Influence of Bt Transgenes on Cell Wall Lignification and Digestibility of Maize Stover for Silage

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ABSTRACT

There have been inconsistent reports that maize (*Zea mays* L.) hybrids with the *Bacillus thuringiensis* (Bt) *cry1 Ab* transgene contain more lignin than non-Bt hybrids of similar genetic background. Our objective was to evaluate the impact of the *cry1 Ab* transgene on lignin concentration (using three different assays), yield, and forage quality traits of maize. Replicated trials were conducted at four locations in Minnesota with 12 commercial hybrids (three MON810 and three Bt11 *cry1 Ab* transgene event hybrids, and respective near-isogenic controls). Whole plants and the fourth elongated, above-ground internodes were harvested at silage maturity. Samples were analyzed for crude protein, starch, neutral detergent fiber (NDF), acid detergent fiber, 24- and 96-h in vitro ruminal NDF digestibility, and lignin (acid detergent, Klason, and acetyl bromide). European corn borers (*Ostrinia nubilalis* Hübner) were not controlled and damage was limited to the non-Bt hybrids, averaging 1.5 internodes plant^{-1} with tunnels. Environment and environment × hybrid interactions affected all measures of maize performance and quality, but comparisons of non-Bt/Bt hybrid pairs, for both whole plants and internodes, found no consistent differences in yield, nutrient content, in vitro ruminal NDF digestibility, or lignin concentration. Differences in lignin concentration were infrequent, small in magnitude, and inconsistent between a few non-Bt/Bt hybrid pairs at individual locations. Two non-Bt/Bt hybrid pairs did not differ in lignin concentration at any location. Contrary to some earlier reports, presence of the *cry1 Ab* transgene did not alter lignin concentration or other forage quality traits of maize stover in commercial maize hybrids.

Commercially available transgenic maize hybrids provide crop producers with herbicide tolerance and insect resistance. Insertion of the *cry1 Ab* transgene for the insecticidal protein from *Bacillus thuringiensis* (Bt) into maize has resulted in hybrids resistant against European corn borer attack (Koziel et al., 1993) and has reduced insecticide application (Benbrook, 2003). These Bt hybrids accounted for approximately 29% of the maize acreage planted in the USA in 2003 (Benbrook, 2003). A summary of 23 livestock performance trials conducted before 2001 concluded that commercially available transgenic crops "are substantially equivalent in composition, are similar in digestibility, and have similar feeding value for livestock." (Clark and Ipharraguerre, 2001). Recent reports showed that milk production by dairy cattle and growth rate of beef steers were comparable when fed non-Bt or Bt maize silages (Barriere et al., 2001; Folmer et al., 2002; Donkin et al., 2003).

In contrast to the results of feeding studies, results of plant anatomical experiments have shown that the Bt trait was associated with increased lignification of the stems and nodes of corn plants. Masoero et al. (1999) reported that a B73 × Mo17 maize hybrid containing the *cry1 Ab* transgene had a 17% greater ADL concentration than its isogenic control; however, in vitro rumen digestibility was not altered. Also, Saxena and Stotzky (2001) reported very large increases (33–97%) in acetyl bromide lignin concentration for lower stem internodes of nine Bt maize hybrids compared with isolines. The higher acetyl bromide lignin concentrations were observed for hybrids containing two independent *cry1 Ab* transgene insertion events (MON810 and Bt11) under both growth chamber and field conditions. These reports are of concern because lignin is known to reduce the availability of cell wall polysaccharides for rumen digestion (Jung and Deetz, 1993) and the possibility of increased lignin concentrations occurring in Bt maize hybrids raises issues concerning the feeding value of silage made from these genetically modified hybrids. Several studies have reported no difference in the lignin concentration of Bt maize silage compared with appropriate genetic controls (Faust, 1999; Barriere et al., 2001; Folmer et al., 2002). However, some of these reports were from nonreplicated, field-scale plantings used in animal production trials (Faust, 1999; Folmer et al., 2002).

Although the Bt trait is utilized in maize hybrids on a significant portion of the agricultural landscape, and lignin has a major effect on forage digestibility, it is inconclusive as to whether the *cry1 Ab* transgenic trait affects lignin and overall forage quality of maize hybrids. Some of the inconsistency in reports may be partially due to limitations in experimental designs employed by researchers (e.g., Saxena and Stotzky, 2001) and failure to test the effects of environment × genotype interactions (e.g., Masoero et al., 1999). Our objective was to determine if *cry1 Ab* transgenic maize hybrids contain more lignin than nontransgenic maize when grown in diverse field environments, and whether the trait affected plant yield and other indicators of feeding value such as in vitro ruminal digestibility, fiber, starch, and crude protein concentration. Because lignin procedures are known to provide different estimates of lignin con-

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Abbreviations: ADF, acid detergent fiber; ADL, acid detergent lignin; Bt, *Bacillus thuringiensis*; DM, dry matter; NDF, neutral detergent fiber.

centration, we compared the acid detergent, Klason, and acetyl bromide methods.

