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Cell Wall Composition of Smooth Bromegrass Plants Selected for Divergent Fiber Concentration

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Neutral detergent fiber (NDF) is considered the single best laboratory predictor of voluntary intake by ruminant livestock, creating interest in using NDF as a selection criterion in forage breeding programs. Because genetic reductions in NDF lead to increases in dry matter digestibility but not to changes in digestibility of the NDF fraction, we postulated that low-NDF plants do not have altered compositions of their cell walls. We tested this hypothesis using clones of smooth bromegrass (*Bromus inermis* Leyss.) with divergent NDF concentrations. High-NDF and low-NDF plants did not differ in cell wall concentrations or in the concentrations of any cell wall component (fucose, arabinose, rhamnose, galactose, glucose, xylose, mannose, uronic acids, and lignin). Instead, low-NDF plants had a cell wall that was more susceptible to solubilization in neutral detergent solution, suggesting that their cell walls were less well-developed as compared to high-NDF plants. NDF should not be used as a substitute for cell wall concentration in forage plants.

KEYWORDS: Neutral sugars; lignin; NDF; *Bromus inermis*

INTRODUCTION

Voluntary intake may be more important than digestibility in limiting animal performance (*1*), accounting for up to 70% of the variation in animal production (*2*). The neutral detergent fiber (NDF) concentration is the most reliable laboratory predictor of voluntary intake potential. Physical distension of the rumen is the major factor limiting voluntary intake of high-producing ruminants on high-forage diets (*3*). For most high-forage diets, intake of fibrous bulk generally causes rumen fill and satiation before the ruminant has maximized its caloric intake, resulting in a reduced plane of nutrition (*4*).

The concentration of NDF can be decreased by selection and breeding and creating new cultivars that possess environmentally stable and repeatable reductions in NDF (*5*−*7*). Selection for reduced NDF concentration increases dry matter digestibility, due to the dilution effect of reduced cell walls relative to cell contents (*5*), which are assumed to be completely digestible (*4*). Conversely, selection for reduced NDF does not result in changes to the digestibility of the NDF fraction (*5*). Increases in digestibility of the NDF fraction generally result from decreases in lignin concentration or decreases in the frequency of etherified ferulates, the latter a measure of covalent bonding between lignin and arabinoxylans (*8*, *9*).

On the basis of these results, we hypothesize that selection for reduced NDF has no substantial effect on the composition of the cell wall. The primary objective of this study was to compare the cell wall composition of smooth bromegrass clones that are characterized by high or low NDF concentrations. The secondary objective was to determine the relationship between the NDF and the cell wall concentration. Many researchers have a tendency to equate NDF to cell wall concentration, and our goal was to determine if this assumption is valid for a small sample of divergent NDF smooth bromegrass clones.

MATERIALS AND METHODS

A total of 320 smooth bromegrass clones from the WB-RP_1 population were evaluated for NDF concentration over two replicates, two harvest dates, and 2 years (*5*). This population was a composite, created by intercrossing 129 clones selected from nine smooth bromegrass cultivars. Eight clones were selected from this study—four with high NDF and four with low NDF. Mean NDF values of high vs low NDF groups were 431 ± 4 vs 352 ± 3 for leaf blades and sheaths at a vegetative growth stage and 647 ± 4 vs 515 ± 10 for whole plants at the heading growth stage. The clones were vegetatively increased in the field in 1999 and 2000.

The eight clones were transplanted into a new experiment in July 2001 at Arlington, WI. The soil type was a Plano silt loam (fine silty, mixed, mesic Typic Argiudoll). Plants were spaced 1.2 m apart in a randomized complete block design with two replicates. Four ramets of each clone were established within each replicate in a split-plot randomization with four harvest dates as the whole-plot factor and eight clones as the subplot factor. Plants were fertilized with nitrogen fertilizer and clipped in 2001 to improve their establishment. Weeds were controlled with pre-emergence herbicide and by hand (*10*).

