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ORIGINAL ARTICLE

MicroRNA expression profiles in the emerging tillers and inflorescence of switchgrass, a major feedstock for biofuel production

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Abstract MicroRNAs are known to regulate almost all developmental processes in plants. These are ubiquitously expressed and most importantly their abundances vary by several orders of magnitude in different tissues and developmental stages. MicroRNA profiles in seedlings and leaves of switchgrass have been reported but not from the inflorescence or tillers. The overall small RNA population in inflorescence differs from the other vegetative organs, and, moreover, miRNAs are important regulators of flowering and flower development, thus inflorescence was chosen. Likewise, emerging tillers were chosen to identify miRNAs that might play a role in tillering, which is an important trait contributing to higher biomass production. The sequencing followed by computational analyses of small RNA libraries generated from inflorescence and emerging tillers revealed the identification of 28 conserved and 4 novel miRNA families. The expression levels of most miRNA families and miRNA variants within a family displayed greater differences between the tissues. Importantly, this study has offered insights into differences in abundances of miRNA families in the inflorescence and tillers. Specifically, the reported miRNA profiles from tillers are a valuable resource to examine which of these miRNAs play important roles in tillering, an important agronomic trait in this bioenergy crop.

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Introduction

With the need for biofuel production from plants that are not being used in producing the human diet, several species such as switchgrass, Sorghum, *Brachypodium* and *Miscanthus* have emerged as promising feedstock species. Of these, switchgrass has received much attention, however, to advance the utility of switchgrass as a major biofuel feedstock, unlocking the molecular switches that controls biomass production (increased foliar number and size, plant height, tiller numbers etc.,) is critically important.

In plants, the importance of miRNA-guided target gene regulation is indispensable for various physio-biochemical processes/pathways associated with the development and maintaining cellular homeostasis under stress conditions (Sunkar and Zhu 2007; Sunkar et al. 2012). Earlier studies have reported the identification miRNAs in switchgrass including their response to PEG-induced osmotic stress and salinity (Matts et al. 2010; Sun et al. 2012; Xie et al. 2014). Additionally, we reported the expression profiles of miRNAs in switchgrass exposed to long-term drought or heat (Hivrale et al. 2016). These studies have explored the miRNA profiles either in leaves or seedlings with or without the stress treatments. It is known that the plant small RNA population especially the small interfering RNA (siRNA) population varies greatly in inflorescence (reproductive tissues) compared to the vegetative tissues. Further, the miRNA profiles and their abundances in inflorescence of switchgrass is unknown. Keeping this in view, we analyzed small RNAs in the inflorescence or flowers of switchgrass.

Biomass production in the case of herbaceous bioenergy plant species is dependent on more tillers, thus tillering is an important trait in switchgrass (Fu et al. 2012; Wang et al. 2012, 2013; Xu et al. 2015). Tillering involves initiation of tiller buds and subsequently their outgrowth (Xu et al. 2015). At the molecular level genes such as the *MONOCULM 1*, *TEOSINTE BRANCHED 1*, *PIN FORMED FAMILY OF TRANSPORTER 1* (*PIN1*), *HIGH TILLERING DWARF/DWARF17*(*D17*), *DWARF 10* (*D10*), *DWARF 14* (*D14*), *DWARF 27* (*D27*), *RICEFLORICULA/LEAFY* (*RFL*) and *MOC3* (*MONOCULM 3*)/*TILLERS ABSENT1*(*TBA1*) are known to regulate the process of tillering in rice and other monocots (Xu et al. 2015). MicroRNAs largely fine-tune the abundances of target gene expression in a tissue- or cell-specific manner. Tillering is controlled by multiple transcription factors and pathways, therefore fine-tuning their expression is critically important, which in turn suggests an important role for miRNAs in the process of tillering. More recently, a role for miR156 and consequently their target genes (*Squamosa promoter-binding transcription factors*) in the tillering of switchgrass has been revealed (Fu et al. 2012; Chuck et al. 2011). However, a role for the other miRNAs in tillering cannot be excluded and this requires a comprehensive miRNA profiling in emerging tillers, which was undertaken in this study.

