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Fructan Content and Synthesis in Leaf Tissues of *Festuca arundinacea*[1]

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ABSTRACT

The concentration of fructan in tall fescue (*Festuca arundinacea* Schreb.) changes during growth and in response to environment. The objective of this research was to compare the fructan concentration and fructosyl-transferase activity of tall fescue leaf tissues. Expanding leaves, inner and outer sheaths, and expanded blades of greenhouse-grown tall fescue plants were assayed for fructan concentration and fructosyl-transferase activity. Leaf sheaths contained significantly more nonstructural carbohydrate than did the expanded blade. Sheaths also contained a greater percentage of fructan with more than six sugar residues (long chain fructan), than either the expanded blade or expanding leaf. Expanding leaves contained a greater concentration of fructose and oligosaccharides than did sheath or blade tissues. Expanding leaves also had the greatest fructosyl-transferase activity measured either as radiolabel incorporated into fructans in tissue pieces or protein extracts. Activity of fructosyl-transferase was greater in expanding leaf tissue than in sheath tissues.

Tall fescue (*Festuca arundinacea* Schreb.) is a cool-season grass that accumulates polymers of fructose called fructans (1, 10, 12, 16). Understanding fructan metabolism in this species is important, since fructans are thought to be one mechanism by which plants adapt to environmental change (10, 12). The fructan concentration and mean mol wt of fructans from leaf and stem tissues of meadow fescue (F. *pratensis* Huds. cv S 215) has been shown to vary with season (11). Stem fructan concentration increased in autumn and winter and decreased in spring. Leaf fructan concentration reached a maximum in late summer and declined through autumn, winter, and spring. The mean mol wt of leaf fructan was smaller than that of the stem, but increased in both tissues during fall and winter before declining with the onset of spring. Fructan concentration of tissues has also been shown to depend on temperature and radiant energy (8). High temperatures and low photon flux density resulted in little accumulation of fructan. The optimum condition for accumulation of fructan in meadow fescue tissue was exposure to high PPFD[2] and low temperature during anthesis. In tall fescue, as in meadow fescue, fructan concentration varies among tissues, and between genotypes (21–23). Young leaf sheath tissues had low fructan concentrations and fructans with low mean mol wt, with both concentration and mean mol wt increasing with tissue age (21). In contrast, increased fructan concentrations of young leaf blade tissues with age was not closely associated with increased mean mol wt of fructans. In addition, net decreases in both concentration and mean mol wt of fructan was observed for old, outermost leaf sheaths in response to defoliation (21). Differences in fructan concentration and mean mol wt imply changes in activity of fructan metabolic enzymes may exist among tissues. Our objective was to compare the fructan concentrations and fructosyl-transferase activities of tall fescue leaf tissues differing in age.

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[2] PPFD, photosynthetic photon flux density; SST, sucrose:sucrose fructosyl-transferase; FFT, fructan:fructan fructosyl-transferase; BLSD, Bayes least significant difference; LCF, long chain fructan.

MATERIALS AND METHODS

Plant Growth. A single tall fescue genotype (FFR 73-237) was used throughout these studies to ensure genetic uniformity of the plant materials. Plants were grown from vegetative tillers in 13-cm diameter pots containing a 1:1 (v/v) mixture of silt loam topsoil and peat. Plants were established in the greenhouse for 6 months prior to tissues being sampled. Daylength was extended to 15 h with 160 μmol · m^{-2} · s^{-1} PPFD using fluorescent and incandescent lamps. Temperature was maintained at 25 ± 5°C. Plants were defoliated leaving a 5-cm stubble at 6-week intervals. Approximately 6 weeks following defoliation the following tissues were obtained: recently expanded leaf blades, the lower 5-cm of the outermost and innermost sheath tissues, and the lower 5-cm of the enclosed expanding leaf (21).

Fructan Content. Tissues were frozen in dry ice, freeze-dried, and stored in a −80°C freezer. Tissues were extracted three times with 80% (v/v) ethanol and twice with deionized water. The extracts were flash evaporated, then reconstituted with distilled water, frozen, thawed, and centrifuged (10 min, 2000*g*). Carbohydrates were separated using a 110 × 1.5-cm column of Bio-Gel P-2 (200–400 mesh) (6). Columns were eluted using 4 g/L NH_4HCO_3. Fractions were assayed colorimetrically for free and combined fructose after reaction with anthrone (7). The concentration of carbohydrates containing one to six sugar residues were compared separately, while carbohydrate pools containing more than six residues were summed and termed long-chain fructan.

