1 **Elucidating the role of polygalacturonase genes in strawberry fruit**
2 **softening**
3
4 **Candelas Paniagua[1], Pablo Ric-Varas[1], Juan A. García-Gago[1], Gloria López-**
5 **Casado[1], Rosario Blanco-Portales[2], Juan Muñoz-Blanco[2], Julia Schückel[3], J. Paul**
6 **Knox[4], Antonio J. Matas[1], Miguel A. Quesada[5], Sara Posé[1], José A. Mercado[1]***
7
8 [1]Instituto de Hortofruticultura Subtropical y Mediterránea "La Mayora" (IHSM-UMA-
9 CSIC), Departamento de Botánica y Fisiología Vegetal, Universidad de Málaga, 29071
10 Málaga, Spain
11 [2]Departamento de Bioquímica y Biología Molecular, Universidad de Córdoba, 14071
12 Córdoba, Spain
13 [3]Department of Plant and Environmental Sciences, University of Copenhagen,
14 Copenhagen, Denmark
15 [4]Centre for Plant Sciences, Faculty of Biological Sciences, University of Leeds, Leeds
16 LS2 9JT, United Kingdom
17 [5]Departamento de Botánica y Fisiología Vegetal, Universidad de Málaga, 29071
18 Málaga, Spain
19
20 C.P candelaspc@uma.es; P.R-V. pabloric@uma.es; J.A.G-G jagarciagago@gmail.com;
21 G.L-C glorialc@uma.es; R.B-P bb2blpor@uco.es; J.M-B bb1mublj@uco.es; J.S.
22 js@glycospot.dk; J.P.K. j.p.knox@leeds.ac.uk; A.J.M. antoniojmatas@uma.es; M.A.Q.
23 quefe@uma.es; S.P. sarapose@uma.es; J.A.M. mercado@uma.es
24
25 ***Corresponding author:** José A. Mercado; mercado@uma.es; Telf. +34 952 13 75 22
26
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34 **Short running title:** Role of PG genes in strawberry fruit softening

35

36 **Highlight**

37 Polygalacturonase genes *FaPG1* and *FaPG2* play key roles in strawberry fruit firmness,
38 disassembling pectins associated to hemicelluloses and cellulose and modulating the
39 expression of genes involved in cell wall remodeling

40

41 **Abstract**

42 To disentangle the role of polygalacturonase (PG) genes in strawberry softening, the
43 two PG genes most expressed in ripe receptacle, *FaPG1* and *FaPG2*, were down-
44 regulated. Transgenic ripe fruits were firmer than wild type when PG genes were
45 silenced individually. Simultaneous silencing of both PG genes by transgene stacking
46 did not result in an additional increase in firmness. Cell walls from ripe fruits were
47 characterized by a carbohydrate microarray. Higher signals of homogalacturonan and
48 rhamnogalacturonan I pectin epitopes in polysaccharide fractions tightly bound to the
49 cell wall were observed in the transgenic genotypes suggesting a lower pectin
50 solubilization. At the transcriptomic level, the suppression of *FaPG1* or *FaPG2* alone
51 induced few transcriptomic changes in the ripe receptacle, but the amount of
52 differentially expressed genes increased notably when both genes were silenced. Many
53 genes encoding cell wall modifying enzymes were down-regulated. The expression of a
54 putative high affinity potassium transporter was induced in all transgenic genotypes,
55 indicating that cell wall weakening, and loss of cell turgor could be linked. These results
56 suggest that, besides the disassembly of pectins tightly linked to the cell wall, PGs could
57 play other roles in strawberry softening, such as the release of oligogalacturonides
58 exerting a positive feedback in softening.

59

60 **Keywords:** cell wall, *Fragaria* × *ananassa*, fruit ripening, fruit softening,
61 oligosaccharides, pectins, polygalacturonase.

62

63 **Abbreviations**

64 HG: homogalacturonan; OGAs: oligogalacturonides; PG: polygalacturonase; PME:
65 pectin methyl esterase; RGI: rhamnogalacturonan I; XG: xyloglucan

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67

68 **Introduction**

69 Cultivated strawberry (*Fragaria* × *ananassa*, Duch.) is one of the most important fruits
70 worldwide due to its delicious sensorial attributes and attractive appearance.
71 Additionally, this fruit is an important source of bioactive compounds with high
72 antioxidant capacity (Hannum, 2004; Zhang *et al.*, 2008; Tulipani *et al.*, 2008). As most
73 soft fruits, one of the main limitations of this crop is the fast softening during fruit
74 ripening, a process that determines a very short postharvest life and elevated economic
75 losses.

76 Cell wall modifying enzymes and proteins are involved in the softening of fleshy
77 fruits through the disassembly of the main components of primary cell walls and the
78 dissolution of middle lamella, decreasing wall strength and cell to cell adhesion
79 (Brummell, 2006; Wang *et al.*, 2018a; Posé *et al.*, 2019). Pectinase enzymes such as
80 polygalacturonases (PG), pectate lyases and β-galactosidases, play key roles in this
81 process. Transgenic silencing of ripening-specific genes encoding these enzymes
82 reduced softening and increased postharvest shelf life in fruits with contrasting texture
83 such as tomato, strawberry and apple (Jiménez-Bermúdez *et al.*, 2002; Quesada *et al.*,
84 2009; Atkinson *et al.*, 2012; Paniagua *et al.*, 2016; Uluisik *et al.*, 2016). Softening of
85 fleshy fruits is also associated to the loss of cell turgor due to transpirational water loss
86 and/or to the accumulation of solutes in the apoplast (Saladié *et al.*, 2007; Wada *et al.*,
87 2009). If there is a relationship between cell wall disassembly and loss of cell turgor it is
88 largely unknown.

89 PGs are the most studied pectinases involved in fruit softening. Some fruits, such as
90 tomato, avocado and peach, show high PG activity that correlates with the rate of
91 softening (Hadfield and Bennett, 1998). By contrast, other fruits display very low PG
92 activity, e.g. strawberry, melon, apple (Mercado *et al.*, 2011). PGs belong to the
93 glycoside hydrolases family 28 and are divided into three main types: exo-, endo- and
94 rhamno-PGs (Markovic and Janecek, 2001; Yang *et al.*, 2018). Exo- (EC 3.2.1.67) and
95 endo-PGs (EC 3.2.1.15) catalyze the hydrolytic cleavage of galacturonic linkages in
96 homogalacturonan (HG) pectin domains, a linear homopolymer of α-(1–4)- linked
97 galacturonic acid (GalA). Plant endo-PGs act preferentially in HG chains with at least
98 four de-methylesterified GalA residues, releasing oligogalactunorides of various length;
99 the exo types remove single GalA residues from the non-reducing end of the HG
100 backbone (Sénéchal *et al.*, 2014). Rhamno-PGs (EC 3.2.1.-) can also be exo or endo-
101 type and catalyze the hydrolysis of GalA-rhamnose bonds in rhamnogalacturonan I

102 (RGI), a highly ramified pectin domain formed by a backbone of the disaccharide α-L-
103 Rha-(1-4)-α-D-GalA, with galactose, arabinose and arabinogalactan neutral side chains
104 attached to the rhamnose resides (Yang *et al.*, 2018).

105 PGs are encoded by large multigene families (Yang *et al.*, 2018). In strawberry fruits,
106 two different polygalacturonase genes have been described, *FaPG1* (accession number
107 AF380299) and *FaPG2* (AY280662) (Redondo-Nevado *et al.*, 2001; Salentijn *et al.*,
108 2003; Villarreal *et al.*, 2008; Quesada *et al.*, 2009). Both genes were expressed at the
109 onset of ripening and maintained a high expression level in ripe fruit. The deduced
110 FaPG1 protein was described as an endo-PG by Redondo-Nevado *et al*. (2011);
111 however, Villarreal et al. (2008) included this protein in the same clade than pollen PG
112 genes, thought to encode exo-PGs. Down-regulation of *FaPG1* reduced strawberry
113 softening (Quesada *et al.*, 2009) and this was related to a lower pectin solubilization and
114 depolymerization and a better preservation of nanostructural features of pectins
115 extracted with a chelating agent or sodium carbonate (Posé *et al.*, 2013, 2015). The
116 atomic force microcopy analysis of pectins from down-regulated *FaPG1* strawberry
117 fruits suggested that this enzyme could degrade HG backbone (Pose *et al*., 2015). As
118 regards *FaPG2*, the active site of the deduced protein differs from the one present in
119 known PGs (Salentijn *et al.*, 2003). The functional analysis of this gene has not been
120 addressed so far. Interestingly, Salentijn *et al.* (2003) found that *FaPG2* was expressed
121 at higher levels in ripe fruits from soft cultivars than in fruits from firm genotypes.
122 Additionally, in a RNAseq study performed in the firm cultivar 'Camarosa', Sánchez-
123 Sevilla *et al.* (2017) detected expression of *FaPG1* but not *FaPG2*. Therefore, it could
124 be likely that both genes exerted an additive effect on fruit softening.

125 The aim of this study was to further evaluate the role of PG genes in strawberry
126 softening. To this purpose, transgenic plants containing an antisense sequence of
127 *FaPG2* as well as double antisense *FaPG1-FaPG2* plants have been obtained. The
128 changes induced by PGs silencing in cell wall composition and the transcriptomic
129 profiles of ripe fruits have been determined.

130

131 **Materials and Methods**

132

133 **Plant material and culture conditions**

134 Leaf disks from micropropagated strawberry, *Fragaria* × *ananassa* Duch. plants, cv.
135 Chandler, were used for genetic transformation to obtain antisense *FaPG2* transgenic

136 plants. Micropropagation medium contained the macroelements of the $N_{30}K$ mineral
137 formulation (Margara, 1984), MS (Murashige and Skoog, 1962) microelements and
138 vitamins and 2.21 µM kinetin (Barceló *et al.*, 1998). Meristems from runners of the
139 antisense *FaPG1* transgenic line PG29, renamed as PGI-29, previously obtained and
140 characterized by Quesada *et al.* (2009), were introduced *in vitro* as described by López-
141 Aranda *et al.* (1994). Leaf discs from PGI-29 micropropagated plants were used to
142 obtain double transgenic *FaPG1-FaPG2* lines (named as PGI/II). Transgenic plants
143 were grown in a confined greenhouse under natural temperature and photoperiod.
144

145 **Binary vectors and generation of transgenic strawberry plants**

146 A 833 bp fragment from a non-conserved region of the *FaPG2* gene was cloned in
147 antisense orientation into the pB7WG2 binary vector (Karimi *et al.*, 2002) under the
148 control of the constitutive promoter *CaMV35S*, using Gateway technology (Invitrogen,
149 Darmstadt, Germany). This vector harbors the *bar* gene for phosphinothricin selection.
150 The plasmid was introduced into the *Agrobacterium tumefaciens* strain AGL1 by
151 electroporation. Genetic transformation was performed as described by Barceló *et al.*
152 (1998). Leaf explants from control, non-transformed, or PGI-29 micropropagated plants
153 were inoculated with a diluted *A. tumefaciens* overnight culture and cultivated in
154 regeneration medium, modified MS medium with $N_{30}K$ macroelements and
155 supplemented with 8.88 µM BA, 2.46 µM IBA and 5 mg/l phosphinothricin.
156 Regenerated shoots were micropropagated and rooted in the presence of 10 mg/l
157 phosphinothricin and acclimated to *ex vitro* conditions.
158

159 **Phenotypic analysis of transgenic plants**

160 Transgenic plants were evaluated during two consecutive years, using each year 8-10
161 plants per line obtained by runner propagation. Non-transformed plants and PGI-29
162 transgenic plants were used as controls. Fruits were harvested at the stage of full
163 ripeness, 100% surface red, from February to June. Fruit quality parameters were
164 evaluated in fruits of uniform size, standard shape, and weight higher than 5 g. Fruit
165 color was measured with a colorimeter (Minolta Chroma Meter CR-400, Osaka, Japan).
166 The L* a* b* color space parameters (lightness, redness, yellowness) were recorded.
167 Soluble solids were measured using a refractometer (Atago N1), and firmness by using
168 a hand penetrometer (Effegi) with a cylindrical needle of 9.62 mm^2 surface. A minimum
169 of 10 ripe fruits per line were evaluated.