Materials and methods

Small RNA library construction and sequence analysis

Switchgrass cultivar Alamo was grown to maturity under greenhouse conditions. Emerging tillers from the young plants and inflorescence from the adult plants were harvested, snap frozen in liquid nitrogen, and stored at − 80°C until total RNA was isolated. Total RNA from the frozen samples was extracted using the Trizol reagent (Invitrogen, USA) following the manufacturer's instructions. Construction of small RNA libraries and their sequence analyses were accomplished as described earlier (Matts et al. 2010; Jagadeeswaran et al. 2012).

Small RNA blot analysis

Small RNA blot analysis was performed as described previously (Matts et al. 2010). In brief, low-molecular weight (LMW) RNA was precipitated using NaCl and PEG from the total RNA. Twenty micrograms of LMW RNA was resolved on a denaturing 15% acrylamide/8 M urea gel, transferred to a hybond-N + (Amersham) membrane, and probed with the labeled ^{32}P oligonucleotides complementary to the miRNA sequence. The membranes were pre-hybridized for at least 1 h, and hybridized overnight with PerfectHYB + buffer (Sigma) at 38 °C and then washed, and exposed to a phosphoscreen. Images were acquired by scanning the phosphoscreen using a Typhoon scanner.

Results and discussion

Sequencing of small RNA libraries provided a glimpse of miRNAs expressed in switchgrass seedlings or leaves (Matts et al. 2010; Sun et al. 2012; Xie et al. 2014; Hivrale et al. 2016). To identify miRNAs and to determine their abundances in inflorescence and emerging tillers of switchgrass, two small RNA libraries were generated from these tissues and sequenced using Illumina sequencing platform. This yielded approximately 10 and 11 million total reads as well as 3,757,827 and 5,175,081 unique reads from emerging tillers and inflorescence libraries, respectively. The degradation products from the rRNAs, tRNAs and from the protein-coding mRNAs were removed and the remaining unique small RNAs were mapped to miRBase to identify known miRNAs.

The small RNAs ranging in size between 18 and 27 nt were analyzed for their abundances, which revealed two peaks (Fig. 1); one at the 21 nt size that largely includes miRNAs and the other at 24 nt size class which mostly comprises heterochromatic siRNAs, and these findings were consistent with small RNA populations from diverse plant species (Jagadeeswaran et al. 2012; Zhang et al. 2011; Li et al. 2013). Although the total reads of 24 nt small RNAs were approximately similar in numbers both in tillers and inflorescence, but the unique read numbers differed greatly between these tissues. The count of unique small RNAs of 24 nt size class was far greater in inflorescence than in tillers (Fig. 1), which suggests a greater diversity of 24-nt long small RNAs in inflorescence but less diverse (greater redundancy) in tillers (Fig. 1). Unlike 24 nt long total reads, the 21 nt long total read count differed greatly between inflorescence and tillers, i.e., inflorescence has much greater frequencies than in tillers. A similar trend was observed for 21 nt long unique small RNA population (Fig. 1). This observation suggests that the overall miRNA abundances were much greater in inflorescence relative to emerging tillers. Consistent with this suggestion, the expression levels of the most miRNA families were several folds greater in inflorescence compared to tillers (Table 1).

In plants, most conserved miRNAs exist as families (multiple loci) with or without sequence variation. Our analysis has revealed the identification of 88 distinct conserved miRNAs belonging to 28 miRNA families (miR156,

