



Cloning and Expression Analysis of the *ScD53* Gene from Sugarcane

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Abstract *D53* is a crucial gene involved in tillering regulation in crops via the strigolactone signaling pathway. The objective of our research was to clone the homologous sugarcane (*Saccharum* L. spp. hybrids) gene of *D53* (*ScD53*) by in silico cloning combined with RT-PCR and rapid amplification of cDNA ends techniques, and the physicochemical properties and structure of *ScD53* protein were analyzed using bioinformatic software and tools. The relationships between the expression of *ScD53* and some plant hormones, bud germination and tillering occurrence of seedlings were also detected using the real-time fluorescence quantitative PCR (FQ-PCR) technique. The homolog was named *ScD53* and possessed a 3918-bp cDNA sequence. Bioinformatic analysis showed that *ScD53* has a complete open reading frame of 3363 bp that encodes 1120 amino acid residues. The *ScD53* protein possesses the conserved domain of the P-loop NTPase and ClpB_D2-small superfamilies, which may be unstable hydrophilic proteins and are likely to be located in the nucleus. The main secondary and tertiary structures of the *ScD53* protein are composed of random coil and alpha helix structures and exhibit high similarity in structure and homology with *D53* proteins from sorghum [*Sorghum bicolor* (L.) Moench] and maize (*Zea mays* L.). The FQ-PCR results showed that the expression of *ScD53* is tissue specific and is differently expressed in the different tissues. The expression of *ScD53* is associated with bud

germination, the high expression of which inhibits bud germination. Plant hormone treatment experiments indicated that the expressions of *ScD53* were induced in bud and leaf tissues by strigolactones, 3-indole acetic acid and kinetin (KT, a cytokinin analog). The high expression of *ScD53* following strigolactone and auxin treatment obviously delayed occurrence of tillers and reduced the number of tillers for the seedlings. However, this was not observed during KT treatment, which implied that the negative effects of this gene on seedlings tillering phenotype were eliminated by the positive influences of other unknown pathways. Our results above help elucidate the function of *ScD53* and provide important information and direction for the use of this gene in improving sugarcane yield through tiller regulation.

Keywords Sugarcane · *ScD53* · Cloning · Expression · Tillering

Introduction

Tillering is one of the most important agronomic traits for gramineous plants, such as (*Oryza sativa* L.), wheat (*Triticum aestivum* L.) and sugarcane (*Saccharum* L. spp. hybrids). It determines the final yield and plant plasticity by influencing the tillering and panicle number of crops (Li et al. 2012). Previous studies have shown that the tillering capability of gramineous plants is jointly regulated by multiple plant hormones, such as auxins, cytokinins and strigolactones (Leyser 2003; Tanaka et al. 2006; Kyozyuka 2007; Mikiyama et al. 2008; Gallavotti et al. 2008; Brewer et al. 2013). The downward movement of auxins and the upward movement of cytokinins and strigolactones form a dynamic equilibrium that regulates crop tillering (Brewer

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et al. 2009; Dun et al. 2009). Interestingly, auxin is not directly involved in tillering regulation, but together with cytokinins and strigolactones executes regulatory functions (Shani et al. 2006; Kyojuka 2007).

The strigolactones are a class of novel, recently discovered plant hormones, first reported by Cook et al. (1966, 1972) in their research on the germination stimuli of seeds of parasitic plants. They were subsequently isolated from numerous plants (Fen and Chen 2011), where they are required for some plant development processes, such as branching, hypocotyl growth, lateral root formation and root hair elongation (Gao et al. 2013). Recently, some key genes from its signal transduction pathways have been successfully cloned, such as *D3/MAX2/RMS4* (Stirnberg et al. 2002; Ishikawa et al. 2005), *D14/D88/HTD2/AtD14/DAD2* (Arite et al. 2009; Gao et al. 2009) and *D53/SMX-L6/7/8* (Jiang et al. 2013; Zhou et al. 2013). Jiang et al. (2013) and Zhou et al. (2013) showed that *D53* plays a key role in the perception and response of strigolactones signaling under the influence of strigolactones. *D53* proteins can interact with *D14* and *D3* proteins to form *D53–D14–SCFD3* protein complexes. These enable the ubiquitin *D53* protein to be specifically degraded by the proteasome system, thereby inducing the expression of downstream target genes and ultimately regulating plant tillering. This implies that the tillering capability of plants can be regulated by regulating the expression of *D53*, which can influence the signal transduction of strigolactones.

Sugarcane (*Saccharum* L. spp. hybrids) is an important global sugar crop, producing more than 70% of the world's sugar, and it is also an efficient source for renewable energy production (Li and Wei 2006; Lee and Bressan 2006). Sugarcane reproduces asexually. Stalks are the primary harvested product, so the number and weight of effective (harvestable) stalks impact the ultimate tonnage yield ha^{-1} (Li et al. 2015a, b). Stalk number per stool is influenced by the tillering traits of the variety, and thus, tillering is regarded as one of sugarcane's most fundamentally important agronomic traits (Vasantha et al. 2012). Improvement in sugarcane is needed through a better understanding of tiller regulation and enhancement. Key genes and metabolic pathways regulating tillering need further analysis and screening to provide the theoretical guidance for improving yields via tillering.

In view of the importance of the *D53* gene in tillering regulation, our objective was to clone the homologous sugarcane gene of *D53* (*ScD53*) by in silico cloning combined with RT-PCR and rapid amplification of cDNA ends (RACE) techniques, and the physicochemical properties and structure of *ScD53* protein were analyzed using bioinformatic software and tools. The relationships between the expression of *ScD53* and some plant hormones, bud germination and tillering occurrence of

seedlings were also detected using the real-time fluorescence quantitative PCR (FQ-PCR) technique. Our findings should contribute toward characterizing the structure, expression and function of this gene, and also provide theoretical information for the further functional analysis and utilization of this gene in the regulation of tillering capability of sugarcane.

Materials and Methods

Materials and Treating Methods

ROC22, the primary sugarcane variety in China, was selected as the research material for this study. Apical meristem tissues were sampled for gene cloning. For all FQ-PCR experiments, three biological replicates were designed, and sampled tissues were immediately frozen in liquid N_2 after sampling and stored at -80°C prior to analysis.

