Cell wall composition as a maize defense mechanism against corn borers

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ABSTRACT

European and Mediterranean corn borers are two of the most economically important insect pests of maize (*Zea mays* L.) in North America and southern Europe, respectively. Cell wall structure and composition were evaluated in pith and rind tissues of resistant and susceptible inbred lines as possible corn borer resistance traits. Composition of cell wall polysaccharides, lignin concentration and composition, and cell wall bound forms of hydroxycinnamic acids were measured. As expected, most of the cell wall components were found at higher concentration in the rind than in the pith tissues, with the exception of galactose and total diferulate esters. Pith of resistant inbred lines had significantly higher concentrations of total cell wall material than susceptible inbred lines, indicating that thickness of cell walls could be the initial barrier against corn borer larvae attack. Higher concentrations of cell wall xylose and 8-*O*-4-coupled diferulate were found in resistant inbreds. Stem tunneling by corn borers was negatively correlated with concentrations of total diferulates, 8-5-diferulate and *p*-coumarate esters. Higher total cell wall, xylose, and diferulate concentrations appear to be possible mechanisms of corn borer resistance.

KEY WORDS*: *Zea mays; Ostrinia nubilalis; Sesamia nonagrioides; Resistance; Hydroxycinnamates; Polysaccharides; Lignin; Pith; Rind

1. Introduction

Maize (*Zea mays* L.) resistance to European corn borer (ECB), *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae) and Mediterranean corn borer (MCB), *Sesamia nonagrioides* Lefèbvre (Lepidoptera: Noctuidae) has been extensively evaluated because these are two of the most economically important insect pests of maize production in North America and southern Europe, respectively (Gianessi et al., 2003; Velasco et al., 2007). Several physical and biochemical characteristics (general plant traits, antibiotic compounds, repellent or attractant metabolites, etc.) have been studied as constitutive resistance mechanisms to corn borers (Malvar et al., 2008). There has also been significant research directed toward cell wall concentration, composition, and structure as possible resistance traits to corn borers (Buendgen et al., 1990; Santiago et al., 2006a). Resistance to ECB has been related to cell wall polysaccharide and lignin content of maize stalks (Ostander and Coors, 1997; Martin et al., 2004).

Cell wall bound forms of hydroxycinnamic acids in cereals consist largely of *p*-coumaric (*p*-CA) and ferulic (FA) acids (Hartley and Jones, 1978). All FA is ester linked to arabinoxylan and some of these FA molecules form additional covalent linkages to lignin (Ralph et al., 1992). While some *p*-CA is similarly esterified to arabinoxylan, the majority of *p*-CA is esterified to syringyl monolignol units of lignin (Ralph et al., 1994). Formation of diferulates (DFA) and higher oligomers of FA has been shown to cross link arabinoxylan chains (Bunzel, 2010). The deposition of DFAs in various tissues (kernel, leaf, pith, rind and nodes) has been shown to be associated with resistance to pests such as ECB (Bergvinson et al., 1997); Southwestern corn borer (*Diatraea grandiosella* Dyar) and sugarcane borer (*Diatraea saccharalis* Fabricius) (Ramputh, 2002); maize weevil (*Sitophilus zeamais* Motschulsky) (Garcia-Lara et al., 2004); MCB (Santiago et al., 2006a,

75 b, 2008) and diseases such as *Gibberella* stalk and ear rot (*Fusarium graminearum*
76 Schwabe*)* (Bily et al., 2003; Santiago et al., 2007). Several quantitative trait loci (QTL) for
77 concentrations of cell wall esterified *p*-CA, esterified and etherified FA, and esterified 5-5
78 DFA and 8-*O*-4 DFA that were identified by Barrière et al. (2008) also co-localized with
79 QTLs identified in other studies for ECB damage (Cardinal et al., 2003).
80 Previous work of our group identified sources of maize resistance to corn borer
81 (Butrón et al., 1999; Ordás et al., 2002). We have shown that resistant inbred lines
82 contained significantly higher concentrations of DFA than susceptible lines (Santiago et al.,
83 2006 a, b); however, it is unknown if other changes in cell wall concentration or
84 composition are associated with corn borer resistance of these maize lines. Our current
85 objectives were (i) to determine the concentration of the cell wall polysaccharide
86 components, lignin concentration and composition, and hydroxycinnamates (*p*-CA, FA, and
87 DFA) in pith and rind tissues of resistant and susceptible inbred lines of maize, and (ii) to
88 examine the role of these cell wall components as maize constitutive defense mechanisms
89 against corn borers.
90

