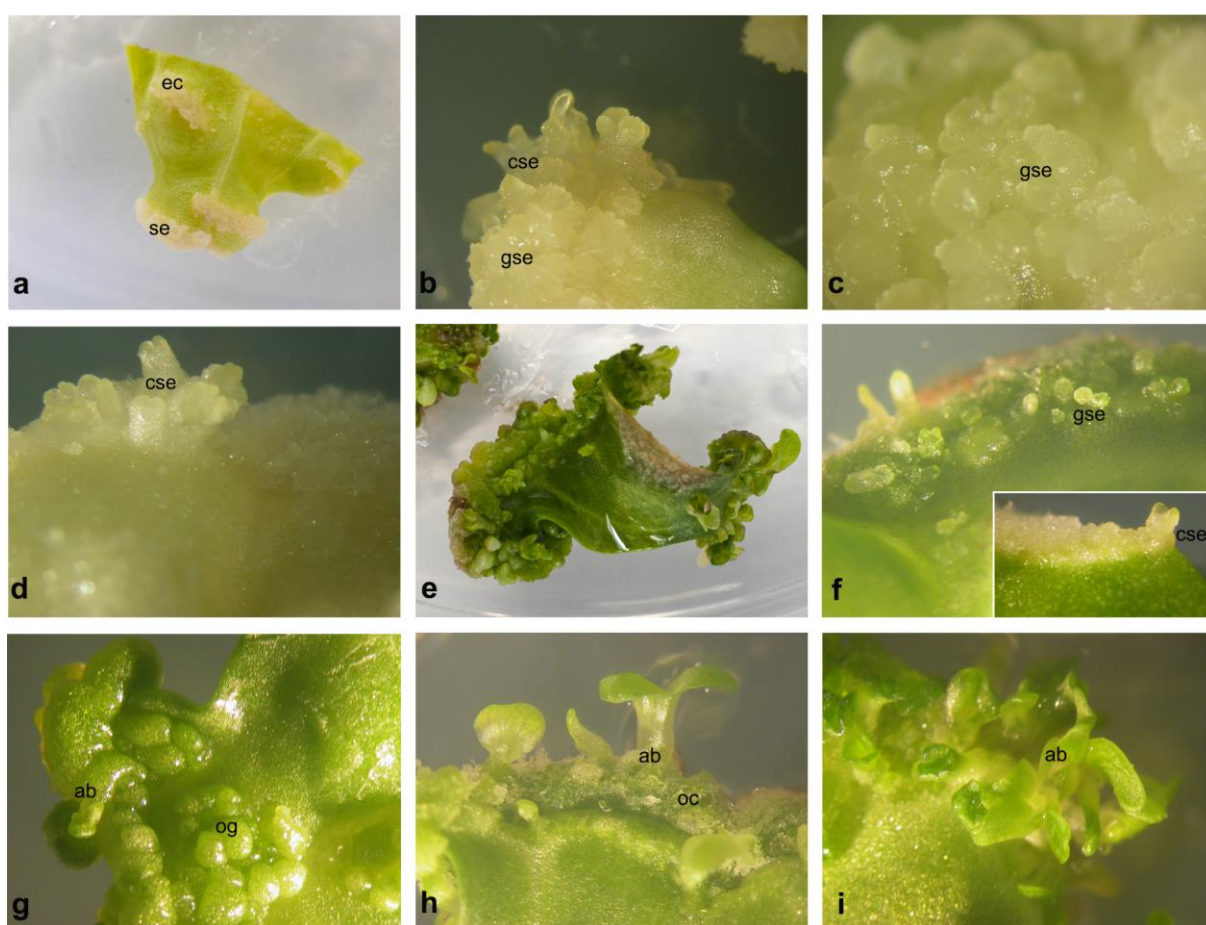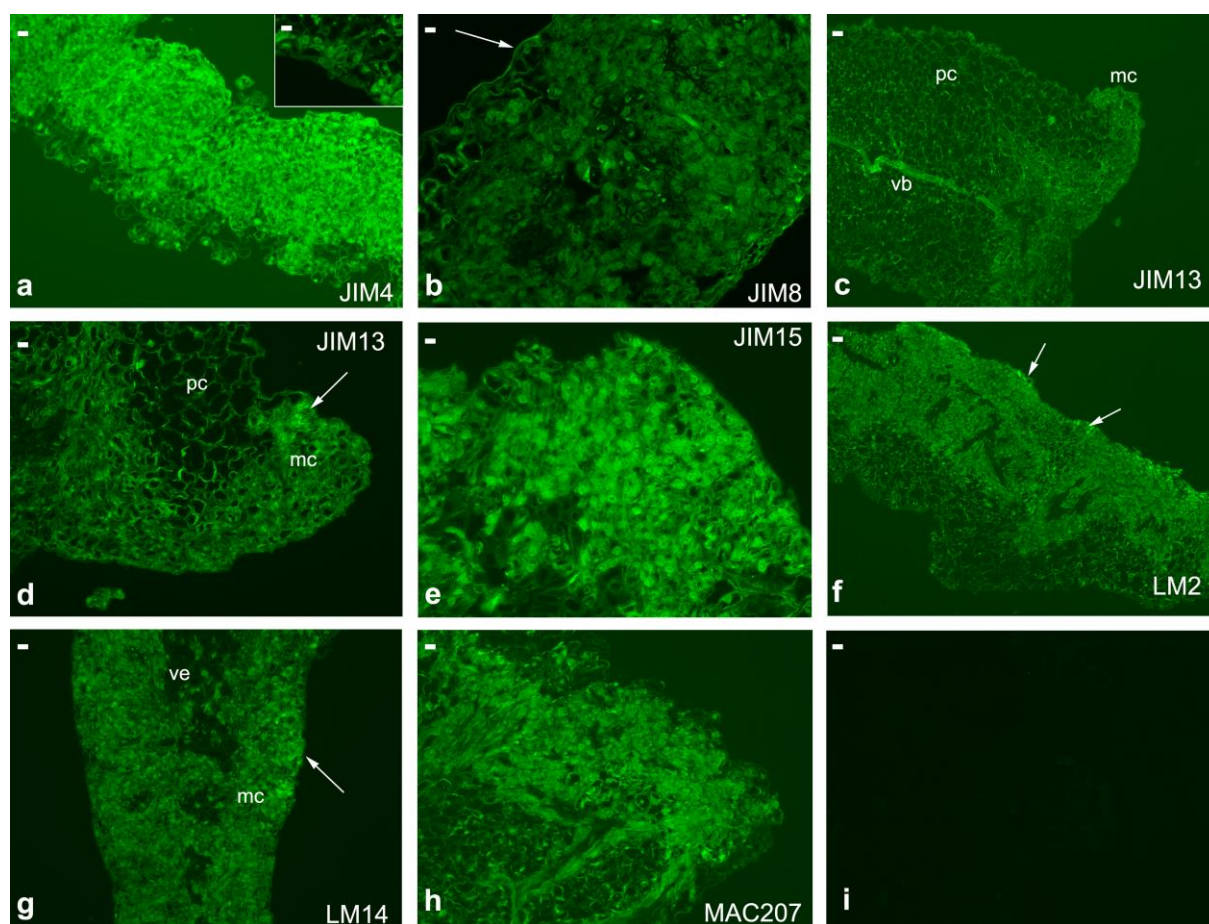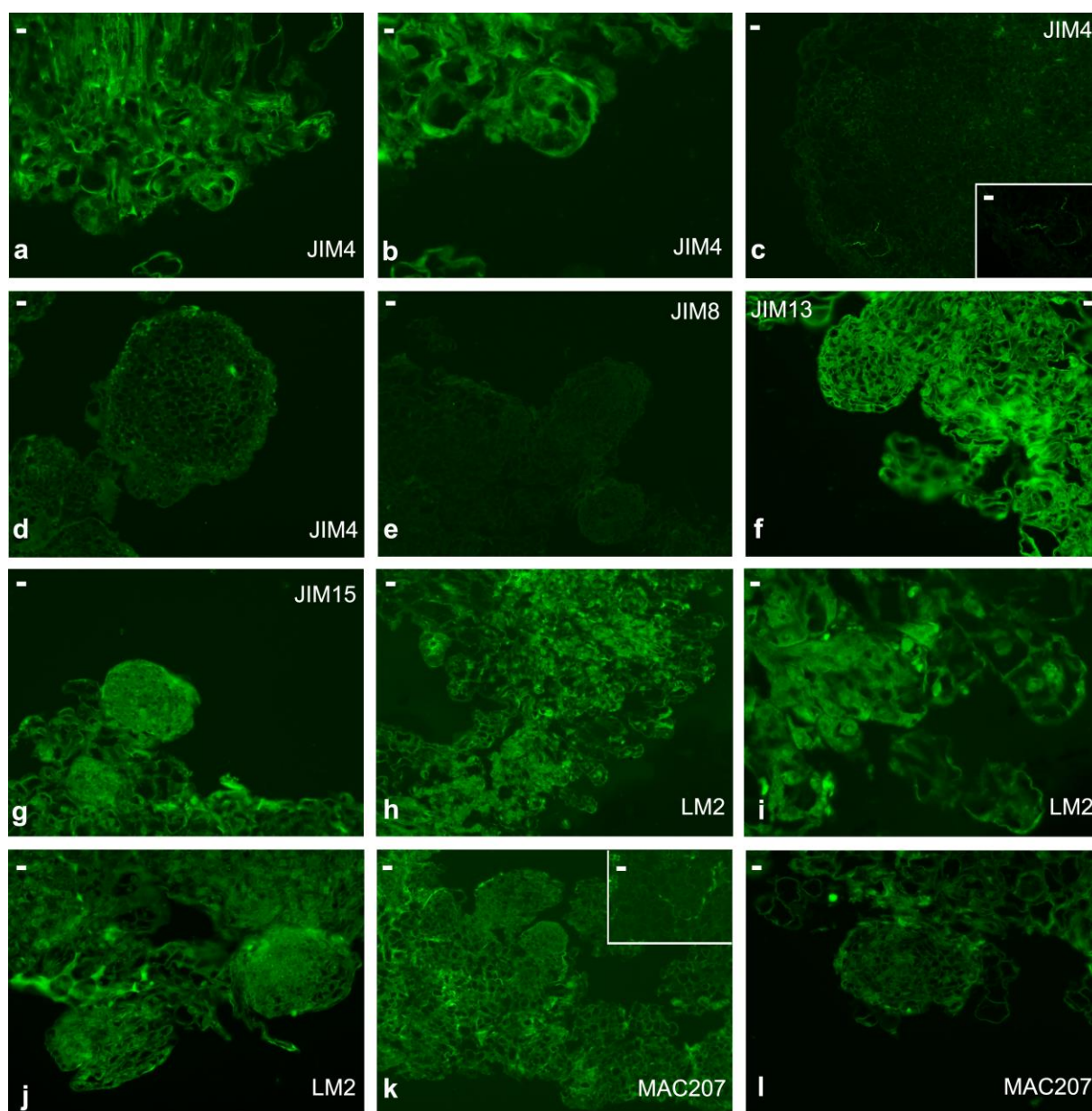
Fig. 2 Immunolocalization of AGP epitopes in meristematic cells of *Centaurium erythraea* Rafn leaf explants. **a** meristematic cells and dividing epidermal cells (**a inset**) with JIM4; **b** meristematic centers and extracellular matrix covering dividing