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Senescence, dormancy and tillering in perennial C4 grasses

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Review

Senescence, dormancy and tillering in perennial C₄ grasses Gautam Sarath^{a,b,*}, Lisa M. Baird^c, Robert B. Mitchell^{a,b}^a USDA-ARS Grain, Forage and Bioenergy Research Unit, Lincoln, NE 68583-0937, United States^b Department of Agronomy and Horticulture, University of Nebraska, Lincoln, NE 68583, United States^c Biology Department, University of San Diego, San Diego, CA 92110, United States

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ABSTRACT

Perennial, temperate, C₄ grasses, such as switchgrass and miscanthus have been tabbed as sources of herbaceous biomass for the production of green fuels and chemicals based on a number of positive agronomic traits. Although there is important literature on the management of these species for biomass production on marginal lands, numerous aspects of their biology are as yet unexplored at the molecular level. Perenniality, a key agronomic trait, is a function of plant dormancy and winter survival of the below-ground parts of the plants. These include the crowns, rhizomes and meristems that will produce tillers. Maintaining meristem viability is critical for the continued survival of the plants. Plant tillers emerge from the dormant crown and rhizome meristems at the start of the growing period in the spring, progress through a phase of vegetative growth, followed by flowering and eventually undergo senescence. There is nutrient mobilization from the aerial portions of the plant to the crowns and rhizomes during tiller senescence. Signals arising from the shoots and from the environment can be expected to be integrated as the plants enter into dormancy. Plant senescence and dormancy have been well studied in several dicot species and offer a potential framework to understand these processes in temperate C₄ perennial grasses. The availability of latitudinally adapted populations for switchgrass presents an opportunity to dissect molecular mechanisms that can impact senescence, dormancy and winter survival. Given the large increase in genomic and other resources for switchgrass, it is anticipated that projected molecular studies with switchgrass will have a broader impact on related species.

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1. Introduction

Perennial, warm-season C_4 -grasses such as switchgrass (*Panicum virgatum*), miscanthus (*Miscanthus* $\times$ *giganteus*), prairie cordgrass (*Spartina pectinata*) and others are being developed as sources for bioenergy in many temperate regions of the world, due to their many positive agronomic features [1]. In addition to high above-ground biomass yields, these plants also sequester significant amounts of carbon below-ground [2]. In general, these perennial grasses are composed of below-ground tissues, the crowns and rhizomes and roots, and above-ground tillers. A tiller consists of a main stem composed of solid nodes and usually, hollow internodes. Each node subtends a leaf and leaf sheath. Depending on the species, axillary buds present on the nodes may develop into sub-ordinate tillers (branches) [3]. Tiller meristems remain vegetative, and transition into a reproductive phase once appropriate flowering signals are received. Again, depending on the species, plants could contain mostly flowering tillers as in switchgrass, or a combination of vegetative and reproductive tillers, for example, big bluestem (*Andropogon gerardii*). Flowering and seed development generally serve as cues for tiller senescence in cereals and other grasses and could be providing similar cues in C_4 perennial grasses and also could be factors in the induction of dormancy in the rhizomes. Irrespective of these variations in tiller composition, in temperate regions of the world, the onset of winter (first killing frost) will result in the death of the above ground tissues. Crowns and rhizomes and associated tiller buds will stay dormant until spring. Ultimately, all photosynthate required for below-ground growth is derived from the shoots, and the seasonal progression of shoot initiation, growth and eventual senescence of the aerial tissues are intimately linked to growth and the imposition of dormancy in the crowns and rhizomes. Reserves stored in the rhizomes will also drive growth in the spring. Appropriate allocation of reserves in the crowns and rhizomes for maintaining cellular integrity during the dormant phase, for driving the elongation of preexisting tiller buds when growth resumes, and for formation of new meristems, rhizomes and roots during the active growth phase are also likely to influence overall health of the below-ground perennial tissues. As compared to annual plants which are primarily dependent on seeds as their perenniating structures, perennials overcome adverse environmental conditions through maintenance of viable meristems associated with storage organs such as rhizomes, roots, stolons, or other plant structures. Growth of meristems during adverse conditions is detrimental to meristem survival, and thus perennials often have developed mechanisms for inducing dormancy during or prior to the adverse conditions in order to prevent meristem growth. This allows the plant to resume growth with prior stored reserves once conditions are favorable. Seasonal dormancy can occur in the summer months (generally induced by lack of water and/or high temperature) [4–6] or in the winter months (generally induced by low light and low temperature limitation; see below). It is possible that summer dormancy in grasses might share some similar molecular pathways to winter dormancy in temperate C_4 grasses (for example signaling responses to senescence, desiccation, and meristem metabolism). However, mapping these relationships will be dependent on the pace at which the appropriate functional genomic resources and experimental datasets become available for the individual species.

Several excellent papers and reviews have appeared over the last few years that have started to map both the physical and physiological networks that underpin senescence and dormancy, for example see [7–16] to name a few. There appears to be a central role for the clock related genes and in genes regulated by plant hormones (notably IAA, ABA and ethylene signaling) in controlling bud dormancy in the dicot systems. Coordinated regulation of these networks could be critical to ensure timely transition to

dormancy and winter survival. Since these networks are fundamental to plant development, it is likely they will be important circuits in temperate warm-season grasses as well. However, it is also possible that networks unique to these grasses exist based on their developmental and evolutionary history. Redundancy and overlap between these signaling pathways can be expected.

