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Seasonal below-ground metabolism in switchgrass

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SUMMARY

Switchgrass (*Panicum virgatum*), a perennial, polyploid, C4 warm-season grass is among the foremost herbaceous species being advanced as a source of biomass for biofuel end uses. At the end of every growing season, the aerial tissues senesce, and the below-ground rhizomes become dormant. Future growth is dependent on the successful over-wintering of the rhizomes. Although the importance of rhizome health to overall year-upon-year plant productivity has been long recognized, there is limited information on seasonal changes occurring during dormancy at both the transcriptome and metabolite levels. Here, global changes in transcriptomes and metabolites were investigated over two growing seasons in rhizomes harvested from field-grown plants. The objectives were: (a) synthesize information on cellular processes that lead to dormancy; and (b) provide models that could account for major metabolic pathways present in dormant switchgrass rhizomes. Overall, metabolism during dormancy appeared to involve discrete but interrelated events. One was a response to abscisic acid that resulted in dehydration, increases in osmolytes and upregulation of autophagic processes, likely through the target of rapamycin complex and sucrose non-fermentative-related kinase-based signaling cascades. Another was a recalibration of energy transduction through apparent reductions in mitochondrial oxidative phosphorylation, increases in substrate level generation of ATP and reducing equivalents, and recycling of N and possibly CO_2 through refixation. Lastly, transcript abundances indicated that cold-related signaling was also occurring. Altogether, these data provide a detailed overview of rhizome metabolism, especially during dormancy, which can be exploited in the future to improve winter survival in switchgrass.

Keywords: *Panicum virgatum*, switchgrass, Accession numbers: SRX1601466, rhizomes, dormancy, transcriptomes, abscisic acid, metabolites, target of rapamycin kinase, autophagy.

INTRODUCTION

Switchgrass (*Panicum virgatum* L.) is a temperate, perennial, polyploid, warm-season grass that has importance as a conservation, forage and biofuel species (Vogel *et al.*, 2011). Over the last ~15 years, concerted research efforts have been devoted to identifying genetic, biochemical and management factors that influence biomass quality of switchgrass to improve its utility as a biofuel crop (Nageswara-Rao *et al.*, 2013; Casler *et al.*, 2015; Anderson *et al.*, 2016; Tubeileh *et al.*, 2016; Grabowski *et al.*, 2017).

Perenniality resides in the ability of the plant to regenerate from dormant tiller buds present at the base of old tillers (crowns) and rhizomes at the beginning of every growing season (Beaty *et al.*, 1978; Sarath *et al.*, 2014), indicating that rhizome health is critical to the long-term survival of the plant (Vogel, 2004). Early season growth of tillers is dependent on stored reserves and, once tillers attain photosynthetic independence, transfer of assimilates from the shoots to the rhizomes and roots will occur

(Sarath *et al.*, 2014; Stewart *et al.*, 2016). Switchgrass can accrue an equivalent weight of C in below-ground biomass as in above-ground biomass (Schmer *et al.*, 2011; Garten, 2012). This robust translocation of C is also matched by the transfer of N and other nutrients to below-ground tissues of the plant (El-Nashaar *et al.*, 2009; Wayman *et al.*, 2013; Wilson *et al.*, 2013; Palmer *et al.*, 2014b; Pedroso *et al.*, 2014; Gorlitsky *et al.*, 2015; Yang *et al.*, 2016). Although bidirectional transport of materials can be expected between the shoots and the rhizomes at all developmental stages, the transfer of remobilized nutrients to rhizomes post-flowering and during aerial senescence could be linked to the onset of rhizome dormancy and winter-hardening in switchgrass (Schwartz and Amasino, 2013; Sarath *et al.*, 2014; Yang *et al.*, 2016).

Most switchgrass tillers that emerge early during the growing season will flower (Mitchell *et al.*, 1997). Flowering is an established plant trigger for monocarpic senescence (Thomas, 2013), and could be expected to bring about senescence-related changes in switchgrass tillers (Palmer *et al.*, 2015). However, reproductive switchgrass tillers will have two competing sink tissues; namely, the developing seeds, and the rhizomes and roots. Physiological mechanisms that regulate resource partitioning between these multiple sinks are essentially unexplored in switchgrass.

Changes in the flux and types of C and N compounds that flow from the shoots to the rhizomes could be part of this dormancy-triggering process. For example, progression of shoot senescence can be anticipated to lower the concentrations of recently assimilated photosynthates relative to amounts of remobilized compounds translocated to the rhizomes. Plants actively monitor cellular C and N status and the flux of hormones and metabolites to effectively coordinate processes such as growth, senescence and dormancy (Forde and Lea, 2007; Anderson *et al.*, 2010; Szal and Podgorska, 2012; Marchi *et al.*, 2013; Sheen, 2014). Together, these data suggest that changes in the amount and type of metabolites (including plant hormones) unloaded into the rhizomes could trigger processes ultimately leading to rhizome dormancy.

In this study, transcriptomic data obtained from rhizomes harvested from field-grown switchgrass (cultivar Summer) plants over two growing seasons were analyzed and merged with both broad and specific metabolite analyses to: (a) synthesize information on cellular processes that lead to dormancy; and (b) provide models that describe metabolic pathways present in dormant switchgrass rhizomes.

RESULTS

Environmental conditions during plant harvests

To compare yearly differences in growing conditions, air and soil temperatures as well as precipitation data were collected. Average air temperature and soil temperature at 10 cm depth were relatively similar during both harvest years (Figure S1a and b), and key shoot developmental events occurred within a similar time-frame (red and black arrows, Figure S1a). Overall, 2010 was wetter as compared with 2011 (Figure S1c). Growing degree days (GDD) analysis indicated that 2010 was a better growing year as compared with 2011 (Figure S1d).

Abscisic acid (ABA) levels were significantly enhanced during dormancy

Levels of ABA, a plant growth hormone associated with dormancy, were measured. Rhizome ABA content was found to be relatively low (~10–25 ng ABA per g rhizome fresh wt) in the rhizomes for the first four harvests, and increased significantly in rhizomes harvested in September (Figure 1a). ABA content increased further by nearly 20-fold to 200–480 ng of ABA per g of rhizome fresh wt from the September to the November harvests (Figure 1a). Although there were differences in total ABA content between each harvest year, the profiles followed identical trends in both harvest years, with low ABA levels in growing rhizomes and high ABA levels in dormant rhizomes.

Rhizome starch and sucrose content change significantly over the course of the growing season

Starch is the primary C storage molecule in many plants, while sucrose could originate from shoots or through breakdown of starch. Starch content (measured as glucose equivalents) was low in rhizomes exiting dormancy in May and increased over the growing season with a peak accumulation in September, which coincides with the onset of rhizome dormancy. Starch content decreased by approximately 20% between the September (pre-dormant) and November (dormant) harvests (Figure 1b). Starch accumulation followed a similar pattern over the two harvest years (Figure 1b), except that the levels of starch were four- to sixfold higher at each harvest date in the second year post-establishment, likely due to enhanced stand growth in the years following establishment (Schmer *et al.*, 2006).

Sucrose levels (measured as glucose equivalents) were low in rhizomes collected during the early part of the season and increased significantly in dormant rhizomes (Figure 1c). Unlike the data observed for starch accumulation, there was minimal variation in sucrose levels at each harvest date across both years.

RNA-Seq mapping statistics

In order to better understand metabolic activity within the rhizome over the growing season, transcriptional activity was assessed using RNAseq. Overall mapping statistics for the two harvest years are shown in Table S1. Sequencing of 33 rhizome samples collected across 11 time points over 2 years yielded over 1.5 billion reads. Total read yields

