

1 **Running Head:** PRX01, PRX44 and PRX73 are peroxidases active in root hair growth

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20

RAPID REPORT

21 **Class III peroxidases PRX01, PRX44, and PRX73 potentially target extensins during root hair** 22 **growth in *Arabidopsis thaliana***

23

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54 Key words: *Arabidopsis*, cell walls, extensins, root hairs, ROS, class-III peroxidases.

55

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57 **Abstract**

58

59 • Root hair cells are important sensors of soil conditions. Expanding several hundred times their
60 original size, root hairs grow towards and absorb water-soluble nutrients. This rapid growth is
61 oscillatory and is mediated by continuous remodelling of the cell wall. Root hair cell walls contain
62 polysaccharides and hydroxyproline-rich glycoproteins including extensins (EXTs).

63

64 • Class-III peroxidases (PRXs) are secreted into the apoplastic space and are thought to trigger either
65 cell wall loosening, mediated by oxygen radical species, or polymerization of cell wall components,
66 including the Tyr-mediated assembly of EXT networks (EXT-PRXs). The precise role of these EXT-
67 PRXs is unknown.

68

69 • Using genetic, biochemical, and modeling approaches, we identified and characterized three root
70 hair-specific putative EXT-PRXs, PRX01, PRX44, and PRX73. The triple mutant *prx01,44,73* and the
71 PRX44 and PRX73 overexpressors had opposite phenotypes with respect to root hair growth,
72 peroxidase activity and ROS production with a clear impact on cell wall thickness.

73

74 • Modeling and docking calculations suggested that these three putative EXT-PRXs may interact with
75 non-*O*-glycosylated sections of EXT peptides that reduce the Tyr-to-Tyr intra-chain distances in EXT
76 aggregates and thereby may enhance Tyr crosslinking. These results suggest that these three
77 putative EXT-PRXs control cell wall properties during the polar expansion of root hair cells.

78

79 Word count: 200

80 Introduction

81 Primary cell walls, composed by a diverse network containing mainly polysaccharides and a small
82 amount of structural glycoproteins, regulate cell elongation, which is crucial for several plant growth
83 and developmental processes. Extensins (EXTs) belong to hydroxyproline (Hyp)-rich glycoprotein
84 (HRGP) superfamily and broadly include related glycoproteins such as proline-rich proteins (PRPs) and
85 leucine-rich repeat extensins (LRXs) with multiple Ser-(Pro)₃₋₅ repeats that may be *O*-glycosylated and
86 contain Tyr (Y)-based motifs (Lampert et al. 2011; Marzol et al. 2018). EXTs require several
87 modifications before they become functional (Lampert et al., 2011; Marzol et al. 2018). After being
88 hydroxylated and *O*-glycosylated in the secretory pathway, the secreted *O*-glycosylated EXTs are
89 crosslinked and insolubilized in the plant cell wall by the oxidative activity of secreted class-III
90 peroxidases (PRXs) on the Tyr-based motifs (Baumberger 2001, 2003; Ringli 2010; Held et al. 2004;
91 Lampert et al., 2011; Chen et al. 2015; Marzol et al. 2018). PRXs are thought to facilitate both intra
92 and inter-molecular covalent Tyr–Tyr crosslinks in EXT networks, possibly through the assembly of
93 triple helices (Velasquez et al. 2015a; Marzol et al. 2018) by generating *isodityrosine* units (IDT) and
94 *pulcherosine*, or *di-isodityrosine* (Di-IDT), respectively (Brady et al., 1996; 1998; Held et al. 2004). In
95 addition, *O*-glycosylation levels in EXTs also affect their insolubilization process in the cell wall (Chen
96 et al. 2015; Velasquez et al. 2015a) since it might influence the EXT interactions with other cell wall
97 components (Nuñez et al., 2009; Valentin et al., 2010). However, the underlying molecular
98 mechanisms of EXT crosslinking and assembly have not been fully determined. It is proposed that *O*-
99 glycosylation levels as well as the presence of Tyr-mediated crosslinking in EXT and related
100 glycoproteins allow them to form a dendritic glycoprotein network in the cell wall. This EXT network
101 affects *de novo* cell wall formation during embryo development (Hall and Cannon 2002; Cannon *et al.*,
102 2008), they are also implicated in roots, petioles and rosette leaves growth (Saito et al 2014; Møller
103 et al. 2017) and in polar cell expansion processes in root hairs (Baumberger 2001, 2003; Ringli 2010;
104 Velasquez et al. 2011; 2012; 2015a,b) as well as in pollen tubes (Fabrice et al. 2018; Sede et al. 2018;
105 Wang et al. 2018).

106
107 Apoplastic class-III PRXs are heme-iron-dependent proteins, members of a large multigenic family in
108 land plants, with 73 members in *Arabidopsis thaliana* (Passardi et al. 2004; Weng and Chapple, 2010).
109 These PRXs catalyze several different classes of reactions. PRX activities coupled to *apo*ROS molecules
110 (*apo*H₂O₂) directly affect the degree of cell wall crosslinking (Dunand et al. 2007) by oxidizing cell wall
111 compounds and leading to stiffening of the cell wall through a peroxidative cycle (PC) (Passardi et al.
112 2004, Cosio & Dunand 2009; Lampert et al. 2011). By contrast, *apo*ROS coupled to PRX activity
113 enhances non-enzymatic cell wall-loosening by producing oxygen radical species (e.g., •OH) and
114 promoting growth in the hydroxylic cycle (HC). In this HC cycle, PRXs catalyze the reaction in which
115 hydroxyl radicals (•OH) are produced from H₂O₂ after O₂•⁻ dismutation. In this manner, some PRXs
116 (e.g. PRX36) may function in weaken plant cell walls by the generated •OH that cleave cell wall
117 polysaccharides in seed mucilage extrusion in epidermal cells in the *Arabidopsis* seed coat (Kunieda

118 et al., 2013). It is unclear how these opposite effects on cell wall polymers are coordinated during
119 plant growth (Passardi et al. 2004, Cosio & Dunand 2009; Lee et al. 2013; Ropollo et al. 2011; Lee et
120 al 2018; Francoz et al. 2019). Finally, PRXs also contribute to the superoxide radical ($O_2^{\bullet-}$) pool by
121 oxidizing singlet oxygen in the oxidative cycle (OC), thereby affecting $_{apo}H_2O_2$ levels. Thus, several PRXs
122 are involved in the oxidative polymerization of monolignols in the apoplast of the lignifying cells in
123 xylem (e.g. PRX017, Cosio et al 2017; PRX72, Herrero et al. 2013), in the root endodermis (e.g. PRX64;
124 Lee et al. 2013; Ropollo et al. 2011), and in petal detachment (Lee et al 2018). In addition, PRXs are
125 able to polymerize other components of the plant cell wall such as suberin (Bernards et al., 1999),
126 pectins (Francoz et al. 2019), and EXTs (Schnabelrauch *et al.*, 1996; Jackson et al., 2001). Although
127 several candidates of PRXs have been associated specifically with EXT-crosslinking (EXT-PRXs) by *in*
128 *vitro* studies (Schnabelrauch et al., 1996; Wojtaszek et al., 1997; Jackson et al., 2001; Price et al., 2003;
129 Pereira et al. 2011; Dong et al., 2015) or based on an immunolabelling extensin study linked to a
130 genetic profile (Jacobowitz et al. 2019), the *in vivo* characterization and mode of action of these EXT-
131 PRXs remain largely unknown. In this work, we used a combination of reverse genetics, molecular and
132 cell biology, computational molecular modeling, and biochemistry to identify three apoplastic PRXs,
133 PRX01, PRX44 and PRX73, as key enzymes possibly potentially involved in Tyr-crosslinking of cell wall
134 EXTs in growing root hair cells. In addition, we propose a hypothetical model in which O-glycosylation
135 levels on the triple helixes of EXTs might regulate the degree of Tyr-crosslinking affecting the
136 expansion properties of cell walls as suggested before based on the extended helical polyproline-II
137 conformation state of EXTs (Stafstrom & Staehelin 1986; Owen et al., 2010; Ishiwata et al., 2014)
138 together with an experimental Atomic Force Microscopic (AFM) analysis of crosslinked EXT3
139 monomers (Cannon et al. 2008) linked to modelling approaches (Velasquez et al. 2015a; Marzol et al
140 2018). Our results open the way for the discovery of similar interactions in EXT assemblies during root
141 hair development and in response to the environmental changes, such fluctuating nutrient availability
142 in the soil.

143

144 **Results and Discussion**

145 In this work, we have chosen to analyze root hair cells because they are an excellent model for tracking
146 cell elongation and identifying PRXs involved in EXT assembly. In previous work, the phenotypes of
147 mutants for PRX01, PRX44 and PRX73 suggested that these PRXs are involved in root hair growth and
148 ROS homeostasis, although their mechanisms of action remained to be characterized (Mangano et al.
149 2017). All three PRXs are under the transcriptional regulation of the root hair specific transcription
150 factor RSL4 (Yi et al. 2010; Mangano et al. 2017). As expected, these three PRXs are also highly co-
151 expressed with other root hair-specific genes encoding cell wall EXTs (e.g., EXT6-7, EXT12-14, and
152 EXT18) and EXT-related glycoproteins (e.g. LRX1 and LRX2), which functions in cell expansion (Ringli
153 2010; Velasquez et al. 2011; Velasquez et al. 2015b) (**Figure S1**). Based on this evidence, we
154 hypothesized that these three PRXs might be EXT-PRXs and catalyze Tyr-crosslinks to assemble EXTs
155 in root hair cell walls.

