The *miR156-SPL4* module predominantly regulates aerial axillary bud formation and controls shoot architecture

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Summary

- Grasses possess basal and aerial axillary buds. Previous studies have largely focused on basal bud (tiller) formation but scarcely touched on aerial buds, which may lead to aerial branch development.
- Genotypes with and without aerial buds were identified in switchgrass (*Panicum virgatum*), a dedicated bioenergy crop. Bud development was characterized using scanning electron microscopy. Microarray, RNA-seq and quantitative reverse transcription polymerase chain reaction (RT-qPCR) were used to identify regulators of bud formation. Gene function was characterized by down-regulation and overexpression.
- Overexpression of *miR156* induced aerial bud formation in switchgrass. Various analyses revealed that *SQUAMOSA PROMOTER BINDING PROTEIN LIKE4* (*SPL4*), one of the miR156 targets, directly regulated aerial axillary bud initiation. Down-regulation of *SPL4* promoted aerial bud formation and increased basal buds, while overexpression of *SPL4* seriously suppressed bud formation and tillering. RNA-seq and RT-qPCR identified potential downstream genes of *SPL4*.
- Unlike all previously reported genes acting as activators of basal bud initiation, *SPL4* acts as a suppressor for the formation of both aerial and basal buds. The *miR156-SPL4* module predominantly regulates aerial bud initiation and partially controls basal bud formation. Genetic manipulation of *SPL4* led to altered plant architecture with increased branching, enhanced regrowth after cutting and improved biomass yield.

Introduction

Branch development directly affects shoot architecture and biomass yield. Axillary buds are the sole originators of various branches (Domagalska & Leyser, 2011). Axillary buds are developed from axillary meristems (AMs) formed in the axil of primary organs (i.e. leaf, bract) (Long & Barton, 2000; Bennett & Leyser, 2006). Two models, detached vs *de novo*, have been proposed to explain the origin of AMs (Grbic & Bleecker, 2000; Long & Barton, 2000; Bennett & Leyser, 2006; Woods *et al.*, 2011). The 'detached meristem' theory suggests that AMs are initiated from a set of cells that bud off from the primary shoot apical meristem (SAM) and retain their meristematic identity in the axil. By contrast, the *de novo* hypothesis suggests that AMs develop from a set of differentiated cells that regain meristematic activity after receiving external signals. It is unclear whether the detached and *de novo* AMs form via different and distinct mechanisms or if the two models are opposite extremes of the same underlying mechanism (Bennett & aLeyser, 2006; Woods *et al.*, 2011).

Axillary meristems often initiate a few leaves before halting their growth to form a dormant axillary bud (Domagalska & Leyser, 2011). The bud may later be activated to form a branch or may remain dormant. In grasses, based on the position of emergence along the shoot axis, axillary buds are classified into two types: basal and aerial. Basal buds arise from the base (also called crown in grasses) of the main shoot of a plant and eventually become basal branches (tillers). Aerial axillary buds arise from elongated internodes in the upper part of the stem and may ultimately become aerial branches, which are also called secondary branches (Oikawa & Kyozuka, 2009; Domagalska & Leyser, 2011).

The development of both basal and aerial buds undergoes two stages: initiation and outgrowth (Kebrom *et al.*, 2013). In the last

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two decades, the outgrowth stage has been extensively studied and the regulatory mechanisms have been well elucidated (McSteen, 2009; Domagalska & Leyser, 2011; Wang & Li, 2011; Guo *et al.*, 2013; Kebrom *et al.*, 2013). Well-known examples include the discovery of strigolactones as endogenous hormones affecting shoot branching and the identification of *TEOSINTE BRANCHED1* (*TB1*) as an integrator of hormonal and environmental signals regulating axillary bud outgrowth (Doebley *et al.*, 1997; Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008; Domagalska & Leyser, 2011). By contrast, our understanding of bud initiation remains rudimentary (Tian *et al.*, 2014; Tanaka *et al.*, 2015). To date, only two classes of genes have been identified in bud initiation regulation. The first class consists of *Lateral suppressor* (*Ls*) and *YUCCA* (*YUC*) (Cheng *et al.*, 2006; Gallavotti *et al.*, 2008). *Ls* was identified in tomato as a result of its failure to initiate axillary buds (Schumacher *et al.*, 1999). Its orthologs *LATERAL SUPPRESSOR* (*LAS*) and *MONOCULM1* (*MOC1*) were characterized in *Arabidopsis* (Greb *et al.*, 2003) and rice (Li *et al.*, 2003). Ls/*LAS*/*MOC1* encode a conserved family of transcriptional regulators that have diverse functions and are involved in signal transductions in the GA and phytochrome A pathways (Tian *et al.*, 2004). Meanwhile, *YUCs* are involved in auxin biosynthesis (McSteen, 2009). The second class includes *LAX PANICLE1* (*LAX1*) (Komatsu *et al.*, 2003) and its orthologs, such as *BARREN STALK1* (*BA1*) in maize (Gallavotti *et al.*, 2004), *REGULATOR OF AXILLARY MERISTEM FORMATION* (*ROX*) in *Arabidopsis* (Yang *et al.*, 2012), and *PINFORMED1* (*PIN1*) and its orthologs (Michniewicz *et al.*, 2007). Genes from the second class are related to auxin transport and redistribution (McSteen, 2009). In dicot plants like tomato, these molecular mechanism studies involve both kinds of buds (Schumacher *et al.*, 1999; Muller *et al.*, 2006; Martin-Trillo *et al.*, 2011). However, in grasses, related research has mainly focused on basal bud formation, yet has hardly explored the subject of aerial buds. Furthermore, all these previously characterized genes act as activators of basal bud initiation.

In monocot plants, basal buds normally continue to develop across initiation and outgrowth stages, while the development of aerial buds varies with species. For instance, aerial buds may develop into aerial branches in *Brachypodium* and many panicoid grasses (Doust, 2007), whereas they typically arrest at an early stage and remain dormant as a result of apical dominance in cereal species such as wheat, barley and rice (Kebrom *et al.*, 2013). However, certain conditions such as long-term heat stress could induce dormant aerial buds to enter the outgrowth stage and form aerial branches in rice. The genetic control of aerial bud formation is largely unknown, and the only report to date is a recent mapping study using domesticated foxtail millet and its wild relative, green millet (Mauro-Herrera & Doust, 2016). This study identified nine quantitative trait loci (QTLs) explaining 42.4% of the phenotypic variance of aerial branching (Mauro-Herrera & Doust, 2016).