MATERIALS AND METHODS

Plant Material

Twelve commercial maize hybrids adapted to Minnesota were planted in replicated trials at four locations. The hybrids included three incorporating the MON810 *cry1 Ab* transgene event (35R58, DKC44-42, DKC53-32) and their respective genetic controls (35R57, DK440, DK537); and three hybrids with the Bt11 *cry1 Ab* transgenic event (N2555Bt, N3030Bt, N45-A6) and their respective controls (N2555, N3030, N45-T5). The non-Bt/Bt hybrid pair 35R57 and 35R58 was isogenic whereas all other hybrid pairs were near-isogenic. Days-to-maturity ratings for these hybrids were 90 to 104 d. Randomized complete block designs, with four replications, were planted at two locations in southeastern Minnesota near LaCrescent and Potsdam (Houston and Wabasha Counties, respectively), and two locations in the central region of Minnesota near St. Martin and Melrose in Stearns County. The current study was planted adjacent to and managed with the 2002 University of Minnesota Corn Silage Hybrid Testing Consortium trial. Soils were a Littleton silt-loam (fine-silty, mixed, superactive, mesic, Aquic Cumulic Hapludolls) at LaCrescent, a Port Byron silt-loam (fine-silty, mixed superactive mesic, Typic Hapludolls) at Potsdam, a Dolen silt-loam (very-fine, mixed isohyperthermic, Typic Dystropepts) at Paynesville, and a Nebish sandy-loam (fine-loamy, mixed, superactive, frigid, Typic Hapludalfs) at Melrose, MN. Hybrids were seeded in 2-row plots, 10 m in length, at a 79 000 seed ha^{-1} population density with 76.2-cm row spacing. Planting dates were 6, 3, 15, and 20 May 2002 at LaCrescent, Potsdam, St. Martin, and Melrose, respectively. Fertilizers were applied according to University of Minnesota Soil Test recommendations for corn silage (Rehm et al., 1993). Urea was applied before planting in 2002 at the LaCrescent, Potsdam, and St. Martin locations (112, 157, and 134 kg N ha^{-1}, respectively). The Melrose location was treated with 175 kg N, 166 kg P, and 362 kg K ha^{-1} in liquid dairy manure in the fall of 2001. Post-emergence herbicides were applied in mid-June at all locations based on the incidence of specific weeds according to University of Minnesota recommendations (Gunsolus, 2003). The Potsdam location was also cultivated on 24 June 2002 for additional weed control. Both locations in Stearns County received a corn rootworm (*Diabrotica virgifera* LeConte) insecticide treatment. The test locations were not treated with insecticide to control European corn borer.

Weather data for the four test locations are shown in Table 1. Beginning in mid-August, field plots were monitored for grain development and whole plant moisture content. Silage maturity stage (about 650 g kg^{-1} whole plant moisture) was the target for harvest of the maize hybrids and 50% kernel milkline was used as the harvest indicator. All plots were harvested at LaCrescent and St. Martin on single days (26 August and 11 September, respectively). Study plots were harvested on two dates at the Potsdam and Melrose locations. Because kernel milkline evaluation indicated that two of the non-Bt/Bt hybrid pairs of longer maturity (35R57 and 35R58; DK537 and DK53-32) were lagging in development, the more fully developed hybrids were harvested 30 August and 13 September (Potsdam and Melrose, respectively) followed by the later maturing hybrids on 3 and 18 September.

Fifteen randomly selected maize plants of normal appearance were harvested from the interior of each plot by cutting at ground level. Three plants at the end of each row were considered border. Ten plants were chopped through a garden-style chipper–shredder, bulked and weighed, and a 1-kg subsample retained for analysis as whole plant samples. The fourth elongated, above-ground internode was excised from the stalks of the remaining five plants by cutting through the stem nodes. These internodes were bulked by plot. Before chopping, the stems of three maize stalks were split-open lengthwise and scored for how many internodes contained tunnels associated with European corn borer damage. The excised internodes were checked for corn borer damage after drying. All samples were dried at 50°C and subsequently ground to pass a 6-mm screen in a Wiley mill followed by grinding through a 1-mm screen in a cyclone-type mill. Whole maize plant sample moisture content was based on 50°C dry matter (DM). Dry matter for chemical composition data was determined by drying a subsample at 100°C for 24 h.

Maize Sample Analysis

Crude protein (CP) and starch concentrations of the whole maize plant samples were estimated using the near-infrared calibration equation developed for the University of Minnesota Corn Silage Hybrid Testing Consortium. The standard errors of prediction for crude protein and starch were 2.8 and 14.8 g kg^{-1} DM, respectively; and the explained variances were 0.76 and 0.93, respectively. All other analyses of whole plant and internode samples were done by wet-chemistry. Neutral detergent fiber (NDF), acid detergent fiber (ADF), and ADL using 72% sulfuric acid were determined sequentially (Van Soest et al., 1991). α-Amylase was included in the NDF extraction and the Ankom filter bag and extractor

Table 1. Mean air temperature and precipitation during the 2002 growing season at experimental locations near LaCrescent, Potsdam, St. Martin, and Melrose, MN.

Location	Month							
	April	May	June	July	August	September	Mean	Total
	°C							
Mean air temperature								
LaCrescent	8.6	13.6	21.7	24.8	21.6	18.6	20.4	
Potsdam	6.7	12.1	20.4	22.8	19.6	17.0	18.7	
St. Martin	5.6	10.8	20.2	22.7	19.4	16.2	18.3	
Melrose	5.6	10.9	19.9	22.6	19.6	16.2	18.3	
	mm							
Precipitation								
LaCrescent	109	33	162	95	69	94		562
Potsdam	107	37	208	128	116	51		647
St. Martin	86	52	125	130	91	169		653
Melrose	30	69	39	155	80	180		553

(Ankom Technology Corp., Fairport, NY)[1] were used for the detergent analysis. The ADL residues were combusted in a muffle furnace at 450°C for 16 h to correct for ash content.

Klason lignin concentration was determined by a two-stage sulfuric acid hydrolysis (Theander et al., 1995). Whole plant and internode samples were treated with α-amylase and amyloglucosidase in 0.1 *M* acetate buffer (pH 5) to hydrolyze starch before addition of ethanol to achieve a final concentration of 80% (v/v). After centrifugation and discarding of the supernatant, the alcohol insoluble residue was subjected to a 12 *M* sulfuric acid treatment for 1 h at 39°C to solubilize cell wall polysaccharides. The sample and sulfuric acid solution were then diluted with water to a concentration of 0.4 *M* sulfuric acid and placed in an autoclave for 1 h at 117°C to hydrolyze the cell wall polysaccharides. Insoluble Klason lignin residues were collected by filtration through a glass fiber filter in a Gooch crucible after the acid hydrolysis and corrected for ash content by combustion.

