Sterile Spikelets Contribute to Yield in Sorghum and Related Grasses

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Short title: Carbon assimilation in sorghum spikelets

One-sentence summary: Apparently non-functional floral structures (spikelets) provide carbon for the developing grain in sorghum and two related wild species, thereby contributing to yield (fitness).

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ABSTRACT

Sorghum (Sorghum bicolor) and its relatives in the grass tribe Andropogoneae bear their flowers in pairs of spikelets, in which one spikelet (seed-bearing, or SS) of the pair produces a seed and the other is sterile or male (staminate). This division of function does not occur in other major cereals such as wheat (Triticum aestivum) or rice (Oryza sativa). Additionally, one bract of the seed-bearing spikelet often produces a long extension, the awn, which is in the same position as, but independently derived from, that of wheat and rice. The function of the sterile spikelet is unknown and that of the awn has not been tested in Andropogoneae. We used radioactive and stable isotopes of carbon, RNA-seq of metabolically important enzymes, and immunolocalization of Rubisco to show that the sterile spikelet assimilates carbon, which is translocated to the largely heterotrophic SS. The awn shows no evidence of photosynthesis. These results apply to distantly related species of Andropogoneae. Removal of sterile spikelets in sorghum significantly decreases seed weight (yield) by ca. 9%. Thus, the sterile spikelet, but not the awn, affects yield in the cultivated species and fitness in the wild ones.
**Introduction**

The grain and bioenergy crop sorghum (*Sorghum bicolor*), along with most of the 1200 species in the grass tribe Andropogoneae, produces more sterile than fertile flowers in the inflorescence (Clayton and Renvoize, 1986; Schneider and Vegetti, 1992; Watson and Dallwitz, 1992 onwards; McKone et al., 1998; LeRoux and Kellogg, 1999; Vegetti, 1999; Hodge and Kellogg, 2014; Gladman et al., 2019). This pattern is counter-intuitive, in that the plant appears to be sacrificing potential reproductive output by taking many of its floral structures "off line." The sterile flowers must therefore have another function maintained through evolution to compensate for the loss of reproductive potential.

The seed of sorghum, like that of all grasses, is enclosed in floral bracts (glumes and lemmas), which together form a terminal unit known as a spikelet (a little spike) (Clayton and Renvoize, 1986; Watson and Dallwitz, 1992 onwards; Kellogg, 2015). The seed-bearing, or sessile, spikelet (SS) sits directly on the inflorescence axis and is paired with a pedicellate (stalked) spikelet (PS) (Figures 1A and 1B). Unlike the seed-bearing SS, which is bisexual, the PS is generally sterile in sorghum, although in some lines the PS produces stamens (Burow et al., 2014; Jiao et al., 2018; Gladman et al., 2019). Presence of a PS, its shape, size and often sex expression appear to be genetically fixed among plants of any given accession (E. A. Kellogg, personal observation).

The function of the PS is unknown in sorghum or in any other member of Andropogoneae, in most of which the PS is also sterile or staminate (Clayton and Renvoize, 1986; Watson and Dallwitz, 1992 onwards; Vegetti, 1999; Kellogg, 2000, 2015; Arthan et al., 2017). Because grasses are wind-pollinated (Niklas, 1987; Friedman and Harder, 2004; Cresswell et al., 2010), the PS cannot function in pollinator attraction, as sterile flowers do in some other angiosperms (Stuessy et al., 1986; Krannitz and Maun, 1991; Nielsen et al., 2002; Jin et al., 2010; Morales et al., 2013). In some wild species, the PS may function in seed dispersal (Davidse, 1987; Doust et al., 2014; Linder et al., 2018) and/or in controlling pollen to ovule ratio (Connor, 1981, 1987). However, the dispersal function is not universal, nor is it relevant in cultivated sorghum, in which the grains are not shed, and pollen production in the PS only occurs in a few accessions.

The spikelet pair in sorghum may also include an awn on the SS (Sieglinger et al., 1934) (Figures 1A and 1B). The awn, when present, is a slender extension of the lemma (floral bract) of the upper flower and may be twisted and geniculate. Like the PS, the presence, shape and size of the awn appear to be genetically fixed within an accession (Jowett, 1968) and its function has not been demonstrated (“the awn is not known to have any protective or physiologic value to the sorghum plant” (Sieglinger et al., 1934)). In some wild Andropogoneae, the awn is hygroscopic and can help orient the spikelet in the soil to enhance germination (Peart, 1979, 1981, 1984; Peart and Clifford, 1987). Awns in wheat and rice are vascularized and assimilate carbon, which is then transferred to the grain (Grundbacher, 1963; Motzo and Giunta, 2002; Li et al., 2006; Simkin et al., 2020), often contributing appreciably to yield; however, wheat and rice have solitary (unpaired) spikelets and the awns may have evolved independently of those in Andropogoneae (Teisher et al., 2017). Carbon assimilation has not been assessed in awns, or more generally in any floral structures of Andropogoneae.

The PS, the awn, or both could produce photosynthate that contributes to grain filling. The PS is green, which suggests that it could be photosynthetically active, but its tiny size (3–6 mm long) makes it unclear whether it could assimilate and export enough carbon to contribute materially to the carbon economy of other floral organs. The SS is slightly longer (ca. 4–9 mm) and more broadly ovate, but does not stay green as long as the PS. By contrast, the awn turns brown soon after heading, suggesting limited capacity for photosynthesis. If so, its function must differ from that of the wheat or rice awn. The awn is also small (4–18 mm), and in most sorghum lines, missing entirely.
Here we test the hypothesis that the PS and awn contribute photosynthate to the developing seed in sorghum and other members of the tribe Andropogoneae. We report a combination of radioactive and stable carbon isotopic labeling and metabolite analyses, RNA-seq experiments, immunolocalization, morphological observations with scanning electron microscopy (SEM), and spikelet removal experiments. Our goal was to assess photosynthetic capacity in these organs and their potential contributions to yield and fitness. Given that Andropogoneae, including sorghum, are C₄ NADP-ME subtype (Hattersley and Watson, 1975, 1992), we also considered whether the anatomy, gene expression, and Rubisco localization in the PS were consistent with a two-cell C₄ shuttle, as suggested for inflorescence tissues in a few other grasses (Pengelly et al., 2011; Rangan et al., 2016, 2016; Henry et al., 2017).

RESULTS

The SS and awn are carbon sinks, the PS is a source

For an initial assessment of carbon assimilation, we used a pulse-chase experiment to assess whether sorghum spikelets or the awn could take up ¹⁴C. Inflorescence branches were exposed to ¹⁴C for one hour. Measurements were standardized by weight (disintegrations per minute per mg) and presented as a percentage of the total counts in spikelets and awns (Figure 2A; Supplemental Table 1). The PS accounted for a significantly greater percentage of ¹⁴CO₂ taken up than the SS or awn (p<0.0000001), with the latter being scarcely detectable. This was true whether the inflorescences were intact (attached), or whether the spikelets and awns were detached and lying on the bottom of the flask, suggesting that relative carbon uptake by the individual structures was consistent regardless of the effects of transpiration (difference non-significant, p>0.997).

Intact inflorescences were also subjected to a pulse-chase with one-hour pulse labeling followed by 24-hour chase period in air (i.e., no additional labeled carbon provided). Over 24 hours, the fraction of counts in the PS decreased and that of the SS increased significantly (0.01<p<0.5), indicating translocation of carbon from one structure to the other and other parts of the plant (Figure 2A, Supplemental Table 1). The fraction in the awn also increased but was still small relative to the spikelets and not significantly different from the 1-hour labeling experiment.

To determine whether our result was specific to sorghum or might apply generally to Andropogoneae, we repeated the experiment with the distantly related species Themeda triandra and Andropogon schirensis, which, like sorghum, have an SS and PS, with an awn on the SS (Figures 1C-1I). These two species represent distinct clades of Andropogoneae and diverged ca. 15 million years ago (Estep et al., 2014). The results were similar to those for sorghum: most ¹⁴C appeared in the PS after 1 hour of labeling, whether attached to the inflorescence or not (difference non-significant, 0.5<p<1.0), but after a 24–hour chase the proportion of label in the PS had decreased and that in the SS had increased significantly (0.0003<p<0.0022 (Andropogon schirensis) and 1x10⁻⁵<p<4x10⁻⁵ (Themeda triandra); Figures 2E and 2I; Supplemental Tables 2 and 3). Awns were scarcely labeled.

The surface morphology of each organ is consistent with what would be expected from the ¹⁴C results. The outer bracts (glumes) of the PS have obvious stomata in all three species (Figures 2B, 2F and 2J), and are similar in this respect to leaves. By contrast, stomata are absent from the surface of the glumes of the SS (Figures 2C, 2G, and 2K), except near the apex in sorghum and on the sides in Andropogon schirensis. No stomata were found on awns (Figure 2D, 2H, and 2L), unlike wheat (Li et al., 2006). SEM data combined with the ¹⁴C data suggest that the PS may contribute photosynthate to the SS, whereas the awn may not.
The PS produces isotopically labeled intermediates of carbon assimilation, SS and awns do not

If the PS is indeed a source of carbon for the grain, it should produce metabolites characteristic of photosynthesis. To test this hypothesis, intact inflorescences were exposed to $^{13}$CO$_2$ for 30s, 60s or 300s, and then key metabolites assessed using LC-MS/MS. Knowing that inflorescence tissues often lack a fully developed C$_4$ cycle, even in C$_4$ plants such as sorghum or maize (Pengelly et al., 2011), we looked for evidence of photosynthetic metabolites, whether from C$_4$ (e.g. malate), C$_3$ (Calvin-Benson cycle intermediates such as PGA and F6P), sucrose (UDPG) or starch (ADPG) biosynthetic pathways. Conversely, we expected that SS and awn should be metabolically distinct, likely heterotrophic. Note that for this analysis, we were not attempting to determine whether the tissue should be classified as C$_3$ or C$_4$, but rather to determine which of several pathways might be active.