epidermal cells (arrow) with JIM8; **c** and **d** cells of explant vascular bundle (**c**), meristematic cells (arrow) and parenchyma cells (**c**, **d**) with JIM13; **e-h** meristematic cells with JIM15 (**e**), LM2 (**f**), LM14 (**g**) and MAC207 (**h**); Arrows point to strong signal in meristematic centers on the surface of explants with LM2 (**f**) and LM14 (**g**); **i** negative control. *mc* meristematic cells, *ve* vascular elements, *pc* parenchyma callus cells, *vb* vascular bundle. Scale bars = 20 µm (**c**, **f**), 10 µm (**a**, **d**, **g**, **h**), 7,5 µm (**a inset**), 5 µm (**b**, **e**).
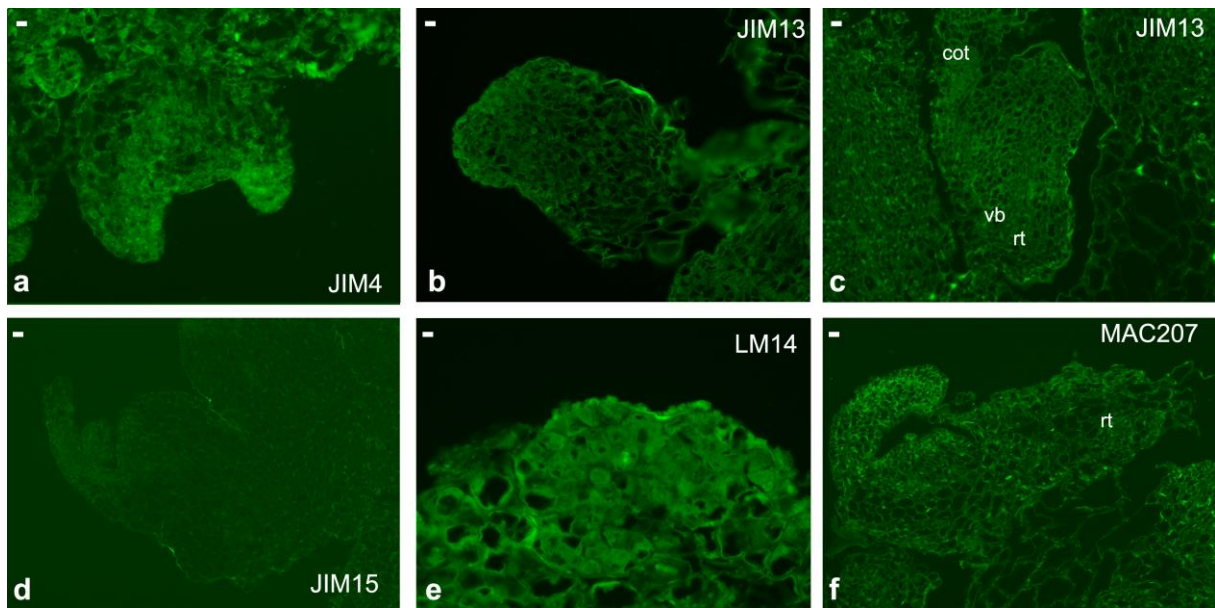
595 **Fig. 3** Immunolocalization of AGP epitopes in proembryos and globular SEs of *Centaurium*

