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 (Ostrander 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 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) (García-Lara et al., 2004), MCB (Santiago et al., 2006a, 2006b) and diseases such as Gibberella stalk and ear rot (Fusarium graminearum Schwabe) (Bily et al., 2003; Santiago et al., 2007). Several quantitative trait loci (QTL) for concentrations of cell wall esterified p-CA, esterified and etherified FA, and esterified 5-5 DFA and 8-O-4 DFA that were identified by Barrière et al. (2008) also co-localized with QTLs identified in other studies for ECB damage (Cardinal et al., 2001).

Previous work of our group identified sources of maize resistance to corn borer (Butrón et al., 1999; Ordás et al., 2002). We have shown that resistant inbred lines contained significantly higher concentrations of DFA than susceptible lines (Santiago et al., 2006a, 2006b), however, it is unknown if other changes in cell wall concentration or composition are associated with corn borer resistance in these maize lines. Our current objectives were (i) to determine the concentration of the cell wall polysaccharide components, lignin concentration and composition, and hydroxycinnamates (p-CA, FA, and DFA) in pith and rind tissues of resistant
and susceptible inbred lines of maize, and (ii) to examine the role of these cell wall components as maize constitutive defense mechanisms against corn borers.

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 as nutrients (Terra et al., 1987). As expected, most of the cell wall components were found at higher concentration when calculated on a dry matter (DM) basis in rind than pith tissues with the exception of 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 the degree of arabinoxylan substitution among tissues (Hazen et al., 2003). Therefore, the high values for xylose and arabinose probably represent a high content of arabinose-substituted xylan (arabinoxylan), although some arabinose may also stem from pectic arabinan side-chains. Lignin content represented 17.8% of the total cell wall mass (Supplemental Table S2). Syringyl units (S) were more common than guaiacyl units (G) in lignin. Pith and rind tissues had approximately the same S/G ratio (1.48 and 1.51, respectively), which was similar to previous data for mature maize stalks (Lapiere, 1993).

Hydroxycinnamates such as FA and p-CA are minor components in plant cell walls (Bunzel, 2010). Our analyses found that ester bound p-CA was the most abundant hydroxycinnamic acid detected in maize pith and rind tissues (2.1% and 2.7% of total cell wall, respectively), with FA (ester plus ether bound) also present in significant quantities (1.4% and 1.5% of total cell wall). Together these monomeric phenolics accounted less than the 5% of the total cell wall in both tissues (Supplemental Table S2). Three different DFAs were identified and quantified: 8-5-DFA, 8-O-4-DFA, and 5-5-DFA. The 8-5-DFA was calculated as the sum of 8-5-open or non cyclic and 8-5-benzofuran or cyclic forms, because it has been reported that 8-5-non-cyclic form may be a product after alkaline hydrolysis of the cyclic form, the only naturally occurring in plant cell walls (Ralph et al., 1994). The dimers in order of abundance for pith tissue were 8-5-DFA (52% of total DFAs measured), 8-O-4-DFA (35%), and 5-5-DFA (13%). Rind tissue differed with the most abundant dimer being 5-5-DFA (41%), followed closely by 8-5-DFA (39%), and 8-O-4-DFA (20%) present in lower proportion (Supplemental Table S2). Overall, DFAs only accounted for 0.1% and 0.0002% of mature maize pith and rind tissue cell walls, respectively.

A higher lignin concentration was observed in rind than pith tissue (70% higher) (Supplemental Table S1). This fact may partially account for lower concentrations of esterified DFAs in the rind than pith (92% less) because some DFA molecules become cross linked to lignin through ether and other covalent linkages, such as occurs for FA (Ralph et al., 1992). Any DFAs that were ether linked to lignin could not be determined by the alkaline hydrolysis method employed in the current study.

