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1           **TBL38 is an atypical homogalacturonan acetyltransferase with a peculiar cell wall**  
2           **microdomain localization in Arabidopsis seed mucilage secretory cells**

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21

22 **Running title** : TBL38 is a cell wall homogalacturonan acetyltransferase  
23

24 **Abstract**

25 Plant cell walls are made of complex polysaccharidic/proteinaceous network whose  
26 biosynthesis and dynamics implicate several cell compartments and impact plant development.  
27 The synthesis and remodeling of homogalacturonan pectins is associated with multiple  
28 developmental processes ranging from growth to response to biotic/abiotic stress. It  
29 encompasses Golgi-localized methylation and acetylation and subsequent demethylation and  
30 deacetylation in the cell wall. In the last decade, our comprehension of plant polysaccharides  
31 acetylation has increased significantly thanks to the study of the TRICHOME  
32 BIREFRINGENCE-LIKE (TBL) protein family. TBLs are mostly described as Golgi-localized

33 acetyltransferases specifically targeting diverse hemicelluloses or pectins. Various *tbl* mutants  
34 showed altered wall mechanical properties and dynamics. Here, we study *TBL38* that is co-  
35 expressed with *PECTIN METHYLESTERASE INHIBITOR6 (PMEI6)* and *PEROXIDASE 36*  
36 (*PRX36*) during the development of Arabidopsis seed mucilage secretory cells (MSCs). We  
37 demonstrate the atypical TBL38 cell wall localization restricted to the PMEI6/PRX36 MSC cell  
38 wall microdomain. A *tbl38* mutant displays an intriguing homogalacturonan immunological  
39 phenotype in this cell wall microdomain and in a MSC surface-enriched abrasion powder. This  
40 fraction was further characterized by mass spectrometry oligosaccharide profiling revealing an  
41 increased homogalacturonan acetylation phenotype. Finally, a recombinant TBL38 is shown to  
42 display pectin acetylerase activity *in vitro*. These results indicate that TBL38 is an atypical  
43 cell wall-localized TBL that displays a homogalacturonan acetylerase activity rather than a  
44 Golgi-localized acetyltransferase activity as observed in previously studied TBLs. TBL38  
45 function during seed development is discussed.

46

47 **Key words:** *Arabidopsis thaliana* seed development, homogalacturonan acetylerase,  
48 mucilage secretory cell, pectin maturation, plant cell wall microdomain remodeling

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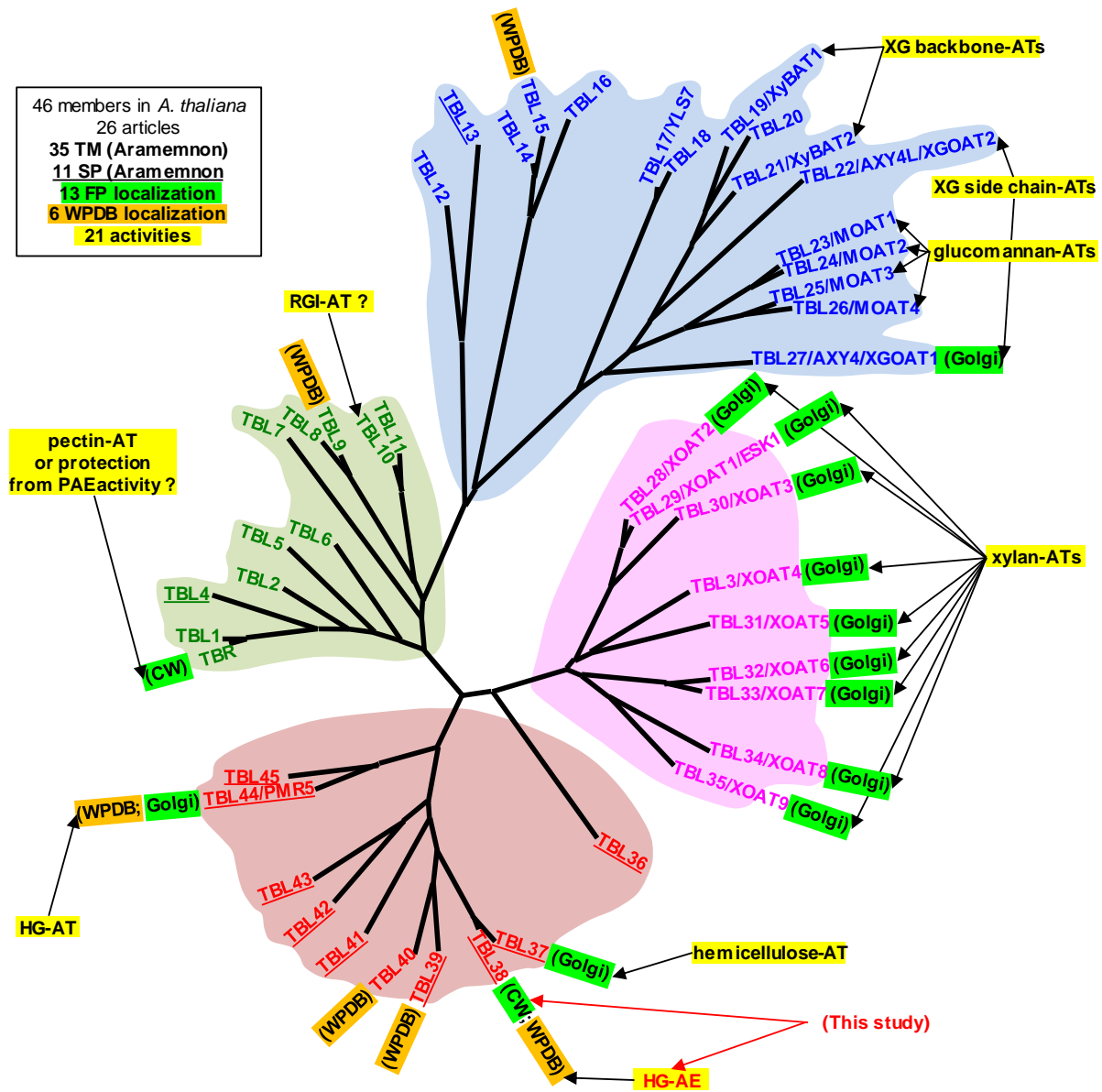
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## 66 **Introduction**

67 Plant cells specifically design, synthesize and constantly remodel their cell wall (CW) to  
68 achieve proper cell shape and function along development to face environmental constraints.  
69 Such dynamics lead to specific composition and properties of CW polymers. Accumulating  
70 evidence of chemical group modifications on particular polysaccharides involved in key  
71 developmental processes can be found in the literature. The importance of pectin  
72 methylesterification dynamics has been largely studied, particularly for homogalacturonans  
73 (HGs). In the Golgi apparatus, HGs are methylated on the COOH of galacturonic acids (GalA)  
74 through methyl-transferase activity (Driouich et al., 2012) and later undergo additional  
75 remodeling once in the CW. Methylated HG can be demethylesterified by pectin methyl  
76 esterases (PMEs) whose activity can be regulated by PME inhibitors (PMEIs). The joint activity  
77 of both enzyme categories is the source of many developmental roles (Wolf et al., 2009).  
78 Additionally, *O*-acetylation of CW polymers such as pectins and hemicelluloses in the Golgi  
79 lumen has been described in multiple studies highlighting three groups of proteins involved in  
80 this process (Gille and Pauly, 2012). In *Arabidopsis thaliana*, (i) ALTERED XYLOGLUCAN  
81 9 (AXY9) is encoded by a unique gene likely indirectly responsible for the non-specific  
82 acetylation of xyloglucan and xylan hemicelluloses (Schultink et al., 2015) leading to smaller  
83 plants when mutated. (ii) The REDUCED WALL *O*-ACETYLATION (RWA) family has four  
84 members in *A. thaliana* which also participate in the *O*-acetylation machinery of xylans,  
85 mannans, xyloglucans and pectins (Manabe et al., 2011; Manabe et al., 2013). (iii) The  
86 TRICHOME BIREFRINGENCE-LIKE (TBL) family comprises 46 members distributed in  
87 four phylogenetic clades in *A. thaliana* that target specific CW polysaccharides (**Figure 1; See**  
88 **Supplemental Table 1**). Originally named based on the observed changes in the birefringence  
89 properties of a mutant of *TRICHOME BIREFRINGENCE* (*TBR*) (Potikha and Delmer, 1995),  
90 21 TBLs have been characterized for their *O*-acetyltransferase activity specific for the  
91 xyloglucan backbone and side chains, xylans, mannans, rhamnogalacturonan I (RG-I) or HGs  
92 (**Figure 1; Supplemental Table 1**, reviewed in (Gille and Pauly, 2012; Pauly and Ramírez,  
93 2018). These activities are highly clade-specific since nine TBLs belonging to a similar clade  
94 displayed xylan-acetyltransferase activity (**Figure 1, Supplemental Table 1**).



95

96 **Figure 1: The TBL family mostly comprises Golgi-localized acetyltransferases specifically acting on various**  
 97 **CW polymers.** The 46 members show high sequence homology and two conserved GDSL and DXXH motifs  
 98 associated with esterase activities. Previous work showed that the esterase activity is not targeted on CW polymers  
 99 but rather allows the formation of an acyl-enzyme intermediate, and described multiple TBLs as participating in  
 100 *O*-acetylation of a wide range of CW polymers in the Golgi with the notable exception of TBR which was  
 101 positioned to the CW. Twenty one recombinant enzymatic activities (yellow), 13 fluorescent protein (FP)  
 102 localization (green) and 6 CW proteome occurrence (<https://www.polebio.lrsv-ups-tlse.fr/WallProtDB/>)  
 103 were reported. <http://aramemnon.uni-koeln.de/> predicts that the N-terminus hydrophobic domains either acts as a  
 104 transmembrane (TM) domain (35 non-underlined proteins) or as a cleaved signal peptide (11 underlined proteins).  
 105 So far, each TBL or TBL phylogenetic cluster seems to specifically act on a CW polysaccharide which assumes  
 106 various roles in development. The phylogenetic tree was adapted from (Bischoff et al., 2010). AE, acylesterase;  
 107 AT, acetyltransferase; PAE, pectin acetyl esterase; HG, homogalacturonan; RGI, rhamnogalacturonan I; XG,  
 108 xyloglucan. See **Supplemental Table 1** for detailed information and references. This study reports on TBL38  
 109 peculiar localization and activity.

110

111

112 Mutations on particular *TBLs* led to (i) defect in CW structure and overall plant development  
113 as observed in *tbr*, *tbl3*, *tbl29* and *tbl37* (Bischoff et al., 2010; Lefebvre et al., 2011; Sun et al.,  
114 2020), and (ii) increased resistance to drought stress (Stranne et al., 2018) or pathogen infection  
115 (Chiniquy et al., 2019) in *tbl10* and *tbl44/pmr5*, respectively (**Supplemental Table 1**). Like  
116 AXY9, TBLs have the conserved GDSL domain which is typically associated with esterase  
117 activity/lipase (Akoh et al., 2004; Lai et al., 2017), and a DXXH motif also found in a  
118 rhamnogalacturonan acetyltransferase of *Aspergillus aculeatus* (Molgaard and Larsen, 2004). The  
119 TBL acetyltransferase activity allows the formation of an acyl-enzyme intermediate necessary for  
120 the *O*-acetyltransferase activity targeting various CW polymers (Gille and Pauly, 2012; Lunin  
121 et al., 2020) (**Figure 1, Supplemental Table 1**). Out of the 23 characterized TBLs, 12 have  
122 been localized to the Golgi alongside with AXY9 and RWAs, supporting their role as *O*-  
123 acetyltransferases (**Figure 1, Supplemental Table 1**). Surprisingly, TBR was localized to the  
124 CW (Sinclair et al., 2017). Yet, although its precise activity is still unknown, an overall decrease  
125 in pectin acetylation was observed in *tbr*, suggesting an acetyltransferase activity that is  
126 surprising considering that the existence of a CW-localized acetyl donor is unclear (Sinclair et  
127 al., 2017). *In silico* prediction of the targeting function of the N-terminal hydrophobic sequence  
128 sorted the 46 TBLs in two groups: 35 with a transmembrane anchoring domain, and 11 with a  
129 cleavable signal peptide. Six TBLs have been identified in CW-enriched fractions without clear  
130 relationship with the *in silico* targeting predictions (**Supplemental Table 1**). Overall,  
131 experimental evidence depicts TBLs as acetyltransferases forming an acyl-enzyme intermediate  
132 to further perform an *O*-acetyltransferase activity targeted on various CW polymers during their  
133 synthesis in the Golgi lumen.