Measurement of Transferase Activity. Estimates of fructosyl-transferase activity were made using two methods. In the first method, proteins were extracted from the four tissues described above. Crude protein extracts were prepared following the procedure of Housley and Daughtry (5) with the following modifications. After grinding in the extraction buffer the tissue slurry was centrifuged at 2000*g* for 15 min, and the supernatant desalted by passage through Bio-Gel P6 (1 × 5 cm column). The Bio-Gel P6 desalting column does not separate proteins from large mol wt fructan. To identify newly synthesized fructan from extracted fructan, uniformly labeled ^{14}C-sucrose was added to the assay buffer (5) to provide a concentration of 40 mM at a specific radioactivity of 2.06 kBq/mmol. Fructosyl-transferase activity was determined by measuring the radiolabel in fructan of higher mol wt than sucrose following gel permeation chromatography on Bio-Gel P2.

In the second method, fructosyl-transferase activity was estimated by floating 2-mm pieces of the tissues on a radiolabeled sucrose solution (40 mM sucrose containing 2.06 kBq/mmol). Following incubation times of 1.5 to 4-h, tissues were transferred to distilled water for 10 to 15 min to rinse radiolabel from the free space. The rinsed tissues were frozen, freeze-dried, weighed, and then extracted in 80% (v/v) ethanol and water as described above. The extracts were concentrated, frozen, thawed, centrifuged, and either spotted on TLC plates (silica gel) or applied to a Bio-Gel P2 column. Radioactivity of each fraction was determined by scintillation spectrometry. Fructosyl-transferase activity for each experiment was calculated by multiplying the accumulated radiolabel in fructans by the specific activity of the sucrose in the incubation media. The slope of the regression line of label incorporated into fructan versus time was used to estimate the fructosyl-transferase for each tissue. Fructans separated on TLC plates (18) were visualized after exposure of the developed TLC plate to x-ray film (Kodak Oximate). The spots were scraped off the plates and radioactivity in the spot determined by scintillation spectrometry. Nonradiolabeled ketoses separated by TLC were visualized by spraying the developed TLC plate with ethanol/HCl/urea (90 ml:5 ml:4 g) and heating the plate to 90°C.

Statistical Analysis. Five replicates of each tissue were extracted and the percentage of fructose equivalents in the separated fractions analyzed using analysis of variance. Where the *F* test was significant ($P < 0.05$), a BLSD was calculated using a *k*-ratio of 100 (17). Differences in the rate of fructosyl-transferase activity were estimated by determining the differences between slopes of regression lines (19) using seven to nine measurements of radiolabel incorporation per tissue.

RESULTS AND DISCUSSION

The composition of the nonstructural carbohydrates extracted from a tissue reflected the metabolism occurring in the tissue. Metabolically, the sheath functions as a reservoir of nonstructural carbohydrates for use during flowering or for regrowth after defoliation. This function was reflected in the high concentrations and the greater proportion of high mol wt fructans extracted from sheath tissues when compared to that of expanded blades (Table I). Both the expanding leaf and the expanded blade had significantly less LCF than did sheath tissues. Expanded blades synthesize sucrose which is transported to sheaths for storage as fructan. This synthetic metabolism of the expanded blade was reflected in a significantly greater percentage of sucrose in this tissue relative to other nonstructural carbohydrates. Expanding leaves contained tissues which, upon differentiation, would have become primarily the expanded blade, but may have contained some sheath tissue. Expanding leaves were entirely enclosed by the whorl of surrounding leaf sheaths and therefore would be expected to import assimilates to support leaf growth. This dependence on imported assimilates and subsequent metabolism was indicated by the significantly greater percentage of fructose extracted from expanding leaves. As others have reported (13, 21), expanding leaf tissues contained a significantly greater percentage of oligosaccharides than did expanded blade tissues. Differences in fructan concentration and composition of expanding leaves and sheath tissues emphasizes the need to separate the lower 5-cm of the 'stem base' into its component parts to prevent confounding of responses of these tissues (21).