Root, stem, leaf, bud and apical meristem tissues were sampled during the tillering period for tissue-specific expression detection of *ScD53*. Single-bud stem cuttings were cultured in a tray containing Hoagland's nutrient solution (Chen et al. 2013). The germinating bud tissues were sampled for expression of *ScD53* at different developmental stages while dormant (day 0), sprouting (day 3), elongating (day 6) and elongating buds with a greenish-yellow sharp leaf (day 9). Other sprouting buds were continuously sprayed for 3 days with 60 mg/L kinetin (KT, a plant growth regulator with the same physiological function as cytokinin), 60 mg/L 3-indole acetic acid (IAA), 10 $\mu\text{mol/L}$ of strigolactones (SLs) and water, and then, these treated bud tissues were also sampled for detecting the expression of *ScD53*.

Single-bud stem cuttings were planted in buckets containing sand in a greenhouse, four buds per bucket. These buds were immersed in Hoagland's nutrient solution every 3 days to ensure adequate nutrition. Once these buds had developed into seedlings with 4–5 leaves, different concentrations of plant hormones (IAA: 100 mg/L, 200 mg/L, 400 mg/L; KT: 100 mg/L, 200 mg/L, 400 mg/L; SLs: 1 $\mu\text{mol/L}$, 10 $\mu\text{mol/L}$, 30 $\mu\text{mol/L}$) were continuously sprayed on the leaves and stem base for screening out the appropriate concentration of plant hormone treatment. Control seedlings were treated with water. The results showed that the KT 400 mg/L solution encouraged earlier tillering, with tillers after 8 days treatment, and the seedlings subsequently produced more tillers than the control. In contrast, 400 mg/L IAA solution and 10 $\mu\text{mol/L}$ SLs solution effectively delayed the occurrence of tillers, which occurred after 16 days and 28 days, respectively, and the subsequent seedlings developed fewer tillers than the

control. Finally, based on the above results, the seedlings were sprayed with 400 mg/L KT solution, 400 mg/L IAA solution and 10 $\mu\text{mol/L}$ SLs solution for 35 days, and then, these tissues from tillering bud and leaf were sampled for detecting the expression of *ScD53* after treating seedlings with different plant hormones.

Cloning of *ScD53*

The total RNA of these sampled tissues was extracted using the TransZol plant RNA kit (Transgen Biotech, China), and the RNA was reverse-transcribed into cDNA as the template for the RT-PCR using the TransScript one-step gDNA removal and cDNA Synthesis SuperMix reverse transcription kit (Transgen Biotech, China). Using electronic cloning technology, the *D53* gene-encoding region sequences from rice and sorghum were used as probes (KF709434.1 and XM_002441614.1), and the expressed sequence tag (EST) sequences with high nucleotide identity were retrieved from the sugarcane EST library on the GenBank database. Vector NTI 11.5 software (Invitrogen, USA) and DNAMAN 5.0 (Lynnon Biosoft, USA) software were used for comparison and splicing to obtain overlapping groups. The overlapping groups were then used to continuously compare and retrieve sugarcane EST sequences until no new sequences were available for splicing and extension, thereby obtaining the final electronic clone sequences. Primer Premier 5.0 software (Premier Biosoft International, Canada) was used to design the RT-PCR primers and 5' RACE gene-specific primers according to electron cloning sequences (Table 1). The TransTaq DNA polymerase high fidelity (HiFi) (Transgen Biotech, China) and ClonTech SMARTER™ RACE cDNA amplification kit (ClonTech, USA) were used in the RT-PCR and RACE experiments for the gene cDNA sequences, following which the coding region primer was designed to amplify the coding region sequence for verification. All of the above PCR products were recovered using the EasyPure quick gel extraction kit (Transgen

Biotech, China), connected to the pEASY-T5 Zero Cloning Kit vector (Transgen Biotech, China), and then transferred into Trans1-T1 phage-resistant chemically competent cells (Transgen Biotech, China). At least three clones were selected for bidirectional sequencing by the Beijing Genomics Institute (BGI, Beijing, China).

Bioinformatic Analysis of *ScD53* Protein

The online ORF Finder tool (<https://www.ncbi.nlm.nih.gov/orffinder/>) was used to analyze the open reading frame of the cDNA sequence of *ScD53* and the presumed amino acid sequence. The protein characteristics of *ScD53*, including the basic physicochemical properties, hydrophilicity or hydrophobicity, conserved domain, sub-cellular orientation, potential signal peptide and function, were predicted using ProtScale (<https://web.expasy.org/protscale/>), InterPro (<http://www.ebi.ac.uk/interpro/search/sequence-search>), TargetP 1.1 (<http://www.cbs.dtu.dk/services/TargetP/>), PSORT (<http://psort.hgc.jp/form.html>), SignalP4.1 (<http://www.cbs.dtu.dk/services/SignalP/>), Prot Param (<https://web.expasy.org/protparam/>) and Protfun (<http://www.cbs.dtu.dk/services/ProtFun/>).

The secondary and tertiary structures of *ScD53* were analyzed using the SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html) and Phyre2 (<http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index>) online tools. The homologs of *ScD53* were retrieved by Blastp in NCBI, and their amino acid sequences were compared with *ScD53* using DNAMAN5.0. The NJ (neighbor-joining) tree for all of the homologs of *ScD53* was constructed with 1000 bootstrap replications using MEGA7.0 software (Kumar et al. 2016).

Expression Analysis of *ScD53*

The FQ-PCR primer was designed according to the coding region sequence of the *ScD53* gene, using Primer Express3.0 (PE Applied Biosystems, USA). The primer

Table 1 Primers used in *ScD53* gene cloning

Primer name	Primer sequence (5'–3')	TM (°C)	Expected amplification product size (bp)	Strategy
ScD53-373-F	TGGGTCATGGGCTGGT	52	2511	RT-PCR
ScD53-2884-R	CTACTATGCACTATGGTGGGT			
ScD53-431-R1	GGGAACCTTGAAAGGAAGGCGAGGT	68	> 400	5' RACE amplification
ScD53-390-R2	CGACCAGCCCATGACCCAGACCGT	60		
UPM	CTAATACGACTCACTATAGGGCAAGCAG TGGTATCAACGCAGAGT	68		
NUPM	AAGCAGTGGTATCAACGCAGAGT	60		
ScD53-cds-F	ATCTAATCCCAATCCAATC	48	3395	Coding region amplification
ScD53-cds-R	TATCACCATCACCAATCCA			

sequences were 5'-AAAGCGTGCCACCGACTGTA-3' and 5'-GGCATGAATCAGGCTCTCCT-3'. The *GAPDH* gene was used as the reference gene (primer sequences: 5'-CACGGCCACTGGAAGCA-3' and 5'-TCCTCAGGGTTCCTGATGCC-3') (Ling et al. 2014). The FQ-PCR system was configured in accordance with the instructions of SYBR premix ExTaqTM II (TaKaRa, Japan) with the following amplification procedure: pre-denaturation at 95 °C for 30 s, then 40 cycles of denaturation at 95 °C for 5 s, followed by annealing and extension at 60 °C for 34 s. All of the PCRs were run on an ABI Viia7 Real-time PCR System, and each experiment was performed with three technical replicates. The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) was used to calculate the relative expression of genes. Analysis of variance (ANOVA) was used to assess the relative expression data, and the diagrams were plotted in Excel 2007 (Microsoft, USA).