156
157 To validate that PRX01, PRX44, and PRX73 are expressed specifically in root hairs, we made
158 transcriptional reporters harboring GFP-tagged fusions of the promoter regions of their genes. In
159 agreement with the *in silico* database (Mangano et al. 2017 and **Figure S1**), all three genes were
160 strongly expressed in root hair cells during cell elongation (**Figure 1A**). Single mutants for these three
161 PRXs showed almost normal root hair growth (Mangano et al. 2017), suggesting a high degree of
162 functional redundancy. Double combinations of *prx44 prx73* (Mangano et al. 2017), *prx01 prx44* and
163 *prx01 prx73* (this study, not shown) as well as the triple null mutant, *prx01 prx44 prx73* showed
164 similarly shorter root hair cells (**Figure 1B**) than what was previously reported for each of the
165 individual *prx* mutants (Mangano et al. 2017). We also obtained two independent lines for each
166 overexpressing PRXs fused to GFP and under the control of a strong *35SCaMV* promoter (PRX^{OE}).
167 Unlike the *prx01 prx44 prx73* triple mutant, the lines overexpressing PRX44 and PRX73 had
168 significantly longer root hairs than the Wt Col-0 control (**Figure 2A–B**). The root hairs of the PRX01^{OE}
169 lines, however, were similar to those of Wt Col-0 (**Figure 2A–B**). We reasoned that the lack of
170 enhanced root hair expansion in the PRX01^{OE} lines could be due to reduced levels of overexpression
171 compared to the PRX44^{OE} and PRX73^{OE} lines. However, based on the GFP signals in intact roots (**Figure**
172 **2C**), we established that PRX01^{OE} and PRX44^{OE} are strongly expressed, whereas PRX73^{OE} showed more
173 moderate expression. Furthermore, the three PRX-GFP-fusion proteins were detected at the expected
174 molecular weights in an immunoblot (**Figure 2D**), indicating that the tagged proteins are stable. The
175 lack of root hair growth enhancement in PRX01^{OE} line might be due to regulatory aspects on the
176 protein activity rather than in the protein level. Together, these results highlight the partially
177 redundant roles of PRX01, PRX44, and PRX73 as positive regulators of polar growth. This is in
178 agreement with the negative effect of SHAM (salicylic hydroxylamino acid), a peroxidase activity
179 inhibitor (Ikeda-Saito, Shelley et al. 1991; Davey and Fenna 1996), on root hair growth (Mangano et
180 al. 2017). Here is important to highlight that a SHAM treatment produce a more drastic effect on root
181 hair growth and on the inhibition on overall peroxidase activity in the roots (Mangano et al. 2017)
182 than the triple mutant *prx01 prx44 prx73*, suggesting the implication of other unidentified PRXs.

183
184 To confirm that our mutant and overexpressing lines had the expected changes in peroxidase activity,
185 we measured *in vitro* total peroxidase activity using a guaiacol oxidation-based assay. The *prx01,44,73*
186 roots showed reduced peroxidase activity (close to 50% reduction) (**Figure 1C**), whereas there was a
187 40–50% increase in PRX73^{OE} and an approximately 20% increase in PRX44^{OE} (**Figure 2E**). Consistent
188 with our root hair growth analysis (**Figure 2A**), PRX01^{OE} showed normal peroxidase activity (**Figure**
189 **2E**). The homeostasis and levels of ROS (mostly H₂O₂) that regulates polar growth of root hair cells
190 (Mangano et al. 2017) is composed by apoplastic ROS (_{apo}ROS) as well as cytoplasmic ROS pools
191 (_{cyt}ROS). Both pools of ROS, their homeostasis and levels are modulated by their transport from the
192 apoplast to the cytoplasmic side by specific aquaporins (PIPs for plasma membrane intrinsic proteins)
193 in plant cells (Dynowski et al., 2008; Hooijmaijers et al., 2012 Rodrigues et al. 2017). We hypothesized

194 that these three PRXs might change the levels of ROS, most probably H₂O₂, for their catalytic functions
195 in the cell wall/apoplast. Therefore, we measured _{cyt}ROS levels by oxidation of H₂DCF-DA and _{apo}ROS
196 levels with the Amplex Ultra Red (AUR) probe in root hair tips. The *prx01,44,73* root hair tips showed
197 lower levels of _{cyt}ROS (**Figure 1D**) but increased _{apo}ROS accumulation (**Figure 1E**) compared to Wt Col-
198 0. The _{apo}ROS levels were similar in PRX01^{OE}, and slightly lower in PRX44^{OE}, and PRX73^{OE} lines when
199 compared to Wt Col-0 (**Figure 2F**). These results suggest that PRX01, PRX44, and PRX73 function as
200 apoplastic regulators of ROS-linked root hair cell elongation.

201
202 Next, to further analyze the ultrastructure of the cell wall in growing root hairs, we analyzed Wt Col,
203 PRX44^{OE}, and *prx01,44,73* triple mutant roots treated or not with SHAM by transmission electron
204 microscopy (**Figure 3A**). Much found thinner cell walls at the root hair tips of PRX44^{OE} ($0.74 \pm \text{SD } 0.24$
205 μm for PRX44^{OE}) and *prx01,44,73* ($0.61 \pm \text{SD } 0.14 \mu\text{m}$) when compared to Wt Col-0 plants ($1.2 \pm \text{SD}$
206 $0.3 \mu\text{m}$ for Wt) (**Figure 3B**). SHAM treatment caused a statistically significant increase in cell wall
207 thickness in the PRX44^{OE} and *prx01,44,73* root hairs (**Figure 3B**), but not in Wt Col-0. This result
208 suggests the importance of peroxidase activity in cell wall structure and highlights that either
209 depletion of PRX01,44,73 (triple mutant) or the overexpression of PRX44 results in an overall
210 reduction in cell wall thickness in growing root hairs. This implies that the constitutive mis-regulation
211 of PRX activity (either reduced/impaired function or overexpression) affects the capacity of root hairs
212 to form normal cell walls and this clearly affects their cell expansion process.

213
214 Then, we designed an EXT reporter to track EXT secretion and PRX-mediated insolubilization in the
215 cell walls during root hair cell elongation. The secreted EXT reporter carries a Tomato tag (SS-TOM-
216 Long-EXT) that is fluorescent under the acidic pH (Shen et al. 2014) that is typical of plant cell walls
217 and apoplastic spaces (Stoddard & Rolland 2018). A secreted Tomato tag (ss-TOM) was used as a
218 control (**Figure S2A**). The EXT domain includes only two Tyr, which are at the C-terminus and
219 separated by 10 amino acids (Stratford et al., 2001). Expression of the EXT reporter was first tested in
220 onion (*Allium cepa*) cells, and then the reporter was stably expressed in Arabidopsis root hairs (**Figure**
221 **S2C-F**). In both cases, plasmolysis was used to retract the plasma membrane from the cell surface to
222 show that the EXT reporter was localized in the cell walls. Using immunoblot analysis, we detected
223 the full-length EXT-Tomato fusion protein, with possible O-glycan modifications, running as higher
224 molecular weight bands than expected (**Figure S2B**). Importantly, the EXT reporter did not interfere
225 with the polar growth of root hairs (**Figure S2D**), and, therefore, could be used to track changes in the
226 *in situ* arrangement of cell wall EXTs. SS-TOM-Long-EXT is clearly secreted in the cell wall of growing
227 root hairs (**Figure S2C**) but remains to be tested if these EXT reporter is mislocalized under an inhibited
228 PRX environment (SHAM treated) or in *prx01,44,73* mutant background.

229
230 We then assessed the level of crosslinking of EXT Tyr residues by measuring peptidyl-tyrosine (Tyr)
231 and isodityrosine (IDT, dimerized Tyr) in EXT extracted from whole roots. We detected a significant

