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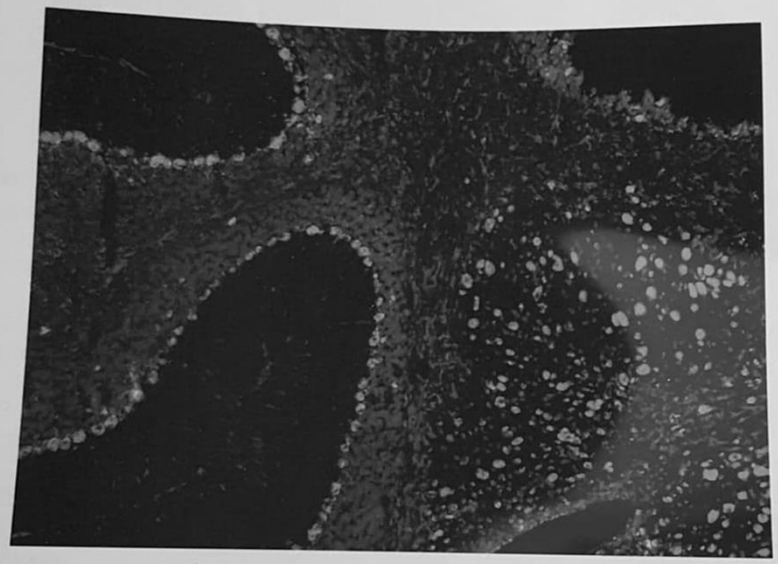

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CORRELATION BETWEEN KEY TRANSCRIPTION FACTORS CONTROLLING LIGNIN BIOSYNTHESIS AND LIGNIFICATION PATTERN WITHIN AN ELONGATING STEM INTERNODE OF *SETARIA*

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Introduction

Plant biomass from C_4 bioenergy crops has emerged as a potential source of renewable energy[1,2]. However lignin, a primary component of plant biomass is complex and challenges to biomass digestibility during the process of biofuel production[2].

In this study, an RNA-Seq investigation performance of the fifth elongating internode of *S. viridis* identified a cohort of candidate Transcription Factors (TFs) likely tobe involved in controlling lignin biosynthesis. RNA-Seq shown that the most of up-regulated transcription factors (TFs) belong to MYB and NAC domain gene families. In particular, *MYB42*, *MYB59-like*, *NAC73* and *NAC63* were expressed most highly in the transitional and maturation zones of the elongating stem internode of *S viridis* where lignin deposition occurs.

Quantitative RT-qPCR was performed to further confirm the expression of the candidate genes highly expressed in the TZ and MZ. Furthermore, the histological staining of the elongation stem internode of *S. viridis* and selected accessions of *S. italica* exhibited a correlation between the lignification patterns within mature stem cell walls and total lignin content.

Methods

Histological staining of the elongating stem internode of *Setaria*

Transverse sections of different developmental zones within the elongating fifth internode of five accessions of *S. italica* were stained with phloroglucinol-HCl[3,4] and viewed under an Axioscope A1 using bright field illumination.

Gene expression analysis by RT-qPCR

- Total RNA was extracted from different developmental zones of the elongating stem internode harvested at 50% grain head emergence using a Trizol method[5,6]. Complementary DNA (cDNA) was generated using RevertAid First Strand cDNA Synthesis Kit under manufacturer 'inductions (Thermo Scientific USA) and it was used as templete for Quantitative-qPCR.
- *MYB42, MYB59-like, NAC63 and NAC73* levels were expressed relative to the average of the combination of three reference genes suitable for normalisation of gene expression in *Setaria*[7] including; *SERINE/THERONINE-PROTEIN PHOSPHATASE 2A (PP2A)*, *5'-ADENYLYLSULFATE REDUCTASE 6 (ASPR6)* and *DUAL SPECIFICITY PHOSPHATASE (DUSP)*.
- The quantitative cycle (Cq) was determined using the Cy0 method[8].

Results

1. Phenotypic characterisation of five selective accessions of *S. italica*

Five accessions of *S. italica*

Representative accession 54 and elongating internode five (5) exhibited four different developmental zones including Meristematic Zone (MeZ), Cell expansion Zone (CeZ), Transitional Zone (TZ) and Maturation Zone (MZ).

Accessions	Plant heights (mm)	Flowering time (days)	Cell wall predicted Lignin (%)*
54	845.25 ± 9.9	33 ± 3	26.78
102	823.5 ± 9.2	30 ± 2	24.67
32	830.5 ± 7.6	32 ± 3	21.3
82	835.75 ± 8.6	31 ± 2	20.45
65	828.25 ± 7.2	33 ± 3	18.7

* Total cell wall lignin (%) was predicted using Partial Least Squared Regression model based on Fourier-transform infrared spectroscopy (FTIR spectra).