As in the $^{14}$C experiments, more carbon was labeled in the PS than in either the SS or awn. In a principal component analysis (PCA) of the metabolite results, the first principal component (PC1) accounted for over 87% of the variance, distinguishing between values for unlabeled metabolites (loading just below 0) and those for labeled metabolites (loading positively) (Figure 3A). By five minutes, metabolite labeling was greatest in the PS, whether considering the weighted average of all isotopologues for each metabolite (i.e., one data point per metabolite per organ and time point; Figure 3A) or individual isotopologues (i.e., three or four data points per metabolite per organ and time point; Figure 3B). Overall effects of organ, time, and organ with time were all significant (generally p ≤ 0.0001) (Supplemental Tables 4A and 5A). Values for the PS were significantly different from those of either of the other two organs (generally p<0.0001) (Supplemental Tables 4B, 4C, 5B and 5C), but the SS and awn were not significantly different from each other. In addition, the five-minute time point was significantly different from the 30 or 60 second pulses for most metabolites (p<0.0001), malate being an important exception. Malate was labeled between 30s and 60s, possibly signifying NADP-ME C$_4$ activity, but not between 60s and 300s, indicating that initial labeling had stopped by 60s (Supplemental Tables 4B and 4C).

At the onset of a $^{13}$CO$_2$ labeling experiment, the drop in the unlabeled isotopologue fraction for central metabolites indicates carbon assimilation. After correction for natural abundance, we see such a drop in metabolites in the PS (Figure 4A) along with increasing average fractional enrichment (Figure 4B), showing that primary metabolites were significantly labeled by 300s. To further account for differing biomass compositions among organs that can confound metabolite pool sizes, we calculated the $^{13}$C atom equivalents formed (Supplemental Figure 1, inspired by (Arrivault et al., 2017)), which further confirmed that the PS is the only organ with significant $^{13}$C labeling and thus carbon assimilation.

Labeling in malate appeared low, with the unlabeled isotopologue fraction, M0, scarcely decreasing between the 30 and 300s time points (Figure 4A; p=0.0132, Supplemental Table 5). However, most plant cells have a large inactive pool of malate (Arrivault et al., 2017), and we hypothesized that this pool might obscure changes in the dynamically labeled portion. By calculating the active malate pool in $^{13}$C, we found rapid incorporation at the 30s and 60s time points in the PS (inset Figures 4A and 4B). The rapid initial rise in the single-labeled active $^{13}$C-malate pool would be expected if there is a functional two-cell C$_4$ shuttle using NADP-ME.

Phosphoglyceric acid (PGA), the immediate product of RuBisCO-based carbon assimilation, and triose phosphate (TP) were rapidly labeled, as shown by the relative drop in M0 and increase in M1 (black and orange dots; Figure 4A), with the remaining unlabeled fraction in triose phosphate constituting < 40% of the total pool in the PS after five minutes. Fructose-6-phosphate (F6P), which is also tied to the Calvin-Benson cycle, showed a similar but weaker labeling pattern. Pyruvate (PYR), a product of glycolysis and several steps removed from
the Calvin-Benson cycle, was labeled \((p \leq 0.0001)\) linearly, as were other hexose and nucleotide hexose phosphates (glucose-6-phosphate (G6P), UDP-glucose (UDPG) and ADP-glucose (ADPG); all \(p \leq 0.0001\); Figures 4A and 4B, Supplemental Figure 1) used in sucrose and starch biosynthesis respectively. The linear slope of labeling in PYR and others within similar time frames indicates the delay due to \(C_4\)-based \(CO_2\) delivery to the Calvin-Benson cycle and has been observed in other \(C_4\) studies (Weissmann et al., 2016; Arrivault et al., 2017) but not in \(C_3\) studies that result in hyperbolic labeling curves with less delay for three carbon intermediates during the same time frame (Ma et al., 2014). We also looked for 2-phosphoglycolate (2PG), glyoxyxylate, and glycolate, intermediates associated with photorespiration that could indicate \(C_3\) metabolism, although low levels of photorespiration may also occur in \(C_4\) plants (Zelitch et al., 2009). Typically, only a subset of these particular metabolites is reliably detected or reported (Weissmann et al., 2016; Arrivault et al., 2017). 2PG was labeled in the PS by 300s, but not in the other organs (Figures 4A and 4B).

To test the generality of our results, we repeated the \(^{13}CO_2\) experiments with *Themeda triandra*. As in sorghum, the PS was clearly distinct from the SS and awn in a PCA. PC1 explained 66% of the variance and the overall pattern of the PCA was similar to that in sorghum (average labeling: Figure 3C, Supplemental Table 6; all isotopologues: Figure 3D, Supplemental Table 7), suggesting that the results may apply to many other members of Andropogoneae. Labeling of individual metabolites was also similar between the two species, except that aspartate (ASP) labeling was significant \((p<0.001)\) and ADPG, PYR, and P5P labeling were non-significant (Supplemental Tables 6, 7).

**Photosynthetic genes are expressed in leaf and PS, but not SS or awn**

To determine if the metabolite data corresponded to underlying patterns of gene expression in the three organ types, we compared transcriptomes for spikelets and awns to those for leaves, focusing on a hand-curated set of 1441 genes encoding enzymes of central carbon metabolism, including photorespiration and also major transporters required for \(C_4\) photosynthesis (hereafter called metabolic genes, Supplemental Data set 1). A PCA of the transcript data separated the leaf and SS along PC1 (Supplemental Figure 2A), whereas values for awn and PS separated most clearly along PC2, a pattern nearly identical to that of a PCA for all transcripts (Supplemental Figure 2B). The PCA indicates distinct differences in carbon metabolism among the four organ types. Of the 1441 metabolic genes, 922 were differentially expressed (DE) in at least one pairwise comparison of organs. A heat map of these 922 transcripts (Supplemental Figure 3) found that genes upregulated in leaves were downregulated in the SS and vice versa. Expression in awns and PSs was clearly different from the other two organs. A distinct set of genes was upregulated in awns, but these appeared unrelated to either photosynthesis or sugar metabolism.

We examined expression of genes encoding the enzymes that directly produced the metabolites measured with \(^{13}C\) and other related central intermediates, a 52-gene subset of the 922 DE genes. Genes were assigned a provisional subcellular location by TargetP (Emanuelsson et al., 2007), although this should be regarded as a preliminary hypothesis. A heat map of this subset (Figure 5) produced a pattern similar to that for the full set of metabolic genes (Supplemental Figure 3). Genes specific to \(C_4\) metabolism, including those encoding the copy of phosphoenolpyruvate carboxylase (PEPC) located on chromosome 10 (#27 in the figure), NADP-dependent malate dehydrogenase (NADP-MDH) and NADP-dependent malic enzyme (NADP-ME) were highly expressed in the leaf, followed by the PS. The gene for the non-\(C_4\) PEPC (chromosome 4, #36 in the figure) was expressed highly in the leaf and PS (Figure 5), whereas the \(C_4\)-based phosphoenolpyruvate carboxykinase (PEPCK) located on chromosome 1 was not detected (and thus not shown in Figure 5), indicating that the sorghum PS, like sorghum leaves, lacks an active PEPCK pathway (Wang et al., 2014; Döring et al., 2016). The small subunit of RuBisCO (transcript #5) was strongly expressed in the leaf, moderately in the spikelets, and scarcely at all in the awn.
Analogous data for *Themeda triandra* produced similar but not identical findings. Because *Themeda triandra* spikelets are subtended by a large leaf-like bract (see below), expression of the 769 metabolic genes in the spikelets was compared to that of the bract rather than to a foliage leaf. PCA of these transcripts placed the awns and PS at opposite sides of PC1 (32.9% of variance); the three replicate bract samples were distinguished from the other organs on PC2 (Supplemental Figure 4). Of the 769 genes, 322 were differentially expressed between at least one pair of organs. Relative expression of these genes showed a different pattern for each of the four organs (Supplemental Figure 5). Because the variability among samples of *Themeda triandra* was higher than that in sorghum, fewer genes were significantly DE but overall trends were similar. Awns in *Themeda triandra* had a distinct set of upregulated genes that were not reflected in photosynthetic metabolism. Of the genes encoding enzymes that could produce the $^{13}$C metabolites, only 24 were DE (Supplemental Figure 6). (Note that *Themeda triandra* lacks a reference genome, so the genes are labeled with the names of their putative sorghum orthologues). Genes related to photosynthesis are upregulated in the leaf-like bract and downregulated in the SS, whereas the situation is opposite for genes involved in starch and sucrose metabolism. The PS is similar to both bracts and SSs, whereas awns have a unique expression profile. Unlike sorghum, genes for aspartate metabolism are DE, and the C$_4$ isoform of PEPCK (#19 in Supplemental Figure 6) is moderately expressed in the PS, SS, and bract, suggesting that this species may have an active PCK-type of C$_4$ photosynthesis and use aspartate to transport carbon between the bundle sheath and mesophyll.