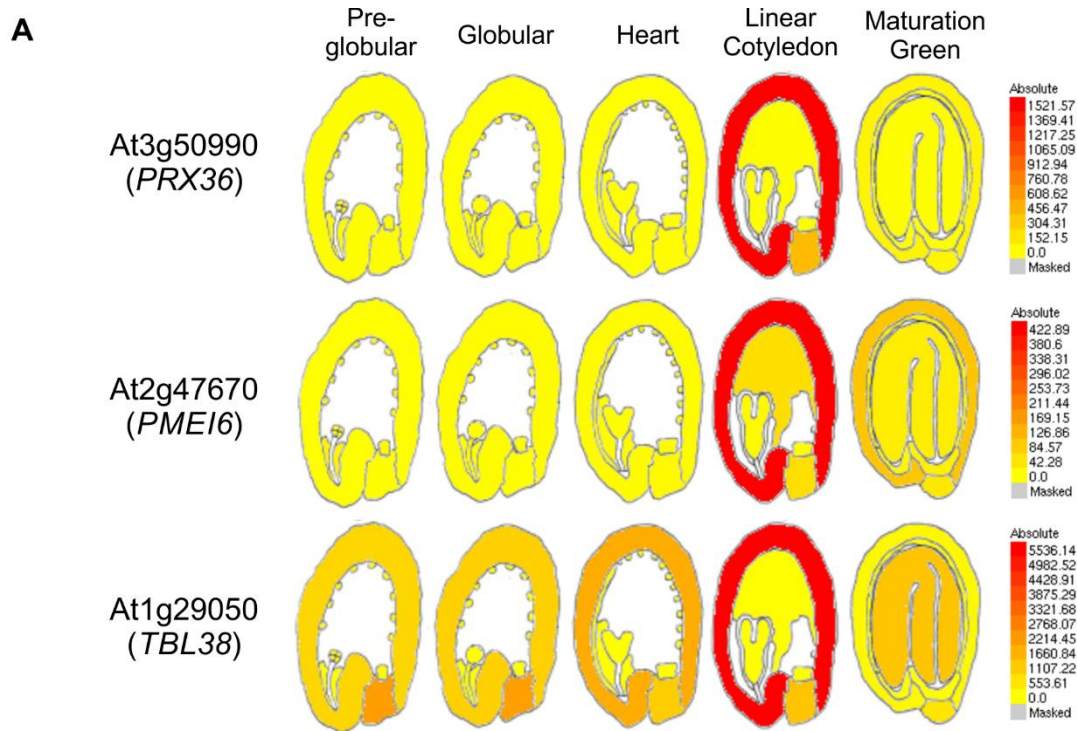
134 Initially, we screened genes involved in *A. thaliana* seed mucilage release with particular  
135 interest on candidates that could modify the esterification status of HGs. The mucilage is a  
136 polysaccharidic hydrogel synthesized by the seed mucilage secretory cells (MSCs) in the outer  
137 most cell layer of the seed coat of the so-called myxospermous species (Francoz et al., 2015;  
138 Viudes et al., 2021; Western, 2012). During *A. thaliana* seed maturation, the HG  
139 methylesterification status is modified through the PECTIN METHYLESTERASE  
140 INHIBITOR6 6 (PMEI6) activity on an unknown PME in a CW microdomain at the top of the  
141 radial primary wall of MSCs, leading to a HG partially methylesterified pattern (Francoz et al.,  
142 2019b; Saez-Aguayo et al., 2013). This pattern acts as an anchoring platform for  
143 PEROXIDASE36 (PRX36) which then induces a local weakening and thinning of the CW  
144 microdomain (Francoz et al., 2019b; Kunieda et al., 2013). After seed inhibition, the swelling

145 mucilage breaks the pre-weakened CW microdomain and is extruded around the seed. We  
146 identified *TBL38* (*At1g29050*) for its high co-expression level with *PRX36* and *PMEI6* during  
147 *A. thaliana* seed coat development. Based on the literature cited above and considering its co-  
148 expression with *PMEI6*, we first hypothesized TBL38 to be a Golgi localized protein acting as  
149 an acetyltransferase onto CW polymers possibly including HGs. Hereafter, we examined  
150 TBL38 localization, activity and putative role in MSCs and uncovered that TBL38 is an atypical  
151 CW-localized HG acetyltransferase rather than a Golgi-localized acetyltransferase. Although  
152 TBLs are not thought to influence the methylesterification status of CW polymers, we  
153 investigated the putative indirect role of TBL38-dependent HG *O*-deacetylation on the HG  
154 methylation status and on PRX36 anchoring, thus adding another layer of complexity on the  
155 fine tuning of HG remodeling during plant development.

## 156 **Results**

### 157 ***TBL38* is co-expressed with *PRX36* and *PMEI6* in mucilage secretory cells at** 158 **intermediate developmental stages of seed development**

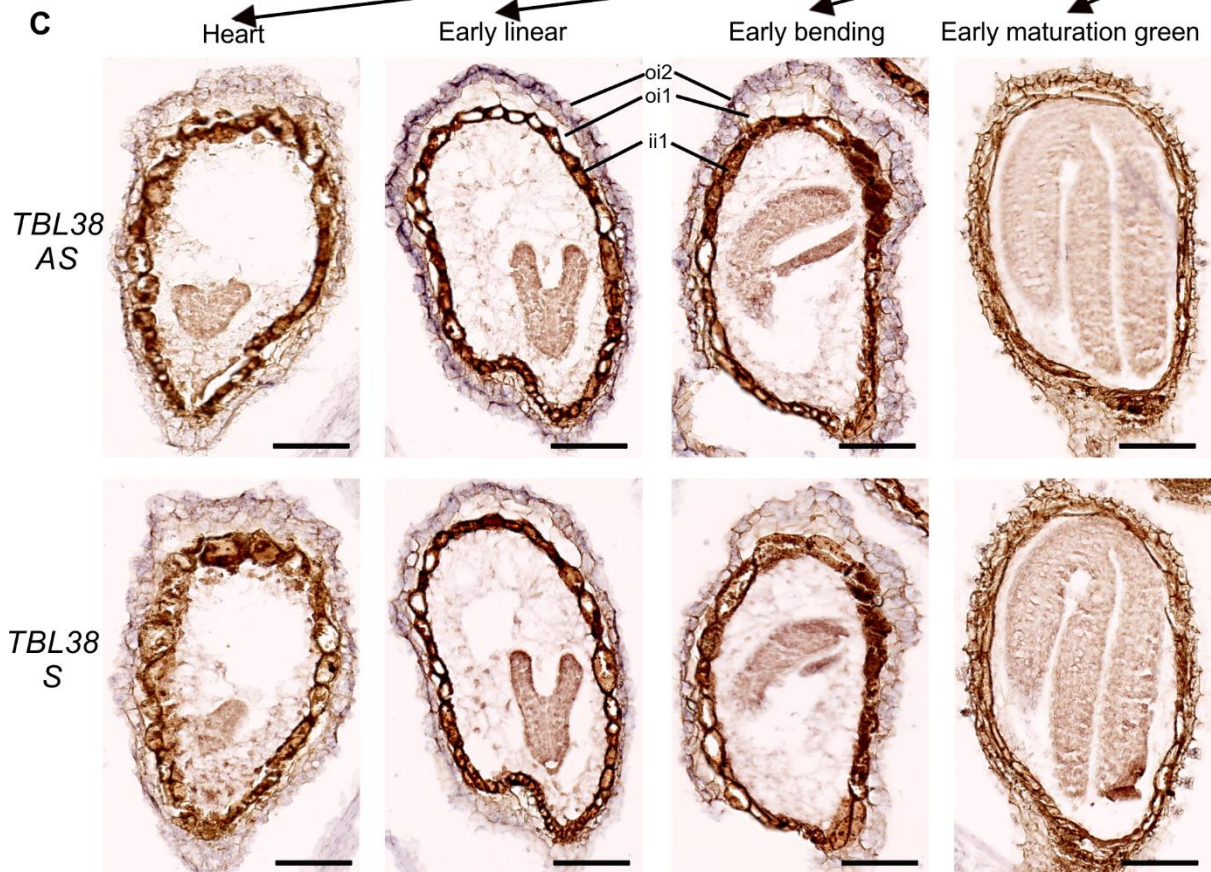
159 Using the tissue-specific seed developmental kinetics GSE12404 dataset (Belmonte et al.,  
160 2013), we previously built a co-expression network centered on *PRX36* in which *PMEI6* came  
161 as the second hit (Francoz et al., 2019b) (**Supplemental Table 2**). We noticed the high co-  
162 expression of *TBL38* which was ranked 13 in this network with a strong specific seed coat  
163 expression at the linear cotyledon stage (**Figure 2A-B; Supplemental Table 2**). We filtered  
164 *TBL* expression data which was available for 34 out of the 46 *TBLs*, although only 23 were  
165 above the detection limit, and confirmed that *TBL38* was likely to be the only *TBL* highly co-  
166 expressed with *PRX36* and *PMEI6* (**Supplemental Table 2**). The seed coat transcriptomic data  
167 encompasses five cell layers: ii1 (inner integument), ii1', ii2, oi1 (outer integument), and oi2,  
168 commonly addressed here as MSCs (Debeaujon et al., 2003; Francoz et al., 2015).



**B**

RK	PCC	Gene Symbol	pre-globular stage					globular stage					heart stage					linear-cotyledon stage					bending cotyledon stage					mature green stage																
			ro EP	ro MCE	ro PEN	ro CZEN	ro CZSC	ro SC	ro WS	g EP	g SUS	g MOEN	g PEN	g CZEN	g CZSC	g SC	g WS	h EP	h MOEN	h PEN	h CZEN	h CZSC	h SC	h WS	lc EP	lc MOEN	lc PEN	lc CZEN	lc CZSC	lc SC	lc WS	bc EP	bc MOEN	bc PEN	bc CZEN	bc CZSC	bc SC	bc WS	mg EP	mg MOEN	mg PEN	mg CZEN	mg CZSC	mg SC
1	1.0000	<i>PRX36</i>	15	27	4	11	8	8	2	4	2	13	2	12	8	2	4	3	3	3	15	14	3	9	4	5	9	12	405	1592	554	1	2	27	392	29	20	7	1	11	3	53	65	9
2	0.9474	<i>PME16</i>	2	15	7	8	2	1	6	2	1	2	1	2	2	6	6	2	8	8	4	10	6	13	10	54	54	1	52	437	236	6	34	14	232	65	52	29	11	19	25	37	93	34
13	0.8160	<i>TBL38</i>	9	11	25	34	2119	878	1094	10	5	81	26	2	2076	1003	1508	8	9	8	55	1064	1790	1012	16	47	7	17	1274	8597	2991	44	1	12731	894	312	1262	10	31	9	582	135	476	

**C**





170 **Figure 2: *TBL38* is co-expressed with *PRX36* and *PMEI6* in the seed coat and transcript localization of**  
171 ***TBL38* is tied to the mucilage secretory cells (outer integument 2).** (A) eFP browser pictogram displaying the  
172 similarity in the spatiotemporal expression of *PRX36*, *PMEI6* and *TBL38* in *A. thaliana* seed coat. Absolute tissue-  
173 specific expression level is represented in color variation from yellow (lowest) to red (highest). Adapted from  
174 <http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi>. (B) *PRX36* co-expression network using the same dataset  
175 highlighting the similar profiles of *PRX36*, *PMEI6* and *TBL38* (see **Supplemental Table 2** for details). (C) *In situ*  
176 hybridization of dig-labeled RNA probes targeting *TBL38* on paraffin sections of developing wild-type seeds.  
177 Anti-sense probes showed highest purple signal in the outermost layer corresponding to MSCs between the early  
178 linear and early bending developmental stages. These signals should be subtracted from the overall background  
179 obtained on serial sections of the same seeds with the sense probe control and natural pigmentation. Bars: 200  $\mu$ m.