170

171 **Molecular analysis of transgenic plants**

172 Genomic DNA was extracted from young strawberry leaves using the Quiagen DNeasy
173 Plant kit. Previously, the plant material had been washed three times with a washing
174 buffer solution as described by Mercado *et al.* (1999). The presence of the transgene in
175 phosphinothricin resistant lines was confirmed by PCR amplification of a 334-bp
176 fragment belonging to the *bar* gene, using the primers 5'-
177 CATCGAGACAAGCACGGTCAACTTC-3' and 5'-
178 ATATCCGAGCGCCTCGTGCATGCG-3'.

179 Total RNA was isolated from independent pools of de-achened ripe fruits in
180 accordance with Gambino *et al.* (2008). The RNA obtained was treated with RNase free
181 DNase I (Roche) and purified through the RNeasy MinElute Cleanup kit (Qiagen).
182 RNA concentration and purity were evaluated using a NanodropTM spectrophotometer
183 ND-1000 (Thermo Scientific) and a 1 % agarose gel electrophoresis. The gene
184 expression analysis of *FaPG1* and *FaPG2* genes were performed by qRT-PCR through
185 a CFX96 Touch Real-Time PCR Detection System, as previously described (Benitez-
186 Burraco *et al.*, 2003). The *FaPG1* gene primer sequences for quantitative amplification
187 were: 5´GCTCCTGGTGACTTTGATGT-3 and 5´-ACTCTACTTGGCGTTGTTGC-3´;
188 and for *FaPG2* gene the primers were: 5´- TTCAGCCTCACCAGCCATT-3 and 5´-
189 TGGAACTGTTGCCACCAGA-3`. The interspacer 26S-18S strawberry RNA
190 housekeeping gene was used for normalization. The expression of each gene was
191 compared with the control gene expression in accordance with Pedersen (2001). A
192 relative quantification was done following the 2–ΔΔCT (Livak) method.

193

194 **Transcriptomic analysis by RNAseq**

195 Total RNA from control and transgenic ripe fruits was extracted as described in the
196 previous section. RNA quantity and integrity were checked by nanodrop, agarose gel
197 electrophoresis and Agilent 2100 bioanalyzer. RNA integrity number (RIN) were >8 for
198 all replicates. Three independent biological replicates were used. Libraries with insert
199 sizes ranging from 150 to 200 bp were constructed and sequenced using an Illumina
200 HiSeq 2000 at the Novogene Bioinformatics Institute, Beijing. More than 80 million
201 reads per sample were generated. These sequences were mapped to the *Fragaria vesca*
202 reference genome v.4.0.a1 using TopHat2. Gene expression level was estimated by
203 counting the reads that map to genes or exons and expressed as fragments per kilobase

204 of transcript sequence per million base pairs sequenced (FPKM). For differential
205 expression analysis, log2FoldChange values were calculated from normalized
206 transgenic and wild type redcounts; a negative binomial distribution was used to
207 estimate the associated probability and those genes with an adjusted p-value <0.05 were
208 considered as differentially expressed gene (DEG). RNA-seq data have been deposited
209 in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under
210 accession number E-MTAB-8736.

211 Genes annotated as PGs in the v.4.0.a1 *F. vesca* genome were searched using the
212 Genome Database for Rosaceae (https://www.rosaceae.org/). Those PG sequences that
213 were detected in the RNAseq study showing a FPKM value higher than 5 in the three
214 biological samples were selected. The Pfam database (https://pfam.xfam.org) was used
215 to predict the conserved domains of these proteins and those lacking a Pfam00295,
216 typical of glycose hydrolase family 28, or a Pfam12708, Pectate Lyase domain 3, were
217 discarded. Multiple sequence alignments of PG proteins were performed with Clustal
218 Omega (Madeira *et al.*, 2019).

219

220 **Cell wall extraction**

221 Fruits from selected lines were harvested at ripe stage (fully red) and stored at -40ºC
222 until use. Frozen fruits from each line were finely milled to powder under liquid
223 nitrogen and extracted with PAW (phenol: acetic acid: water, 2:1:1, w: v: v) to avoid
224 action of cell wall degrading enzymes, following the protocol of Redgwell *et al.* (1992).
225 Briefly, 10 g of fruit powder were extracted wit 20 ml of PAW. After centrifugation at
226 4000 *g*, pellets were de-starched by aqueous DMSO 90% treatment, and the final
227 residue lyophilised, considered the cell wall extract.

228

229 **Carbohydrate microarray**

230 Comprehensive Microarray Polymer Profiling (CoMPP) was performed using the
231 protocol described in Kračun *et al.* (2016). High-throughput cell wall fractions were
232 obtained by sequential homogenization with glass beads using a tissue lyser (Retsch
233 MM400 mixer mill) for the next solvent series: sterile water; 0.1 M Na_2CO_3; 4 M KOH
234 and cadoxen (31% 1,2-diaminoethane with 0.78 M cadmium oxide, (v:v)). Both alkaline
235 fractions included 0.1% $NaBH_4$ freshly added just before use. For the first water-soluble
236 fraction, 10 mg of cell wall extract were homogenised in tissue lyser with 500 µl of
237 water at 30 Hz shaking for 20 min, followed by gentle rocking 1h at RT. After

238 centrifugation at 2700 *g* for 15 min, supernatants were saved in a fresh tube and stored
239 as water-fraction, while pellets were further extracted with the next solvent, following
240 the same extraction steps. Supernatants of each fraction were diluted four times (first
241 dilution 1:1 and five-fold for the following dilutions) and printed as 4 technical
242 replicates. All transgenic and wild-type samples were printed simultaneously on the
243 same sheet of nitrocellulose as adjacent arrays using an ArrayJet Sprint (ArrayJet,
244 Roslin, UK) and quantified as previously described (Kračun *et al.*, 2017). In brief, the
245 printed nitrocellulose sheets were probed with the primary mAbs diluted (1/10) in
246 phosphate-buffered saline (PBS) containing 5% w/v milk powder (MPBS). Secondary
247 anti-rat or anti-mouse antibodies conjugated to alkaline phosphatase (Sigma) were
248 diluted (1/5000) in MPBS. Primary mAb used in this study (Table S1) are from
249 PlantProbes (Leeds, UK) except INRA-RU1 and INRA-RU2 that were kindly provided
250 by M.C. Ralet (Biopolymères Interactions Assemblages, Nantes, France). Developed
251 microarrays were scanned (CanoScan 8800F), converted to TIFFs, and signals were
252 processed by ImaGene 6.0 microarray analysis software (BioDiscovery), as described
253 before (Moller *et al.*, 2007). The mean spot signals obtained from four experiments are
254 presented in heat maps in which colour intensity was correlated to signal. The highest
255 signal in each dataset was set to 100, and all other values were normalised accordingly.
256 A cut-off value of 5 was applied.
257
258 **Statistical analysis**
259 Data were subjected to analysis of variance (ANOVA) using IBM SPSS Statistics
260 software, version 25. Levene test for homogeneity of variance was performed prior to
261 ANOVA. Tukey and Kruskal-Wallis tests were used for mean separation in the case of
262 homogeneous and non-homogeneous variances, respectively. Normalised values
263 obtained in the carbohydrate microarray experiment were subjected to Principal
264 Component Analysis (PCA) and hierarchical clustering using the R package
265 FactoMineR. All tests were performed at $P = 0.05$.
266
267 **Results**
268 **Identification of PG genes expressed in ripe fruits**
269 A total of 82 sequences were annotated as PG genes in the *F. vesca* whole genome v4.0
270 (Supplementary Data 1). The transcriptomic analysis of receptacle tissue from ripe fruits
271 of *F.* × *ananassa*, cv. 'Chandler', by RNAseq showed that besides FvH4_6g41380.1

272 (homologous to *FaPG1* in *F.* × *ananassa*) and FvH4_7g15040.1 (*FaPG2*), eight
273 additional putative PG genes were expressed in this tissue. Four of these sequences
274 were discarded since their encoded protein lacked a glycoside hydrolase family 28
275 domain (Pfam00295), characteristics of PGs, or a Pectate Lyase 3 domain (Pfam12708),
276 as it is found in *QRT3* Arabidopsis PG and its homologs (Yang *et al.*, 2018)
277 (Supplementary Data 1). The coding sequences of the expressed PG genes ranged from
278 1218 to 1476 bp, with protein lengths ranging from 405 to 491 amino acids. Fungal and
279 plant PG genes contain at least one of the four conserved motifs known as SPNTDG
280 (motif I), GDDC (motif II), CGPGHGISIGSLG (motif III), and RIK (motif IV) (Yang
281 *et al.*, 2018). *FaPG1* and FvH4_2g16950.1 contained the four domains, while the other
282 genes had lost one (*FaPG2*), two (FvH4_1g09050.1) or three conserved domains
283 (FvH4_5g04440.1 and FvH4_6g22970.1) (Table S2). A multiple sequence alignment
284 was performed using Clustal Omega (Fig. S1). The cladogram generated by the
285 alignment separated PG genes in two main clades, one formed by *FaPG1* and
286 FvH4_2g16950.1, and the other group composed by the rest of PGs (Fig. S2). *FaPG1*
287 and *FaPG2* were the most divergent proteins.

288 *FaPG1* and *FaPG2* were the most expressed PGs in ripe receptacle of cv.
289 'Chandler', being *FaPG1* expression level about 7 fold higher than that of *FaPG2* (Fig.
290 1). The other PG genes showed very low expression levels in the RNAseq study.

291

292 **Recovery of *FaPG2* and *FaPG1-FaPG2* antisense plants**

293 Twenty-four independent phosphinothricin resistant shoots were recovered when
294 explants from control plants were transformed with the binary vector harboring the
295 antisense *FaPG2* (named as PGII- lines). The transformation experiment using leaf
296 explants from the transgenic line PGI-29 yielded 12 independent antisense *FaPG1-*
297 *FaPG2* lines (PGI/II- lines). Genomic DNA was extracted from leaves of these lines
298 and used to amplify a 334 bp DNA fragment corresponding to the *bar* gene. All
299 transgenic plants showed the correct amplification of the transgene whereas no
300 amplification signal was detected in controls, non-transformed and PGI-29 line (results
301 not shown).