Switchgrass (*Panicum virgatum*) is a C_4 perennial tetraploid bunchgrass that has been developed into a dedicated biofuel crop because of its high biomass yield, low agricultural input requirements and the ability to grow in marginal lands (Schmer *et al.*, 2008; Hardin *et al.*, 2013). Extensive efforts have been made to genetically improve switchgrass productivity and reduce biomass recalcitrance (Fu *et al.*, 2011, 2012; Shen *et al.*, 2013; Baxter *et al.*, 2014; Dumitrache *et al.*, 2017; Li *et al.*, 2017; Liu *et al.*, 2017). We have shown that overexpression of a *microRNA156* (*miR156*) precursor in switchgrass resulted in various morphological alterations, and the degree of the morphological changes depends on the miR156 level (Fu *et al.*, 2012). *miR156* is one of the master regulators of plant vegetative phase transition via suppressing its targets *SQUAMOSA PROMOTER BINDING PROTEIN LIKEs* (*SPLs*) (Wu & Poethig, 2006). Among the 19 *SPLs* identified in rice, *OsSPL14* has been shown to inhibit tillering in rice, even though the regulation is not from basal bud formation but from the prolonged plastochron (Wang & Li, 2011).

As an outcrossing species, switchgrass is self-incompatible; individual seeds within a cultivar may represent different genotypes (Wang & Ge, 2006). This means that it is almost impossible to maintain a unique genotype through seeds in switchgrass. For this reason, node culture, a method of mass vegetative propagation (micropropagation), was developed (Alexandrova *et al.*, 1996). While doing experiments on node culture, we observed that some genotypes were not responsive to micropropagation. Upon detailed microscopic examination, we found that these genotypes were completely devoid of aerial buds. Such genotypes provide unique materials for the study of aerial bud development in monocot species. At the same time, we unexpectedly observed improved branching and aerial bud development from our previously generated transgenic switchgrass plants overexpressing *miR156* (Fu *et al.*, 2012). This prompted us to test the effect of *miR156* overexpression in a defined genotype devoid of aerial buds. Indeed, overexpression of *miR156* in such a genotype successfully induced aerial bud formation. Further studies led to the identification of a specific downstream gene, *SPL4*, which directly regulates axillary bud formation. In contrast to all previously characterized genes that act as activators of bud initiation, *SPL4* acts as an inhibitor. Furthermore, while the *miR156-SPL4* module predominantly controls aerial bud formation, it also partially regulates basal bud development. Genetic manipulation of the *miR156-SPL4* module led to altered shoot architecture, improved biomass yield and accelerated regrowth after cutting, thus offering the potential for enhancing agricultural productivity.

Materials and Methods

Plant materials and growth conditions

All genotypes (AP13, ST2 and NFCX1) used in this study are derived from the lowland-type switchgrass (*Panicum virgatum* L.) cv Alamo ($2n = 4 \times = 36$). Plants were grown in three replicates in the glasshouse at 26°C with 16 h light (390 μmol m^{-2} s^{-1}). The identification of switchgrass development stages and the harvest of samples followed the criteria described by Hardin *et al.* (2013).

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Gene constructs and transformation

The *miR156* overexpression transgenic lines were created by transforming a previously described *OsmiR156b* construct (Fu *et al.*, 2012) into the switchgrass genotype NFCX1 following the established protocol (Fu *et al.*, 2011). Forty-six independent transgenic lines were produced.

Based on the information obtained from partial expressed sequence tags (SPL4a's accession number is KanlCTG20060 and SPL4b's accession number is KanlSGLT49238 at the Switchgrass Functional Genomics Server: https://switchgrassgenomics.noble.org/index.php), the full-length coding sequences of *PvSPL4a* and *4b* were isolated by 5′- and 3′-rapid amplification of cDNA ends following protocols from the manufacturer (Invitrogen) using the primers listed in Table S1. The amplified PCR products were cloned into the pGEM-T Easy Vector (Promega) and verified by Sanger sequencing. The full mRNA sequences of *PvSPL4a* and *PvSPL4b* have been deposited in the NCBI GenBank (accession numbers: MF067411 and MF067412).

For overexpression, the coding sequence of *PvSPL4a* was amplified (see primers in Supporting Information Table S1) and cloned into the pANIC10A gateway vector driven by the *ZmUbi1* promoter (Mann *et al.*, 2012). The verified constructs were used to transform the genotype NFCX1 and 32 independent transgenic lines were generated.

To knockdown *PvSPL4*, an RNAi binary vector was constructed using the pANIC12A gateway vector (Mann *et al.*, 2012). A 443 bp SPL4 cDNA fragment selected from the conserved domain was amplified by PCR (see primers in Table S1) and cloned into the pANIC12A vector. The verified constructs were used to transform NFCX1, and 42 independent transgenic lines were created.

Gene expression quantification

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was performed to analyze the transcript abundance of various genes. Total RNA was extracted from various tissues by Tri-Reagent (Invitrogen) and subjected to reverse transcription with the Superscript III Kit (Invitrogen). SYBR Green (Applied Biosystems, Foster City, CA, USA) was used as the reporter dye. The primers used for RT-qPCR are listed in Table S1. *Ubq1* (GenBank accession number FL899020) was used as an internal control. The normalized data was statistically treated using Student's *t*-test.

The mature miR156 level was quantified by using the stem-loop RT-PCR procedure (Cui *et al.*, 2014). The miR156-specific stem-loop primers are listed in Table S1.

Microarray analysis of miR156 transgenic lines

Total RNA samples from duplicate biological replicates of the selected miR156 transgenic events and the wild-type (WT) NFCX1 (WT-NFCX1) were extracted from node 2 and node 4 meristems at E5 stage using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich). Five hundred nanograms of RNA were amplified and labeled using the GeneChip 3′ IVT Express Kit (Affymetrix, Santa Clara, CA, USA) and hybridized to Affymetrix switchgrass cDNA chips. Data normalization was conducted by using the robust multiarray average (RMA). Data analysis of differentially expressed probe sets on the chip was performed by associative analysis as described by Dozmorov & Centola (2003). Hierarchical analysis was used to identify genes with a positive correlation between phenotype and gene expression.

Characterization of plant growth and development

Tiller number and fresh biomass were measured from three biological replicates of each line when plants reached R1 stage. The harvested biomass was dried in an oven at 45°C for 96 h to measure the dried biomass. The data were statistically analyzed using Student's *t*-test.

Microscopy analysis and photography

Axillary buds and related node samples were harvested and immediately fixed in 3% glutaraldehyde (in 25 mM phosphate buffer, pH 7.0) overnight and dehydrated in a graded ethanol series. The fixed and dried samples were observed using a Hitachi TM-3000 scanning electron microscope (SEM, Tokyo, Japan). Light microscopy was performed using a Nikon SMZ 1500 stereomicroscope (Tokyo, Japan).