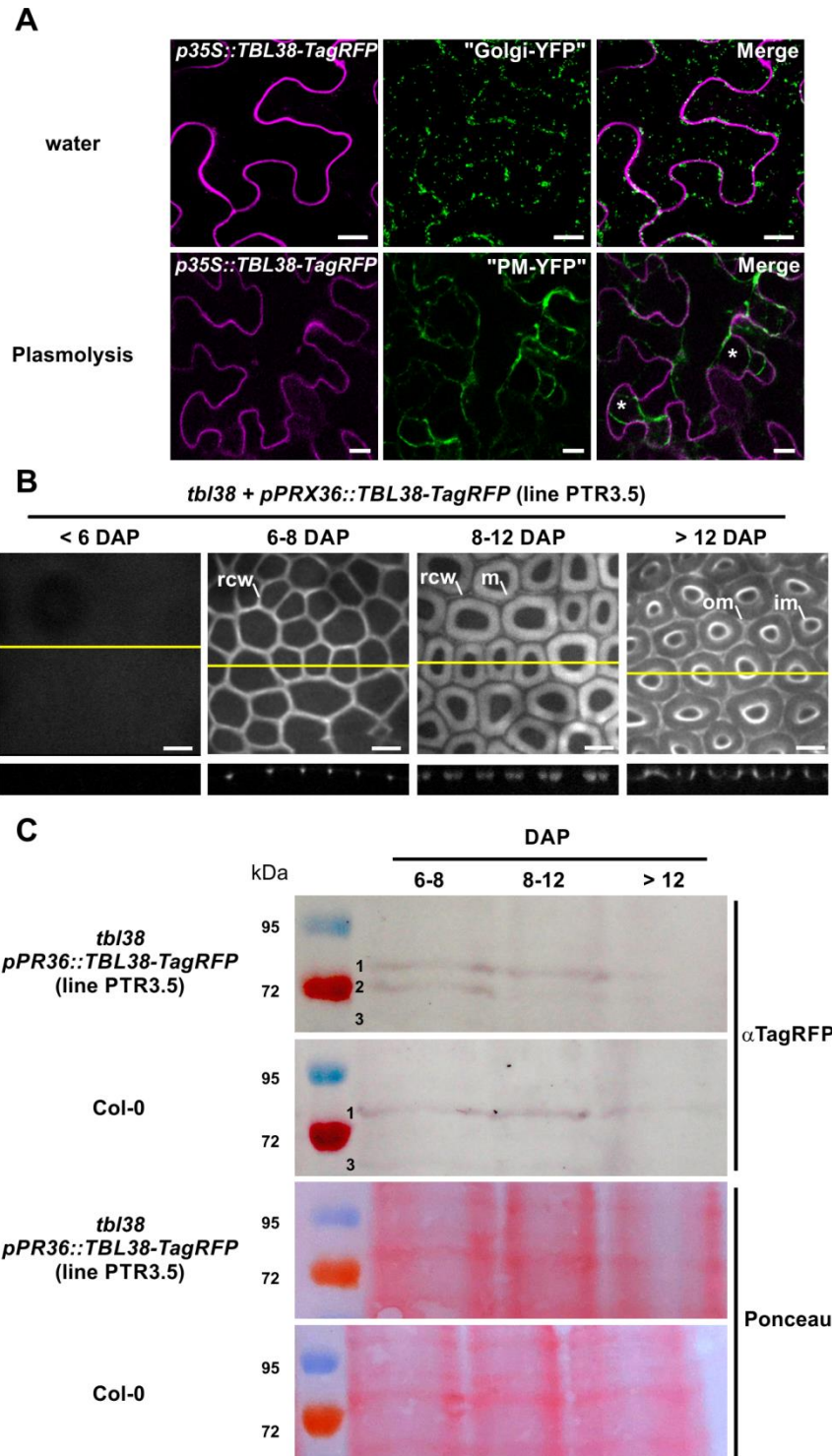
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181 Therefore, we determined MSC-specific *TBL38* expression using *in situ* hybridization on tissue  
182 array paraffin sections encompassing the whole developmental kinetics of wild-type seeds.  
183 *TBL38* anti-sense probes displayed a clear purple/violet hybridization signal in MSCs,  
184 particularly between the early linear and early bending cotyledon developmental stages (**Figure**  
185 **2C**). No such signal could be observed at earlier and later stages with the anti-sense probe and  
186 at all developmental stages with the sense probe used as negative control to account for the  
187 overall non-specific background and natural pigmentation. We could associate the specific  
188 *TBL38* anti-sense probe signal to MSCs excluding the chalazal tissues, in agreement with -and  
189 refining- the transcriptomic data (**Figure 2A-C**).

190

### 191 ***TBL38*-TagRFP is transiently localized to the *PRX36*-*PMEI6* outer periclinal/radial cell** 192 **wall microdomain**

193 We generated a *p35S::TBL38-TagRFP* construct to transiently transform *N. benthamiana*  
194 leaves. The *TBL38*-TagRFP fluorescence did not colocalize with a Golgi-YFP marker, but  
195 rather displayed a cellular delineation (**Figure 3A**). Under plasmolysis conditions, we could  
196 clearly attribute the *TBL38*-TagRFP signal to the CW separating from the receding plasma  
197 membrane YFP marker signals (**Figure 3A**). We concluded that *TBL38* is a CW-localized  
198 protein similarly to TBR and contrarily to the 12 other Golgi-localized TBLs (Sinclair et al.,  
199 2017) (**Figure 1, Supplemental Table 1**). We then moved on to assess protein localization in  
200 *A. thaliana* seed MSCs. As seed development advances, MSCs become mostly made of  
201 different CWs, namely the primary CW, the mucilage and the columella (Francoz et al., 2015).  
202 Within MSCs, increasing evidence have positioned CW proteins in only one or two types of  
203 CW (Francoz et al., 2019b; McGee et al., 2019; Sola et al., 2019) or in sub-layer (microdomain)  
204 of one CW (Dauphin et al., 2022; Francoz et al., 2019b).



205

206 **Figure 3: TBL38 is a cell wall protein transiently localized to the radial primary CW microdomain of MSCs.**  
 207 (A) Confocal microscopy visualization of both TBL38-TagRFP and a Golgi-YFP marker transiently  
 208 overexpressed in *N. benthamiana* leaves indicates that TBL38 is not a Golgi-localized protein. Under plasmolysis,  
 209 the TBL38-TagRFP signal and the plasma membrane-YFP marker signal are separated, indicating the localization  
 210 of TBL38-TagRFP to the CW. First row images are extracted from a maximum projection of 17 stacks. Second  
 211 row images are a representative view of a single confocal plan. Bars: 10  $\mu$ m. (B) Confocal spinning disk  
 212 observations of TBL38-TagRFP in *A. thaliana* developing seeds. At the onset of *pPRX36* activity (Francoz et al.,  
 213 2019b), TBL38-TagRFP fluorescence was positioned to the radial primary CW of MSCs. Orthogonal projection  
 214 refined this localization to the CW microdomain consisting in the top of the radial primary CW similarly to PRX36-  
 215 TagRFP (Francoz et al., 2019b). However, contrary to the previously observed stable localization of PRX36-  
 216 TagRFP along seed development, TBL38-TagRFP fluorescence was delocalized to the future mucilage pocket at

217 8-12 DAP and eventually to the outer and inner margins of the mucilage-filled pocket at > 12 DAP. Sum intensity  
218 projections were done using similarly sized projection of 30 Z stacks with no singular edition. rcw, radial CW  
219 microdomain; m, mucilage pocket; im and om, inner and outer margins of mucilage pocket, respectively. Bars: 25  
220  $\mu\text{m}$ . (see **Supplemental Figure 1** for full kinetics performed on three individual transformed lines). (C)  $\alpha\text{Tag}$ -  
221 RFP western blot performed using total protein extracts from developing seeds harvested at stages corresponding  
222 to the three sequential patterns of fluorescence shows that the band #2 corresponding to the TBL38-TagRFP fusion  
223 protein is only seen at the early stage when TBL38-TagRFP is localized to the CW microdomain. Non-specific  
224 bands #1 and #3 are also present in Col-0 samples (see **Supplemental Figure 2** for full membrane visualization).

225

226 To localize TBL38 in *A. thaliana* MSCs, we generated a *pPRX36:TBL38-TagRFP* construct  
227 which was stably transformed in *tbl38* plants. We localized TBL38-TagRFP to the radial  
228 primary CW of MSCs at the onset of *pPRX36* activity at early developmental stages (**Figure**  
229 **3B; Supplemental Figure 1**). Orthogonal projection allowed to refine TBL38-positioning at  
230 the top of the radial primary CW, corresponding to the CW microdomain encompassing PRX36  
231 (Francoz et al., 2019b). Intriguingly, while PRX36-TagRFP was stably accumulated at this  
232 location using the same promoter (Francoz et al., 2019b), TBL38-TagRFP appeared to be  
233 naturally delocalized to the future mucilage pocket and eventually pushed toward the inner and  
234 outer margins of the pocket when filled with mucilage after 12 DAP (**Figure 3B; Supplemental**  
235 **Figure 1**).

236 We accurately sampled developing seeds at stages corresponding to the three above mentioned  
237 fluorescence patterns and performed a  $\alpha\text{TagRFP}$  western blot (**Figure 3C; Supplemental**  
238 **Figure 2**). Excluding the non-specific bands (#1 and 3) also observed with similarly staged  
239 Col-0 seeds, a single specific band (#2) corresponding to the apparent molecular mass of  
240 TBL38-TagRFP fusion protein (70 kDa) was only observed at 6-8 DAP when TBL38-TagRFP  
241 was localized to the CW microdomain. The absence of such specific band when the  
242 fluorescence was later localized to the mucilage pocket suggests that this additional  
243 fluorescence pattern rather corresponds to the delocalized TagRFP possibly cleaved from  
244 TBL38. This hypothesis is strengthened by the appearance of a 28 kDa band that may  
245 correspond to cleaved TagRFP at 8-12 DAP that unfortunately migrated with an additional non-  
246 specific band of similar molecular mass also observed in Col-0 after 12 DAP (**Supplemental**  
247 **Figure 2**).

248 Altogether, expression of TBL38-TagRFP in both *N. benthamiana* and *A. thaliana* showed an  
249 unexpected CW localization making TBL38 the second TBL localized to the CW with TBR.  
250 Because its localization to the outer radial CW microdomain was strikingly similar to that of  
251 PRX36, we investigated TBL38 involvement in the above mentioned PRX36-PMEI6 molecular  
252 module and particularly its influence on HG modification.

253

254 **The mucilage release and the anchoring of PRX36 are not significantly modified in *tbl38***

255 Mutation in either *PRX36* or *PMEI6* results in impaired mucilage release because the primary  
256 CW microdomain improperly brakes up upon seed imbibition (Francoz et al., 2019b; Kunieda  
257 et al., 2013; Saez-Aguayo et al., 2013). Because *TBL38* and *PRX36* gene products have similar  
258 spatio-temporal expression and positioning, we assessed whether mucilage release was affected  
259 in mutated seeds. We isolated a homozygous KO line for *tbl38* (**Supplemental Figure 3**) and  
260 used it for comparison with Col-0 wild type seeds using ruthenium red staining of adherent  
261 mucilage. Visually, we did not see any differences between WT and *tbl38* seeds (**Supplemental**  
262 **Figure 4A**), contrarily to impaired mucilage previously observed in *prx36* or *pmei6* (Francoz  
263 et al., 2019b; Kunieda et al., 2013; Saez-Aguayo et al., 2013). Quantification of adherent  
264 mucilage area and circularity on > 1,000 seeds confirmed the similarity of the patterns of Col-  
265 0 and *tbl38* (**Supplemental Figure 4B**). We did not see any obvious change in mucilage  
266 staining intensity as sometimes observed in mutated background responsible for mucilage  
267 modification (Turbant et al., 2016).

268 Since PRX36 anchoring to a specific HG platform and subsequent CW weakening are required  
269 for proper mucilage release, we investigated PRX36 positioning in *tbl38*. For this, we used the  
270  $\alpha$ PRX36 antibody on paraffin sections of developing seeds from Col-0 or *tbl38*. Anti-PRX36  
271 similarly labeled the top of the radial primary CW in Col-0 and *tbl38* at all developmental stages  
272 (**Supplemental Figure 4C**). *tbl38* did not show the loss of signal previously observed in *prx36*  
273 or *pmei6* (Francoz et al., 2019b). To further validate this result, we generated a  
274 *pPRX36::PRX36-TagRFP* construct, which we expressed in *prx36* and *tbl38* backgrounds.  
275 Observation of the PRX36-TagRFP signal was made on developing seeds aged from 6-to-12  
276 DAP, a period which encompasses the onset of the *PRX36* promoter activity up to the last stage  
277 of the clearly visible PRX36-TagRFP fluorescence signal (Francoz et al., 2019b). PRX36-  
278 TagRFP showed very similar signal localization at the top of the radial primary CW in both  
279 *tbl38* and *prx36* (**Supplemental Figure 4D**). Again, no delocalization of the signal was  
280 observed in *tbl38* contrary to the PRX36-TagRFP delocalization in the mucilage pocket  
281 previously observed in *pmei6* (Francoz et al., 2019b).