The significant lack of cellular and molecular data on these processes in temperate C_4 grasses is currently a challenge. Meeting this challenge will require detailed biochemical, molecular and cellular analyses of shoots and below-ground tissues collected over a growing season, and harvested from divergent switchgrass populations. Placing changes in gene expression datasets within the framework of plant developmental events (tillering, flowering, senescence, etc.) will yield gene co-expression networks and possibly identify key transcription factors coordinating these vital responses. To identify tissue specific processes required for dormancy induction and release in perennial grasses, similar studies will be needed on the physiology and biochemistry on the different plant organs at these important plant developmental events, along with metabolite profiling of tissues whenever possible. Specific roles of key genes will need to be validated by transgenic routes. These studies can be performed in switchgrass [17,18], but they can be relatively time consuming. Potentially other model systems such as *Brachypodium*, *Setaria italica* and *Panicum halli* could be used which are easier to transform and analyze [19–21]. Ultimately, these datasets will provide genes (targets) and pathways within the developmental biology of the plants that have a strong impact on the agronomic traits of most importance for the sustainable production of biomass from these grasses. Although not considered in this review, there is a large body of literature on other C_4 crop grasses, such as sorghum, sugarcane and maize that can provide additional insights.

2. Impacts of germplasm and harvest management on switchgrass productivity

Under optimal management, currently available switchgrass germplasm can become productive in the first year of establishment and reach full yield capacity in the second year of growth. With appropriate input, specifically N, it is possible to maintain biomass yields for considerable periods of time (>5 years) [22,23]. Under different soil types that have been evaluated, P and K were not as critical as N for maintaining biomass yields, although this can be expected to be soil dependent [24].

The number of times switchgrass and related species can be harvested will be dependent on the genotype $\times$ environment interactions. In most parts of the temperate world, current projections are for a single harvest post killing frost, with approximately 50–60% of shoot N remobilized to the rhizomes [25–27]. Under specific conditions, essentially long growing season with reasonable moisture (either through rain or irrigation) can make several harvests possible. Under these situations, the first harvest generally has the highest biomass yields [25]. Although yields are a major driver for production, biomass quality and sustainability of production are important factors as well [25,28]. Lastly, where the effects of repeated harvests during a season on switchgrass stand counts and yield have been evaluated, most studies support a single harvest as an optimal means to maintain stand counts and plant persistence [25,26,29]. Similar scenarios are projected for other target perennial grasses grown in a temperate climate under rain-fed conditions [30]. These data indicate that nutrient cycling from the shoots to the below-ground tissues directly impacts meristems on the crowns and rhizomes, and potentially winter-survival of the plant. In a large-scale analysis of different grasses across the southern half of the USA [31] yield components were dependent on the interactions between the environment and genotype. As an

example, colder winter temperatures favored upland switchgrasses as compared to the lowland cultivar Alamo. However, cv Alamo out yielded the upland cultivars within its optimal regions for growth [31].

Based on the results of the timing and number of harvests discussed above, it would appear that these temperate grasses need to translocate a certain minimal amount of C and N from the shoots to the crowns, rhizomes and roots over the course of a growing season to provide the needed buffer for maintaining perennality [29,32]. Too little C transfer as a consequence of low tillering and too little N transfer from the shoots due to perturbation of senescence and/or multiple harvests may lead to lowered viability of below-ground tissues and associated attrition in the numbers of tiller meristems that are formed and/or survive. Cumulative losses accruing from repeated cycles of lowered tillering and lowered accumulation of storage reserves from a loss in photosynthetic tissues can be expected to eventually lead to the death of the plant. For herbaceous biomass crops, even a small loss in yields can have negative economic consequences [33].

Different strategies can be employed to improve biomass yields and perennality. These can include various combinations of conventional breeding, improved harvest and management, and biotechnology. At present, two strategies have been exploited to increase switchgrass biomass yields under field conditions. Of particular interest is the use of southerly adapted populations in more northern locations. For switchgrass, the tetraploid southern adapted populations have considerably greater yield potential than the populations of northern origin [34]. Greater yield of the southern adapted germplasm is generally maintained when moved to a more northern site; however, these plants can suffer from significant winter-kill [35,36]. The other means has been through conventional breeding by selection of high-yielding germplasm [37] or through capturing heterosis present among switchgrass populations [36]. If heterosis is found when plants of different latitudinal adaptations are crossed, it is possible to gain improved winter-survival along with increased biomass yields.

3. Molecular mechanisms impacting senescence, dormancy and tiller buds in switchgrass

As described earlier, switchgrass populations adapted to different latitudes behave differently when moved one plant hardiness zone north or south. It is also possible to compromise switchgrass plant fitness (winter survival) by repeated selection for increased in vitro dry matter forage digestibility (as a proxy for ruminant digestion; [38]) in northerly adapted populations as a consequence of lowering lignin content of the biomass [39]. Although such selection did not lead to changes in the timing of plant developmental responses, such as green-up and flowering dates, populations with lower plant lignin had greater winter kill. These findings suggest that potentially aerial senescence, dormancy and winter-survival in switchgrass, and possibly other warm-season C_4 temperate grasses, can be uncoupled from each other, and existing switchgrass germplasm can be exploited to understand some of these relationships.

The latitudinally adapted germplasm can provide data on the interactions between flowering and dormancy. Non-adapted plants can provide information on the mechanisms that impact winter-survival relative to the adapted and non-adapted genotypes. Finally, adapted populations and plants that have contrasting winter-survival will offer an opportunity to distinguish molecular and cellular changes that have occurred in the rhizomes as a consequence of selection of improved ruminant digestibility of the aerial biomass. In a broad sense these datasets will be applicable to related grasses which have been historically utilized as forages.