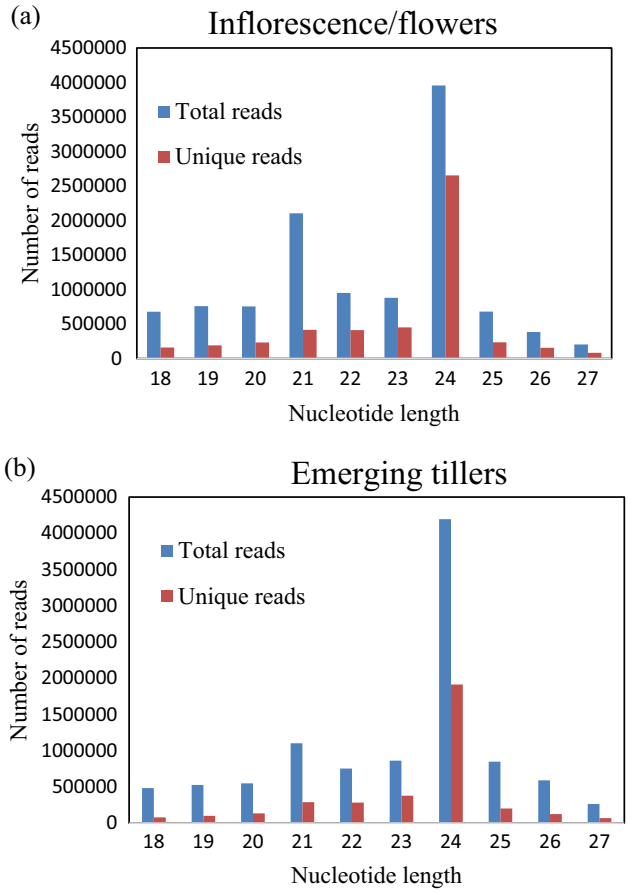

Fig. 1 Size distribution and abundance of small RNAs in inflorescence and emerging tillers libraries

miR159, miR160, miR162, miR164, miR165/166, miR167, miR168, miR169, mIR170/171, miR172, miR319, miR390, miR393, miR394, miR395; miR396, miR397, miR398, miR399, miR408, miR444, miR528, miR529, miR535, miR827, miR2118, and miR5025) in both tissues of switchgrass. The identified distinct miRNAs and their frequencies in inflorescence and emerging tillers was shown in Table 1. On the basis of number of miRNA variants that differ at least by one or more nucleotides for each family, miR169 was found to be the largest family represented by 12 members in these tissues of switchgrass (Table 1). This was followed by miR170/171 and miR399 families with 6 members each; four miRNA families (miR156, miR165/166, miR393 and miR2118) were represented by 5 members each and another four miRNA families (miR164, miR167, miR172 and miR395) were represented by 4 members each in these tissues of switchgrass. The miRNA families such as miR160, miR319, miR393, miR397, miR398, miR444, miR529, miR1432 were only represented by 2 members each whereas miR162, miR168, miR390, miR394, miR408, miR528, miR535, miR827 and miR5025 were represented by only one member in these two tissues of switchgrass (Table 1).

The overall abundances of almost all miRNA families were differed between inflorescence and emerging tillers. In general, most miRNA families exhibited relatively much higher levels in the inflorescence than in tillers. For instance, miR165/166 family was most abundantly expressed, followed by miR168, miR156 and miR167 in the flowers (Table 1). Although these miRNA families expressed at much lower levels in emerging tillers, a similar trend was observed with respect to ranking of their abundances (Table 1). Likewise, the abundances of miR172 family that regulates flower development, were at least five-fold higher in inflorescence compared to tillers. Similarly, miR159, miR164, miR170/172, miR827 and miR396 exhibited approximately similar abundances (about 7000 RPTM) in inflorescence, while their levels were far low (ranged between half to one-fourth levels) in emerging tillers (Table 1). By contrast, miR528 levels were almost four-fold greater in emerging tillers compared to inflorescence (Table 1). Additionally, few miRNA families such as miR169, miR397, miR408 and miR529 were also displayed slightly higher abundances in tillers than in inflorescence. Only miR393 family displayed similar abundances between inflorescence and tillers. Thus, the majority of miRNA families were more abundantly expressed in inflorescence and only a small number of families were expressed at higher levels in tillers.

Within the two small RNA libraries (inflorescence and emerging tillers), miR165/miR166 family had the highest abundances. The miR165/166 family targets HD-ZIP transcription factors that are essential for specification of adaxial/abaxial (dorso-ventral) polarity. This polarity is established through the polarized expression of *HD-ZIPIII* transcription factors that specify adaxial/upper cell fate (Emery et al. 2003; Juarez et al. 2004; Nogueira et al. 2007). In Arabidopsis and maize, the adaxial-specific expression of *HD-ZIPIII* family members is delineated by the expression pattern of miR166 (Juarez et al. 2004; Kidner and Martienssen 2004; Nogueira et al. 2007). HD-ZIP factors are also implicated in regulating floral development (Jung and Park 2007). Given the fact that *HD-ZIPIII* family members play diverse roles in leaf and flower development, it was not surprising that their abundance was the highest in inflorescence library. However, the highest abundance in emerging tillers was noteworthy and this requires further attention to scrutinize if miR165/166-HD-ZIP circuitry plays a role in tillering.

miR444, miR528 and miR1432 are well-characterized monocot-specific miRNA families (Sunkar and Jagadeeswaran 2008). Of these, miR528 had the highest abundances in the emerging tillers and deserves further attention. The targets for miR528 include Cu/Zn SODs,