Concentration of acetyl bromide lignin was determined as outlined by Hatfield and coworkers (Hatfield et al., 1999; Fukushima and Hatfield, 2001). Starch-free, 80% (v/v) ethanol insoluble cell wall residues were prepared from maize samples as described above for the Klason lignin procedure. A freshly prepared solution of 25% acetyl bromide in glacial acetic acid (v/v) was added to the sample and incubated at 50°C for 2 h. After cooling samples, three volumes of acetic acid were added and the samples were mixed. A 0.5 mL aliquot was removed after centrifugation and combined with 2.5 mL acetic acid and 1.5 mL of 0.3 *M* NaOH. An additional 5 mL of acetic acid and 0.5 mL of 0.5 *M* hydroxylamine were then added. Absorbance of the resulting solution was determined at 280 nm. All steps of the acetyl bromide lignin method were performed under an exhaust hood and all vessels were capped. A maize stover lignin was used for the standard curve to quantify acetyl bromide lignin concentration of the experimental samples. The acidic dioxane procedure outlined by Fukushima and Hatfield (2001) was used to isolate the maize lignin. This lignin standard was isolated from a bulked sample of six whole maize plants (minus ears) harvested at full physiological maturity. Because these maize plants were grown from a sample of bulked remnant seed from a maize variety trial, the genetic identity of the maize plants used for the lignin isolation was unknown.

In vitro ruminal NDF digestibility was determined after 24- and 96-h incubations with rumen fluid using Ankom filter bags in the Daisy Oven (Jung and Lamb, 2003). Whole maize plant samples were pre-treated with porcine α-amylase (product number A-3176; Sigma, St. Louis, MO) over-night, in 0.1 *M* acetate buffer (pH 5) at 39°C, to remove starch which interferes with whole maize plant in vitro digestion using Ankom filter bags (H.G. Jung, unpublished). Empty Ankom filter bags were included in each jar of the Daisy Ovens during each in vitro rumen digestion run. Filter bags were rinsed under cold tap water for 30 min upon removal from the Daisy Oven before extraction with neutral detergent. Neutral detergent fiber digestibility was calculated based on the starting sample weight and the sample's NDF concentration, and the residual NDF remaining after the in vitro ruminal digestion corrected for rumen inoculum source NDF that entered empty bags.

[1] Mention of a proprietary product does not constitute a recommendation or warranty of the product by the USDA or the University of Minnesota, and does not imply approval to the exclusion of other suitable products.

Statistical Analysis

All laboratory analyses were done in duplicate and the data are reported on a dry matter basis. Data means were calculated for each field plot and used in the statistical analysis. The analysis of variance was conducted as a randomized complete block design with a split-plot arrangement of treatments. Location was the main plot, and hybrid and the location × hybrid interaction were the subplot parameters. Treatment means were compared by the least significant difference method when a significant *F*-test ($P < 0.05$) was detected.

RESULTS

Performance of Maize Hybrids

European corn borer damage to whole maize plants tended ($P < 0.10$) to be greater at Melrose than at Potsdam and St. Martin (21, 10, and 11% of plants had tunnels in one or more stalk internodes, respectively) with LaCrescent being intermediate (17%). Significant hybrid differences were observed for corn borer damage of whole plants across locations (data not shown). The non-Bt hybrids showed signs of damage (23 to 35% of plants sampled) whereas the Bt hybrids showed no damage, except for N2555Bt where one plant had some possible stalk tunneling. Of the non-Bt plants that had stalk tunneling, one to four internodes in the stem were affected with a mean of 1.5 internodes plant^{-1} having tunnels. For the excised internodes collected for analysis, 5 to 10% of the internodes from non-Bt hybrids had tunnels, whereas none of the internodes from the Bt hybrids had tunnels.

Yield (calculated on a per plant basis) was different ($P < 0.05$) among locations, hybrids, and for the location × hybrid interaction. Potsdam maize plants were the heaviest, LaCrescent and St. Martin were intermediate, and whole maize plants grown at Melrose were the lightest (Fig. 1). Across locations, the Bt hybrids DKC44-42 (274 g plant^{-1}) and DKC53-32 (292 g plant^{-1}) yielded more than the corresponding non-Bt near-isolines (DK440, 256 g plant^{-1} and DK537, 273 g plant^{-1}). However, when non-Bt/Bt hybrid pair contrasts were evaluated for individual test locations, only DKC53-32

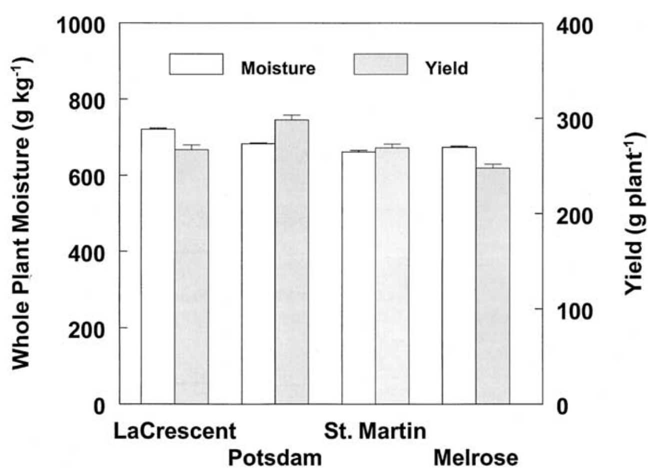

Fig. 1. Whole maize plant moisture content and yield at four test locations in Minnesota during 2002. Data were averaged across 12 maize hybrids and four plots per location.

at Potsdam had greater yields than its non-Bt counterpart. In contrast, at St. Martin only the non-Bt hybrid N3030 yielded more than its Bt near-isoline N3030Bt (280 and 232 g plant^{-1}, respectively).