282 So far, the particular PMEI6-specific HG methylation pattern that exists at the top of the radial  
283 primary CW linked proper mucilage release with PRX36 anchoring (Francoz et al., 2019b).  
284 Our results did not show such a relationship between TBL38, mucilage release and PRX36

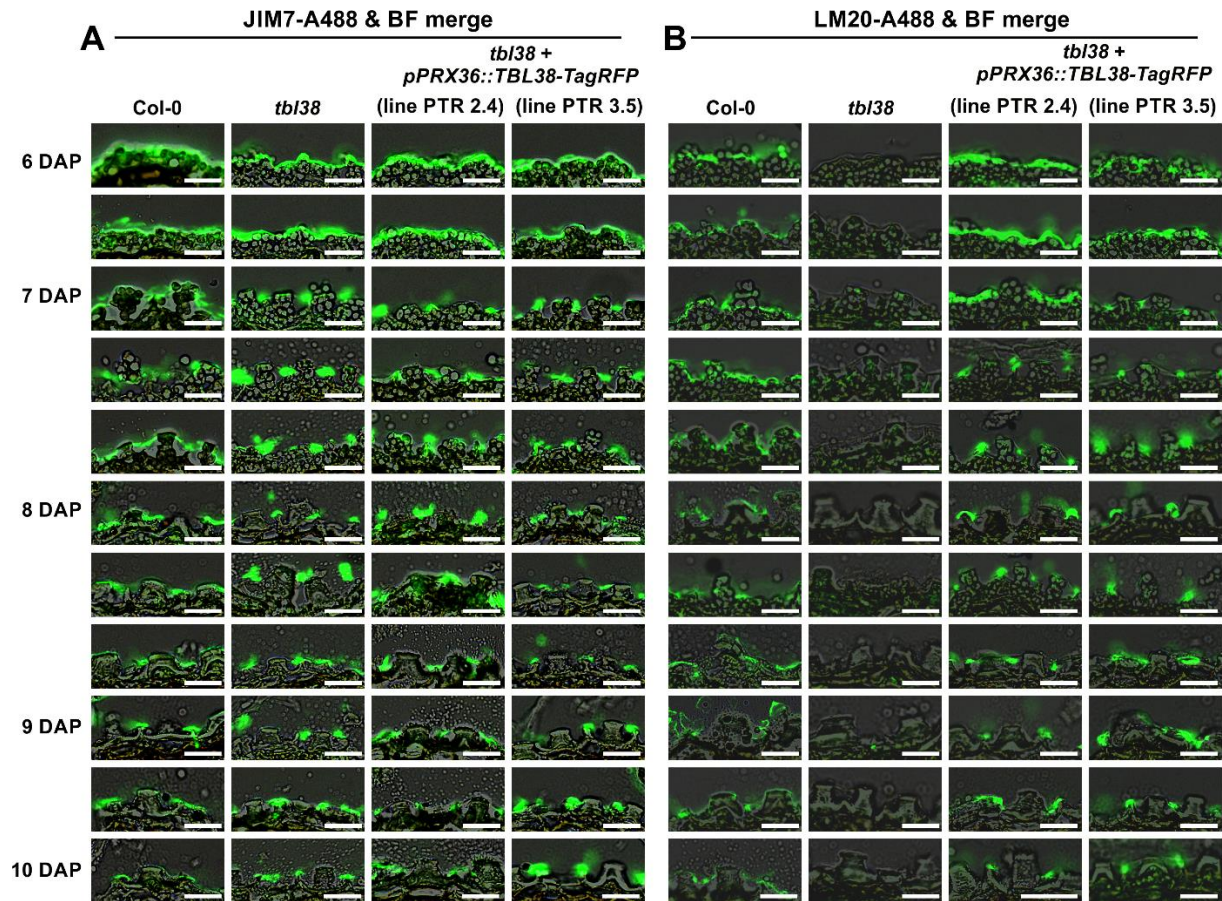
285 positioning, suggesting that no changes in the HG methylation pattern occur in *tbl38*. However,  
286 TBL38 could be involved in acetylation of CW polymers similarly to other described TBLs.  
287 Because changes in HG acetylation could lead to different HG methylesterification status,  
288 possibly through indirect modulation of PME activity or hindering the dimerization of pectin  
289 chains (Kohn and Furda, 1968; Ralet et al., 2003), we first studied the HG methylation pattern  
290 in *tbl38*.

291

292 **The partially methylated homogalacturonan JIM7 epitope corresponding to the PRX36-**  
293 **PMEI6 CW microdomain is maintained in *tbl38*, while the supposed similar LM20 epitope**  
294 **is surprisingly lost**

295 The specific HG platform enabling PRX36 anchoring, is generated through the inhibition of an  
296 unknown PME by PMEI6 leading to a partially methylesterified pattern in HGs at the top of  
297 the radial primary CW. This molecular pattern could be labeled by both JIM7 and LM20, two  
298 monoclonal antibodies routinely used to characterize partially methylesterified HGs, and these  
299 labelings were lost in *pmei6* (Francoz et al., 2019b). Thus, we incubated JIM7 and LM20 on  
300 paraffin sections of developing seeds from *tbl38* and Col-0.

301 As a control, JIM7 signals were correctly positioned at the top of the radial primary CW in Col-  
302 0 developmental kinetics, particularly as MSC development advanced to later stages (**Figure**  
303 **4A**). Very similar JIM7 labeling was observed in *tbl38* (**Figure 4A**) contrary to the loss of  
304 signal previously observed in *pmei6* (Francoz et al., 2019b). A similar pattern was observed  
305 with LM20 along the developmental kinetics of the Col-0 control genotype (**Figure 4B**).  
306 However, the LM20 labeling was intriguingly lost in *tbl38* (**Figure 4B**). This was related to the  
307 *tbl38* mutation since LM20 labeling was restored along the developmental kinetics of *tbl38*  
308 plants complemented with a *pPRX36::TBL38-TagRFP* construct (**Figure 4B**). While both  
309 antibodies are generally used in parallel to similarly label partially methylesterified HG, their  
310 partially characterized epitopes may be somehow different (Clausen et al., 2003;  
311 Verhertbruggen et al., 2009). Overall, we concluded that *tbl38* mutation resulted in lack of the  
312 LM20 signal, but not of that of JIM7. Yet, it was still unclear if this could be due to putative  
313 changes in the acetylation of HGs which may prevent LM20 binding through steric hindrance,  
314 or to changes in HG methylation as indirect effect of acetylation. Regardless, we sought to  
315 investigate TBL38 biochemical function in MSCs.



317 **Figure 4: LM20 but not JIM7 labeling is lost in *tbl38*.** (A) In Col-0, JIM7 labeling of partially methylesterified  
318 HG is visible as early as 6 DAP at the MSC surface and is restricted to the top of the radial primary CW  
319 microdomain of more mature MSCs (7-10 DAP). A very similar labeling pattern occurs in *tbl38* as well as in two  
320 complemented lines (*tbl38* transformed with *pPRX36::TBL38-TagRFP*). (B) LM20 was previously thought to bind  
321 to similar epitopes as JIM7 in MSCs since both epitopes were lost in *pmei6* (Francoz et al., 2019b). However,  
322 contrary to JIM7, the LM20 labeling at the top of the radial primary CW microdomain was lost in *tbl38* and  
323 restored in the two tested complemented lines. 40 x scans of fluorescence and brightfield channels were merged.  
324 The same parameters were used to analyze each image which are a representative of  $n > 20$  MSCs at each  
325 developmental stage. Additional stages positioned between the staged ranks are shown. Bars: 25 $\mu$ m.

326

### 327 **TBL38 displays an atypical acetylsterase activity towards CW polymers**

328 We took advantage of MSC surface accessibility to generate a MSC surface-enriched CW  
329 abrasion powder from dry seeds using home-made abrasion columns (see **Methods**). The  
330 resulting homogeneous powder (about 1 mg per 50 mg seeds) was collected through a nylon  
331 mesh and subsequently analyzed (**Supplemental Figure 5A-F**). We confirmed that the  
332 abrasion process was restricted to the MSC surface as MSC CW autofluorescence was strongly  
333 reduced but could still be barely observed in abraded seeds (**Supplemental Figure 5G-H**)  
334 which released residual amount of adherent mucilage when imbibed (**Supplemental Figure 5I-**  
335 **J**). Although the overall MSC surface morphology was affected, we could still see the base of  
336 broken columella and radial CW (**Supplemental Figure 5M-N**). We successfully recovered a

337 surface-abraded powder containing the top of the radial primary wall, considering the loss of  
338 LM20 signal in Col-0 abraded seeds (**Supplemental Figure 5K-L**). We validated the method  
339 since JIM7 and LM20 dot blot signal intensities in *pmei6* were both about 5 % as compared to  
340 Col-0 (**Figure 5A, B**), in agreement with the previously observed loss of JIM7 and LM20  
341 immunofluorescence signals in development kinetics of *pmei6* MSCs (Francoz et al., 2019b).  
342 Again, in agreement with the present immunofluorescence study on development kinetics  
343 (**Figure 4**), the JIM7 signal intensity from dry seed surface abraded fractions was very similar  
344 in all genotypes except *pmei6*, since *tbl38* and three complemented lines all showed 91-95 %  
345 of Col-0 intensity (**Figure 5A**). However, the LM20 signal intensity in *tbl38* was only 4 % of  
346 that in Col-0 control while the three complemented lines showed a strong -though partial- signal  
347 restoration with 66-79 % of Col-0 intensity (**Figure 5B**).

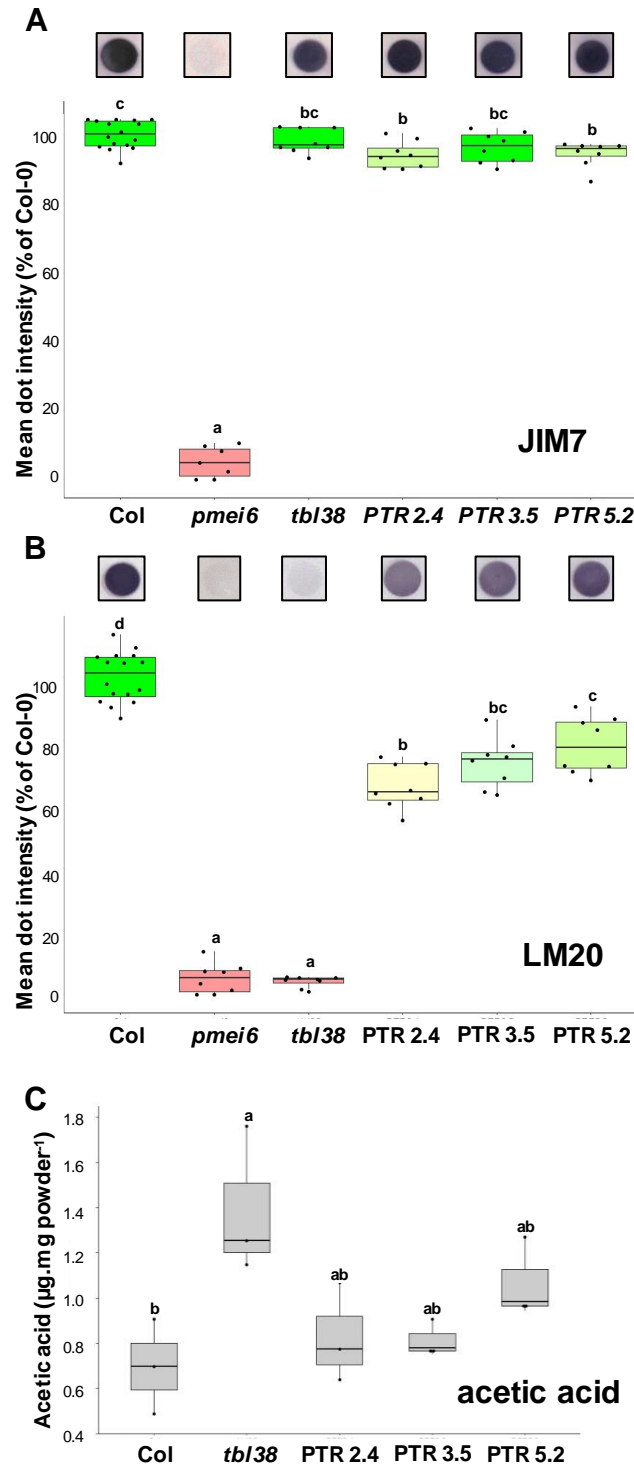
348 So far, all the described TBLs were either directly characterized as acetyltransferases or  
349 associated with lower acetylation content of CW polymers in mutated backgrounds  
350 (**Supplemental Table 1**). Therefore, we quantified the acetylation of CW polymers using the  
351 surface-enriched CW abrasion powders. Surprisingly, the mean amount of released acetic acid  
352 in *tbl38* was 199 % of that in Col-0 and the level dropped back to 117-153 % of that of Col-0  
353 in the three complemented lines (**Figure 5C**). This suggested that TBL38 activity was akin to  
354 an acylesterase rather than the previously described acetyltransferase of other characterized  
355 TBLs. Because the increased acetylation level in *tbl38* could be achieved through acetylation  
356 of multiple polymers, we sought to identify TBL38 substrates in these fractions.