**Rubisco is expressed in both bundle sheath and mesophyll cells**

If the PS and SS were similar to C$_4$ leaves, we would expect that Rubisco would be restricted to cells of the bundle sheath and absent in the inter-vascular regions (Dengler and Nelson, 1999). In addition, veins would be separated by no more than two mesophyll cells. Immunolocalization showed abundant Rubisco in bundle sheath cells of both the PS and SS, but also in most of the intervening cells (Figure 6; controls in Supplemental Figure 7). Veins are separated by more than two mesophyll cells, suggesting that a C$_4$ shuttle might be operating around the veins but that other mesophyll cells may be relying on C$_3$-like metabolism. In the PS, this interpretation is consistent with the $^{13}$C and gene expression data, which show some labeling of the C$_4$ metabolite malate as well as labeling of C$_3$ intermediates of the Calvin-Benson cycle plus 2-PG indicating photorespiration, and expression of C$_4$- and C$_3$-related enzymes.

In the SS, Rubisco-expressing cells are restricted to the adaxial (inner) side of the glume. This tissue is not green, and the plastids do not fluoresce in the red confocal channel (compare Figures 6D vs. 6F, and 6G vs. 6H; red color in Figures 6H and 6J appears to be non-specific fluorescence of thick-walled cells). By contrast, the abaxial (outer) side is made up of several layers of sclerenchymatous cells. The thickness of the outer layers of the SS glume, the lack of stomata (Figure 2C) and chlorophyll, and the lack of labeled $^{13}$C intermediates in the metabolite experiments (Figure 3) together suggest that the SS is assimilating relatively little carbon from the surrounding air (except possibly near the very tip where it is similar to the PS; not shown). We speculate that the Rubisco-expressing chloroplasts in the inner cells of the glume instead could be refixing respired CO$_2$, as is common in floral and fruiting structures (Simkin et al., 2020), although validating this would require a different set of experiments than those presented here.

**The PS contributes significantly to seed weight (yield)**

To test the influence of PS function on plant grain yield, individual PSs were removed from alternating panicle branches at anthesis. Four genotypes differing in inflorescence architecture and relative size of the PS and SS (Figures 7A–7D) were tested, with 20 treated plants (spikelets removed) and 20 untreated plants per genotype, grown in a randomized complete block design. In 78 of the 80 treated plants, seed size was smaller on branches
from which PSs had been removed; one plant failed to set seed and in the other seed size was larger on the
branches with the PS removed. The overall effect of treatment was significant (p=0.000263), with seeds from
which the adjacent PS was removed being significantly smaller than those on unmanipulated plants (Figures 7E
and 7F, Supplemental Table 8). Differences among genotypes accounted for the greatest fraction (41%) of the
overall variance, which was expected given substantial differences in seed size, panicle architecture, and time to
maturity (Supplemental Table 8). Within any given genotype, however, treatment effects were highly
significant. Overall, the PS contributed 8.82% to seed weight, with values of 8.08, 9.72, 13.61 for each
individual genotype (Supplemental Table 8). The effect in accession 597971, 7.80%, was non-significant as a
result of highly variable seed filling in all plants and particularly high variance in the control plants; however,
the result was highly significant (p=8.73e-08) if only the manipulated plants were considered (Figure 7F).

**Bracts enhance carbon assimilation in wild species**

The contribution and position of photosynthetic organs to seed production was also considered by analyzing the
bracts in *Themeda triandra* and *Andropogon schirensis*, which are much closer to the spikelets than the flag
leaves of sorghum. Our $^{14}$C pulse-chase experiments tested whether these played a role in carbon assimilation.

In *Andropogon schirensis*, the upper modified leaf (“bract”) is reduced largely to a sheath, with only a short
blade (Figure 1E). As expected, this modified leaf assimilated $^{14}$C, although the difference in percent counts
between the pulse and chase for the attached bract was non-significant (p=0.9987; Figure 8A, Supplemental
Table 9). The leaf sheath bears stomata throughout (Figure 8B), as expected. Stem segments including the flag
leaf node and the lowermost inflorescence node contained some $^{14}$C but there was little signal from the
internode in between, suggesting that little carbon was being transported. The leaf is thus not a major source of
photosynthate for the inflorescence within the timeframe and conditions of this experiment (anthesis), and the
increased $^{14}$C in SSs during the chase was largely accounted for by the drop in PS values.

By contrast, the bract of *Themeda triandra* is closely connected to the spikelets, separated by an internode of
only 2–4.5 mm (Figure 1I). The bract accounted for 64–71% of the fixed $^{14}$C in the 1-hour pulse (Supplemental
Table 10), and that percentage dropped to 25% in the chase (p<0.0001), with a corresponding increase in the
amount in the SS (p<0.01, Figure 8C, Supplemental Table 10). The epidermal pattern of the bract is leaf-like,
with extensive stomata (Figure 8D). Results of $^{13}$C assimilation were qualitatively similar to those for $^{14}$C
(Figures 3E and 3F), and also similar to those for *Andropogon schirensis*, with the bract being significantly
different from the other three organs (Supplemental Tables 11 and 12). Thus, adding a leaf-like structure close
to the spikelets can enhance carbon assimilation in the form of PS or bract and relative contribution is
dependent on architecture and location.

**DISCUSSION**

Our data suggest that carbon capture in sterile spikelets may help compensate for the approximately 50%
reduction in meristems caused by having non-seed-bearing spikelets paired with seed-bearing ones, as
summarized in Figure 9. It is unexpected that one set of floral structures would contribute carbon to the
metabolism of other flowers; previously, flowers and inflorescences were reported only to contribute fixed
carbon to their own metabolism (e.g., (Bazzaz and Carlson, 1979; Vemmos and Goldwin, 1994; Antlfinger and
Wendel, 1997; Aschan and Pfanz, 2003; Earley et al., 2009)). Reproductive tissues of oilseeds are also often
green and may assimilate or re-assimilate carbon (King et al., 1998; Furbank et al., 2004; Ruuska et al., 2004;
Schwender et al., 2004; Goffman et al., 2005; Allen et al., 2009) and use photosynthetic light energy for ATP or
reductant production (Browne and Slack, 1985; Asokanthan et al., 1997; Ruuska et al., 2004; Rolletschek et al.,
A number of these studies focused on the contribution of sunlight to metabolically demanding processes such as fatty acid biosynthesis that generate large amounts of CO$_2$.

The PS exhibits autotrophic metabolism with labeled metabolites and transcript accumulation qualitatively similar to, but less significant than, a leaf. It can assimilate CO$_2$ whether attached or detached from other structures, consistent with the leaf-like epidermal morphology of the glumes, with stomata distributed over most of the surface (Figures 2B, 2F and 2J). The PS contributes 8–13% to yield (Figures 7E and 7F), an estimate consistent with earlier reports that photosynthesis from the entire sorghum inflorescence may account for 6–18% of yield (Fischer and Wilson, 1971; Evans and Wardlaw, 1976). We can now attribute that contribution to yield to a particular component of the inflorescence.

By contrast, the SS in sorghum appears to be largely heterotrophic at anthesis. Carbon assimilation is lower than in the PS, but still easily detected and stomata are much less common on the surface of the glumes (Figure 2C). Rubisco is strongly expressed in both bundle sheath and mesophyll cells, but only on the adaxial (internal) side of the glumes (Figure 6). Expression of transcripts encoding proteins involved in photosynthesis was low in the SS, whereas genes involved in starch synthesis were relatively high (Figure 5, Supplemental Figure 3). Decarboxylating enzymes, found in other experiments to be upregulated in tobacco (Nicotiana tabacum) stems (Hibberd and Quick, 2002), were not upregulated in the SS, providing no evidence for decarboxylation of transported four carbon compounds.

We hypothesized initially that awns in sorghum should be similar to those in wheat, barley, and rice (e.g., Bort et al., 1996; Li et al., 2006; Maydup et al., 2010; Sanchez-Bragado et al., 2014; Hu et al., 2018) and references therein), which are large, green, and provide photosynthate to the developing grain. However, no evidence of CO$_2$ assimilation was found in awns whether isolated or intact (Figure 2A), none of the metabolites were labeled (Figures 3 and 4), and no stomata were present (Figure 2D). While the awns exhibit distinct gene expression patterns (Figure 5, Supplemental Figures 2 and 3), they are generally more similar to the SS than to either the PS or the leaf or bract. Awns in Andropogoneae are derived independently from those in wheat, barley or rice (Teisher et al., 2017), and, as shown here, differ in structure and physiology.

The results for sorghum apply to other members of the tribe Andropogoneae, as shown by data on the unrelated species Andropogon schirensis and Themeda triandra. In both species, the SS is bisexual as in sorghum, has a firm outer glume, and bears an awn (Figure 1). The PS in these species is staminate (male). PSs in these species are photosynthetic and export carbon to the heterotrophic SS at anthesis (Figures 2E and 2F). The awns do not assimilate carbon and lack any evidence of carbon assimilation (Figures 2E and 2F, 3C-3F), although, as in sorghum, in Themeda triandra they produce awn-specific gene transcripts (Supplemental Figures 4, 5, and 6).

Because the structure of the spikelet pair is conserved among most of ca. 1200 species of Andropogoneae (maize being an exception)(Clayton and Renvoize, 1986; Watson and Dallwitz, 1992onwards; Veggetti, 1999; Kellogg, 2000, 2015; Arthan et al., 2017), we can infer that the function of the PS as a nurse tissue has been fixed and maintained by natural selection over ca. 15 million years of evolution. The proximity of PSs to seeds may enable delivery of photoassimilates during stress such as drought-induced reduction in transpiration, as described for wheat (Hu et al., 2018; Simkin et al., 2020).