RNA-seq analysis of the regulation mechanism of bud development

Total RNA samples from selected PvSPL4-RNAi (SPL4Ri) transgenic events and the WT-NFCX1 were extracted from node 4 meristems at E5 stage using a Spectrum™ Plant Total RNA Kit (Sigma-Aldrich). RNA-seq library was constructed with TruSeq Stranded mRNA Library Prep Kit following the protocol from the manufacturer (Illumina Inc., San Diego, CA, USA). Sequencing was performed using the Hiseq 2000 Sequencing System (Illumina) at 100 bp paired reads. All reads were quality-trimmed before mapping, removing bases from the end of the read until two consecutive bases with quality scores of 30 or higher were found. Reads < 30 bases long after trimming were discarded, along with their mate pair. The trimmed reads were then mapped to the *Panicum virgatum* v1.1 genome sequence (http://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Pvirgatum) using TOPHAT 2.0.12 with 24 threads, an average mate inner distance of 100 bp, mate distance standard deviation of 50, and a maximum intron length of 25 000 bp. Transcripts were assembled and quantified using CUFFLINKS v.2.2.1 with the default assembly parameters. The transcripts identified in all samples in the study were then compiled into a unified set of transcripts and compared with the *Panicum virgatum* v1.1 annotated transcripts using CUFFCOMPARE v.2.2.1. Differential expression testing was performed using the default settings of CUFFDIFF v.2.2.1.

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Results

Micropropagation by node culture is based on the formation of aerial buds

Three switchgrass genotypes, ST2, AP13 and NFCX1, were selected from the commonly used switchgrass cv Alamo, and subjected to node culture tests. The three genotypes were very similar in morphology (Fig. S1) but exhibited different node culture results. Both ST2 and AP13 were easily propagated while NFCX1 had no response to node culture (Figs 1a–f, S2). After examining node morphological structures, we found that both ST2 and AP13 displayed intact aerial buds enclosed between the culm and the leaf sheath in each node, especially in the lower (older) nodes (Figs 1g, S3). However, no aerial buds were found in any nodes of NFCX1 (Figs 1h, S3). Meanwhile, all the three genotypes had similar basal buds (Fig. S3). The results indicated an association between the lack of aerial buds and the failure of node culture in the genotype NFCX1.

Basal and aerial buds have similar structure but also exhibit differences

Although basal and aerial buds arose from different positions (Fig. 2a), they had similar appearance and structure in both AP13 and ST2 (Fig. 2b,c). SEM observation showed that both types of buds have a few foliage leaves surrounding the apical meristems, and the buds are enclosed by the prophylls (Figs 2d–g, S4). On the other hand, differences were also observed in the two types of buds: aerial buds develop into a flatter shape because they arise

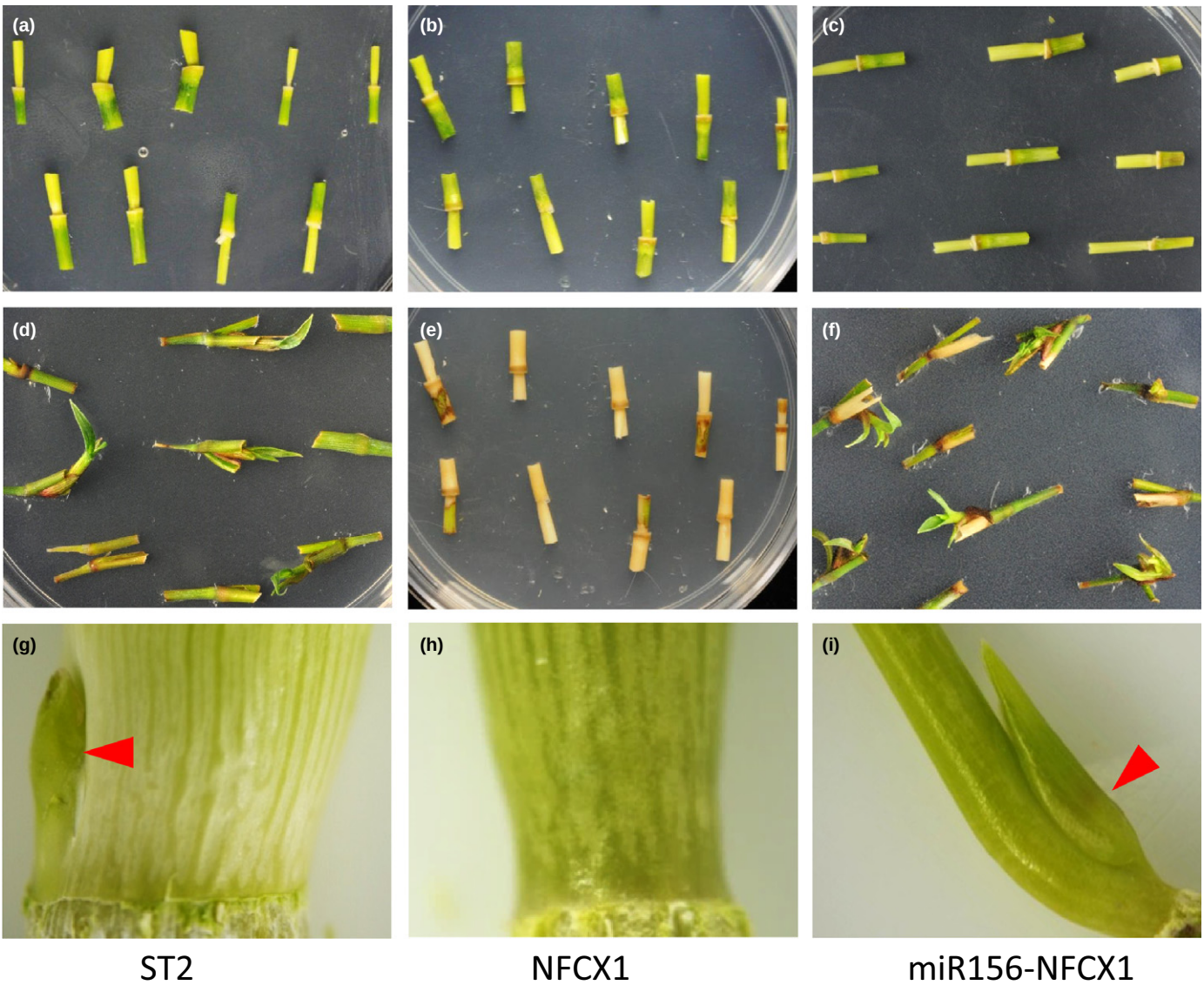

Fig. 1 Responses of different switchgrass genotypes to node culture. Nodal segments harvested from switchgrass genotypes ST2 (a), NFCX1 (b) and an overexpressor of *miR156* (c) in NFCX1 background for node culture. Shoot formation from the nodal segments after 12 d in culture (d–f). Shoots emerged from ST2 (d) and miR156-NFCX1 (f), but no shoots were formed from NFCX1 (e). Formation of aerial axillary buds (g–i). Buds were formed in ST2 (g) and miR156-NFCX1 (i), but no aerial axillary buds were found in NFCX1 (h). Red arrowheads, aerial axillary buds.