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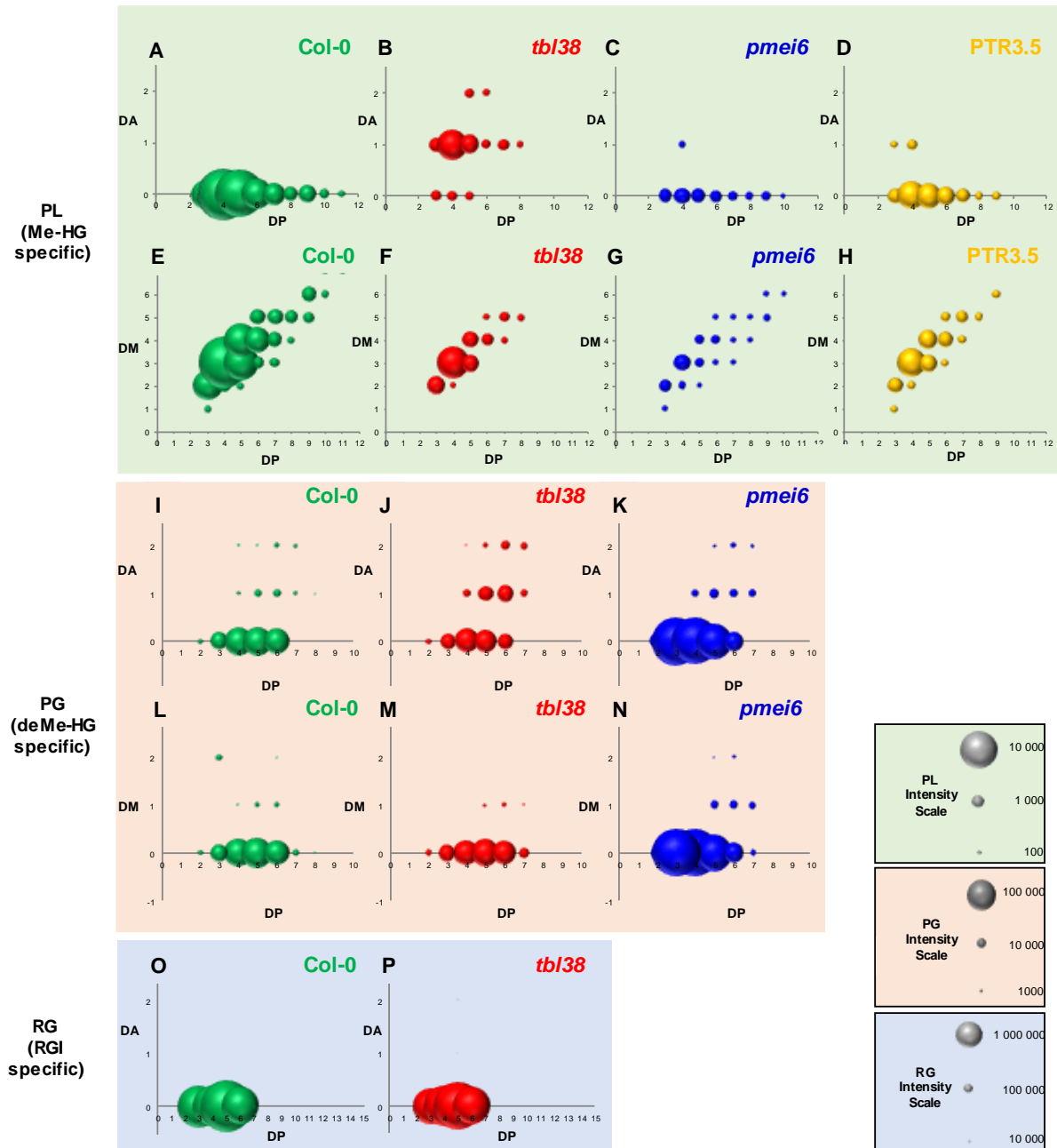
360



361

362 **Figure 5: Dry seed surface enriched fraction shows a decreased LM20 epitope signal intensity and an**  
 363 **increased acetic acid content in *tbl38* compared to Col-0, and the wild type phenotype is restored in**  
 364 **complemented lines. JIM7 (A) and LM20 (B) immuno dot blot of dry seed surface CW-enriched fraction from**  
 365 **Col-0, *pmei6*, *tbl38* and three *tbl38* complemented lines expressing *pPRX36*:TBL38-TagRFP (PTR2.4, 3.5 and**  
 366 **5.2). On top of the panels are shown screen shots of individual dots from the same nitrocellulose membrane. On**  
 367 **the bottom of the panel are shown box plot representations of the data analysis. (C) Box plot representation of the**  
 368 **acetic acid content released following acid hydrolysis of dry seed surface CW enriched fraction from Col-0, *tbl38***  
 369 **and three *tbl38* complemented lines expressing *pPRX36*::*TBL38-TagRFP* (PTR2.4, 3.5 and 5.2). Three biological**  
 370 **replicates each with at least two technical replicates were analyzed. Letters above the bars show the statistical**  
 371 **distribution of the results using ANOVA and TUKEY HSD tests.**





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**Figure 6: Enzymatic profiling reveals the HG acetylation qualitative phenotype of *tbl38* and the quantitative HG methylation phenotype of *pmei6*.** MSC surface abrasion powders of Col-0, *tbl38*, *pmei6* and *tbl38* transformed with *pPRX36::TBL38-TagRFP* (line PTR3.5) were digested with pectin lyase (PL) specific for highly methylesterified HG (A-H; **Supplemental Table 3**), polygalacturonase (PG) specific for low-methylesterified HG (I-N; **Supplemental Table 4**) or rhamnogalacturonan hydrolase (RG) specific for RGI backbone (O-P; **Supplemental Table 5**). The identified HG species (A-N) or RGI species (O-P) were sorted according to their degree of acetylation (DA), degree of methylation (DM) and degree of polymerization (DP). The mean intensities were plotted either as DA vs DP or DM vs DP, regardless their DM and DA, respectively. See **Supplemental Table 3B, 4A and 5B** for details on the data treatment. The individual scales are provided on the Figure. Note the specific HG acetylation phenotype of *tbl38* with PL (B), the qualitative complementation of PTR3.5 with PL (D), the absence of RGI acetylation phenotype for *tbl38* (P) and the quantitative HG methylation phenotype of *pmei6* (G, N). These results argue for an HG acetyltransferase role of TBL38 and are in consistent with the PME1 activity of PME16 (Saez-Aguayo et al., 2013).

## 386 **TBL38 is an homogalacturonan acetyltransferase**

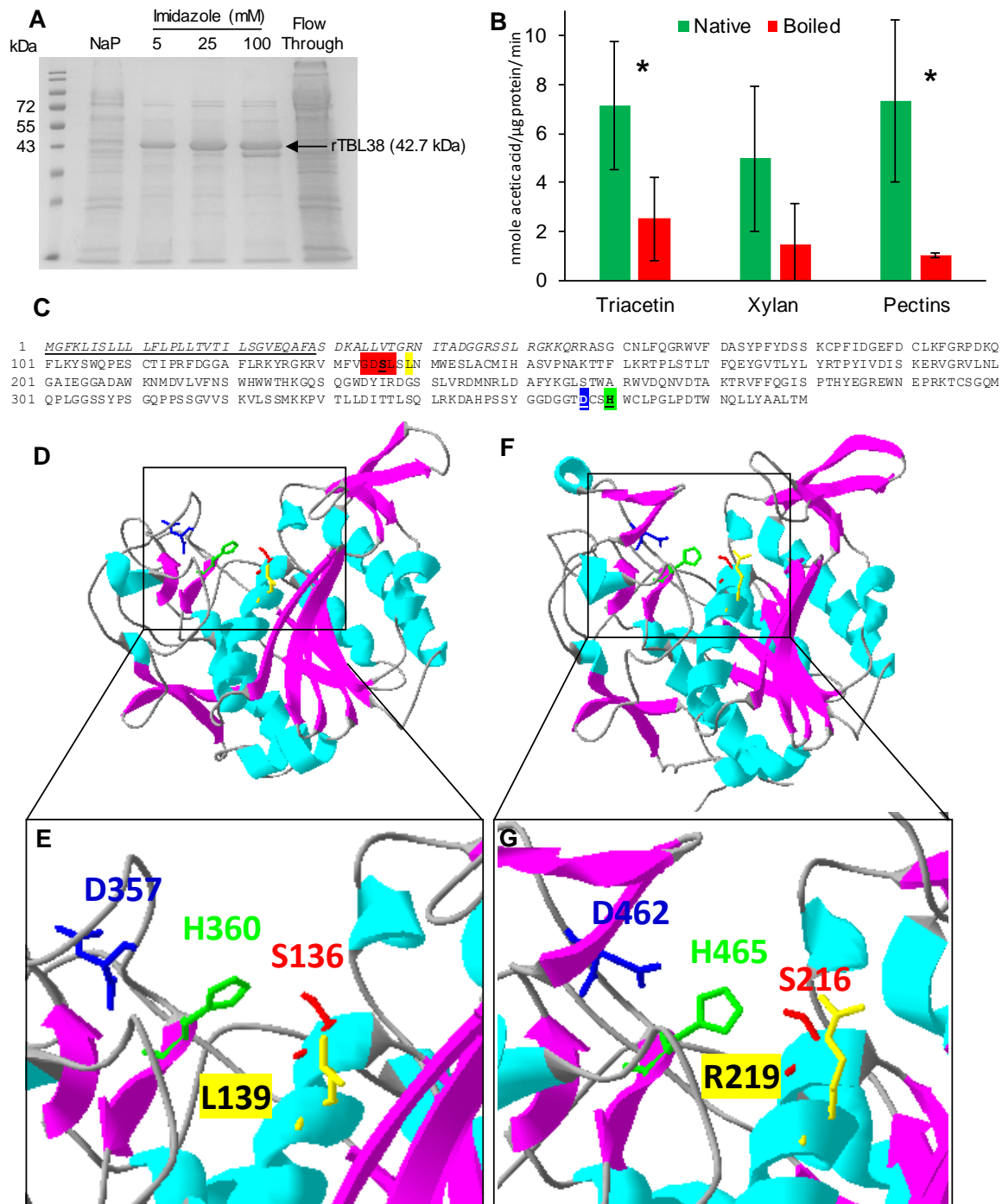
387 Surface-enriched CW abrasion powders were enzymatically hydrolyzed with a pectin lyase  
388 (PL) or a polygalacturonase (PG), displaying specificity toward highly and lowly  
389 methylesterified HGs, respectively. The resulting oligogalacturonates (OGAs) from Col-0,  
390 *tbl38* as well as *pmei6* and the complemented line PTR3.5 used as additional controls, have  
391 then been analyzed by mass spectrometry, which allowed to determine the degree of  
392 polymerization (DP), degree of methylation (DM) and degree of acetylation (DA). Technical  
393 repeats showed the low standard deviation of the intensities of identified species validating the  
394 approach despite the low amount of biological material (**Supplemental Table 3A**). We first  
395 used a PL having a high affinity for HG with a high DM. The intensity of the identified OGA  
396 species was summed for the identified OGA species of same DA and DP regardless the DM, or  
397 same DM and DP regardless the DA (**Supplemental Table 3B**) and for each genotype, the  
398 results were plotted as DA vs DP (**Figure 6A-D**), or DM vs DP (**Figure 6E-H**). We observed  
399 a clear qualitative HG acetylation phenotype in *tbl38* as compared to Col-0 and *pmei6* (**Figure**  
400 **6A-C**) and a qualitative complementation of PTR3.5 (**Figure 6D**). This was in agreement and  
401 refined the high acetylation phenotype observed in *tbl38* with the enzymatic assay (**Figure 5C**)  
402 since new OGA species of higher DA appeared in *tbl38* as compared to the three other  
403 genotypes. This strongly suggests that TBL38 could be an HG acetyltransferase. Conversely, a  
404 quantitative HG methylation phenotype was observed for *pmei6* as compared to Col-0, *tbl38*  
405 and PTR3.5 (**Figure 6E-H**).