2.2. Environmental effects

Concentrations on a DM basis of most pith cell wall components were significantly different between locations, except for p-CA, FA ethers, and total DFA (data not shown). These differences in cell wall component abundance were probably due to the different growth environments at the two locations. Usually Zaragoza is warmer and drier than Pontevedra during the growing season. Although temperature profiles in 2008 were similar for the locations, Pontevedra had greater precipitation overall (data not shown). Pith tissue at Zaragoza had higher total cell wall and individual cell wall component concentrations, except Klasson lignin and 5-5-DFA which were not different between locations (data not shown). As total cell wall concentration differed between locations, we examined the data on a cell wall basis to determine if composition of the walls varied independently of total cell wall accumulation. Zaragoza still had higher concentrations of uronic acids, arabinose, galactose, and glucose on a cell wall basis, however, Klasson lignin was greatest at Pontevedra (data not shown). Lower stem internodes should have completed cell wall development by 30-d post-flowering (Jung, 2003), therefore, differences between locations suggest that growing conditions affected cell wall development.

Based on biomass productivity, which did not differ based on measured of plant height, neither location was more stressful than the other (data not shown). Corn borer tem tunneling was different between the two locations, with MCB showing larger tunnels at Pontevedra than ECB tunneling at Zaragoza (Table 2). However, this difference in tunneling could reflect differences between the damage potential of these two corn borer species rather than environmental impacts on cell wall development.

Although differences for locations were confirmed for most cell wall components, a genotype × location interaction was only significant for uronic acid concentration. The interaction was due to the EP42 inbred line which had higher uronic acid concentration at Pontevedra than at Zaragoza (data not shown). Also, the A509 inbred line had the second highest uronic acid concentration at Zaragoza but the lowest concentration at Pontevedra.

2.3. Genotypic effects

Analysis by location for stem tunneling data for corn borer damage showed that resistant and susceptible lines differed for length
of ECB tunnels at Zaragoza; however, damage caused by MCB at the Pontevedra location during the year of evaluation did not differ between resistant and susceptible lines (Table 2). Lower temperatures during the year of evaluation adversely affected development of MCB larvae (Velasco et al., 2007), causing shorter tunneling than expected based on previous studies (Butrón et al., 2009; Sandoya et al., 2008). These results do not alter the resistance classification of the lines which is based on their resistance or susceptibility response to corn borers across multiple evaluations (Butrón et al., 1999; Ordás et al., 2002).

Significant differences among genotypes were observed for concentration of all cell wall components (on DM basis) except FA and 5-5-DFA concentration (Table 3). In the contrast for resistant vs. susceptible lines, resistant lines had significantly higher xylose, arabinose, Klasen lignin, FA ethers, 8-O-4-DFAs, p-CAs, and total cell wall material than susceptible lines (Tables 2 and 3). The resistant line EP39 had the highest concentrations of most wall components. Clearly, the resistant group had more total cell wall material, particularly those components abundant in secondary cell wall components. In an attempt to attribute differential impact of individual cell wall components on resistance, independent of total cell wall concentration, we also evaluated the composition data on a cell wall basis. The resistant lines had significantly more xylose and 8-O-4-DFAs on a cell wall basis and significantly less glucose, mannose, and uronic acids, and esterified FA (data not shown). Klasen lignin concentration in cell wall material was similar among resistant and susceptible groups of inbreds (data not shown). Xylose concentration was 19% greater in the resistant than susceptible lines whereas 8-O-4-DFAs was 46% greater for the resistant lines. These results suggest that 8-O-4-DFAs could play a greater role in corn borer resistance than 5-5 DFA or ferulate which did not differ between resistant and susceptible maize inbreds. Higher concentration of 8-O-4-DFAs could lead to more fortified cell walls and therefore more resistant genotypes. Molecular modelling experiments by Hatfield et al. (1999) showed that 8-coupled DFA cross links arabinoxylans inter-molecularly while some 5-5-linked DFA could also form intra-molecular linkages when arabinose residues bearing ferulate units are positioned three xylose residues apart on the same xylan chain. Although Obel et al. (2003) suggested that 8-5-DFAs could also cross link intra-molecularly, as far as we know 8-0-4 DFAs can only link two separate arabinoxylan chains. However, at present there are no definitive data as to which of the DFAs