Plants were fertilized with 90 kg N ha^{-1} in early spring 2003. Plants were harvested on one of four harvest dates in May and June 2003. The four harvest dates and growth stages (*11*) were May 17 (vegetative, two collared leaves, V2), May 27 (boot, R0), June 6 (heading, R3), and June 18 (anthesis, R4). Plant samples were cut at a height of 9

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cm, separated into their components, placed in paper bags, dried at 60 °C, ground through a 1 mm screen of a Wiley type mill, and reground through a 1 mm screen of a cyclone mill. Leaf blades were removed from the stem by cutting at the collar, leaf sheaths were removed from the stem by peeling and cutting at the node, and panicles were removed from the stem by cutting at the lowest panicle node.

Cell walls were isolated using a modified Uppsala procedure (*12*). Briefly, samples were weighed into dry preweighed 50 mL centrifuge tubes and extracted with a series of solvents and treatments to remove cytoplasmic materials and starch. The extraction sequence consisted of cold acetate buffer (50 mM, pH 5.0, 5 °C), starch removal (50 mM TRIS-acetate buffer, pH 6.5, 10 U amylase and 10 U amyloglucosidase, 55 °C), 80% ethanol, and acetone. For each solvent or treatment, sample tubes were centrifuged at 3100*g* for 15 min and the supernatant was removed from the pellet. After the amylase/amyloglucosidase treatment, samples were made 80% ethanol by adding 100% ethanol to precipitate solubilized cell wall carbohydrates. For all ethanol extractions, samples were suspended in ethanol and sonicated for 15 min before centrifugation to remove solubilized materials. After the final acetone wash, insoluble residues were allowed to air-dry to remove all acetone traces before oven drying at 55 °C to obtain a recovered weight. The final dried alcohol insoluble residue constituted the cell wall preparation (total cell wall = TCW).

Subsamples (100 mg) of the cell wall preparation were hydrolyzed with a two-step sulfuric acid treatment (*13*) as modified in our laboratory (*14*). Total uronosyls were determined on the final hydrolyzate using galacturonic acid as a standard. Previous work has shown that either galacturonic or glucuronic acid serves as an adequate standard, with relatively little bias, in such a mixed carbohydrate system (*15*). Neutral sugars were determined using a Dionex BioLC carbohydrate system using 2-deoxyglucose as an internal standard (*16*). Lignin values were determined using the acetyl bromide method with extracted bromegrass lignin as a standard (*17*). Samples were analyzed for NDF (*18*), omitting the sodium sulfite and α-amylase steps.

Data were analyzed by analysis of variance, using contrasts to test the linear and quadratic effects of harvest dates, the differences between high NDF and low NDF group means, and the interactions between these effects (*19*). Blocks and harvest dates were treated as random effects, and clones were treated as a fixed effect. Repeatability of clone means was computed as $R = s_C^2/(s_C^2 + s_e^2/r)$, where s_C^2 and s_e^2 = the variance components for clones and error, respectively, and r = number of replicates. Correlations between cell wall components within plant parts were based on means of the eight clones. Concordance of clone rankings across the three plant parts and across the seven neutral sugars was tested by Kendall's τ, for which a value of 1 indicates complete agreement in ranking and 0 indicates no agreement in ranking (*20*). A significance level of $P < 0.05$ was chosen as the maximum tolerable type I error rate for presentation of results.

RESULTS

All changes in cell wall components of smooth bromegrass plant parts were consistent across clones, as indicated by lack of clone × maturity stage interactions (data not shown). There were no differences among clones in the rate at which any cell wall component changed with advancing maturity. Therefore, all results pertaining to variation among smooth bromegrass clones were presented as means over maturity stages, and all results pertaining to maturity stages were presented as means over clones.