Results

Cloning the cDNA Sequence of *ScD53*

A total of 21 sugarcane EST sequences with a nucleotide identity of more than 90% were obtained using the Blast tool in the GenBank database (Table 2). A spliced sequence fragment of 3204 bp was obtained by electronic cloning splicing technology, it contained a partial 3'UTR region with a length of 422 bp and an incomplete encoding region at the 5'-end by comparing these reference sequences (KF709434.1 and XM_002441614.1) from the *D53* genes of rice and sorghum, and the results showed the spliced sequence. Using the RT-PCR primers, a fragment of 2521 bp was obtained (Fig. 1a), which had 99.97% nucleotide identity with the previous spliced sequence, thereby confirming the validity of the electronic cloning sequence. The 5' RACE experiment yielded a 1132-bp PCR product (Fig. 1b). The entire cDNA sequence (3918 bp) was obtained by splicing the above sequences using Vector NTI 11.5 and DNAMAN 5.0. Amplification of the coding region resulted in a correct fragment of 3395 bp (Fig. 1c). This cDNA sequence, named *ScD53*, contains a 133-bp 5' UTR sequence, a 3363-bp coding region sequence and a 422-bp 3' UTR sequence, and was uploaded to GenBank with the accession number: KX893542.

Bioinformatic Analysis of *ScD53* Protein

Physicochemical Properties of ScD53 Protein

ORF Finder deduced that *ScD53* protein encoded a total of 1120 amino acids (as shown in Fig. 2). Analysis of the physical and chemical properties of protein using the

ProtParam and ProtScale tools showed that the molecular formula of *ScD53* was $C_{5284}H_{8453}N_{1561}O_{1649}S_{45}$, with a molecular weight of 121.68 kDa and theoretical pI of 6.94. Its instability index > 40 (54.83) indicated that *ScD53* was an unstable protein with an aliphatic index of 81.60 and a total grand average of hydropathicity index (GRAVY) was -0.324 . The 141st amino acid had the highest hydrophobicity score of 2.411, thereby possessing the strongest hydrophobicity, while the 110th and 744th amino acids had the lowest hydrophobicity score of -2.989 , indicating that these two amino acids were mostly hydrophilic. A total of 61.69% of the amino acids were hydrophilic, implying that the *ScD53* protein may be a hydrophilic protein.

Signal Peptide Prediction and Subcellular Localization of ScD53 Protein

The predicted results using SignalP4.1 showed that there was a high score in the 34th amino acid position for C-score, but no high score for S-score and Y-score appeared in the same position simultaneously (Fig. 3), and the D-cutoff value of 0.222 was less than 0.450. These results implied that no signal peptide was found in *ScD53* protein. In addition, because the mean S-score of 0.237 was less than 0.5, *ScD53* protein was presumed to be a non-secretory protein. Localization analysis in cells using PSORT software indicated that *ScD53* was probably located in the nucleus with a high probability value of 97.0%.

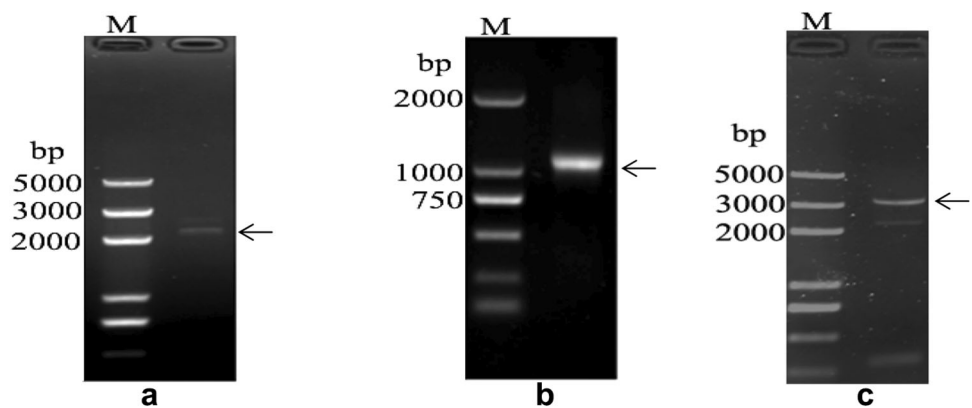
Structure and Function Prediction of *ScD53*

The conserved domain analysis using the conserved domain search tool in NCBI indicated that the structure of *ScD53* included the domain of the p-loop NTPase superfamily and ClpB_D2-small superfamily, and possessed an AAA-specific binding site and three non-specific binding sites (ClpA, PRK10865, AAA_2). The secondary structure of *ScD53* protein, which was predicted by using SOPMA online software, consisted mainly of random coil, alpha helix, extended strand and beta turn, respectively, accounting for 51.34%, 33.21%, 11.61% and 3.84% of the secondary structure. The tertiary structure of *ScD53* was constructed according to the folds recognition model using Phyre2 software, which indicated that the spatial structure of *ScD53* was dominated by random coil and alpha helix (see Fig. 4). Comparison of the spatial structure to other plant D53/D53-like proteins indicated that these D53 proteins from sorghum, maize, and rice are similar to *ScD53*. The functional prediction using ProtFun2.2 revealed that *ScD53* might constitute an enzyme protein that participates in coenzyme biosynthesis.