302

303 **Phenotypical and molecular analysis of transgenic lines**

304 Transgenic plants were evaluated during two consecutive growing seasons, using each
305 year daughter plants derived from runner propagation. Fig. 2A shows the aspect of some

306 of the transgenic lines obtained. In general, vegetative growth pattern of transgenic
307 plants was similar to wild type. However, some of the lines did not flower or produced a
308 very low number of fruits. Fruit quality parameters measured during the first year of
309 greenhouse evaluation are shown in Table 1. Most of the PGII and PGI/II transgenic
310 lines yielded fruit smaller than controls, showing a reduction in fruit weight and size.
311 Minor differences in skin fruit color were detected; only line PGII-2 showed significant
312 increments in color parameters, denoting a more intense red color. As regards soluble
313 solids (SS), 30 and 50% of PGII and PGI/II lines, respectively, showed higher SS values
314 than controls. Firmness of ripe fruits in controls and both transgenic genotypes is shown
315 in Fig. 2B. In the case of antisense *FaPG2*, PGII lines, 5 out of the 10 lines analyzed
316 produced fruits significantly firmer than control non-transformed plants; the increase in
317 firmness obtained in these lines was similar or slightly higher than that observed in PGI-
318 29 transgenic control. As expected, all antisense *FaPG1-FaPG2* plants yielded fruits
319 significantly firmer than control non-transformed plants; however, firmness values were
320 significantly higher than PGI-29 only in three lines, PGI/II-2, PGI/II-9 and PGI/II-16.
321 The results obtained during the second year of agronomical evaluation were similar
322 to those previously described (Table S3). Fruit firmness was significantly higher than
323 control non-transformed plants in PGII-4, PGII-5, PGII-7 and PGII-8 (Fig. 2B). In the
324 case of double transgenic lines, some of them produced fruits slightly firmer than PGI-
325 29 although the differences were not significant. Overall, two antisense *FaPG2* lines
326 produced fruits firmer than control during the two growing seasons but none *FaPG1-*
327 *FaPG2* yielded fruits significantly firmer than PGI-29 controls. Based on fruit yield and
328 fruit quality parameters, the transgenic lines PGII-5, PGII-7, PGII-8, PGI/II-9, PGI/II-
329 15 and PGI/II-16 were selected for further analysis.
330 The expression levels of *FaPG1* and *FaPG2* in ripe fruits from transgenic selected
331 lines were estimated by qPCR and compared with control non-transformed fruits. In
332 PGI-29 line, *FaPG1* gene was strongly down-regulated, > 99%, whereas the level of
333 *FaPG2* mRNA was similar to control (Quesada *et al.*, 2009). The three PGII lines
334 analyzed showed a strong *FaPG2* silencing >90%, but *FaPG1* expression was not
335 significantly modified (Fig. 2C). Double transgenic lines showed a strong silencing of
336 both PG genes, except for PGI/II-15 line which showed only a 46% reduction in *FaPG2*
337 mRNA level (Fig. 2C).
338
339 **Cell wall analysis by carbohydrate microarray**

340 Cell walls from ripe fruits of control and selected transgenic lines was isolated and
341 sequentially extracted with different solvents to yield fractions enriched in loosely and
342 tightly bound pectins (water and Na_2CO_3, respectively), xyloglucans (4 M KOH) and
343 recalcitrant matrix polysaccharides highly imbricated into cellulose microfibrils
344 (cadoxen). These samples were printed in nitrocellulose and probed with a set of
345 monoclonal antibodies (mAbs) directed to different cell wall epitopes (Table S1). A
346 heatmap showing the relative abundance of cell wall glycans recognized by the mAbs is
347 shown in Fig. 3. LM27, a mAb against heteroxylan from grass cell walls (Cornuault *et*
348 *al.*, 2015) was used as negative control, and, as expected, no signal was detected in any
349 sample. JIM7 and LM5 which bind to methyl-esterified HG and RGI-galactan,
350 respectively, showed the strongest signals in the array; by contrast, LM10 (xylan
351 epitope) and LM2 (arabinogalactan-protein epitope) yielded a weak signal in all the
352 samples. Down-regulation of PG genes substantially modified the pectin profiles when
353 compared with control non-transgenic fruits. In water soluble fractions, the abundance
354 of methyl esterified pectins from HG recognized by JIM7 was significantly higher in
355 antisense PG lines than in the control. This cell wall fraction showed a weak signal of
356 mAbs that bind to RGI pectin domain in most of the samples, except for PGII-5 line.
357 Sodium carbonate fraction was rich in non-methylesterified HG (LM18 and LM19) and
358 RGI (especially LM5 that recognizes galactans, and RU1 and RU2 that bind to the RGI
359 backbone). The abundance of these epitopes was higher in all transgenic lines than in
360 the control. KOH and cadoxen fractions also contained significant amount of pectins,
361 and, as observed in the other fractions, mAbs associated to HG and RGI showed
362 stronger signals in transgenic lines than in the control. LM15 and LM25 recognize
363 different xyloglucan (XG) motifs. These epitopes were abundant in the KOH and
364 cadoxen fractions and did not appear in the pectin fractions. mAbs directed to xylan
365 (LM10, LM11 and LM28) followed a similar trend. Minor differences in XG and xylan
366 contents were detected between control and transgenic lines, although LM25 and LM28
367 signals were slightly higher in the control in both fractions. Arabinogalactan protein
368 epitopes were especially abundant in the KOH fraction of the transgenic lines. Finally,
369 the mAbs JIM20 and LM21, directed to extensin and mannan, respectively, showed
370 weak signals in all the samples.

371 The Principal Component Analysis (PCA) performed in pectin enriched fractions
372 (water and sodium carbonate) showed that the first dimension explained more than 85%
373 of the total variance in the samples (Fig. 4). This dimension was positively correlated

374 with RGI epitopes abundance in both fractions, as well as methyl-esterified and de-
375 methyl-esterified pectins in the case of water and sodium carbonate fractions,
376 respectively. The first principal component in the PCA of matrix glycan fractions (KOH
377 and cadoxen) also accounted for a large proportion of total variability, >60% (Fig. 5). In
378 both cases, this dimension was positively correlated with the content of RGI (LM6-M,
379 LM5, RU1 and RU2 mAbs) and HG (LM18 and LM19) related epitopes. XyG
380 antibodies (LM15 and LM25) were the main contributors to the second dimension in
381 KOH fraction, while in the case of cadoxen fraction, this dimension was related to the
382 presence of extensin, mannans and xylan epitopes. In the four cell wall fractions, the
383 factor score plots clearly separated transgenic samples from wild type along the first
384 dimension, indicating an enrichment on HG and RGI in transgenic fruits.
385 The whole set of microarray data was subjected to hierarchical clustering (Fig. S3).
386 Samples were grouped into four main clades that, in the case of transgenic fruits,
387 corresponded with the four cell wall fractions analyzed. By contrast, in control fruits,
388 water and sodium carbonate pectin fractions were grouped in the same cluster than
389 water samples from transgenic PG fruits while KOH and cadoxen matrix glycan
390 fractions were grouped with cadoxen from transgenic fruits.
391
392 **Transcriptomic analysis of transgenic fruits**
393 RNA was isolated from ripe receptacle of control and selected transgenic lines PGI-29,
394 PGII-5, PGI/II-9 and PGI/II-16, to determine transcriptomic changes through RNAseq
395 analysis. An average of 87,714,847 clean reads per sample was obtained. These
396 sequences were mapped to the *F. vesca* reference genome Fv_v4.0.a1. Table S4 shows
397 an overview of the mapping results. The mean number of transcripts detected in the
398 RNAseq analysis was 21,616, representing a 75% of the genes predicted in the *F. vesca*
399 genome. The number of differentially expressed genes (DEGs) relative to WT was low
400 in PGII-5 and especially PGI-29 samples, 72 and 15 DEGs, respectively; most of these
401 DEGS were down regulated (Fig. S4). The simultaneous silencing of both PG genes
402 induced more extensive transcriptomic changes in PGI/II genotypes, i.e. 283 and 316
403 DEGS were found in PGI/II-9 and PGI/II-16 lines, respectively, more than 61% of these
404 DEGs corresponding to up regulated genes. DEGs were annotated and classified using
405 Blast2Go software and the NCBI nr and EMBL-Ebi InterPro databases. The gene
406 ontology annotation results are provided in Fig. S5. In all the genotypes, most enriched
407 GO terms, level 2, were membrane and membrane part in cellular component, catalytic

408 activity and binding in molecular function and metabolic and cellular process in
409 biological process. The top 15 biological process GO terms in up and down-regulated
410 DEGs for PGI/II-16 sample is displayed in Fig. 6A. Similar distributions of gene
411 annotations were found in the other genotypes (results not shown). A complete list of
412 DEGs in each transgenic genotype and their annotation can be found in Supplementary
413 Data 2.

414 Transgenic lines with the two PG genes silenced shared 63 DEGs, around 22% of the
415 genes showing differential expression in these samples (Fig. 6B). Twenty-four DEGs
416 overlapped in PGII-5 and PGI/II genotypes. Interestingly, four DEGs, three of them
417 upregulated and one down-regulated, were common to all the genotypes. The
418 upregulated genes corresponded to a potassium transporter 5-like (FvH4_6g30550.1)
419 and two unknown genes (FvH4_1g09170.1, FvH4_5g29210.1), whereas the down-
420 regulated gene was a putative RNA cytidine acetyltransferase 1-like (FvH4_6g25830.1).
421 All these DEGs showed high fold change values when compared with wild type; even
422 more, the potassium transporter transcript was not detected in control fruit (Fig. S6).

423 To further investigate the impact of PG downregulation in cell wall remodeling,
424 genes involved in cell wall metabolism were identified among all DEGs detected in the
425 four transgenic genotypes (Table 2). These DEGs included several pectinesterases, β-
426 galactosidases, endo-glucanases, xyloglucan galactosyltransferases and cellulose
427 synthases, single genes encoding N-glycan glucosidase, expansin and xylose isomerase
428 and two genes encoding structural proteins, a glycine rich structural protein and an
429 arabinogalactan protein. Three of these genes had previously been characterized in
430 strawberry fruit, the pectin methyl esterase *FaPE1* (FvH4_1g09610.1; Osorio *et al.*,
431 2008), the β-galactosidase *FaβGal4* (FvH4_2g27470.1; Paniagua *et al.*, 2016) and the
432 expansin *FaExp4* (FvH4_4g31140.1; Dotto *et al.*, 2006). A heat map showing the
433 normalized expression levels of these cell wall genes is shown in Fig. 7. In general, the
434 expression levels for most of the genes followed the same trend in the four transgenic
435 lines. Most pectinesterase, β-galactosidase, endo-glucanase and cellulose synthase genes
436 were downregulated in the transgenic fruits when compared with control; however,
437 xyloglucan galactosyltransferase and arabinogalactan protein were upregulated.