232 increase in peptidyl-Tyr in the *prx01,44,73* triple mutant relative to Wt Col-0, and slightly higher levels
233 of IDT in EXTs extracted from the PRX73^{OE} line (**Table 1**). By contrast, we identified strong
234 downregulation of Tyr- and IDT-levels in the EXT under-*O*-glycosylation mutants *p4h5 sergt1-1*, and
235 *sergt1-1 rra3* (**Table 1**). In these two double mutants, root hair growth is drastically inhibited
236 (Velasquez et al. 2015a). PROLYL 4-HYDROXYLASE (P4H5), PEPTIDYL-SER GALACTOSYLTRANSFERASE
237 (SGT1/SERGT1), and REDUCE RESIDUAL ARABINOSE 3 (RRA3) are key enzymes that modify EXT
238 hydroxylation (P4H5) and EXT *O*-glycosylation (SERGT1 and RRA3) (Marzol et al. 2018). Specifically, it
239 was shown that P4H5 is a 2-oxoglutarate (2OG) dioxygenase that catalyze the formation of trans-4-
240 hydroxyproline (Hyp/O) from peptidyl-proline preferentially in an EXT context allowing these proteins
241 to be *O*-glycosylated (Velasquez et al. 2011; Velasquez et al. 2015b). In the case of RRA3, together
242 with RRA1–RRA2 homologous proteins (Egelund et al., 2007; Velasquez et al., 2011), they are thought
243 to transfer the second arabinose to the sort glycan (composed by 4–5 units of L-arabinofuranose)
244 attached to the Hyp in the EXT peptides. SERGT1 add the single galactose units to the serine in the
245 repetitive motif of Ser-(Pro)_{3–5} present in EXT and EXT-related proteins (Saito et al. 2014). These
246 results are consistent with the notion that *O*-glycans strongly affect EXT Tyr crosslinking, as was
247 previously suggested based on the drastically reduced root hair growth of the under-glycosylation
248 mutants and *in vitro* crosslinking rates (Velasquez et al 2015a,b; Chen et al. 2015). We hypothesize
249 that absent or low *O*-glycosylation of EXTs or an increase in PRX levels may trigger a reduction in the
250 amount of peptidyl-Tyr and IDT levels in EXTs, with a putative concomitant increase in the amounts
251 of higher-order Tyr crosslinks (trimers as Pulcherosine and tetramers as Di-IDT), thus inhibiting root
252 hair growth. For technical reasons we could not measure the Pulcherosine and Di-IDT levels described
253 before in EXTs (Brady et al., 1996; 1998; Held et al. 2004) to test this hypothesis. Further research is
254 needed to decipher the *in vivo* regulation of Tyr crosslinking of EXTs by these three PRXs in plant cells.
255

256 A major limitation in our understanding of how EXTs function in plant cell walls is the lack of a realistic
257 full-length EXT protein model. We used coarse-grained molecular dynamics to build a larger model of
258 a triple-helix EXT sequence, that includes 10 conserved repeats (SPPPPYVYSSPPPPYSPSPKVYYK, 250
259 aminoacids in each polypeptide chain) (**Figure S3A–B**). Parameters for the *O*-glycosylated form of EXT
260 were developed in this work (**Figure S4**). The EXT molecules were modeled in two different states: as
261 a non-glycosylated trimeric helical conformation similar to animal collagen and in the *O*-glycosylated
262 state, with 4 arabinose monosaccharides in each hydroxyproline. Those two states were simulated
263 restraining both ends of the polypeptide chains, to model a fully extended helix (consistent with an
264 “indefinitely long-EXT”), and without that restriction, to evaluate the conformation that an isolated
265 10-repeat triple helix would adopt. The results indicate the importance of the triple-helix
266 conformation in the overall stability of the protein and especially in the conservation of its fibril-like
267 structure, in agreement with shorter-repeats single helix simulations performed previously
268 (Velasquez et al. 2015a; Marzol et al. 2018). The total volume of the extended systems triple helix was
269 measured in both glycosylation states (**Table S1**), differentiating EXT-protein-only and EXT-

270 protein+glycan volumes for the fully *O*-glycosylated EXT state. We observed that the EXT-protein-only
271 volume was significantly augmented by the presence of the oligosaccharide moieties, indicating that
272 *O*-glycans increase the distance between peptide chains in the EXT triple helix. We report the average
273 diameters for those systems (**Table S1**), which are consistent with the diameters previously reported
274 based on Atomic Force Microscopy (AFM) (images (Cannon et al. 2008). Additionally, *O*-glycosylation
275 contributes to an increase in the average distance between the side chains of tyrosine residues,
276 decreasing the proportion of tyrosine side chains that are close enough to lead to crosslinked EXT
277 chains (**Figure S3C**). Current experimental and modeling lines of evidence are in agreement with the
278 proposed role of proline-hydroxylation and carbohydrate moieties in keeping the EXT molecule in an
279 extended helical polyproline-II conformation state (Stafstrom & Staehelin 1986; Owen et al., 2010;
280 Ishiwata et al., 2014). This extended conformation might allow EXTs to interact properly with each
281 other and with other components in the apoplast, including PRXs and pectins, to form a proper cell
282 wall network (Nuñez et al., 2009; Valentin et al., 2010).

283
284 To test if these three PRXs (PRX01, PRX44, and PRX73) might be able to interact with single-chain
285 EXTs, we performed homology modeling with GvEP1, an EXT-PRX that is able to crosslink EXTs *in vitro*
286 (Jackson et al., 2001; Pereira et al. 2011). In addition, we included PRX64, as a PRX described for lignin
287 polymerization in the root endodermis (Lee et al. 2013) and PRX36, which is able to bind
288 homogalacturonan pectin in the seed coat (Francoz et al. 2019) as controls. By *docking* analysis, we
289 obtained interaction energies (Kcal/mol) for all of them. We analyzed docking with four different short
290 EXT peptides: a non-hydroxylated peptide, a hydroxylated peptide, an arabinosylated peptide and an
291 arabino-galactosylated peptide. As mentioned earlier, it was previously shown that mutants carrying
292 under-*O*-glycosylated EXTs have severe defects in root hair growth (Velasquez et al. 2011; Velasquez
293 et al. 2015a). Our docking results for the different PRXs show consistent interaction energy differences
294 that depend on the EXT glycosylation state, being higher for non-*O*-glycosylated species. In addition,
295 *O*-glycosylated EXT variants docked in a rather dispersed way while non-*O*-glycosylated variants
296 preferentially docked in a grooved area (**Figure 4A–C**). Furthermore, **Figure 4A** shows how a non-*O*-
297 glycosylated peptide binds through a groove, leaving one Tyr docked in a cavity and very close to the
298 heme iron (5Å), with a second Tyr a few Angstroms away. The arrangement and distances between
299 the tyrosines suggest that this could be an active site where Tyr crosslinking takes place. Although it
300 is not possible to compare the interaction energies obtained with the different EXT species among
301 docking runs, a general trend can be observed in **Figure 4C**. In general, we observed higher interaction
302 energies (more negative values) for hydroxylated EXT species, followed by non-hydroxylated EXTs,
303 and then by *O*-glycosylated EXT variants. When we compared interaction energies among different
304 PRXs interacting with EXT substrates with the same degree of *O*-glycosylation, we observed that
305 PRX73 displayed the highest interaction activity with the non-hydroxylated EXT species, followed by
306 PRX01 and then PRX44. For the hydroxylated EXT variant, the order was PRX44>PRX73>PRX01. PRX44
307 displayed the highest interaction energy with the *O*-glycosylated species. All together, these results

308 are consistent with the constitutive root hair growth effect observed for PRX44^{OE} and PRX73^{OE} and
309 with non-glycosylated EXT being the substrate of peroxidation. Overall, this possibly indicates that
310 PRX44 and PRX73 might interact with EXT substrates and possibly catalyze Tyr-crosslinking in open
311 regions of the EXT backbones with little or no *O*-glycosylation. This is in agreement with previous
312 studies that suggested that high levels of *O*-glycosylation in certain EXT segments physically restrict
313 EXT lateral alignments, possibly by acting as a branching point (Cannon et al.2008; Velasquez et al.,
314 2015a; Marzol et al. 2018).

315
316 To examine the evolution of PRX01, PRX44, and PRX73, we performed comprehensive phylogenetic
317 analyses of Class-III peroxidases across diverse land plant lineages. Under low selective pressure to
318 maintain substrate specificity, EXT-PRX activities might have evolved multiple times in parallel during
319 land plant evolution through gene duplication followed by neofunctionalization or
320 subfunctionalization. PRX01, PRX44, and PRX73 belong to three independent orthologous groups
321 (**Figure S5**) and orthologs for each *A. thaliana* PRX have been detected in available Brassicaceae
322 genomes and in various Angiosperm and Gymnosperm families, but not from Lycophytes and from
323 non-vascular land plants. Thus, these three PRX sequences were the result of ancestral duplications
324 before the divergence between Gymnosperms and Angiosperms but after the emergence of the
325 Tracheophytes (**Figure S5**). Orthologs of the three PRX genes have only been detected in true root
326 containing organisms and these three PRXs are expressed in roots and root hairs, as are most of their
327 orthologous sequences (where expression data are available) (**Figure S6**). This strongly supports the
328 hypothesis that the three independent orthogroups have conserved functions in roots. With the
329 exception of PRX73, which belongs to a cluster containing the putative EXT-PRX from tomato
330 (*Solanum lycopersicum*; LePRX38), the other two PRX sequences did not cluster with sequences
331 already described as putative EXT-PRXs, such as PRX09 and PRX40 (Jacobowitz et al. 2019). Indeed,
332 the other known EXT-PRXs (identified mostly based on *in vitro* evidence) are not clustered together,
333 but are widely distributed in the tree (**Figure S5**). This analysis suggests that plant EXT-PRXs might
334 have evolved several times in parallel during Tracheophyte evolution.