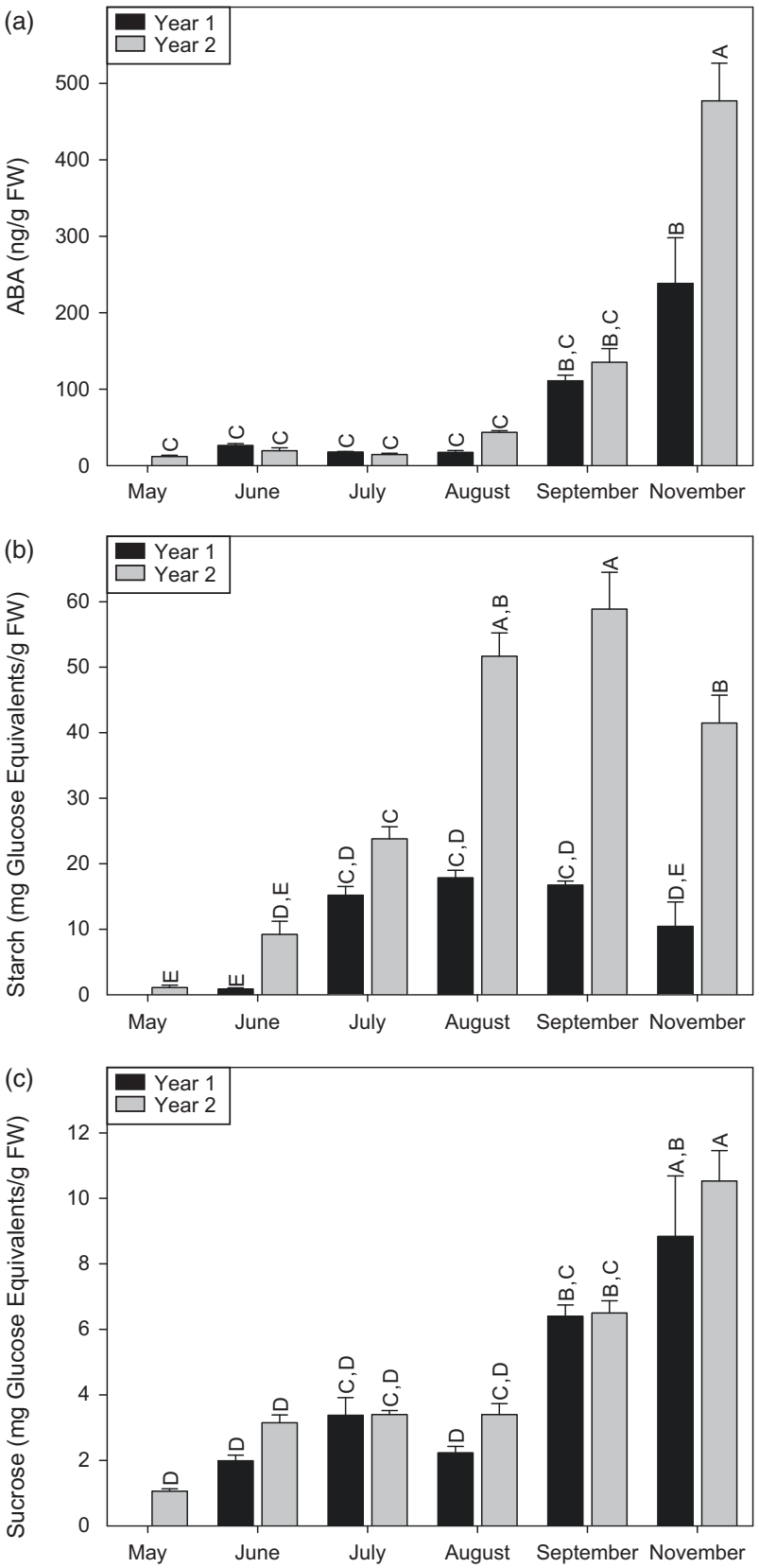


Figure 1. Seasonal changes in rhizome abscisic acid (ABA) (a), starch (b) and sucrose (c) content. In year 1 (2010) gray bars, and in year 2 (2011) black bars. Error bars are (standard errors). Different letters over bars indicates significant differences at $P \leq 0.05$.

were greater for 2010 samples (~50.7 million per sample) than for 2011 samples (~43.5 million per sample). Nevertheless, similar percentages of reads mapped to the genome (76.5%) and annotated gene regions (64.7%) across both years (Table S1).

Discriminant analysis effectively differentiates transcriptomes of pre-dormant and dormant rhizomes

The transcriptome profiles of all harvest dates were separated by discriminant analysis using principal components of expressed transcripts as covariates (Figure 2a). The first two canonical components accounted for 87.4% of the total variance and differentiated the transcriptomes into six main groups that corresponded with specific aerial plant developmental stages across both years. The May harvests collected at tiller emergence (purple triangles, Figure 2a) were differentiated from the transcriptional profiles of all other developmental time points and appeared to represent an intermediate between the end of the growing season and the beginning of active vegetative growth. The only two transcriptome profiles that overlapped with one another included those sampled from the July and August harvests. The greatest discrimination was observed between the August (gold symbols) and September (green symbols) rhizome harvests based on the first canonical axis, which accounted for 78.6% of the variance. The November transcriptomes (blue symbols) were also well differentiated (Figure 2a).

Differentially expressed genes (DEGs) cluster with developmental stage

Hierarchical clustering analysis of the DEGs from both harvest years is depicted in Figure 2b. The DEGs from the entire dataset could be broadly segregated into 10 distinct clusters based on their expression patterns across the different harvest dates (labeled C1–C10; Figure 2b). About 15 570 genes were significantly upregulated during periods of active rhizome metabolism (May–July harvests, C1–C4; Figure 2b), while rhizomes harvested in May contained 4349 genes that were exclusively expressed at significantly higher levels relative to the rest of the harvest dates (C3 and C8; Figure 2b). Clusters C5–C7 predominantly contained genes that were upregulated at various time points during pre-dormancy, which included genes that were expressed highly in 2010 and 2011 September (C5), and genes expressed highly in August 2011 and September of both years (C6 and C7). DEGs in dormant rhizomes were similar across both harvest years and contained over 11 607 genes (C5, C9 and C10; Figure 2b).

Starch and sucrose metabolism are seasonally regulated

Of the six copies of starch synthase genes (*StarSyn*) expressed in rhizomes, two copies were most highly expressed in June, and two additional copies were strongly upregulated in November as compared with the other time points (Figure 3a). Dominant expression observed for one *StarSyn* copy in September also coincided with maximal expression of two glucose-1-phosphate adenylyltransferase

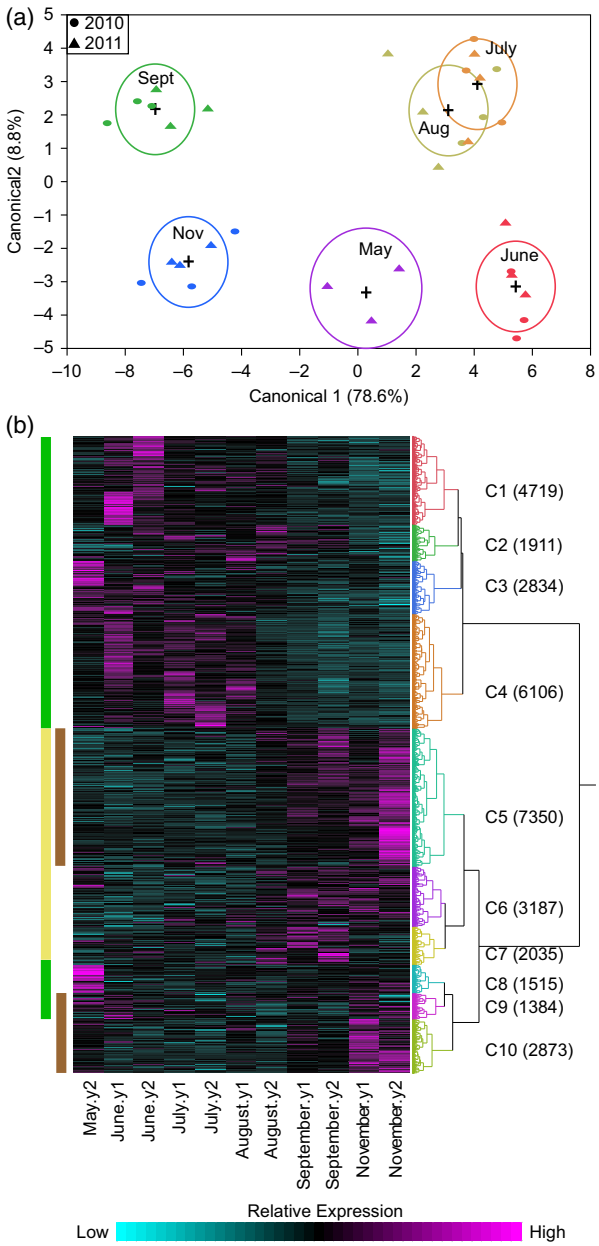


Figure 2. Global transcriptome analyses of switchgrass rhizomes.
(a) Discriminant analysis of all transcriptomes. Harvest dates are shown in different colors; 2010 samples, ovals; 2011 samples, triangles. May samples were collected only in 2011.
(b) Heatmap of all differentially expressed genes (DEGs). Bars on the left indicate approximate rhizome growth phases (green bars; May–July); pre-dormant (yellow bar; August and September) and dormant (brown bars; November). Clusters (C) with associated numbers of DEGS (brackets) are shown to the right. Relative expression is shown as a gradation from low (cyan) to high (magenta).