2. Results and discussion

2.1. Pith vs. rind cell-wall composition

Pith and rind tissues were both analyzed in order to gather comprehensive data regarding maize stalk cell walls; however, the following data presentation is focused primarily on pith tissue because that is the tissue where corn borer larvae tunnel and feed. It has been assumed that the cell wall polysaccharides are indigestible to Lepidopterans larvae, which utilize mainly soluble carbohydrates and proteins as nutrients (Terra et al., 1987). As expected, most of the cell wall components were found at higher concentration, dry matter (DM) basis, in rind than pith tissues with the exception of the galactose, uronic acids, and total DFAs (Supplemental Table S1). Compositional analysis showed that cell wall components accounted for 320 g kg^{-1} DM in pith tissue and 580 g kg^{-1} DM in rind tissue (Supplemental Table S1), indicating higher concentrations of cytoplasmic components (proteins, lipids, ash, organic acids, etc.) in pith tissue. This greater concentration of total cell wall material in rind may explain why corn borer larvae, particularly MCB larvae, enter the stem through the base of the internode where the intercalary meristem is located and the cells are the least developed (Santiago et al., 2003, Barros et al., 2010). While total cell wall concentration was numerically higher in resistant lines for both tissues, the statistical contrast of resistant vs. susceptible lines was only significant for pith tissues. All inbred lines had higher rind cell wall concentrations than the cell wall concentration in pith tissue of resistant lines.

In pith tissues glucose was the predominant constituent of the cell wall polysaccharides (58%), followed by xylose (27%), uronic acids (6%), arabinose (5%), mannose (2%), and galactose (2%) (Supplemental Table S2). Arabinoxylan is the major hemicellulose component in most cereal cell walls, although there are large differences in

115 the degree of arabinose substitution among tissues (Hazen et al., 2003). Therefore, the high
116 values for xylose and arabinose probably represent a high content of arabinose-substituted
117 xylan (arabinoxylan), although some arabinose may also stem from pectic arabinan side-
118 chains. Lignin content represented 17.8 % of the total cell wall mass (Supplemental Table
119 S2). Syringyl monolignol units (S) were more common than guaiacyl units (G) in lignin.
120 Pith and rind tissues had approximately the same S/G ratio (1.48 and 1.51, respectively),
121 which was similar to the previous data for mature maize stalks (Lapierre, 1993).
122 Hydroxycinnamates such as FA and *p*-CA are minor components in plant cell walls
123 (Bunzel, 2010). Our analyses found that ester bound *p*-CA were the most abundant
124 hydroxycinnamic acid detected in maize pith and rind tissues (2.1 and 2.7% of total cell
125 wall, respectively), with FA (ester plus ether bound) also present in significant quantities
126 (1.4 and 1.5% of total cell wall). Together these monomeric phenolics accounted less than
127 the 5% of the total cell wall in both tissues (Supplemental Table S2). Three different DFAs
128 were identified and quantified; 8-5- 5-5-, and 8-*O*-4-coupled DFA. The dimers in order of
129 abundance for pith tissue were 8-5-DFA (52% of total DFAs measured), 8-*O*-4-DFA
130 (35%), and 5-5-DFA (13%). Rind tissue differed with the most abundant dimer being 5-5-
131 DFA (41%), followed closely by 8-5-DFA (39%), and 8-*O*-4-DFA (20%) present in lower
132 amounts (Supplemental Table S2). Overall, DFAs only accounted for 0.1% and 0.0002% of
133 mature maize pith and rind tissue cell walls, respectively.
134 A higher lignin concentration was observed in rind than pith tissue (70% higher)
135 (Supplemental Table S1). This fact may partially account for lower concentrations of
136 esterified DFAs in the rind than pith (92% less) because some DFA molecules become
137 cross liked to lignin through ether and other covalent linkages, such as occurs for FA

138 (Ralph et al., 1992). The DFAs ether linked to lignin were not determined by the alkaline
139 hydrolysis method employed in the current study.
140 *2.2. Environmental effects*
141 Locations differed significantly for the DM basis concentrations of most pith cell
142 wall components, but not for several hydroxycinnamate fractions (*p*-CA, FA ethers, and
143 total DFA) (data not shown). These differences in cell wall component abundance were
144 probably due to the different growth environments at the two locations. Usually Zaragoza is
145 warmer and drier than Pontevedra during the growing season. Although temperature
146 profiles in 2008 were similar for the locations, Pontevedra had greater precipitation overall
147 (data not shown). Pith tissue at Zaragoza had higher total cell wall and individual cell wall
148 component concentrations, except Klason lignin and 5-5-DFA which were not different
149 between locations (data not shown). Because total cell wall concentration differed between
150 locations, we examined the data on a cell wall basis to determine if composition of the
151 walls varied independently of total wall accumulation. Zaragoza still had higher
152 concentrations of uronic acids, arabinose, galactose, and glucose on a cell wall basis;
153 however, Klason lignin was greatest at Pontevedra (data not shown). Lower stem
154 internodes should have completed cell wall development by 30-d post-flowering (Jung,
155 2003); therefore, differences between locations suggest that growing conditions affected
156 cell wall development.
157 Based on biomass productivity, which did not differ based on measured of plant
158 height, neither location was more stressful than the other (data not shown). Corn borer tem
159 tunneling was different between the two locations, with MCB showing larger tunnels at
160 Pontevedra than ECB tunneling at Zaragoza (Table 1). However, this difference in