335
336 Based on the results shown in this work, we propose a working model in which PRX01, PRX44, and
337 PRX73 (and possibly other PRXs) control root hair growth by channelling H₂O₂ consumption and
338 affecting the cell wall hardening process. In this polar growing cells, it is known that H₂O₂ is primary
339 derived from by the respiratory burst oxidase homolog C (RBOHC), and to a lower extent from RBOHH
340 and RBOHJ activities that produce superoxide ions (Monshausen et al. 2007; Tajeda et al. 2008;
341 Mangano et al. 2017) that are further converted chemically or enzymatically to H₂O₂. Then, part of
342 H₂O₂ might be transported from the apoplast to the cytoplasm side by specific PIPs as it was shown
343 to occur in several plant cell types (e.g. in stomata and epidermal cells) in response to diverse stimuli
344 (Dynowski et al., 2008; Hooijmaijers et al., 2012; Rodrigues et al. 2017). When apoplastic PRX protein
345 levels are low, which is linked to reduced peroxidase activity as in the triple mutant *prx01,44,73*, high

346 levels of H₂O₂ accumulate in the apoplast, triggering through the oxidative cycle (OC) a cell wall
347 loosening effect that affects growth homeostasis and inhibits expansion by decreasing root hair
348 growth and cell wall thickness (**Figure S7**). Concomitantly, deficient PRX activity in the apoplast also
349 triggers lower H₂O₂ levels in the cytoplasm of growing root hairs. This is in agreement with the fact
350 that exogenously supplied H₂O₂ inhibited root hair polar expansion, whereas treatment with ROS
351 scavengers (e.g., ascorbic acid) caused root hair bursting (Orman-ligeza et al. 2016), reinforcing the
352 notion that apoROS modulates cell growth by impacting cell-wall properties (Mangano et al. 2017). Our
353 results suggest that either low or high levels of apoplastic Class-III PRXs in the root hair cell walls might
354 affect the homeostasis of ROS and cell wall thickness with a clear effect on cell expansion. Still several
355 aspects of this model proposed here remains to be tested.

356

357 **Conclusions**

358 Currently, several of the 73 apoplastic Class-III PRXs in *Arabidopsis thaliana* have no assigned
359 biological function. In this work, we have characterized three related EXT-PRXs, PRX01, PRX44, and
360 PRX73 that function in ROS homeostasis and potentially in EXT assembly during root hair growth.
361 These PRXs might control Tyr crosslinking in EXTs and related glycoproteins and modify its secretion
362 and assembly in the nascent tip cell walls. Using modeling and docking approaches, we were able to
363 measure the interactions of these PRXs with single chain EXT substrates. All these lines of evidence
364 indicate that PRX01, PRX44, and PRX73 are important enzymes that could be involved in EXT assembly
365 during root hair growth. From an evolutionary perspective, all the putative EXT-PRXs (previously
366 identified based on *in vitro* evidence or immunolabeling) do not cluster together in the phylogenetic
367 tree of Class-III PRXs, suggesting that plant-related EXT-PRXs might have evolved several times in
368 parallel during Tracheophyte evolution. Interestingly, as a convergent evolutionary extracellular
369 assembly, hydroxyproline-rich collagen Class-IV, similar to the green EXT lineage and related
370 glycoproteins, is also crosslinked by the activity of a specific class of animal heme peroxidases (named
371 peroxidasin or PXDN) to form insoluble extracellular networks (Vanacore et al. 2009; Bhave et al.
372 2012). While the biophysical properties of collagen IV allow the correct development and function of
373 multicellular tissues in all animal phyla (Brown et al. 2017), EXT assemblies also have key functions in
374 several plant cell expansion and morphogenesis processes (Baumberger 2001, 2003; Hall and Cannon
375 et al. 2002; Cannon *et al.*, 2008; Ringli 2010; Lamport et al., 2011; Velasquez et al. 2015a,b; Fabrice et
376 al. 2018; Sede et al. 2018; Marzol et al. 2018). This might imply that crosslinked extracellular matrices
377 based on hydroxyproline-rich polymers (e.g., collagens and EXTs) have evolved more than once during
378 eukaryotic evolution, providing mechanical support to single and multiple cellular tissues. Further
379 analyses are required to establish how these described EXT-PRXs catalyze Tyr crosslinks on EXTs at
380 the molecular level and how this assembly process is regulated during polar cell expansion.

381 **Experimental Procedures**

382
383 **Plant and growth conditions.** *Arabidopsis thaliana* Columbia-0 (Col-0) was used as the wild Class (Wt)
384 genotype in all experiments. All mutants and transgenic lines tested are in this genetic background.
385 Seedlings were germinated on agar plates in a Percival incubator at 22°C in a growth room with 16h
386 light/8h dark cycles for 10 days at 140 $\mu\text{mol m}^{-2}\text{s}^{-1}$ light intensity. Plants were transferred to soil for
387 growth under the same conditions. For identification of T-DNA knockout lines, genomic DNA was
388 extracted from rosette leaves. Confirmation by PCR of a single and multiple T-DNA insertions in the
389 target PRX genes were performed using an insertion-specific LBb1.3 primers in addition to one gene-
390 specific primer. To ensure gene disruptions, PCR was also run using two gene-specific primers,
391 expecting bands corresponding to fragments larger than in WT. We isolated homozygous lines for
392 PRX01 (AT1G05240, *prx01-2*, Salk_103597), PRX44 (AT4G26010, *prx44-2*, Salk_057222) and PRX73
393 (AT5G67400, *prx73-3*, Salk_009296). SERGT1 (*sergt1-1* SALK_054682), *rra3* (GABI_233B05)
394 (Velasquez et al., 2011) and *p4h5* T-DNA mutant (Velasquez et al., 2011) were isolated and described
395 previously. Double and triple mutants were generated by manual crosses of the corresponding single
396 mutants (Velasquez et al., 2015a). All the mutant lines used in this study are described in **Table S2**.

397
398 **PRX::GFP and 35S::PRX-GFP lines.** Vectors based on the Gateway cloning technology (Invitrogen)
399 were used for all manipulations. Constitutive expression of PRXs-GFP tagged lines were achieved in
400 plant destination vector pMDC83. cDNA PRXs sequences were PCR-amplified with AttB recombination
401 sites. PCR products were then recombined first in pDONOR207 and transferred into pGWB83. To
402 generate transcriptional reporter, the PRXs promoter regions (2Kb) was amplified and recombined
403 first in pDONOR207 and transferred into pMDC111. All the transgenic lines used in this study are
404 described in **Table S2**.

405
406 **SS-TOM and SS-TOM-Long-EXT constructs.** The binary vector pART27, encoding tdTomato secreted
407 with the secretory signal sequence from tomato polygalacturonase and expressed by the constitutive
408 CaMV 35S promoter (pART-SS-TOM), was the kind gift of Dr. Jocelyn Rose, Cornell University. The
409 entire reporter protein construct was excised from pART-SS-TOM by digesting with *NotI*. The resulting
410 fragments were gel-purified with the QIAquick Gel Extraction Kit and ligated using T4 DNA Ligase (New
411 England Biolabs) into dephosphorylated pBlueScript KS+ that had also been digested with *NotI* and
412 gel-purified to make pBS-SS-TOM. The plasmid was confirmed by sequencing with primers 35S-FP (5'-
413 CCTTCGCAAGACCCTCCTC-3') and OCS-RP (5'-CGTGCACAACAGAATTGAAAGC-3'). The sequence of the
414 EXT domain from *SIPEX1* (NCBI accession AF159296) was synthesized and cloned by GenScript into
415 pUC57 (pUC57-EXT). The plasmid pBS-SS-TOM-Long-EXT was made by digesting pUC57-EXT and pBS-
416 SS-TOM with *NdeI* and *SgrAI*, followed by gel purification of the 2243 bp band from pUC57-EXT and
417 the 5545 bp band from pBS-SS-TOM, and ligation of the two gel-purified fragments. The pBS-SS-TOM-
418 Long-EXT plasmid was confirmed by sequencing with 35S-FP, OCS-RP, and tdt-seq-FP (5'-

419 CCCGTTCAATTGCCTGGT-3'). Both pBS plasmids were also confirmed by digestion. The binary vector
420 pART-SS-TOM-Long-EXT was made by gel purifying the *NotI* insert fragment from the pBS-SS-TOM-
421 Long EXT plasmid and ligating it with pART-SS-TOM backbone that had been digested with *NotI*, gel
422 purified, and dephosphorylated. This plasmid was confirmed by sequencing. The construct SS-TOM
423 and SS-TOM-Long-EXT were transformed into *Arabidopsis* plants. The secretory sequence (SS) from
424 tomato polygalacturonase is MVIQRNSILLIIIFASSISTCRSGT (2.8kDa) and the EXT-Long domain
425 sequence with six alanine cluster is
426 BAAAAAAAACTLPSLKNFTFSKNIFESMDETCRPSSESKQVKIDGNENCLGGRSEQRTEKECFPVVSKPVDCSKGHCG
427 VSREGQSPKDPKTVTTPPKPSTPTTPKPNPSPPPPKTLPPPKTSPPPPVHSPPPPVASPPPPVHSPPPVASPPPP
428 VHSPPPPVASPPPPVHSPPPVASPPPPVHSPPPVHSPPPVHSPPPVHSPPPVHSPPPVHSPPPVHSPPPVHSPPP
429 PVHSPPPVHSPPPVHSPPPVHSPPPVHSPPPVHSPPPVHSPPPVHSPPPVHSPPPVHSPPPVHSPPPVHSPPPVHSP
430 PPPVASPPPPVHSPPPVHSPPPVHSPPPVHSPPPVHSPPPVHSPPPVHSPPPVHSPPPVHSPPPVHSPPPVHSPPPVHSP
431 PPPVHSPPPVHSPPPVHSPPPVHSPPPVHSPPPVHSPPPVHSPPPVHSPPPVHSPPPVHSPPPVHSPPPVHSPPPVHSP
432 TOM-EXT-Long Mw is 97.4 kDa. All the transgenic lines used in this study are described in **Table S2**.