Table 1 Conserved miRNA families and their normalized abundances (RPTM-reads per ten million) in flowers and emerging tillers of switchgrass

miRNA	miRNA sequence	Flower	Flower (RPTM)	Tillers	Tillers (RPTM)
miR156a	UUGACAGAAGAGAGCGAGCAC	13,540	11,510	4437	4159
miR156b	UGACAGAAGAGAGAGAGCAC	43	37	132	124
miR156c	UGACAGAAGAGAGUGAGCAC	17,098	14,535	14,131	13,244
miR156d	UUGACAGAAGAGAGUGAGCAC	18,343	15,593	15,741	14,753
miR156e	UGACAGAAGAGAGUGAGCAU	950	808	509	477
miR159a	UUGCAUGACCGAGGAGCUGC	31	26	11	10
miR159b	UUUGGAUUGAAGGGAGCUCUG	9302	7907	3858	3616
miR159c	UUGCAUGACCGAGGAGCUGC	31	26	11	10
miR160a	UGCCUGGCUCCCUGUAUGCCA	1826	1552	209	196
miR160b	UGCCUGGCUCCCUGUAUGCU	32	27	0	0
miR162a	UCGAUAAACCUCUGCAUCCAG	234	199	64	60
miR164a	UGGAGAAGCAGGGCACGUGCA	6794	5775	4708	4413
miR164b	UGGAGAAGCAGGGUACGUGCA	157	133	36	34
miR164c	UGGAGAAGCAGGGCACGUGCU	1070	910	280	262
miR164d	UGGAGAAGCAGGACACGUGAG	1134	964	90	84
miR165	UCGGACCAGGCUUCAUCCCCC	1259	1070	203	190
miR166a	UCGGACCAGGCUUCAUUCCCC	675,372	574,120	146,865	137,648
miR166b	UCGGACCAGGCUUCAUUCCUC	58,024	49,325	10,106	9472
miR166c	UCGGACCAGGCUUCAAUCCCU	13,932	11,843	2274	2131
miR166d	UUCGGACCAGGCUUCAUUCCC	16,206	13,776	6147	5761
miR167a	UGAAGCUGCCAGCAUGAUCUGA	8122	6904	6497	6089
miR167b	UGAAGCUGCCAGCAUGAUCUA	1577	1341	817	766
miR167c	UGAAGCUGCCAGCAUGAUCU	679	577	280	262
miR167d	UGAAGCUGCCAGCAUGAUCUG	1219	1036	650	609
miR167e	UGAAACUGUCACAGCAUGAUC	0	0	0	0
miR168a	UCGCUUGGUGCAGAUCGGGAC	107,613	91,480	36,876	34,562
miR169a	UUAGCCAAGAAUGGCUUGCCUA	2	2	4	4
miR169b	UAGCCAAGGAUGACUUGCCUG	90	77	98	92
miR169c	UAGCCAAGGAUGACUUGCCGG	24	20	45	42
miR169d	UAGCCAAGGAUGAUUUGCCUGU	4	3	3	3
miR169e	AGCCAAGGAUGACUUGCCGGC	49	42	66	62
miR169f	UAGCCAAGGAUGACUUGCCUA	50	43	64	60
miR169 g	CAGCCAAGGAUGACUUGCCGG	357	303	382	358
miR169 h	UAGCCAAGGAUGACUUGCCUACA	70	60	90	84
miR169i	CAGCCAAGGAUGACUUGCCGA	29	25	76	71
miR169j	UAGCCAAGAAUGAUUUGCCUGU	17	14	4	4
miR169 k	UUAGCCAAGAAUGACUUGCCUA	29	25	41	38
miR169 l	UAGCCAAGAAUGACUUGCCUA	15	13	14	13
miR170a	UGAUUGAGCCGUGCCAAUAUC	1402	1192	1754	1644
miR171a	UUGAGCCGCGUCAAUAUCUCC	267	227	109	102
miR171b	UGAUUGAGCCGCGCCAAUAUC	11	9	2	2
miR171c	UGAGCCGAACCAAUAUCACUC	4	3	1	1
miR171d	CGAUUGAGCCGUGCCAAUAUC	27	23	13	12
miR171e	UGAUUGAGCCGUGCCAAUAUC	1409	1198	1764	1653
miR172a	AGAAUCUUGAUGAUGCUGCAU	4812	4091	1445	1354
miR172b	GGAAUCUUGAUGAUGCUGCAU	3981	3384	400	375
miR172c	AGAAUCCUGAUGAUGCUGCAU	9	8	4	4
miR172d	UGAAUCUUGAUGAUGCUGCA	0	0	1	1