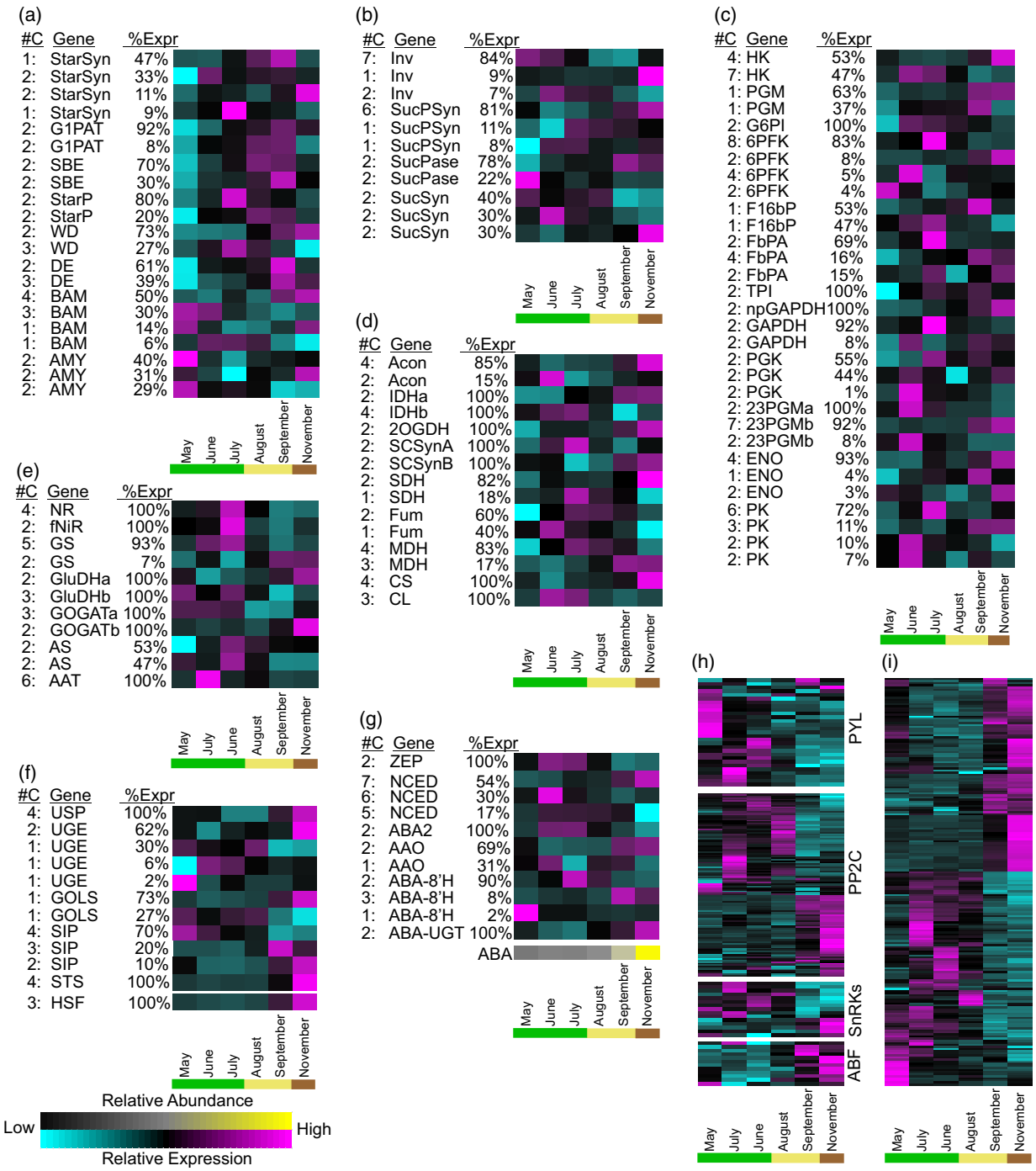


Figure 3. Heatmaps of expression profiles for genes associated with different metabolic pathways.
(a) Starch.
(b) Sucrose.
(c) Glycolysis.
(d) Tricarboxylic acid.
(e) Nitrate assimilation.
(f) Galactinol and raffinose family oligosaccharides (RFO) biosynthesis.
(g) Abscisic acid (ABA) biosynthesis and degradation.
(h) ABA signal transduction.
(i) Genes responsive to ABA. Gene abbreviations are provided in Data S1. For (a–g) #C, number of copies for an identified gene sharing the same expression profile; %Expr, percent of transcripts associated with all copies of a gene with the same expression profile. In (g), tissue ABA content (data from Figure 1a) is summarized as a heatmap, with gray indicating low and yellow indicating high ABA. Other details are as described in Figure 2.

genes (*G1PAT*; Figure 3a) and four genes coding for starch branching enzymes (*SBE*; Figure 3a). These transcriptional activities mirrored the peak starch accumulation observed in rhizomes harvested in September (Figure 1b). Two genes encoding starch phosphorylase (*StarP*; Figure 3a) were significantly upregulated in July, whereas transcripts for two other *StarPs* were greatest in rhizomes harvested in August and September (Figure 3a). Coinciding with reductions in starch levels observed during dormancy, genes encoding enzymes required for starch catabolism, including glucan/phosphoglucan water kinases (*WD*), debranching enzymes (*DE*) and three β-amylases (*BAM*), were significantly upregulated in the pre-dormant and dormant rhizomes. Genes encoding α-amylases (*AMY*) were expressed across all developmental stages, although two and one genes were upregulated at green-up and at dormancy, respectively (Figure 3a). In plants, starch is predominately broken down to maltose subunits by BAMs (Lu and Sharkey, 2006), which could account for the greater overall transcript levels of *BAMs* relative to *AMY* in dormant switchgrass rhizomes.

Sucrose synthesis and breakdown are dynamic processes in plant cells, and are responsive to a variety of abiotic and biotic stresses (Ruan, 2014). Analysis of the expression profiles of genes associated with sucrose metabolism in switchgrass rhizomes indicated significant linkage to seasonal changes in rhizome metabolism.

A total of 10 invertase (*Inv)* encoding gene-copies were found to be differentially expressed in rhizomes, with one *Inv* gene most highly expressed in dormant rhizomes (November, Figure 3b). Six of the eight sucrose-6-phosphate synthase (*SucPSyn*) copies (Figure 3b) detected were most highly expressed in dormant rhizomes, consistent with the higher levels of sucrose detected at dormancy. In contrast, the other two *SucPSyns* were expressed at similar levels during July, August and September (Figure 3b). Two of four sucrose-6-phosphate phosphatases (*SucPase*; Figure 3b) were significantly upregulated in the September and November samples. In contrast, six sucrose synthase genes (*SucSyn*) were variably expressed (three groups of two copies) over the growing season (Figure 3b).

Genes associated with glycolysis are differentially regulated over the growing season

Glycolysis is central to carbon metabolism in plant tissues, and changes in flux through this pathway commonly occur in response to many environmental triggers (Plaxton, 1996; van Dongen *et al*., 2011). Switchgrass rhizomes appeared to be no exception to this scenario. A total of 89 gene-copies encoding enzymes involved in glycolysis were expressed in at least one developmental (harvest) stage (Figure 3c). In general, there appeared to be a bimodal expression of the genes linked to this pathway. One set of copies was strongly upregulated during the growth phases (June and July harvests; Figure 3c, green bar), and the other set was upregulated during the pre-dormant and dormant phases (September and November harvests; Figure 3c, yellow and brown bars). Notable significant differences were observed for some glycolysis pathway genes, which could be reflective of changes in plant metabolism accompanying dormancy. For example, two copies of phosphoglucomutase (*PGM*) were significantly upregulated in dormant rhizomes. PGM catalyzes the interconversion of glucose-1-phosphate to glucose-6-phosphate, which is an intermediate for the synthesis of sucrose and related metabolites. Similarly, transcripts encoding non-phosphorylating GAPDH (*npGAPDH*) and five copies of genes encoding enolase (*ENO*) were strongly upregulated at the end of the year (Figure 3c).

Gene expression in the TCA and nitrogen assimilatory pathways reveals significant remodeling of primary metabolism during dormancy

Similar to the expression profiles observed for genes associated with glycolysis, genes encoding enzymes required for the TCA cycle and N-assimilation largely followed a bimodal expression pattern. Of significance to rhizome metabolism during dormancy, several copies of genes encoding aconitase (*Acon*), isocitrate dehydrogenases (*IDHa*, NADP+dependent), 2-oxoglutarate dehydrogenase (*2OGDH*) and citrate synthase (*CS*) were significantly upregulated in dormant rhizomes. In contrast, genes encoding citrate lyase (*CL*) were upregulated in actively growing rhizomes collected in June and July (Figure 3d).

Expression patterns for genes associated with N assimilation were suggestive of significant redirection of N metabolism from one of active N assimilation from inorganic NO_3^- during growth (June–July) to recycling of N from amino acids during dormancy (Figure 3e). Supporting these hypotheses, transcripts encoding nitrate (*NR*) and ferrodoxin-dependent nitrite reductases (*fNiR*), asparagine synthetases (*AS*), aspartate aminotransferases (*AAT*) and several glutamine synthetases (*GS*) were significantly upregulated in the July samples and subsequently down-regulated as the growing season progressed. Genes encoding the two other major enzymes in N metabolism, namely glutamate-oxoglutarate amino transferase (*GOGATb*, ferrodoxin dependent) and glutamate dehydrogenase (*GluDHa*, NADP+ dependent), were strongly upregulated in dormant rhizomes (Figure 3e).

Identification of putative HSF transcription factors controlling genes associated with the biosynthesis of galactinol and raffinose family oligosaccharides (RFO) during dormancy

Raffinose family oligosaccharides can accumulate in seeds and during stress in other plant organs, where they can function to protect cells from dehydration and reactive

oxygen species. In addition, they can be readily transported in the phloem (Sengupta *et al.*, 2015). Transcript evidence indicated a significant upregulation of genes associated with RFO biosynthesis during dormancy. Gene-copies encoding UDP-sugar pyrophosphorylase (*USP*), UDP-glucose-4-epimerases (*UGE*), one copy of galactinol synthase (*GOLS*), raffinose synthases (*SIP*) and stachyose synthases (*STS*) were highly upregulated during dormancy (Figure 3f). In Arabidopsis, *GOLS* expression is controlled by a heat-shock transcription factor (HSFA2; Nishizawa *et al.*, 2006). Three HSFA2 homologs were significantly upregulated in September and November samples, suggesting that they may be involved in regulating RFO accumulation in switchgrass (Figure 3f).