Table 2 Sugarcane EST sequences used in silicon cloning

EST name	GenBank accession number	EST name	GenBank accession number	EST name	GenBank accession number
EST1	BQ536100.1	EST8	CA173483.1	EST15	CA242253.1
EST2	CA066330.1	EST9	CA193834.1	EST16	CA244790.1
EST3	CA067240.1	EST10	CA199988.1	EST17	DV638883.1
EST4	CA106787.1	EST11	CA203128.1	EST18	DV640809.1
EST5	CA162162.1	EST12	CA215284.1	EST19	CA067163.1
EST6	CA170492.1	EST13	CA222254.1	EST20	CA157360.1
EST7	CA171494.1	EST14	CA222772.1	EST21	CA165861.1

Fig. 1 Amplification images of the *ScD53* gene. **a** The amplification product of RT-PCR; **b** the amplification product of 5'-RACE; **c** the amplification product of code region; arrow: the amplification product



ScD53 Homology Analysis

The evolutionary relationships between ScD53 and other plant D53 proteins were compared by a neighbor-joining phylogenetic tree of D53 proteins from 13 monocotyledons and five dicotyledons using MEGA7.0 (Fig. 5). The tree showed that all D53 proteins were clustered into two large groups, one of which was composed of dicotyledonous D53 proteins, while the other was composed of monocotyledonous D53 proteins, indicating that the D53 proteins from monocotyledons had high homology. This large group containing all of D53 proteins from monocotyledons was divided into two subgroups. One subgroup included the D53 proteins from Orchidaceae, Palmae and Musaceae, and the other subgroup was composed of D53 proteins from Gramineae. Thus, D53 proteins from Gramineous are closely related, and ScD53 protein was most closely related to sorghum D53 protein, followed by maize and millet D53 proteins. And homology analysis using the Blastp program in NCBI showed that ScD53 has 93%, 82%, 80% and 69% identity with these D53 proteins of sorghum, maize, foxtail [*Setaria italic* (L.) P. Beauv.], and rice, respectively. According to the comparison map of the amino acid sequences of D53 proteins from sugarcane, sorghum, maize, foxtail and rice (Fig. 6), the region from the 1st to

460th amino acids was relatively conserved, whereas the middle and posterior regions were less conserved.

Expression Analysis of *ScD53* Gene

The *ScD53* gene of ROC22 was expressed at different levels in the root, stem, leaf, bud and apical meristem (Fig. 7). The highest expression was detected in the stem, which was significantly higher than that in the other tissues ($P < 0.05$), followed by the apical meristem and bud, and low expression was detected in the leaves and roots, indicating that the expression of this gene exhibits certain tissue specificity.

The *ScD53* gene was differentially expressed in sugarcane bud tissues according to developmental stage (Fig. 8a). The highest expression was detected in the dormant buds, which was significantly higher than sprouting buds, elongation buds and sharp leaf buds. These results indicated that the high expression of *ScD53*, as in dormant buds, can inhibit the germination of sugarcane buds.

After three days of treatment with 60 mg/L KT, 60 mg/L IAA, and 10 μ mol/L SLs, both IAA and SLs inhibited the continued development of the sprouting buds, whereas KT had no significant influence on the growth of sprouting buds in comparison with the control. Following hormone