161 tunneling could reflect species differences between the damage potential of these two corn
162 borers rather than environmental impacts on cell wall development.
163 Although differences for locations were confirmed for most cell wall components, a
164 genotype × location interaction was only significant for uronic acid concentration. The
165 interaction was due to the EP42 inbred line which had higher uronic acid concentration at
166 Pontevedra than at Zaragoza (data not shown). Also, the A509 inbred line had the second
167 highest uronic acid concentration at Zaragoza but the lowest concentration at Pontevedra.
168 *2.3. Genotypic effects*
169 As expected based on previous research (Butrón et al., 1999; Ordás et al. 2002),
170 corn borer damage was greatest for the EP42 inbred line, and inbred EP39 showed the least
171 extensive tunneling (Table 2). The resistant vs. susceptible contrast indicated that the two
172 inbreds in the resistant group had shorter tunnel lengths than did the susceptible group of
173 inbreds (Table 2).
174 Because the plants selected for the compositional analysis were not infested or
175 damaged by corn borers, data analysis for cell wall traits was performed combining
176 locations. Significant differences among genotypes were observed for concentration of all
177 cell wall components (on DM basis) except FA ether and 5-5-DFA concentration (Table 3).
178 In the contrast for resistant vs. susceptible lines, resistant lines had significantly higher
179 xylose, arabinose, Klason lignin, FA ethers, 8-*O*-4-DFA, *p*-CA, and total cell wall material
180 than susceptible lines (Table 2, 3). The resistant line EP39 had the highest concentrations of
181 most wall components. Clearly, the resistant group had more total cell wall material,
182 particularly those components abundant in secondary walls (glucose, xylose, and lignin),
183 which suggests that cell wall thickness may be an important barrier that corn borers must

184 mechanically rupture in order to gain access to the cytoplasmic nutrients contained within
185 pith tissues.
186 In an attempt to attribute differential impact of individual cell wall components on
187 resistance, independent of total cell wall concentration, we also evaluated the composition
188 data on a cell wall basis. The resistant lines had significantly more xylose and 8-*O*-4-DFA
189 on a cell wall basis and significantly less glucose, mannose, and uronic acids, and esterified
190 FA (data not shown). Klason lignin concentration in cell wall material was similar among
191 resistant and susceptible groups of inbreds (data not shown). Xylose concentration was
192 19% greater in the resistant than susceptible lines whereas 8-*O*-4-DFA was 46% greater for
193 the resistant lines. This suggests that 8-*O*-4-DFA could play a more significant role as a
194 resistance mechanism against corn borers. Molecular modelling experiments suggested that
195 the 5-5-linked DFA could be formed intra-molecularly within a single arabinoxylan
196 molecule (Hatfield and Ralph, 1999). Allerdings et al. (2004) isolated and identified a di-
197 arabinosyl 8-*O*-4-DFA (Ara-FA-8-*O*-4-FA-Ara) from maize bran, suggesting inter-
198 molecular coupling of two different arabinoxylans by this specific DFA. Because 8-8-, 8-5-
199 , and 8-*O*-4-coupled DFAs do not undergo 8-β´-coupling and de-esterification, they assume
200 a greater role in cell wall stiffening by forming stable cross-links between xylan chains and
201 between xylans and lignin (Grabber et al., 2002). In addition, 8-5-DFA ($P < 0.08$) and total
202 DFAs ($P < 0.07$) showed a trend toward higher concentrations in the resistant maize
203 inbreds. However, 5-5-coupled DFA concentration was not different ($P = 0.82$) between
204 resistant and susceptible groups. Previous research by our group (Santiago et al., 2006a)
205 also found higher concentrations of 8-*O*-4- and 8-5-DFAs in the pith tissue of a larger
206 group of MCB resistant lines. Higher DFA ester concentrations in the resistant lines would
207 be expected to lead to more DFA ethers and other cross links to lignin (currently under