Developing models (at a molecular/cellular level) into these differential mechanisms with respect to senescence, dormancy and winter survival are now possible based on the availability of germplasm and genomic resources and the rapid expansion of high-throughput sequencing platforms. Understanding the how and why of these processes has important basic and applied outcomes for switchgrass and related temperate, warm-season C_4 grasses. Results can be used to extend the latitudinal range of high-yielding germplasm, developing greater data on the important molecular circuitry that integrate shoot and rhizome metabolism, and by identifying molecular markers that can expedite breeding of elite germplasm for specific end uses.

3.1. Using latitudinally adapted and non-adapted populations to dissect interactions between aerial growth, senescence, onset of dormancy and winter survival

Switchgrass is a recent addition to the genomic arena. However, the rapid growth in genomic and genetic resources (discussed later) presents an opportunity to address the interactions between of key plant developmental events and responses to the environment at the molecular level. It should be noted that the depth of molecular analyses of developmental events in switchgrass are still relatively few. This situation generally extends to other temperate C_4 perennial grasses as well, indicating that it is somewhat difficult to extrapolate observational data (such as bud banks, tiller numbers and biomass yields) to their underlying molecular controls. Using these datasets can however inform molecular studies, especially when populations or plants or clones have been compared for similar responses at the same site or across geographic locations.

In this context, latitudinally adapted switchgrasses offer an excellent model to derive molecular data based upon their contrasting growth responses to the environment. Using these genotypes/populations it should be possible to discern metabolic changes occurring in the rhizomes/crowns at specific shoot developmental changes. It can be anticipated that some of these changes will be specific for each population based on their zone of adaptation. Other changes might be similar (say response to flowering) but should occur earlier in the northerly adapted germplasm relative to the southerly adapted germplasm which will flower later. It should also be possible to differentiate changes impacted by the timing and extent of aerial senescence on below-ground metabolism. Data taken following post-killing frost can provide information on the strategies utilized by plants belonging to the different latitudinal adaptation zones to cold temperatures. The onset, timing, and extent of these responses could be essential to winter survival.

A schematic of the plant development of northerly and southerly adapted populations of switchgrass grown at a northerly site are shown in Fig. 1A. Growth of the aerial switchgrass tissues occurs in spring, and tillers can arise from a range of axillary buds (henceforth referred to as tiller buds), that are present on rhizomes, at the base of old tillers and in the crown region [40]. New phytomers are produced until the apical meristem becomes reproductive [3]. In northerly adapted germplasm (Fig. 1A, solid black line) the reproductive phase occurs about one month or more sooner as compared to the non-adapted southerly populations (dashed line). This extra period of vegetative growth in the non-adapted plants allows for greater accumulation of above ground biomass, resulting in yield gains as compared to the northerly adapted germplasm [34].

In most northerly adapted genotypes, above ground tissues will have senesced or be mostly senescent prior to a killing frost [25]. However, non-adapted plants do not fully cycle through aerial senescence and rhizome dormancy prior to the first killing frost in northern sites (Fig. 1B) which can result in substantial stand losses over winter due to death of the perennating structures. Repeated selection of non-adapted germplasm for winter survival

Fig. 1. Seasonal changes in shoot growth (A) and approximate time frames for shoot developmental stages (B) in latitudinally adapted and non-adapted switchgrass (~40° N). (A) The increase in shoot biomass in adapted (solid line) and non-adapted (dashed line) ecotypes as a function of the growing season. Peak biomass occurs near the time of heading and decreases thereafter. All above ground growth stops with the first killing frost (gray bar). Months of the year are shown as a single letter, starting with April. (B) Spring green up occurs approximately at the same time in both ecotypes, however, the (vegetative) growth stage is shorter in the adapted ecotypes which transition into a reproductive phase about 25–35 days earlier than the non-adapted ecotypes. The senescent phase is generally well-defined in adapted germplasm, and most frequently the above ground biomass is dead prior to the killing frost. This phase is not as obvious in southerly adapted germplasm grown in northerly latitudes of the USA.

can identify plants that survive in field nurseries over the course of several years. These plants generally serve as progenitors for the development of stable populations of originally non-adapted germplasm [36]. Even with this long-term selection strategy, occasional severe winters and/or ones accompanied by freeze–thaw cycles in spring can cause significant winter-kill in provisionally “adapted” southerly germplasm (unpublished observations). These observations indicate that some key cellular mechanisms that can confer sustainable (>10 years+) winter-survival might be differentially regulated (or even absent) in plants adapted to different latitudes and could be a productive area for future research. Similarly, it should be possible to select for later flowering in northerly adapted germplasm to achieve increases in biomass yields without potentially sacrificing winter survival. Other mechanisms driven by plant developmental events such as flowering and nutrient remobilization are considered next.

The expected changes in the crowns and rhizomes for plants belonging to different zones of adaptation, but grown at a northern site are shown in Fig. 2. For northerly adapted germplasm it can be expected that crown and rhizome growth continues in parallel with above ground tissues when grown at northern sites. Growth in the below ground tissues begins to slow once tillers enter a senescent phase [29]. Senescence (in most plants) is normally accompanied by lowered translocation of photosynthates from the shoots and increased amounts of nutrients including N remobilized from the shoots to (sinks) which will include the rhizomes in perennial grasses. Although the exact timing of this process is not known, flowering and nitrogen remobilization are likely to be linked to the onset of dormancy [41]. Since aerial tissues in latitudinally adapted plants are essentially senesced prior to a killing frost [25], it is possible that dormancy of the crowns and rhizomes is set prior to this time (Fig. 2A), and continued cold temperatures probably result in strengthening the cold acclimation of these tissues [12]. Insufficient acclimation probably also results in greater tissue damage to the rhizomes and tiller buds in non-adapted germplasms.