ABA-responsive gene expression is a significant event impacting rhizome dormancy in switchgrass

Expression profiles of genes associated with ABA metabolism and response were undertaken to verify a relationship to the significant changes in ABA content detected in rhizomes (Figure 1a). Expression profiles of genes associated with ABA biosynthesis only partially explained the gradual increases in ABA levels observed throughout the growing season (Figure 3g). For example, peak expression of *ZEAXANTHIN EPOXIDASE* (*ZEP*) occurred during the June and July harvests, whereas *9-CIS-EPOXYCAROTENOID DIOXYGENASES* (*NCED*) were significantly upregulated in rhizomes collected in June or November. Genes encoding switchgrass homologs for *ABA2* (*ABSCISIC ACID REDUCTASE*) were more enriched in the June/July harvests, whereas transcripts for *AAO* (*ABSCISIC ACID ALDEHYDE OXIDASE*) were enriched in both growing and dormant rhizomes (Figure 3g). ABA can be degraded by cytochrome P450 hydroxylases and sequestered via conjugation with glucose (Seiler *et al.*, 2011; Dong *et al.*, 2014). Expression of six putative *ABA-8′ hydroxylases* was detected in rhizomes, with two of these copies having maximal expression in June and July. This observation suggests that catabolism of ABA was enhanced during growth stages and possibly minimized at later stages of the growing season, consistent with measured ABA levels (Figure 1a). Similarly, two putative ABA-UDP-glucosyl transferase-encoding genes were highly expressed in rhizomes obtained from August to November (*ABA-UGT*; Figure 3g), raising the possibility that ABA was being sequestered in an inactive form in rhizome cells prior to entry into dormancy.

Abscisic acid signaling is transduced by receptors that bind to this hormone and subsequently activate ABA-responsive signaling cascades (Miyakawa *et al.*, 2013). The primary receptors of ABA are encoded by *PYRABACTIN RESISTANCE 1* and related genes (Miyakawa *et al.*, 2013), collectively abbreviated as *PYL* (Figure 3h). Expression of 28 switchgrass *PYL* homologs was detected in rhizomes, and a majority of these genes were more highly expressed in early parts of the growing seasons, with two *PYLs* upregulated specifically in September and another one in November (Figure 3h). Expression of 122 *PROTEIN PHOSPHATASE 2C* (*PP2C*) genes was also observed (Data S1), with 30 genes having the highest expression in dormant rhizomes (Figure 3h). Similarly, seven of 15 putative sucrose non-fermenting related kinase (*SnRKs)* and 10 of 12 ABRE-binding factors (*ABF)* encoding genes were significantly upregulated in dormant rhizomes, consistent with an increase in ABA content.

The switchgrass genome contains 820 genes with similarity to 282 Arabidopsis genes that definitively responded to ABA (Lumba *et al.*, 2014). A total of 416 of these switchgrass ABA-responsive homologs (267 induced, 149 repressed) was differentially expressed as shown in Figure 2b. Among these ABA-responsive DEGs, 90/267 genes were strongly upregulated and 106/149 genes were significantly downregulated in dormant rhizomes (Figure 3i).

Cold-responsive genes are induced in dormant rhizomes

Both air and soil temperatures decreased towards the end of the growing season. Temperatures were above freezing in September (Figure S1), but a killing-frost was experienced prior to the November harvests of both years. Although aerial tissues had largely senesced in Summer plants prior to the killing-frost, it was likely that genes linked to cold stress could have been induced in rhizomes. Plants respond to cold/freezing temperatures by complex cellular pathways (Lissarre *et al.*, 2010; Zhou *et al.*, 2011). Among these pathways, signaling cascades associated with C-repeat binding factors (CBF) have been studied extensively (Weston *et al.*, 2008; Zhou *et al.*, 2011). CBFs are transcription factors that control the expression of cold responsive genes (COR) and are, in turn, positively regulated by inducers of CBF expression (ICE). CBFs belong to the large family of apetella2/ethylene-responsive element-binding proteins (AP2/EREBP), which also contain the dehydration responsive element-binding proteins (DREB) class of transcription factors (Agarwal *et al.*, 2006). ICE belongs to the basic helix-loop-helix (bHLH) family of transcription factors (Zhou *et al.*, 2011).

The expression profiles of all bHLH and AP2/EREBP encoding genes and putative CORs identified in the rhizome DEGs are shown in Figure S2. A simplified linear model for the induction of the COR genes is also shown (Figure S2a). Notably, two separate groups of bHLH genes were upregulated in pre-dormant and dormant rhizomes, respectively (Figure S2b). Examination of these upregulated genes indicated that ICE-like proteins encoded by genes *Pavir.Hb00982*, *Pavir.Eb03736* and *Pavir.Ea03858* (Data S1) were most abundantly induced during pre-dormancy and downregulated during dormancy, marking them as potential switchgrass ICE homologs. Similarly,

eight switchgrass genes encoding DREB-like proteins were differentially induced. *Pavir.J37967* and *Pavir.Ea00429* encoding DREB-2A-like proteins were upregulated in dormant rhizomes. Five DREB1A-like proteins encoded by *Pavir.J03892*, *Pavir.Ba01280*, *Pavir.Ba01281*, *Pavir.J03893* and *Pavir.J03891* were significantly downregulated in dormant rhizomes, suggesting a minimal role in cold-related signaling. A DREB1C homolog encoded by *Pavir.J35991* was also significantly downregulated in pre-dormant and dormant rhizomes (Figure S2b; Data S1).

A large number of COR encoding genes was strongly upregulated in dormant rhizomes (Figure S2c), which also included the dehydrins and LEA-genes. Overall, these analyses indicated that orderly progression to dormancy in switchgrass rhizomes includes a cold-responsive component, in addition to ABA-related signaling.

Networks of transcription factors are associated with seasonal aspects of rhizome metabolism

Network analysis confirmed that rhizome transcriptomes undergo significant remodeling over the course of a growing season (Figure 4a). Six out of a total of 23 co-expression modules identified were strongly associated with harvest dates that corresponded to specific stages of shoot and rhizome development. For example, module M1 contained 10 558 co-expressed genes that were highly expressed in dormant rhizomes (M1, orange, Figure 4a), and was strongly separated from clusters containing 1709 genes upregulated during green-up (May, M5, magenta, Figure 4a); and another module containing 3053 genes upregulated during periods of active rhizome growth (June–August, M3, red, Figure 4a). One cluster of 1507 co-expressed genes appeared to link the transition of rhizomes growth to dormancy (September, M6, cyan, Figure 4a). Another cluster contained 287 genes highly expressed in rhizomes at green-up and dormancy (M15, yellow, Figure 4a), and a co-expression module containing 53 genes with higher co-expression in dormant rhizomes relative to expression profiles at green-up (M23, green, Figure 4a).

A varied number of switchgrass transcription factors (TFs) were found in modules M1 (190), M3 (141), M5 (44), M6 (22), M15 (10) and M23 (2), respectively (Figure 4c). Annotations of Arabidopsis TFs orthologous to the switchgrass TFs identified in the six modules were used to develop a gene-reference-into-function (GeneRIF) analysis using text mining terms shown in Table S2. GeneRIF analysis indicated that switchgrass orthologs to Arabidopsis TFs associated with 'End of the Year' and 'Other Hormones' (non-ABA) text mining categories were significantly enriched in M1 (November samples), whereas those associated with 'Other Hormones' and 'Tissues' were significantly enriched in M5 (May samples). No significant enrichments were found in the other modules. These data indicate that many of these TFs are likely involved in regulating seasonal aspects of rhizome metabolism.

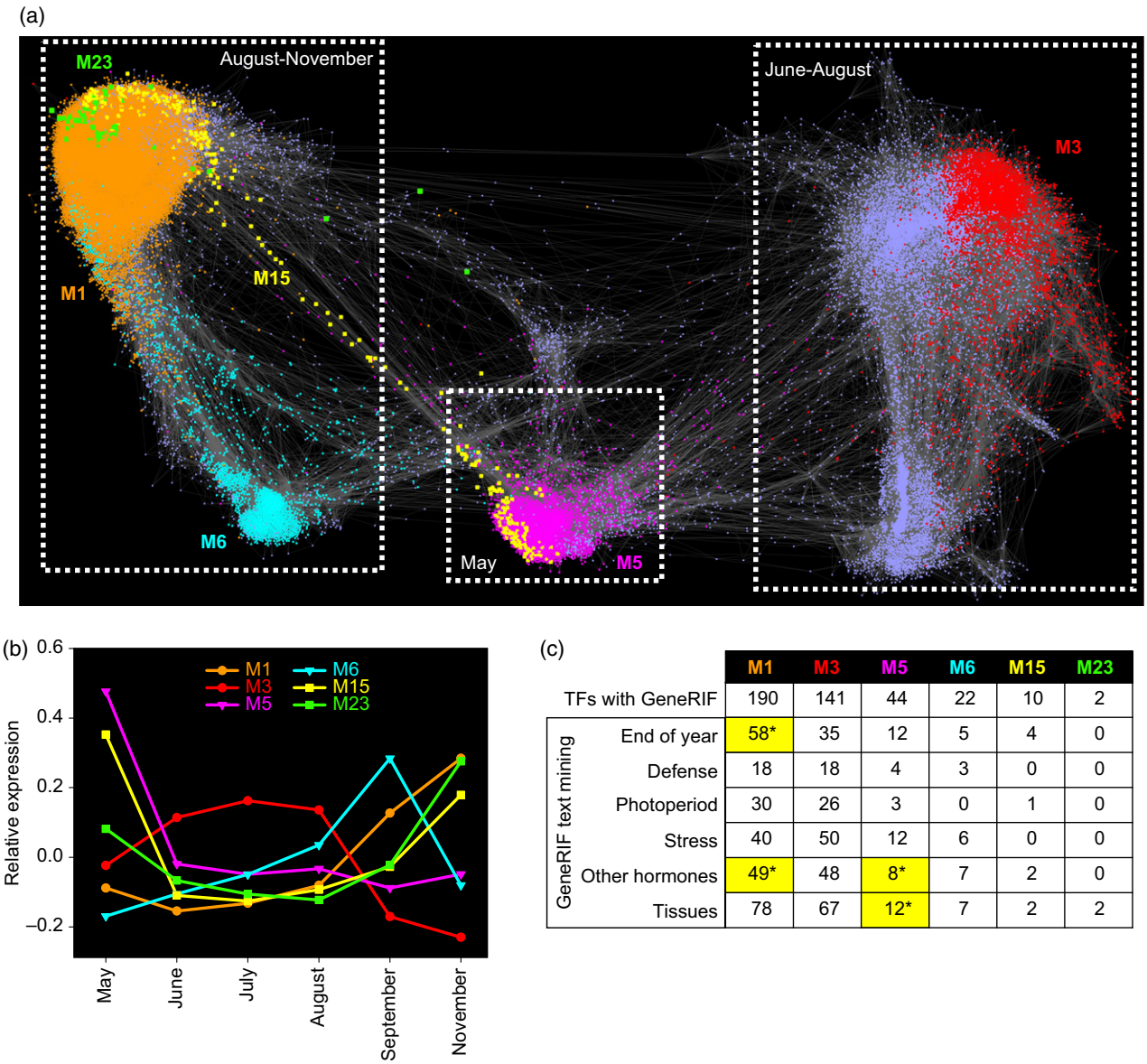
Metabolite profiles reflect the seasonal changes in rhizome transcriptomes