The concentrations of oligosaccharides and LCF in the various tissues (Table I) should be indicative of the fructosyl-transferase activity of the tissues. Expanding leaf tissue would be expected to possess the greatest fructosyl-transferase activity, and the outer sheath the least, while that of expanded blade and inner sheath would be intermediate. To estimate fructosyl-transferase activity, slices of the four tissues above were incubated in 40 mM ^{14}C-sucrose for varying periods of time. The rate of radiolabel accumulation in oligosaccharides and LCF was used as an estimate of the fructosyl-transferase activity for the various tissues (Fig. 1). The slope of the regression line for the expanding leaf (Fig. 1A) was significantly greater than either the inner (Fig. 1C) or outer sheath (Fig. 1D). The slope of the regression line for the inner sheath was numerically greater than that of the outer sheath, but the difference was not statistically significant ($P \leq 0.05$). Surprisingly, the slope of the expanded blade (Fig. 1B) was also significantly greater than that of either sheath. The activity determined in this manner includes both the time necessary for substrate uptake from the incubation media into tissues, as well as the time for fructosyl-transferase activity to synthesize fructans. The nonzero intercept of the regression lines may have resulted from the time necessary for accumulation of radioactive substrate in the vacuole, the site of fructan synthesis (4, 24, 25). This would result in the regression equation having a negative intercept as was found for the expanding leaf and expanded blade (Fig. 1, A and B). To test the influence of tissue size on fructosyl-transferase activity estimated using this technique, smaller pieces of expanding leaf tissue were incubated in radiolabeled sucrose. Smaller tissue pieces (0.2 mm^2) gave higher estimates of fructosyl-transferase activity than pieces cut into 1 mm^2 sections (2.56 *versus* 0.88 μmol fructose transferred · (g fresh wt · h)$^{-1}$, respectively). Therefore, activities obtained using incubation of tissue slices in radiolabeled sucrose may significantly underestimate the actual fructosyl-transferase activity. In addition, the activity of fructosyl-transferase would be underestimated if tissues contained a large endogenous pool of sucrose. Data of Table I indicates that expanding leaves and outer sheaths contained about 40% less sucrose than did expanded blades and inner sheaths. However, no consistent association between tissue fructosyl-transferase activities (Fig. 1) and tissue sucrose concentrations (Table I) were apparent.

Estimates of fructosyl-transferase activity were also measured using protein extracts from each tissue. Radiolabeled sucrose (40 μmol) was added to the assay buffer and the incorporation of ^{14}C into oligosaccharides and LCF measured. When compared to results obtained with incubation of intact tissues, activity measurements with protein extracts were higher for the expanding leaf and inner sheath, and lower for the expanded blade, while that of the outer sheath was about the same (Table II). The

Table I. *Fructose Equivalents of Various Sugars Expressed as a Percentage of Total Extracted Carbohydrate from Tissues of Tall Fescue*

	Fructose	Sucrose	Short-Chain Fructans				LCF	Carbohydrate Concentration
			Tri	Tetra	Penta	Hexa		
	% of nonstructural carbohydrate							*mg/g dry wt*
Outer sheath	1.9	5.7	0.9	0.6	0.6	0.5	89.7	232.6
Inner sheath	1.8	10.3	3.0	2.9	2.4	1.9	77.0	204.8
Expanding leaf	16.9	11.8	8.8	9.6	10.0	10.7	33.2	124.4
Expanded blade	2.9	74.7	1.6	0.2	0.1	0.3	20.2	34.1
BLSD[a]	2.4	9.6	1.5	1.0	2.5	4.2	26.9	108.2

[a]Using a *k* ratio = 100, P = 0.05.