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121 ATCCAATCCGCCAATCCGACCGCGGTGCCGGCCGGGGCCGAGTCGCTCGCCC GGGCCGCGTCAAGCGGCCTGACCGCCGCGTGCCTCGCGCGCCGGCCGCGGCAGCGCCAGACCAC
   M P T P V P A A R Q C L A P A A V T A L D A A V A S A R R R A H A Q T T
241 CTCGCTCCACTCATGCTCGCTCTGTCCTCCACCCGCCGGGCGCTGCTCCGCGAGCGGCTGCGCGCCGCCGACGCGCGCGCTACTCCCGCGCCCTCAGACTCAAGGCGCTCGAGCT
   S L H L I A S L L S P T A A P L L R D A L A R A R S A A Y S P R L Q L K A L D L
361 CTCGCTTCGCGCTCCTCCGACCGCTCCCTCCAGCAGCAATGACAGCAGGACCGCCCGCTGGCCAACTCCCTCATGCGCGCATCAAGCGCTCCAGCGCCAAACCCAGCCGCGCCAAACCC
   C F A V S L D R L P S S S N D Q H E P P V A N S L M A A I K R S Q A N Q R R N P
481 GGACACGTTCCACTTCTACAAACCAGCATCCACC TCCCAACCGCGCTCAAGGTGACGCTCTCCCACTCGTCTCGCCATCTCTGACGACCGCCGCTGTCAGCGCGCTTCTAACGA
   D T F H F Y N H Q P S T S P N A V K V D L S H L V L A I L D D P L V S R V F N D
601 CCGCGGCTTCGCGACCAACGAGTCAAGGTGGCATCTCCGCGCCGGCGCCGCGCTGCGCGCTGCTGCGCGCGCGCTCCCAACGCGCGCGCGCGCGCGCGCTTCTTCTCTGCGACTT
   A G F R S N E I K V A I L R P A P P V P L L A R G L P T R A R P P P L F L C S F
721 CCGCGCGCGCGACGACCGACTCGCTCCGCGCGGGCGCGCGCGGGGAGGACAACTGCGCGCGCATCAACCGACATCTCGCGCGCGCGAGCAACCCCAATGCTCGCGCGCTCG
   A A A D D A V P S P A G A A P G E D N C R R I T D V I L A R G R N P M L V G V G
841 GGCGCGGCTCGCGCGCGGAGACTTGGCAACCGGTGCGCGTACCGCATCTCCCGGTTCAGTTCATCAACGGACCCTCTGCGCGTGGCGCGCGCGCGCGCGCGCGCTTCTGGCT
   A A S A A A D A D F A N A S P Y R I L P V S H Q T D L L A V A A A A P T T P G S G L
961 CATCTTACAGACTCGGTGACCTCAAGGACTGGTCCC GACGAGCCGACCTGCGAGCGCGGTGCGCGCGGTGGCGGAGTCAAGCGGCTTCTGAGAGCGCGCGCGCGCGCGCGCT
   I F S I G D L K D L V P D E A D L Q D A A R R V V A E V T R L L E T R R A A S R
1081 CCAGACGCTTGGTTCATGCGCTGTCGCGCACTACGAGACTACTCGCTCTCTTTCAGTTCCCGCTGCTGACAAAGACTGGGAATCCAGCTCTGCTGCAATCAACCGCGGTGG
   Q T V W W M G W S A T Y E T Y L A F L S K F P L V D K D W E L Q L L P I T A V R
1201 CCGCGCGCGCGCTGCAACCCGGACTCGCTCTCCGACTCCAGCAGCCAGCGGTTGCTGCCTTGCTCATGCCCTGCCACTACAAGTTTCATGAGCGGTTGTTCTTCTTCGTCGACTTTAT
   D A G P A P G L V P P P A P A T T V A A L S M P A T T S F M E S F V P F G G F M
1321 GTGCGATACCTATGCAACAAATTCGCCCTCGAGCGCCCTCGGATGTCAACAGTCGCAACGATAGATAGAGCAAGAAAGTTCGCAACTATCATTAGGAAAGTGCATTACAGCTGAAGCTCA
   C D T Y A A S C P Q A L R C Q C N D R Y E Q E V A T I R G S G I T A E A H
1441 CCAAGAAAGTCTACCTTCCATGTCGAGAATGGCAGCATGATGGTCTCAACAATGGGTTGATGACACTCAAGGTTAGAGCGCATAGATGGTATTGAGTACAAAAATGTAATCTGAA
   Q E G L P S M L Q N G S M M G P N N G F D A L K V R G D Q M V L S T K I L N L K
1561 GAAGAAGTGAAGTACTGCCCTACGACTCCACCAAGGTTGCAATAGSACTAACAGAGATCTTCCAGTTATTTCACATCAAATTTGATGTTCCAGTTGACAGCGAAAGATGTGCANA
   K K W N E Y C L R L H O G C N R I N R D P C O L F P H O I D V R V L E R C A A N
1681 TCCAACCAAGTTCACAGTTCAGTTCAGCTTCAAAAGGAGGTTATTAGACTTCTCCGAGTCTCTCCACATCAACACAACTGCAAAAGGATATTTCGGCGCATCTATTTCACCCA
   P N Q S S Q S V A L Q R E V I R P S A V S S P H T N T T A K S I S A P S I S T Q
1801 AATGAATCGAGACTTGATGTAATGCAACTCAAGTCAAGCAATCAAAAGGATGTAAGCCCTCCAAAGATAGGCGTTGTCATCCCAACATAGCAACTCAAAATGTGCAACCCGGAAGA
   M N A D L V L N L Q V R Q S K S D E P L Q D R A V P S Q H S H S S H C D H P E D
1921 TCATGCGTCACCACTTCGCTGCCCTTGCCTGCTGAGGACTGACTGCTGTTGGCCACCCCTCGTGATCTTCTTCAAGGATTCAGTAAATGTCATGTGTAAGCATGTAGAGGATGCTGAAGG
   H A S P S S A A P V A T D L V L A T P R G S S K D S S N A L C K H V E D E A E S
2041 GTCGATCCAGTGTATGCCAAGAAGGTTGATGATCTGAAATTTAAGCCTCTCACTTCTTCGACAGCCCTTACACTTGCCTTACAGGATTCTCAAATTTGGATCAAATTCACCTAGTGC
   S I Q L M P K K V D D L N L K P P H F S A Q P Y T C F R S S S N W D Q T S P S A