Polar metabolites from rhizomes were extracted and profiled by GCMS, and then spectra were matched to existing databases for identification and quantitation. All putative metabolite features were subjected to discriminant analysis (Figure 5a). Canonical axis 1 accounted for 52% of the variation and separated the metabolomes found in May from all the others (purple ovals, Figure 5a). Canonical axis 2 accounted for 35% of the variance and separated the other harvests into clusters that largely distinguished the September (green ovals, Figure 5a) and November (blue ovals, Figure 5a) harvests from one another and from the June–August periods. There was considerable overlap in the June–August metabolomes sampled from actively growing rhizomes. These broad-scale aspects of the metabolomes were to a large degree consistent with the changes observed in the transcriptomes, indicating that shifts in metabolite concentration occurring over the time course corresponded well with the transcriptional changes driven by rhizome developmental status.

Metabolites with a quality score > 65 as detected by the GCMS software and present in samples harvested across both years were compared (Figure 5b) and analyzed for significant correlations to the specific transcriptome network modules described in Figure 4 (Table S3). In general there were significant positive correlations of metabolites that included several amino acids, organic acids, sugars and sugar alcohols with module 1 (orange dots), module 6 (cyan dots), module 15 (yellow dots) and module 23 (green dots; Figure 5b). All three of these modules were associated with pre-dormant or dormant rhizomes. Associations for other modules were limited to ornithine with module 1 (red dot) and a few assorted metabolites with module 5 (magenta dots). These data were largely consistent with metabolites present in Summer rhizomes harvested at the time of aerial senescence, but before the onset of dormancy (Palmer *et al.*, 2014a), and suggested that metabolite accumulation especially at the pre-dormant and dormant stages could be a result of the up/downregulation of several metabolic processes observed in the transcriptome analyses.

DISCUSSION

The extensive transcriptomic datasets combined with the metabolite analyses indicated a significant redirection in rhizome metabolism as the plants transitioned to dormancy. These changes appear to be linked to both the phenological status of the shoots, and to rhizome ABA, starch and sucrose content.



(c)

		M1	M3	M5	M6	M15	M23
TFs with GeneRIF		190	141	44	22	10	2
GeneRIF text mining	End of year	58*	35	12	5	4	0
	Defense	18	18	4	3	0	0
	Photoperiod	30	26	3	0	1	0
	Stress	40	50	12	6	0	0
	Other hormones	49*	48	8*	7	2	0
	Tissues	78	67	12*	7	2	2

Figure 4. Gene co-expression network analysis.
(a) Network is shown where each node is a gene (30,295) and each edge is a weighted co-expression measure between two genes (770,881). Select co-expression modules are indicated with a letter and color. White boxes provide association of modules with harvest dates.
(b) Expression profiles for the six modules highlighted in (a) coincident with harvest dates (months).
(c) Numbers of switchgrass transcription factors (TF) with Arabidopsis orthologs identified with a specific gene reference into function (GeneRIF) associated with each module. Yellow highlights indicate significant enrichment using Fisher's exact test.

Gene-expression network analyses corroborated the seasonal aspects of switchgrass growth with the May early season transcriptomes occupying a unique expression space, likely linked to an exit from dormancy and entry into growth of aerial tissues. There was minimal separation of the expression networks in rhizomes sampled during periods of active aerial growth in June–July, indicating limited changes to the transcriptomes over this time period. Although water availability appeared to have influenced rhizome transcriptomes at the time of seed fill (August 2010 versus August 2011) in the two collection years, differences in the August transcriptomes did not appear to impact the transcriptomes observed in September (predormant; aerial senescence) and November (dormant) harvests. Conceivably, rhizome metabolism in September is impacted from cooler night temperatures, a loss and/or change in assimilates originating from the aerial tissues, and changes triggered in response to increased concentrations of ABA and sucrose. Many shoot-derived compounds including sucrose and ABA act as signaling molecules

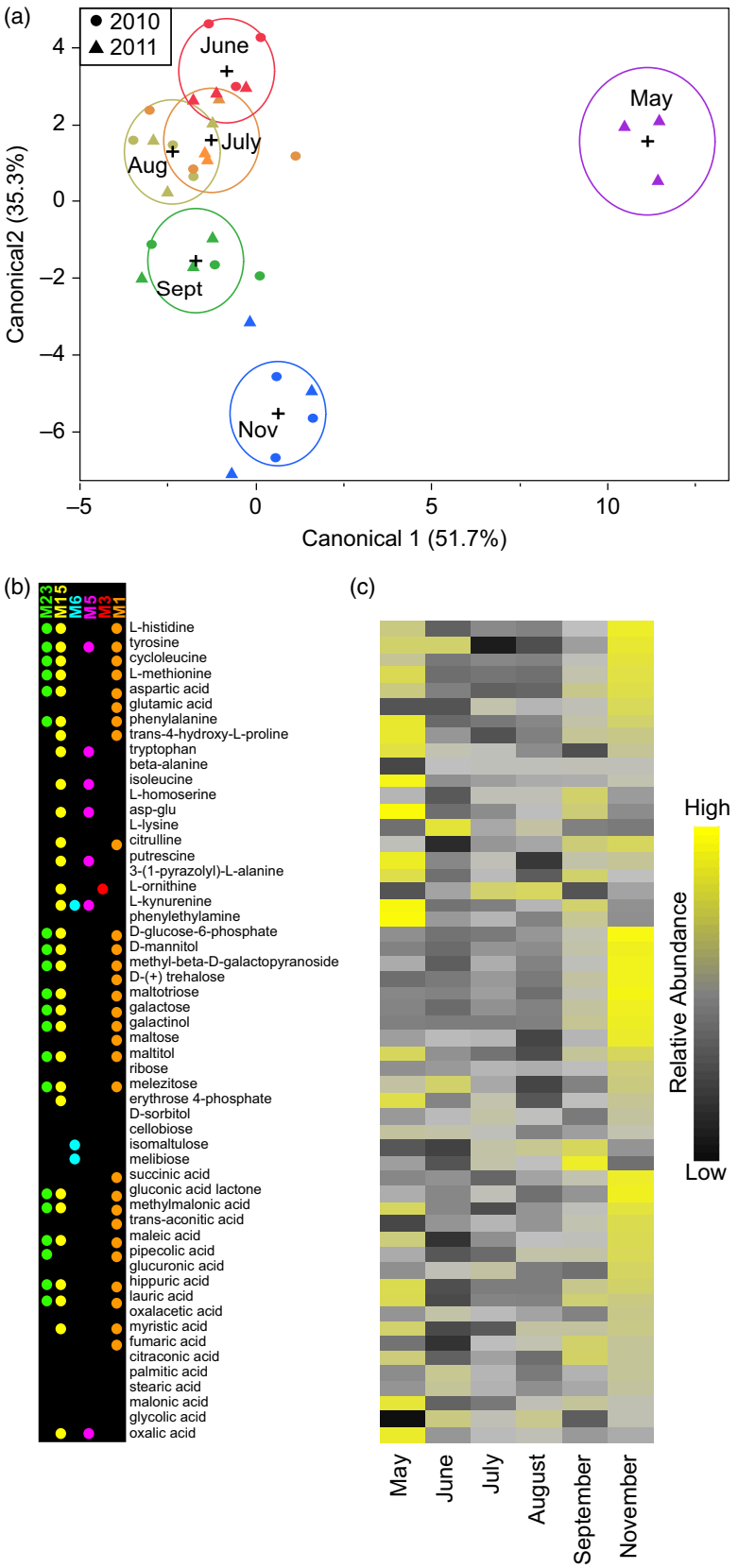


Figure 5. Metabolite profiling of switchgrass rhizomes.
(a) Discriminant analysis of all metabolite features identified with individual samples collected in 2010 (circles) and 2011 (triangles). Harvests associated with similar shoot development from both years are shown inside colored ovals.
(b) Significant positive associations of metabolites (colored dots) to specific gene co-expression modules given in Figure 4.
(c) Corresponding heatmap for metabolites. Relative abundance values are shown as black = low and yellow = high.