208 investigation), hence the cell wall would be more fortified through these additional cross
209 links and corn borer larvae would have greater difficulty accessing pith tissue nutrients. A
210 previous study suggested a role for ester linked DFA in greater cell wall adhesion,
211 increasing the tissue mechanical strength in Chinese water chestnut (*Eleocharis dulcis*
212 [Burm. f.] Trin. ex Henschel) tissues (Parker, et al., 2003). A similar hypothesis regarding
213 maize cell wall fortification against ECB was suggested by Bergvinson et al. (1997).
214 Whether the resistant lines had higher 8-coupled DFAs and total DFA
215 concentrations ($P < 0.10$) because FA biosynthesis was greater than in susceptible lines or
216 if the spatial arrangement of FA esters was closer for resistant line arabinoxylans allowing
217 more frequent dimerization is unknown. The arabinose-to-xylose molar ratio was
218 significantly higher ($P < 0.01$) in susceptible lines, but the total ferulates (FA plus DFA)-to-
219 arabinose molar ratio was not different ($P > 0.10$) between resistant and susceptible
220 genotypes (data not shown). These results jointly with the lower concentration of ferulate
221 esters on a cell-wall basis in the resistant lines suggest that the hypothesis of closer FA
222 ester spatial distribution in the resistant genotypes may be more likely.
223 *2.4. Regression analyses*
224 Stepwise multiple linear regression analysis was performed in order to better
225 understand the relationship between corn borer damage and cell wall components. Tunnel
226 length was the dependent variable, while cell wall components that were significant ($P <$
227 0.05) or tended towards significance ($P < 0.10$) in the resistant vs. susceptible contrasts
228 were included as independent variables (total cell wall, xylose, arabinose, Klason lignin,
229 FA ether, total FA, 8-5-DFA, 8-*O*-4-DFA, total DFAs, and *p*-CA). Because different corn
230 borer species were used to infest the maize inbreds at the two locations, the multiple
231 regression analysis was conducted separately by location.

The combination of total DFAs and 8-5-DFA explained 100% of stem tunneling variation at Pontevedra, with total DFAs alone explaining 86% of the variation (Table 4). Total DFAs had a negative regression coefficient whereas 8-5-DFA had a positive regression coefficient. Such contrasting signs for regression coefficients are common when one independent variables accounts for most of the variation in the dependent variable; resulting in an error for the relationship between the less important independent variable with the dependent variable. This mathematical artifact was corroborated by removing all DFAs from the regression analysis except 8-5-DFA and re-running the regression analysis. Subsequently, a negative regression coefficient was found for 8-5-DFA explaining 78% of the tunnel length variation. Because 8-5-DFAs accounted for half of all DFAs in pith tissue, our results suggest that higher concentrations of 8-β′coupled DFAs are related to length reductions of MCB tunnels.

At the Zaragoza location only *p*-CA was included in the regression model with a negative regression coefficient explaining 94% of ECB stem tunneling variation (Table 4). This suggests that higher concentrations of *p*-CA are related to shorter ECB tunneling. *p*-Coumaric acid is primarily esterified to syringyl monolignol units as a terminal molecule on lignin polymers and does not function as a cross linking agent between wall matrix polymers (Ralph et al., 1994; Hatfield et al., 2010). Although *p*-CA concentration is a good indicator of lignin deposition in grasses associated with maturity (Morrison et al., 1998); it is not obvious how *p*-CA would affect cell wall rigidity and toughness, and thereby corn borer resistance.

3. Conclusions

Pith tissue cell walls may play a role as a defense mechanism of maize against corn borers in three ways: (i) thickness of the cell walls, resistant lines had significantly more

256 total cell wall material than susceptible inbred lines; (ii) monosaccharide composition of
257 cell wall polysaccharides, higher concentration of xylose in resistant inbred lines; and (iii)
258 increased cell wall stiffening in the resistant genotypes, ability of the 8-coupled DFAs to
259 cross link arabinoxylan chains. A plant breeding project is currently underway to develop
260 maize lines with divergent concentrations of DFAs in order to test the role of these cross
261 linking agents on corn borer resistance.

4. Experimental

4.1. Plant materials and experimental design

Four maize inbred lines were selected for their consistent resistance or susceptibility to corn borers across multiple evaluations (Table 1). Inbred lines were grown at two locations in Spain, Pontevedra and Zaragoza, in 2008. Pontevedra (42º 30´ N, 8º 46´ W) is a coastal location in northwestern Spain and is approximately at sea level, whereas Zaragoza (41º 44´ N, 0º 47´´ W) is inland and 250 m above sea level. The most abundant corn borer at Pontevedra is MCB whereas ECB is predominant at Zaragoza (Malvar et al., 1993). The field experimental design at both locations was a randomized complete block design with three replicates. Each plot had two rows spaced 0.80 m apart and each row consisted of 25 two-kernel hills spaced 0.21 m apart. After thinning to one plant per hill, plant density was approximately 60000 plants ha^{-1}. Cultural operations, fertilization and weed control were carried out according to local practices and crop requirements.