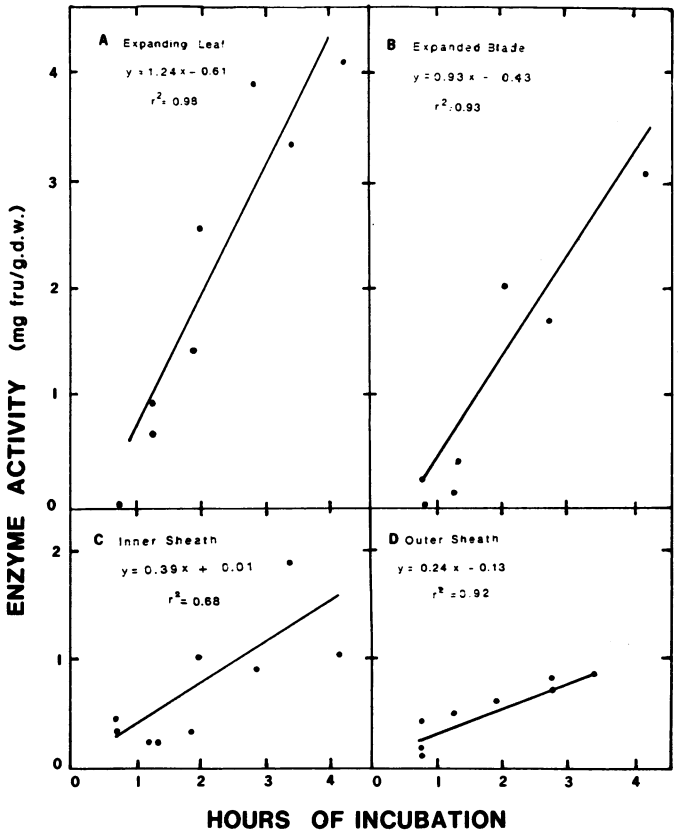

FIG. 1. Estimate of fructosyl-transferase activity (mg fructose transferred to fructans per g of dry weight) of leaf tissue of tall fescue: A, expanding leaf; B, expanded blade; C, inner sheath; D, outer sheath. Fructose transferred to fructans was determined by measuring the radiolabel incorporated into chromatographically separated fructans following incubation of tissue on 40 mM ^{14}C-sucrose for varying periods of time. The slope of the regression line was used to estimate the rate of fructan synthesis.

results indicate that the difference in transferase activity of the expanding leaf blade and sheath tissues was probably due to varying rates of enzyme activity and not due to differences in substrate accumulation. When compared to tissue slices, the fourfold lower fructosyl-transferase activity of the protein extracts from expanded blades might have resulted from a difference in the amount of enzyme present. In leaf tissue the fructosyl-transferase activity has been shown to increase as sucrose concentration increased (6, 24) and to decrease in darkness (25). Diurnal changes in fructosyl-transferase activity in expanded blade tissue could have caused the differences observed between protein extraction and tissue incubation. Experiments are in progress to assess the cyclic nature of fructosyl-transferase activity in leaf tissues of tall fescue.

The estimates of fructosyl-transferase activity in Table II (0.27–2.69 μmol fructose([g fresh wt · h]$^{-1}$) were generally lower than those reported for wheat (*Triticum aestivum*) kernels (0.7–20 μmol fructose[g fresh wt · h]$^{-1}$, Ref. 5), *Lolium temulentum* leaf blades (7.7–44.1 μmol fructose[g fresh wt · h]$^{-1}$, Ref. 10; 0.8–5.0 μmol fructose[g fresh wt · h]$^{-1}$, Ref. 6), and barley (*Hordeum vulgare*) leaf blades (0.09–0.89 nkat/ml; Ref. 25). The assay mixture for our experiments contained 40 mM sucrose instead of the 600 mM sucrose used by other researchers (5, 6, 10, 25). The fructosyl-transferase activity of the expanding leaf was 48-fold higher at 600 mM sucrose (42 μmol fructose[g fresh wt · h]$^{-1}$).

Table II. *Comparison of Tall Fescue Fructosyltransferase Activity Estimated by Incubating Tissue Slices, by Extracting Proteins and by Extracting Fructans Over Time*

Tissue	Tissue Slices	Protein Extract	Tissue Extraction
	μmol fructose transferred/g fresh wt · h		
Outer sheath	0.30	0.27	
Inner sheath	0.44	0.79	2.56
Expanding leaf	0.88	2.69	
Expanded blade	0.99	0.24	

On this basis, the activities reported are well within the range of those found in other Gramineae. Inner sheath fructosyl-transferase activity, estimated using fructan accumulation over time (21), averaged 2.56 μmol fructose[g fresh wt · h]$^{-1}$ which is five-fold greater than that estimated using tissue pieces incubated in 40 mM sucrose (Table II). The difference in activity may be attributable, in part, to differences in sucrose concentration at the site of fructan synthesis.