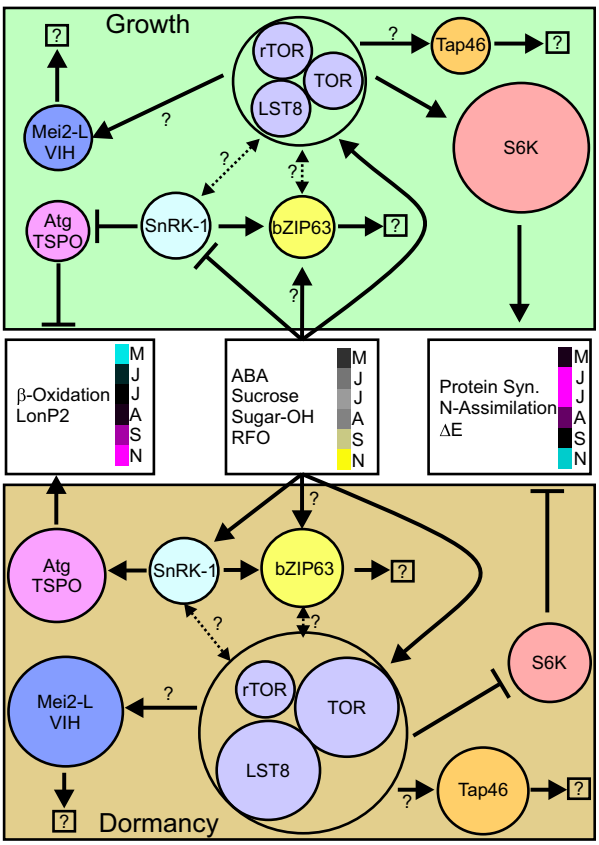


Figure 6. Model for integration of switchgrass rhizome metabolism through TOR signaling adapted from Dobrenel *et al.* (2016). This model is based on transcript data for components of the TOR complex (light blue circles) and key targets found in switchgrass rhizomes during growth (green, upper panel), and dormant stages (brown, bottom panel). Middle panel boxes summarize expression profiles of genes associated with β-oxidation, and the peroxisomal Lon protease 2 (*LonP2*), tissue levels of ABA, sucrose, raffinose family oligosaccharides (RFOs) and sugar alcohols, and expression profiles of genes associated with protein synthesis (Protein Syn.), N-assimilation and mitochondrial energy metabolism (ΔE). Relative expression is shown from low (cyan) to high (magenta). Relative metabolite abundance values are shown as low (black) and high (yellow). Single arrows indicate activation, and blocked arrows indicate repression. The size of circles represents relative transcript abundance between 'Growth' and 'Dormancy' stages. All data and gene annotations associated with this figure are given in Data S1.

(Wang *et al.*, 2012; Nakashima and Yamaguchi-Shinozaki, 2013; Hausler *et al.*, 2014; Ruan, 2014) and impact a variety of root responses. Seasonal changes in the levels of these compounds in rhizomes appear to play a significant role in switchgrass biology as well.

Pathway enrichment for the DEGs associated with each of the six key co-expression modules described in Figure 4 provided more support for distinct changes in rhizome metabolism during pre-dormancy and dormancy. The greatest (> 50%) enrichment of DEGs assigned to a specific pathway was only observed in modules 6 and 1 associated with pre-dormant and dormant stages, respectively (Table S4). Approximately 75% of the genes with putative roles in the ribosomal function pathways were found in module 6, plausibly in readiness for the massive changes to the transcriptome occurring during dormancy. Similarly, dormancy-associated module 1 contained 33 KEGG pathways that were significantly enriched (Table S4), including 'proteasome', 'lipoic acid metabolism', 'β-oxidation', 'lysine degradation', 'autophagy' and 'citrate cycle'. These pathways appear to be important components of dormancy and are suggestive of scavenging and/or reutilization of N and C, potentially in response to increasing tissue ABA content.

Abscisic acid regulates seed and bud dormancy and responses to abiotic stress via a receptor-based mechanism (Nambara *et al.*, 2010; Sonnewald and Sonnewald, 2014; Vishwakarma *et al.*, 2017). The large increase in ABA levels in dormant rhizomes implicated ABA as a key regulator of rhizome dormancy in switchgrass. This regulatory role was supported by a significant upregulation of switchgrass genes orthologous to ABA-responsive genes in Arabidopsis (Lumba *et al.*, 2014). These included a number of *PYLs*, *PP2Cs*, *SnRKs* and *ABFs*, which are essential for ABA signaling, and downstream targets of ABA such as *LEAs*, *HSF 2A* and *GOLS*. Several metabolites associated with ABA, such as RFOs and sugar alcohols, were also elevated in dormant rhizomes, providing a strong connection between tissue ABA levels and changes in cellular metabolism during rhizome dormancy in switchgrass.

Variation in the expression profiles of the genes critical for ABA biosynthesis made it difficult to determine which pathways were responsible for enhancing ABA levels during September and November. However, ABA can be readily transported, sequestered and metabolized in plants (Dong *et al.*, 2015; Verslues, 2016). Plausibly, ABA biosynthesis in rhizomes, translocation of ABA from senescing shoots in September, increased ABA stability due to downregulation of genes associated with ABA catabolism, and conversion of conjugated forms of ABA (not measured) all likely contributed to increased rhizome ABA content at the end of the growing season.

Sucrose is expected to be another important compound impacting switchgrass rhizome dormancy. Sucrose biosynthesis in pre-dormant and dormant rhizomes appears to be linked to starch breakdown. Transcripts for several starch-metabolizing enzymes were upregulated in parallel to transcripts encoding enzymes required for sucrose biosynthesis. Thus, in dormant switchgrass rhizomes, sucrose probably acts as a hub metabolite, an osmolyte, an intermediate in RFO biosynthesis, a source of energy and potentially as a signaling molecule (Wind *et al.*, 2010). Among the genes anticipated to be regulated by sucrose are sucrose and phosphate transporters (Wind *et al.*, 2010). Several putative switchgrass sucrose and phosphate transporters were upregulated in dormant rhizomes (Figure S3a). Future experiments will be needed to unravel the

role of sucrose in signaling pathways in switchgrass rhizome dormancy.

The large-scale changes to the transcriptome and metabolome including changes in ABA levels, especially during the pre-dormant and dormant phases of switchgrass rhizomes, suggested that control of energy and nutrient utilization could be important determinants during dormancy. The target of rapamycin kinase (TOR) occupies a central position in energy and nutrient metabolism in plants, and is interactively linked to SnRK-regulated pathways (Dobrenel *et al.*, 2013, 2016; Henriques *et al.*, 2014). SnRK activity in plant cells is controlled by tissue ABA content via PP2Cs, effectively linking TOR, SnRK and ABA (Dobrenel *et al.*, 2016). The TOR complex (TORC) consists of three components, namely TOR, regulatory associated protein of TOR (RAPTOR), and the small lethal with SEC13 protein (LST8). Depending on internal and external cues and phosphorylation status, the TORC can interact with, and negatively or positively control, a large number of cellular processes (Dobrenel *et al.*, 2016; Pu *et al.*, 2017). In summarizing a large body of information, Dobrenel *et al.* (2016) proposed a model where high energy and nutrient status increases TORC activity, which results in higher levels of nitrate assimilation, RFO and ABA. In turn, high ABA, low energy or starvation induces the SnRK pathway, which negatively regulates nitrate assimilation, and concomitantly increases autophagy and cellular levels of sucrose, starch triglycerides and TCA intermediates (Dobrenel *et al.*, 2016).

The switchgrass genome encodes a number of TORC and TORC-regulated gene orthologs whose expression profiles were determined (Figure S3b) and then incorporated into a model (Figure 6) integrated with changes in ABA, sucrose and sugar alcohol levels. The core components of the TORC/SnRK pathways during growth stages of switchgrass rhizomes (Figure 6, top panel) were largely in agreement with the models proposed by Dobrenel *et al.* (2016). Tissue levels of ABA, sucrose and RFOs were low in switchgrass rhizomes during the growth phase (Figure 6), which could activate TORC (arrow) and repress SnRK (blocked arrow) signaling. A key target for TORC is the ribosomal S-6-kinase (S6K). Transcripts for S6K, and pathways impacted by S6K including those associated with protein synthesis (Protein Syn.), N-assimilation and improved cell energetics (ΔE) were upregulated, whereas genes controlled by SnRK were downregulated during rhizome growth phases (Figure 6). SnRK-regulated genes included those required for autophagy (Atg) and an ortholog to the small translocator protein (TSPO). Whereas the model for rhizome metabolism regulated by TORC during 'growth phases' was congruent with those proposed for annual plants, transcript and metabolic evidence found in pre-dormant and dormant switchgrass rhizomes were less consistent with the Dobrenel *et al.* (2016) models (Figure 6, bottom panel).