433
434 **Root hair phenotype.** For quantitative analysis of root hair phenotypes in *prx01,44,73* mutant,
435 35S:PRX-GFP lines and Wt Col-0, 200 fully elongated root hairs were measured (n roots= 20-30) from
436 seedlings grown on vertical plates for 10 days. Values are reported as the mean \pm SD using the Image
437 J software. Measurements were made after 7 days. Images were captured with an Olympus SZX7
438 Zoom microscope equipped with a Q-Colors digital camera.

439
440 **Confocal imaging.** Root hairs were ratio imaged with the Zeiss LSM 710 laser scanning confocal
441 microscope (Carl Zeiss) using a 40X oil-immersion, 1.2 numerical aperture. EGFP (473–505nm)
442 emission was collected using a 458-nm primary dichroic mirror and the meta-detector of the
443 microscope. Bright-field images were acquired simultaneously using the transmission detector of the
444 microscope. Fluorescence intensity was measured in 7 μ m ROI (Region Of Interest) at the root hair
445 apex.

446
447 **Peroxidase activity.** Soluble proteins were extracted from roots grown on agar plates in a Percival
448 incubator at 22°C in a growth room for 10 days at 140 μ mol m⁻²s⁻¹ light intensity by grinding in 20mM
449 HEPES, pH 7.0, containing 1 mM EGTA, 10mM ascorbic acid, and PVP PolyclarAT (100mg g⁻¹ fresh
450 material; Sigma, Buchs, Switzerland). The extract was centrifuged twice for 10 min at 10,000 g. Each
451 extract was assayed for protein levels with the Bio-Rad assay (Bio-Rad). PRX activity was measured at
452 25°C by following the oxidation of 8 mM guaiacol (Fluka) at 470 nm in the presence of 2 mM H₂O₂
453 (Carlo Erba) in a phosphate buffer (200 mM, pH6.0). Values are the mean of three replicates \pm SD.

454
455 **Cytoplasmic ROS (cytROS) measurements.** 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA) is
456 as a cell-permeable fluorogenic probe to quantify reactive oxygen species (ROS). H₂DCFDA diffuses

457 into cells and is deacetylated by cellular esterases to form 2',7'-dichlorodihydrofluorescein (H₂DCF).
458 In the presence of ROS, predominantly H₂O₂, H₂DCF is rapidly oxidized to 2',7'-dichlorofluorescein
459 (DCF), which is highly fluorescent, with excitation and emission wavelengths of 498 and 522 nm,
460 respectively. To measure cytoplasmic ROS in root hairs cells, growth of Arabidopsis seeds on a plate
461 was done with 1% sterile agar for 8 d in a chamber at 22°C with continuous light. These seedlings were
462 incubated in darkness on a slide for 10 min with 50 μM H₂DCFDA at room temperature. Samples were
463 observed with Zeiss Imager A2 Epifluorescence. A 10× objective was used, 0.30 N.A., and exposure
464 time 80-500ms. Images were analyzed using ImageJ 1.50b software. To measure ROS mean, a circular
465 region of interest (ROI) (r=2.5) was chosen in the tip zone of the root hair. All root hairs of six seedlings
466 per genotype were analyzed. The reported values are the mean ± standard deviation (mean ± SD).

467
468 **Apoplastic ROS (apoROS) measurements.** To measure apoplastic ROS in root hair cells, roots of 7-day-
469 old seedlings were incubated with 50 μM Amplex™ UltraRed Reagent (AUR, Molecular Probes) for 20
470 min in dark conditions and rinsed with liquid MS. Root hairs were imaged with a Zeiss LSM5 Pascal
471 laser scanning confocal microscope. The fluorescence emission of oxidized AUR in the apoplast of root
472 hair cells was observed between 585 and 610 nm using 543 nm argon laser excitation, 40X objective,
473 N/A=1.2. The intensity of fluorescence was quantified on digital images using ImageJ software.
474 Quantification of the AUR probing fluorescence signal was restricted to apoplastic spaces at the root
475 hair tip (as shown in **Figure 1**). The measurements were performed in three independent experiments
476 (n = 6) with the same microscopic settings.

477
478 **Phylogenetic analysis.** 73 class-III PRX protein sequences from *A. thaliana*, two putative lignin class-
479 III PRXs from *Zinnia elegans* and 4 putative Extensin class-III PRXs from *Lupinus album*, *Lycopersicon*
480 *esculentum*, *Phaseolus vulgaris* and *Vitis vinifera*, have been aligned with ClustalW and the tree
481 constructed using the Neighbor-Joining method (Saitou and Nei, 1987). The analyses were conducted
482 in MEGA7 (Kumar, 2016). All protein sequences are available using their ID number
483 (<http://peroxibase.toulouse.inra.fr>) (Savelli et al., 2019).

484
485 **Co-expression analysis network.** Co-expression networks for *RSL4* root hair genes were identified
486 from PlaNet (<http://aranet.mpimp-golm.mpg.de>) and trimmed to facilitate readability (Mutwill et al.
487 2011). Each co-expression of interest was confirmed independently using the expression angler tool
488 from Botany Array Resource BAR ([http://bar.utoronto.ca/ntools/cgi-](http://bar.utoronto.ca/ntools/cgi-bin/ntools_expression_angler.cgi)
489 [bin/ntools_expression_angler.cgi](http://bar.utoronto.ca/ntools/cgi-bin/ntools_expression_angler.cgi)) and ATTED-II (<http://atted.jp>). Only those genes that are
490 connected with genes of interest are included.

491
492 **Tyr-crosslinking analysis.** Alcohol-insoluble residues of root tissues obtained from *PRX01,44,73*
493 mutants, Col-0 and 35Sp::PRX44-GFP lines were hydrolyzed in 6 N HCl (aqueous) with 10 mM phenol
494 (2 mg ml⁻¹; 110 °C; 20 h). Hydrolysates were dried under a steady stream of nitrogen (gas) and then

495 re-dissolved at 10 mg ml⁻¹ in water. The hydrolysates were fractionated by gel permeation
496 chromatography on a polyhydroxyethyl A column (inner diameter, 9.4 x 200 mm, 10 nm pore size,
497 Poly LC Inc., Columbia, MD) equilibrated in 50 mM formic acid and eluted isocratically at a flow rate
498 of 0.8 ml min⁻¹. UV absorbance was monitored at 280 nm. The amounts of Tyr and IDT in the
499 hydrolysates were then determined by comparison with peak areas of authentic Tyr and IDT
500 standards. Response factors were determined from three level calibrations with the Tyr and IDT
501 standards.

502
503 **Immuno-blot Analysis.** Plant material (100 mg of root from 15 days old seedlings grown as indicated
504 before) was collected in a microfuge tube and ground in liquid nitrogen with 400 mL of protein
505 extraction buffer (125 mM Tris-Cl, pH. 4.4, 2% [w/v] SDS, 10% [v/v] glycerol, 6M UREA, 1% [v/v] b-
506 mercaptoethanol, 1mM PMSF). Samples were immediately transferred to ice. After 4° centrifugations
507 at 13000 rpm for 20 min, supernatant was move to a new 1.5 ml tube and equal volumes of Laemmli
508 buffer (125 mM Tris-Cl, pH. 7.4, 4% [w/v] SDS, 40% [v/v] glycerol, 10% [v/v] β-mercaptoethanol,
509 0.002% [w/v] bromphenol blue) were added. The samples (0.5–1.0 mg/mL of protein) were boiled for
510 5 min and 30 mL were loaded on 10% SDS-PAGE. The proteins were separated by electrophoresis and
511 transferred to nitrocellulose membranes. Anti-GFP mouse IgG (clones 7.1 and 13.1; Roche Applied
512 Science) was used at a dilution of 1:2,000 and it was visualized by incubation with goat anti-mouse
513 IgG secondary antibodies conjugated to horseradish peroxidase (1:2,000) followed by a
514 chemiluminescence reaction (Clarity Western ECL Substrate; Bio-rad). For the SS-TOM lines analysis,
515 proteins were extracted in 2x SDS buffer (4% SDS, 125mM Tris pH 6.8, 20% glicerol, 0.01%
516 bromophenol blue, 50 mM dithiothreitol [DTT]), using 10 µl of buffer per mg of plant tissues of Wt
517 Col-0, transgenic lines 35S:SS-TOM and 35S:SS-TOM-Long-EXT. Two transgenic lines were analyzed.
518 10 µl of supernatant of each protein extract were run into a 12% polyacrylamide gel during one hour
519 at 200 V, and then transferred to a PVDF membrane. PVDF was blocked with 5% milk in TBST (Tris-HCl
520 10 mM, pH 7,4, NaCl 150 mM, Tween-20 al 0,05%) for 1 hour at 4°C and then washed four times
521 during 15 min in TBST. An anti-RFP (A00682, GenScript) was used as primary antibody overnight at
522 4°C. Four washes of 15 min each in TBST at room temperature and then it was incubated two hours
523 with a secondary antibody anti-rabbit (goat) conjugated with alkaline phosphatase (A3687, Sigma), in
524 a 1:2,500 dilution with TBST. Four washes of 15 min each in TBST at room temperature. Finally, 10 ml
525 of alkaline phosphatase (100mM Tris-HCl pH 9.5, 100 mM NaCl, 3 mM MgCl₂) containing 80 µl NBT
526 (Sigma) (35mg/ml in 70% DMSO and 30 µl de BCIP (Sigma) (50 mg/ml in 100% de DMSO) were used.