596 *erythraea* Rafn. **a-d** early proembryo (**a, b**), proembryogenic nodule (**c**) and globular somatic

597 embryo (**d**) with JIM4; **e-g** globular somatic embryo with JIM8 (**e**), JIM13 (**f**) and JIM15 (**g**);

598 **h-j** formation of SEs in embryogenic callus (**h, i**) and globular embryo (**j**) with LM2; **k** and **l**

599 formation of SEs in embryogenic callus (**k, k** inset) and globular embryo (**l**) with MAC207.

600 Scale bars = 15 μm (**k** inset), 10 μm (**c, d, e, h, k**), 7,5 μm (**c inset**), 5μm (**a, f, g, j, l**), 2,5μm

601 (**b, i**).

Fig. 4 Immunolocalization of AGP epitopes in late developmental stages of *Centaurium erythraea* Rafn SEs **a** early cotyledonary somatic embryo with JIM4; **b-d** cotyledonary somatic embryo with JIM13 (**b, c**) and JIM15 (**d**); **e** heart-shaped somatic embryo with LM14; **f** cotyledonary somatic embryo with MAC207; *cot* cotyledon, *vb* vascular bundle, *rt* root meristem. Scale bars = 10 μm (**c, d, f**), 5 μm (**a, b**), 2,5 μm (**e**).

Fig. 5 Immunolocalization of AGP epitopes in developmental stages of *Centaurium erythraea*

618 Rafn ISO. **a-d** initials of AB with JIM8 (**a**), LM2 (**b**), MAC207 (**c**, **c inset**) and JIM 15 (**d**); **e-**

619 **h** AB with LM2 (**e**), JIM4 (**f**), JIM8 (**g**) and JIM15 (**h**); **i** and **i inset** regenerated AB with a

620 well-developed first leaf and second leaf which developed later with MAC207; **j-l** fully

621 formed AB with well-defined shoot apex and leaf primordia with JIM4 (**j**, **k**) and MAC207

622 (**l**). *am* apical meristem, *lp* leaf primordia. Scale bars = 20μm (**j, i inset**)**,** 10μm (**b-l, c inset**),