Although our goal was to harvest the hybrids at 650 g kg^{-1} moisture, mean moisture content of harvested whole maize plants was somewhat higher (mean = 685 g kg^{-1}). Both location and the location × hybrid interaction were significant for whole plant moisture. Maize from LaCrescent had the highest moisture content, followed by maize from Potsdam (Fig. 1). Maize grown at St. Martin had the lowest moisture content while Melrose maize overlapped both St. Martin and Potsdam whole plants for moisture content. While the location × hybrid interaction was significant, in only one case was the non-Bt/Bt hybrid pair contrast significant. DK440 contained less moisture than its Bt near-isoline DKC44-42 at St. Martin (63.0 and 65.7%, respectively).

Lignin

Lignin concentration estimates for whole maize plants and excised internodes differed among the three lignin analysis methods (Fig. 2). For both internode and whole plant samples, the Klason lignin values were the highest and ADL results were the lowest. Acetyl bromide estimates of lignin concentration were slightly lower than Klason lignin results. Concentration estimates of lignin from all three methods were correlated with one another for internodes (Klason vs. ADL, r = 0.83; Klason vs. acetyl bromide, r = 0.55; ADL vs. acetyl bromide, r = 0.63; $P < 0.01$); but only the ADL and acetyl bromide lignin methods were correlated for whole plants (r = 0.43, $P < 0.01$). The reason for this disparity between the two types of maize samples for correlations among lignin analysis methods is unknown but may be related to the smaller range of lignin values in the whole plant samples compared with the internodes.

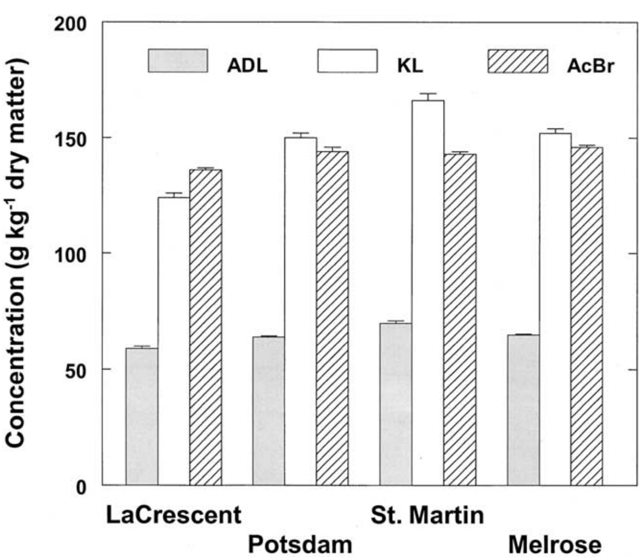

Fig. 2. Acid detergent (ADL), Klason (KL), and acetyl bromide (AcBr) lignin concentration estimates for whole maize plants at four test locations in Minnesota during 2002. Data were averaged across 12 maize hybrids and four plots per location.

Locations differed for all three lignin concentration estimates for internodes, but only ADL and acetyl bromide lignin estimates were affected by location for whole maize plants (Table 2). Acid detergent lignin and acetyl bromide lignin concentrations of whole plants from LaCrescent were greater than similar samples from Melrose and St. Martin. Potsdam whole plants had similar acetyl bromide lignin concentrations as those from LaCrescent whereas ADL values were similar to the other two locations (Fig. 2). For internodes, LaCrescent samples had lower ADL and acetyl bromide lignin concentrations than internodes from the other three locations, but ADL results for internodes from St. Martin were higher than for Potsdam and Melrose while acetyl bromide lignin concentrations did not differ among these three locations (data not shown).

Hybrids differed for the ADL estimate of lignin concentration of whole maize plants, but hybrids did not differ for Klason and acetyl bromide lignin estimates (Table 2). For the non-Bt/Bt hybrid contrasts, no differences were found for ADL concentration of whole plants (Table 3). In contrast to whole plants, all three lignin estimates indicated differences among maize hybrids for internode lignin concentration although only one non-Bt/Bt pair contrast was significant (ADL for DK440 vs. DKC44-42; Table 3).

No location × hybrid interaction for any of the three lignin estimates was significant for whole maize plants; however, the location × hybrid interaction was significant for all three lignin estimates of internodes (Table 2). The Bt hybrid DKC44-42 contained less ADL in its internodes than the DK440 near-isoline at Melrose and St. Martin (66 and 66 g kg^{-1} DM vs. 71 and 75 g kg^{-1} DM, respectively). Similarly, the Bt hybrid DKC44-42 had less Klason lignin in internodes than DK440 at Melrose (149 vs. 170 g kg^{-1} DM, respectively). However, the Bt hybrid DKC55-32 contained more internode ADL than its near-isoline DK537 at St. Martin (68 vs. 60 g kg^{-1} DM, respectively). Hybrid DKC53-32 also contained more acetyl bromide lignin than DK537 in internodes at Melrose (154 vs. 142 g kg^{-1} DM, respectively). The acetyl bromide lignin estimate for internodes of hybrid N2555Bt (147 g kg^{-1} DM) was greater than for the non-Bt hybrid N2555 (132 g kg^{-1} DM) at Potsdam. None of the remaining non-Bt/Bt hybrid pair contrasts were significant for internode samples.

Protein, Starch, and Fiber

Chemical composition of whole plants and the fourth elongated, above-ground internode was significantly affected by environment. Test location was significant for crude protein, starch, NDF, and ADF concentrations (Table 2). Whole plants grown at St. Martin had the greatest starch concentration and lowest crude protein concentration, whereas maize at LaCrescent had the opposite pattern of starch and crude protein concentrations (Fig. 3). Maize at Potsdam and Melrose was intermediate for these quality traits. Neutral detergent fiber and ADF concentrations of whole plants were lower at LaCrescent than for the other three locations. Similarly,

Table 2. Mean squares from analysis of variance for yield, chemical composition, and in vitro fiber digestibility of whole plants and the fourth elongated, above-ground internode of 12 maize hybrids grown at four locations in Minnesota during 2002 and harvested at silage maturity stage.