To accurately define each genotype's silking time, plots were checked until 50% of plants were showing silks. At silking, five plants in each plot were artificially infested with MCB in Pontevedra and ECB in Zaragoza by placing an egg mass between the shank of the main ear and the stem. This infestation treatment has been demonstrated to be sufficient to guarantee corn borer damage (Butrón el at., 1999). Natural corn borer infestation and damage during the year of evaluation at both locations was minor. At harvest, the stem of infested plants was split longitudinally and corn borer tunnel length was measured (Table 1). Five to eight non-infested plants were collected for stalk composition analysis. Based on previous studies (Jung, 2003; Santiago et al., 2006a) samples for analysis were collected 30 d after silking when internode elongation had ceased. Pith and rind material were manually separated from the second and third elongated, above-ground internodes, frozen (–20 ºC),

lyophilized, and ground through a 0.75 mm screen in a Pulverisette 14 rotor mill (Fritsch GmbH, Oberstein, Germany) [Mention of a proprietary product does not constitute a recommendation or warranty of the product by Misión Biológica de Galicia, USDA, or the University of Minnesota, and does not imply approval to the exclusion of other suitable products].

4.2. Chemical determinations

4.2.1. Cell wall polysaccharide analysis

The Uppsala Dietary Fiber method (Theander et al., 1995) was used to measure cell wall polysaccharide components and lignin. A starch free, alcohol-insoluble residue was prepared according to Theander and Westerlund (1986). Acetate buffer (5 ml, 0.1 M, pH 5.0) and 0.1 ml of heat-stable α-amylase Termamyl 120 L (EC 3.2.1.1, from *Bacilus licheniformis*, 120 KNU/g) (Sigma Chemical Co., St. Louis, MO, USA) were added to 100 mg samples and heated at 90 ºC for 60 min. After the mixture cooled to 50 ºC, 0.2 ml of amyloglucosidase AMG 300 L (EC 3.2.1.3, from *Aspergillus niger*, 300 AGU/g) (Sigma) was added to the samples which were then heated for 3 h at 60 ºC. Sufficient 95% ethanol was subsequently added to achieve a final concentration of 80% ethanol and the sample was held at 4 ºC overnight. The crude cell wall preparation was recovered by centrifugation, washed twice with 80% ethanol and once with acetone, and allowed to air dry under a hood. Samples were suspended in 12 M sulfuric acid at 30 ºC for 1 h, followed by dilution with water to 0.3 M sulfuric acid and heating in an autoclave for 1 h at 117 ºC to hydrolyze the cell wall polysaccharides. After acid hydrolysis, the neutral sugar components (glucose, xylose, arabinose, mannose, and galactose) in the filtrate were quantified by gas chromatography as alditol acetate derivatives (Theander et al., 1995). Inositol was used as an internal standard to correct for volume variation. Neutral sugar data

were converted to an anhydro-sugar basis. The acidic sugars (glucuronic, galacturonic, and 4-*O*-methylglucuronic acids) were measured as total uronic acids by the colorimetric method of Ahmed and Labavitch (1977), in aliquots of the 0.3 M sulfuric acid solution sampled before heating, using glucuronic acid as the reference standard.

4.2.2. Lignin analysis

Klason lignin was determined as the insoluble residue from the two-stage acid hydrolysis retained on a glass fibre filter mat in a coarse-porosity Gooch crucible and corrected for ash content by combustion in a muffle furnace for 6 h at 450 ºC. Monolignol composition of maize lignin was determined by pyrolysis-gas chromatography-mass spectral analysis as described by Ralph and Hatfield (1991). The S/G ratio of lignin was calculated using data normalized for the guaiacol yield from each sample (Jung and Buxton, 1994).

4.2.3. Cell wall hydroxycinnamic acids analysis

Ester-linked FA and *p*-CA monomers were extracted from similar starch-free, alcohol insoluble residues with 2 M NaOH at 39 ºC for 24 h (Jung and Shalita-Jones, 1990). Alkaline extracts were acidified to pH 1.5-1.6 with concentrated phosphoric acid. The acidified extract was filtered through a Whatman filter 0.45 µm pore size, loaded on a C18 solid-phase extraction column (Supelco, Inc., Bellefonte, PA, USA), the column was washed with 2 ml of the same NaOH/phosphoric acid solution (pH 1.6) as that of the samples, and the hydroxycinnamic acids were eluted with two 2.5 ml 50% methanol washes. The eluted samples were brought to a final volume of 10 ml and stored at -20 °C until they were analyzed. The FA and *p*-CA released by the alkaline extraction were analyzed with an Agilent 1100 high pressure liquid chromatography (HPLC) system (Agilent Technologies, Wilmington, DE, USA) fitted with a diode array detector and a