Transcript levels for *TOR* and *LST8* increased significantly in pre-dormant and dormant rhizomes, whereas those for *RAPTOR* (rTOR) decreased at the same sampling dates. Similarly, transcript levels for genes encoding *TAP46*, *VIH* and *Mei2-like* (Mei2-L, Figure 6), which are direct targets of TOR in other plants (Dobrenel *et al.*, 2016) increased during dormancy along with expression of the TF, *bZIP63*. These TORC-impacted genes are, in most instances, activated during growth in other plants, and growth processes impacted by these genes might be expected to be inactive during switchgrass rhizome dormancy. In Arabidopsis, genes encoding bZIP63, TAP46 and TSPO are responsive to ABA and/or sugar alcohols (Matiolli *et al.*, 2011; Hu *et al.*, 2014). Increased levels of ABA and/or sugar alcohols during switchgrass rhizome dormancy could have similarly induced *bZIP63*, *TAP46* and *TSPO* transcription in dormant switchgrass rhizomes, although the mechanism of this integration with TORC signaling and dormancy is unknown.

Pathway enrichment, especially in dormant rhizomes, supports the theory that SnRK could link TORC and autophagy during switchgrass rhizome dormancy (Figure 6). For example, elevated levels of SnRKs and ABA could activate genes such as *TSPO* and the peroxisomal marker *LonP2* (Goto-Yamada *et al.*, 2014) that are required for autophagy and efficient scavenging and reutilization of metabolites (Figure S3b). Conceivably, greater abundance of TOR, LST8, TAP46, bZIP63 and SnRK in dormant rhizomes may regulate cellular metabolism to maintain appropriate activity levels of competing pathways, such as protein synthesis and autophagy, as well as reinforce ABA-dependent signaling during the dormant period.

Based on the datasets presented in this study, a putative integrated model of metabolism in dormant switchgrass rhizomes was developed (Figure 7). In Figure 7a, a simplified model of primary metabolism during dormancy is proposed. In Figure 7b, the observed levels of metabolites (black/yellow bars) and transcript abundances of select key genes (cyan/magenta bars) that support the model are given.

Starch is expected to be the primary driver of rhizome metabolism during dormancy. Starch accumulation was highest during August and September, and mirrored the upregulation of genes encoding enzymes required for starch biosynthesis, such as *StarSyn*. Concomitant reduction in starch levels in dormant rhizomes correlated with increased transcript levels of several genes encoding enzymes required for starch degradation, including water dikinases (*WD*). Further breakdown of starch by BAM (1) yields maltose and ultimately glucose-6-phosphate (Glc6P), and levels of these two compounds increased significantly in dormant rhizomes (Figures 5b and 7b), as did transcript levels of genes encoding BAM and HK (Figure 3). Levels of maltose and Glc6P were also positively correlated to gene

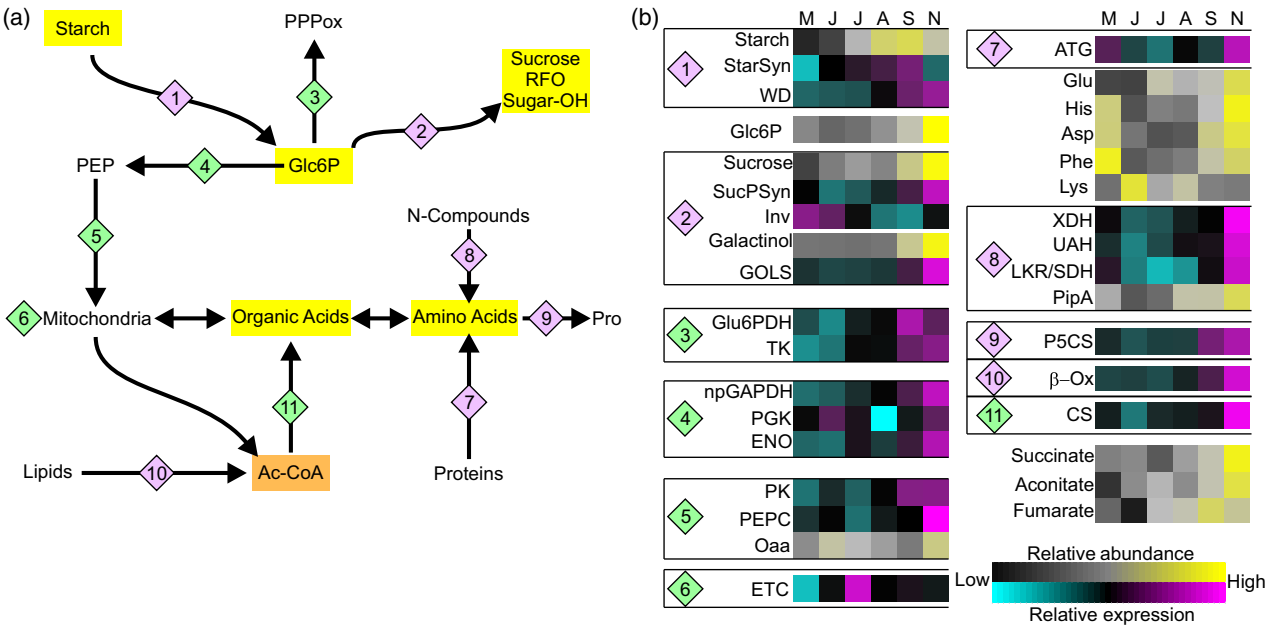


Figure 7. Predicted cellular metabolism in dormant switchgrass rhizomes.
(a) Predicted connections in primary metabolism. Metabolites enriched in dormant rhizomes are shown with yellow boxes. Purple diamonds indicate specific metabolic processes associated with each identified step based on transcript evidence. Green diamonds indicate transcript evidence for processes that can generate substrate-level ATP and/or reducing equivalents.
(b) Associations between metabolite levels (where detected; black, low; yellow high) and select key genes associated with each pathway. Other abbreviations as described in (a) and in the text.

co-expression module 1 found in dormant rhizomes (Figures 4 and 5b), providing further support for their importance during dormancy. Glc6P is required for the biosynthesis of sucrose, RFOs and sugar alcohols (2). Levels of sucrose, RFOs, sugar alcohols and expression levels of genes associated with their biosynthetic pathways were greatest in dormant rhizomes as well. In addition, for *GOLS* a strong correlation was found to a specific group of *HSFA2* transcription factors. *HSFA2* is regulated by ABA in Arabidopsis (Nishizawa *et al.*, 2006), and a similar regulatory circuit appears to be functional in switchgrass.

The data discussed above suggest an efficient transfer of stored carbon from plastids to RFOs and sugar alcohols in the cytoplasm during dormancy. However, cytosolic Glc6P is an intermediate of other metabolic pathways that enhance substrate-level generation of ATP and reducing equivalents. Transcripts for several genes associated with the oxidative pentose phosphate pathway (PPPox; 3), including Glc6P-dehydrogenase (Glc6PDH) and transketolases (TK, Figure 7b) were enriched in dormant rhizomes, providing evidence for the utilization of Glc6P to generate reducing equivalents via the PPPox pathway, and potentially feed resulting 3PGA into glycolysis.

Phospho*enol*pyruvate (PEP; 4) derived from Glc6P can improve substrate level generation of both ATP and reducing equivalents under stress (Chastain *et al.*, 2011). In dormant switchgrass rhizomes, PEP appears to be generated by ENO. Transcripts of genes encoding *ENO* were massively upregulated in dormant rhizomes, along with transcripts encoding *npGAPDH*. NPGAPDH converts 3P-glyceraldehyde to 3P-glyceric acid and generates NADPH (Rius *et al.*, 2006). In turn, 3P-glycerate can be readily converted by phosphoglycerate mutases (transcripts also elevated in dormant rhizomes) to 2P-glycerate, the substrate for ENO. This cycle could conceivably generate significant amounts of substrate level ATP via the catalytic activities of pyruvate kinase (PK; Figures 5 and 7b). PEP can be converted to oxaloacetic acid (Oaa) through the catalytic activities of PEPcarboxylase. Oaa, and expression levels of PEPcarboxylases encoded by *Pavir.Ab00954* and *Pavir.Aa02787*, were significantly higher in dormant rhizomes, providing support for this pathway. Recapturing portions of respired CO_2 could potentially improve overall energetics during dormancy and increase substrate level production of reducing equivalents through decarboxylation of malate. As an example, *Pavir.Ea02983*, encoding a putative cytosolic NADP-malic enzyme, was massively upregulated in dormant switchgrass rhizomes.

Substantial increases in substrate level formation of ATP and reducing equivalents were strongly supported by evidence of modulation of mitochondrial metabolism (6) during dormancy. Plant mitochondrial function is malleable and responsive to energy demands, ROS and other stresses (Shingaki-Wells *et al.*, 2014; Wilson, 2015; Igamberdiev and Eprintsev, 2016). Shifts in mitochondrial function likely occurred in dormant rhizomes as genes coding for TCA

cycle enzymes were downregulated, potentially lowering rates of oxidative phosphorylation. In support of this finding, expression of genes encoding proteins needed for mitochondrial electron transport were downregulated in dormant rhizomes (Figures 6, 7b and S3d), along with the significant upregulation of an alternative oxidase encoded by *Pavir.Ab02811*. Lowered mitochondrial activities were also evident in increased organic acid and amino acid pools as rhizomes transitioned to a dormant phase. Overall, it is possible that lowered aerobic respiration occurring in dormant rhizomes likely modulated the generation of ROS through lowered O_2 consumption and changed the relative levels of several metabolites related to the tricarboxylic acid cycle. These changes in aerobic respiration could potentially regulate TORC signaling through changes in nutrient levels and energy status of the cells.