Trait†	Parameter					
	Replicate (R)	Location (L)	R × L	Hybrid (H)	L × H	Residual
Whole plant						
Yield	166.9	20 826.9**	479.0	5 841.9**	878.0*	518.2
Crude protein	3.0	1 976.7**	42.0	35.1**	24.7**	13.2
Starch	309.4	38 048.2**	1 518.6	2 309.8*	1 840.5	1 220.9
NDF	91.9	21 413.8**	1 132.0	1 549.4	1 850.0	1 326.4
ADF	108.2	10 443.5**	391.8	763.7	718.0	589.7
ADL	16.0	207.5**	20.3	31.7**	17.0	12.8
Klason lignin	360.3	4 052.1	2 215.6	196.4	122.4	121.7
Acetyl bromide lignin	70.0	563.7**	70.1	87.21	124.6	83.5
In vitro NDF digestibility						
24 h	3 159.1	11 523.4*	2 463.9	1 370.4	1 750.8**	944.2
96 h	62.2	12 050.3**	970.5	2 270.9**	742.0	591.2
Internode						
NDF	878.5	54 739.4**	4 793.1	12 298.0**	4 326.9**	1 653.1
ADF	106.6	22 971.0**	2 083.8	5 886.7**	2 384.7**	913.9
ADL	41.7	973.7**	94.0	172.0**	67.8**	33.9
Klason lignin	600.5	14 439.2**	1 333.5	385.3**	187.9*	121.6
Acetyl bromide lignin	357.5	1 010.3*	251.3	135.8*	115.9**	60.0
In vitro NDF digestibility						
24 h	91.1	9 037.3**	1242.7	1 652.9**	474.9	344.5
96 h	1 145.3	14 432.4**	1 364.8	21 680.0**	404.5	318.0

* Probability level of 0.05.
** Probability level of 0.01.
† NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin.

internodes from LaCrescent contained less NDF and ADF than from the other three locations (data not shown).

Maize hybrids differed in whole plant crude protein and starch, but not NDF or ADF, concentrations across locations (Table 4). Among the non-Bt/Bt hybrid pairs, a difference was only detected between N3030 and N3030Bt for crude protein concentration (76 and 73 g kg^{-1} DM, respectively). For these quality traits, only crude protein had a significant location × hybrid interaction (Table 2); however, no non-Bt/Bt hybrid pair comparisons were significant at individual test locations. In contrast to the results for whole plants, significant hybrid effects on NDF and ADF concentrations were observed for internodes across locations (Table 2), but of the non-Bt/Bt hybrid pair contrasts, only DK537 and DKC53-32 were different for NDF and ADF concentration (Table 5). Significant location × hybrid interactions occurred for NDF and ADF concentration of internodes (Table 2). The non-Bt hybrid DK537 had lower NDF concentrations at the LaCrescent and St. Martin locations (646 and 735 g kg^{-1} DM, respectively) than the Bt near-isoline DKC53-32 (710 and 815 g kg^{-1} DM, respectively). The same hybrids and locations also differed in ADF concentration (data not shown).

In Vitro Fiber Digestibility

Digestibility of maize NDF after 24-h in vitro incubations with rumen fluid accounted for approximately 60% of the digestion observed after 96 h. As expected, in vitro ruminal NDF digestibility of internodes was lower than for whole maize plant samples. Hybrid differences were observed for 96-h but not 24-h in vitro NDF digestibility of whole maize plants whereas both measures of fiber digestibility differed among hybrids for internodes (Table 4 and 5). However, none of the non-

Table 3. Mean lignin concentrations (across locations) for whole plants and the fourth elongated, above-ground internodes of maize hybrids as determined by three lignin analysis methods.

Hybrid	Bt event	Whole plant			Internode		
		Acid detergent	Klason	Acetyl bromide†	Acid detergent	Klason	Acetyl bromide
		g kg^{-1} dry matter					
35R57	None	22	107	98	67	153	142
35R58	MON810	23	108	100	66	148	143
DK440	None	22	106	102	69	156	144
DKC44-42	MON810	20	106	101	63	149	145
DK537	None	20	104	100	57	139	137
DKC53-32	MON810	19	103	103	60	143	141
N2555	None	23	108	106	64	145	139
N2555Bt	Bt11	23	116	101	66	147	143
N3030	None	22	107	102	62	150	141
N3030Bt	Bt11	21	106	99	65	155	142
N45T5	None	23	112	105	69	145	147
N45-A6	Bt11	23	110	103	66	149	146
SE		9	3	2	1	3	2
LSD 0.05		3	NS‡	NS	4	8	5

† An isolated maize stover lignin was used as the standard.
‡ NS, nonsignificant ($P > 0.05$).

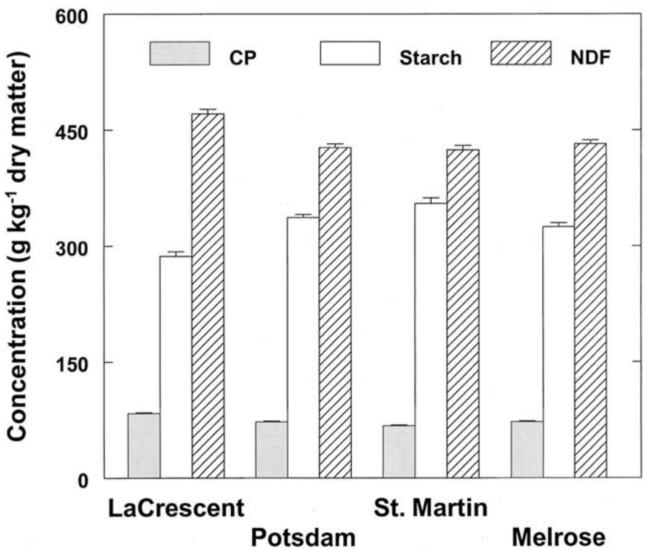

Fig. 3. Whole maize plant crude protein (CP), starch, and neutral detergent fiber (NDF) concentrations at four test locations in Minnesota during 2002. Data were averaged across 12 maize hybrids and four plots per location.