527
528 **Transmission electron microscopy of root hair cell walls.** Seeds were germinated on 0.2x MS, 1%
529 sucrose, 0.8% agar. Seven days after germination, seedlings were transferred to new 0.2x MS, 1%
530 sucrose, 0.8% agar plates with or without 100 µM SHAM. After 4 additional days, 1-mm root segments
531 with root hairs were fix in 2% glutaraldehyde in 0.1M cacodylate buffer pH7.4. Samples were rinsed
532 in cacodylate buffer and post-fixed in 2% OsO₄. After dehydration in ethanol and acetone, samples

533 were infiltrated in Epon resin (Ted Pella, Redding, CA). Polymerization was performed at 60°C.
534 Sections were stained with 2% uranyl acetate in 70% methanol followed by Reynold's lead citrate
535 (2.6% lead nitrate and 3.5% sodium citrate [pH 12.0]) and observed in a Tecnai 12 electron
536 microscope. Quantitative analysis of cell wall thickness was performed using FIJI.

537
538 **Modeling and molecular docking between PRXs and EXTs.** Modeling and molecular docking: cDNA
539 sequences of PRXs were retrieved from TAIR (PRX01: AT1G05240, PRX36: AT3G50990, PRX44:
540 AT4G26010, PRX64: AT5G42180, PRX73: AT5G67400) and NCBI Nucleotide DB (PRX24Gv:Vitis vinifera
541 peroxidase 24, GvEP1, LOC100254434). Homology modeling was performed for all PRXs using
542 modeller 9.14 (Sali et al. 1993), using the crystal structures 1PA2, 3HDL, 1QO4 and 1HCH as templates,
543 available at the protein data bank. 100 structures were generated for each protein and the best
544 scoring one (according to DOPE score) was picked. The *receptor* for the docking runs was generated
545 by the prepare_receptor4 script from autodock suite, adding hydrogens and constructing bonds.
546 Peptides based on the sequence PYYSPSPKVYYPPSSYVYPPPPS were used, replacing proline by
547 hydroxyproline, and/or adding *O*-Hyp glycosylation with up to four arabinoses per hydroxyproline in
548 the fully glycosylated peptide and a galactose on the serine, as it is usual in plant *O*-Hyp
549 <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5045529/>. Ligand starting structure was generated
550 as the most stable structure by molecular dynamics (Velasquez et al. 2015a). All ligand bonds were
551 set to be able to rotate. Docking was performed in two steps, using Autodock vina (Trott et al. 2010).
552 First, an exploratory search over the whole protein surface (exhaustiveness 4) was done, followed by
553 a more exhaustive one (exhaustiveness 8), reducing the search space to a 75x75x75 box centered over
554 the most frequent binding site found in the former run.

555
556 **EXT conformational coarse-grained model.** The use of coarse-grained (CG) molecular dynamics (MD)
557 allowed collection of long timescale trajectories. System reduction is significant when compared to all
558 atom models, approximately reducing on order of magnitude in particle number. In addition, a longer
559 integration time step can be used. Protein residues and coarse grained solvent parameters
560 correspond to the SIRAH model (Darré et al. 2015), while ad hoc specific glycan parameters were
561 developed. The CG force field parameters developed correspond to arabinofuranose and
562 galactopyranose (**Figure S5**). Triple helix systems were simulated both, in the non-glycosylated and
563 fully *O*-glycosylated states, where all the hydroxyprolines are bound to a tetrasaccharide of
564 arabinofuranoses, and specific serine residues contain one galactopyranose molecule. They were
565 immersed in WT4 GC solvent box that was constructed to be 2 nm apart from the extensin fiber, and
566 periodic boundary conditions were employed. Coarse grained ions were also included to achieve
567 electroneutrality and 0.15 M ionic strength. All simulations were performed using the GROMACS MD
568 package at constant temperature and pressure, using the Berendsen thermostat (respectively) and
569 Parrinello-Rahman barostat (Parrinello and Rahman 1981), and a 10 fs time step. The obtained
570 trajectories were analysed using the Mdtraj python package (McGibbon et al, 2015) and visualized

571 with Visual Molecular Dynamics (VMD) 1.9.1 (Humphrey et al. 1996). Volume measurements were
572 performed using a Convex Hull algorithm implemented in NumPy (Oliphant 2006), and average
573 diameter calculations were derived from this quantity using simple geometric arguments.

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581

582 **Author Contribution**

583 E.M and C.B performed most of the experiments and analysed the data. P.R. and C.D. analysed the
584 peroxidase activity and performed phylogenetic analysis. J.W.M-E and M.H. analysed the Tyr-
585 crosslinking on EXTs. A.A.A. and A.D.N performed the docking experiments and analysed this data.
586 M.B. and L.C. perform the EXT modelling and analysed this data. M.F. and P.B generated the EXT
587 reporter lines and performed the immune-blot analysis of SS-TOM and SS-TOM-Long-EXT lines.
588 J.M.P., D.R.R.G., Y.d.C.R.G., S.M., and F.B.H. analysed the data. J.P., J.P-V., and M.S.O. performed the
589 transmission electron microscopy analysis. J.M.E. designed research, analysed the data, supervised
590 the project, and wrote the paper. All authors commented on the results and the manuscript. This
591 manuscript has not been published and is not under consideration for publication elsewhere. All the
592 authors have read the manuscript and have approved this submission.

593

594 **Competing financial interest**

595 The authors declare no competing financial interests. Correspondence and requests for materials
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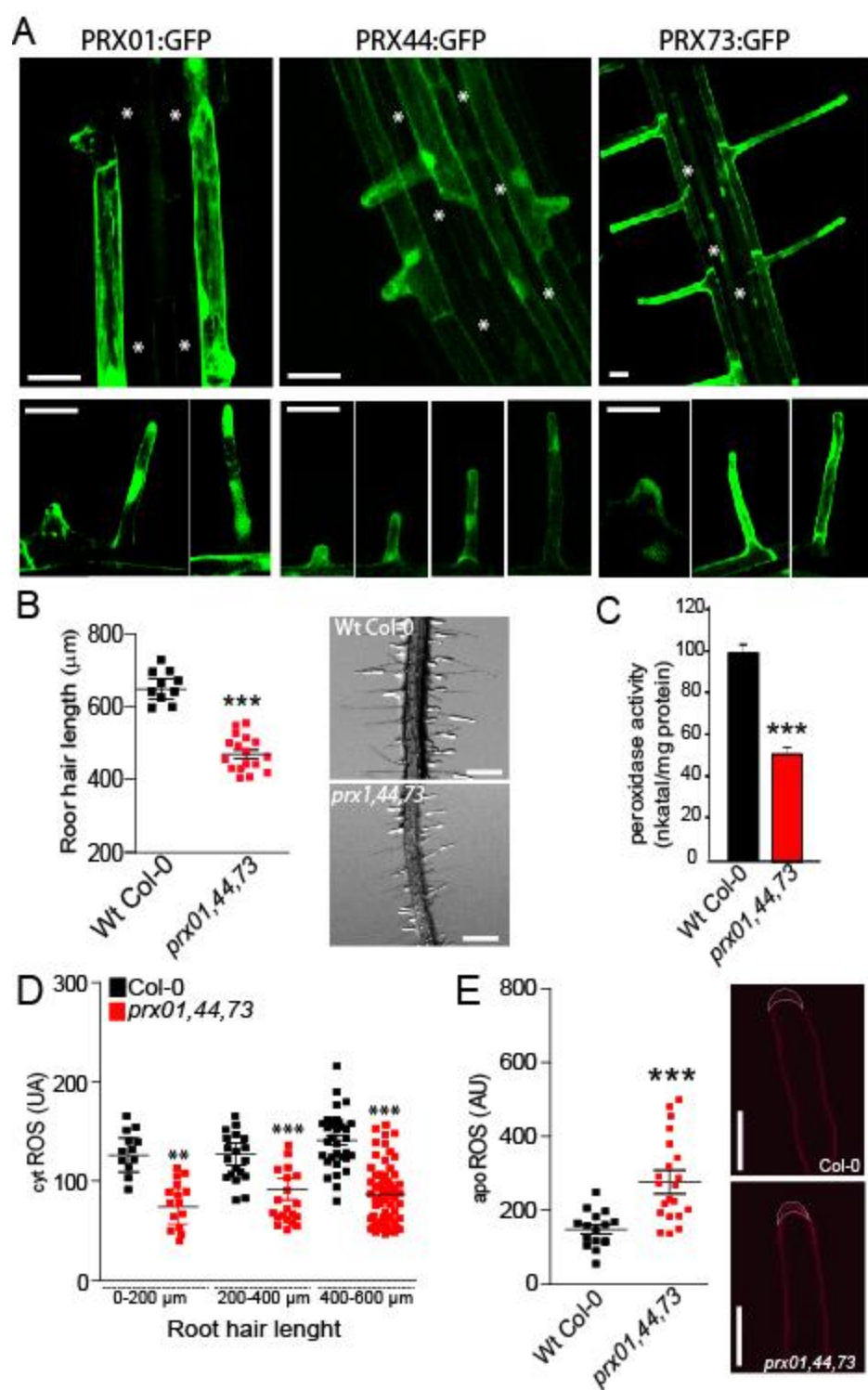
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782 **Figure 1. Characterization of root hair-specific PRX01, PRX44 and PRX73 expression and mutant**
783 **analysis.**