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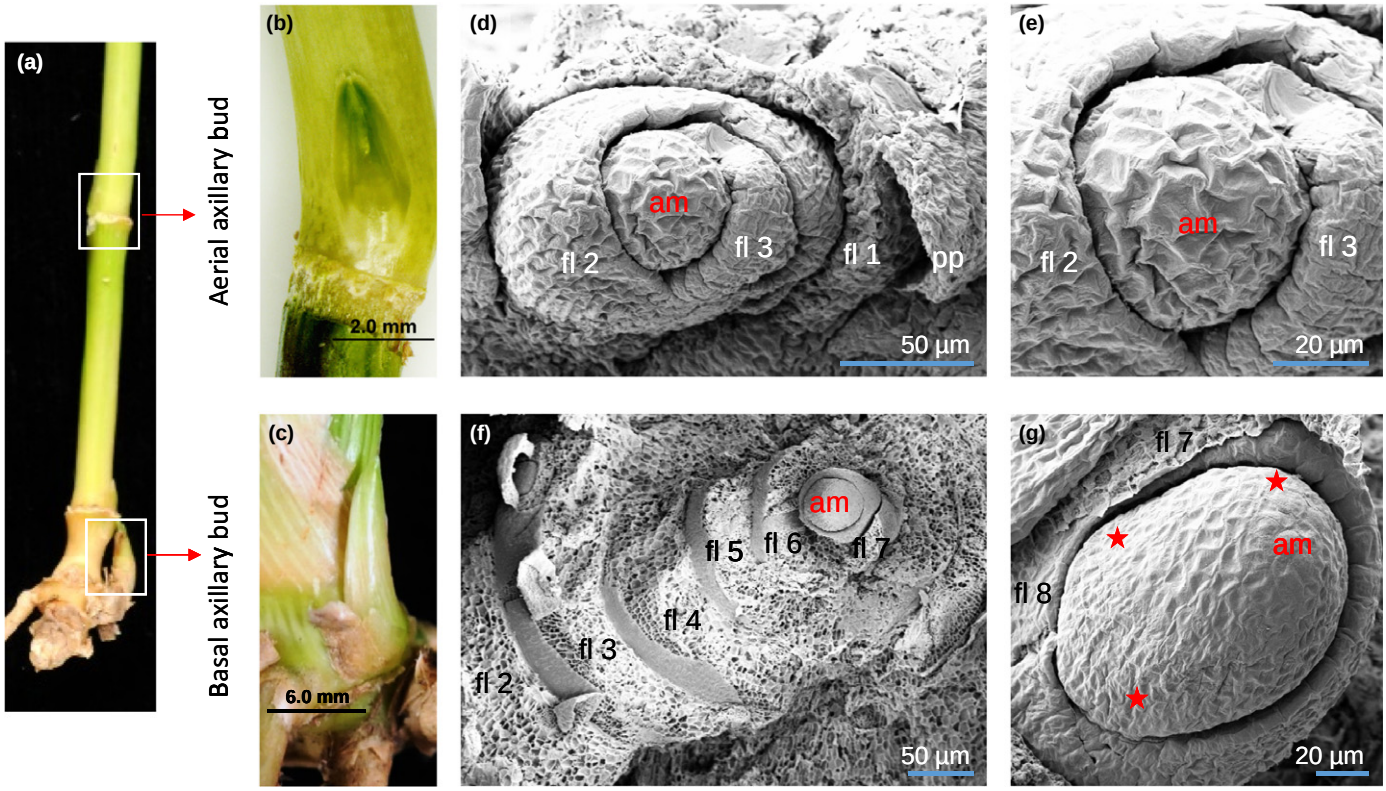

Fig. 2 External appearance and interior structure of aerial and basal axillary buds in switchgrass. (a) A switchgrass (genotype ST2) tiller shows different nodes after removing the leaf sheath. (b) Aerial axillary bud formed in the elongated internode. (c) Basal axillary bud formed in the nonelongated internode. (d) Scanning electron micrograph (SEM) of an aerial bud with partial foliage leaves removed. (e) Apical meristem of an aerial bud. (f) SEM of a basal bud with foliage leaves removed. (g) Apical meristem of a basal bud. Red stars, the leaf primordia. am, apical meristem; pp, prophyll; fl, foliage leaf.

from elongated internodes and are tightly hugged by the leaf sheath against the culm (Fig. S4a,b), whereas basal buds are not bound by this space limitation and are more rounded (Fig. S4c, d); aerial buds are smaller with only two to five foliage leaves (Fig. 2d) while basal buds are much bigger and may develop seven to nine foliage leaves (Fig. 2f). Prominent differences were observed between the apical meristems of the two types of buds. The basal bud apical meristem exhibits very early stages of leaf initiation (Fig. 2g), whereas leaf primordia have not visibly initiated on the aerial bud meristem (Fig. 2e). In terms of shape and leaf initiation, the basal bud axillary meristem more closely resembles the SAM than does the aerial axillary meristem (Fig. S5). All these together indicate that aerial bud development is arrested and becomes dormant after initiation. By contrast, basal buds continue developing to the outgrowth stage and subsequently form tillers. The similarities and distinctions between basal and aerial buds imply that they share certain regulatory mechanisms, but the activating mechanisms are different.

Axillary bud formation is regulated by *miR156*

Overexpression of *miR156* was found to significantly increase tiller numbers in rice (Xie *et al.*, 2006) and switchgrass (Chuck *et al.*, 2012; Fu *et al.*, 2012); however, the authors did not investigate axillary bud development. Because tillers are derived from basal buds, the results suggest that *miR156* plays a role in basal bud development. To elucidate whether *miR156* is also involved in aerial bud formation, we overexpressed *miR156* in the genotype NFCX1 (Fig. S6a) which has no aerial buds. Examination of the transgenics showed that aerial buds were successfully induced (Fig. 3a) along with an increase in basal buds (Fig. S6c). Furthermore, both initiation and elaboration of aerial buds were highly correlated with the *miR156* levels in these transgenic lines (Fig. 3b). In contrast to the WT-NFCX1, the transgenics allowed us to successfully carry out node culture and regenerate shoots (Fig. 1c,f). These results demonstrated that *miR156* directly regulates both aerial and basal bud formation. In addition, the results further confirmed that successful node culture is closely associated with the presence of aerial buds.

Microarray and hierarchical analyses identified specific *miR156* targets associated with aerial bud formation

Three *miR156* transgenic lines that varied in aerial bud formation (from total absence to well-developed buds; Fig. 3a) were selected for microarray analysis. From each line, tillers at E5 stage (with five visible internodes; see Fig. S3) were harvested and the node axillary meristems excised separately from two different nodes (node 2 with developed bud and node 4 with initiating bud primordia) from each tiller. Table S2 lists the 16 samples employed