There was relatively little variation among clones for plant part composition, none of which was related to differences between high NDF and low NDF groups. All plants were composed of 100% leaf blades at the vegetative growth stage (day 17 in **Figure 1**). Plant part compositions for the remaining three growth stages were 51% blade, 26% sheath, and 23% stem at jointing (day 27); 32% blade, 23% sheath, 26% stem, and 18% panicle at heading (day 37); and 19% blade, 14% sheath, 40% stem, and 28% panicle at anthesis (day 49).

All cell wall components changed in one or more plant parts as plants matured, except for total uronosyls. Most of these responses were linear, with only one notable exception, the mannose concentration of leaf blades (**Figure 1**). In leaf blades, fucose, rhamnose, galactose, xylose, mannose, and lignin increased, while glucose decreased with advancing maturity. In leaf sheaths, fucose and lignin increased, while glucose, mannose, and total neutral sugars decreased with advancing maturity. In stems, glucose and lignin increased, while fucose, arabinose, rhamnose, galactose, xylose, and mannose decreased with advancing maturity.

All carbohydrate fractions of the cell wall were characterized by differential rates of change with advancing maturity, including increases in some plant parts and decreases in other plant parts (**Figure 1**). These differential responses most likely reflect differential development of primary cell walls, with higher concentrations of pectins vs. branched xylans, and secondary cell walls, with higher concentrations of cellulose and more linear xylans. Lignin was the only cell wall component that increased consistently with advancing maturity in all three plant parts, with twice as great a rate of increase in leaf sheaths as compared to leaf blades and more than three times as great a rate of increase in stems as compared to leaf sheaths.

The concentration of NDF increased with advancing maturity in all three plant parts, but the concentration of TCW increased with advancing maturity only in stems (**Figure 2**). The rate of increase in NDF was twice as great in leaf sheaths as in leaf blades, while the rate of increase in TCW of stems was over three times as great as for NDF of stems. As a result of these changes or lack thereof, the difference between TCW and NDF (soluble cell wall = SCW) decreased in leaf blades and leaf sheaths (-0.73 and -3.03 mg g^{-1} $days^{-1}$, respectively; $P < 0.05$ and 0.01, respectively) and increased in stems (0.96 mg g^{-1} $days^{-1}$; $P < 0.01$) with advancing maturity.

Smooth bromegrass clone means differed in all cell wall components of leaf blades, except for uronosyls (**Table 1**). Excluding uronosyls, repeatabilities for cell wall components of leaf blades were moderate to high, ranging from 0.59 to 0.94. Differences among clone means were large for most variables measured on leaf blades, typically exceeding 3−4 times the LSD value. In contrast, clone means differed for only five or three cell wall components of leaf sheaths and stems, respectively. Repeatabilities for cell wall components of leaf sheaths and stems were low to moderate, rarely exceeding 0.70. Generally, the range among clone means was greatest for leaf blades and least for stems.

Differences among clones selected for high NDF vs low NDF were repeatable with respect to the original selection experiment, with high NDF clones exceeding low NDF clones in NDF by 10.1, 5.6, and 6.7%, respectively (**Table 2**). High NDF and low NDF clone groups did not differ for any other variable, except SCW, which was 21−84% greater in low NDF clones as compared to high NDF clones. Selection for low NDF did not affect total cell wall concentration or composition but did result in considerably greater disagreement between NDF and TCW values, i.e., increases in SCW.

The correlation between NDF and TCW ranged from -0.68 for stems to 0.26 for leaf sheaths (data not shown). The concentration of NDF was not correlated with any of the cell wall components, either within or averaged across plant parts. The lignin concentration was not correlated with any of the individual neutral sugar concentrations within or averaged across plant parts. All of the individual neutral sugars tended to be positively correlated with each other, but the individual cor-