The small photosynthetic PS boosts fitness or yield but placing a leaf-like structure in the inflorescence closer than the flag leaf appears to be even more favorable. The bract in Themeda triandra assimilates considerably more carbon than the PS and transfers that carbon to the SS (Figure 8C). Metabolically and anatomically it is leaf-like. The bract in Andropogon schirensis is also photosynthetically active but appears to transfer little carbon to the developing inflorescence (Figure 8A). This may reflect the distance of translocation within the
short time frames of these experiments. While the distance from the flag leaf to the panicle in sorghum is generally at least 30 cm and often twice that, the corresponding distance in Andropogon schirensis is 14–21 cm, and that in Themeda triandra is 2–4.5 mm (Figures 1E and 11).

Although the PS is undoubtedly photosynthetic, it appears to be neither wholly C₃ nor C₄. A number of photosynthetic intermediates were significantly labeled in the PS and the relative expression of photosynthetic enzymes is higher in the PS than the SS or awns, but lower than in leaves or bracts, respectively (Figure 5, Supplemental Figures 3, 5 and 6), though these data do not distinguish the mode of carbon assimilation (Hibberd and Furbank, 2016). Rubisco is present in the bundle sheath (as in a C₄ tissue) but also throughout the mesophyll (as in C₃ tissue) (Figure 6). Veins in the PS and SS are also separated by more than two cells (Figure 6), suggesting the possibility of a limited C₄ shuttle, as demonstrated in other inflorescence-related structures (Langdale et al., 1988; Pengelly et al., 2011). We also found labeled 2-phosphoglycolate in PSs and some unlabeled 2-phosphoglycolate in other organs indicating limited photorespiration, consistent with either C₃ or C₄ RuBisCO-based assimilation, though 2-phosphoglycolate phosphatase, an enzyme specific to the photorespiratory pathway, was not differentially expressed.

In summary, despite its small size, the PS in sorghum and Andropogoneae can make a significant contribution to yield by fixing carbon and translocating it to the SS, which holds the developing seed. Awns, in contrast, are carbon sinks, with limited metabolic activity. These results reflect millions of years of evolution, over which the spikelet pair has been selected and conserved. The PS may thus have contributed to fitness in natural populations and could also be a useful target for sorghum improvement in agricultural settings.

**METHODS**

**Plant material**

*Sorghum bicolor* accessions BTx623 (PI 564163), SO85 (PI 534096), Jola Nandyal (PI 534021), and SAP-170 (PI 597971) were obtained via the USDA Germplasm Resource Information Network (GRIN). All except SO85 are members of the Sorghum Association Panel (Casa et al., 2008). BTx623 is the line from which the reference genome sequence was obtained (Paterson et al., 2009; McCormick et al., 2018). Additional experiments were conducted on *Themeda triandra* (PI 208197) and *Andropogon schirensis* (Pasquet s.n.).

The three species represent different major clades of Andropogoneae (Estep et al., 2014). In addition, they differ in inflorescence structure. In *Sorghum bicolor*, the pedicellate spikelets are usually sterile, whereas in the other two species, the pedicellate spikelets are staminate (Figures 1A, 1C, and 1G). In *Themeda triandra*, the sessile spikelet is associated with two staminate, pedicellate spikelets and is subtended by two additional pairs of staminate spikelets for a total of six staminate spikelets per sessile bisexual spikelet (Figure 1F, 1G, and 1H). For the purposes of this experiment, all six were considered similar and were treated together. In addition, the set of spikelets in *Themeda triandra* is closely subtended and partially enclosed by a large bract (Figure 1I). In *Andropogon schirensis*, the uppermost leaf, which we refer to here as a bract, is reduced to a sheath and minimal blade (Figure 1E). The distance between the node of the leaf and the inflorescence node is 14–21 cm. *Sorghum bicolor* has no bracts or inflorescence-associated leaves. In all three species, only the sessile, seed-bearing spikelet bears an awn.

**Pulse-chase experiments, ¹⁴C**

*Labeling.* To determine which structures assimilated and fixed CO₂, we traced the localization of ¹⁴C in *Sorghum bicolor* SO85, *Themeda triandra*, and *Andropogon schirensis*. Plants were collected at anthesis in the greenhouse between 10 and 11 AM. Culms were cut with a razorblade and placed directly into tap water before
being transferred to 250 ml Erlenmeyer flasks containing filter paper and 1 mL of water, with two flasks per species. One flask (A) contained one (Themeda, Sorghum) or two (Andropogon) intact inflorescences, and was used for the 24-hour pulse-chase experiments. The second flask (B) for each species held one or two intact inflorescences and was used for the 1-hour pulse experiment. Flask B also contained an additional inflorescence dissected into sessile spikelets, pedicellate spikelets, awns, and bracts or leaves, to determine $^{14}$C assimilation without connection to the rest of the inflorescence. Each experiment used ca. 40–80 mg of tissue, which for Themeda triandra equated to approximately 15 awns, 22 bracts, 16–20 sessile spikelets, and 40 staminate spikelets. For Andropogon schirensis a similar amount of biomass required approximately 25 awns, 22 sessile spikelets, 30 staminate spikelets, or 1 to 2 leaves. For Sorghum bicolor we used 2.5 cm of one leaf, ~6–8 sessile spikelets, ~30 staminate spikelets, and all available awns from one inflorescence.

A plastic tube containing 12.5 microcuries of $^{14}$C sodium bicarbonate was placed in each flask and maintained in an upright position by attachment to a plastic rod. Flasks were capped with airtight septa closures and 1 mL 6 N H$_2$SO$_4$ was added directly to each plastic tube using a syringe, releasing a pulse of $^{14}$CO$_2$ into the flask. All samples were incubated for one hour in a growth chamber at ~350µE.

Processing. After one hour, all flasks were purged with air (30–60 sec) to remove the $^{14}$CO$_2$. The radioactive gas stream was captured in a reservoir containing 2L of 2N KOH. For flask A for each species, the airtight septum and closure were replaced with a sponge top prior to incubation in the growth chamber under continuous illumination for the 24-hour chase period. The contents of flask B were analyzed immediately. Detached awns, sessile and pedicellate spikelets, and leaves were separated, weighed, and transferred to tubes containing cold methanol:chloroform (7:3, v:v) and steel beads. Simultaneously, the intact inflorescence was dissected, and individual components treated identically to the detached samples. Tissues were homogenized at 30 cycles/second in a bead homogenizer for two 5-minute intervals and were stored at –20°C for 48 hours. After 24 hours, samples from flask A were dissected, weighed and processed in the same way and stored at –20°C for 24 hours.

All samples were extracted sequentially. The first extraction was based on 1.5 mL 7:3 (v:v) methanol:chloroform that was used to homogenize and store tissues after labeling. 200 uL from the methanol:chloroform extract was then combined with 5 mL of scintillation fluid (HIONIC FLUOR, Perkin Elmer). The remaining extract was removed from the residual biomass and water (2 mL) was added. The biomass was bead homogenized as before, centrifuged at low to medium speed (~5000 rpm) for several minutes at room temperature, and 200 uL was combined with 5mL scintillation fluid. Residual biomass was then treated with tissue solubilizer (ScintiGest), incubated overnight at 60°C and prepared for scintillation identically to prior extracts. Scintillation counting in disintegrations per minute (DPM) was performed on a Beckman Coulter LS-6000TA Scintillation Counter and included recording $^{14}$C photon emissions for five minutes. After background subtraction from a blank that contained identical amounts of solvent and scintillation cocktail, total radioactivity per amount of tissue was calculated by accounting for differences in volume and mass.

Analysis of $^{14}$C data. The total $^{14}$C assimilation (DPM/mg) for each organ was calculated by summing the counts from the three serial extractions and accounting for the total volumes of each extraction. Then the percent of label within awn, pedicellate and sessile spikelets was determined by calculating the fractional $^{14}$C assimilation in each organ. Experiments were repeated three times over a ten-month span using individual plants harvested from a greenhouse. Percent label for each organ was averaged across experiments and means and standard deviations plotted in MS Excel. Significance was assessed using analysis of variance (Type 1 ANOVA, balanced design).
**13C labeling in planta**

**Labeling.** 13C isotopic labeling studies were carried out on inflorescences of *Themeda triandra* and *Sorghum bicolor* accession SO85. *Themeda* and *Sorghum* plants were grown in the greenhouse until anthesis. The intact inflorescence was placed in a deflated plastic bag (inflated volume ca. 2–3 liters). A 10 mL serological pipet was fed into the bag with its tip near the apex of the inflorescence. The other end of the pipet was connected by hose to a tank of synthetic air comprising $^{13}$CO$_2$/N$_2$/O$_2$ at a ratio of 0.033:78:21.967. For each timed treatment, the bag was rapidly inflated (~15 L/min), and then the flow of gas was decreased to approximately 2 L/min. Structures were labeled for 30, 60 or 300 seconds. Three replicate experiments were done for each time point for each species. For sorghum, each replicate was a single inflorescence from a single plant, making a total of nine inflorescences (3 reps × 3 time points). For *Themeda*, each replicate included all flowering structures on a single tiller from a single very large plant, making a total of nine tillers (3 reps × 3 time points). All experiments were conducted in the morning between 10 and 11 AM to minimize circadian effects. The synthetic air pumped into the bag flowed from the release of the pipet at the tip along the inflorescence structures and exited the bag at the point where the bag opening was grasped around the culm. At the end of the labeling period the inflorescence was cut immediately and dropped in a large pool of liquid nitrogen in a Styrofoam box (22 × 33 × 15 cm) to quench metabolism. During labeling and quenching plant tissues were exposed to greenhouse light levels between 250–400 µE m$^{-2}$s$^{-1}$ from a combination of metal halide and high pressure sodium lights.