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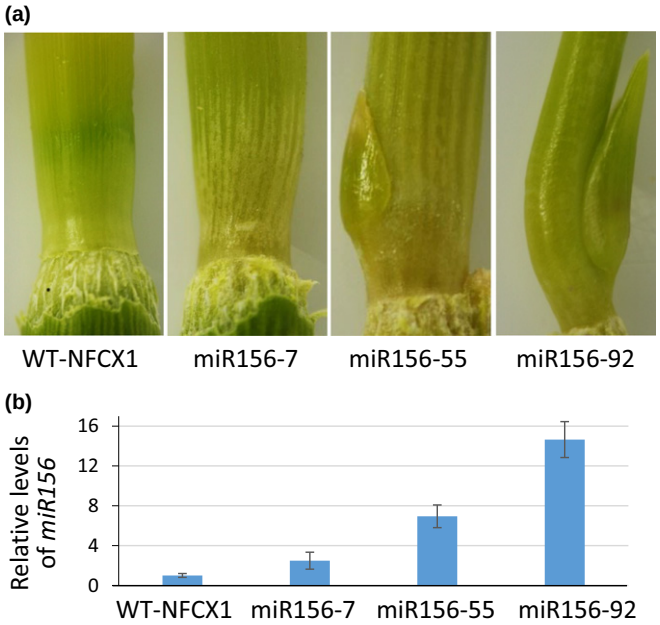

Fig. 3 Aerial axillary bud formation in *miR156*-overexpressing transgenic switchgrass plants (miR156). (a) Developmental status of aerial axillary buds in different miR156 plants. (b) Relative levels of *miR156*. Values represent mean ± SD of three biological replicates.

in this study. Microarray analysis revealed that 4963 genes exhibited significant differences among the 16 samples. The abundance of 605 genes changed more than twofold in the events with well-developed buds compared with the events lacking buds and the wild-type control. As axillary bud formation was established in node 2 yet still underway in node 4, the bud regulation genes showed differential expression in node 4 only. Applying this criterion, hierarchical analysis further identified 48 genes that were strongly down-regulated in developing aerial buds of the lines highly expressing *miR156* compared with lines with low expression of *miR156* and with the WT (Table S3). By contrast, expression of these genes showed only moderate down-regulation or up-regulation in the fully differentiated buds (Table S3). Further gene annotation revealed that three of the 48 genes, *PvSPL2*, *PvSPL4* and *PvSPL5*, contain the target sequence of *miR156* (Fig. S7).

The expression of *PvSPL4* and *5* is highly correlated with aerial bud and tiller formation

To decipher which *SPL* is the direct regulator of axillary bud formation, we first investigated expression profiles of these genes in various tissues of WT-NFCX1 using RT-qPCR. The RT-qPCR results showed that *PvSPL2* was expressed in all tissues at low levels, while both *PvSPL4* and *5* were highly and predominantly expressed in node and shoot apical meristems (Fig. S8). We further investigated the expression of *PvSPL4* and *5* in all meristems including SAM, node aerial axillary meristems (AAMs) and inflorescence meristems (InMs) of different genotypes that vary in aerial bud formation (Fig. S9) using RT-qPCR. *PvSPL4* and *5* did not show any difference in either shoot apical meristems or inflorescence meristems between genotypes, but exhibited significantly higher expression in the aerial axillary meristems of NFCX1 compared with other genotypes (Fig. 4). This difference is highly associated with the variation of aerial bud formation among these genotypes. Meanwhile, none of the other *SPLs* showed such differences (Fig. S9). Furthermore, the investigation of *PvSPL4* and *5* expression in nodes of four WT and four transgenic lines with various axillary bud formations showed that plants with lower *PvSPL4* and *5* levels possess aerial buds and more tillers, whereas plants with higher *PvSPL4* and *5* levels have no aerial buds and fewer tillers (Figs S3, S6, S10). Statistical analysis revealed that the expression of *PvSPL4* and *5* is highly correlated with aerial bud formation and tillering (Fig. S10c,d). Taken together, the results suggest that *PvSPL4* and *5* directly regulate axillary bud formation.

After cloning full-length cDNA sequences of *PvSPL4* and *5*, we found that they are very similar in the coding region but different in the 5′ untranslated region (Fig. S11). BLAST search against the switchgrass genome (http://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Pvirgatum) showed that *PvSPL4* and *5* are located at the same locus on chromosome 6. In addition, *PvSPL4* and *5* exhibit very similar expression patterns in various tissues and genotypes. All these results suggest that *PvSPL4* and *5* are paralogs. We therefore renamed them *PvSPL4a* and *PvSPL4b*, respectively.

Down-regulation of *PvSPL4* induces aerial bud formation

The *PvSPL4*-RNAi construct was introduced into NFCX1 calli and transgenic plants (SPL4Ri) were produced. RT-qPCR analysis of the youngest leaf at the E1 stage showed that the expression levels of both *PvSPL4a* and *4b* were dramatically decreased, whereas the other *SPLs* had no significant expression changes in the SPL4Ri lines (Fig. S12). Examination of the nodes revealed that aerial axillary buds were induced in all transgenic lines with a significant decrease of *PvSPL4* expression (Fig. S13).

More detailed observation with SEM showed that while WT-NFCX1 does not produce any aerial axillary buds (Fig. 5a), transgenic plants with > 90% reduction of *PvSPL4* levels exhibited well-developed aerial buds with two foliage leaves in the upper (younger) buds (Figs 5, S14a–c). The results indicate that aerial bud development in the heavily down-regulated SPL4Ri lines is more advanced than in WT ST2 and AP13, because only the older aerial buds in ST2 and AP13 displayed two foliage leaves (Fig. S4b), whereas the younger buds exhibited just one foliage leaf (Fig. S14d–f). On the other hand, SPL4Ri plants with a < 70% decrease of *PvSPL4* level failed to restore the formation of aerial buds (Fig. 5a), suggesting that in these plants the *PvSPL4* expression level was still high enough to suppress aerial bud initiation completely.

Down-regulation of *PvSPL4* significantly increases tiller number, biomass yield and regrowth

In addition to aerial bud formation, SPL4Ri plants also showed significant improvement in tiller numbers and biomass yield

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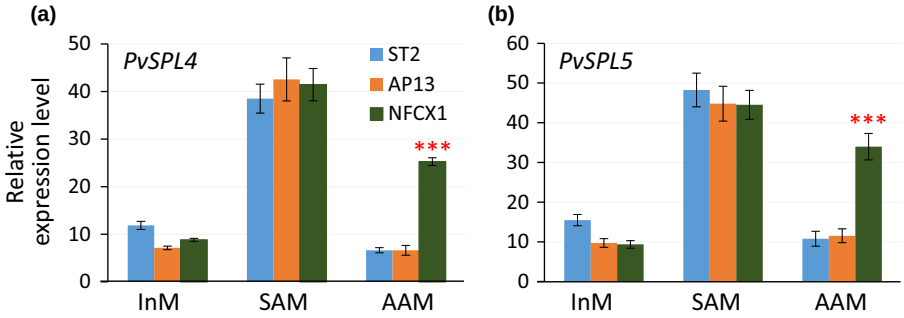

Fig. 4 Transcript abundance of *PvSPL4* (a) and *PvSPL5* (b) in switchgrass determined by quantitative reverse transcription polymerase chain reaction (RT-qPCR). Higher transcript abundances were detected in NFCX1 node aerial axillary meristems. InM, inflorescence meristems; SAM, shoot apical meristems; AAM, node aerial axillary meristems. Values represent means ± SD of three biological replicates and were statistically analyzed (*t*-test): ***, $P < 0.001$.