334 Spherisorb-ODS2, C_{18}, 5 µm column (Waters Corp. Millford, MA, USA). Samples (20 µl)
335 were eluded with a 97.7:0.3:2.0 (vol/vol) water-glacial acetic acid-butanol solvent for 15
336 min, followed by a methanol wash of the column, at a flow rate of 1.8 ml min^{-1} (Jung and
337 Shalita-Jones, 1990). Hydroxycinnamic acids were detected at 320 nm and quantified using
338 the external calibration method.
339 Total (ester- and ether-linked) FA monomers in the cell wall were extracted with 4
340 M NaOH for 2 h at 170 ºC from starch-free, alcohol-insoluble residues (Iiyama et al.,
341 1990). Alkaline extracts were treated as described above to isolate and quantify
342 hydroxycinnamic acids. Ether-linked FA was calculated as the difference between total and
343 ester-linked FA concentrations of each sample (Iiyama et al., 1990).
344 Ester bound DFAs were extracted based on a procedure previously described
345 (Santiago et al., 2006a) with minor modifications. One gram of ground material was
346 extracted with 30 ml of 80% methanol. The suspension was homogenized for 30 s with a
347 Heidolph mixer (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) before
348 being centrifuged at 1000*g* for 10 min. After centrifugation, the pellet containing ester-
349 bound phenols incorporated in the cell wall was shaken in 20 ml of 2 M NaOH under
350 nitrogen flow and darkness for 4 h. The pH of alkali-treated samples was lowered to 2.0
351 with 6 N HCl. After centrifugation, the supernatant was collected and the pellet washed
352 twice with distilled water (10 ml each). Supernatants were pooled and then extracted twice
353 with ethyl acetate (40 ml each). Collected organic fractions were combined and reduced to
354 dryness using a Speed Vac (Thermo Fisher Scientific Inc., MA, USA) for 5 h. The final
355 extract was dissolved in 3 ml of HPLC-grade methanol. All of the extracts were stored at -
356 20 °C prior to HPLC analysis. Samples were filtered through a 2 µm pore poly
357 (tetrafluoroethylene) filter (Chromatographic Specialties, Brockville, ON, Canada) before

358 analysis. Analyses were performed using a 2690 Waters Separations Module (Waters,
359 Milford, MA, USA) equipped with a Waters 996 photodiode array detector and a Waters
360 YMC ODSAM narrow-bore column (100 × 2 mm i.d.; 3 μm particle size). Elution
361 conditions with a mobile phase system of acetonitrile (solvent A) and trifluoroacetic acid
362 (0.05%) in water (solvent B) were as follows: initial conditions 10:90 (A/B), changing to
363 30:70 in 3.5 min, then to 32:68 in 6.5 min, then to 100:0 in 4 min, then isocratic elution
364 with 100:0 for 4.5 min, finally returning to the initial conditions in 3 min. The mobile phase
365 flow rate was 0.3 ml min^{-1}, the total analysis time was 21.5 min, and the sample injection
366 volume was 4 μL.
367 Retention times and UV spectra were compared with freshly prepared standard
368 solutions of 5-5-DFA, kindly provided by the group of Dr. J.T. Arnason (University of
369 Ottawa, Ontario, Canada). The absorption UV spectra of other DFAs were compared with
370 previously published spectra (Waldron et al., 1996) and absorbance at 325 nm was used for
371 quantification.
372 Total cell wall concentration was calculated as the sum of glucose, xylose,
373 arabinose, mannose, galactose, uronic acids, Klason lignin, total FA, and ester-linked *p*-CA.
374 The DFAs were not included in the cell wall concentration calculation because some
375 samples were lost and their minor contribution to total cell wall concentration. All
376 compositional analyses were done in duplicate except for the analysis of DFAs, where only
377 20% of the samples were replicated, and monolignol composition analysis. All data were
378 corrected to a DM basis by drying ground stalk tissue samples overnight at 100 ºC.
379 *4.3. Statistical analysis*
380 Analysis of variance for cell wall components was computed using the PROC GLM
381 procedure of the SAS software package (SAS Institute, 2007). Pith and rind tissues were

382 analyzed separately. Only field replication was considered a random factor. Comparisons of
383 means among inbred line genotypes were made by the least significant difference method.
384 The resistant and susceptible inbred pair groups were compared using an orthogonal
385 contrast. To examine relationships between extent of corn borer stem tunneling and cell
386 wall components, multiple linear regressions by location were calculated for inbred line
387 mean data using the PROC REG procedure of SAS.
388

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531

532 Table 1. Pedigree, stem tunneling by corn borers, and resistance classification of four maize
533 inbred lines used in the current study.

Genotypes	Pedigree	Stem tunneling[a] (cm)		Group[b]
		ECB	MCB	
A509	A78 x A109	1.4	17.5	R
EP39	Fino	0.0	7.7	R
EP42	Tomiño	8.3	24.5	S
EP47	(EP4 x A239) EP4[2]	13.7	13.3	S

534

535 [a] Stem tunneling observed after artificial infestation with European corn borer (ECB) and
536 Mediterranean corn borer (MCB) in 2008 trials.

537 [b] R, resistant; S, susceptible. Classification based on tunneling by ECB and MCB (Butrón et
538 al., 1999; Ordás et al., 2002).