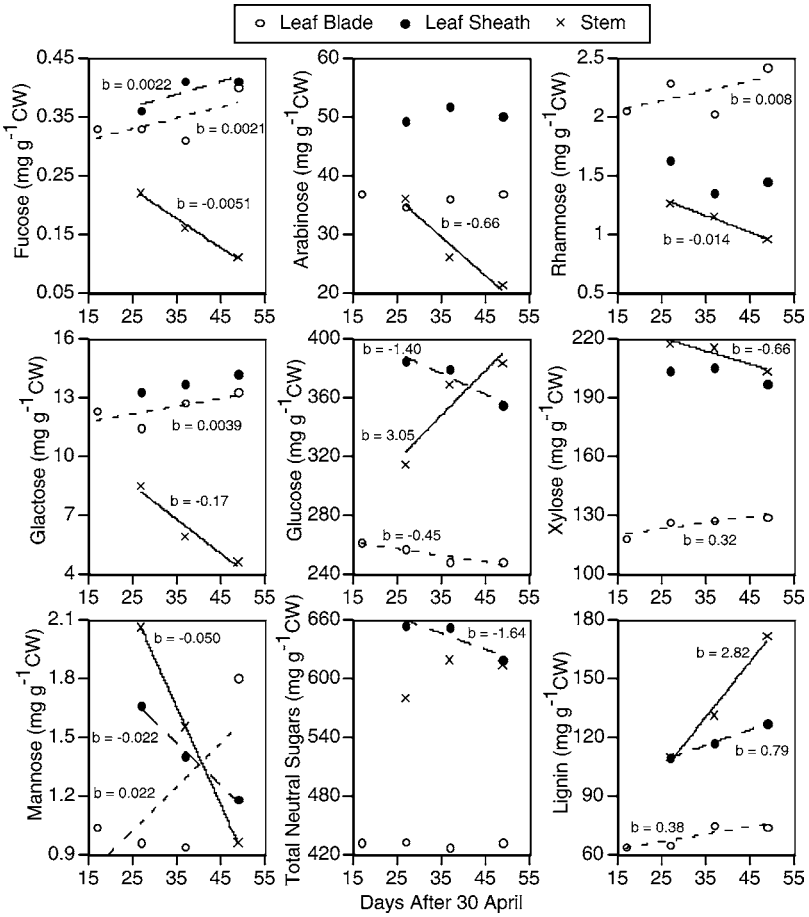

Figure 1. Changes in cell wall components of smooth bromegrass leaf blades, leaf sheaths, and stems as a function of advancing maturity. Linear regression coefficients (*b*) are shown only for those regressions that were significant at $P < 0.05$. Note that the scale is not constant among the components of the figure. Units of measurement on regression coefficients are mg g^{-1} CW $days^{-1}$.

relations were not sufficiently large to be individually significant. However, the overall positive correlations among the seven neutral sugars resulted in significant values of Kendall's τ for leaf blades ($\tau = 0.76$; $P = 0.03$) and stems ($\tau = 0.86$; $P = 0.01$) across all seven neutral sugars. Glucose and xylose were the only neutral sugars correlated with total neutral sugars ($r = 0.74$ and 0.71, respectively; $P < 0.05$), but xylose was the only neutral sugar correlated with TCW ($r = 0.85$; $P < 0.01$). There was a general pattern of positive correspondence across plant parts for each individual variable, particularly for the concentrations of individual neutral sugars, NDF, and TCW (**Table 3**).

DISCUSSION

Eight smooth bromegrass clones were selected for divergent NDF concentration from within a single population. Differences among these clones were highly repeatable across replicates, maturity stages, and plant parts. These results confirm that the NDF trait is highly repeatable and that genetic variation for NDF is relatively insensitive to external factors, such as the environment in which the plants are grown and the stage of maturity at which they are harvested (*5*).

This study demonstrated genetic variation of a different sort for the first time in this species. Genetic variation was observed for all cell wall monosaccharides, for total neutral sugars, and for cell wall concentration. This variation was completely unrelated to the selection pressure placed on these plants for NDF concentration. Large differences in NDF, sufficient to have nutritional implications for ruminant livestock (*1*, *5*), were not associated with any changes to cell wall concentration or composition.