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Stem tunnel (cm)</th>
<th>Total (g kg⁻¹ dry matter basis)</th>
<th>Polysaccharide components (g kg⁻¹ dry matter basis)</th>
<th>Lignin (g kg⁻¹ dry matter basis)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pontevedra</td>
<td>Zaragoza</td>
<td>Cell wall</td>
<td></td>
</tr>
<tr>
<td>A505 (R)</td>
<td>17.53</td>
<td>1.40ab</td>
<td>333b</td>
<td></td>
</tr>
<tr>
<td>EP39 (R)</td>
<td>7.67</td>
<td>0.00ab</td>
<td>440b</td>
<td></td>
</tr>
<tr>
<td>EP42 (S)</td>
<td>24.50</td>
<td>8.27b</td>
<td>335b</td>
<td></td>
</tr>
<tr>
<td>EP47 (S)</td>
<td>13.30</td>
<td>13.67b</td>
<td>299b</td>
<td></td>
</tr>
<tr>
<td>LSD (P &lt; 0.05)</td>
<td>17.49</td>
<td>12.75</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>Mean resistant group</td>
<td>12.60</td>
<td>0.70</td>
<td>386</td>
<td></td>
</tr>
<tr>
<td>Mean susceptible group</td>
<td>18.90</td>
<td>10.97</td>
<td>317</td>
<td></td>
</tr>
<tr>
<td>Contrast R vs S</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

NS, non significant (P > 0.05).

** Significant at P < 0.05.

### Table 2

Means by location for stem tunneling, and combined means over locations for concentration of total cell wall, cell wall polysaccharide components, and lignin concentration and composition in the pith tissue of four inbred lines grown at Pontevedra and Zaragoza, Spain in 2008.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Ferulates (g kg⁻¹ DM)</th>
<th>Esterified ferulates (g kg⁻¹ DM)</th>
<th>p-Coumarates (g kg⁻¹ DM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A505 (R)</td>
<td>2.45</td>
<td>2.26</td>
<td>4.75</td>
</tr>
<tr>
<td>EP39 (R)</td>
<td>3.41</td>
<td>2.34</td>
<td>5.75</td>
</tr>
<tr>
<td>EP42 (S)</td>
<td>2.63</td>
<td>1.63</td>
<td>4.26</td>
</tr>
<tr>
<td>EP47 (S)</td>
<td>3.16</td>
<td>1.65</td>
<td>4.81</td>
</tr>
<tr>
<td>LSD (P &lt; 0.05)</td>
<td>0.57</td>
<td>0.88</td>
<td>1.02</td>
</tr>
<tr>
<td>Mean resistant group</td>
<td>2.95</td>
<td>2.29</td>
<td>5.25</td>
</tr>
<tr>
<td>Mean susceptible group</td>
<td>2.90</td>
<td>1.64</td>
<td>4.54</td>
</tr>
<tr>
<td>Contrast R vs S</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>
form intra and/or intermolecular cross-links. Grabber et al. (2002) also showed that 8-coupled DFAs may assume a greater role in cell wall stiffening than 5-5 DFA and ferulic acid because a substantial proportion of 5-5 DFA and ferulic acid mediated cross links between xylans and lignin could be lost via de-esterification, if 8-β-lactone structures with monolignols were the major product of 8-β-lactone coupling during lignification. However, it is not known whether these 8-β-lactone structures are actually present in the plant or whether they are artifacts created during the analytical procedure.

In addition, 8-5-DFA (P < 0.08) and total DFAs (P < 0.07) showed a trend toward higher concentrations in the resistant maize inbreds. However, 5-5-coupled DFA concentration was not different (P = 0.82) between resistant and susceptible groups. Previous research by our group (Santiago et al., 2006a) also found higher concentrations of 8-0-4- and 8-5-DFAs in the pith tissue of a larger group of MCB resistant lines. Higher DFA ester concentrations in the resistant lines would be expected to lead to more DFA ethers (currently under investigation), hence the cell wall would be more fortified through these additional cross links and corn borer larvae would have greater difficulty accessing pith tissue nutrients. A previous study suggested a role for ester linked DFA in greater cell wall adhesion, increasing the tissue mechanical strength in Chinese water chestnut (Eleocarhis dulcis [Burm. f.] Trin. ex Henschel) tissues (Parker et al., 2003). A similar hypothesis regarding maize cell wall fortification against ECB was suggested by Bergvinson et al. (1997).