We propose three possible growth models for crowns and rhizomes for the southerly adapted germplasm grown in northern

sites which are amenable for field-testing. These are indicated as curves 1, 2 and 3 in Fig. 2B. In curve 1, crowns, rhizomes and associated tiller buds continue to grow until all above-ground growth is terminated by a killing frost. Such plants are unlikely to overwinter in more northern locations. For plants predicted to display curves 2 and 3 for their crown and rhizome growth, it is expected that at least a proportion of these plants adapted to more southerly latitudes will successfully over-winter in northern sites. In plants displaying putative crown and rhizome growth shown in curve 2, there is some transition toward dormancy and these could be driven by both internal and external signals. A proportion of these plants can be selected for winter-survival (for example see [36]). Plants falling under curve 3 can be expected to have a timely transition to dormancy and relatively robust winter survival, and probably occur in northern-adapted lowland populations. Most frequently, for switchgrass, the measure of winter-survival is evaluated by taking stand counts at green-up (see Fig. 1) over one or more years for individual plants or swards planted in field nurseries [35,39]. The stand-count data is generally analyzed with heading, yield and related data to obtain correlations between these measures. Having germplasm with contrasting responses and developmental patterns appears to hold some promise for uncovering the molecular control of these processes in switchgrass.

Initial studies using high-throughput sequencing of tissues collected from field grown switchgrass plants of a northerly adapted cultivar (cv Summer) at physiological maturity provided some data into the metabolism of the crowns and rhizomes [42], but interpretation of this dataset was compromised by the lack of adequate genome annotation at that time. Now the switchgrass genome is in its second iteration (v 1.1; www.phytozome.org) and will provide a significant resource in the future.

Expanding studies to cover crowns and rhizomes collected at specific plant developmental points across adapted and non-adapted populations will be useful and are currently ongoing for switchgrass (Sarath, unpublished). Other datasets on the onset of senescence and related cellular changes in aboveground tissues can add significantly to the overall physiological and molecular

Fig. 2. Predicted seasonal changes in the crowns and rhizomes in latitudinally adapted (A) and non-adapted (B) plants. (A) In adapted plants, crowns and rhizomes and associated buds begin the transition to dormancy with the onset of tiller senescence, and can be expected to be dormant prior to a killing frost (gray bar below months of the year). Months of the year are shown as a single letter, starting with April. In non-adapted germplasm, we suggest that crown and rhizome dormancy could take several different routes (curves 1–3 in panel B). These scenarios are explained in greater details in the text. The approximate plant developmental phase is shown above the graphs in Panels A and B. See Fig. 1 for more details.

understanding of these processes. The goals would be to tease apart responses in crowns and rhizomes that are shared or not-shared across adapted and non-adapted populations, and how developmental changes in the shoots affect below-ground processes. Additional possibilities are to select for late-flowering adapted plants which can be mated with the non-adapted germplasm in order to utilize as much of the growing season as possible without sacrificing perenniality. A detailed analysis of crown and rhizome metabolism within the framework of specific shoot developmental changes (flowering, seed set, etc.) in these plants could help to establish the genes important during the transition to dormancy. They will also help establish when senescence is initiated in the shoots. Again the variation in the date of flowering in the non-adapted populations can be exploited to select for plants with late flowering dates from plants with early flowering times. Using this divergent pool of plants, the adaptation of progeny plants from half-sib families with different flowering times to a specific latitudinal zone can be tested. Such studies might also provide some evidence for the relative plasticity in molecular networks controlling these plant responses.

A dis-linkage between senescence and dormancy could provide yield gains when late senescence does not impact transition to dormancy or the transfer of N and other nutrients from the shoots. Lowered N of biomass is important (see above) from both the production and conversion aspects.

3.2. Tiller bud development and rhizome nutrient status in temperate *C₄* grasses

Much deeper cellular and molecular knowledge of bud initiation, formation and growth are also needed. Here, a combination of tools from bioinformatics, deep transcriptomic sequencing and plants with altered/compromised tiller bud formation and extension will be useful. Several key genes involved in meristem organization, identity and elongation have been studied in both monocots and dicots [43–48]. For switchgrass, seedlings can be selected for single or multiple tillers at an early stage of growth [49].

Potentially these or similar populations can be used to understand the controls of tiller bud initiation and elongation at a molecular level.

Rhizomes and crowns are the biggest sources of tiller buds for new growth every year [40,50,51]. Recruitment of these buds to produce tillers directly impacts the net productivity in native prairies, since primary productivity appears to be a function of tiller densities rather than tiller size [52]. These studies indicate that similar dynamics could control biomass productivity under cropping conditions. Indeed, first year stand establishment is the major limitation to the economics of producing switchgrass biomass [22,33].