The large amount of genetic variation for cell wall composition indicated that there is considerable plasticity in the monosaccharide composition of smooth bromegrass cell walls. This characteristic has also been observed in several species of *Panicum* (*21*, *22*). Strong negative correlations between glucose and xylose (*21*, *22*) suggest genetic variability for the cellulose:hemicellulose ratio. Numerous heteropolymers exist within cell walls of angiosperms (*23*), and there may be some evolutionary advantage to have some flexibility in both monosaccharide and polysaccharide composition. Cell wall synthesis is a highly dynamic process regulated by long-term turnover, alteration, and reorganization of specific polymers during plant development (*24*).

This genetic variation in monosaccharide composition may also be an indication of genetic variation for anatomical organization or the relative proportions of different types of cells. Smooth bromegrass, particularly leaf blades, contains a large amount of genetic variability in the relative composition of various cell types (*25*) that are known to possess large differences in monosaccharide composition (*26*, *27*). The greater degree of genetic variation for cell wall components of leaf blades, as compared to leaf sheaths and stems, mirrors results for anatomical traits of smooth bromegrass (*25*). In the latter study, variation in several types of leaf blade cells explained much of the genetic variability in dry matter digestibility, but there was little variability for anatomical traits of stems.

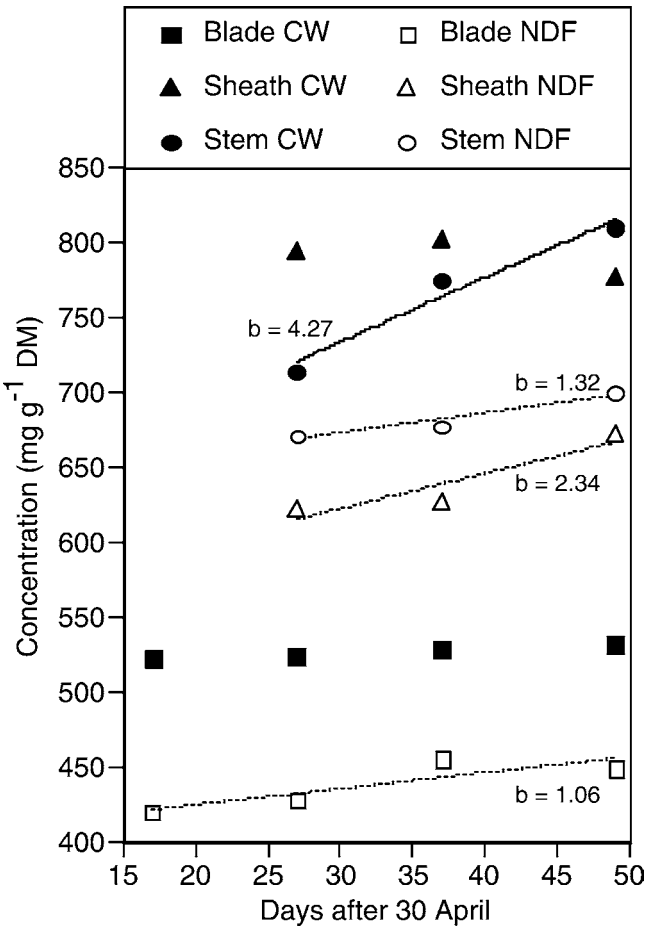

Figure 2. Changes in cell wall or NDF concentration in smooth bromegrass plant parts as a function of advancing maturity. Linear regression coefficients (*b*) are shown only for those regressions that were significant at $P < 0.05$. Units of measurement on regression coefficients are mg g^{-1} DM $days^{-1}$.