2161 TCTGCAATCAGCAGTTCAGGAGGTGCTTCTGCCCTTTGGCCAAATGCGAGAGGCTTCACCCCCTCGCAGCACAAAGTTATGATTTGAGCAATTAACAAGCTACTCATGGAACGACTGTGTTAA
   L H S A A S G G A F G W Q R P S L A A Q S Y D L S N Y K L M E R L F K
2281 GGCTGTGGGAGCGAGGAGGAAAGCGCTTGAGTCTATTGTGTCATCCTGTGCGGTGACAGTCAACCGAGAGCGGTGCTGTTGGTCAAAACAAAGAAGATGACATATGTTTATGTATG
   A V G R Q E E A L S A I C A S I V R C R S T E R R R G A N K K N D I W F S F Y G
2401 CCCGATAGCATGCCAAGCGGAGAGTGGGTTTGGACTTGGCTGAGCTGATGCAGATAGATCAGAGAATCTGATATATCTGGACCTAAGCTTCCATGACTGGGGTAAOCCCAATTTCCAG
   P D S I A K R R V G V A L A E L M H D S S E N L I Y L D L S L H D W G N P N F R
2521 AGGAAGCGTGCACCAGCTTGATCTGTAAGAGTTGAGAAGGAAGCGGACAGTATGATCTGACAAATGTGACAAAGCTGACTGCTTGTTCAGGAGAGCTGATTCATGCCAT
   G K R A T I S E E L R R K R R S V T F L D N V D K A D C L K H V E D E A E S
2641 GGAGACTGGCAGTCAACAAAGACTTGCATGGGGAGCGGTGGCTGACCTTAATGACTCGATTGGTGTGTGTCTACAAGAATGATCCAAGGATGCAAGATGCTCTCTTGGGATGAAGA
   E T G R Y K D L H G G R V A D L N D S I V V L S T R M I Q G C Q D A S L G M E E
2761 GGGCAATGTTTTTCAGAAAGAAAGTTGTCGACTCGTGGAAATCAACTGAAGATCATAGTTGAGCGGGCCAGCAACATCAGTGGAGGCGCCGAGGAAAGTGTAGTGTTCCTC
   G N V F S E E K V V A A R G N Q L K I I V E P G T A N I S G G P G K V V V S S
2881 AAGGCATTCTTGAAGAACAAATCAAGCATCTTGTACTCCAGTCTCTTCAAGTAAAGCGGAAGCTCAGATCTCTGATGACAAAGAAAGACAGCAAGTCAACGAGCATCTCAAGCGACT
   R H S L R N N Q A S L Y S S S F S K R K L Q I S D E Q E K T A E S P S T S K R L
3001 GCATGAAGCATCAAGCGCTCCATTCGACTTGAACCTCCAGCGGACGAGCGGAAATCCAGGATGTTGATGACGACAGCAGTACGACCCAGGCACTCTGCTGAGTACGAGGGGATC
   H R T S S V P F D L N L P G D E A E V Q D G D D D S S S S H G N S S G D P E G S
3121 TGTGGCAACTCTTAGCATAGTGGACGAGTCAATCAATTCAGCCATTTGACTTGGGAACTTTTGAAGATATCTGCAAGGAGTTTGAATACTATGAGCAAGACTCTTAGGCTC
   V G N L L R S V D E S I R F K P F D F G K L C E D I L O E F S N T M S K T L G S
3241 CAGGTGACAGGTGAGATGAGGCTGTCGCTTATGGAGCAAGTGTGGCAGCGGATCGGATCCGATCACATGAGAAGAGCGCTGTCGCGCATGTTGGAGGACAGGTTGTTCGCCAGG
   R C R L E I D A V A M E Q V V A A A W A S D S H E K R P V R T W V E Q V F A R S
3361 CCTTGAGCAGCTGAAGGTCAAGTGTGAAGATTTGAGCAGCTGACTCTGAGATTGTGCTGTGAGCATGAAAACCGCGTGAAGAAGAGCGTTTGGACCTTCTCTCCTCGAGAAAT
   L E Q L K V R C K N L S S C T L R L V A C E H E T P V K E D G F G P F L P S R I
3481 AATTCGGATTGGTATGGTGATATGTTCTGTATAGGGTTGCAATTATGCTAGCTGA
   I L D W *
```

Fig. 2 Nucleotide sequence and deduced amino acid sequence of the *ScD53* gene. The initiation codon is underlined, *stand for termination codon

treatment, the expression of *ScD53* appeared to increase significantly in comparison with the control (Fig. 8b). After continuously spraying the seedlings with 400 mg/L KT, 400 mg/L IAA and 10 µmol/L SLs solution for 35 days, all three plant hormones significantly induced the expression of *ScD53* in the tillering buds and leaves compared to the control (Fig. 8c, d). The highest expression was observed in the KT treatment, followed by the IAA treatment. The above results indicate that these three exogenous hormones positively regulate the expression of *ScD53*.

Discussion

Tillering is an important component of plant phenotypic plasticity and is also an important protection mechanism for plants to adapt to the surrounding environment and avoid injury (Gao et al. 2013). In agricultural crops, tillering is largely responsible for crop biomass or yield and thus is considered to be one of the most important agronomic traits. Tillering is mainly derived from the growth and development of the axillary buds at the leaf axils of

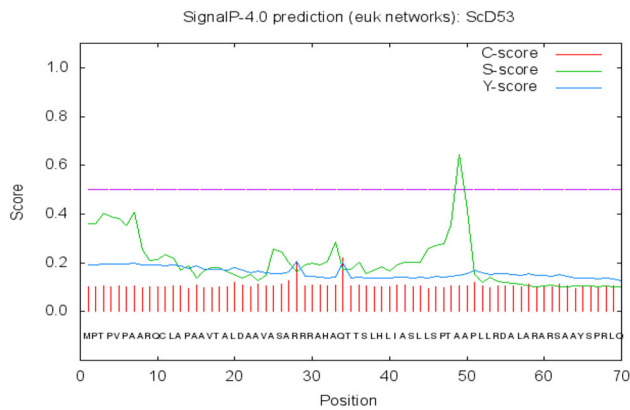


Fig. 3 Signal peptide prediction of ScD53 based on neural networks method