438

439 **Discussion**

440 **Several PG genes are expressed in ripe strawberry fruit**

441 In this research, besides the previously identified PG genes, *FaPG1* and *FaPG2*, four
442 additional PG genes were found to be expressed in ripe 'Chandler' receptacle although
443 at very low levels. The expression level of *FaPG1* was significantly higher than that of
444 *FaPG2*, as previously observed by Quesada *et al.* (2009). These two genes would likely
445 account for most PG activity during ripening of 'Chandler' fruit. Sánchez-Sevilla *et al.*
446 (2017) also detected expression of four out of the six PG genes described in this
447 research in ripe 'Camarosa' fruit, being *FaPG1* the most expressed; however, they did
448 not report the expression of neither *FaPG2* nor FvH4_2g16950.1.

449 Four conserved motifs are present in fungal and plant PGs genes (Yang *et al.*, 2018).
450 These motifs are thought to be components of the catalytic site (motifs I and II)
451 (Rexovabenkova, 1990), participate in the catalytic reaction (III) (Markovic and
452 Janecek, 2001), or ionically interact with the carboxylate group of pectate substrate (IV)
453 (Bussink *et al.*, 1991). In apple, most PG proteins contained the four domains or had
454 lost individual amino acids from these motifs (Chen *et al.*, 2016), a fact that contrasts
455 with the results described in this paper where only two out of the six PGs analyzed
456 (*FaPG1* and FvH4_2g16950.1) displayed the four conserved domains. *FaPG2* had lost
457 the motif III. According to Park *et al.* (2008), this motif is the less conserved and it is
458 absent in rhamno-PGs.

459

460 **Silencing of *FaPG1* and *FaPG2* increases pectins associated to xyloglucan and**
461 **cadoxen fractions and reduces strawberry softening**

462 Previous works showed that the antisense down-regulation of *FaPG1* reduced
463 strawberry softening without affecting other quality traits (Quesada *et al.*, 2009; Julio-
464 González *et al.*, 2018). At the cell wall level, *FaPG1* silencing reduced pectin
465 solubilization and depolymerization of the more tightly bound pectin fractions (Posé *et*
466 *al.*, 2013). Similar changes were observed in apple (Atkinson *et al.*, 2012) and tomato
467 (Carrington *et al*., 1993) when silencing a PG gene, although, in the case of tomato,
468 firmness of ripe fruit was unaffected. The silencing of *FaPG2* also reduced strawberry
469 fruit softening; however, the simultaneous down-regulation of both genes did not
470 produce a synergistic effect on fruit texture, and the level of increase in firmness was
471 similar in the three transgenic genotypes. This result might indicate some level of
472 redundancy of PG genes in pectin disassembly during strawberry ripening. The
473 important role of pectate lyase in cell wall disassembly (Jiménez-Bermúdez *et al.*,

474 2002), an enzyme that also degrades HG, could also prevent a deeper effect of PGs
475 silencing in fruit softening.
476 The cell wall changes detected in the glycome microarray were similar in *FaPG1*,
477 *FaPG2* and *FaPG1-FaPG2* silenced fruits and minor differences were detected among
478 independent transgenic lines. The changes induced by PGs silencing involved the
479 increase of methyl-esterified HG in the water soluble fraction and the enrichment of HG
480 and RGI pectin epitopes in polysaccharide fractions tightly bound to the cell wall
481 (sodium carbonate, KOH and cadoxen). These results confirm previous observations of
482 a lower pectin disassembly in antisense PG plants.
483 Pectin solubilization is a common event in the softening of many fleshy fruits
484 (Brummell, 2006; Mercado *et al.*, 2011), including strawberry (Redgwell *et al.*, 1997;
485 Paniagua *et al.*, 2017a). It is though that pectin solubilization occurs at the expense of
486 more tightly bound pectins, mainly sodium carbonate soluble pectins (Paniagua *et al.*,
487 2014). The depolymerization of covalently bound pectins mediated by pectinase
488 enzymes has been proposed as one of the probable causes of pectin solubilization
489 (Paniagua *et al.*, 2014). The most striking difference between control and transgenic
490 fruits observed in the carbohydrate microarray was the presence of large amounts of
491 pectins, both HG (recognized by LM18 and LM19) and RGI (RGI backbone recognized
492 by RU1-RU2 and galactan and arabinan neutral sidechains recognized by LM5 and
493 LM6-M, respectively) in the cadoxen fraction from antisense PG fruits. Cadoxen,
494 cadmium oxide with diaminoethane, is used to yield cellulosic polymers (Fry, 2000)
495 and low amount of pectins would be expected in this fraction, as observed in the control
496 fruits. However, this was not the case for antisense PG samples and the clustering of
497 glycome microarray data clearly separated these fractions from their corresponding
498 cadoxen control fraction. An increasing number of evidences indicates that a fraction of
499 pectins could be linked and/or entrapped into cellulose microfibrils. In a glycan
500 microarray of different Arabidopsis tissues, Moller *et al.* (2007) found that arabinan but
501 not HG were present in the cadoxen fraction, suggesting the close interaction between
502 side chain of pectins and cellulosic network. Natural arabinan or galactan-cellulose
503 composites have been isolated (Zykwinska *et al.*, 2005; Vignon *et al.*, 2004). In apple
504 fruit, Oechslin *et al.* (2003) identified a pectin domain associated to cellulose,
505 containing a RGI backbone with xylogalacturonan, linear galactan and highly ramified
506 arabinan side chains. HG does not have the ability to bind to cellulose in vitro
507 (Zykwinska *et al.*, 2005), therefore, the presence of HG in the cadoxen fractions from

508 antisense PG strawberry fruits could be explained if these pectin domains were linked to
509 cellulose through RGI. Along this side, the AFM characterization of sodium carbonate
510 soluble pectins from strawberry fruits digested with fungal endo-PG suggested the
511 presence of complex pectin molecules formed by an HG unit linked to a RGI core
512 (Paniagua *et al.*, 2017b). On the other hand, solid-state nuclear magnetic resonance
513 spectroscopy has provided strong evidences of extensive pectin-cellulose interactions in
514 native Arabidopsis cell walls. Around 25-50% of surface cellulose binds pectins, while
515 the cellulose-xyloglucan contacts are more limited than previously thought, less than
516 10% (Dick-Pérez *et al.*, 2011; Wang *et al.*, 2015; Wang and Hong, 2016). This last
517 result is in accordance with the biomechanical hot-spot model proposed by Park and
518 Cosgrove (2012) who found that only a small fraction of xyloglucans has load-bearing
519 function. These structural evidences suggest a prominent role of pectin-cellulose
520 interactions in the biomechanical properties of the cell wall. Along this line, it can be
521 hypothesized that the silencing of PG genes increased the amount of pectins linked to
522 the cellulose network and this would probably result in strengthened parenchyma cell
523 walls leading to firmer fruits.
524

525 **Changes in gene expression induced by PG genes downregulation**

526 Few transcriptomic changes induced by the downregulation of *FaPG1* or *FaPG2* were
527 found when these genes were silenced independently. This result reinforces the
528 hypothesis that both PG genes could play a redundant role in the last step of the pectin
529 disassembly process associated to fruit softening. However, the number of differentially
530 expressed genes increased notably in the PGI/II transgenic lines where both PGs were
531 simultaneously silenced. Several transcription factors, some stress response genes and
532 other genes encoding cell wall modifying enzymes were included among these DEGs. It
533 is therefore likely that PGs also participate in other signaling processes as discussed
534 below.

535 In the strawberry down-regulated PG lines, the expression of several genes involved
536 in the synthesis or modification of different cell wall polysaccharides was changed,
537 being the transcript levels of most of them lower than in control fruit. Among these cell
538 wall DEGs, it is remarkable the group of PMEs where four PME genes were
539 downregulated and one upregulated. Consequently, a higher signal of esterified HG
540 (JIM7) was obtained in the water fraction of all transgenic lines. This was an
541 unexpected result since it is generally accepted that the action of PMEs precedes that of

542 PGs during fruit ripening because PGs need at least four consecutive de-esterified GalA
543 residues to act (Sénéchal *et al.*, 2014). In strawberry, the degree of pectin de-
544 methylesterification (Draye and Van Cutsem, 2008; Osorio *et al.*, 2011) and PG activity
545 (Figueroa *et al.*, 2010; Ramos *et al.*, 2018) increased from the green to the red stage;
546 however, conflicting results regarding PME activity have been reported, some authors
547 described that PME activity diminished as the fruit ripen (Draye and Van Cutsem, 2008;
548 Figueroa *et al.*, 2010) while others found the contrary result (Osorio *et al.*, 2011). Cell
549 wall degradation was not in synchrony with pectin de-esterification in other fruits and
550 alternative roles to PME enzymes have been proposed, such as the activation of cell
551 wall loosening enzymes or proteins by decreasing pH through H^+ release (Redgwell *et*
552 *al.*, 1990; Bidhendi and Geitmann, 2016). Besides PME genes, PGs downregulation
553 also reduced the level of some β-galactosidase and endo-glucanase genes, including
554 *FaβGal4*, a ripening-related β-Galactosidase whose suppression reduced strawberry
555 fruit softening (Paniagua *et al.*, 2016). In other studies, it was also observed that the
556 manipulation of a cell wall gene affected the expression of other unrelated genes,
557 suggesting that a sort of unknown feedback regulation at the cell wall level might occur.
558 Brummell and Harpster (2001) found that the suppression of the expansin *LeExp1* gene
559 expression did not affect the mRNA accumulation of other expansin related genes;
560 however, *PG* gene expression was significantly reduced in these fruits. The transgenic
561 expression of a persimmon xyloglucan endotransglucosylase/hydrolase gene in tomato
562 accelerated fruit ripening likely due to the overexpression of genes involved in ethylene
563 biosynthesis (Han et al., 2016). β-xylosidase genes were up-regulated in transgenic *F.*
564 *vesca* fruits overexpressing the pectin methyl esterase gene *FaPE1* (Osorio *et al*., 2011).
565 It is well known that endoPGs release oligogalacturonides (OGAs) that might act as
566 developmental signals in pathogen defense and morphogenesis processes (Caffall and
567 Mohnen, 2009). As a working hypothesis, our results could indicate that PGs, besides
568 their structural role as pectin depolymerizing enzymes, might release OGAs that, in a
569 positive feedback, trigger cell wall disassembly. Along this line, it has been found that
570 OGAs generated in vitro by hydrolysis of pectins promote ripening in tomato and other
571 fruits (Dumville and Fry, 2000).