Fructans in plants occur as phleins, β(2-6)-linked fructose, inulins, β(2-1)-linked fructose, or as mixed-linked polymers of fructose. They are thought to be synthesized in two steps by the enzymes SST and FFT. The fructosyl-transferase activity as reported reflects the activity of both SST and FFT. In the first step of fructan synthesis, a trisaccharide is synthesized from sucrose by SST (10). The trisaccharides, neokestose, kestose, and isokestose are possible products of this reaction. In most plant systems, isokestose has been the product isolated as a result of SST activity (10), although structural analysis of products of SST has not often been attempted. In asparagus (*Asparagus officinalis* L.) roots, all three trisaccharides were synthesized by SST (14, 15). In tall fescue, two trisaccarides are usually observed. Neither the autoradiographs or sprayed TLC plates (Fig. 2) indicated the presence of two trisaccharides. The limited resolution of the different trisaccharides was likely a result of loading large amounts of sugars onto the TLC plate in an attempt to visualize the individual oligosaccharide pools. The fructans isolated from tall fescue appear to be branched (2). The ratio of 6-linked to 1-linked fructose in tall fescue was about 3.26. It is possible that the usually observed two trisaccharides could be associated with this branching phenomena, or a result of invertase fructosyl-transferase activity (26).

Fructans having higher mol wt than the trisaccharide are thought to be synthesized by FFT. The substrates are reported to be a trisaccharide and/or oligofructans, rather than sucrose (10). Fructosyl-transferase activity reported here was dependent on synthesis of new trisaccharide from radiolabeled sucrose, and as such, may be a more accurate estimate of SST, than FFT activity. The synthetic patterns observed on the TLC plates indicate a flow of synthesis through oligosaccharides possessing 5 fructose moieties DP5 (Fig. 2). The DP5 pool consistently contained more radioactivity than other oligosaccharides, with the exception of the DP2 pool in the expanding leaf (Fig. 2a). Radiolabel beyond DP5 accumulated primarily in LCF, with only minor accumulation in DP6 for the expanding leaf (Fig. 2a). The differences observed in accumulated radiolabel does not indicate movement towards a dynamic (3) or isotopic (9) equilibrium. Perhaps the short assay time (4 h) prevented the establishment of such an equilibrium. The data indicates that oligosaccharide synthesis beyond DP5 or 6 either does not normally occur or that the pools turn over so rapidly that detection using these methods was not possible. Radiolabel tended to accumulate in oligosaccharides even though small amounts of fructose containing sugars were detected on the TLC plates. This difference was most obvious in the expanded blade tissue (Fig. 2b). The pattern of radiolabel