(Fig. 6a). Interestingly, the increases were even more prominent in the second harvest after cutting with a three- and twofold gain in tiller number and biomass yield, respectively (Figs 6a, S15). Essentially, SPL4Ri plants showed dramatically accelerated regrowth after cutting (Fig. 6b). Examinations from independent cutback experiments revealed that WT plants produced one or two basal buds in each stem after cutting, whereas SPL4Ri lines produced two to four basal buds (Fig. 6c–e). This difference alone enabled SPL4Ri lines to form more new tillers than the WT. Furthermore, we were surprised to find that new branches are also induced in the SPL4Ri plants (Fig. 6c). The production of branches is unusual because WT switchgrass genotypes generally do not produce branches. The combined effects of more tillers and the formation of branches contributed to the significant increase in regrowth and faster regeneration in the SPL4Ri plants than in the WT.

Overexpression of *PvSPL4* suppresses bud formation and decreases tiller number

Opposite to the effect of *PvSPL4* down-regulation, overexpression of *PvSPL4* under control of the *ZmUbi1* promoter resulted in a dramatic decrease in bud formation and tillering (Fig. S16a–c). WT plants generally possess 16–25 tillers at the R1 stage; by contrast, overexpression plants had only two to four tillers (Fig. S16a,e). Regrowth of the *PvSPL4* overexpression plants was also reduced compared with the WT. Consistently, biomass yield was significantly decreased in the *PvSPL4* overexpression plants (Fig. S16f). These results further confirmed that SPL4 directly suppresses both aerial and basal axillary buds formation.

RNA-seq analysis identified possible downstream regulation genes of axillary bud formation

To investigate the regulatory mechanisms of *PvSPL4*, representative SPL4Ri lines with different *PvSPL4* expression levels and bud developmental status (Fig. 5) were selected for RNA-seq analysis. A total of 294 015 transcripts were identified by blasting against the *Panicum virgatum* v1.1 genome sequence (http://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Pvirgatum); 68 073 transcripts passed the Cuffdiff 2.2.1 test and 15 639 were

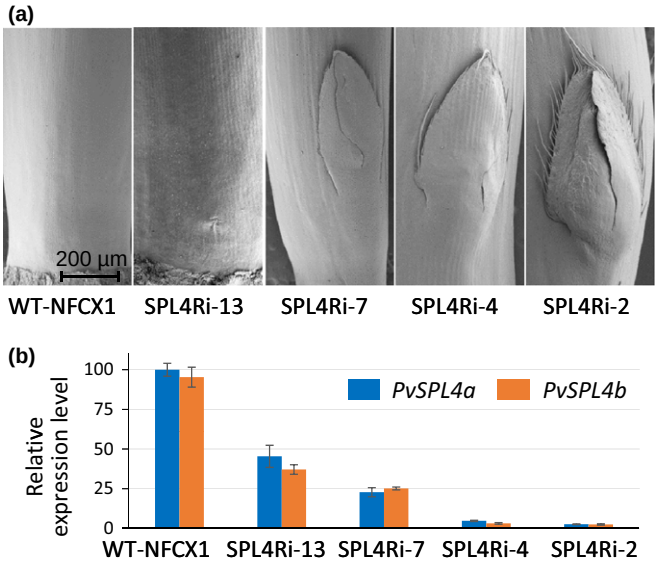

Fig. 5 Aerial axillary bud formation in *PvSPL4-RNAi* transgenic switchgrass plants (SPL4Ri). (a) Scanning electron micrograph observation of aerial axillary bud development in SPL4Ri plants. (b) Expression levels of *PvSPL4s*. Values represent means ± SD of three biological replicates.

identified with significant differences in various samples. Differential analysis further identified 132 up-regulated genes (Table S4) and 501 down-regulated genes (Table S5) with abundance changing more than twofold in transgenic plants relative to WT-NFCX1. Nine up-regulated and nine down-regulated genes were selected and subjected to RT-qPCR verification; 94.4% (17 out of 18) of the tested genes showed consistent results with RNA-seq (Fig. S17), indicating the high reliability of RNA-seq analysis. Among the 132 up-regulated genes, many genes are involved in carbohydrate and lipid biosynthesis/metabolic processes, which would be expected with the additional energy requirements of axillary meristem formation. Specifically, *LAX1* and *YABBY* are strongly up-regulated in PvSPL4-RNAi lines. *LAX1* was undetectable in the WT but substantially up-regulated in the node meristems of transgenic plants (Table S4). *YABBY* is the second highest up-regulated gene with greater than 70- and 50-fold increases in RNA-seq and RT-qPCR analyses, respectively (Fig. S17). Similar to *LAX1*, *MOC1* was also significantly

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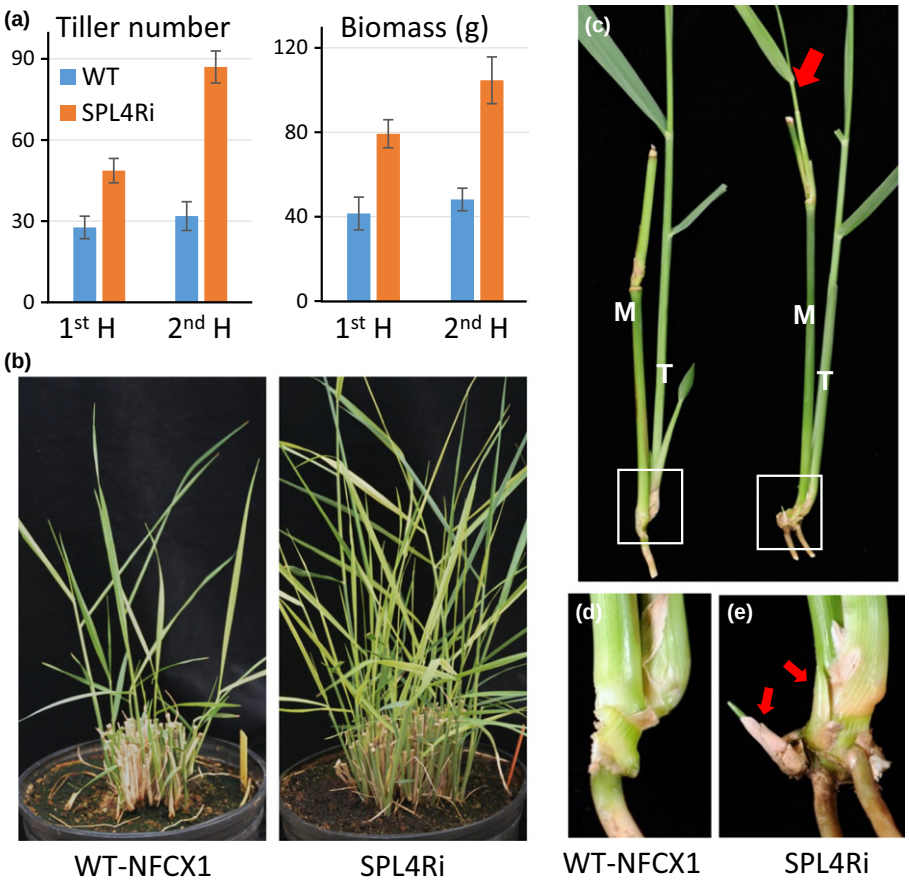