572 Although fruit softening is largely attributed to changes in cell wall structure, loss of
573 cell turgor as result of transpirational water loss and/or solute accumulation in the
574 apoplast can also contribute to the reduction in firmness, especially in berry fruits
575 (Saladié *et al.*, 2007; Wada *et al.*, 2009; Castellarin *et al.*, 2015). The genetic basis of

576 this reduction in turgor are largely unknown but it is conceivable that membrane
577 transporters were involved in the process. In strawberry fruit, turgor changes during
578 ripening have received little attention. However, the important role of ion channels on
579 fruit ripening has recently been demonstrated (Song *et al.* 2017; Wang *et al.*, 2018b). In
580 this research, we found that all antisense PG transgenic lines analyzed showed a strong
581 induction of a putative potassium transporter like-5 gene. This protein is a high affinity
582 H^+:K^+ symport transporter (HAK) belonging to the KUP/KT/HAK transporters family,
583 that are expressed mainly in roots, but also in shoots, in K^+ starved plants (Rubio *et al.*,
584 2000). Two genes encoding KUP/KT/HAK-type were identified in grape berries that
585 were expressed mainly in the skin at high levels during the initial phases of fruit
586 development and declining after the initiation of ripening (Davies *et al.*, 2006).
587 Similarly, 16 putative KUP/KT/HAK transporters were detected in peach fruit and the
588 expression of some of them was related to fruit growth and firmness (Song *et al.*, 2015).
589 In strawberry fruit, no other HAK gene has been previously described. The potassium
590 transporter gene induced in antisense PG plants could contribute to the uptake of K^+ in
591 transgenic ripe fruit, maintaining the solute potential and the turgor pressure of
592 parenchyma fruit cells. This fact would contribute to the higher firmness of the
593 transgenic fruits jointly with the lower disassembly of cell wall pectins. This hypothesis
594 implies that both processes leading to fruit softening, cell wall disassembly and loss of
595 cell turgor, could be linked by an unknown signaling pathway where cell wall integrity
596 would be an upstream event.
597
598 **Conclusions**
599 Although at least 6 PG genes are expressed in ripe receptacle, *FaPG2* and especially
600 *FaPG1* accounted for most PG transcripts in this tissue. Fruit softening was reduced at
601 similar level when silencing one of these genes. However, the simultaneous silencing of
602 both PGs did not induce a synergistic or additive effect on fruit softening. The increased
603 firmness in transgenic fruits could be due to a lower degradation of pectins associated to
604 xyloglucans and/or cellulose, a fact that would results in a higher cell wall mechanical
605 strength. Besides their structural role as pectin degrading enzymes, the transcriptomic
606 study indicates that both PG genes could release oligogalacturonides that would act as
607 positive signals in strawberry fruit softening. Many other genes involved in cell wall
608 remodeling were down-regulated as results of *FaPG1* or *FaPG2* silencing, standing out
609 the PME gene family that was extensively downregulated. On the other hand, it is

610 remarkable that all transgenic lines analyzed overexpressed a gene encoding a high
611 affinity K^+ transporter. This transporter could be involved in the maintenance of turgor
612 pressure in ripe transgenic fruits, contributing to their higher firmness. Overall, the
613 results obtained indicate that PG genes are excellent targets to modulate fruit softening
614 in strawberry, a crop of a high economic impact.
615
616 **Supplementary data**
617
618 **Supplementary Data 1.** Genes annotated as PG in the *F. vesca* genome.
619
620 **Supplementary Data 2.** Differentially expressed genes (DEGs) found in each
621 transgenic genotype.
622
623 **Table S1.** Monoclonal antibodies used in the carbohydrate microarray.
624
625 **Table S2.** Conserved domains in PG genes expressed in ripe receptacle.
626
627 **Table S3.** Characteristics of ripe fruits in control and transgenic plants.
628
629 **Table S4.** Summary of RNAseq mapping results.
630
631 **Fig. S1.** Multiple sequence alignment of the PG peptides expressed in strawberry ripe
632 receptacle by Clustal Omega.
633
634 **Fig. S2.** Clustering guide-tree generated by the multiple alignment of the PG protein
635 sequences expressed in ripe strawberry receptacle.
636
637 **Fig. S3.** PCA and hierarchical clustering analysis of carbohydrate microarray data.
638
639 **Fig. S4.** Vulcano plots representing the distribution of differentially expressed genes in
640 transgenic ripe fruits.
641
642 **Fig. S5.** Functional classification of differentially expressed genes in transgenic ripe
643 fruits according to the gene ontology (GO) terms, level 2.

644

645 **Fig. S6.** Fold change values in genes differentially expressed that were common to the

646 four transgenic genotypes analyzed.

647

648 **Data availability**

649 RNA-seq data that support the findings of this study are openly available in the

650 ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession

651 number E-MTAB-8736

652

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659

660 **Author contribution**

661 Conceptualization, JPK, JM-B, AJM, MAQ, JAM; Data curation, PR-V, GL-C, AJM;

662 Formal analysis, AJM, JAM; Funding acquisition, AJM, MAQ, JAM; Investigation,

663 CP, PR-V, JAG-G, GL-C, RB-P, JS, SP; Writing-original draft preparation, CP, JAM;

664 Writing-review and editing, SP, JPK, JAM.

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Table 1. Characteristics of ripe fruits in control and transgenic plants. Data represent means±SD of a minimum of 10 fruits per line, evaluated during the first year of analysis. Lines that are significantly different from the control by Mann-Whitney *U* test with sequential Bonferroni correction at *P*=0.05 are indicated with asterisks. PGI: antisense *FaPG1* transgenic line; PGII: antisense *FaPG2* transgenic lines; PGI/II: antisense *FaPG1-FaPG2* transgenic lines. L*: lightness; a*: redness; b*: yellowness.

Genotype	Weight (g)	Length (mm)	Width (mm)	Color			Soluble solids
				L*	a*	b*	
Control	11.9±3.4	35.8±4.4	27.3±3.6	36.1±3.5	38.4±3.7	21.0±4.2	6.9±1.5
PGI-29	12.6±3.8	36.7± 3.6	28.4±4.8	36.0±2.7	38.0±4.8	20.6±5.0	7.9±2.0
PGII-1	6.2±3.0*	24.3±5.4*	23.3±3.9*	38.5±4.5	39.7± 4.1	22.9±5.6	8.5±1.8
PGII-2	8.9±2.5	29.8±5.1*	25.9±3.1	39.7±4.8*	40.7±4.4*	25.0±5.8*	8.5± 2.0
PGII-3	6.3±1.8*	26.1±3.8*	23.5±2.6	38.9±4.2	38.8±5.3	24.7±4.4*	8.2±1.2
PGII-4	10.4±3.6	30.2±3.4	28.0±3.4	37.2±4.4	36.7± 4.0	20.1±4.9	8.2±2.0
PGII-5	10.5±4.0	35.6±7.6	26.4±2.9	37.2±4.4	38.8±3.4	21.4±4.7	7.6±1.7
PGII-7	6.7±0.7*	26.5±1.9*	24.6±2.0	38.5±3.8	38.7±4.0	22.8±3.8	7.9±1.1
PGII-8	8.2±2.1*	29.0±3.0*	25.3±3.3	36.0±3.2	34.9±6.3	19.4±3.0	9.0±2.0*
PGII-10	8.8±2.6*	31.2±2.7*	25.0±2.9	37.3±3.7	37.2±4.2	21.7±4.4	8.3±2.3
PGII-14	11.8±3.4	34.3±4.8	28.7±4.3	38.2±4.3	39.7± 4.0	22.4±5.9	9.3±2.1*
PGII-16	10.5±2.7	34.3±6.4	26.3±3.2	38.4±3.8*	38.3±4.6	22.3±5.5	9.0±2.1*
PGI/II-2	7.6±1.9*	24.6±3.3*	26.4±2.4	37.6±3.9	39.9±4.2	22.7±4.3	8.5±1.9*
PGI/II-5	7.4±2.1*	28.3±3.3*	24.7±3.4	39.0±4.1*	38.7±3.3	23.0±4.3	8.7±1.5*
PGI/II-9	10.5±2.6	33.0±5.2	27.7±3.1	37.3±4.1	39.2±3.4	22.4±4.0	9.5±1.8*
PGI/II-11	10.3± 2.5	32.2±4.5	27.1±2.6	36.7±4.4	39.5±4.5	21.5±4.3	8.0±2.2
PGI/II-13	10.4±3.7	29.6±3.9*	27.3±4.2	36.9±5.0	40.0±3.9	21.9±5.0	9.0±2.1*
PGI/II-14	9.7±2.1	32.7±9.1	26.1±2.0	36.5±4.2	38.9±4.4	21.5±4.8	8.3±1.7
PGI/II-15	12.3±3.9	31.8±5.1	29.2±4.3	37.2±2.7	40.7±2.3	23.5±3.2	7.9±1.6
PGI/II-16	11.4±3.6	29.2±5.3	29.0±3.1	36.6±2.9	35.2±5.5*	19.3±4.1	8.2±1.7

Table 2. Genes detected in the RNAseq study that were differentially expressed when compared with WT and could be involved in cell wall remodeling. The asterisk indicates statistically significant differences with WT at P=0.05.

Gene	Description	log2 Fold change			
		PGI-29	**PGII-5**	**PGI/II-9**	**PGI/II-16**
FvH4_6g41380.1	polygalacturonase (*FaPG1*)	-1.76*	0	-1.93*	-2.11*
FvH4_7g15040.1	polygalacturonase (*FaPG2*)	-0.65	-1.05*	-1.44*	-1.77*
FvH4_1g09610.1	pectinesterase (*FaPE1*)	-0.35	-1.15*	-1.59*	-1.05*
FvH4_2g25960.1	probable pectinesterase/pectinesterase inhibitor 7	-0.78	-0.06	-1.41*	-0.16
FvH4_4g12140.1	probable pectinesterase/pectinesterase inhibitor 35	-1.49*	-0.88	-1.81*	-2.3*
FvH4_6g35830.1	pectinesterase/pectinesterase inhibitor U1	-0.5	-0.74	-1.36*	-0.71
FvH4_7g11320.1	pectinesterase 2	1	0.81	1.76*	1.56
FvH4_1g26380.1	beta-galactosidase 15-like	-1.07	-2.44	-Inf*	-1.47
FvH4_2g27470.1	beta-galactosidase 16 (*FaβGal4*)	-0.26	-1.64*	-1.08*	-0.13
FvH4_4g10980.1	beta-galactosidase-like isoform X1	-0.35	-1.21	-3.21*	-1.72
FvH4_6g26960.1	glucosidase 2 subunit beta (N-glycan processing)	-1.23	-0.23	-1.26*	-1.36*
FvH4_1g21000.1	probable xyloglucan galactosyltransferase GT17	1.03	0.73	1.94*	1.44
FvH4_4g13220.1	probable xyloglucan galactosyltransferase GT14	0.57	0.55	1.10	1.30*
FvH4_2g23970.1	endo-1,3;1,4-beta-D-glucanase isoform X2	-0.57	-0.03	-0.54	-1.03*
FvH4_3g06030.1	glucan endo-1,3-beta-glucosidase 7-like isoform X2	2.43	3.41	4.13	4.59*
FvH4_3g23380.1	endoglucanase 11-like	-0.78	-0.99	-0.82	-1.41*
FvH4_4g35090.1	probable endo-1,3(4)-beta-glucanase ARB_01444	-0.75	-0.29	2.99*	-0.58
FvH4_7g27790.1	endoglucanase 8-like	-0.47	-1.36	-2.13*	-0.84
FvH4_4g31140.1	expansin 2 (*FaExp4*)	0.65	0.32	-0.01	0.92*
FvH4_4g32520.1	xylose isomerase	-0.24	-0.58	-0.66	-1.10*
FvH4_3g07410.1	WD repeat-containing protein 53 (Cellulose synthase (UDP-forming)	-0.97	-0.72	-2.05*	-1.62*
FvH4_3g06590.1	alpha,alpha-trehalose-phosphate synthase [UDP-forming] 1-like	-0.37	-0.21	-0.92*	-0.13
FvH4_6g42780.1	cellulose synthase-like protein H1 isoform X1	-0.51	-0.62	-1.28*	-0.71
FvH4_6g42840.1	cellulose synthase-like protein H1	0.58	0.64	0.79	1.38*
FvH4_3g23040.1	glycine-rich cell wall structural protein	0.2	-0.99	-0.21	-1.76*
FvH4_2g04630.1	classical arabinogalactan protein 9	0.31	0.07	0.23	1.33*

Figure legends

Figure 1. Expression of PG genes in ripe strawberry. Gene expression values were obtained in a RNAseq study performed in ripe receptacle and correspond to fragments per kilobase of transcript sequence per million base pairs sequenced (FPKM). Columns with different letters indicate significant differences by Tukey test at P=0.05.