539 Table 2. Means for stem tunneling by corn borers and concentration of total cell wall, cell wall polysaccharide components, and lignin

540 concentration and composition in the pith tissue of four inbred lines grown at Pontevedra and Zaragoza, Spain in 2008.

Genotypes	Stem Tunnel	Total Cell Wall	Polysaccharides components[d]						Lignin[e]	
			Glc	Xyl	Ara	Man	Gal	UA	KL	S/G
	(cm)	----------(g kg^{-1} DM)----------								ratio
A509 (R)	9.47^{ab}	333^{b}	136^{b}	81^{ab}	13^{b}	4.0^{b}	4.3^{b}	16^{b}	65^{a}	1.46^{b}
EP39 (R)	3.48^{b}	440^{a}	207^{a}	100^{a}	18^{a}	5.0^{a}	6.6^{a}	21^{a}	69^{a}	1.38^{c}
EP42 (S)	16.38^{a}	335^{b}	160^{b}	66^{bc}	12^{b}	4.6^{ab}	4.3^{b}	17^{b}	60^{ab}	1.99^{a}
EP47 (S)	13.48^{a}	299^{b}	142^{b}	59^{c}	13^{b}	4.1^{b}	4.7^{b}	16^{b}	50^{b}	1.39^{bc}
LSD ($P \leq 0.05$)	9.64	65	29	19	4	0.9	1.6	3	10	0.36
Mean Resistant group	6.65	386	172	90	16	4.5	5.4	19	67	1.37
Mean Susceptible group	14.93	317	151	63	12	4.3	4.5	17	55	1.64
Contrast R vs. S[f]	*	*	NS	***	*	NS	NS	NS	**	NS

541

542 [abc]Inbred line means in the same column not sharing a superscript differ ($P < 0.05$).

543 [d] Glc, glucose; Xyl, xylose; Ara, arabinose; Man, mannose; Gal, galactose; UA, uronic acids,

544 [e]KL, Klason lignin S/G, syringyl-to-guaiacyl monolignol ratio.

[f] Orthogonal contrast of resistant (R) and susceptible (S) lines. NS, non significant ($P > 0.05$); *, **, and *** significant at $P < 0.05$, 0.01, and 0.001, respectively.

549 Table 3. Means for esterified and etherified ferulates, and esterified diferulates and *p*-coumarates in the pith tissue of four inbred lines grown at

550 Pontevedra and Zaragoza, Spain in 2008.

Genotypes	Ferulates			Diferulates[d]				*p*-Coumarates
	Esters	Ethers	Total	8-5-	8-*O*-4-	5-5-	Total	Ester
	(g kg^{-1} DM)							
A509 (R)	2.49^{c}	2.26	4.75^{ab}	0.10^{b}	0.08^{b}	0.02	0.21^{b}	8.82^{a}
EP39 (R)	3.41^{a}	2.34	5.75^{a}	0.17^{a}	0.12^{a}	0.04	0.33^{a}	7.47^{a}
EP42 (S)	2.63^{bc}	1.63	4.26^{b}	0.09^{b}	0.05^{c}	0.02	0.16^{b}	7.04^{a}
EP47 (S)	3.16^{ab}	1.65	4.81^{ab}	0.11^{b}	0.07^{bc}	0.04	0.22^{b}	5.13^{b}
LSD ($P \leq 0.05$)	0.57	0.88	1.02	0.03	0.02	0.02	0.07	1.90
Mean Resistant group	2.95	2.29	5.25	0.13	0.09	0.03	0.26	8.15
Mean Susceptible group	2.9	1.64	4.54	0.10	0.06	0.03	0.19	6.08
Contrast R vs. S[e]	NS	*	NS	NS	**	NS	NS	**

551

552 [abc]Inbred line means in the same column not sharing a superscript differ ($P < 0.05$).

553 [d] 8-5-DFA (or 8-5-cyclic DFA) was calculated as the sum of 8-5-linear (open or non cyclic) and 8-5-benzofuran forms of this DFA because both

554 are derived from the same precursor in the plant.

555 [e] Orthogonal contrast of resistant (R) and susceptible (S) lines. NS, non significant (P >0.05); *, **, and *** significant at P < 0.05, 0.01, and

556 0.001, respectively.

Table 4. Stepwise multiple linear regression results for corn borer stem tunnel length as a function of cell wall component concentrations in the pith tissue of four inbred lines grown at Pontevedra and Zaragoza, Spain in 2008.

Dependent variable	Location-Species[a]	Intercept	Independent variable	b coefficient	Pr < F[b]	R^2
stem tunnel length	Pontevedra-MCB	38.17	Total DFAs	-511	0.07	0.86
			8-5-DFA	816	0.05	0.14
stem tunnel length	Zaragoza-ECB	24.52	*p*-CA ester	-3	0.03	0.94

[a] Mediterranean corn borer (MCB) and European corn borer (ECB).