In native prairies, bud banks in a perennial grass showed two maxima, one in March and one in September [52]. It can be anticipated that a similar scenario could occur in cultivated stands of switchgrass, indicating that molecular control of bud formation and timing should associate with critical events happening prior to spring green up in April/May (see Fig. 1) and with the onset of tiller senescence in August/September (see Fig. 2). Tiller senescence is correlated with nitrogen mobilization from shoots to roots in switchgrass [29,53] and is likely to be influenced by flowering [41]. In a related species, flowering tillers of big bluestem produced more buds than non-flowering tillers, which was attributed to bigger bud size and potentially weaker apical dominance in flowering tillers [50]. However, it is tempting to speculate that larger buds could be partly attributed to differential nutrient remobilization in flowering versus non-flowering tillers arising from changes in shoot physiology. Tiller bud growth has been studied in prairie *C₃* species and *C₄* grasses in response to different nitrogen and light regimes. For two *C₄* grasses, no apparent interactions were seen between nitrogen and light on bud dormancy or growth [51]. In these experiments, nitrogen was added to dormant rhizomes pieces, and it is uncertain as to how much nitrogen was actually absorbed by the rhizomes prior to the onset of tiller growth from dormant buds. The authors have suggested that alternate constraints may control bud break in *C₄* grasses. Although, these data do not allow discrimination between different mechanisms impacting bud growth, nor provide any cellular/molecular information, they suggest that

Fig. 3. Scanning electron micrographs of the crown and rhizomes obtained from field grown plants following a killing frost in 2012. (A) Section of crown/rhizome region for a southerly adapted cv Kanlow plant. Arrows show a large number of starch-filled cells on either side of a vascular bundle (VB). White bar is 200 μm . (B) Section of a rhizome from a northerly adapted cv Summer plant. White bar is 30 μm . Inset (C) shows a starch grain at higher magnification. White bar is 5 μm .

the nutritional status of the rhizomes could play a key role in the formation and maintenance of tiller buds in C_4 grasses. In seeded Bermuda grass, stolon starch content was positively correlated to bud outgrowth following a dormant period [54] providing indirect evidence for a role in the rhizome nutritional state to perenniality in other grasses such as switchgrass. In an interesting study with *Miscanthus $\times$ giganteus* [32], starch content in roots and rhizomes actually decreased when plants were grown at elevated CO_2 ($550 \mu\text{mol mol}^{-1} \text{CO}_2$) concentration as compared to plants grown at an ambient CO_2 ($394 \mu\text{mol mol}^{-1} \text{CO}_2$) suggesting that regrowth potential could be significantly impacted in plants with lowered rhizome starch content. This study highlights the importance of rhizome nutritional status on tillering potential, and the possible negative aspects of increased CO_2 concentrations on perennial temperate C_4 grasses.

Switchgrass rhizomes (collected post-frost from field grown plants) from both southerly (cv Kanlow, Fig. 3A) and northerly adapted populations (cv Summer, Fig. 3B) appear to store abundant amounts of starch. Since shoot translocated sugars are the primary source of carbon for the root system, they can be expected to play a role in rhizome metabolism. Sugar-sensing is an important circuit in plant cells [13,55,56], and seasonal changes in the flux in both the amounts and type of sugars could be important triggers of cell development and arrest in rhizomes. These triggers

could lead to internal signaling cascades based on well-defined mechanisms involving sucrose non-fermenting-1 related kinase (SnRK-1), hexokinase, invertase, glucose levels and their intersection with hormone signaling [55,57–61]. Presumably a shift in the balance from starch biosynthesis during most of the period of active shoot growth toward starch degradation once shoot senescence is initiated will also impact hexose pools within the rhizomes. The assumption is that starch will be the primary energy currency in dormant rhizomes, and provide the needed carbon for maintaining meristem health and viability over winter. It is not known if these patterns are perturbed in non-adapted germplasm. Starch storage in rhizomes probably also helps maintain a relatively high state of desiccation during dormancy, similar to the situations seen in many seeds.

In other plants, dormancy and related cellular changes such as tissue water status, chilling and meristem growth are linked through the actions of the DAM (dormancy associated MADS box) and DREB (dehydration-responsive element binding proteins) genes [62–65], indicating that switchgrass orthologs of these genes will be reasonable targets for future study.

3.3. Uncoupling dormancy from winter-survival in switchgrass

Based on much of the existing literature (see above) and studies on genetically related switchgrass plants [39] winter survival can be uncoupled from dormancy. Adapted switchgrass populations can be bred for increased ruminant digestibility of biomass [66,67]. However, with every cycle of selection for improved biomass quality (increased digestibility), plants begin to exhibit increased susceptibility to winter-kill, without a major change in other aspects of plant development [39,68]. Lignin content, a critical plant component, was significantly negatively correlated with improved ruminant digestibility of grass biomass [69]. Plant lignin content affects fitness, and decreasing lignin content can have a deleterious effect on plant growth and development under field conditions [70]. Within the focus of a bioenergy perspective, lowering lignin in biomass has remained a major goal [71] for improving the conversion efficiency of biomass into liquid fuels through biochemical routes. However, for other conversion strategies or platforms biomass with higher lignin content might be beneficial [28] or without an effect [72].

Nevertheless, there is importance of the divergent switchgrass populations described above to understanding the molecular underpinnings of winter survival. The question is why lowering lignin content in the aerial biomass, predominately in the stems [73], impacts the health and survival of the rhizomes?