Resistant lines had greater feruloylarabinobioxytan concentration (total FA plus total DFA plus arabinoxylan) than susceptible lines on both a dry matter (P < 0.07) and cell wall (P < 0.01) basis. However, the degree of xylan substitution with arabinose (arabinose-to-xylene ratio) and the degree of feruloylation of arabinose (total FA plus DFA-to-arabinose ratio) did not differ (P > 0.10) between the resistant and susceptible lines. As a result, the xylan cross linking (total FA plus DFA-to-xylene ratio) was not different (P > 0.10) between the two groups of maize lines. We suggest that the higher 8-coupled DFAs and total DFA concentrations (P < 0.10) in the resistant lines resulted from the higher concentration of feruloylarabinobioxytan chains in the cell wall, allowing more frequent dimerization between FA residues to occur, because more feruloylarabinobioxytan polymers present per unit of cell wall probably placed FA molecules in closer physical proximity.

### 2.4. Regression analyses

Stepwise multiple linear regression analysis was performed in order to better understand the relationship between corn borer damage and cell wall components. Tunnel length was the dependent variable, while cell wall components that were significant (P < 0.05) or tended towards significance (P < 0.10) in the resistant vs. susceptible contrasts were included as independent variables (total cell wall, xylose, arabinose, Klasson lignin, FA ether, total FA, 8-5-DFA, 8-0-A-4-DFA, total DFAs, and p-CA). As different corn borer species were used to infest the maize inbreds at the two locations, the multiple regression analysis was conducted separately by location in case ECB and MCB respond differently to cell wall structure with regard to extent of tunneling.

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. As 8-5-DFAs accounted for half of all DFAs in pith tissue, our results suggest that higher concentrations of 8-coupled DFAs are related to length reductions of MCB tunnels.

At the Zaragoza location only p-CA was retained 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. In maize, p-CA is primarily esterified to syringyl 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). 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. These results suggest that ECB damage is impacted by maize cell wall structure, but the nature of this effect is unclear.

### 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 total cell wall material than susceptible inbred lines; (ii) monosaccharide composition of cell wall polysaccharides, higher concentration of xylose in resistant inbred lines; and (iii) increased cell wall stiffening in the resistant genotypes, ability of the 8-coupled DFAs to cross link arabinobioxytan chains. A plant breeding project is currently underway to develop maize lines with divergent concentrations of DFAs in order to test the role of these cross 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 (42° 30’ N, 8° 46’ W) is a coastal location in northwestern Spain and is approximately at sea level, whereas Zaragoza (41° 44’ N, 0° 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 60 000 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 et al., 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 2). 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 °C), 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 ß-amylase Termamyl 120 L (EC 3.2.1.1, from Bacillus licheniformis, 120 KNU/g) (Sigma Chemical Co., St. Louis, MO, USA) were added to 100 mg samples and heated at 90 °C for 60 min. After the mixture cooled to 50 °C, 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 °C. Sufficient 95% ethanol was subsequently added to achieve a final concentration of 80% ethanol and the sample was held at 4 °C 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 °C for 1 h, followed by dilution with water to 0.3 M sulfuric acid and heating in an autoclave for 1 h at 117 °C 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 a monosaccharide 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 °C. Monomeric composition of maize lignin was determined by pyrolysis–gas chromatography–mass spectral analysis as described by Ralph and Hatfield (1991). The total ion abundance data for six syringyl compounds (2,6-dimethoxyphenol; 2,6-dimethoxy-4-methylphenyl; 4-ethyl-2,6-dimethoxyphenol; 2,6-dimethoxyvinylphenol; 2,6-dimethoxy-4-propenylphenol; and syringaldehyde) and five guaiacyl compounds (guaiacol, 4-ethylguaiacol, 4-methylguaiacol, isoeugenol, and vanillin) were collected. The ion abundance data for each compound was normalized for the guaiacol yield from the guaiacol yield from each sample and then the S/G ratio was calculated by summation of the syringyl and guaiacyl compounds (Jung and Buxton, 1994).

4.2.3. Cell wall hydroxycinnamic acids analysis

Ester-linked FA and p-CAs were extracted from similar starch-free, alcohol-insoluble residues with 2 M NaOH at 39 °C 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-CAs 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 Spherisorb ODS2, C18, 5 µm column (Waters Corp., Millford, MA, USA). Samples (20 µl) were eluted with a 97.7:0.3:2.0 (vol/vol) water–glacial acetic acid–butanol solvent for 15 min, followed by a methanol wash of the column, at a flow rate of 1.8 ml min⁻¹ (Jung and Shalita-Jones, 1990). Hydroxycinnamic acids were detected at 320 nm and quantified using the external calibration method.