[b] F, test statistic used to reject or fail to reject the null hypothesis; Pr, probability of obtaining the F test statistic, assuming that the null hypothesis is true.

563 Supplemental Table S1. Concentration on dry matter basis (g/kg DM) of cell wall polysaccharide components, lignin and hydroxycinnmates in

564 pith and rind tissues. Data are averaged over four inbred lines grown at Pontevedra and Zaragoza, Spain in 2008 (N=24).

	Polysaccharides components[a]							Lignin	*p*-CA	FA			DFAs				
	Total	Glc	Xylose	Ara	Man	Gal	UA	KL	ester	Total	ester	ether	Total	8-5-	8-*O*-4-	5-5-	CW
										Pith Tissue							
MEAN[b]	278	161	76	14	4.4	5.0	18	61	7.11	4.89	2.92	1.97	0.208	0.109	0.071	0.027	319.4
S.D	74	43	23	5	1.0	2.1	5	11	2.01	1.00	0.64	0.77	0.071	0.041	0.025	0.017	50.2
MIN	182	101	43	7	2.7	2.2	11	41	3.97	3.42	1.73	0.59	0.064	0.029	0.026	0.010	237.5
MAX	455	264	121	25	7.0	10.0	30	80	10.86	7.30	4.13	3.69	0.376	0.208	0.133	0.057	433.1
										Rind Tissue							
MEAN	451	256	156	14	5.2	4.3	16	104	16.27	8.70	3.40	5.30	0.017	0.007	0.003	0.006	582.6
S.D.	70	35	33	3	1.0	1.4	2	15	2.32	0.93	0.40	0.81	0.012	0.007	0.004	0.006	84.1
MIN	343	201	113	9	3.1	2.0	10	76	10.69	7.08	2.53	4.03	0.001	0.000	0.001	0.000	463.6
MAX	664	362	251	21	7.1	6.9	20	145	19.78	10.05	4.10	6.32	0.047	0.027	0.015	0.019	842.0

565

566 [a] Glc, glucose; Xyl, xylose; Ara, arabinose; Man, mannose; Gal, galactose; UA, uronic acids. KL, Klason lignin; *p*-CA, *p*-coumaric acid; FA,

567 ferulic acid; DFAs, diferulic acids; CW, total cell wall concentration was the sum of all components except DFAs.

568 [b] MEAN, arithmetic mean; SD, standard deviation; MIN, minimum value; MAX, maximum value;

569 Supplemental Table S2. Cell wall proportion of polysaccharide components, lignin, and hydroxicinnamates in pith and rind tissues. Data are

570 averaged over four inbred lines grown in Pontevedra and Zaragoza, Spain in 2008 (N=24).

	Polysaccharides components[a]							Lignin	*p*-CA	FA			DFAs			
	Total	Glc	Xylose	Ara	Man	Gal	UA	KL	ester	Total	ester	ether	Total	8-5-	8-*O*-4-	5-5-
	%CW	----------% polysaccharides----------						---------- %CW----------		-------%FA-------			%CW	----------%DFA----------		
									Pith Tissue							
MEAN[b]	78.7	58.1	27.4	4.9	1.6	1.7	6.3	17.8	2.1	1.4	60.5	39.5	0.07	52.3	34.5	13.2
S.D.	4.1	3.27	3.3	0.9	0.3	0.4	0.8	3.6	0.6	0.2	11.4	11.4	0.03	6.8	4.3	7.3
MIN	69.4	52.2	21.8	3.8	1.1	1.2	5.0	12.8	1.2	1.0	43.5	14.0	0.02	40.5	28.3	5.7
MAX	84.4	63.4	33.0	4.9	2.4	1.7	8.5	26.1	3.1	2.0	86.0	56.5	0.10	61.0	43.6	31.2
									Rind Tissue							
MEAN	77.6	56.9	34.4	3.0	1.1	0.9	3.5	18.1	2.7	1.5	39.3	60.7	0.003	39.0	20.3	40.7
S.D.	2.3	2.7	2.4	0.5	0.1	0.3	0.5	2.2	0.4	0.2	4.7	4.7	0.002	24.7	16.6	33.2
MIN	74.0	52.0	29.4	2.3	0.8	0.5	2.5	13.5	2.2	1.2	31.6	51.1	0.0001	2.1	3.1	0.1
MAX	82.2	62.3	39.0	4.1	1.3	1.3	4.6	22.0	3.7	1.8	48.9	68.4	0.009	81.1	79.4	90.0

571

572 [a] Glc, glucose; Xyl, xylose; Ara, arabinose; Man, mannose; Gal, galactose; UA, uronic acids. KL, Klason lignin; *p*-CA, *p*-coumaric acid; FA,

573 ferulic acid; DFAs, diferulic acids.

574 [b] MEAN, arithmetic mean; SD, standard deviation; MIN, minimum value; MAX, maximum value;