**Processing.** Frozen tissue was dissected in liquid nitrogen to separate sessile and pedicellate spikelets, awns, and bracts. Each sample was then ground in liquid nitrogen with a mortar and pestle, extracted with methanol:chloroform (7:3 v:v) and then through addition of water to segregate polar and non-polar phases as described previously (Ma et al., 2014; Ma et al., 2017). PIPES (12 nmol) was added to each sample as an internal standard.

**LC-MS/MS.** A QTRAP 6500 tandem mass spectrometer linked to two Shimadzu LC-20AD pumps working in coordination with a SIL-20AC/HT autosampler was used to assess and quantify the isotopic labeling in intermediates of primary metabolism. Extract from ca. 5% of the total harvested sample was injected. Standards for metabolites were run separately to establish retention time and, in some cases, confirm identification of isomers. Separation on LC involved an ion pair method (Ma et al., 2017) with a flowrate of 300 microliters/min and a binary gradient buffer combination with Buffer A: 11 mM acetic acid with 10 mM tributylamine as the ion pair and Buffer B: 100% methanol. The method differed from prior work as the ramp profile was shortened with 3 min equilibration at 5% B, 10 min ramp to 35% B, 2 min ramp to 95% B, hold for 3 min, return to 5% B within 2 min, and equilibration there for 11 min resulting in a total run time of 31 min. The source inlet temperature (550°C), curtain gas (35 psi) and auxiliary gases (both set to 60 psi) were chosen based on optimal peak response. Declustering potential, collision energy and collision exit potential for individual mass transition pairs of multiple reaction monitoring were based on prior work (Ma et al., 2014; Ma et al., 2017).

**Analysis of mass spectrometry data.** Peak intensities for individual mass traces that represent a precursor-product ion combination were measured using Analyst 1.6 (AB Sciex). The relative percent combination of isotopologues was calculated, as well as 13C average labeling that is defined as $\left(\frac{1}{n}\sum_{i=0}^{n} m_i \cdot i \right)$ with n equal to the number of carbons and $m_i$ defining the relative isotopologue abundance for each of the $i$ isotopologues measured. Estimates were corrected for natural abundance, following (Fernandez et al., 1996) and using the INCA computational platform (Young, 2014). Data were additionally compared through plots of the isotopologue distributions and 13C atom equivalents formed. All isotope labeling plots included standard deviations. Average labeling data was initially analyzed in raw, uncorrected form with PCA to assess
reproducibility of results. Statistical significance of results was assessed with ANOVA (Type 1, balanced design). Both PCA and ANOVA used standard programs in R, as presented in the text and figures.

Estimate of $^{13}$C atom equivalents formed: The $^{13}$C average labeling and $^{13}$C isotopologue distributions reflect the dynamics of isotope incorporation into different organs, and capture more information than static measurements of metabolite levels alone; however, relative labeling comparisons cannot fully account for the variation in biomass composition and differences in active and inactive pools between organs that dilute the rate of $^{13}$C labeling. Full comparisons of one metabolite to another in the strictest sense require a computational flux model, which would be technically challenging and well beyond the scope of this study given the small organs investigated here. In Supplemental Figure 1, the $^{13}$C atom equivalent plots provide a comparison of individual metabolites across the three organs compared in this study. The $^{13}$C isotopologues were normalized by the ratio of total metabolite peak signal to the median peak signal of all metabolites examined within that run, then weighted for the number of carbons per isotopologue and summed to get atom equivalents.

Malate active pool estimates: The unlabeled fraction in malate leveled off by 60s and did not vary significantly to 300s indicative of an inactive pool (Ma et al., 2014; Arrivault et al., 2017; Allen and Young, 2020). The active pool of malate was calculated by subtracting the unlabeled fraction that remains at long time points from all unlabeled fractions during the pulse and rescaling the unlabeled and labeled isotopologues.

Rubisco immunolocalization
Sorghum spikelets at anthesis were fixed and sectioned with the same method used for histology (Ruzin, 1999). Immunofluorescence assays were adapted from Lucas et al. (Lucas et al., 1995). Specifically, sections were deparaffinized with histoclear for 10 min twice, and rehydrated with 100%, 100%, 90%, 70%, 50% and 30% ethanol. After brief washing in water and PBS, the slides were treated with 0.1 mg/ml Proteinase K solution in PBS buffer for 10 min at room temperature, washed in PBS for three times (5 min each), and incubated in blocking reagent (2% dry milk in PBS) for 2h at room temperature. The slides were then probed with anti Rubisco large subunit (rabbit) (AS03 037, Agrisera, Vännäs, Sweden) with 1:250 dilution for 4 h at room temperature, followed by two washes with blocking reagents (the second wash was overnight at 4°C). Samples were then incubated with goat anti-rabbit IgG secondary antibody, Alexa Fluor 488 (ThermoFisher, USA), with 1:100 dilution for 2 h at room temperature, followed by two washes in blocking reagent for 15 min each, and stored in PBS buffer.

Fluorescence was obtained using a 20X HC PL APO (N.A. 0.7) objective lens on an inverted Leica TCS SP8-X confocal microscope (Leica Microsystems) using the 405, 499 and 649 nm laser excitation lines. Alexa Fluor 488 labeled Rubisco signal was detected by PMT3 detector using an emission window of 509–585 nm. Lignin and chlorophyll autofluorescence were detected in parallel by HyD2 detector using emission windows of 415–485 nm and 661–779 nm, respectively. Bright field images were obtained through the transmitted light detector. Images from individual channels were merged using Leica Application Suite software.

Sorghum spikelet removal experiments
Removal of pedicellate spikelets. The impact on grain weight of removing pedicellate spikelets was tested in four accessions (genotypes) of *Sorghum bicolor*: SAP-170 (PI 597971), BTx623 (PI 564163), Jola Nandyal (534021), and SO85 (PI 534096), referred to in this manuscript by their PI numbers except for BTx623, which is the line used for the reference genome sequence. 40 seedlings of each genotype, total 160 plants, were grown in individual pots in a greenhouse at the Donald Danforth Plant Science Center in 2019, under metal halide and high pressure sodium lights set to 14-hour days, 28°C daytime and 22°C nighttime temperatures, and relative humidity 40% days and 50% nights. For each accession, 20 plants were left unmanipulated (designated “on”, or control plants). The other 20 plants were manipulated (designated “off”). When the plants reached anthesis,
pedicellate spikelets were removed with forceps from alternating branches of the treated plant, which thus had a mix of treated and untreated branches (designated “off-off” and “off-on” respectively). By removing pedicellate spikelets from alternating branches, variation along the inflorescence was averaged out. The untreated branches of the manipulated plants (“off-on”) thus served as an internal control. Treated branches were marked. Plants were grown to maturity, the inflorescence removed and dried at 40°C for 4–5 days, and spikelets harvested. Caryopses (“seeds”) were removed from the glumes and floral bracts.

**Data analysis:** Seeds for genotypes BTx623 and 534021 were counted with a Seedburo 800 Count-a-Pak seed counter at the USDA in Columbia, Missouri. Seeds for genotypes 597971 and 534096 were too small for reliable counts on the seed counter, so were spread on a flatbed scanner (Epson Perfection V550 or Epson WorkForce DS-50000, 300dpi resolution, greyscale), imaged and weighed. The resulting JPG files were converted to binary images using Fiji (Abramoff et al., 2004) and the Analyze Particles tool used to count the seeds in each image. Total seed weight was divided by the number of seeds to estimate the weight of 100 seeds. The statistical effect of pedicellate spikelet removal on seed weight was assessed using a mixed effect linear model (**lmer** function in R packages **lme4** (Bates et al., 2014) and **lmerTest** (Kuznetsova et al., 2015), where block, row, and column were specified as fixed effects, and genotype was treated as a random effect. All data were visualized using **ggplot2** (Wickham, 2016).

**Removal of awns:** Preliminary experiments found that the effect of awn removal was appreciably less that than of spikelet removal, with a non-significant effect of 1% or less depending on genotype. Limited resources prevented us from carrying out an awn removal experiment large enough to demonstrate whether an effect that small actually exists.

**Scanning electron microscopy (SEM)**

Plant material was fixed in formalin:acetic acid:50% alcohol (10:5:85 by volume)(FAA) for a minimum of 24 hours and then transferred to 50% ethanol. Material was then taken through a standard ethanol dehydration series (70,80,90,95,100,100,100% EtOH) with at least 24 hours per stage. Samples were dried in a SamDri-780 critical point drier at the Washington University Center for Cellular Imaging, coated with gold palladium, and imaged on a Zeiss Merlin Field Emission SEM at 5.0 kV. Photographs were adjusted for brightness, contrast, and input levels using Adobe Photoshop.