623 5 μm (**a**, **c inset**).
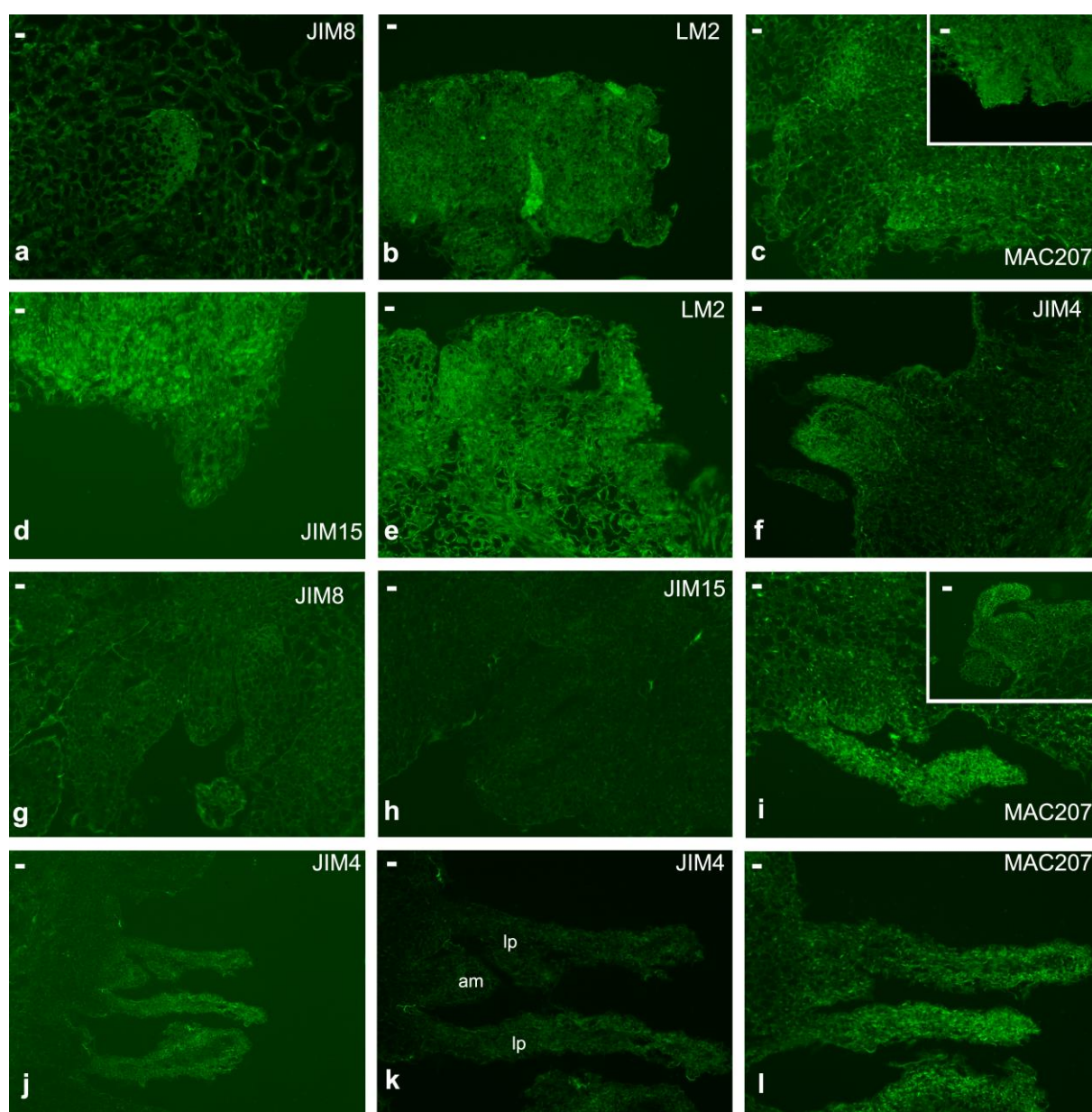
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625   Table 1. Primary antibodies used in this study, the epitopes they recognize and relevant references

Antibodies	Epitopes	References
JIM4	β-D-GlcpA-(1→3)-α-D-GalpA-(1→2)-L-Rha	Knox *et al.* (1989) and Yates *et al.* (1996)
JIM8	AG	Pennel *et al.*(1991) and McCabe *et al.*(1997)
JIM13	β-D-GlcpA-(1→3)-α-D-GalpA-(1→2)-L-Rha	Knox *et al.* (1991) and Yates *et al.* (1996)
JIM15	β-D-GlcpA, epitope structure unknown	Knox *et al.* (1991) and Yates *et al.* (1996)
LM2	β-D-GlcpA, AGP	Smallwood *et al.* (1996), Yates *et al.* (1996) and Ruprecht *et al.* (2017)
LM14	AGP	Moller *et al*. (2008)
MAC 207	β-D-GlcpA-(1 → 3)-α-D-GalpA-(1 → 2)-L-Rha, AGP	Bradley *et al.* (1988), Pennel *et al.* (1989) and Yates *et al.* (1996)

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