Numerous studies of grasses have demonstrated circumstantial evidence that increased dry matter digestibility, arising from reduced NDF and/or reduced lignin concentration, is often associated with wider leaf blades (*28*−*31*). High digestibility of smooth bromegrass was associated with a reduced frequency and total cross-sectional area of vascular bundles per leaf cross-section and per unit of leaf width (*25*, *32*). Although there has been limited research demonstrating genetic variation for anatomical composition of grasses, it seems likely that anatomical differences could account for much of the genetic variation observed in monosaccharide composition of these species. Various types of cells, such as epidermis, mesophyll, schlerenchyma, xylem, and phloem, have different functions and therefore can be expected to have different monosaccharide and polysaccharide compositions. For example, because arabinoxylans are responsible for cross-linking with ferulates, linking these polysaccharides to lignin (*33*), we expect to find highly lignified cells enriched with arabinoxylans (*26*, *27*).

Genetic selection for low NDF concentration appears to have acted on a detergent soluble fraction of the cell wall, increasing the soluble portion of the cell wall without changing the cell wall concentration (**Figure 3**). The supernatant following neutral detergent treatment contains a soluble fraction of cell wall polysaccharides that may include pectins and hemicelluloses, the most soluble and nutritionally available components of the cell wall (*23*).

Selection for low NDF concentration is a mechanism for increasing in vitro dry matter digestibility but not digestibility of the NDF fraction (*5*). This earlier study led to the hypothesis that increased dry matter digestibility resulted largely as a dilution effect in which low NDF plants had relatively less indigestible cell wall (equated to NDF residue). The current study has identified a second factor partly responsible for this increase in IVDMD, an increase in the highly soluble and nutritionally available portion of the cell wall. Genetic variation in NDF concentration arises, not from inherent variability in cell wall monosaccharides or lignin, but from genetic variability in the solubility of the cell wall, a characteristic that appears to be more related to cell wall structure than cell wall composition.

Treatment of grass cell walls with a variety of solvents results in isolation of lignin−carbohydrate complexes that are constitutively similar, both qualitatively and quantitatively, to intact cell walls (*34*, *35*). These results led Morrison to postulate that these lignin−carbohydrate complexes are functional units of the cell wall, remaining intact regardless of the solubilization or enzymatic degradation treatment (*35*). Lignin acts as a physical barrier, limiting access of microbial enzymes to their substrate polysaccharides (*36*, *37*). Covalent ester linkages between arabinoxylans and ferulates and ether linkages between ferulates and lignin are nonlabile in neutral detergent solution, largely leaving the functional lignin−carbohydrate complex intact in the neutral detergent residue. Solubilization of neutral sugars appears to occur in direct proportion to their presence in the cell wall. That this characteristic is clearly under genetic control, is highly heritable, and responds easily to genetic selection (*5*) supports Morrison's contention that lignin−carbohydrate complexes are the functional unit of grass cell walls. These complexes are highly organized, specialized across different cell types, and probably have variable functions within the plant. The tendency of these lignin−carbohydrate complexes to remain intact during solubilization or enzymatic hydrolysis points to their dual and dichotomous role in ruminant nutrition, as a source of fiber to maintain rumen function (*1*−*4*) and as an antiherbivory mechanism, preventing overconsumption of herbaceous plants by grazing livestock (*4*, *10*).

Despite the dynamic nature of cell wall synthesis, we observed a general pattern of positive correlation among the seven neutral sugars found in smooth bromegrass cell walls and that individual neutral sugars tend to be positively correlated in concentration across plant parts. These correlation patterns indicate that differential neutral−sugar composition of smooth bromegrass cell walls cannot explain the differential solubility of cell walls in low vs high NDF plants. Rather, it appears likely that low NDF plants have a cell wall that is not as highly developed as the cell wall in high NDF plants. The important components of the cell wall are all present in low NDF plants, but their greater solubility suggests a reduced state of structural development due, more likely, to delayed structural development of covalent linkages with lignin. A high, positive, genetic correlation between NDF and etherified ferulates of several grass species indicates that low NDF plants have reduced ferulate cross-linking between lignin and arabinoxylans, an indicator of cell wall ontogeny (*9*). Because this species is highly photoperiod sensitive, all plants proceed to reproductive maturity at the same rate, indicating that there is genetic variability for a rate of cell wall development that is independent of the rate of reproductive maturity. Retarded development of the cell wall at a given stage of reproductive maturity, particularly in stem tissue, may be responsible for the drastic reductions in fitness observed in smooth bromegrass populations selected for low