plants (Rameau et al. 2015), and thus, the development of the axillary bud plays a key role in the forming of tillering. Axillary bud development is strongly influenced by both external environmental factors and internal factors (Wang et al. 2014). In the absence of genetic factors, endogenous hormones are considered to be closely involved in the axillary bud development of plants (Mcsteeen 2009). Before 2008, IAA and CTK were thought to be the main regulatory hormones of axillary bud development in plants. After

that, a new class of phytohormone, named strigolactones, was discovered, and also shown to participate in the regulation of axillary bud development (Gomez-Roldan et al. 2008; Umehara et al. 2008), the regulation of axillary bud development is conserved in monocots and dicots (Stanga et al. 2013). Some mutations occurring in these genes involved in the synthetic pathway of SLs significantly affected tiller formation, including *MAX3/CCD7/RMS5/D17/DAD3* (Booker et al. 2004; Drummond et al. 2009), *MAX4/CCD8/RMS1/D10/DAD1* (Foo et al. 2005; Sorefan et al. 2003; Snowden et al. 2005; Arite et al. 2007) and *D27* (Lin et al. 2009). In addition, some genes involved in the signal transduction pathways of SLs also played a very important role in the regulation of plant tillering or branch control, namely *D3/MAX2/RMS4* (Stirnberg et al. 2002), *D14/D88/HTD2/AtD14/DAD2* (Arite et al. 2009; Gao et al. 2009) and *D53/SMX-L6/7/8* (Jiang et al. 2013; Zhou et al. 2013). *D3/MAX2/RMS4* and *D14/D88/HTD2/AtD14/DAD2* are two important SLs signaling molecules that interact with *D53/SMX-L6/7/8*, which is an inhibiting factor of SLs, to form protein complexes that ubiquitinate the inhibiting factor and cause it to be further specifically degraded by the proteasome, thereby inducing the expression of downstream target genes and the response of SLs signaling. The

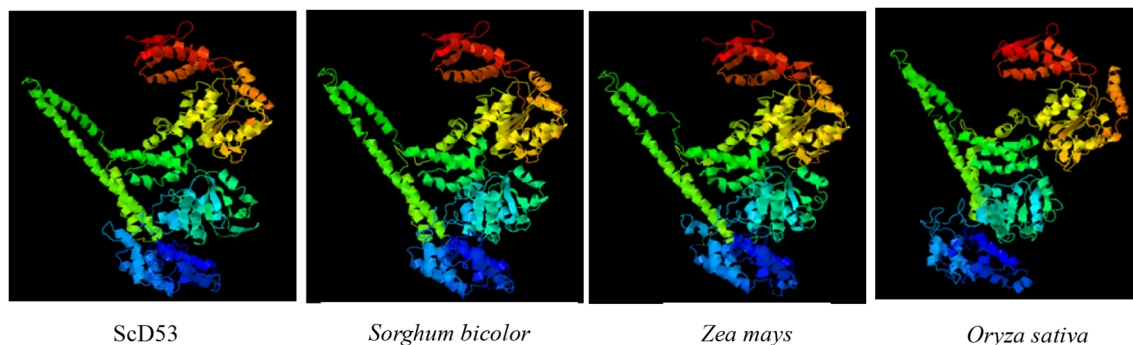


Fig. 4 Comparison of tertiary structure of ScD53 with D53 proteins of sorghum, maize and rice

Fig. 5 Neighbor-joining phylogenetic tree of D53 proteins from different plants species

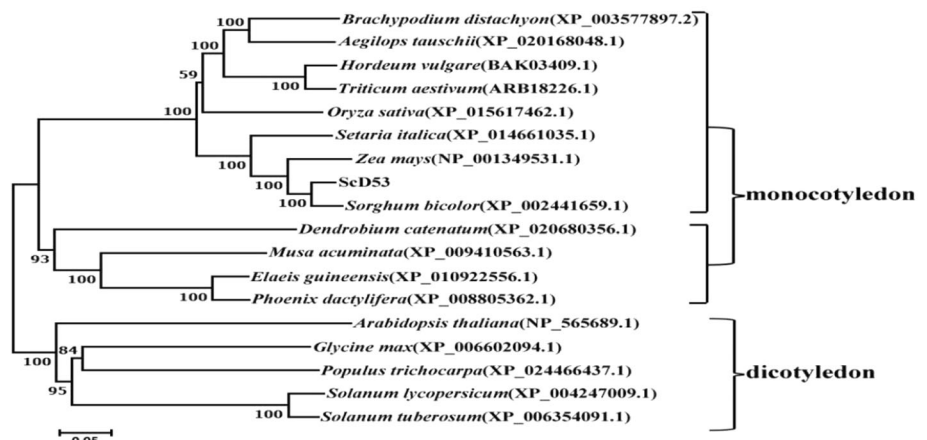
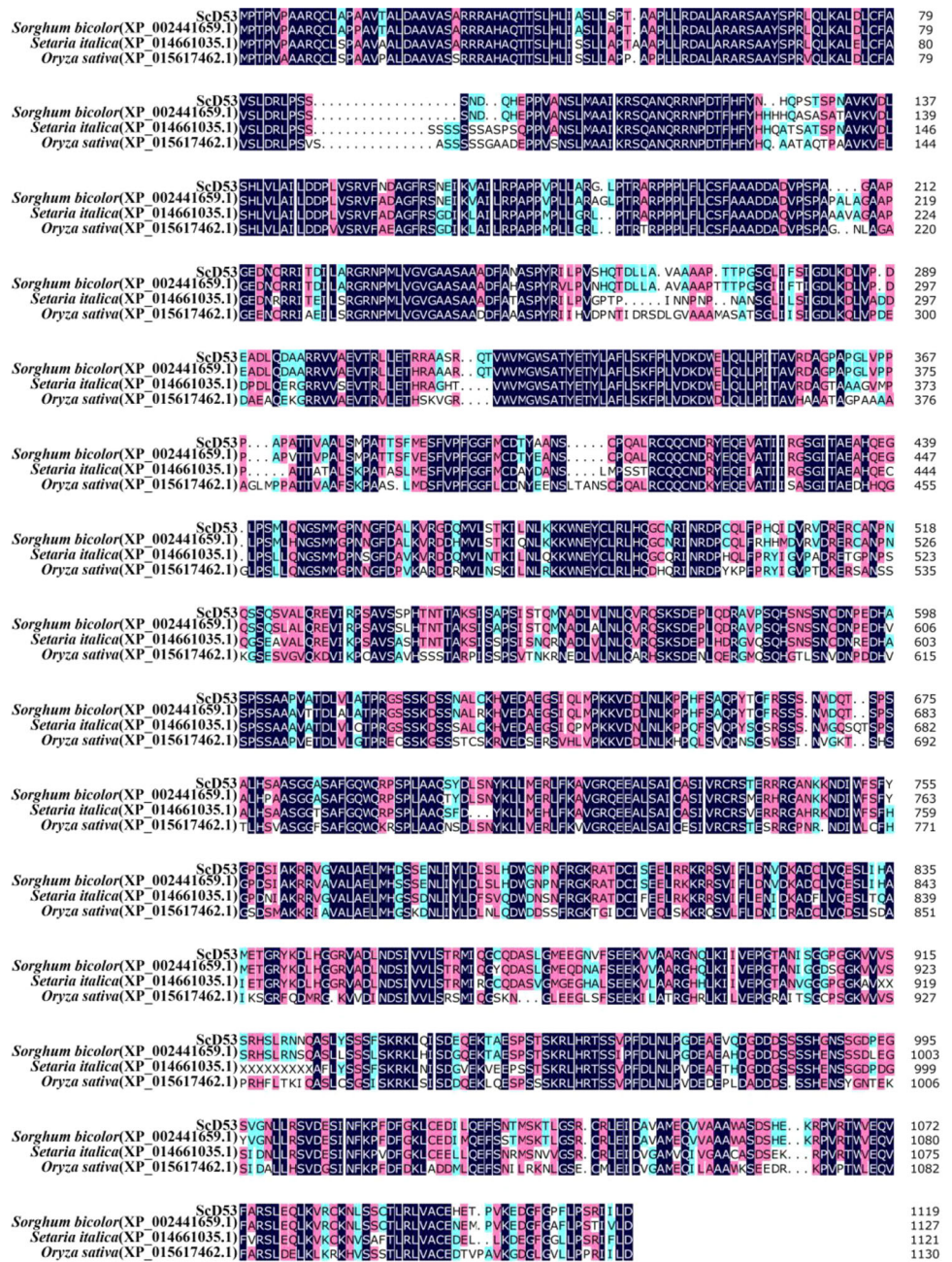


Fig. 6 Amino acid sequence comparison of D53 proteins from sugarcane and other plant species



inhibiting factor thus plays a key role in the SLs signaling pathway (Wang et al. 2015; Li et al. 2015a, b).

In this study, the homolog of *D53* was successfully cloned from a sugarcane variety and was found to encode 1120 amino acids, which is slightly smaller than the 1131 and 1142 amino acids of the *D53* proteins from rice and sorghum, but greater than the 678 amino acids of the *D53* protein from *Narenga porphyrocoma* (Liu et al. 2017). The conservative domain of ScD53 is similar to the *D53* proteins from rice and sorghum, all of which contain a p-loop NTPase superfamily domain, a ClpB_D2-small superfamily domain, one AAA-specific binding site and two non-

specific binding sites (ClpA, PRK10865) (Jiang et al. 2013; Zhou et al. 2013), except for the one non-specific AAA_2 binding site occurring only in the ScD53 and sorghum *D53* protein. Homology analysis demonstrated that ScD53 has the highest homology with sorghum *D53* (a 93% identity), followed by the *D53* proteins of maize and millet, which corroborate the findings of Zhou et al. (2013) and Jiang et al. (2013).

ScD53 exhibited particularly high expression in the stem and low expression in the root, which is consistent with the expression of *D53* in rice (Jiang et al. 2013). Previous studies have indicated that the expression of *D53* in the rice