406 The same analysis and data processing were performed with PG which has an affinity for lowly  
407 methylated HG (**Figure 6I-N; Supplemental Table 4A-D**). The DA vs DP profiles of Col-0,  
408 *tbl38* and *pmei6* were qualitatively similar though a slight increase of species of  $DA > 0$   
409 occurred for *tbl38* (**Figure 6I-K; Supplemental Table 4A-D**). The DM vs DP plots showed a  
410 quantitative phenotype of *pmei6* as compared to Col-0 and *tbl38* in agreement with the PME1  
411 activity of PME16 (Saez-Aguayo et al., 2013) (**Figure 6L-N**). We also noticed an about ten-  
412 fold higher intensity of OGA species of low DM identified with PG (**Figure 6I-N;**  
413 **Supplemental Table 4A**) vs those of high DM species identified with PL (**Figure 6A-H;**  
414 **Supplemental Table 3B**). This could reflect the nature of the major HGs in MSCs (low DM  
415 and low DA) and/or be related to a possible lower activity of PL towards HGs with relatively  
416 high DA (Zeuner et al., 2020). To strengthen the HG high acetylation phenotype of *tbl38*, we  
417 additionally compared Col-0 and *tbl38* rhamnogalacturonate acetylation profiles to see whether  
418 TBL38 could also target the abundant RGI present in seed mucilage (**Figure 6O-P;**

419 **Supplemental Table 5A**). No increased acetylation phenotype occurred in *tbl38* for RGI since  
420 the very strong intensity of the identified RGI species that were non-acetylated had similar  
421 profiles in both Col-0 and *tbl38*. This result demonstrates the specificity of the acetylation  
422 phenotype of *tbl38* towards HGs rather than RGI.

423 Finally, we produced recombinant TBL38 in *Pichia pastoris* (**Figure 7A**) and tested its acetyl  
424 esterase activity *in vitro* towards three acetylated substrates. Albeit low, we detected acetyl  
425 esterase activity using acetylated pectins from sugar beet and to a lesser extent with the generic  
426 acetylated substrate triacetin, while no significant activity could be detected using acetylated  
427 xylans (**Figure 7B**).

428



429

430 **Figure 7: Pectin acetyl esterase activity of recombinant TBL38 and molecular modeling of TBL38 displaying**  
 431 **its putative catalytic triad and substrate binding pocket.** (A) SDS-PAGE showing the production of  
 432 recombinant TBL38 (rTBL38) in *Pichia pastoris*. (B) *In vitro* enzymatic activity on acetylated substrates. The  
 433 activity of the purified rTBL38 (100 mM imidazole fraction) was assayed using triacetin, acetylated xylans and  
 434 sugar beet pectins as substrates and the ACET enzymatic acetic acid assay kit. Boiled samples were used as  
 435 negative control, mean values  $\pm$  SD, \*,  $p$ -value < 0.05. (C) Amino acid sequence of TBL38 (At1g29050). The  
 436 predicted signal peptide (<https://services.healthtech.dtu.dk/services/SignalP-6.0/>) is underlined. The amino acids  
 437 in italics correspond to the 15% of the protein not covered by the molecular modeling performed in (D). GDSL  
 438 and DxxS tetrads that are highly conserved among the 46 TBR/TBLs including TBL38 (Bischoff et al., 2010) are

439 highlighted in red and blue, respectively. The amino acids participating to the putative catalytic triad S136-H360-  
440 D357 appear in bold underlined, in red, green and blue, respectively. L139 in TBL38 underlined in yellow replaces  
441 R219 in TBL29/ESK1/XOAT1 that was demonstrated to be necessary for acetyltransferase activity (Lunin et al.,  
442 2020). **(D)** The TBL38 structural model proposed in this study is a Phyre2 prediction (Kelley et al., 2015) with the  
443 highest ranking score (Confidence: 100.0%; Coverage: 85%). The template was the crystallographic structure (X-  
444 Ray diffraction, 1.85 Å) of *A. thaliana* TBL29 (At3g55990; PDB 6CCI; Phyre2 c6cciA) (Lunin et al., 2020). **(E)**  
445 Zoom on the putative catalytic triad S136-H360-D357 originally identified in Ser proteases (Dodson and  
446 Wlodawer, 1998), and necessary for the acyl-enzyme formation and acetyltransferase activity of TBL29 (Lunin et  
447 al., 2020). **(F and G)** structure and zoom on the catalytic triad of TBL29 for comparison (Lunin et al., 2020). Note  
448 that R219 necessary for acetyltransferase activity in TBL29 (Lunin et al., 2020) is replaced by L139 in TBL38.

449

## 450 **Discussion**

### 451 **TBL38 is atypically positioned in the PRX36/PMEI6 cell wall microdomain of MSCs** 452 **with a transient pattern**

453 Originally, we searched for genes involved in the PMEI6-JIM7/LM20 HG epitopes-PRX36  
454 molecular module (Francoz et al., 2019b) with a particular interest in HG remodeling enzymes.  
455 Using spatio-temporal co-expression with *PRX36*, we selected *TBL38* for its putative role in  
456 HG acetylation. Previously described TBLs are mostly anchored to the Golgi membrane  
457 (**Supplemental Table 1**) with the exception of TBR (Sinclair et al., 2017). Although the authors  
458 did not refute the possibility of some TBR-GFP signal in the Golgi, they concluded that TBR  
459 was a CW protein. The Golgi experimental localization matches with the N-terminal  
460 transmembrane domain prediction for 10 TBLs (TBL03, 27, 28, 29, 30, 31, 32, 33, 34, 35)  
461 while the Golgi-localized TBL37 has a predicted cleavable signal peptide (akin to a secreted  
462 CW protein), and conversely, TBR that has been localized to the CW has a predicted N-terminal  
463 transmembrane domain (**Supplemental Table 1**). Six TBLs including TBL38 have been  
464 identified in CW proteomes but these proteins did not match perfectly to the targeting  
465 prediction: there is a good match with cleavable signal peptide prediction for TBL9, 38, 39 and  
466 45, but a bad match with transmembrane domain prediction for TBL15 and TBL40  
467 (**Supplemental Table 1**). Here, we clearly localized TBL38-TagRFP in the CW of tobacco  
468 leaves or *A. thaliana* MSCs (**Figure 3**) in agreement with TBL38 presence in a CW proteome  
469 and its predicted cleavable signal peptide (**Supplemental Table 1**). In MSCs, we showed that  
470 this targeting is restricted to the CW microdomain harboring the PMEI6-JIM7/LM20 HG  
471 epitopes-PRX36 molecular module with an intriguing transient localization pattern (6-8 DAP).  
472 The localization to the CW microdomain occurs as soon as the fusion protein is produced just

473 after the onset of *PRX36* promoter activity. We chose this promoter considering the strong co-  
474 expression of *PRX36* and *TBL38* and because it worked well with stable localization in various  
475 CW microdomains in previous studies (Francoz et al., 2019b; Kunieda et al., 2013; McGee et  
476 al., 2019; Sola et al., 2019). The physiological relevance of the transient localization pattern of  
477 TBL38-TagRFP is attested by the fact that the PTR lines displayed functional complementation  
478 in our different assays discussed hereafter. Then, the early delocalization of the fusion protein  
479 could be related to the loss of the putative anchoring motif (acetylated HG) as soon as TBL38  
480 performed its acetyltransferase activity, leading to degradation of the protein.

481

### 482 **TBL38 is a HG acetyltransferase**

483 So far, all described TBLs have been associated with a Golgi-localized acetyltransferase  
484 activity, either on hemicelluloses or pectins (**Figure 1; Supplemental Table 1**). Therefore, the  
485 question arose on whether the CW-localization of TBL38 could lead to similar activity. Yet,  
486 both acetic acid quantification and mass spectrometry analysis, as well as *in vitro* enzymatic  
487 assays on rTBL38 indicate that TBL38 is an acetyltransferase targeting acetylated HGs as opposed  
488 to other TBLs (**Figures 5-7**). Sequence analysis of the TBL family highlighted the conservation  
489 of two characteristic domains: the GDS(L) and DXXH motifs commonly found in esterases  
490 (Akoh et al., 2004; Molgaard and Larsen, 2004). Crystallography analysis of  
491 TBL29/ESK1/XOAT1 pointed to the spatial reunion of these two domains forming the  
492 conserved Ser-His-Asp catalytic site, Ser coming from GDS(L) and Asp and His coming from  
493 DXXH (Lunin et al., 2020). TBL29 displayed an xylan acetyl transferase activity involving the  
494 formation of an acyl-enzyme intermediate on Ser129 (Lunin et al., 2020). Therefore, the  
495 acetyltransferase activity of TBL29 necessitated a generic acetyltransferase activity on both the  
496 acetyl donor and the acyl-enzyme intermediate. Based on the crystallography structure of  
497 TBL29, we built an *in silico* model of TBL38 to determine the position of the above mentioned  
498 amino acids. The predicted catalytic site was correctly positioned in a putative polysaccharide  
499 binding groove (**Figure 7D, E**). Interestingly, in TBL29, the nearby R219 (**Figure 7F-G**) was  
500 shown to stabilize the catalysis (Lunin et al., 2020) as R219A site mutation led to loss of transfer  
501 of acetyl groups for the acetyl transferase activity without loss of the acetyltransferase activity  
502 necessary for the acyl-enzyme formation (Lunin et al., 2020). TBL multiple alignment (Lunin  
503 et al., 2020) shows that 9 TBLs, including TBL38, do not have this particular Arg residue:  
504 TBL12 (E135), TBL30 (Y156), TBL37 (L143), TBL38 (L139) (**Figure 7C-E**), TBL39 (T125),

505 TBL40 (L123), TBL41 (L112), TBL42 (N115), TBL43 (L122). All the other TBLs have an  
506 Arg except TBL44/PMR5 which possess another polar positive residue at this position (K145)  
507 and an acetyltransferase activity (Chiniquy et al., 2019). Among these 9 TBLs with no polar  
508 positive residue at this position, 4 correspond to the 6 TBLs identified in CW proteomes  
509 (**Supplemental Table 1**). It is therefore tempting to postulate that the absence of this residue  
510 could fit with the absence of a (Golgi-localized) acetyltransferase activity and the presence of  
511 a (CW-localized) acetylerase activity as for TBL38. However, this cannot be the sole  
512 determinant since TBL37 and TBL30/XOAT3 both display a Golgi-localized acetyltransferase  
513 activity (Sun et al., 2020; Zhong et al., 2017). The characterization of TBL12, TBL39-43 could  
514 help in deciphering the importance of this residue at this specific position. Since we could not  
515 identify in TBL38 a particular motif or conformation which could explain such opposite  
516 esterase vs transferase activity, we propose that the opposite activity of TBL38 is primarily  
517 related to its CW localization. Indeed, TBL-dependent polysaccharide acetylation is done in  
518 two consecutive steps: hydrolysis of the acetyl group from the donor as evidence by the  
519 formation of the Acyl-enzyme intermediate, followed by transfer of the *O*-acetyl moiety to its  
520 polysaccharide target. To our knowledge, neither acetyl-CoA nor another putative acetyl donor  
521 is present in the CW, which could prevent the CW-localized TBL38 to complete the successive  
522 steps of the transferase reaction, the enzyme could then rather function as a direct acetylerase  
523 on acetylated HGs. It should be noted that an esterase activity targeted on CW polymers was  
524 not observed for TBR despite its CW localization. *tbr* was associated with lower acetylation  
525 level of HGs (the opposite phenotype of *tbl38*), possibly through TBR-mediated protection of  
526 HG from pectin acetylerases (Sinclair et al., 2017). Although is it unclear how such a  
527 mechanism could play out, it is difficult to explain the differences between the measured  
528 acetylation levels. Characterization of additional TBLs with regards to subcellular localization  
529 and acetylerase activity is required for a better understanding of TBL roles.