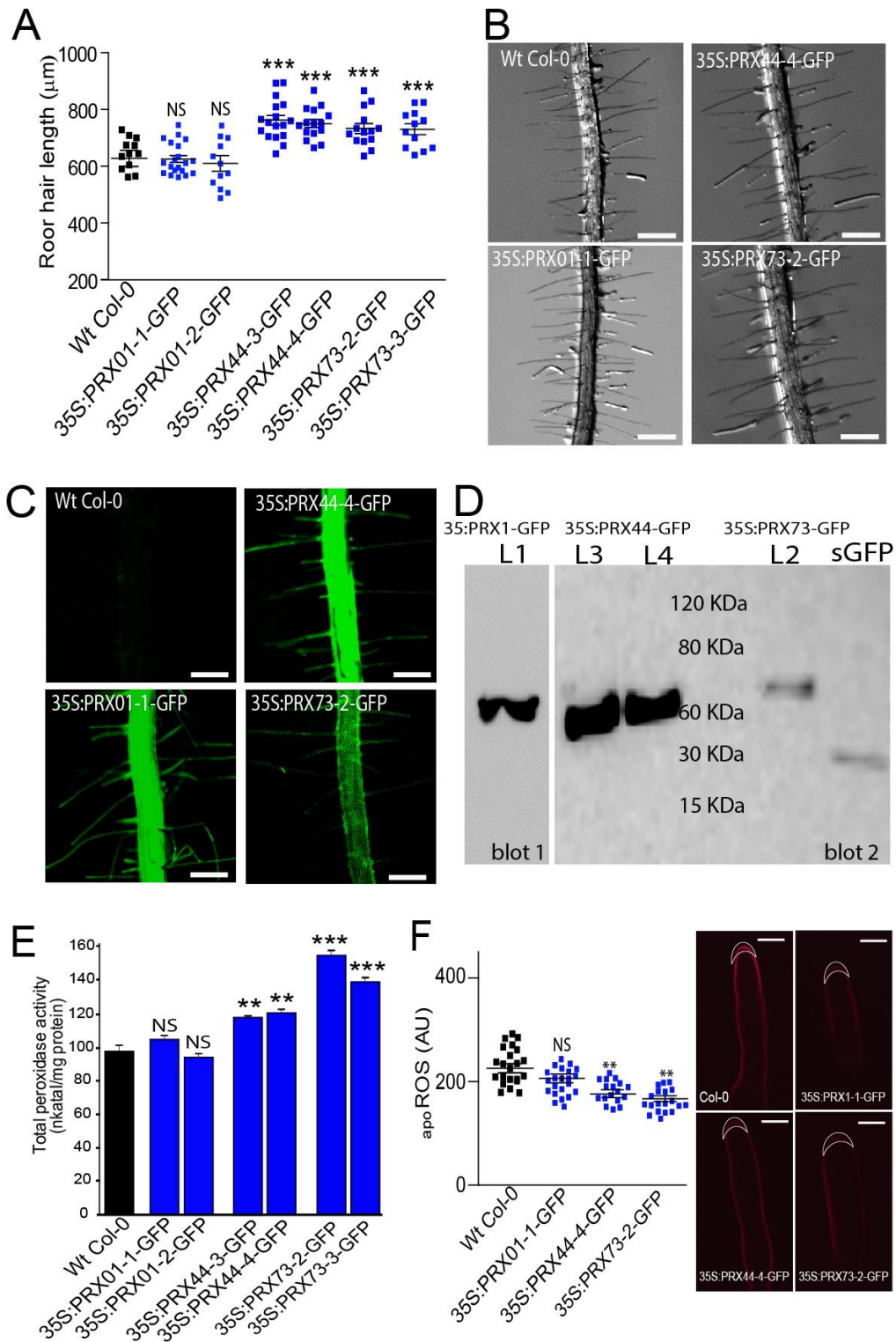
784 **(A)** GFP-tagged transcriptional reporters of PRX01, PRX44 and PRX73 show expression in the root
785 elongation zone and specifically in root hairs (bottom). Scale bar = 20 μ m. (*) indicates atrichoblast
786 cell layers, which lack GFP expression.

787 **(B)** Root hair length phenotype of Wt and the *prx01,44,73* triple mutant. Left, box-plot of root hair
788 length. Horizontal lines show the means. P-value determined by one-way ANOVA, (***) $P < 0.001$.
789 Right, bright-field images exemplifying the root hair phenotype in each genotype. Scale bars, 1 mm.

790 **(C)** Peroxidase activity in Wt and *prx01,44,73* triple mutant roots. Enzyme activity values (expressed
791 as nkatal/mg protein) are shown as the mean of three replicates \pm SD. P-value determined by one-
792 way ANOVA, (***) $P < 0.001$.

793 **(D)** Cytoplasmic ROS levels measured with H₂DCF-DA in Wt and *prx01,44,73* triple mutant root hairs.
794 Horizontal lines show the means. P-values determined by one-way ANOVA, (***) $P < 0.001$ and (**)
795 $P < 0.01$.

796 **(E)** Apoplastic ROS levels measured with Amplex™ UltraRed (AUR) in Wt and *prx01,44,73* triple
797 mutant root hairs. ROS signal was quantified from the root hair cell tip. Left, box-plot of apoROS
798 values. Horizontal lines show the means. P-value determined by one-way ANOVA, (***) $P < 0.001$.
799 Right, fluorescence images exemplifying apoROS detection in root hair apoplast.



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Figure 2. Over-expression of PRX44 and PRX73 promotes root hair growth and higher root peroxidase activity.

804 **(A)** Root hair length phenotype of Wt and PRX^{OE} lines (in Wt background). Box-plot of root hair length.
805 Horizontal lines show the means. P-values determined by one-way ANOVA, (***) P<0.001, (NS) not
806 significantly different.

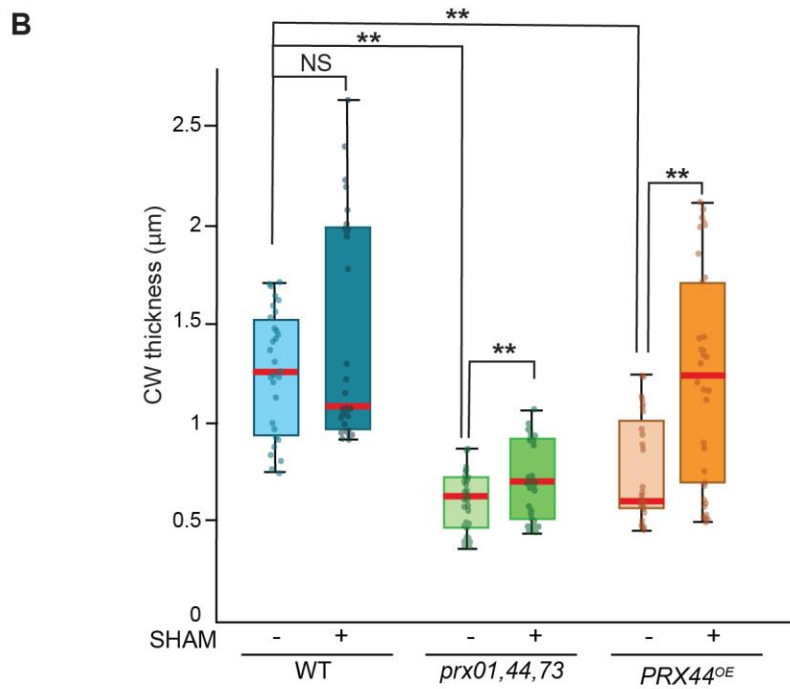
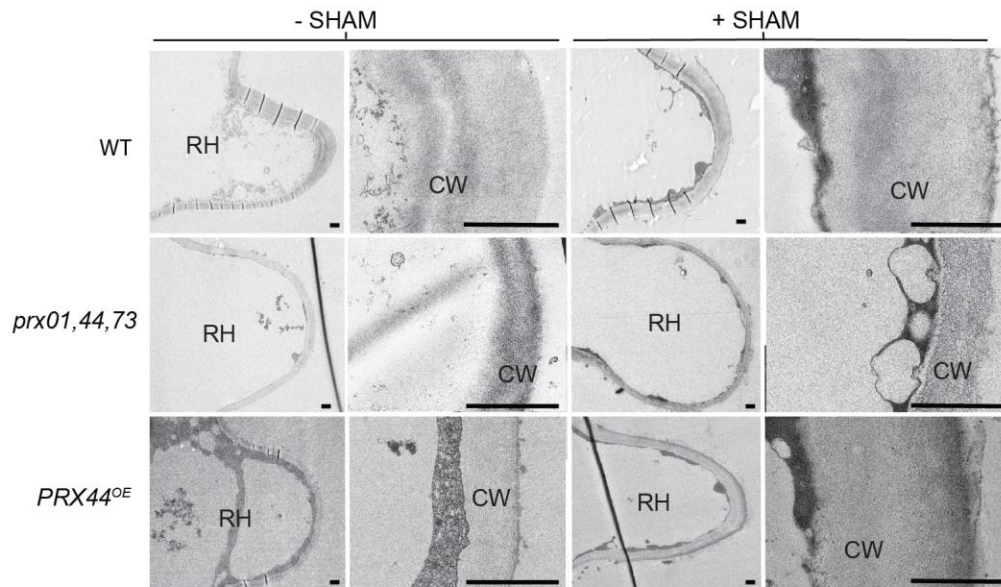
807 **(B)** Bright-field images exemplifying the root hair phenotype analyzed in Figure 2A. Scale bar = 0.5
808 mm.