Fig. 7 Relative expression of *ScD53* gene in different sugarcane tissues. Vertical bars represent the standard error of mean for each tissue. Bars with a common letter do not differ at $P < 0.05$

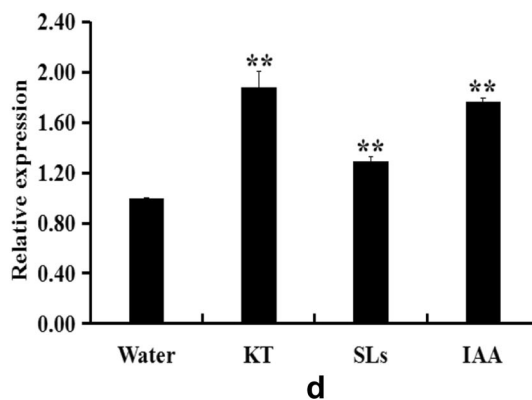
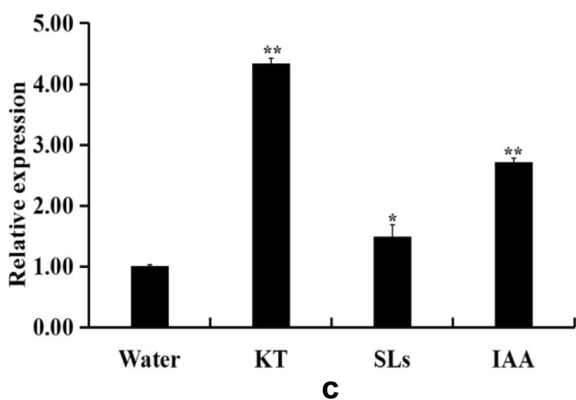
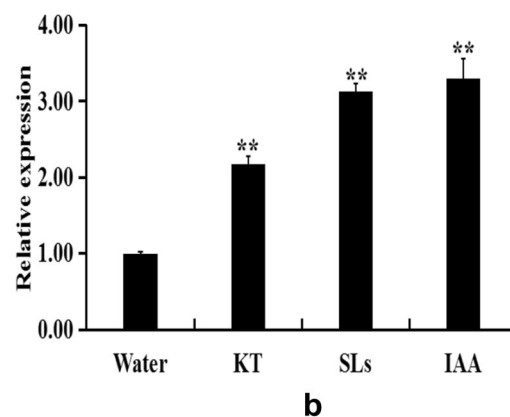
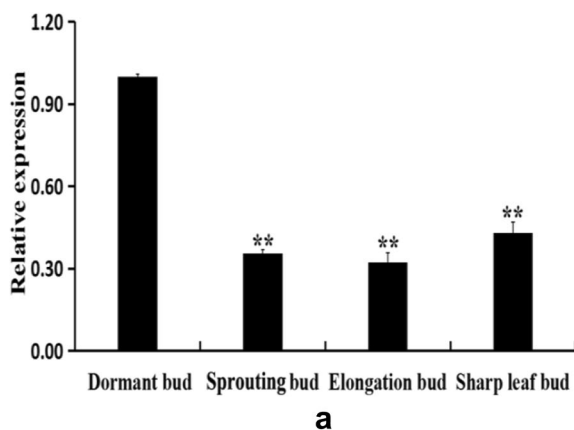
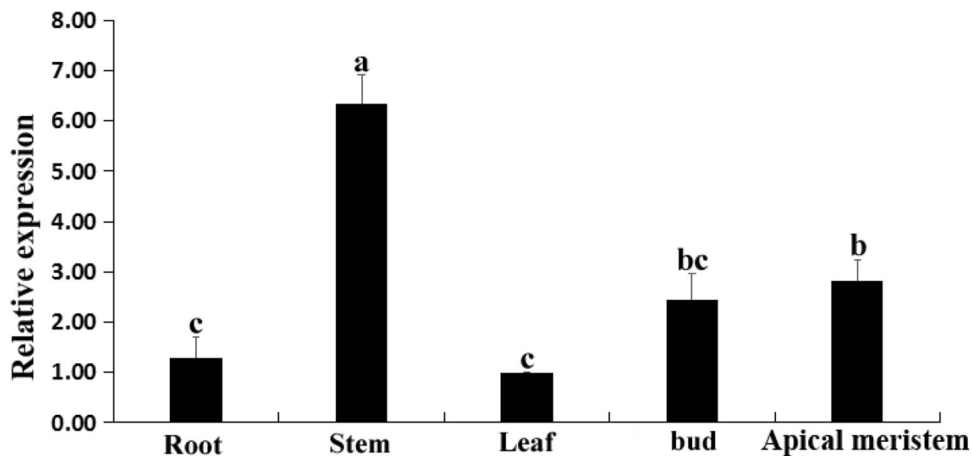


Fig. 8 Relative expression of *ScD53* gene at different developmental stages of buds, and as affected by plant hormone treatment of sprouting buds and seedlings. **a** the relative expression at different developmental stages of buds; **b** the relative expression in buds after treating sprouting buds with different plant hormones; **c** the relative

expression in tillering buds after treating seedlings with different plant hormones; **d** the relative expression in leaves after treating seedlings with different plant hormones; the error bars represent the standard error of each treating group, *significant difference from control at $P < 0.05$; **significant difference at $P < 0.01$

d53 mutant was significantly down-regulated compared with that of wild type, and the low expression of *D53* alleviated axillary bud germination inhibition and increased the number of tillers (Jiang et al. 2013; Zhou et al. 2013). In this study, *ScD53* was highly expressed in

dormant buds compared to germinating and developing buds, demonstrating that *ScD53* may have a negative regulatory role in bud germination of sugarcane.

In addition to environmental factors, plant tillers (branches) are also regulated by plant hormones (Durbak

et al. 2012). Plant IAA produced at the apical meristem can be transported to the stem base in a polar manner where they inhibit the germination of axillary buds through two purported mechanisms, namely the auxin transport channel formation hypothesis or the secondary messenger hypothesis (Kebrom 2017). Both SLs and CTK are currently thought to be important secondary messengers as they regulate axillary bud development together with IAA (Rashotte et al. 2003; Rameau et al. 2015). We demonstrated by plant hormone treatments that sugarcane bud development was regulated by KT (in a similar manner to CTK), IAA and SLs, which is consistent with results in other crops (Rameau et al. 2015). Our FQ-PCR experiments showed that the expression of *ScD53* was also significantly induced by the three hormones, similar to that observed in the *D53* homologs of rice and *Arabidopsis* (Jiang et al. 2013; Zhou et al. 2013; Soundappan et al. 2015; Wang et al. 2015). While KT induced the expression of *ScD53*, high expression of this gene did not inhibit the further bud development, suggesting that other pathways may eliminate the negative effect of *ScD53*. Our results help elucidate the function of *ScD53* and provide important information and direction for the use of this gene in improving sugarcane yield through tiller regulation. Further studies are needed to illustrate the molecular mechanisms of *ScD53* in tillering regulation of sugarcane, and this will help to understand the molecular basis of sugarcane tillering trait.

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Authors' Contributions ALL carried out the experiment and wrote the manuscript. XJL, CJL, HBL, QYZ, XQL participated in experimental work. XLL conceived and designed the study.

Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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