Figure 2. (A) Aspect of control and some transgenic lines. Bars correspond to 4.5 cm in the pictures of the potted plants and 0.8 cm in the figures of the fruits. (B) Firmness of ripe fruits in control and transgenic strawberry plants evaluated during the first and second year of analysis. The asterisks indicate significant differences with control non-transformed fruits in the case of PGII genotypes or with PGI-29 in the case of PGI/II lines, by Mann-Whitney U test with sequential Bonferroni correction at P = 0.05. (C) Expression of *FaPG1* and *FaPG2* in ripe strawberry fruits from selected transgenic lines estimated by qRT-PCR. For each PG gene, expression levels were normalized to the control. Columns with different letters indicate significant differences by Kruskal-Wallis test at P = 0.05. C: control, non-transformed fruits; PGII: antisense *FaPG2* transgenic lines; PGI/II: antisense *FaPG1-FaPG2* transgenic lines.

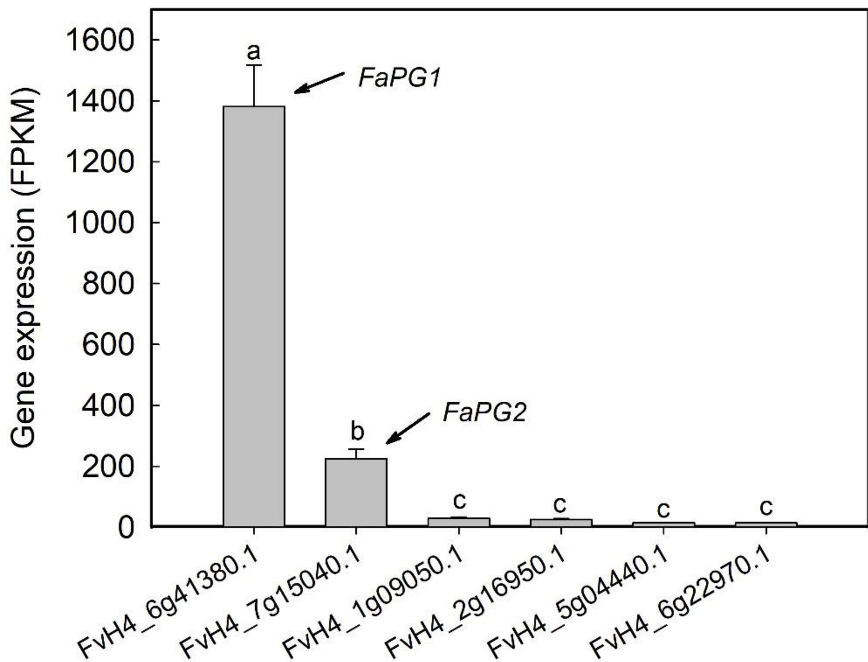
Figure 3. Heat map showing the relative abundance of cell wall epitopes recognized by various mAbs in cell wall fractions extracted from ripe strawberry fruits of control and antisense PG lines. A value of 100 was assigned to the highest mean spot signal and all other signals were adjusted accordingly. WT: control non-transformed fruit; PGI-29: antisense *FaPG1* line; PGII: antisense *FaPG2* lines; PGI/II: antisense *FaPG1-FaPG2* lines; HG: homogalacturonan; RGI: rhamnogalacturonan I; XG: xyloglucan; AGP: arabinogalactan proteins.

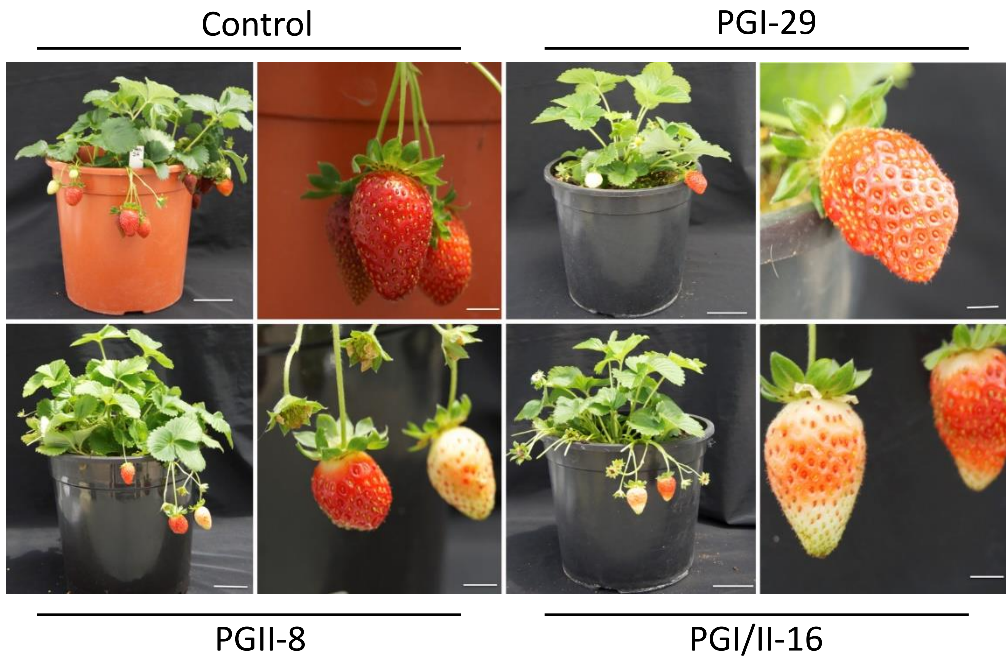
Figure 4. PCA analysis of carbohydrate microarray data from water and sodium carbonate cell wall fractions. The factor score plots are shown in the left and the variable plots in the right. The color scale in the variable plots represents the average contribution of the variables to the variation explained by the two principal components. WT: control fruit; PGI-29: antisense *FaPG1* line; PGII-5 and PGII-8: antisense *FaPG2* lines; PGI/II-9 and PGI/II-16: antisense *FaPG1-FaPG2* lines.

Figure 5. PCA analysis of carbohydrate microarray data from KOH and cadoxen cell wall fractions. The factor score plots are shown in the left and the variable plots in the right. The color scale in the variable plots represents the average contribution of the variables to the variation explained by the two principal components. WT: control fruit; PGI-29: antisense *FaPG1* line; PGII-5 and PGII-8: antisense *FaPG2* lines; PGI/II-9 and PGI/II-16: antisense *FaPG1-FaPG2* lines.

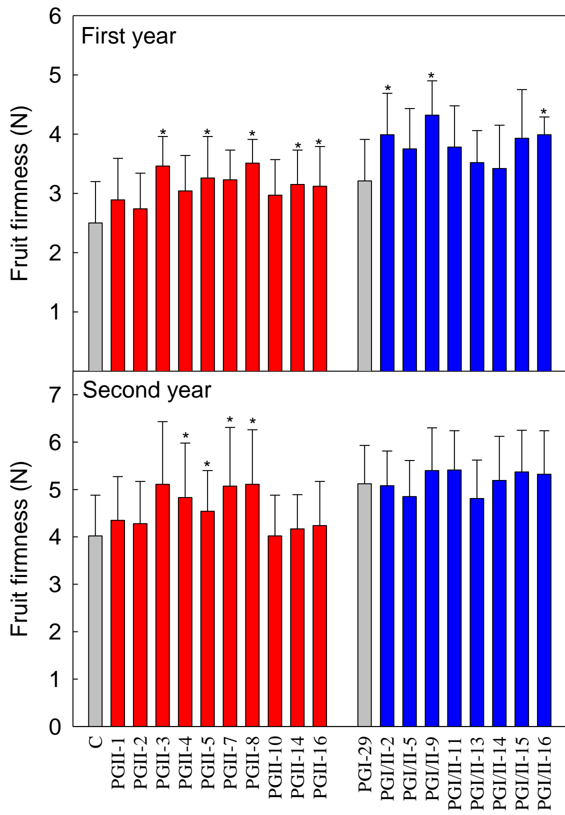
Figure 6. (A) Top 15 more enriched biological processes in up and down-regulated differently expressed genes (DEGs) relative to WT in ripe fruits from transgenic PGI/II-16 line expressing antisense sequences for *FaPG1* and *FaPG2* genes. (B) Venn diagrams for up and down-regulated DEGs relative to WT in ripe fruits from the different antisense PG lines.

Figure 7. Heat map showing the fold change expression values of genes involved in cell wall remodeling detected in the RNAseq study. Expression values were compared to control, non-transformed fruits. PGI-29: antisense *FaPG1* line; PGII-5 and PGII-8: antisense *FaPG2* lines; PGI/II-9 and PGI/II-16: antisense *FaPG1-FaPG2* lines.

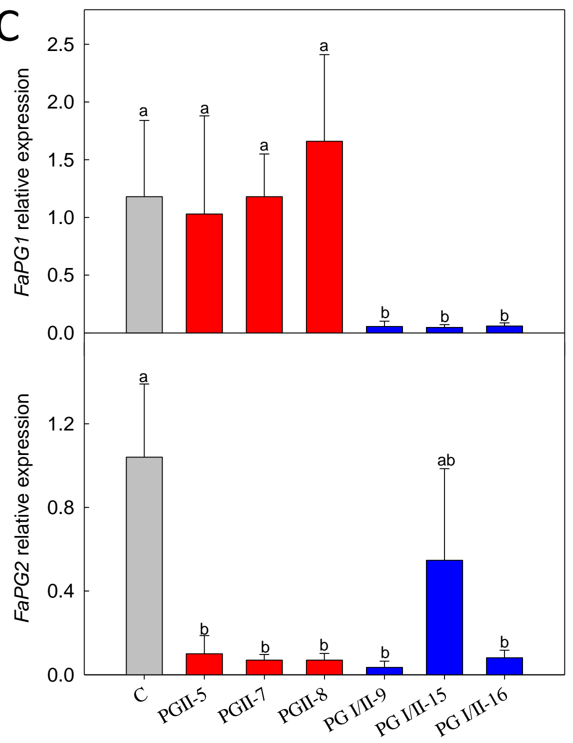

Gene expression (FPKM)
1600
1400
1200
1000
800
600
400
200
0
a
b
c
c
c
c
FaPG1
FaPG2
FvH4_6g41380.1
FvH4_7g15040.1
FvH4_1g09050.1
FvH4_2g16950.1
FvH4_5g04440.1
FvH4_6g22970.1