Table 1 continued

miRNA	miRNA sequence	Flower	Flower (RPTM)	Tillers	Tillers (RPTM)
miR319a	AUUGGAUGGCGCGGGAGCUAA	510	434	14	13
miR319b	UUGGACUGAAGGGUGCUCCCU	1558	1324	120	112
miR390a	AAGCUCAGGAGGGAUAGCGCC	69	59	13	12
miR393a	CUCCAAAGGGAUCGCAUUGAU	137	116	123	115
miR393b	UCCAAAGGGAUCGCAUUGU	4	3	1	1
miR394a	UUGGCAUUCUGUCCACCUCC	430	366	37	35
miR395a	UGAAGUGUUUGGGGGAACUC	255	217	178	167
miR395b	UGAAGUGUUUGGGGGGAACUC	30	26	9	8
miR395c	UGAAGUGCUUGGGGGAACUC	12	10	29	27
miR395d	UGAAGUGUUUGGGAGAACUC	5	4	7	7
miR396a	UUCCACAGCUUUCUUGAACUG	3110	2644	397	372
miR396b	UUCCACAGCUUUCUUGAACUU	1491	1267	288	270
miR396c	UUCAAUAAAGCUGUGGGAAA	125	106	44	41
miR396d	UCCACAGGCUUUCUUGAACUG	1710	1454	291	273
miR396e	UCCACAGGCUUUCUUGAAC	787	669	131	123
miR397a	UUGAGUGCAGCGUUGAUGAGC	270	230	372	349
miR397b	UCAUUGAGUGCAGCGUUGAUG	4	3	2	2
miR398a	UGUGUUCUCAGGUCACCCCUU	1	1	0	0
miR398b	UGUGUUCUCAGGUCGCCCCCG	9	8	13	12
miR399a	UGCCAAAGGAGAGCUGCCCUG	31	26	0	0
miR399b	UGCCAAAGGAGAUUUGCCCAG	68	58	1	1
miR399c	UGCCAAAGGAGAAUUGCCCUG	61	52	2	2
miR399d	UGCCAAAGGAGAUUUGCCCGG	10	9	0	0
miR399e	UGCCAAAGGAGAGUUGCCCUG	93	79	6	6
miR399f	UGCCAAAGGAGAGUUGCCCUA	74	63	5	5
miR408a	CAGGGAUGAGGCAGAGCAUGG	91	77	111	104
miR444a	UUGUGGCUUUCUUGCAAGUUG	71	60	6	6
miR444b	UGCAGUUGUUGCCUCAAGCUU	956	813	58	54
miR528a	UGGAAGGGGCAUGCAGAGGAG	1043	887	3739	3504
miR529a	AGAAGAGAGAGAGUACAGCCU	132	112	230	216
miR529b	AGAAGAGAGAGGGUACAGCCU	0	0	0	0
miR529c	AGAAGAGAGAGAGUACAGCUU	454	386	47	44
miR535a	UGACAACGAGAGAGAGCACGC	1443	1227	352	330
miR827	UUAGAUGACCAUCAGCAAACA	9113	7747	1958	1835
miR1432a	CUCAGGAGAGAUGACACCGAC	7	6	13	12
miR1432b	CUCAGGAGAGAUGACACCGAU	8	7	3	3
miR2118a	UUCCUGAUGCCUCCCAUGCCUA	10	9	7	7
miR2118c	UUCCUGAUGCCUCCUAUGCCUA	12	10	2	2
miR2118d	UUCCCGGUGCCUCCCAUUCCUA	2	2	1	1
miR2118e	UUCCCGAUGCCUCCCAUUCCUA	29	25	16	15
miR2118 h	UUUCCGAUGCCUCCCAUUCCUA	14	12	15	14
miR5025	AUAAUUUGGGACGGAGGGAGU	1	1	0	0

which suggests that suppressing transcripts of ROS scavenging Cu/Zn SODs is important in tillers. This in turn could maintain a threshold level of ROS, which might be important for ROS signaling in tillers. Further studies are needed to examine if this is indeed true or not. miR444 family was more abundantly expressed in the inflorescence while miR1432 expressed at very low levels in both tissues (Table 1). Other partially conserved miRNA families

identified in these libraries include miR827 and miR2118 that were reported from some of the monocots and dicots (Jagadeeswaran et al. 2009; Arenas-Huertero et al. 2009; Johnson et al. 2009). The miR827 was expressed at much higher levels (at least four-fold greater) in inflorescence compared to tillers.