Five accessions of *S. italica* namely 54, 102, 32, 82 and 65 were selected as they exhibited similar phenotypic traits (A and C), but were found to deposit different contents of lignin in the stem (C).

2. Expression of four selected transcription factors in the elongating stem internode of *S. viridis*

	MeZ	CeZ (fold-change)		TZ (fold-change)		MZ (fold-change)	
		RNA-seq[1]	RT-qPCR[2]	RNA-seq	RT-qPCR	RNA-seq	RT-qPCR
MYB42	-	-1.95 ± 0.4	1.66 ± 0.12	4.79 ± 0.56	5.43 ± 0.74	5.06 ± 0.52	5.72 ± 0.45
MYB59-like	-	-1.29 ± 0.42	1.22 ± 0.16	0.30 ± 0.02	2.70 ± 0.41	1.7 ± 0.35	2.78 ± 0.15
NAC63	-	-0.36 ± 0.06	1.27 ± 0.15	2.05 ± 0.07	3.51 ± 0.31	1.87 ± 0.18	3.55 ± 0.28
NAC73	-	-3.6 ± 0.58	1.07 ± 0.03	3.51 ± 0.38	3.53 ± 0.32	3.55 ± 0.49	4.03 ± 0.25

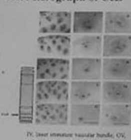
Quantitative-qPCR showed that *SevirMYB42*, *SevirMYB59-like*, *SevirNAC63* and *SevirNAC73* were expressed in four developmental zones of the *S. viridis* internode examined, consistent with previous observations from RNA-Seq analysis.

From RNA-Seq, there was a slight decrease from MeZ to CeZ, but had dramatic rises in their expression from CeZ to TZ, with *SevirMYB42* experiencing the highest fold changes.

From RT-qPCR performance, expression levels of all four genes were low and stable in the MeZ and CeZ. From the CeZ to the TZ, there was a highly expressed and similar trend of expression for those four genes compared to those from RNA-Seq analysis. From TZ to MZ, while expression of *SevirMYB42* continued increasing, expressions of *SevirMYB59-like*, *SevirNAC63* and *SevirNAC73* remained high and stable

3. Anatomical investigation of the elongating stem internode of *S. italica*

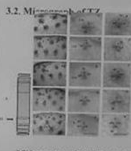
3.1. Micrograph of CeZ



IV, inner immature vascular bundle; OV, outer immature vascular bundle; PX, protoxylem; IX, immature xylem.

- Micrographs of fresh hand-cut transverse sections of CeZ from the elongating stem internode of five accessions of *S. italica*. Purple/red staining indicated the presence of lignin.
- In the CeZ, minimal lignification was evident in protoxylem of the inner layer of immature vascular bundles. No different lignification was observed among those five accessions.

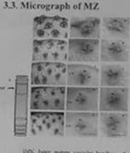
3.2. Micrograph of TZ



IMV, Inner mature vascular bundles; S, Small outer vascular bundles; L, Large outer vascular bundles; P, phloem; BS, bundle sheath; MX, metaxylem; TCW, thickened cell walls. Bars = 50µm.

- The TZ appeared rather more developed with lignified cells found in various elements including protoxylem, metaxylem,, and bundle sheath of outer vascular bundles.
- Bundle sheath of large outer vascular bundles and thickenrd cell wall of accession 54, 102 and 32 were clearly lignified (B to J), whilst a minimal lignification was observed from accessions 82 and 65 respectively (K to P). 27.5

3.3. Micrograph of MZ



IMV, Inner mature vascular bundles; S, Small outer vascular bundles; L, Large outer vascular bundles; P, phloem; BS, bundle sheath; MX, metaxylem; TCW, thickened cell walls. Bars = 50µm.

- With acropetal development of the 5th internode, a sharp increase in cell wall lignification of the bundle sheath surrounding the inner and outer mature vascular bundles (IMV, S and L), the walls of MZ and TCW were clearly evident in MZ (B-P).
- Accession 54 and 102 exhibited heavily lignification in IVB, BS, L, TCW, appearing dark purple in colour, whilst slight exhibition of lignin was recorded from BS of outer bundle sheath of accessions 82 and 65 respectively (LO).

Conclusions and future work

- RNA-Seq and RT-qPCR analyses shew that *MYB42, MYB59-like, NAC63 and NAC73* were up-regulated and highly expressed in TZ and MZ where increased lignin deposition was observed.
- A strong correlation between cell wall total lignin (%) and lignification pattern within an elongating internode of the five selective accessions of *S. italica* was observed.
- Further work will focus on the analysis of lignin content and performance of Quantitative-qPCR of the candidate TFs in the CeZ, TZ and MZ of the S. italic accessions in order to demonstrate a clear correlation between TFs expression and level of lignification in the elongating internode of *Setaria*

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