**RNA-Seq**

To complement $^{13}$C mass spec measurements of metabolites, expression levels for genes controlling photosynthesis and sugar and starch metabolism were estimated with RNA-seq. As with the $^{13}$C experiments, material of *Sorghum bicolor* and *Themeda triandra* was harvested at anthesis and immediately frozen in liquid nitrogen and dissected while frozen. Material was harvested between 10 and 11 AM to minimize circadian effects. For sorghum, mature leaves and inflorescences were harvested from three plants and used as individual biological replicates. Inflorescences were hand dissected into pedicellate spikelet, sessile spikelet, and awn samples. For *Themeda triandra*, inflorescences from three tillers from one individual were dissected into bract, pedicellate spikelet, sessile spikelet, and awn, each tiller representing a biological replicate. Each of the 24 samples (2 species × 4 organs × 3 biological replicates) was individually ground to a fine powder using liquid nitrogen. To extract total RNA, ca. 100 mg of tissue was added to 1 mL TRIzol reagent (15596026, Thermo Fisher Scientific) and the samples were vortexed. Next, 0.2 mL of chloroform was added, the samples were shaken for 15 s and incubated at room temperature for 3 min followed by centrifugation at 13,000 g for 15 min at 4°C and the upper aqueous phase was removed and added to 0.5 mL of isopropanol. The sample was incubated on ice for 10 min and centrifuged as above to obtain a nucleic acid pellet. The pellet was washed in 70% (v/v) ethanol, air-dried briefly, and dissolved in 100 μL DEPC-treated water. Total RNA was cleaned and concentrated using an RNeasy kit (74104, QIAGEN) including on-column DNase treatment (79254, QIAGEN).
Total RNA was quantified with a Qubit RNA HS kit (Q32852, Thermo Fisher Scientific). At least one sample for each organ was run on a RNA Pico Bioanalyzer microfluidics chip (Agilent) to calculate RNA integrity numbers (RIN). All RIN were in the range 7.8–9.5.

The 24 RNA sequencing (RNA-Seq) libraries were prepared using SENSE mRNA library prep kit V2 for Illumina (001.24, Lexogen) with 500 ng input for all samples except for the three Themeda awn samples (160, 420, and 350 ng, respectively). The protocol was adjusted to produce a mean insert size of ~413 bp. Each library was indexed uniquely and amplified by 12 cycles of PCR for all samples except for the three Themeda awn samples, which were amplified using 14 cycles. Final libraries were quantified using a Qubit DNA HS kit (Q32854, Thermo Fisher Scientific). Select libraries were also run on a High Sensitivity DNA Bioanalyzer microfluidic chip (Agilent) to confirm library size in the desired range (~400 bp). Libraries were pooled by organism (3 biological replicates × 4 organs, each), resulting in 2 pools of 12 libraries at ~0.8 nM per library (10 nM final pool concentration). These pools were sequenced using Illumina HiSeq4000 paired end (2 x 150 bp) technology at Michigan State University Genomics facility (https://rtsf.natsci.msu.edu/genomics/sequencing-services/).

Data analysis. See Supplemental Table 13 for numbers of transcripts at each step.

a) Read-mapping in sorghum. Reads for Sorghum bicolor were trimmed using Trimmomatic (Bolger et al., 2014) and mapped to the sorghum reference genome (Phytozome, Sbicolor_454_v3.1.1) using HISAT (Kim et al., 2015). To create expression level analysis, htseq-count was used to count the number of reads per gene for each sample (Anders et al., 2015).

b) Read-mapping and identification of putative orthologs in Themeda. Reads for Themeda triandra were downloaded and submitted to Trinity (Grabherr et al., 2011) for automated trimming, quality filtering, assembly, and expression quantification. CDS and peptides were extracted using trandecoder (Haas et al., 2013). Putative orthologs of the metabolic genes in sorghum were identified using OrthoFinder (Emms and Kelly, 2015) on the longest isoform of peptide sequences from the sorghum and maize genomes, and the predicted Themeda peptides. If an orthogroup contained a sorghum metabolism gene as identified in the sorghum transcriptome analysis, the Themeda peptides in that group were blasted against the sorghum peptides. If the Themeda gene’s best match was a sorghum metabolism gene it was considered that gene’s putative ortholog. The list of Themeda metabolism genes generated from this approach was then used for downstream analyses. Read counts per gene were generated with htsq-count (Anders et al., 2015).

c) Normalization for library size and log2. All normalization was performed in R (RCoreTeam, 2013). Raw transcript counts for each sample were normalized for library size across all samples for Sorghum bicolor and Themeda triandra using the calcNormFactors function in the edgeR package (Robinson et al., 2010) and filtered to remove transcripts with fewer than 1 normalized read count in at least 3 samples, effectively discarding unexpressed transcripts. Next, library size-normalized read counts were log2-transformed. This step led to 17232 unique transcripts in sorghum and 20826 in Themeda.

d) Construction of the metabolic gene set. We developed a custom list of genes involved in carbon metabolism to enable comparison between the RNA-seq data and the carbon isotope data. Manually annotated pathway descriptions are based on the Kyoto Encyclopedia of Genes and Genome (KEGG) pathway (Kanehisa, 2019; Kanehisa and Goto, 2000; Kanehisa et al., 2019) and biochemical textbook descriptions (Buchanan et al., 2002) supported by comparative organ expression profile data. Enzyme Commission (EC) numbers of enzymes involved in carbon metabolism were retrieved from the KEGG pathway database (https://www.genome.jp/kegg/kegg3a.html) for glycolysis/gluconeogenesis, the tricarboxylic acid (TCA) cycle, pentose phosphate pathway, pentose glucuronate interconversions, fructose/mannose interconversions, galactose metabolism, starch sucrose metabolism, sugar nucleotide metabolism, pyruvate metabolism, glyoxylate/dicarboxylate metabolism, oxidative phosphorylation, photosynthesis, Calvin Benson cycle, etc. In instances where an enzyme could not be unambiguously assigned to a pathway, TargetP (Emanuelsson et al.,
2007), a protein localization prediction tool, was used to determine possible organellar targeting and to guide descriptions. Genes not obviously targeted to chloroplasts or mitochondria were assumed to be cytoplasmic. A subset of C₄ genes was identified based on phylogenetic literature and comparative genomic analysis (Wang et al., 2009; Huang et al., 2017). Expression levels of genes encoding these enzymes for each species and tissue were then extracted from the full transcriptomes using custom Python scripts (https://github.com/ekellogg-lab/pedicellate-spiketlet-carbon), giving us a total of 3505 loci related to carbon metabolism. The intersection of this list with the log₂ normalized filtered transcripts left us with 1441 transcripts in sorghum and 769 in Themeda. We refer to this list as our set of metabolic genes.

e) Identification of DE genes. Differential expression analysis was performed in R (RCORETEAM, 2013). Tukey tests were performed on the expression values of the metabolic gene set using the function TukeyHSD in conjunction with ANOVA (Analysis of Variance) to generate all possible pairwise organ comparisons for each transcript in our metabolically-relevant gene list. The p-values generated by this approach were additionally corrected for multiple testing using p.adjust with method set to “BH” (Benjamini-Hochberg). 922 DE genes were identified in sorghum with at least one significant pairwise organ comparison (BH-corrected p-value < 0.05), and 322 DE genes were identified in Themeda.

f) Data display. All normalized transcripts and the normalized set of metabolic genes were analyzed by Principal Component Analysis (PCA) using the function prcomp with log₂-transformed expression values, and visualized with functions in the ggplot2 package (Wickham, 2016). DE genes were displayed as expression heatmaps and were plotted using pheatmap (Kolde, 2015) with the ward.D2 clustering option and row scaling.

g) Genes to compare to ¹³C data. To connect the RNA-seq and ¹³C data directly, we generated a list of 36 enzymes that could produce the metabolites we assayed with ¹³C; some of these are encoded by more than one gene. We then used this list of enzymes to generate a small focused subset of the DE metabolic genes, giving us 52 and 24 DE genes in sorghum and Themeda, respectively.

Data availability: Raw reads from the RNA-seq experiment have been deposited at the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (accession numbers SAMN15618964 - SAMN15618987; BioProject ID PRJNA648104). Data matrices for downstream analyses have been deposited at Dryad (datadryad.org; doi.org/10.5061/dryad.jsxksn06v).

Accession Numbers See Supplemental dataset 1.

Supplemental Data
Supplemental Figure 1. ¹³C atom equivalents produced over time.
Supplemental Figure 2. Sorghum bicolor, PCA of gene expression data.
Supplemental Figure 3. Sorghum bicolor, heat map of the 922 differentially expressed metabolic genes, by organ.
Supplemental Figure 4. Themeda triandra, PCA of gene expression data for the 769 select metabolic genes.
Supplemental Figure 5. Themeda triandra, heat map of 322 differentially expressed metabolic genes, by organ.
Supplemental Figure 6. Themeda triandra, relative expression of 24 genes encoding biosynthetic enzymes immediately responsible for producing the metabolites labeled with ¹³C.
Supplemental Figure 7. Immunolocalization, negative control with Alexa 488 secondary antibody only and no Rubisco antibody.
Supplemental Table 1. Sorghum bicolor, ¹⁴C data and analyses for pulse-chase experiments.
Supplemental Table 2. Andropogon schiresis, ¹⁴C data and analyses for pulse-chase experiments.
Supplemental Table 3. Themeda triandra, ¹⁴C data and analyses for pulse-chase experiments.
Supplemental Table 4. Sorghum bicolor, ¹³C data, p values for ANOVA (Type 1, two way), average labeling.
Supplemental Table 5. *Sorghum bicolor*, $^{13}$C data, p values for ANOVA (Type 1, two way), isotopomers of each metabolite analyzed separately.