530

### 531 **HG fine tuning of esterification is affected in *tbl38***

532 Although the control of HG acetylation and methylation is achieved by different proteins, proof  
533 of the direct and indirect interplay between both chemical groups was given. Indeed, HG  
534 acetylation could inhibit PME activity by steric hindrance (Celus et al., 2018; Kohn and Furda,  
535 1968). Additionally, methyl ester groups are preferentially added on non-acetylated  
536 galacturonic acid moieties, which may explain the overall low level of HG acetylation observed

537 in our results with Col-0 and in most plants (Pérez et al., 2000; Ralet et al., 2005), (**Figures 6**  
538 **and 7**). Although we did not quantify HG methylesterification, the total number of identified  
539 species was particularly low in *tbl38*, when using a PL that has high affinity for highly  
540 methylesterified HGs (**Figure 6**). Even if we cannot exclude the possibility that the acetyl  
541 groups in *tbl38* negatively impact the PL activity, this could be associated with reduced HG  
542 methylesterification in *tbl38* considering the lack of LM20 labeling in *tbl38*. It should be noted,  
543 however, that the combined use of PG/PL might not give the full representation of HG species,  
544 since randomly methylated HG of DM around 50% are mediocre substrates for both PL and PG  
545 (Ralet et al., 2012). Additionally, JIM7 antibody still labels *tbl38* seeds suggesting that the lack  
546 of LM20 binding might rather be due to steric hindrance caused by HG acetylation, rather than  
547 changes in the pattern of methylesterification. Therefore, LM20 could display specificity for  
548 partially methylesterified and non-acetylated HGs, while JIM7 would be specific for partially  
549 methylesterified HGs regardless of the acetylation status. Interestingly, nanofibrils of HGs  
550 could be observed with LM20 but not with JIM7 in epidermis anticlinal CW from pavement  
551 cells (Haas et al., 2020; Haas et al., 2021). Considering the proposed different specificities of  
552 the two antibodies, one interpretation would be that the reduced steric hindrance and/or altered  
553 HG gelling properties (Ralet et al., 2003) related to the absence of acetyl groups would be  
554 necessary for proper assembly of HGs in nanofibrils. Evaluation of PME activity in *tbl38*  
555 developing MSCs could give clues as to TBL38 indirect influence on HG methylesterification.  
556 Regardless of the DM, our results confirmed increased amounts of HG acetylation in *tbl38* seed  
557 surface extracts. Contrary to methylesterification, acetylation does not influence the charge  
558 density and therefore the cation binding capacity of HGs (Dronnet et al., 1996; Ralet et al.,  
559 2003). However, experimental evidences have consistently associated highly acetylated HGs  
560 with reduced affinity to cations binding such as  $\text{Ca}^{2+}$  (Celus et al., 2018; Ralet et al., 2003).  
561 Conceptually, this could be due to direct influence of acetylation on conformation, access or  
562 cations binding properties, but also indirect effect on the PME activity as stated above  
563 (Oosterveld et al., 2000; Renard and Jarvis, 1999).

564

#### 565 **Increased HG acetylation does not influence PRX36 anchoring**

566 Our results have identified TBL38 as a CW localized HG acetylase with increased HG  
567 acetylation level in *tbl38* and no obvious effect on PRX36 positioning, despite the  
568 colocalization of TBL38 and PRX36 in the same CW microdomain. Previous work has



569 demonstrated that PRX36 binding to HG was dependent on both methyl and  $\text{Ca}^{2+}$  as the PRX36-  
570 TagRFP localization was severely impacted in *pmei6* or using EDTA, respectively (Francoz et  
571 al., 2019b). To better understand the absence of PRX36 delocalization in *tbl38*, we investigated  
572 the implication of acetylated HGs on PRX36 docking using an *in silico* model based on PRX53  
573 crystallographic data (Oosterveld et al., 2000). The previous *in silico* docking study tested the  
574 five OGAs of DP6 used to define JIM7 specificity (Clausen et al., 2003; Francoz et al., 2019b).  
575 It turned out that the three OGAs (so-called Clausen 3, 4 and to a lesser extend 5) that were the  
576 best recognized by JIM7 could be used for *in silico* docking on a PRX36 valley that was  
577 validated by site-directed mutagenesis (Francoz et al., 2019b). Here, we first randomly screened  
578 *in silico* PRX36 anchoring on 124 OGAs representing all the combinations of  
579 methylesterification from DP2 to DP6 (**Supplemental Table 6**). The *in silico* models consisted  
580 in nine poses of decreasing affinity (increasing energy level). To rationalize the distribution of  
581 these poses on the protein surface, we systematically measured the root mean square deviation  
582 (RMSD) showing the distance between the best pose and the eight other poses. We integrated  
583 the sum of energy levels and sum of RMSD for each OGA as a proxy of affinity and distances  
584 (**Supplemental Table 6**; see Methods). The 124 OGAs could then be sorted in a rationale -  
585 though of course imperfect manner- for their ability to bind to the PRX36 valley. This strongly  
586 reinforced the previous docking experiments made with *a priori* with Clausen1-5 OGAs  
587 (Francoz et al., 2019b) since Clausen 4 and Clausen 3 ranked 1 and 2 (**Supplemental Figure**  
588 **6**; **Supplemental Table 6**). Finally, we tested the impact on *in silico* docking of the addition of  
589 2-*O* or 3-*O* acetyl on the non-methylated galacturonic acids of Clausen 3 and 4 (**Supplemental**  
590 **Figure 6**). The addition of acetyl on hit number 2 (Clausen 3) strongly impaired PRX36 docking  
591 capacity, which does not fit with the observed results (no obvious modification of PRX36  
592 anchoring and no mucilage release phenotype in *tbl38*). However, the addition of acetyl on hit  
593 number 1 (Clausen 4) did not have a strong impact on the docking capacity (**Supplemental**  
594 **Figure 6**). In turn, this is consistent with the immunolocalization of  $\alpha$ PRX36 and confocal  
595 imaging of PRX36-TagRFP in *tbl38* background (**Supplemental Figure 4**) which did not show  
596 any obvious mislocalization of PRX36, suggesting that the Clausen 4 model might have a  
597 higher affinity towards PRX36 *in muro*.

598

599 In light of our results, we conclude that TBL38 acts as an HG acetylase in the PME16-  
600 JIM7/LM20 HG epitopes-PRX36 CW microdomain with no effect on PRX36 anchoring  
601 observed in *tbl38* contrarily to *pmei6*. The uncommon acetylase activity could be related

602 to the localization to the CW where acetyl-CoA is absent. We could hypothesize that TBL38  
603 could have an acetyltransferase activity if artificially targeted in a different environment such  
604 as the Golgi considering the presence of the canonical amino acids required for  
605 acetyltransferase activity. It remains to be understood why evolution has selected  
606 acetyltransferase-capable protein to deacetylate HGs instead of traditionally described PAEs,  
607 though in *A. thaliana*, no PAE have yet been linked to HG deacetylation. We were not able to  
608 associate the increased HG acetylation observed in *tbl38* to any clear developmental phenotype.  
609 However, considering the localization of this process to a remote CW microdomain, additional  
610 tools will be required to search for subtle developmental phenotypes, similarly to the  
611 development of the new original abrasive method allowing the characterization of the  
612 biochemical phenotype. Beyond this case study, this work paves the way for further  
613 understanding of the role of fine tuning of complex CW polysaccharides.

614

## 615 **Methods**

### 616 **Plant material and growth conditions**

617 *A. thaliana* mutants were ordered from the NASC for *tbl38*: SAIL\_34\_C03  
618 (<https://arabidopsis.info/>; **Supplemental Figure 3**), or were previously available for *prx36*  
619 (SAIL\_194\_G03 (Kunieda et al., 2013)) and *pmei6* (SM\_3.19557 (Saez-Aguayo et al., 2013)).  
620 Homozygous lines for *tbl38* were identified by PCR (**Supplemental Figure 3; Supplemental**  
621 **Table 7**). The knock out status of *tbl38* was determined by RT-PCR (**Supplemental Figure 3;**  
622 **Supplemental Table 7**). *A. thaliana* culture was performed as previously described (Francoz  
623 et al., 2019b).

624 *Nicotiana benthamiana* were cultivated in a growth chamber under a 16 h day / 8 h night cycle  
625 at 23°C (Neon (86.90  $\mu\text{mol}/\text{m}^2/\text{s}$ )) / 22°C upon 70 % humidity. They were transplanted after  
626 14 days and fertilized each week.

### 627 **Transcriptomic data mining**

628 *PRX36*, *PMEI6* and *TBL38* seed-specific expression profiles were obtained using the seed data  
629 source of eFP browser (<http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi?dataSource=Seed>).  
630 Tissue-specific seed development transcriptomic data (Belmonte et al., 2013) was used to build  
631 the *PRX36* co-expression network (Francoz et al., 2019b). The *TBL* family (Bischoff et al.,  
632 2010) was filtered out.

### 633 **Bioinformatic analysis of the TBL family**

634 Previous *TBL38* phylogeny (Bischoff et al., 2010) was used. The occurrence of TBLs in cell  
635 wall proteomes was assessed using <http://www.polebio.lrsv.ups-tlse.fr/WallProtDB/>. The  
636 topological prediction of the N-terminal hydrophobic sequence as being a transmembrane  
637 domain or a signal peptide was determined using <http://aramemnon.uni-koeln.de/>.

### 638 **Ruthenium Red Mucilage Release Test, Image Analysis and Statistical Analysis**

639 High-throughput adherent mucilage release semi-quantitative phenotyping used the ruthenium  
640 red (**Supplemental Table 8**) staining method previously described allowing to calculate  
641 adherent mucilage area and circularity (Francoz et al., 2019b). The images were analyzed using  
642 ImageJ 1.8 (<https://imagej.nih.gov/ij/>) without edition of native images using an updated  
643 ImageJ script that facilitates cleaning of the data and that allows their automatic storage (see  
644 **Supplemental Methods** for more details).

### 645 **TagRFP reporting constructs, plant transformation and selection**

646 The primers used for vector construction are listed in **Supplemental Table 7**. Full length cDNA  
647 clones and custom-ordered DNA plasmids used as template DNA for further cloning are listed  
648 in **Supplemental Table 9**. Level 0 GoldenGate generated constructs are listed in **Supplemental**  
649 **Table 10**. Level 1 GoldenGate plasmids assembled in pL1V-R2 vector and finalized Level 2  
650 GoldenGate generated constructs are detailed in **Supplemental Table 11**. Level 1 plasmid was  
651 pICH47811 “pL1V-R2”pL1V-R2 vector (Weber et al., 2011). Level 2 plasmid was EC15027  
652 “pL2V-HYG” with pICH471744 “pL1M-ELE-2” as a linker-containing plasmid (gift from Dr  
653 Pierre-Marc Delaux, LRSV, Auzeville-Tolosane, France). Every construct was checked by  
654 restriction analysis and sequencing prior to its transfer into *Agrobacterium tumefaciens*  
655 GV3101::pMP90 strain (Koncz and Schell, 1986). The transformed bacteria were grown in the  
656 presence of 15 µg.mL<sup>-1</sup> gentamycin, 50 µg.mL<sup>-1</sup> rifampicin and 25 µg.mL<sup>-1</sup> hygromycin. *A.*  
657 *thaliana* plants were transformed by floral-dipping (Clough and Bent, 1998) or spraying flower  
658 buds with an Agromix containing 0.05 % (v/v) Silwet L-77 (De Sangosse 2000235).  
659 *proPRX36::PRX36-TagRFP* construct was transformed in *prx36* and *tbl38* plants. The  
660 *proPRX36::TBL38-TagRFP* construct was transformed in *tbl38* plants. These last  
661 complemented lines were referred as PTR2.4, PTR3.5 and PTR5.2 in the manuscript. Seeds  
662 were selected on Murashige and Skoog (MS) medium containing 25 µg.mL<sup>-1</sup> hygromycin.  
663 Three independent homozygous transformed plant lines were studied for each construct.