As shown in Fig. 4, living tillers are intimately connected to the rhizomes, and tiller bases contain vascular bundles (VB, Fig. 4A) that appear to be at or close to the interface between the tiller and the rhizome. Such an arrangement could be important for the effective bidirectional transport of nutrients and minerals in and out of the rhizomes. Changes in metabolite flux can then be sensed rapidly and could permit timely or even a rapid adjustment in rhizome metabolism. Since lignin is a major component of the tracheid and vessel cell walls, it is possible that the low lignin plants are more susceptible to freezing damage as a result of compromised wall strength in xylary cells. Plausibly, perturbation of the phenylpropanoid pathway [74] that supplies not only monolignols for lignin biosynthesis, but also a host of other intermediates important to plant development and defense [75] in the low-lignin populations results in increased damage from other biotic and abiotic stressors. Discrimination between these and other mechanisms will be useful to the understanding of freezing tolerance in switchgrass rhizomes. Although lowering the water potential of cells is a frequent companion to freezing tolerance (and dormancy), accumulation of a range of solutes such as proline,

Fig. 4. Scanning electron micrographs of crowns and rhizomes collected from field grown switchgrass plants following a killing frost. (A) Image of the rhizome with attached tiller from the non-adapted cv Kanlow. The tiller is indicated and the white arrow points toward the aerial portion of the tiller. The rhizome–tiller interface appears to be delineated by vasculature (VB). (B) Image of a rhizome containing a “dead tiller hole” from an adapted cv Summer plant. The approximate boundary between the living and dead portions of the rhizome is indicated by a white line, and the dead cells are shown with black arrows. White bars in Panels A and B are 200 µm. (C) Higher magnification image of the dead cell ghosts that ring the dead tiller hole shown in Panel B. White bar is 20 µm.

fructans and mannitol, and proteins such as the ice recrystallization inhibition proteins (IRIPs) [76,77] can be important as well. Cold-responsive IRIPs have been documented in the genome of the model grass *Brachypodium* [78], but have yet to be reported from a temperate warm-season C_4 grass. A preliminary search of the switchgrass genome using a *Lolium perenne* IRIP protein sequence (GenBank: ACN38303.1) did not yield a good match. It is possible that IRIPs or related proteins have yet to be annotated in the switchgrass genome. However, the switchgrass genome contains several leucine-rich receptor kinases, including phytosulfokine receptor kinase like proteins which have been postulated to be ancestral to the IRIP genes [79].

In several other plants, such as grasses with summer dormancy or tree buds with winter dormancy, spontaneous mutations or divergent selection can yield plants which lack dormancy [4,64,80–82]. Depending on the environmental stress that is placed on these plants, responses can be neutral, positive or negative. However, the basal mechanisms affecting these changes appear to come from specific genes (*DAM* genes in peach; [64]) which are of importance to dormancy in other dicot species or from a suite of traits that primarily affect the onset leaf senescence in grasses experiencing summer dormancy [4,6,81]. These studies point to

potential mechanisms that tie into the overall integration of timely aerial senescence to the onset of dormancy in the perennating structures, and provide targets for study in the temperate C_4 perennial grasses.

Another point to consider is the consequence of tiller senescence on the physical structure of the rhizome. Once a tiller senesces, or is destroyed in some other manner (for example harvesting), tissues associated with the tiller appear to become lost/degraded resulting in a hole within the rhizome (Fig. 4B). Surrounding this “dead tiller hole” are several layers of dead cells (Fig. 4B), which appear to be cell ghosts at higher magnification (Fig. 4C). Assuming these observations are part of a normal developmental cycle, this region of the rhizome could become an important component in determining rhizome health. Tiller holes could be points of entry of pathogens, and potentially the dead cells surrounding these parts of the rhizome could provide an effective barrier against colonization by pathogens. Several layers of dead cell could also serve as insulation from freezing injury. A possibility is that if tiller death occurs suddenly as in the non-adapted genotypes during a killing frost (see Fig. 2) it could predispose rhizomes to injury.

3.4. Molecular studies on rhizomatousness in grasses

Plants such as rice (*Oryza sativa*) and sorghum (*Sorghum bicolor*) which have closely related perennial relatives [83] have provided a means to understand the origins of perenniality in grasses. Other studies have identified quantitative trait loci that control rhizomatousness and over-wintering capacity in sorghum species [84]. Within this experiment, the authors found that rhizome formation was a key but not the only factor that controlled winter survival, again providing indirect evidence that many aspects of dormancy and winter-survival can be separated. More recently, other researchers have begun the task of dissecting the genes and co-expressed gene sets that might control rhizomatousness in monocots [85–89]. These studies have identified transcripts for several genes including transcription factors that are selectively enriched in rhizomes relative to shoots. It is still too early to know which of these differentially expressed genes are critical for rhizome development. However, they provide important datasets that can be applied to the future study of the temperate warm-season C_4 grasses.

4. Integration of signals that might trigger dormancy in warm-season temperate C_4 grasses

Under natural conditions the transcriptional response of the plant to internal and external events drives the systemic outcomes, such as senescence, transition to dormancy and acclimation to winter. How these cues are integrated becomes an important aspect of understanding the molecular biology of the biofuel grasses. Both internal and external cues (Fig. 5) can be expected to impact rhizome metabolism and nutritional health. Whether these cues drive cellular processes in switchgrass and related species in a manner similar to other systems that exhibit dormancy and winter-survival also needs to be investigated.

Contrary to dicots, where bud dormancy is enforced relatively early during development and remobilized nutrients from leaves are sequestered in the stems; in temperate warm-season grasses, tiller bud initiation in the rhizomes appears to be a continuous process and ceases relatively late in the growing season. Remobilized nutrients are sequestered in the crowns, rhizomes and roots or partially returned to the soil [90,91]. Since these below ground parts are essential to perenniality, it is likely that several signal-perception networks will need to work in concert for eliciting and then imposing dormancy, and thereby maintaining

Fig. 5. Anticipated cellular changes in the crowns and rhizomes in switchgrass as a function of developmental and environmental cues toward the end of the growing season. These signals, both internal and external, can be expected to modify tissue metabolism required for the orderly transition to a dormant phase.