Supplemental Table 6. *Themeda triandra*, $^{13}$C data, p values for ANOVA (Type 1, two way), average labeling.

Supplemental Table 7. *Themeda triandra*, $^{13}$C data, p values for ANOVA (Type 1, two way), isotopomers of each metabolite analyzed separately.

Supplemental Table 8. General linear model for effect of removal of pedicellate spikelet.

Supplemental Table 9. *Andropogon schirensis*, $^{14}$C data and analyses for pulse-chase experiments, including bract.

Supplemental Table 10. *Themeda triandra*, $^{14}$C data and analyses for pulse-chase experiments, including bract.

Supplemental Table 11. *Themeda triandra*, including the bract; $^{13}$C data, p values for ANOVA (Type 1, two way), average labeling.

Supplemental Table 12. *Themeda triandra*, including bract, $^{13}$C data, p values for ANOVA (Type 1, two way), isotopomers of each metabolite analyzed separately.

Supplemental Table 13. Numbers of unique transcripts after successive filters and related figures.

Supplemental Data set 1. Expression values of genes.

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Figure 1. Spikelet pair structures. *Sorghum bicolor* (A, B), *Andropogon schirensis* (C, D, E), and *Themeda triandra* (F, G, H, I). A, C, G. Spikelet pairs, marked by dashed lines. Sex expression of each spikelet is indicated. The sessile spikelet includes a bisexual flower and bears the seed, and also bears a twisted awn from the lemma (floral bract). A. Terminal spikelet (sorghum) is morphologically identical to a pedicellate spikelet. Pedicellate spikelet may be either sterile (most commonly) or staminate. B. Spikelet pair of sorghum accession SAP-15 (PI 656014). C. Pedicellate spikelet is staminate. D. Spikelet pair of *Andropogon schirensis*. E. Inflorescence of *Andropogon schirensis*, showing two branches, each bearing 9–10 spikelet pairs. The uppermost leaf bears a reduced blade (arrow). F. Spikelet pair of *Themeda triandra*, showing the dark indurate sessile spikelet, with two greenish pedicellate spikelets behind. G. Inflorescence structure in *Themeda triandra*, with three spikelet pairs and a terminal spikelet that is morphologically identical to the pedicellate spikelet. Spikelets in the proximal two pairs (both pedicellate and sessile) are all staminate. H. Proximal spikelet pairs of *Themeda triandra*, here called PS and STS since all four are staminate. I. Inflorescence branch of *Themeda triandra*, showing the spikelet complex as in G, subtended by a leaf-like bract. SS, sessile spikelet; PS, pedicellate spikelet; STS, staminate sessile spikelet. Scale bars B, D, F, H = 1 mm; E = 1 cm; I = 5 mm. T.A. and E.A.K. took the photos; E.A.K. created the figures.
Figure 2. Results of $^{14}$C pulse-chase experiments and distribution of stomata. *Sorghum bicolor* (A–D), *Andropogon schirensis* (E–H), and *Themeda triandra* (I–L). A, E, I. Percent dpm/mg for each organ after 1-hour exposure to $^{14}$C with organs removed from the axis (detached), inflorescence intact (attached), or after 24-hour chase (chase). Plot includes mean percentages and standard deviations (n=3). B, F, J. Abaxial epidermis of pedicellate spikelets showing rows of stomata (arrows) and bicellular microhairs (m), and prickles (p). C, G, K. Abaxial epidermis of sessile spikelet showing no stomata, but bicellular microhairs (m) and silica bodies (sb), as well as large pits (pit) and macrohairs (mac) in *Themeda triandra* (K). D, H, L. Awn showing no stomata or other epidermal structures, except for prickles in sorghum (D) and *Andropogon schirensis* (H), and long microhairs in *Themeda triandra* (L). Scale = 50 µm; note that B and C are more highly magnified than D. For panels A, E, and I, T.A., D.K.A., and E.A.K. did the experiments, T.A. ran the assays, D.K.A. and E.A.K. did the data analysis and created the figures. E.A.K. took the images for the other panels (SEM).
Figure 3. Principal components analysis of $^13$C labeled metabolites. *Sorghum bicolor* (A, B) and *Themeda triandra* (C–F). Values for awn and SS are not significantly different in either species for any time point, nor for most individual metabolites. A, C, E. Average labeling. Each point is the weighted average of all labeled isotopologues for a given metabolite, organ, and time point. B, D, F. All isotopologues. A. Values for PS are significantly different from the other organs. See Supplemental Table 4. B. Awn and SS are significantly different for P5P and UDPG but not otherwise; PS is significantly different from the other two organs except for pyruvate. Values at 30 and 60 seconds not significantly different for most metabolites but the 300 second time point is distinct. See Supplemental Table 5. C. Values for PS are significantly different for six of nine metabolites, with the greatest difference in labeling at 300 sec. See Supplemental Table 6. D. Awn and SS are significantly different for P5P but not otherwise; PS is significantly different from the other two organs except for P5P, PGA, and PYR. Values at 30 and 60 seconds are similar for most metabolites but the 300 second time point is distinct. See Supplemental Table 7. E, F. Values including bract; data for awn, SS and PS are the same as those in C. E. Bract is significantly different from all other organs for six out of eight metabolites. Values for PS are significantly different from awn and SS for only ASP and PYR. The 300 sec time point is significantly different from others. See Supplemental Table 11. F. Results are similar to those for average labeling, with bract being significantly different from all other organs for six out of eight metabolites and isotopologues, and the greatest difference in labeling occurring at 300 sec. See Supplemental Table 12. Organs are distinguished by color, and time points by shape. A, awn; SS, sessile spikelet; PS, pedicellate spikelet; 30, values at 30 sec of labeling; 60, values at 60 sec of labeling; 300, values at 300 sec of labeling. T.A., D.K.A., and E.A.K. did the experiments, T.A., D.K.A. and V.C. generated the LC-MS/MS data, D.K.A. and V.C. did the data analyses, V.C. and E.A.K. created the figures.
Figure 4. $^{13}$C labeling for individual metabolites at three time points. A. $^{13}$C isotopologue distribution. B. Average labeling. 2PG, 2-phosphoglycolate; ADPG, ADP-glucose; F6P, fructose-6-phosphate; G6P, glucose-6-phosphate; MAL, malate; PGA, phosphoglycerate; P5P, pentose 5 phosphates; PYR, pyruvate; TP, triose phosphates; UDPG, UDP-glucose. Points are mean fractions for isotopologue distributions and average labeling, bars are standard deviations, n=3. Colors distinguish the three organs. Most label accumulation occurs in the pedicellate spikelet and can be seen at 300 seconds. Insets represent the calculation of the active pool for malate. T.A., D.K.A., and E.A.K. did the experiments, T.A., D.K.A. and V.C. generated the LC-MS/MS data, D.K.A. did the data analyses, D.K.A. and E.A.K. created the figures.
**Sorghum bicolor**