Fig. 6 Phenotype and agronomic performance of *PvSPL4-RNAi* transgenic switchgrass plants (SPL4Ri). (a) Down-regulation of *PvSPL4* in WT-NFCX1 significantly increased tiller number and biomass yield, especially in secondary harvest after cutting. 1st H, the first harvest; 2nd H, the second harvest. Values represent means ± SD of three biological replicates. (b) Down-regulation of *PvSPL4* enhanced plant regrowth after cutting. (c) New stem (branch, red arrow) produced from the outgrowth of the aerial axillary bud in SPL4Ri plant. M, main stem; T, tiller. (d, e) Enlargements of the regions framed in white in (c). Red arrows in (e), more basal axillary buds formed in the SPL4Ri plant.

up-regulated during bud initiation, indicating that both *LAX1* and *MOC1* are involved in the *PvSPL4* regulation pathway.

PvSPL4 acts upstream of *LAX1* and *MOC1*

As RNA-seq analysis indicated that down-regulation of *PvSPL4* dramatically up-regulated *LAX1* and *MOC1*, well-known activators of basal bud formation, we further investigated the relationship of *PvSPL4* and *LAX1* and *MOC1* in switchgrass transgenic plants. Consistent with RNA-seq results, RT-qPCR analysis showed that both *LAX1* and *MOC1* expression were significantly up-regulated in the *PvSPL4* knockdown plants (SPL4Ri). We further investigated expression of the two genes in *PvSPL4* overexpression plants (SPL4OE) and found that both *LAX1* and *MOC1* were dramatically down-regulated (Fig. 7). Taken together, these results indicated that the expression of *LAX1* and *MOC1* was negatively correlated with *PvSPL4*.

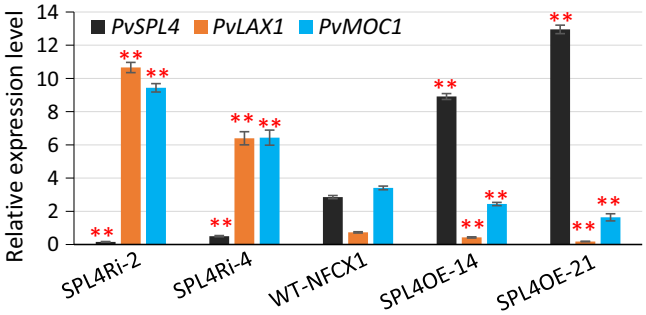

Fig. 7 Expressions of *LAX1* and *MOC1* are significantly up-regulated in *PvSPL4-RNAi* transgenic switchgrass plants (SPL4Ri) and are down-regulated in *PvSPL4* -overexpressing plants (SPL4OE). Node aerial axillary meristems were subjected for analysis. Values represent means ± SD of three biological replicates and were statistically analyzed (*t*-test): **, $P < 0.01$.

Discussion

The architecture of a plant affects its ability to compete for resources and impacts its agronomic performance. The complexity and adaptability of plant architecture depends on the establishment of new axes of growth through the production of secondary axillary meristems (McSteen & Leyser, 2005). Tillering is a common trait that determines architecture in major monocot crops (e.g. wheat, rice, barley). A number of genes that control basal

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bud and tiller development have been identified (Greb *et al.*, 2003; Li *et al.*, 2003; Wang & Li, 2011; Kebrom *et al.*, 2013). By contrast, aerial buds largely remain dormant in domesticated species and are not obviously noticeable. In certain less domesticated species, such as *Brachypodium* and a number of panicoid grasses (Doust, 2007), aerial buds continue to develop and contribute to the formation of plant architecture. Very limited information is available on the genetic regulation of aerial bud formation in monocots, although nine QTLs for aerial branching have been identified in millet (Mauro-Herrera & Doust, 2016). The study also showed that aerial branching QTLs often overlap with tillering QTLs, although some aerial branching QTL regions are independent (Mauro-Herrera & Doust, 2016). Coregulation of tillering and panicle branching has been reported in rice, such as by *MOC1* and *LAX1*; both genes are involved in the formation of tillers and panicles (McSteen, 2009; Wang & Li, 2011; Kebrom *et al.*, 2013). On the other hand, most genes regulate only one process. For example, *OsCKX2* and *SP1* regulate panicle branching but have no effects on tillering (Ashikari *et al.*, 2005; Li *et al.*, 2009; Luo *et al.*, 2012). In the case of *OsSPL14*, this gene regulates tillering and panicle branching in an opposite manner (Jiao *et al.*, 2010; Miura *et al.*, 2010). In the current study, we found some switchgrass genotypes, including NFCX1, that have only basal buds but no aerial buds. Overexpression of *miR156* induced aerial bud formation in NFCX1. Further investigation revealed that *PvSPL4* is a specific target of *miR156*, which negatively regulates axillary bud development. Knockdown of *PvSPL4* significantly promoted aerial bud formation along with an increase of basal buds. Consistently, overexpression of *PvSPL4* dramatically suppressed axillary bud development but did not completely inhibit basal bud formation. These results showed that *PvSPL4* works as a suppressor, and the *miR156-SPL4* module predominantly controls aerial bud formation but only partially regulates basal bud formation. Meanwhile, the *miR156-SPL4* module has no significant impact on panicle branching (Fig. S18).

Nineteen *SPLs* have been identified in rice (Wang *et al.*, 2009) and 16 in *Arabidopsis* (Xie *et al.*, *2006*). The *SPL* family is highly conserved across monocots and eudicots (Wang *et al.*, 2009), but each individual member may function divergently in the regulation of various processes. For example, *AtSPL3/4/5* redundantly regulate developmental aging and floral transition via directly activating *FRUITFULL*, *LEAFY* and *APETALA1* (Yamaguchi *et al.*, 2009; Jung *et al.*, 2016); *AtSPL9* controls the initiation of axillary meristems in cauline leaf axils via directly suppressing *LAS* (Tian *et al.*, 2014); *OsSPL13* and *OsSPL16* regulate grain size and shape (Wang *et al.*, 2015; Si *et al.*, 2016); and *OsSPL14* promotes panicle branching (Jiao *et al.*, 2010; Miura *et al.*, 2010). Phylogenetic analysis indicates that *PvSPL4* is distant from any *SPLs* in *Arabidopsis*, but it has two orthologs in rice: *OsSPL17* and *OsSPL14* (Fig. S19; Table S6). The function of *OsSPL17* has not yet been reported. *OsSPL14* has been shown to decrease tiller number (basal branching) even though it predominantly promotes panicle branching (Jiao *et al.*, 2010). However, *OsSPL14* suppresses basal branching via prolonging the plastochron but not by inhibiting basal bud initiation or outgrowth (Wang & Li, 2011). Our study revealed that *PvSPL4a* and *4b* specifically regulate axillary bud formation, particularly the initiation of aerial buds; meanwhile, they do not affect panicle formation. Apparently, the role of *PvSPL4* in switchgrass is different from its orthologs in *Arabidopsis* and rice.