Bt/Bt hybrid pair contrasts were different for NDF digestibility. The location × hybrid interaction parameter was only significant for 24-h in vitro NDF digestibility of whole maize plants. In two non-Bt/Bt hybrid pair comparisons, the Bt hybrid (DK53-32, 405 g kg^{-1} and N3030Bt, 406 g kg^{-1}) was more digestible than the near-isogenic non-Bt hybrid (DK537, 360 g kg^{-1} and N3030, 353 g kg^{-1}) at Melrose. Whole plants of the non-Bt hybrid DK440 at LaCrescent had greater 24-h in vitro NDF digestibility than the Bt near-isoline DKC44-42 (401 and 350 g kg^{-1}, respectively). No other non-Bt/Bt hybrid contrasts were significant for fiber digestion at individual test locations.

Similar to the effect of location on all other forage quality traits, in vitro ruminal NDF digestibility differed among the locations for both whole plant and internode maize samples (Table 2). Across all hybrids, whole plant in vitro 24- and 96-h NDF digestibilities were greater at Melrose than St. Martin while LaCrescent and Potsdam whole plants were similar to Melrose for 24-h in vitro NDF digestibility but lower than Melrose for 96-h digestibility (Fig. 4). In contrast, the in vitro NDF digestibilities of internodes from LaCrescent were higher than from all other locations (data not shown).

Table 5. Maize hybrid means across experimental locations for chemical composition and in vitro ruminal fiber digestibility of the fourth elongated, above-ground internode.

Hybrid	Bt event	NDF†	ADF‡	In vitro digestibility 24 h	96 h
		— g kg^{-1} dry matter —		— g kg^{-1} —	
35R57	None	713	481	174	291
35R58	MON810	711	480	182	298
DK440	None	771	512	175	326
DKC44-42	MON810	777	511	183	333
DK537	None	724	478	193	370
DKC53-32	MON810	764	508	185	375
N2555	None	706	464	181	292
N2555Bt	Bt11	708	468	176	285
N3030	None	699	454	181	295
N3030Bt	Bt11	709	465	171	286
N45T5	None	718	489	159	267
N45-A6	Bt11	715	487	158	263
SE		10	8	5	4
LSD 0.05		28	21	13	13

† NDF, neutral detergent fiber.
‡ ADF, acid detergent fiber.

DISCUSSION

We concluded that there were no meaningful biological effects of either *cry1 Ab* transgene event (MON810 or Bt11) on lignin concentration or other quality traits of maize in commercial maize hybrids grown under limited European corn borer stress. Lignin concentration, as measured by three analytical methods, was rarely different between non-Bt/Bt hybrid pairs in the current study. In only one case (ADL concentration of internodes from DK440 and DKC44-42) was the non-Bt/Bt hybrid pair difference significant across locations, and in that instance lignin concentration of the non-Bt hybrid was greater than the Bt hybrid. The Bt hybrids were both higher and lower in lignin concentration than their non-Bt hybrid counterparts for the few cases where differ-

Table 4. Hybrid means across experimental locations for whole maize plant chemical composition and in vitro ruminal fiber digestibility.

Hybrid	Bt event	Crude protein	Starch	NDF†	ADF‡	In vitro NDF digestibility 24 h	96 h
		g kg^{-1} dry matter				g kg^{-1}	
35R57	None	75	324	424	225	363	560
35R58	MON810	76	317	437	234	352	559
DK440	None	73	347	440	231	358	567
DKC44-42	MON810	72	341	437	228	356	581
DK537	None	74	331	441	235	358	579
DKC53-32	MON810	75	328	437	233	367	581
N2555	None	77	325	431	229	352	551
N2555Bt	Bt11	76	325	436	232	349	547
N3030	None	76	328	435	227	350	567
N3030Bt	Bt11	73	333	431	227	354	561
N45T5	None	74	306	456	246	329	555
N45-A6	Bt11	74	306	459	247	357	549
SE		1	9	9	6	8	6
LSD 0.05		2	24	NS§	NS	NS	17

† NDF, neutral detergent fiber.
‡ ADF, acid detergent fiber.
§ NS, nonsignificant ($P > 0.05$).

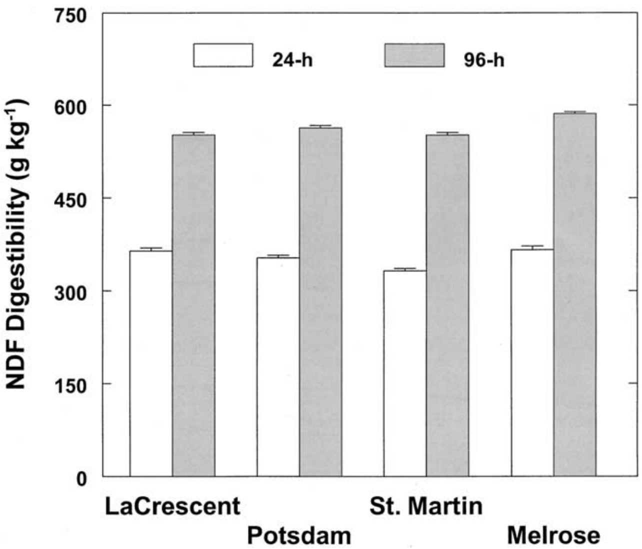

Fig. 4. Whole maize plant 24- and 96-h in vitro NDF digestibility at four test locations in Minnesota during 2002. Data were averaged across 12 maize hybrids and four plots per location.