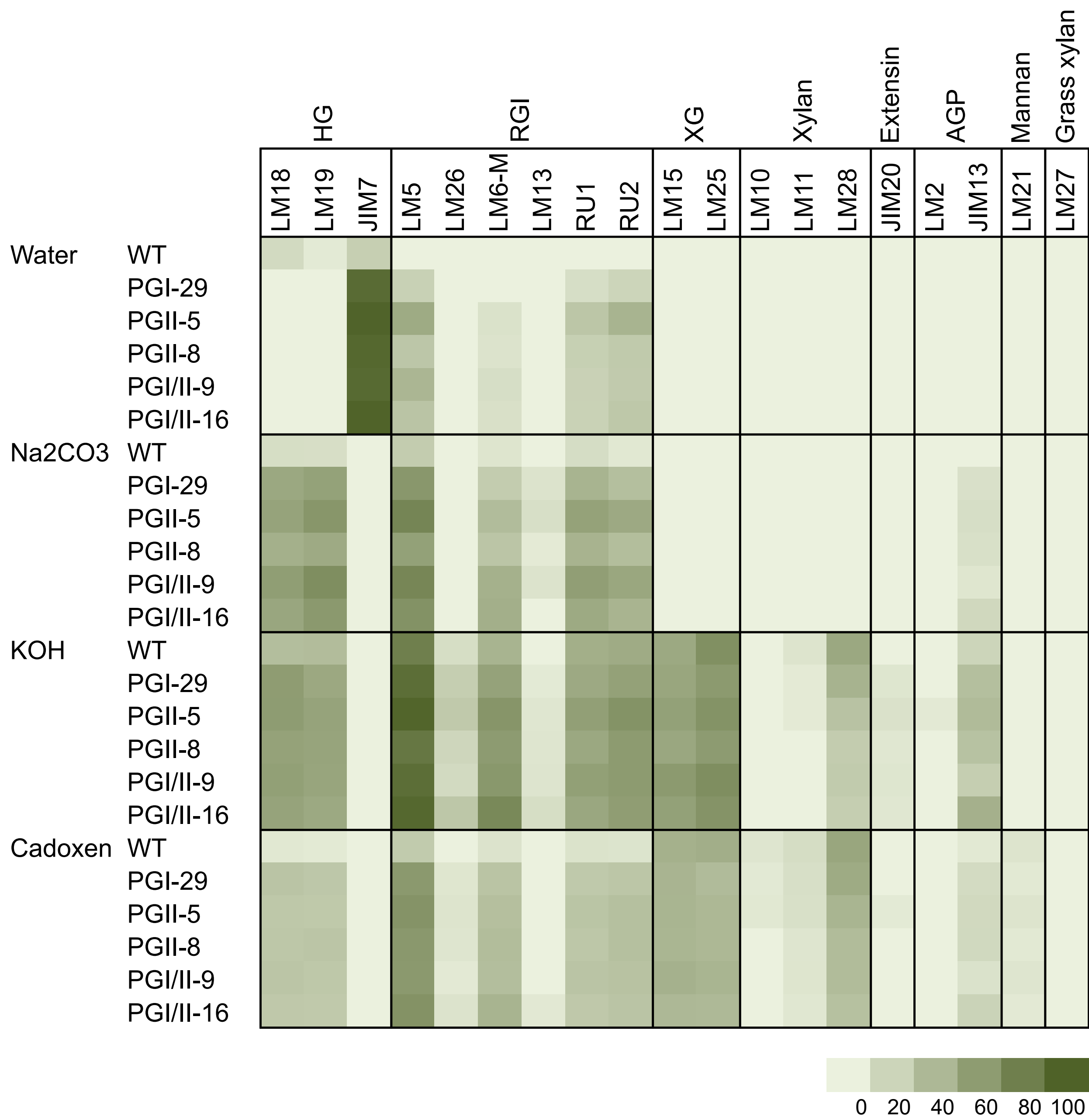
A
Control
PGI-29
PGII-8
PGI/II-16



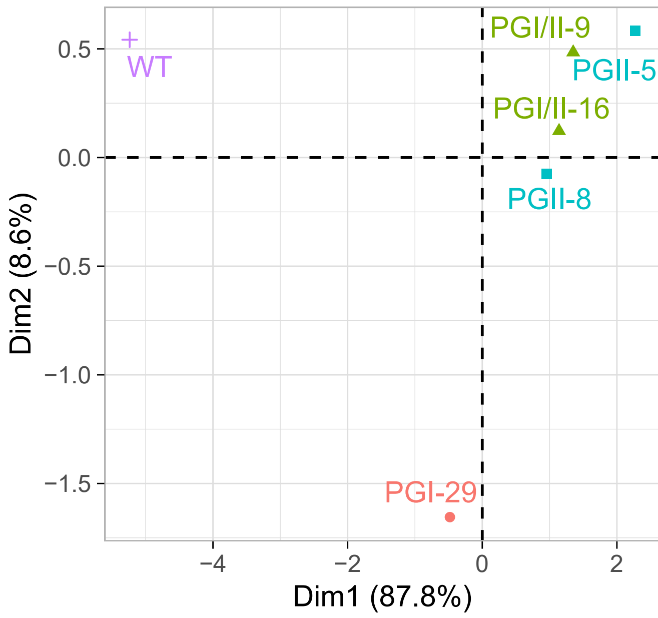
B
First year
Second year
Fruit firmness (N)
Fruit firmness (N)
C
PGII-1
PGII-2
PGII-3
PGII-4
PGII-5
PGII-7
PGII-8
PGII-10
PGII-14
PGII-16
PGI-29
PGI/II-2
PGI/II-5
PGI/II-9
PGI/II-11
PGI/II-13
PGI/II-14
PGI/II-15
PGI/II-16

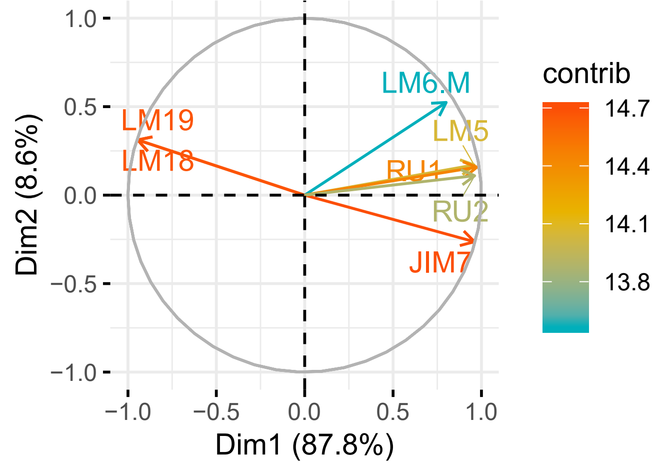


C
FaPG1 relative expression
FaPG2 relative expression
C
PGII-5
PGII-7
PGII-8
PG I/II-9
PG I/II-15
PG I/II-16

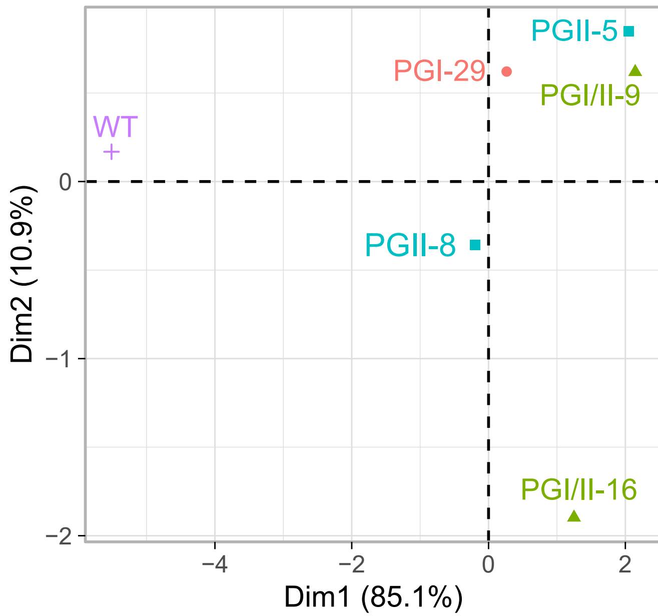

HG
RGI
XG
Xylan
Extensin
AGP
Mannan
Grass xylan
LM18
LM19
JIM7
LM5
LM26
LM6-M
LM13
RU1
RU2
LM15
LM25
LM10
LM11
LM28
JIM20
LM2
JIM13
LM21
LM27
Water
Na2CO3
KOH
Cadoxen
WT
PGI-29
PGII-5
PGII-8
PGI/II-9
PGI/II-16
0
20
40
60
80
100