664 The *pro35S::TBL38-TagRFP* construct was transiently co-transformed with either G-yb CD3-  
665 966 sialyltransferase-YFP Golgi marker or pm-yb-CD3-1006 aquaporin-YFP plasma  
666 membrane marker (Nelson et al., 2007) in 30-day old *N. benthamiana* leaves. The final  
667 inoculum consisted in a mix of various *A. tumefaciens* lines: 10 % (v/v) subcellular YFP-marker  
668 line, 80% (v/v) fusion protein-Tag-RFP line of interest, and 10 % (v/v) P19 silencing inhibitor-  
669 containing vector line. Leaf infiltration was done on the abaxial face through the stomata with  
670 a 1 mL syringe and pieces of leaves were mounted and analyzed under confocal microscopy 48  
671 h-post agro-infiltration.

### 672 **Arabidopsis silique fixation, paraffin tissue array embedding and microtomy**

673 The protocol was as previously described (Francoz et al., 2019b), with minor improvement  
674 using biopsy foam enabling handling more samples (see **Supplemental Methods** for more  
675 details).

### 676 **In situ RNA hybridization**

677 *In situ* RNA hybridization experiments were performed as described (Francoz et al., 2019a;  
678 Francoz et al., 2016). More details are provided in **Supplemental Methods**.

### 679 **Immunofluorescence on tissue-array sections**

680 We used our previously described protocol (Francoz et al., 2019b) with anti-PRX36 primary  
681 antibodies and goat-anti rabbit-A488 secondary antibodies (**Supplemental Table 8**) hybridized  
682 on serial sections of Col-0 and *tbl38* paraffin-embedded seed developmental kinetics. Serial  
683 sections of Col-0, *tbl38*, PTR2.4 and PTR3.5 developmental kinetics were similarly labeled  
684 with JIM7 or LM20 primary antibodies followed by goat anti-rat A488 secondary antibodies  
685 (**Supplemental Table 8**). More details are provided in **Supplemental Methods**.

### 686 **Confocal spinning disk microscopy of seed development kinetics of *A. thaliana* stable lines**

687 Developing siliques taken from plants stably expressing the different TagRFP constructs in  
688 various genetic backgrounds were dissected and the replums containing the seeds were mounted  
689 under a coverslip in distilled water. Images were taken with a PLAN APO 20x/0.75 dry  
690 objective using the confocal spinning disk microscope from Perkin Elmer driven by the  
691 Volocity 6.3.0 software and equipped with a Yokogawa CSU-X1 scan head, two EmCCD  
692 Hamamatsu C9100-13 cameras (Hamamatsu, Hamamatsu City, Japan) and a 580 nm beam  
693 splitter to separate dual staining on the two cameras as previously shown (Francoz et al., 2019b).  
694 Details of image processing are provided in the **Supplemental Methods**.

695 **Confocal microscopy of leaves of *N. benthamiana* transient transformants**

696 Transiently transformed *N. benthamiana* leaves were observed 48 h post agroinfiltration using  
697 an upright confocal laser scanning microscope (LEICA SP8) with a 40 x apochromatic water  
698 immersion lens. TagRFP and YFP fluorescences were imaged with the following settings:  
699 excitation: 561 nm / emission: 582–622 nm; excitation 514 nm / emission: 527-530 nm,  
700 respectively. Z stacks (maximum intensity) were performed using ImageJ.

701 **SDS-PAGE and anti-TagRFP western-blot from *tbl38* complemented lines (PTR)**

702 The TagRFP fluorescence patterns of PTR lines were monitored using spinning disk confocal  
703 microscopy along developmental kinetics of seeds from staged floral stem. The seeds that  
704 displayed the early (6-8 DAP), medium (8-12 DAP) and late (> 12 DAP) fluorescence patterns  
705 were carefully extracted from the dissected siliques (3-4 siliques per fluorescence pattern) and  
706 pooled in separated 2 mL tubes with a metallic grind ball before freezing in liquid nitrogen.  
707 Total proteins were extracted and analyzed by western blot as previously described (Francoz et  
708 al., 2019b), using anti-TagRFP primary and anti-rabbit-AP secondary antibodies  
709 (**Supplemental Table 8**) with minor modifications as detailed in **Supplemental Methods**.

710 **Dry seed MSC surface wall abrasive fraction**

711 Abrasion columns were a home-made design allowing for homogenous dry seed MSC surface  
712 wall abrasive enrichment using plastic columns and collector tubes from the GeneJET Plasmid  
713 Miniprep Kit (Thermo Scientific K0502). The preparation of the columns, the abrasion  
714 procedure and the characterization of the efficiency are described in detail in **Supplemental**  
715 **Methods**.

716 **Immuno dotblot and semi-quantitative analysis from dry seed MSC abrasive fractions**

717 Powder resulting of MSC surface abrasion was chemically extracted in the collector tube with  
718 extraction buffer (80  $\mu$ L of 5 mM sodium acetate pH 4.6 containing 1  $\mu$ L.mL<sup>-1</sup> of plant protease  
719 inhibitor cocktail (Sigma P9599) per mg of powder). The collector tube with cap and a metallic  
720 grind ball was shaken at 250 rpm during 1 h at 4°C to ensure proper extraction. After a 5 min  
721 centrifugation at 14,400 rpm, the supernatant was recovered, diluted (1  $\mu$ L in 50  $\mu$ L milliQ-  
722 H<sub>2</sub>O) and deposited into sample wells onto a nitrocellulose membrane disposed into a 96-well  
723 Bio-Dot microfiltration apparatus (BioRad) previously soaked with in TBS (0.02 M Tris-HCl  
724 pH 7.5, 0.15 M NaCl) for 10 min. A 1 min 30 s of vacuum insured homogeneous transfer among  
725 the wells. The membrane was briefly air-dried to ensure proper adsorption. The detailed

726 protocol of incubation with JIM7 and LM20 primary antibodies followed by goat anti-rabbit  
727 IgG-AP secondary antibodies as well as the semi-quantitative analysis of the results are  
728 provided in **Supplemental methods**.

### 729 **Acetyl group deesterification and acetic acid semi-quantitative analysis from dry seed** 730 **MSC abrasive fractions**

731 The acetylation of cell wall polymers in MSC surface wall abrasive samples was analyzed  
732 directly in the collection tube following adaptation of a previously described method (Stranne  
733 et al., 2018) using the K-ACET enzymatic Acetic Acid Assay Kit (Megazyme). The detailed  
734 protocol is provided in **Supplemental Methods**.

### 735 **Structural oligosaccharide composition of dry seed MSC abrasive fractions by IP-RP-** 736 **UHPC-MS analysis**

737 MSC surface wall powders were resuspended in 1 mL distilled water for 1 h. An aliquot (450  
738  $\mu\text{L}$ ) was mixed with 50  $\mu\text{L}$  of 500 mM sodium acetate buffer pH 5.0 and polysaccharides were  
739 hydrolyzed for 24 h at 40°C under 500 rpm shaking with either a pectin lyase (PL) specific for  
740 methylesterified HGs (Ralet et al., 2012), a polygalacturonase (PG) (Safran et al., 2023) specific  
741 for non-methylesterified HGs or a rhamnogalacturonase (RG, Swiss-Prot Q00018, provided by  
742 Novozymes, Copenhagen, Denmark) specific for the RGI backbone (Ralet et al., 2010).  
743 Resulting OGA digests were filtered on 0.45  $\mu\text{m}$  and analyzed by IP-RP-UHPC-MS.  
744 Acquisitions were performed on a Select Series Cyclic IMS (Waters, Wilmslow, UK) coupled  
745 with a UHPLC system (Acquity H-Class Plus, Waters, Manchester, UK). The chromatographic  
746 separations were performed on an hypersil GOLD<sup>TM</sup> (100 mm  $\times$  1 mm, packed with 1.9  $\mu\text{m}$   
747 porosity particles; Thermo-Fisher Scientific, Bremen, Germany). A ternary gradient was used  
748 (A: Milli-Q water, B: 100% methanol, and C: 20 mM heptylammonium formate, pH 6), from  
749 2 % to 25 % of solvent B for 10 min, then up to 73 % at 23.5 min and maintained at 73 % for 4  
750 min. Percentage of solvent C was kept constant at 25%. The flow rate was 0.175 mL min<sup>-1</sup>, and  
751 the column was heated to 45°C. Spectra were recorded in positive electrospray ionization (ESI)  
752 ionization mode in the m/z range 150–2000, with the TOF operating in the V-mode. The source  
753 parameters were as follows: capillary voltage 2.8 kV; cone voltage 120 V; source temperature  
754 100°C; desolvation temperature 350°C; desolvation gas 350 L/h; and nebulization gas 6 bar.  
755 Data were recorded with the Quartz software (Waters embedded software, release 5) and  
756 processed using Mass Lynx 4.2 (Waters) and MzMine software (Pluskal et al., 2010). MzMine  
757 was used to produce a database of annotated structures (m/z; retention time; intensity). The

758 identified species (charge states and anomers were merged) were annotated according to their  
759 degree of polymerization (DP), degree of methylesterification (DM) and degree of acetylation  
760 (DA). The data was analyzed using Microsoft Excel.

### 761 **Production of recombinant TBL38 in *Pichia pastoris* and enzymatic activity assays**

762 The coding sequence of TBL38 (Q8VY22) was codon-optimized for *Pichia pastoris* and  
763 synthesized without signal peptide in frame with His-tag in pPCIZ- $\alpha$ B by ProteoGenix  
764 (Schiltigheim, France). rTBL38 was produced in *P. pastoris* following the previously described  
765 protocol (Lemaire et al., 2020). Activity assays of rTBL38 was performed with the acetic acid  
766 assay kit (K-ACETRM, Megazyme) using the activity buffer at 40°C and with three different  
767 acetylated substrates: 100 mM Triacetine, 10 mg.mL<sup>-1</sup> xylan (24 % acetylation), and 10 mg.mL<sup>-1</sup>  
768 <sup>1</sup> sugar beet pectins (31 % acetylation). The detailed protocol is provided in **Supplemental**  
769 **Methods**.

### 770 ***In silico* model and docking simulations**

771 Homology models for PRX36 and TBL38 (UniProt accession number Q9SD46 and Q8VY22,  
772 respectively) were built with the Phyre2 server (Kelley et al., 2015). For PRX36, the template  
773 was the crystallographic structure (X-Ray diffraction, 1.45 Å) of *A. thaliana* PRX53  
774 (At5g06720; Protein Data Bank no.1PA2) (Ostergaard et al., 2000). For TBL38, the template  
775 was the crystallographic structure (X-Ray diffraction, 1.85 Å) of *A. thaliana*  
776 TBL29/ESK1/XOAT1 (Lunin et al., 2020). For comparison with the TBL38 model, the TBL29  
777 structure was drawn as well using the 6cci pdb file (Lunin et al., 2020) visualized and analyzed  
778 with Swiss-PdbViewer (<http://www.expasy.org/spdbv/>) (Guex and Peitsch, 1997).

779 For PRX36 docking experiments, the  $\alpha$ -D-(1-4) polygalacturonic acid structural model  
780 (Braccini et al., 1999) was retrieved from the Glyco3D portal (Perez et al., 2015)  
781 <http://glyco3d.cermav.cnrs.fr/mol.php?type=polysaccharide&molecule=2504>). It was  
782 modified to initially build the five hexagalacturonates models used to establish JIM7 specificity  
783 (Clausen et al., 2003). This selection was extended to the 124 oligogalacturonates (OGAs) from  
784 DP2 to DP6 covering all the theoretical combination of methylation (64 OGAs of DP6 + 32  
785 OGAs of DP5 + 16 OGAs of DP4 + 8 OGAs of DP3 + 4 OGAs of DP2). AutoDock Tools  
786 (Morris et al., 2009) and AutoDock Vina (Trott and Olson, 2010) were used for simulating the  
787 binding of the 124 OGAs to PRX36 within a search box encompassing the whole target protein  
788 enabling to recover the nine best poses for each OGA. Details on data processing are provided  
789 in **Supplemental Methods**.

790

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800 **Author contributions**

801 Conceptualization, V.B.; Methodology, all authors; Investigation, B.G.D, D.R., M.R., P.R.,  
802 C.C., S.O., J. M.-B., P.T., A.G. and V.B.; Writing – Original Draft Writing, B.G.D. and V.B.;  
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806



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