Within each miRNA family, the abundances of different members appear to vary greatly, which could occur in a tissue- or cell-specific manner. The abundances of different members can be assessed from their normalized frequencies. Interestingly, a few variants/members of the same miRNA family were more abundantly expressed than the other variants. For instance, miR159 that was represented by 3 members in both tissues but only one of them was abundantly expressed (7907 and 3616 RPTM in inflorescence and tillers, respectively) whereas the other two members of miR159 family were barely expressed in these tissues. Similarly, some of the loci belonging to miR165/166 were expressed as abundant as 575,000 RPTM in inflorescence and 137,000 RPTM in tillers whereas the other members of this family were expressed at varying levels (moderate to low) in both tissues (Table 1). Interestingly, the abundances of specific members of miR170/171 family were greater in tillers compared to inflorescence.

Identification of novel miRNAs in inflorescence and tillers of switchgrass

Novel miRNAs have been reported from diverse plant species including switchgrass. Here we used two tissues (emerging tillers and inflorescence) that have not been used in previous studies, therefore, we surveyed the small RNA libraries for identifying novel miRNAs as suggested by Meyers et al. (2008). Our analysis revealed at least 4 small RNAs that could be annotated as novel miRNAs based on sequencing of miRNA* reads (Table 2). Fold-back structures for the novel miRNA precursors could be predicted using their precursor sequences (Fig. 2). Interestingly, the frequency of a novel miRNA#1 was substantially higher and even greater than the frequency of several highly conserved miRNAs such as miR396, miR393 and miR398. Additionally, we also assessed for their conservation using BLAST searches against the NCBI EST database. This could identify homologs of some of these novel miRNAs in other monocots such as maize, sorghum, sugarcane, rice and *Cenchrus ciliaris*.

Temporal expression analyses of miR529 and miR535 as well as novel miRNAs

The abundances of most conserved miRNAs greatly differed between different tissues and developmental stages of switchgrass (lower and upper leaves from seedlings and mature adult plants, stems from seedlings and adult plants as well as roots and inflorescence) as analyzed by small RNA blot analyses (Matts et al. 2010). In this study, we have sequenced two semi-conserved miR529 and miR535 families from both tissues of switchgrass (Table 1). To examine their expression patterns in the above mentioned eight different tissues, small RNA blot analyses were performed (Fig. 3). The analyses revealed that the miR529 was abundantly expressed in the inflorescence and stems but barely detectable in leaves of both seedlings and adult plants (Fig. 3). On the other hand, miR535 was expressed at moderate levels in leaves but at much lower levels in stem (Fig. 3). We also analyzed the expression pattern of three novel miRNAs in these tissues of switchgrass. Novel miRNA#1 was abundantly expressed in leaves, stem and root tissues of switchgrass (Fig. 3). Novel miRNA#2could be detected in inflorescence and stems of seedlings as well as in leaves. Similarly, the novel miRNA#4 was barely detectable in stem of adult plants as well as upper leaves of both seedlings and adult plants but it could not be detected in other tissues (Fig. 3). The small RNA blot analyses revealed that the novel miRNA#1 levels were much higher compared to the novel miRNAs #2 and #4. Indeed, the high abundances of novel miRNA#1 was consistent between both the sequencing profiles (Table 1) and small RNA blot assay (Fig. 3).