It has been debated whether distinct or shared mechanisms control the development of detached and *de novo* AMs (Grbic & Bleecker, 2000; Long & Barton, 2000; Bennett & Leyser, 2006; Woods *et al.*, 2011). Generally, dicot and monocot plants were considered to have detached and *de novo* AMs, respectively (Oikawa & Kyozuka, 2009; Woods *et al.*, 2011). The conserved roles of *LAX1/BA1* and *MOC1/LAS* in AM development of both monocots and eudicots support the hypothesis that the same underlying mechanisms regulate the development of different AMs (Bennett & Leyser, 2006; Woods *et al.*, 2011). Meanwhile, there are still many exceptions. For example, the effect of *ROX* on AM development in Arabidopsis was found to be different from its orthologs *LAX1/BA1*. *LAX1* and *BA1* strongly control both vegetative and floral AM development in rice (Komatsu *et al.*, 2003) and maize (Gallavotti *et al.*, 2004), while *ROX* only partially modulates AM at the start of vegetative development under short photoperiod, and it does not affect flower development (Yang *et al.*, 2012). Previous studies in monocots, especially in rice, were mainly focused on basal bud formation. In this study, we observed that aerial bud formation differed from that of basal buds in switchgrass. Multiple basal buds were produced at different time points on a single stem, suggesting that basal buds did develop from *de novo* AMs. By contrast, aerial buds always appeared as soon as the youngest leaf axil was formed, indicating that they were most probably not produced *de novo* but from detached AMs. As *PvSPL4* inhibits both aerial and basal bud initiation, even considering the less significant impact on basal buds, our observation lends further support to the hypothesis that the development of detached and *de novo* AMs is controlled by a common underlying regulatory mechanism.

To date, a number of the genes (*MOC1* orthologs, *YUCs*, *LAX1* orthologs, *PIN1* orthologs) have been identified as activators of basal bud formation (tillering) in different species. No gene related to aerial bud formation has been identified in grasses. Unlike all previously reported genes, the *PvSPL4* reported here functions as a suppressor; it not only inhibits the development of aerial branches but also partially suppresses tillering capacity. Our RNA-seq and RT-qPCR analyses suggest that *PvSPL4* acts upstream of *MOC1* and *LAX1*. Consistently, *AtSPL9* has been shown to directly suppress *LAS* (Tian *et al.*, 2014), the *MOC1* ortholog in *Arabidopsis*. Recently, in rice, *TAB 1* was found to be required for axillary bud formation and to act downstream of *MOC1* or *LAX1* (Tanaka *et al.*, 2015). The detailed interactive relationships between *PvSPL4* and *LAX1*, *MOC1* or *TAB 1* may be of interest for future research.

On the applied side, down-regulation of *SPL4* alters shoot architecture and improves biomass yield in switchgrass. Compared with miR156-overexpressing plants in which dwarf and delayed flowering were observed (Fu *et al.*, 2011), the *PvSPL4* knockdown transgenics did not exhibit any of these undesirable phenotypes. Another interesting characteristic observed from the

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PvSPL4 knockdown plants is improved regrowth after cutting (Fig. 6b). This trait is particularly beneficial for perennial crop species that are harvested multiple times during the growing season and could realize improvements in yield following each harvest. Therefore, our research offers a new strategy to improve biomass yield and plant regrowth.

In summary, the study revealed a new mechanism in the regulation of aerial branch and tiller formation. We demonstrated that *miR156-SPL4* is a new module that predominantly controls aerial bud initiation and partially regulates the development of basal buds. Furthermore, we showed for the first time that *SPL4* is a suppressor of aerial bud formation. Genetic manipulation of *SPL4* offers an effective approach to enhance biomass productivity of important agricultural and biofuel crops.

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Author contributions

J.G. and Z-Y.W. planned and designed the research. J.G., C.F., S.L., C.T., S.D., A.F., Y.G., Y.T., Q.J., P.R.L. and J.W. performed experiments and analyzed data. J.G., A.F. and Z-Y.W. wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Morphological performance of switchgrass genotypes ST2, AP13 and NFCX1.

Fig. S2 Node culture results of switchgrass genotypes ST2, AP13 and NFCX1.

Fig. S3 Axillary buds development in different switchgrass genotypes.

Fig. S4 External appearance and interior structure of aerial and basal axillary buds of switchgrass (genotype ST2).

Fig. S5 Anatomical comparison of shoot apical meristem, aerial and basal axillary buds of switchgrass.

Fig. S6 Two-month-old wild-type and transgenic switchgrass plants overexpressing *miR156* (miR156).

Fig. S7 Sequence analyses of *PvSPL2*, *PvSPL4*, *PvSPL5* and *miR156*.

Fig. S8 Expression profiles of *PvSPL2*, *PvSPL4* and *PvSPL5* in switchgrass genotype NFCX1.

Fig. S9 Expression of *PvSPL1*, *2*, *3*, *6*, *7* and *8* in various meristem tissues of different switchgrass genotypes.

Fig. S10 Relationship between tiller development and the expression levels of *PvSPL4* and *5*.

Fig. S11 Sequence analysis of *PvSPL4* and *5*.

Fig. S12 *PvSPL4-RNAi* transgenic switchgrass plants (SPL4Ri) and expression levels of *PvSPLs* in these plants.

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Fig. S13 Aerial axillary buds of different nodes of *PvSPL4-RNAi* transgenic switchgrass plants (SPL4Ri).

Fig. S14 Comparison of the internal structures of aerial axillary buds of the 3rd node of different switchgrass plants.

Fig. S15 Tiller number and biomass yield of wild-type and transgenic switchgrass lines.

Fig. S16 Morphological traits of transgenic switchgrass plants overexpressing *PvSPL4* (PvSPL4OE).

Fig. S17 RT-qPCR validation of selected differentially expressed genes revealed by RNA-seq analysis.

Fig. S18 Panicle branches of WT-NFCX1 and corresponding *PvSPL4-RNAi* transgenic switchgrass plants (SPL4Ri).

Fig. S19 Phylogenetic relationships among SPL gene family members in *Arabidopsis*, rice, and *PvSPL4*.

Table S1 Primers used in this study

Table S2 Samples used for microarray analysis in this study

Table S3 Forty-eight genes identified by hierarchical analysis that exhibited a strong correlation between gene expression and bud developmental status

Table S4 Up-regulated genes with abundance changed more than twofold in transgenic switchgrass plants relative to the WT-NFCX1

Table S5 Down-regulated genes with abundance changed more than twofold in transgenic switchgrass plants relative to the WT-NFCX1

Table S6 Accession numbers of genes used for the phylogenetic analysis in Fig. S19

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