Table 1. Minimum, Maximum, LSD, *P* Values, and Repeatability for Cell Wall Components Measured on Eight Smooth Bromegrass Clones (Means over Two Replicates and Three or Four Harvest Dates)

plant part/ statistic	cell wall component[a]												
	Fuc	Ara	Rha	Gal	Glu	Xyl	Man	TNS	TU	lignin	TCW[b]	NDF[b]	SCW[b]
						leaf blades							
minimum	0.3	33.4	2.0	11.3	234.6	115.1	1.0	399.9	25.3	58.5	493.6	409.7	32.2
maximum	0.4	40.2	2.6	14.1	266.4	136.1	1.7	459.3	28.8	81.9	570.0	463.3	148.5
LSD (0.05)	0.1	2.0	0.3	0.8	11.7	6.6	0.3	18.8	2.7	7.0	20.4	25.9	30.5
P value	<0.01	<0.01	0.04	<0.01	<0.01	<0.01	<0.01	<0.01	0.17	0.01	<0.01	<0.01	<0.01
repeatability	0.75	0.94	0.59	0.91	0.83	0.82	0.76	0.84	0.38	0.69	0.90	0.80	0.91
						leaf sheaths							
minimum	0.4	46.7	1.3	12.2	359.9	189.6	1.3	626.9	29.5	103.7	777.1	631.1	118.4
maximum	0.5	54.3	1.6	15.5	387.8	213.0	1.7	655.2	35.5	125.1	807.0	681.8	166.7
LSD (0.05)	0.1	5.0	0.3	1.7	8.9	10.8	0.4	18.3	4.1	12.8	20.6	32.5	26.1
P value	0.07	0.23	0.66	0.03	<0.01	0.04	0.50	0.11	0.08	0.09	0.43	0.01	0.01
repeatability	0.55	0.32	0.00	0.64	0.81	0.62	0.00	0.48	0.54	0.51	0.05	0.69	0.73
						stems							
minimum	0.1	25.8	1.0	5.3	342.3	199.4	1.0	578.3	23.9	134.5	743.2	630.3	36.7
maximum	0.2	29.9	1.2	7.2	362.8	220.4	2.0	610.1	27.2	140.5	775.3	706.5	140.4
LSD (0.05)	0.1	2.1	0.2	0.6	13.6	10.0	0.3	18.1	3.5	12.8	20.8	33.5	40.3
P value	0.57	0.68	0.41	0.03	0.97	0.82	<0.01	0.98	0.52	0.94	0.98	<0.01	0.09
repeatability	0.00	0.00	0.06	0.65	0.00	0.00	0.87	0.00	0.00	0.00	0.00	0.85	0.51

[a] Fuc, fucose; Ara, arabinose; Rha, rhamnose; Gal, galactose; Glu, glucose; Xyl, xylose; Man, mannose; TNS, total neutral sugars; and TU, total uronosyls (mg g^{-1} CW). [b] SCW = TCW − NDF (mg g^{-1} DM).

Table 2. TCW, NDF, and SCW Concentrations of Smooth Bromegrass Plants Selected for Low or High NDF Concentrations (mg g^{-1} DM)[a]

plant part	group	TCW	NDF	SCW
leaf blade	low NDF	527.4	417.5	109.9
leaf blade	high NDF	526.9	459.6**	67.4*
leaf sheath	low NDF	789.3	623.2	149.3
leaf sheath	high NDF	794.1	657.9*	122.8*
stem	low NDF	772.2	661.5	127.6
stem	high NDF	761.1	705.6**	68.8**

[a] *,**High NDF vs low NDF group means significantly different at $P < 0.05$ or 0.01.