perenniality (Fig. 5). Some of these cues are from the aerial tissues, such as changes in the flux of nutrients and signaling compounds (plant hormones, sugars, etc.) based on the developmental stage of the plant (vegetative growth, flowering, and senescence). The response of plants to a number of these molecules is well established [13,92,93] and not discussed in detail here. However, it is important to consider that the total amount of newly assimilated photosynthate will decline over time, and products of remobilization resulting from scavenging of nutrients from senescing tissues will increase over time. Unloading of these compounds at the tiller/rhizome interface (see Fig. 4A) will likely be monitored by a range of cellular systems that respond to changes in the flux of these shoot-translocated molecules. Signals coming from the environment, such as short days and colder temperatures will need to be integrated with these internal cues. Plants respond to changes in light quality and quantity essentially using conserved pathways such as phytochrome-based signaling [12,94,95]. The nature of the cold-sensing mechanisms appears to be more variable and possibly species or even biotype specific [39,95]. Nonetheless, these signals must be integrated within the larger framework of crown and rhizome growth. This will lead to the arrest of meristems both external and internal to the crowns and rhizomes. Other changes include the accumulation of storage compounds such as starch (Fig. 3) and in the redirection of metabolism to ensure successful winter-survival. Crowns and rhizomes in these grasses face another hurdle of it being beneficial to isolate the dead or dying tiller interface from the rest of the rhizome (Fig. 4B and C). The timing of this event and the mechanisms controlling this process are unknown, but could have important consequences on rhizome survival and warding off biotic and abiotic stresses.

5. Overlapping pathways with dissimilar outputs leading to senescence or dormancy?

A possible grouping of plant developmental phases that result in specific types of physiological outputs in switchgrass tillers during aerial growth and senescence as compared to processes occurring during rhizome growth and dormancy are shown in Fig. 6. In this model, aerial growth begins in spring when tiller meristems that were dormant (arrested state) begin a phase of growth and expansion, followed by growth arrest once seeds have been set and senescence has started. Metabolic redirection can be expected to occur in the senescing shoots as sink-driven (seeds and rhizomes) competition for metabolites ensue. For leaves and stems, at the end of their annual growth cycle, metabolic redirection occurs from catabolic processes involved in nutrient remobilization and transfer to sinks. The net result of this senescent process leads to the

eventual death and/or separation from the plant, consistent with aspects of “monocarpic” senescence at the individual tiller level. A preliminary anatomical investigation of the tiller–rhizome interface shown in Fig. 4 appears to be consistent with this supposition. It is possible that immature or non-flowering tillers are photosynthetically self-sufficient and will die when a killing-frost occurs. In switchgrass, where most tillers that emerge early in the season will flower, redirection of nutrients from immature tillers may not be essential for rhizome health. However, in grasses such as big bluestem where many tillers can remain vegetative, the relative contribution of nutrients from flowering and non-flowering tillers could be important for rhizome health. Nevertheless, resource partitioning for tiller bud meristems appears to be adequate in both species, since adapted germplasm can be maintained in the fields for extended periods of time.

In the case of rhizomes, the model (Fig. 6) starts toward the end of the growing season, when rhizomes will be transitioning from a period of active growth (see Fig. 2) toward dormancy in response both internal and external cues (see Fig. 5). The integrated response to these cues can be expected to lead to metabolic redirection associated with possible changes in the rates of starch breakdown versus starch synthesis and an increase in the metabolites and other machinery needed for winter protection. At some point during this process, both rhizome growth and tiller meristem initiation and growth become arrested. Once these events have occurred, plant metabolism can be anticipated to change to one that maintains cell viability, using stored reserves such as starch and breakdown of proteins and fats. Elegant studies in poplar have shown several key changes that occur during bud dormancy. These include downregulation in the expression of specific transcription factor, CENL-1 [16], the poplar ortholog of the Arabidopsis TERMINAL FLOWER 1 which is required for stem elongation. Other events include changes in the types of lipid bodies in the apices of buds, which could be an important aspect of intracellular signaling [14], and by the formation of callose plugs that symplastically isolate bud meristems from the surrounding tissues. Meristems remain isolated until the advent of conditions that favor dormancy release. Once these conditions are reached, callose plugs are degraded and the symplastic connection is reestablished leading to bud elongation [12]. Whether similar mechanisms exist in dormant tiller buds in perennial grasses is not known, but would be worth investigating. Given the complexities of the interactions between plant growth, tiller bud development, and dormancy decoding these individual events for the different types of emerged and buds embedded within the rhizomes could prove to be challenging.

However, based on the developmental patterns shown in Fig. 6, it is interesting to speculate that common mechanisms might

Fig. 6. Overlap between aerial growth and senescence and rhizome growth and dormancy in temperate C_4 perennial grasses. Aerial growth (light gray oval) occurs once the tiller meristems (arrested mode) begin growth and expansion during spring (see Fig. 1 for approximate timelines). Tiller growth in reproductive tillers stops at physiological maturity when seeds dehisce. Metabolic redirection of assimilates and at later times scavenged compounds occurs as leaves and stems senesce, followed by death and separation from the below-ground parts of the plant (see Fig. 2). In contrast, crown and rhizome (dark gray oval) growth proceeds and begins to change as external cues (see Fig. 5) are received, leading to metabolic redirection in preparation for dormancy. Growth and meristem arrest occur, with the key difference to maintain cell viability of dormant tissues during the winter months. We suggest that the developmental programs in common could share common mechanisms, but that plant might have evolved means to recruit new partners in the different tissues to engender tissue specific outcomes.