The analyses of two independent small RNA libraries sequenced from inflorescence and emerging tillers revealed the identification of 28 conserved and 4 novel miRNA families in switchgrass. Targets for most conserved

Table 2 Identification of novel miRNAs and their normalized abundances in the inflorescence and tillers of switchgrass

miRNA	miRNA sequence	Normalized abundances in inflorescence (RPTM)	Normalized abundances in tillers (RPTM)
Novel#1	UGGGCUGUAAAUCGCGAGACGA	3086	3496
Novel#2	CGUAGAGGCAGCAGCUGCAUA	32	63
Novel#3	CCGUUCGCGACGUUCCUGGAG	52	36
Novel#4	AAAUAUAUGACGUUUAGGACA	128	450

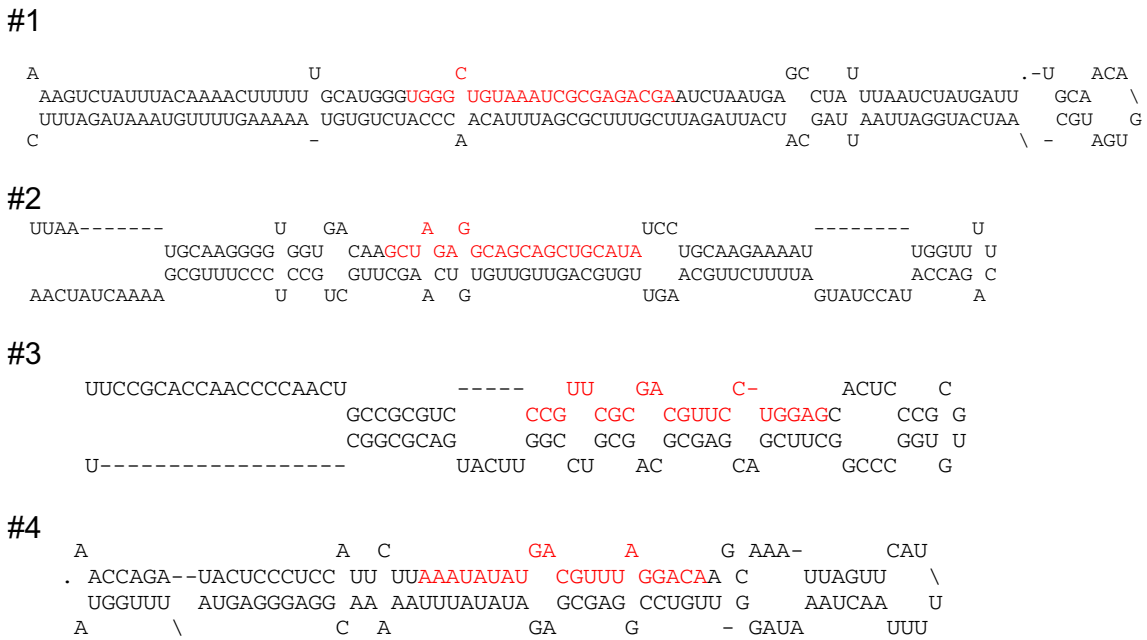

Fig. 2 Predicted fold-back structures using the novel miRNA precursor sequences

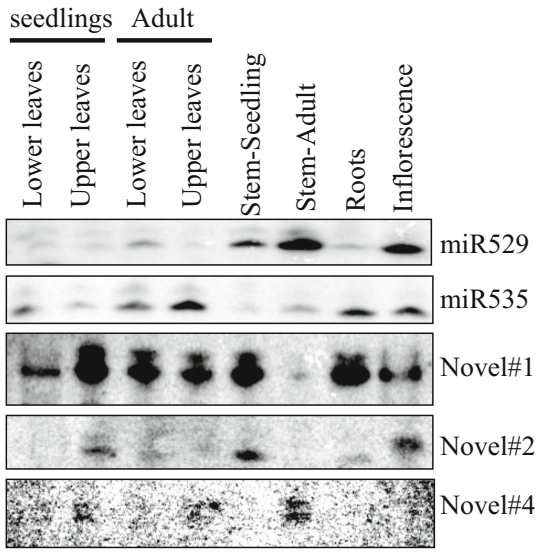

Fig. 3 Temporal expression analyses of miR529 and miR535 as well as novel miRNAs in different tissues of switchgrass

miRNAs have been predicted and some of them have been confirmed (Matts et al. 2010; Hivrale et al. 2016). Identification of most conserved miRNA homologs in these tissues of switchgrass suggests the possibility that the functions described to these miRNAs from other plant species likely to be true for switchgrass also. In general, this study offered insights into which miRNA families and which variants within a miRNA family were more abundantly expressed in inflorescence and tillers. It was shown earlier that miR156 overexpression significantly improves biomass production of switchgrass (Fu et al. 2012; Chuck et al. 2011). Identification of various conserved and novel miRNAs in tillers of switchgrass forms a valuable resource to examine which of these miRNAs have roles in tillering.

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