Relative expression of select metabolic genes

| enzyme       | pathway (location) |
|--------------|--------------------|
| 1) UTP−G1P UdT | sucrase (cy)       |
| 2) Starch bio  | starch (cy)        |
| 3) Starch bio  | starch (ch)        |
| 4) Starch bio  | starch (ch)        |
| 5) Rubisco ssu | Calvin-Benson (ch) |
| 6) F16BP aldo  | glycolysis (cy)    |
| 7) F16BP aldo  | glycolysis (mi)    |
| 8) GDH         | OA/AA (cy)         |
| 9) GS          | OA/AA (ch)         |
| 10) PGM        | glycolysis (mi)    |
| 11) SuSY       | sucrose (cy)       |
| 12) F16BPase   | glycolysis (cy)    |
| 13) PPE        | Calvin-Benson/OPPP (cy) |
| 14) TPI        | Calvin-Benson (ch) |
| 15) Starch bio | starch (ch)        |
| 16) G1P−AdT    | starch (ch)        |
| 17) PGI        | glycolysis (ch)    |
| 18) S17BPase   | Calvin-Benson (ch) |
| 19) PGK        | Calvin-Benson (ch) |
| 20) PPI        | Calvin-Benson (ch) |
| 21) Starch bio | starch (ch)        |
| 22) NADP−ME    | C4 (ch)            |
| 23) NADP−MDH   | C4 (ch)            |
| 24) F16BP aldo | glycolysis (cy)    |
| 25) PEPC       | OA/AA (cy)         |
| 26) SuSY       | sucrose (cy)       |
| 27) PEPC       | C4/OA/AA (ch)      |
| 28) GAPDH      | glycolysis (cy)    |
| 29) PPE        | OPPP (cy)          |
| 30) PEPC       | OA/AA (cy)         |
| 31) UTP−G1P UdT| sucrase (cy)       |
| 32) G1P−AdT    | starch (ch)        |
| 33) TPI        | glycolysis (ch)    |
| 34) F16BP aldo | glycolysis (cy)    |
| 35) ALAAT      | alanine (cy)       |
| 36) PEPC       | OA/AA (mi)         |
| 37) F16BP aldo | glycolysis (cy)    |
| 38) Starch bio | starch (ch)        |
| 39) PPI        | OPPP (cy)          |
| 40) PPI        | OPPP (cy)          |
| 41) TK         | OPPP (ch)          |
| 42) NAD−GS     | OA/AA (ch)         |
| 43) F16BP aldo | glycolysis (cy)    |
| 44) PEPC       | OA/AA (cy)         |
| 45) PEPC       | OA/AA (cy)         |
| 46) TK         | OPPP (ch)          |
| 47) F16BP aldo | glycolysis (cy)    |
| 48) PGM        | glycolysis (ch)    |
| 49) Starch bio | starch (ch)        |
| 50) Starch bio | starch (ch)        |
| 51) GAPDH      | glycolysis (ch)    |
| 52) GAPDH      | glycolysis (ch)    |
Figure 5. *Sorghum bicolor*. Relative expression of genes encoding biosynthetic enzymes immediately responsible for producing the metabolites labeled with $^{13}$C, a subset extracted from full set of 922 DE metabolic genes in Supplemental Figure 3. Colors reflect scaled z-scores of log2-normalized expression values. Labels of genes indicate enzyme name, biochemical process, and subcellular localization. SS, sessile spikelet; PS, pedicellate spikelet; cy, cytosolic localized; ch, chloroplast localized; mi, mitochondrial localized; OA/AA, Organic Acid/Amino Acid metabolism; 1) UTP-G1P UdT, UTP−glucose-1-phosphate−uridyltransferase, Sobic.002G291200.1; 2) Starch synthase, Sobic.001G239500.2; 3) Starch synthase, Sobic.010G047700.1; 4) Starch synthase, Sobic.002G116000.1; 5) Ribulose-1,5-bisphosphate carboxylase/oxygenase, small subunit, Sobic.005G042000.1; 6) F16BP aldo, Fructose-1,6-bis-phosphate aldolase, Sobic.005G056400.1; 7) F16BP aldo, Fructose-1,6-bis-phosphate aldolase, Sobic.008G053200.1; 8) GDH, glutamate dehydrogenase, Sobic.003G188400.1; 9) GS, Glutamine synthetase, Sobic.006G249400.1; 10) PGI, Phosphoglucoisomerase, Sobic.002G116000.1; 11) SuSY, sucrose phosphate synthase, Sobic.004G068400.1; 12) F16BPase, fructose-1,6-bisphosphatase, Sobic.003G367500.1; 13) PPE, Phosphopentose epimerase, Sobic.001G491000.1; 14) TPI, triose phosphate isomerase, Sobic.002G277100.1; 15) Starch synthase, Sobic.006G221000.1; 16) G1P-AdT, glucose-1-phosphate adenyltransferase, Sobic.007G101500.1; 17) PGK, Phosphoglycerate kinase, Sobic.009G183700.1; 18) S17BPase, sedoheptulose 1,7-bisphosphatase, Sobic.003G359100.1; 19) PK, Phosphoketolase kinase, Sobic.009G183700.1; 20) PPI, phosphopentose isomerase, Sobic.001G069000.1; 21) Starch synthase, Sobic.004G238600.1; 22) NADP−ME, NADP-malic enzyme, Sobic.003G306200.1; 23) NAD−MDH, NADP-malate dehydrogenase, Sobic.007G166300.1; 24) F16BPase, fructose-1,6-bisphosphatase, Sobic.010G18300.1; 25) PEPC, phosphoenol pyruvate carboxykinase, Sobic.004G338000.1; 26) SuSY, sucrose phosphate synthase, Sobic.003G403300.1; 27) PEPC, phosphoenol pyruvate carboxykinase, Sobic.010G160700.1; 28) GAPDH, glyceraldehyde-3-phosphate dehydrogenase, Sobic.005G159000.1; 29) PPE, Phosphopentose epimerase, Sobic.002G257300.1; 30) PEPC, phosphoenol pyruvate carboxykinase, Sobic.004G106900.1; 31) UTP−G1P UdT, Sobic.006G213100.1; 32) G1P-AdT, glucose−1-phosphate adenyltransferase, Sobic.002G160400.1; 33) TPI, triose phosphate isomerase, Sobic.003G072300.2; 34) F16BP aldo, Fructose-1,6-bis-phosphate aldolase, Sobic.004G146000.1; 35) ALAAT, alanine amino transferase, Sobic.001G260701.1; 36) PEPC, phosphoenol pyruvate carboxykinase, Sobic.006G198400.2; 37) F16BP aldo, Fructose-1,6-bisphosphatase, Sobic.003G393900.1; 38) Starch synthase, Sobic.007G068200.1; 39) PPI, Phosphopentose isomerase, Sobic.003G182400.1; 40) PPI, phosphopentose isomerase, Sobic.008G135701.1; 41) TK, transketolase, Sobic.010G024000.2; 42) NAD−GS, Sobic.003G258800.1; 43) F16BP aldo, Fructose-1,6-bisphosphatase aldolase, Sobic.003G096000.2; 44) PEPC, phosphoenol pyruvate carboxykinase, Sobic.003G301800.1; 45) PEPC, phosphoenol pyruvate carboxykinase, Sobic.002G167000.1; 46) TK, Transketolase, Sobic.009G062800.1; 47) F16BP aldo, Fructose-1,6-bisphosphatase aldolase, Sobic.009G242700.1; 48) PGM, Phosphoglucomutase, Sobic.001G116500.1; 49) Starch synthase, Sobic.010G022600.1; 50) Starch synthase, Sobic.010G022600.1; 51) GAPDH, glyceraldehyde-3-phosphate dehydrogenase, Sobic.004G056400.1; 52) GAPDH, glyceraldehyde-3-phosphate dehydrogenase, Sobic.004G205100.1.

T.A., D.K.A., and E.A.K. collected the samples, T.A. prepared RNA for sequencing, T.A., D.M.G. and V.C. analyzed the data, V.C. did the statistical analyses, V.C. and E.A.K. created the figures.
Figure 6. Immunofluorescence of Rubisco large subunit (RbcL) in sorghum spikelets. A,C,E,G,I. Cross section of glume of pedicellate spikelet (PS). B,D,F,H,J. Cross section of glume of sessile spikelet (SS). A, B. Brightfield. C, D. Anti-RbcL with Alexa 488 as the secondary antibody; emission window 509–585 nm. E,F. Lignin autofluorescence; emission window 415–485 nm. G, H. Chlorophyll autofluorescence; emission window 661–779 nm. Autofluorescence in sclerenchyma in H is non-specific. I, J. Merge of Rubisco, chlorophyll and lignin channels. Black arrowheads, vascular bundles; inner (adax), facing the inside of the spikelet; outer (abax), facing the outside of the spikelet; bs and white arrows, bundle sheath; scl, sclerenchyma. Scale bars = 50 µm. Sectioning, immunolocalization and photomicroscopy by Y.Y.
Figure 7. Spikelet removal experiments. A–D, representative spikelet pair for each accession. A. Jola Nandyal (534021). B. SO85 (PI 534096). C. SAP-170 (PI 597971). D. BTx623 (PI 564163). Scale bar = 1 mm. All spikelets to the same scale. E,F. Results of removal experiments. 100-seed weight (g) from control (unmanipulated) plants, and manipulated plants. In the latter plants, pedicellate spikelets from some branches were untouched (on) and those from other branches were removed (off). E. Results from all genotypes combined, showing an 8.82% reduction in average weight with spikelet removal. F. Average seed weight for each genotype analyzed separately. Effect sizes for 534021, 534096 and BTx623 were 8.08, 13.61, and 9.72, respectively. Effect size for 597971, 7.80%, was non-significant because of high variance in the control plants; however, a comparison of seeds from only the manipulated plants (bracketed) was highly significant. *** = p < 0.001; * = p <0.05. Box plot center line, median; upper and lower limits of boxes, 1st and 3rd quartiles; whiskers, up to (down to) 1.5x the interquartile range; points, outliers. See also Supplemental Table 8. ps, pedicellate spikelet; ss, seed-bearing spikelet, a, awn. Images in panels A–D by T.A., experiment conducted by T.A., L.M.J., Y.Y., D.K.A., and E.A.K., data analysis by V.C., box plots by V.C. and E.A.K.
Figure 8. Results of $^{14}$C pulse-chase experiments and distribution of stomata in bracts. *Andropogon schirensis* (A, B) and *Themeda triandra* (C, D). A, C. $^{14}$C results including bract. Percent dpm for each organ after 1-hour exposure to $^{14}$C with organs removed from the axis (detached), inflorescence intact (attached), or after 24-hour chase. Plot includes mean percentages and standard deviations; $n=3$. A. Values for bracts and PS are significantly lower and higher, respectively, when attached to the axis rather than detached, whereas values for SS did not differ significantly. Percent counts in the bract were not significantly different after the 24-hour chase as compared to attached, but were significant relative to detached. Percentages in the PS significantly decreased relative to attached but not relative to detached. See also Supplemental Table 9. After 1 hour, most counts are in the bract when organs are detached from the stem but in the PS when they are attached. C. Values for detached and attached organs after 1 hour are not significantly different. Values for bract and SS significantly decrease and increase, respectively, after the 24-hour chase. Values for PS are not significantly different. See also Supplemental Table 10. B, D. Abaxial epidermis of bract showing rows of stomata (arrows) and prickles (p). Scale = 50 µm. Author contributions as in Figure 2.
Figure 9. Summary of biochemical pathways assessed by $^{13}$C and RNA-seq data. Numbered dots correspond to enzymes whose expression is shown in Figure 5. Colored squares reflect percent average label in $^{13}$C assays of metabolites. Upper part of figure shows autotrophic metabolism and is enriched in the leaf and PS; lower part of figure shows heterotrophic metabolism, enriched in the SS. Figure by D.K.A.
Sterile Spikelets Contribute to Yield in Sorghum and Related Grasses
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