underlie aerial senescence and rhizome dormancy. As an example, the initial stages of plant senescence are not that different than the initial changes accompanying dormancy, i.e. arrest of growth and changes in metabolism. However, divergence between these two processes exists, in that plant senescence is normally a unidirectional process eventually leading to the death of the senescent tissue, whereas dormancy and exit from dormancy are cyclical processes, without any apparent loss of tissue function. For instance, transcription factors belonging to the no-apical meristem (NAM)/ATAF/cup shaped cotyledon (CUC) collectively called NACs, are a large group of plant-specific proteins that play important roles in plant processes [96,97]. In the model plant *Arabidopsis*, overexpression of specific NACs can hasten or delay leaf senescence, although these genes are also usually transcribed during leaf development [98–100]. NACs are also important to the remobilization of nutrients during senescence [101]. However, orthologous NACs may or may not have the same function in different plants [101]. Using bioinformatics analyses, 92 potential NACs have been identified in switchgrass [96]. Some of these switchgrass NACs were orthologous to genes shown to be important to cell wall deposition and environmental responses in other plants. More recently transgenic switchgrass plants containing an *Arabidopsis* NAC gene (LONG VEGETATIVE PHASE ONE, AtLOV1) were shown to be altered in many different plant traits, including delayed flowering in the transgenic plants [102]. These results suggest that AtLOV1 can form productive interactions with native switchgrass proteins and change developmental outcomes. Although we currently have no knowledge of the switchgrass proteins that interact with AtLOV1, these data would suggest that the interacting partners (or lack thereof) to specific transcription factors including NACs might bring about ordering of the transcriptional machinery to favor different outcomes. Potentially, association of transcription factors central to plant developmental events, such as a specific NAC, with different protein partners could initiate senescence in the leaf, and lead to the onset of dormancy in rhizomes (see Fig. 6). X-ray crystallographic studies have shown that NACs (*Arabidopsis* NAC019) is present as a dimer in solution and each monomer binds to DNA

through a major groove [103]. This binding of the NAC protein to its cognate DNA element could be constrained (if two binding sites are present) or could be more flexible if only one cognate DNA binding site is present on the promoter. Changes in the flexibility of the protein bound to DNA have been suggested to be important to biological role of NACs and WRKYs which are another large class of plant transcription factors [103].

Many of the NAC proteins are organized with a characteristic DNA binding domain at the N-terminus and a variable C-terminal domain that serves a regulatory role [96,97], allowing multiple protein–protein interactions potentially leading to differential outcomes. It is possible that transcript and protein abundances of the same key genes in the tillers versus the rhizomes might have a significant impact on the metabolic and cellular fates of these tissues by activating or inhibiting specific cellular pathways (Fig. 6). Many other families of plant transcription factors possess similar structures (a relatively conserved DNA-binding domain(s)) and variable N-terminal or C-terminal regions which can impart specificity to protein–protein interactions [104,105]. Placing the switchgrass transcription factor orthologs within the framework of plant development can be anticipated to be productive.

6. Switchgrass as a temperate C_4 warm-season grass model

Switchgrass was selected as a potential herbaceous biofuel feedstock by the US Department of Energy in the 1990s based on a number of different criteria and field evaluations at different locations of the USA [106]. Other aspects of plant growth, development, breeding and agronomy have been reviewed [40,107]. Since that time, considerable resources have been devoted to improving the genetic, genomic and functional knowledge available for this species that can permit its use as a temperate warm-season grass model from both a biological and energy-conversion perspective. The intent here is to provide a snapshot of these research efforts to document how switchgrass can serve as a temperate C_4 warm-season grass model.

Switchgrass breeding and cultivar development has been ongoing since the late 1930s, although the principal focus was on the development of plants destined for forage or conservation end-uses [40]. More recent work has focused on the development of bioenergy-type germplasm and related management, harvest and, conversion procedures [107–111].

Functional genomic research into switchgrass was first initiated with a small-scale Sanger sequencing of cDNA clones [112], followed by a large scale sequencing of over 400 K clones by the US-Department of Energy Joint Genomes Institute that provided the first extensive data on the switchgrass transcriptome [113]. These studies served as a bridge to the current sequencing and annotation of the switchgrass genome available at www.phytozome.org [114], the use of microarrays [115,116], and next generation sequencing [42,117] for understanding different aspects of plant development and responses to the environment. Parallel work on the genetics and genomics of switchgrass has resulted in a deeper understanding of genome organization, linkage groups, single nucleotide polymorphisms (SNPs) and molecular markers associated with specific linkage groups, for example: [118–120]. These studies along with conventional breeding efforts [34,111,121] portend rapid development of switchgrass germplasm for specific end uses.

Conventional and transgenic approaches have been utilized to generate switchgrasses with improved quality, based primarily on the reduction of lignin content as a means to improve ethanol yields through fermentation of biomass [73,122–125], and possibly for increased yields or plant development using transgenic routes [126,127]. The application of biotechnology for switchgrass improvement has been reviewed recently [17]. There is also a large body of literature on many of the downstream aspects for the utilization of switchgrass biomass for conversion to fuels using biochemical or thermochemical platforms. These conversion studies are not reviewed here, but add to the total wealth of information that has become available and indicate that switchgrass could serve as an excellent model for related bioenergy grasses.

Some aspects of switchgrass research still pose challenges. It is a polyploid plant with limited self-fertility, needing several years of field evaluation of germplasm generated in conventional breeding programs. Although new methods continue to be pioneered for developing transgenics, it is likely that in the near-term other related diploid grasses will be easier to manipulate for genetic engineering. Nonetheless, switchgrass may be the best system for the study of perenniality and related issues in temperate warm-season grasses.

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