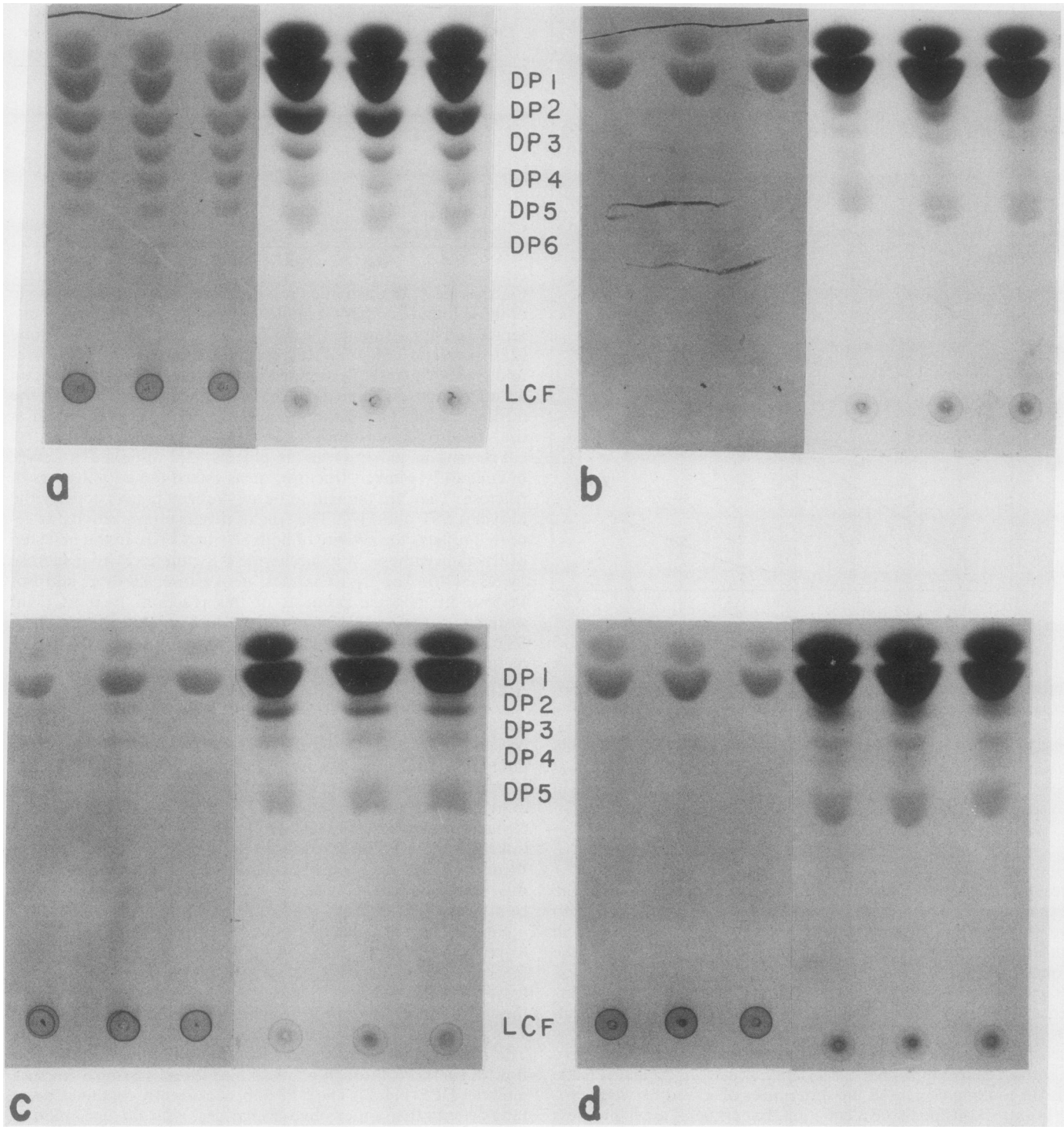

FIG. 2. TLC separation of extracts from tissues incubated on ^{14}C-sucrose. TLC plate sprayed with urea-HCl to identify ketose containing compounds (left 3 lanes) compared with the x-ray chromatogram of the TLC plate (right 3 lanes). Expanding leaf labeled (a), expanded blade (b), inner sheath (c), and outer sheath (d). Degree of polymerization (DP) refers to number of fructose moieties (sucrose = DP1), LCF fructans at origin.

incorporation supports the data of Table II in that the expanding leaf possesses greater amounts of extractable oligosaccharide pools, while the inner (Fig. 2c) and outer (Fig. 2d) sheath contain greater radiolabel in LCF. The synthetic pattern observed in tall fescue is different from that of wheat leaf blades, which synthesize only oligosaccharides of DP7 or less (TL Housley, unpublished results). This pattern is also different from that of timothy (*Phleum pratense*), where it appears that only trisaccharides and LCF are synthesized (20). The relationships between FFT activity and these differing patterns of oligosaccharide and LCF synthesis are not understood. It is possible that FFT represents a class of fructosyl-transferases which differ in spec-

ificity for oligosaccharide substrates used in the synthesis of LCF. Until a series of oligosaccharide substrates are available, factors influencing FFT activity cannot be critically evaluated.

The data presented in this paper indicate that fructan synthetic activity is greatest in the expanding leaf, and least in the outer sheath (Table II). The expanding leaf also contains the largest concentration of oligosaccharides with the outer sheath having the greatest LCF concentration. The synthetic activity of the inner sheath is intermediate between the outer sheath and the expanding leaf. Synthetic activity of the expanded blade was the most sensitive to changes in the method of assay. The data also indicate that as the leaf expands and differentiates into sheath and blade tissues there is a change in fructan synthetic activity that is associated with the metabolic activities and physiologic function of the mature tissues. Synthetic activity of the blade probably varies in response to sucrose supply, while that of the sheath decreases during ontongeny, but continues to produce LCF. In the sheaths and expanded blade, the role of fructans appears to be as a temporary storage pool for carbohydrate. In the expanding blade the oligosaccharides may also function as an osmoticum to support cell expansion.

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