ences were observed at individual test locations. The same pattern of few differences between non-Bt and Bt hybrid pairs was observed for all other measures of maize performance, other than European corn borer damage, and quality traits. Damage to the non-Bt hybrids from corn borer was limited in this study as indicated by the equivalent whole maize yields, starch, crude protein, and fiber digestibility for non-Bt/Bt hybrid pairs. Two hybrid pairs (35R57 and 35R58; N45T5 and N45-A6) were never different in performance or quality at any location. Because lignin is a component of plant cell walls and NDF is a reasonable estimate of cell wall concentration for mature grasses, we also evaluated lignin concentration estimates expressed on a NDF basis. The results from expression of lignin on a NDF basis resulted in the same conclusions as when lignin was expressed on a DM basis (data not shown), which was expected because few differences in NDF concentration were noted between non-Bt/Bt hybrid pairs. Whether the few location specific differences noted for lignin concentration between non-Bt/Bt hybrid pairs were due to less environmental stability of some genetic backgrounds or less than complete isogenic hybrid pairs is not known.

Our results are in contrast to the consistently and substantially higher lignin concentration of Bt hybrids compared with their non-Bt genetic controls reported by Saxena and Stotzky (2001). Using the acetyl bromide lignin method, Saxena and Stotzky (2001) found that a group of seven Bt maize hybrids grown in the field contained 72 $\pm$ 2 g lignin kg^{-1} DM, whereas the corresponding non-Bt hybrids only had 49 $\pm$ 1 g lignin kg^{-1} DM in internodes collected between the third and fourth stem nodes above the soil surface. Data for lignin concentration from internodes of these same seven hybrid pairs, plus two additional non-Bt/Bt hybrid pairs, grown in a growth chamber resulted in mean acetyl bromide lignin concentrations of 65 $\pm$ 1 g lignin kg^{-1} DM for the Bt hybrids and 38 $\pm$ 2 g lignin kg^{-1} DM for the non-Bt controls. The divergent acetyl bromide lignin concentrations between non-Bt and Bt hybrid pairs were observed for both the MON810 and Bt11 transgenic events. Masoero et al. (1999) reported a greater ADL concentration in a Bt hybrid (73 g kg^{-1} DM) than its isogenic control (63 g kg^{-1} DM), although the *cry1 Ab* transgene insertion event was not identified. The same non-Bt/Bt hybrid pairs as used in the previous reports were not evaluated in the current study because we used commercial hybrids available to producers in our region.

Because the gene for production of the Bt toxin is not part of the biosynthetic pathway to lignin, there should be no direct mechanism by which this transgene could alter lignin concentration of maize. Random insertion of the *cry1 Ab* transgene into the maize genome could by chance alter lignin biosynthesis if the transgene were integrated into a gene associated with the lignin pathway. However, increased lignin concentration was reported in maize hybrids with two independent *cry1 Ab* transgene insertion events (Saxena and Stotzky, 2001). It is highly improbable that the *cry1 Ab* transgene would be randomly inserted twice into the same regulatory pathway of lignin biosynthesis. Monolignol production for lignin biosynthesis begins with deamination of phenylalanine to cinnamic acid (Boudet, 1998); therefore, if production of the Cry1 Ab protein in transgenic maize severely depletes the phenylalanine metabolic pool it might be conceivable that precursors for lignin biosynthesis would be reduced. However, such a result should reduce lignin concentration of Bt hybrids rather than increase lignin. The normal development and protein content of the Bt maize hybrids in the current study strongly suggests that the *cry1 Ab* transgene did not significantly influence the phenylalanine pool.

A plausible mechanism whereby the *cry1 Ab* transgene might result in greater lignin concentration of maize stover is if European corn borer damage to non-Bt hybrids was sufficient to impede sucrose translocation from the stem to grain for starch synthesis because of disrupted vascular tissue. This would result in a reduction in lignin concentration of the non-Bt maize stover due to dilution by sucrose. In our study, corn borer damage was limited and did not affect grain development as indicated by similar starch concentrations of whole maize plants. Saxena and Stotzky (2001) did not report damage by European corn borer in their field experiment and such damage under growth chamber conditions would be unlikely. Masoero et al. (1999) found no difference in starch concentration of the non-Bt/Bt hybrid pair for which lignin concentration was reported to be greater in the Bt hybrid, suggesting that sucrose translocation was not impeded by corn borer damage.

Limited histological data were provided by Saxena and Stotzky (2001). The degree of sclerenchyma tissue development around vascular bundles in the rind portion of the internode shown in a Bt hybrid internode cross-section was much greater than for a non-Bt internode cross-section. The auto-fluorescence signal indicating the presence of phenolic compounds was also greater for the Bt maize cross-section and toluidine blue staining was greater for the Bt hybrid cross-section. The authors did not indicate the specific hybrids from which the illustrated cross-sections were made. While the toluidine blue stain has been useful for visualizing those maize tissues which are susceptible to ruminal digestion (F.M. Engels, pers. com.), this stain is not commonly regarded as an indicator of degree of lignification. No histological data were collected for our study.

Concentrations of acetyl bromide lignin for internodes in the current study were substantially greater (126–154 g kg^{-1} DM) than reported by Saxena and Stotzky (2001). A portion of the difference for acetyl bromide lignin concentrations between the two studies may have resulted from the use of an isolated maize lignin as the calibration standard in the current study and use of a commercial alkali lignin by the preceding study (see discussion below). We sampled the fourth elongated above-ground internode which was one or two internodes higher on the stem than the internode sampled by Saxena and Stotzky (2001). Use of an internode higher on the stem would tend to result in lower rather than higher lignin concentrations (Morrison et al., 1998). We chose to evaluate this particular internode because of previous experience with the development of this internode position in three other non-Bt maize hybrids (Jung, 2003). Data for the fourth internode of these other maize hybrids suggest that the maize sampled by Saxena and Stotzky (2001) after 90 to 97 d of growth was unusually immature. Klason lignin concentrations similar to the acetyl bromide lignin concentrations reported by Saxena and Stotzky (2001) were observed by 63 d after planting in another study (H.G. Jung, unpublished). Because Klason and acetyl bromide lignin concentrations of internodes were of similar magnitude and significantly correlated in the current study, comparison of Klason and acetyl bromide lignin results across studies may be valid.