Transcripts associated with a number of KEGG pathways indicative of C and N reutilization were significantly enriched in dormant rhizomes (Table S3), and included 'autophagy' and 'lysine degradation'. As an example, expression profiles of genes encoding proteins required for autophagy are shown (Figures 7b and S3b) to highlight the potential for greater N-recycling of proteins and other N-compounds during dormancy (Figures 7b, 8). Protein degradation generates amino acids, and several amino acids and their derivatives were enriched both at green-up and at dormancy, suggestive of protein catabolism and/or slower reutilization for protein synthesis, consistent with low or no rhizome growth in May and November, respectively. Pipecolic acid (PipA; Figure 7b, 8) derived from lysine catabolism was enriched in dormant rhizomes, whereas lysine was most abundant in growing rhizomes. Transcripts encoding a lysine-degrading enzyme, lysine ketoglutarate reductase/saccharophine dehydrogenase (LKR/SDH), were significantly upregulated during dormancy (Figure 7b). Similarly, genes encoding key enzymes needed for nucleic acid and ureide metabolism including xanthine dehydrogenase (XDH) and ureidoglycine amidohydrolases (UAH; Figures 7b, 8, and S3e) were significantly upregulated during dormancy. The end product of N-metabolism appears to be the recapture of ammonia into amino acids.

Glutamate (Glu) was enriched in dormant rhizomes, as were transcripts encoding GDHa and GOGATb (Figure 3) potentially allowing cellular redox balancing as well as permitting recapture of ammonia. It is likely that a portion of Glu was diverted for the biosynthesis of proline (Pro; 9). Genes encoding enzymes required for Pro biosynthesis, such as pyrroline-5-carboxylate synthase (P5CS, Figure 7b, 9) were significantly upregulated during dormancy. Pro is an important cellular protectant during dehydration stress and its biosynthesis is regulated by tissue ABA content (Vishwakarma *et al.*, 2017).

Fatty acids appear to provide additional carbon skeletons, primarily as Ac-CoA derived via β-oxidation. Genes encoding proteins needed for β-oxidation were significantly elevated during dormancy (β-ox; Figures 7b, 10, and S3c). Ac-CoA was previously identified as an important metabolic hub in Summer rhizomes (Palmer *et al.*, 2014a), and these findings have been extended here. Biosynthesis of citrate (Cit) in the cytosol during dormancy is likely to be a major sink for Ac-CoA. Genes encoding CS (Figure 7b, 11), Acon and IDHa (Figure 3d) were significantly upregulated during dormancy and, conversely, genes encoding CL, isocitrate lyase (ICL) and malate synthase (MS) were downregulated or expressed at low levels, indicating that Cit biosynthesis and eventual formation of 2-oxoglutarate (2-OG) was enhanced during dormancy. The formation of 2-OG from isocitrate appears to be specifically catalyzed by NADP+-dependent IDH, providing more substrate level redox equivalents. Succinate, aconitate and fumarate, metabolites that can be derived from Cit, were elevated in dormant rhizomes. The complex relationships between these different TCA cycle organic acids in plants have been elaborated recently, and have been suggested to impact the redox and energy balance in cells (Igamberdiev and Eprintsev, 2016). Potentially, these interrelationships are impacted by TORC in dormant switchgrass rhizomes.

Overall, metabolism during switchgrass rhizome dormancy appeared to involve discrete but interrelated events. One represented an ABA-driven response that resulted in dehydration, increased cellular osmolytes and upregulation of autophagic processes, likely through TORC and SnRK-based signaling cascades. The other trigger involved a recalibration of energy transduction through apparent reductions in mitochondrial oxidative phosphorylation, increased substrate level generation of ATP and reducing equivalents, and recycling of N and possibly CO_2 through refixation. Lastly, expression profiles indicated that cold-related signaling was occurring, and resulted in the significant upregulation of switchgrass COR genes, although their specific regulation and function needs further investigation. Altogether, these data would support the notion that there is an active monitoring of the internal and external environment by the plant even during the dormant phase.

EXPERIMENTAL PROCEDURES

Plant materials and harvest

Switchgrass plants of cv Summer were raised as seedlings in a greenhouse and planted into field nurseries in June 2009. Rhizome samples from three individual plants were harvested at each time point across six time points in 2010 and 2011. Harvest dates coincided with shoot developmental stages (Moore *et al.*, 1991), corresponding to approximately green-up (V1/V2; May), late vegetative/early reproductive (En/R0; June), anthesis (R4; July), hard dough seed (S2–S4; August), physiological maturity/shoot senescence (S4/S5; September), and after a killing-frost (November). Rhizomes at green-up (May) were collected only in 2011. All other

stages were collected in both years. Different individual plants were harvested at each sampling date from the same field location. Each sample was kept separate. Flash-frozen rhizomes were cryogenically ground and stored at −80°C (Palmer *et al.*, 2012). Aliquots of ground rhizomes were used for all downstream analyses.

Profiling of RNA and polar metabolites

Total RNA, protein and polar metabolites were extracted and analyzed as described (Palmer *et al.*, 2014a; Donze-Reiner *et al.*, 2017). For RNA-Seq experiments, four indexed samples were sequenced per lane on an Illumina HiSeq2000 system with 100-bp single end reads (Palmer *et al.*, 2015).

Starch and sucrose

Starch and sucrose were measured as follows: 50 ± 1 mg of cryogenically pulverized tissue was extracted twice with 80% hot ethanol and separated by centrifugation to yield a supernatant fraction and a pellet. The pooled ethanolic supernatants were dried, redissolved in 300 μL of water and analyzed for sucrose (as glucose equivalents) after incubation with 5 U invertase (Sigma I4504) for 45 min at 55°C. The pellet was washed twice with acetone, air-dried and analyzed for starch following digestions with 67 U AMY (Sigma A3403) in 400 μL 50 mM sodium acetate buffer pH 5 for 30 min at 90°C, and subsequent addition of 4.2 U amyloglucosidase (Sigma A7255) in 100 μL of 50 mM sodium acetate pH 5 at 55°C for 60 min. Released glucose was monitored using glucose test strips and a glucose meter (Roche Accu-Check Aviva; FitzGerald and Vermerris, 2005).

Plant hormone analysis

Triplicate 50 ± 2 mg aliquots of each sample were analyzed for ABA content using mass spectrometry, essentially as described earlier (Pan *et al.*, 2008; Schmitz *et al.*, 2015).

RNA-seq and bioinformatics

RNA-seq and related bioinformatic analyses were performed as described by Palmer *et al.* (2014b), with the exception that the reads were mapped to the switchgrass genome v1.1 available at www.phytozome.org (Goodstein *et al.*, 2012). DEGs present in samples harvested at the six time points over 2 years were identified with a likelihood ratio test using the DESeq2 package (Anders and Huber, 2010; Love *et al.*, 2014) in the R environment (Team, 2011) and based on a false discovery rate threshold of < 0.05 and $\log_2$ fold change threshold of > 1. Co-expression network analysis was performed as previously described by Donze-Reiner *et al.* (2017). KEGG annotations were used to assign DEGs to biochemical pathways adapted from the KEGG (www.genome.jp/KEGG/) database (Kanehisa *et al.*, 2014). In heatmaps, read counts of genes with the same protein annotation (for example, sucrose synthase or pyruvate kinase, etc.) and expression patterns across harvest dates were pooled. The expression patterns of several KEGG pathways were compared across the three primary growth stages of rhizomes arbitrarily assigned as growth, green; pre-dormant, yellow; and dormant, brown, using a heatmap analysis (Donze-Reiner *et al.*, 2017). Read counts for each individual gene and their corresponding gene identifications are given in Data S1.

Statistical analyses

ANOVA and related statistical analyses were performed in SAS (Cary, NC, USA) PROC MIXED v.9.2 (SAS Institute, 2002–2008) or in Excel. Principal component analysis was done using the 'prcomp' function in R (Team, 2011). Discriminant analysis plots and hierarchical clustering heatmaps were created using JMP® Version 9.0 (SAS Institute, Cary, NC, USA, 1989–2007). Other analyses were as described in Donze-Reiner *et al.* (2017) and Rinerson *et al.* (2015).

ACCESSION NUMBERS

SRX1601466. More details provided in Table S1.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Environmental data. (a) Average air temperature. (b) Average soil temperature at 10 cm depth. (c) Cumulative precipitation. (d) Cumulative growing degree days (GDD). Red = 2010; black = 2011. Arrows = sample collection times.

Figure S2. Heatmaps of DEGs associated with cold responsiveness. (a) Simplified model used for discovery. (b) bHLH and AP2 transcription factors. (c) COR.

Figure S3. Additional heatmaps of DEGs. (a) Sucrose and phosphate transporters. (b) Genes aligned with TORC model shown in Figure 6. (c) Fatty acid degradation and β-oxidation. (d) Mitochondrial electron transport chain. (e) Ureide and glyoxylate metabolism.

Table S1. RNA-Seq read mapping statistics

Table S2. Arabidopsis GeneRIF text mining terms

Table S3. Metabolites significantly correlated with select co-expression modules

Table S4. Co-expression module KEGG pathway enrichments

Data S1. Gene IDs for all figures.

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