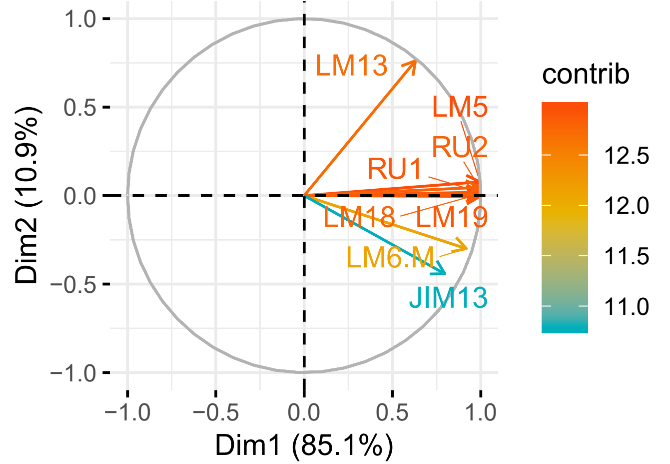
Water fraction



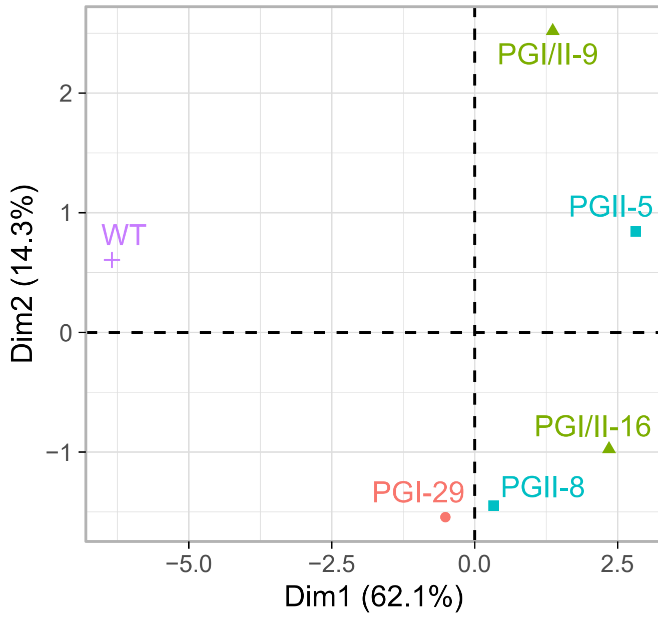


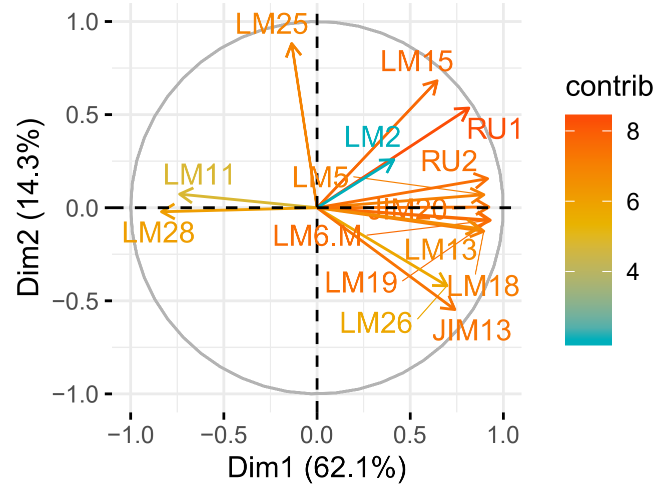
Sodium carbonate fraction



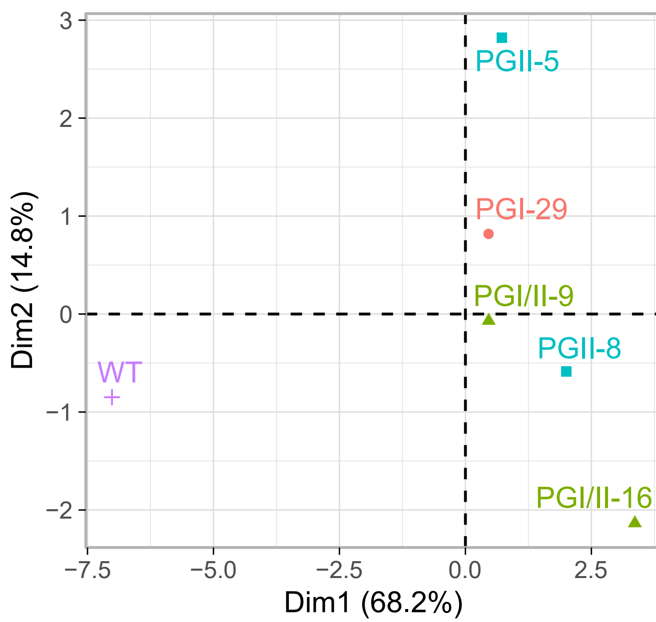


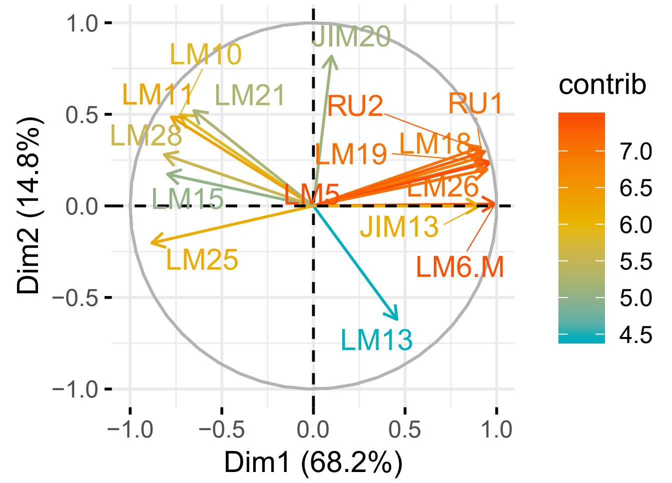
KOH fraction



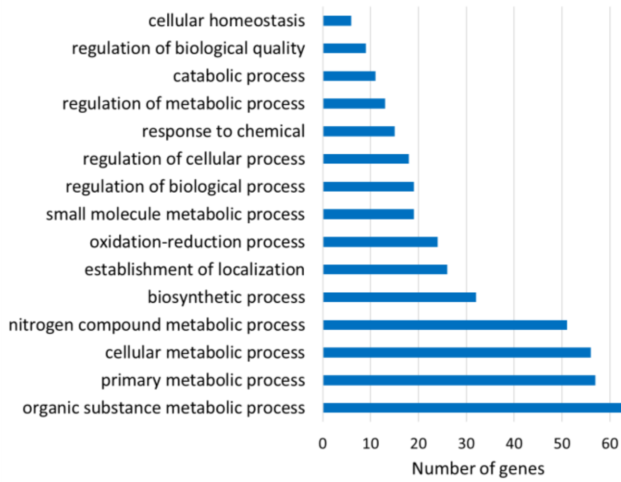


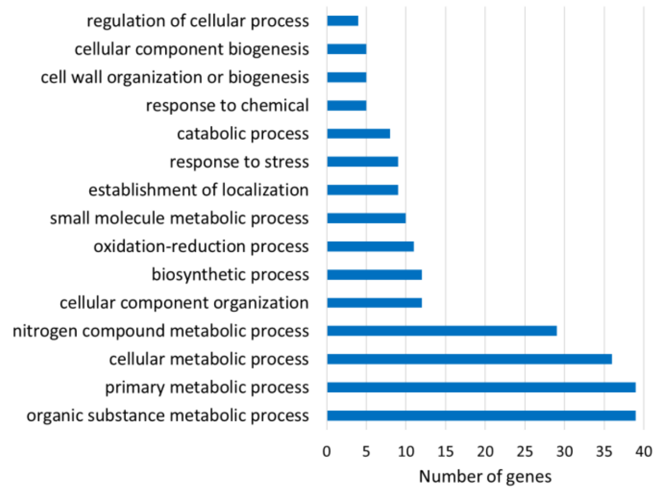
Cadoxen fraction



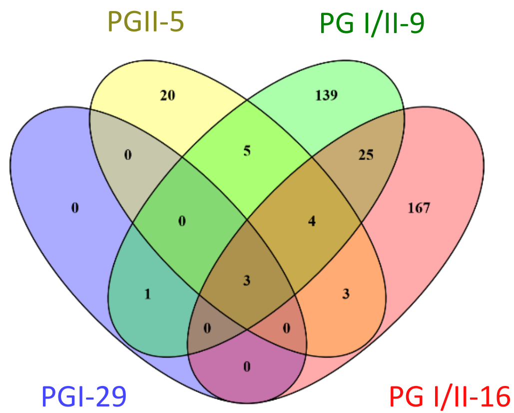


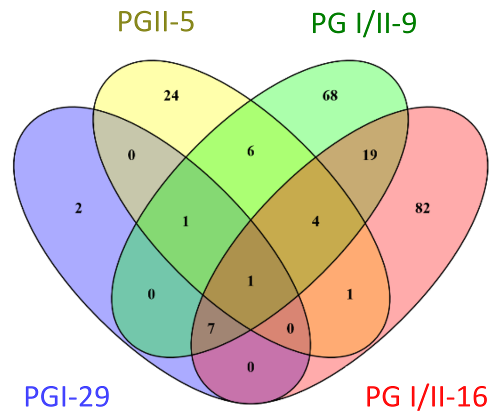
A

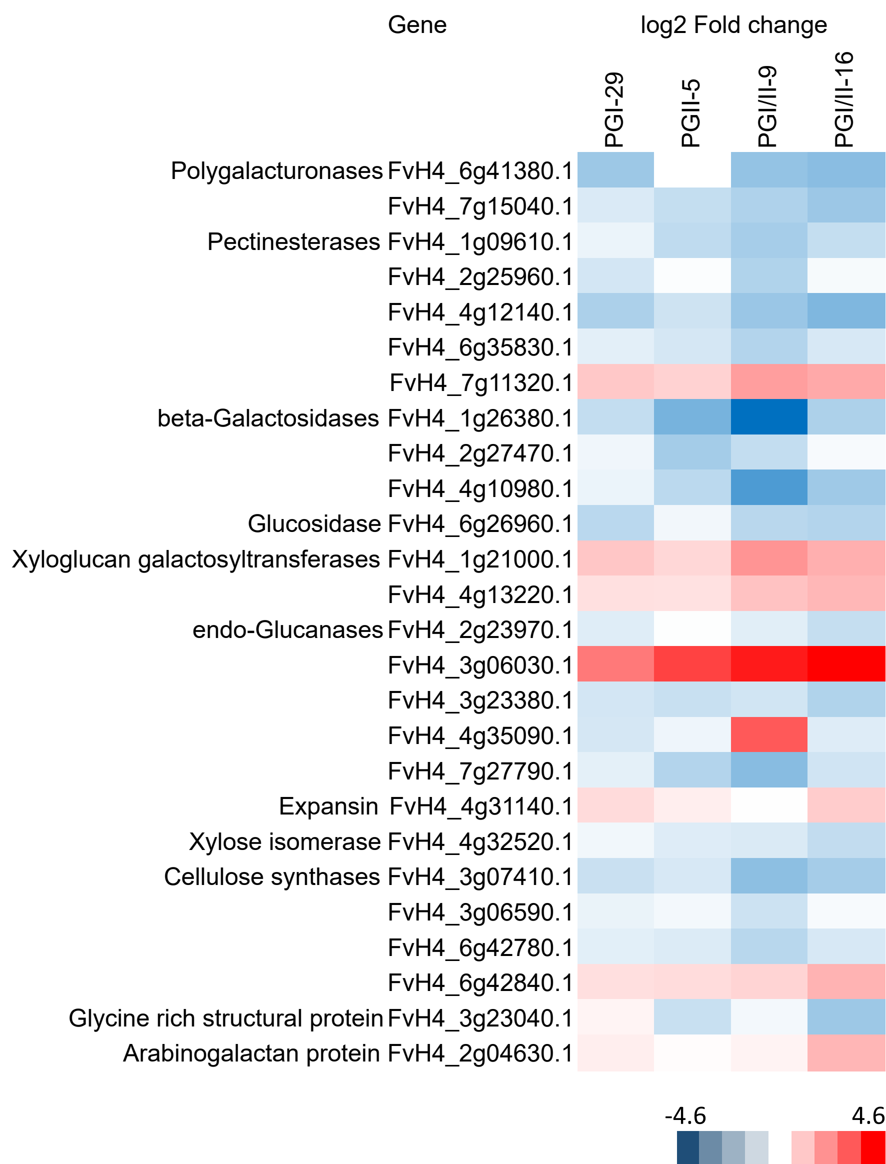




B






Gene
log2 Fold change
PGI-29
PGII-5
PGI/II-9
PGI/II-16
Polygalacturonases FvH4_6g41380.1
FvH4_7g15040.1
Pectinesterases FvH4_1g09610.1
FvH4_2g25960.1
FvH4_4g12140.1
FvH4_6g35830.1
FvH4_7g11320.1
beta-Galactosidases FvH4_1g26380.1
FvH4_2g27470.1
FvH4_4g10980.1
Glucosidase FvH4_6g26960.1
Xyloglucan galactosyltransferases FvH4_1g21000.1
FvH4_4g13220.1
endo-Glucanases FvH4_2g23970.1
FvH4_3g06030.1
FvH4_3g23380.1
FvH4_4g35090.1
FvH4_7g27790.1
Expansin FvH4_4g31140.1
Xylose isomerase FvH4_4g32520.1
Cellulose synthases FvH4_3g07410.1
FvH4_3g06590.1
FvH4_6g42780.1
FvH4_6g42840.1
Glycine rich structural protein FvH4_3g23040.1
Arabinogalactan protein FvH4_2g04630.1
-4.6
4.6