Differential availability of nutrients to support normal plant growth may account for some of the differences in lignin concentration noted between studies. In our study, maize plantings were fertilized to levels recommended for commercial maize production. In contrast, Saxena and Stotzky (2001) did not fertilize which probably resulted in stunted growth due to deficiency of N and possibly other nutrients. Young wheat (*Triticum aestivum* L.) plants contained higher acetyl bromide lignin concentrations when grown with low levels of N fertilization (Brown et al., 1984). Leaves of several *Poa* species grown under low N supply exhibited a modified lignin composition (reduction in the syringyl-to-guaiacyl monolignol ratio) compared with these grasses grown with high levels of N availability (Van Arendonk et al., 1997).

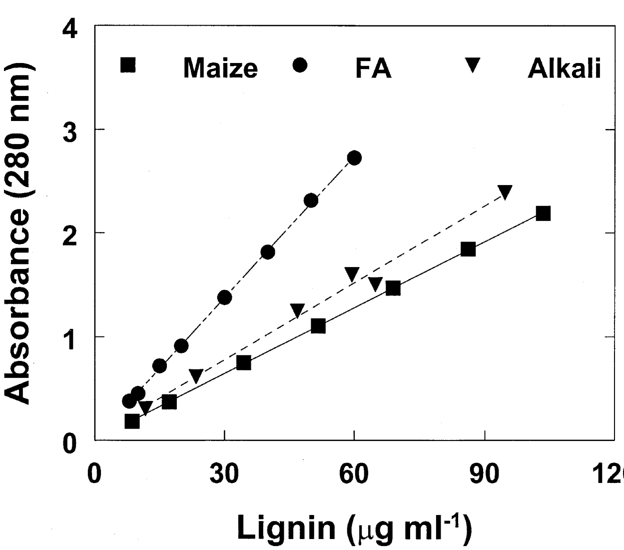

Fig. 5. Absorbance at 280 nm for an isolated maize stover lignin, alkali lignin (catalog number 37 095-9; Aldrich, Milwaukee, WI), and ferulic acid (FA) in the acetyl bromide lignin method using the procedure of Hatfield et al. (1999).

Unlike the ADL and Klason lignin methods, which are gravimetric, the acetyl bromide lignin method is colorimetric and requires use of a lignin standard for quantification. Saxena and Stotzky (2001) used a commercial alkali lignin as their standard (G. Stotzky, pers. comm.). We utilized a lignin preparation that was isolated from maize stover. A comparison of these two lignin standards indicated that the absorbance at 280 nm in the acetyl bromide method associated with a known amount of maize or alkali lignin was similar but deviated increasingly at higher lignin concentrations (Fig. 5). The impact of this differential response of the maize and alkali lignins in the acetyl bromide method was that lignin concentration estimates of internodes from the current study were approximately 28% lower when alkali lignin was used compared with maize lignin (data not shown). However, these differences in estimated lignin concentration using the alkali lignin standard would not alter the conclusions concerning the impact of the *cry1 Ab* transgene in Bt hybrids.

It is critical that the actual lignin content of any standard used in the acetyl bromide lignin method be determined. Two lots of commercial alkali lignin we tested contained vastly different Klason lignin concentrations (671 and 901 g lignin kg^{-1} DM). Alternatively, a standard absorbance value for lignin in the acetyl bromide method as determined in previous work can be utilized (Iiyama and Wallis, 1990; Morrison, 1972). Simple phenolics such as ferulic acid are not appropriate standards for the acetyl bromide method because of their very high extinction coefficients relative to lignins (Morrison and Stewart, 1995), a result that was verified in the current study (Fig. 5).

The observation that Klason lignin concentrations were much greater than ADL concentrations for the same maize samples was in agreement with previous reports (Goto et al., 1992; Hatfield et al., 1994). Lowry et al. (1994) concluded that acid-soluble lignin is lost in the ADL procedure and that this is an especially large problem with grass species. It has been definitively demonstrated that Klason lignin is a more accurate quantitative measure of lignin concentration in forages than the ADL method (Jung et al., 1999). The slightly lower results for acetyl bromide lignin estimates compared with Klason lignin values in the current study corroborate the results of Iiyama and Wallis (1988, 1990). The lack of agreement among lignin procedures highlights the empirical nature of all quantitative lignin analysis methods.

CONCLUSIONS

Contrary to previous reports that maize hybrids expressing a transgene for production of the *B. thuringiensis* Cry1 Ab protein have significantly greater concentrations of lignin than normal maize hybrids, a multi-environment field study in Minnesota of six commercial non-Bt/Bt near-isogenic hybrid pairs did not detect consistent differences in lignin concentration of lower stem internodes or whole plants. The lack of an effect of the *cry1 Ab* transgene on lignin concentration was observed with three different lignin analysis methods (acid detergent, Klason, and acetyl bromide lignins) and was true for two independent *cry1 Ab* insertion events (MON810 and Bt11). Whole plant maize yield, concentration of crude protein, starch, and fiber, and in vitro ruminal fiber digestibility were also not affected by the Bt transgene. All forage quality and yield data support the conclusion that presence of the *cry1 Ab* transgene does not alter the chemical composition or ruminal fiber digestibility of maize. Producers can use maize hybrids containing the Bt trait to reduce the costs and environmental consequences of pesticide application for European corn borer control without affecting forage quality or yield.

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