809 **(C)** Expression of GFP-tagged 35S:PRX01, 35S:PRX44 and 35S:PRX73 in root hair cells.

810 **(D)** Western blot of PRX01-GFP, PRX44-GFP and PRX73-GFP. Soluble GFP (sGFP) was used as control.
811 The predicted molecular weights are 62.6 KDa for PRX01-GFP, 60.8 KDa for PRX44-GFP, 62.9 KDa for
812 PRX73 and 27 KDa for sGFP.

813 **(E)** Assays of total peroxidase activity in Wt and PRXs^{OE} lines (in Wt background). Enzyme activity
814 (expressed in nkatal/mg protein) was determined by a guaiacol oxidation-based assay. Values are the
815 mean of three replicates ± SD. P-values determined by one-way ANOVA, (***) P<0.001, (**) P<0.01,
816 (NS) not significantly different.

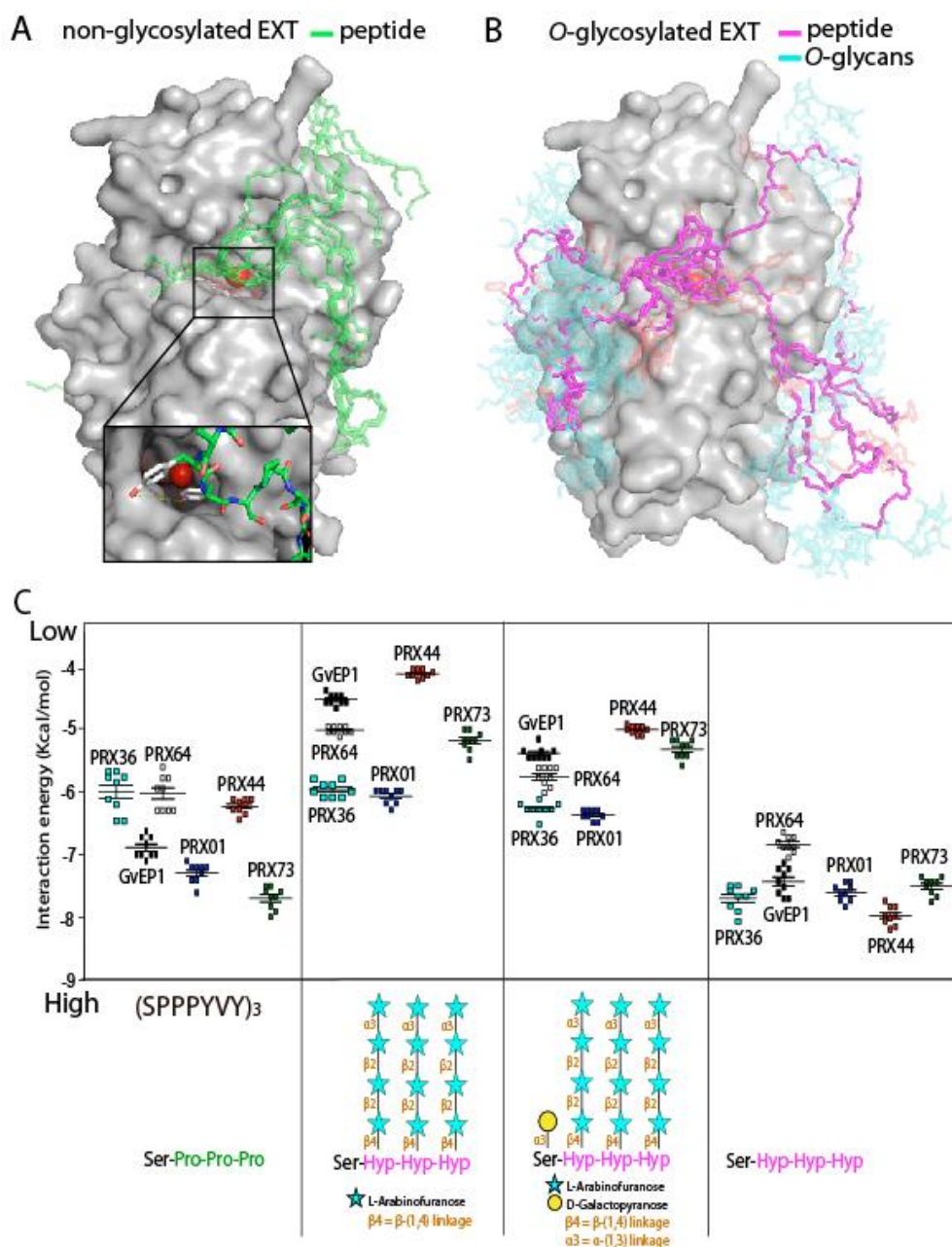
817 **(F)** Apoplastic ROS levels measured with Amplex™ UltraRed (AUR) in Wt and PRX^{OE} lines (in Wt
818 background). ROS signal was quantified from the root hair cell tip. Left, box plot of apoROS values.
819 Horizontal lines show the means. P-values determined by one-way ANOVA, (**) P<0.01, (NS) not
820 significantly different. Right, fluorescence images exemplifying apoROS detection in root hair
821 apoplast. Scale bar = 10 μm.



823 **Figure 3. Effect of PRX expression on cell wall thickness in root hair tips.**

824 **(A)** Transmission electron micrographs of root hair tips from *Wt*, *prx1,44,73* triple mutant, and
825 *PRX44^{OE}* with (+) and without (-) peroxidase inhibitor SHAM. For each genotype and treatment, a
826 representative overview of a root hair (RH) and a detail of the cell wall at the root hair tip (CW) is
827 shown. Scale bar = 1 μ m.

828 **(B)** Box and whisker plot showing cell wall thickness measured at the root hair tip of the three
829 genotypes with or without SHAM treatment. (**) $P < 0.001$ determined by t-test. (NS) not significantly
830 different.



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833 **Figure 4. Interaction by an *in silico* docking approach of PRX01, PRX44 and PRX73 with EXT peptides.**

834 **(A,B)** Ten docking results for each EXT O-glycosylation state are shown superimposed on the PRX44
835 protein surface to evaluate the consistence of docking sites.

836 **(A)** Model of PRX44 (protein surface shown in gray) complexed to a non-O-glycosylated EXT substrate
837 (SPPPYVY)₃ (in green, depicted as sticks). Heme is depicted as thin sticks while iron is a red sphere.
838 Bottom inset, two close tyrosine residues dock near to the possible active site of PRX44.

839 **(B)** Model of PRX44 (protein surface shown in gray) complexed to an O-glycosylated-EXT substrate
840 (protein backbone shown in magenta, and O-glycans shown in light blue, both depicted as sticks).

841 Heme is depicted as thin sticks while iron is a red sphere. Arabino-galactosylated EXT peptide =
842 [(SOOOYVY)₃-AG].

843 (C) Comparison of the binding energy of different peroxidases to EXT substrates with different degrees
844 of *O*-glycosylation. A non-hydroxylated EXT peptide (SPPPYVY)₃, a hydroxylated but not *O*-
845 glycosylated EXT peptide [(SOOOYVY)₃; O=hydroxyproline], only arabinosylated EXT-peptide
846 [(SOOOYVY)₃-A], and arabino-galactosylated EXT peptide [(SOOOYVY)₃-AG] were analyzed.

847 **Table 1.** Peptidyl-Tyr and iso-dityrosine (IDT) contents in cell walls isolated from Wt, *prx01,44,73* triple
 848 mutant, PRX^{OE} lines and mutant lines with under-glycosylated EXTs. P-values were determined by one-
 849 way ANOVA, (***) P<0.001, (**) P<0.01. STD=Standard Deviation. Values significantly different than
 850 Wt are highlighted in blue if higher and in light blue if lower than Wt Col-0.
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	ng Tyr/ μ g CW (STD)	ng IDT/ μ g CW (STD)
Wt Col-0	7.799 \pm 0.26	0.853 \pm 0.08
<i>prx01 prx44 prx73</i>	9.588 \pm 0.31**	0.963 \pm 0.02
PRX44 ^{OE}	8.649 \pm 0.07	0.953 \pm 0.04
PRX73 ^{OE}	8.700 \pm 0.12	1.042 \pm 0.02**
<i>under O-glycosylated EXTs</i>		
<i>sergt1-1 rra3</i>	3.530 \pm 0.08***	0.235 \pm 0.01***
<i>p4h5 sergt1-1</i>	3.766 \pm 0.06***	0.225 \pm 0.02***

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