Table 3. Spearman Rank Correlation Coefficients between Plant Parts and Kendall's Coefficient of Concordance (τ) across Leaf Blades, Leaf Sheaths, and Stems for Cell Wall Components Measured on Eight Smooth Bromegrass Clones[a]

cell wall component	leaf blade vs leaf sheath	leaf blade vs stem	leaf sheath vs stem	Kendall's τ
fucose	0.72*	0.39	0.60	0.67*
arabinose	0.38	0.81*	0.71*	0.76*
rhamnose	–0.30	0.86**	0.00	0.45
galactose	0.74*	0.81*	0.74*	0.84*
glucose	–0.17	0.57	0.21	0.47
xylose	0.48	0.52	0.62	0.69*
mannose	0.39	–0.44	0.27	0.38
total neutral sugars	0.10	0.64	0.43	0.59
total uronosyls	–0.12	–0.05	–0.43	0.20
lignin	0.45	–0.83**	–0.14	0.22
TCW	0.33	0.14	–0.10	0.42
NDF	0.86**	0.90**	0.76*	0.89**
SCW	0.12	0.76*	0.62	0.67*

[a] *,**Significantly different from zero at $P < 0.05$ or 0.01.

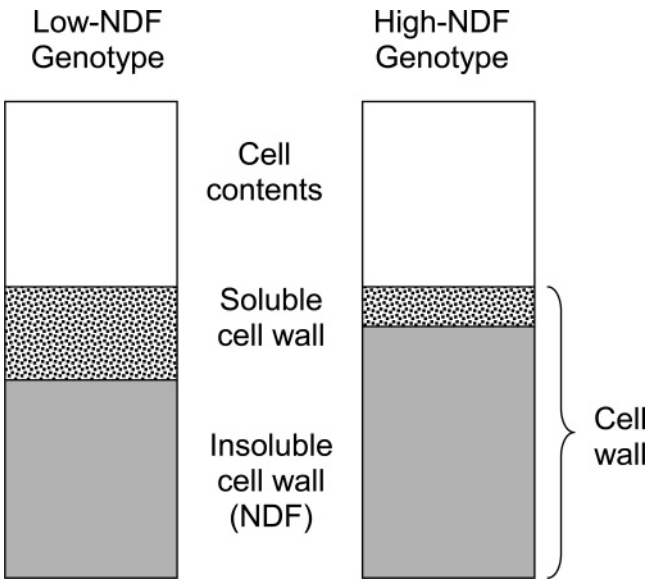

Figure 3. Diagrammatic representation of the cell contents and cell wall of a low NDF and a high NDF genotype, illustrating the difference in NDF concentration and the hypothesized contribution of soluble and insoluble portions of the cell wall.

NDF concentration (*5*, *38*). Because SCW of stem tissue continued to increase with advancing maturity up to the anthesis growth stage, there does not appear to be any potential for low NDF genotypes to "catch up" with high NDF genotypes in development of their stem cell walls. However, for leaf blades and sheaths, the decline in SCW with advancing maturity suggests a possible convergence between low NDF and high NDF plants sometime after anthesis.

Finally, numerous researchers have used NDF as an approximate measure of cell wall concentration, largely on the basis of the assumption that any difference or bias of cell wall concentration estimated from NDF is constant across genotypes, particularly when they are measured at a constant stage of maturity (*8*). Our research demonstrates that this assumption is invalid for smooth bromegrass, calling into question its validity for all other forage species as well. A large amount of forage and livestock nutrition-related research is dependent on an ability to express cell wall components on a cell wall basis (*9*), suggesting there is a need for a reliable, rapid, and repeatable method for estimating cell wall concentrations.

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