Total (ester- and ether-linked) FA monomers in the cell wall were extracted with 4 M NaOH for 2 h at 170 °C from starch-free, alcohol-insoluble residues (Iiyama et al., 1990). Alkaline extracts were treated as described above to isolate and quantify hydroxycinnamic acids. Ether-linked FA was calculated as the difference between total and ester-linked FA concentrations of each sample (Iiyama et al., 1990).

Ester bound DFAs were extracted based on a procedure previously described (Santiago et al., 2006a) with minor modifications. One gram of ground material was extracted with 30 ml of 80% methanol. The suspension was homogenized for 30 s with a Heidolph mixer (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) before being centrifuged at 1000g for 10 min. After centrifugation, the pellet containing ester-bound phenols incorporated in the cell wall was shaken in 20 ml of 2 M NaOH under nitrogen flow and darkness for 4 h. The pH of alkali-treated samples was lowered to 2.0 with 6 N HCl. After centrifugation, the supernatant was collected and the pellet washed twice with distilled water (10 ml each). Supernatants were pooled and then extracted twice with ethyl acetate (40 ml each). Collected organic fractions were combined and reduced to dryness using a Speed Vac (Thermo Fisher Scientific Inc., MA, USA) for 5 h. The final extract was dissolved in 3 ml of HPLC-grade methanol. All of the extracts were stored at −20 °C prior to HPLC analysis. Samples were filtered through a 2 µm pore poly (tetrafluoroethylene) filter (Chromatographic Specialties, Brockville, ON, Canada) before analysis. Analyses were performed using a 2690 Waters Separations Module (Waters, Milford, MA, USA) equipped with a Waters 995 photodiode array detector and a Waters YMC ODSAM narrow-bore column (100 × 2 mm i.d., 3 µm particle size). Elution conditions with a mobile phase system of acetonitrile (solvent A) and trifluoroacetic acid.
Retention times and UV spectra were compared with freshly prepared standard solutions of 5-5-DFA, kindly provided by the group of Dr. J.T. Arnason (University of Ottawa, Ontario, Canada). The absorption UV spectra of other DFAs were compared with previously published spectra (Waldron et al., 1996) and absorbance at 325 nm was used for quantification.

Total cell wall concentration was calculated as the sum of glucose, xylose, arabinose, mannose, galactose, uronic acids, Klason lignin, total FA, and ester-linked p-CA. The DFAs were not included in the cell wall concentration calculation because some samples were lost and their minor contribution to total cell wall concentration. While most compositional analyses were done in duplicate, only single lab replication was possible for S/G ratio determination and analysis of DFAs (20% of the samples were analyzed in duplicate) because of limited sample quantity of the isolated tissues. However, the repeatability of the DFA and S/G analytical methods is high and previous experience has shown that statistically significant differences can be detected in replicated field trials with non-replicated laboratory analyses. All data were corrected to a DM basis by drying ground stalk tissue samples overnight at 100 °C.

4.3. Statistical analysis

Combined analysis of variance by location was performed for cell wall composition data, while analysis of variance by location was done for damage data (stem tunnel length) because each location was infested with different species of corn borer. All analyses were computed using the PROC GLM procedure of the SAS software package (SAS Institute, 2007). Pith and rind tissues were analyzed separately. Only field replication was considered a random factor. Comparisons of means among inbred line genotypes were made using an orthogonal contrast. To examine relationships between extent of corn borer stem tunneling and cell wall components, multiple linear regressions by location were calculated for inbred line mean data using the PROC REG procedure of SAS.

Acknowledgments

We appreciate the valuable training and technical assistance provided by Ted Jeo in sample analysis. This research was supported by the National Plan for Research and Development of Spain (Projects Cod. AGL2006-13140, AGL 2009-09611). J. Barros-Rios acknowledges a grant from the Ministry of Science and Innovation (Projects Cod. AGL2006-13140, AGL 2009-09611). R. Santiago acknowledges postdoctoral contracts: “Juan de la Cierva” partially financed by the European Social Fund, and “Isidro Parga Pondal”.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2011.01.004.

References


