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Mechanical stimulation reprograms the sorghum internode transcriptome and broadly alters hormone homeostasis

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ABSTRACT

Stem structural failure, or lodging, affects many crops including sorghum, and can cause large yield losses. Lodging is typically caused by mechanical forces associated with severe weather like high winds, but exposure to sub-catastrophic forces may strengthen stems and improve lodging resistance. The responses of sorghum internodes at different developmental stages were examined at 2 and 26 h after initiating moderate mechanical stimulation with an automated apparatus. Transcriptome profiling revealed that mechanical stimulation altered the expression of over 900 genes, including transcription factors, cell wall-related and hormone signaling-related genes. IAA, GA_1 and ABA abundances generally declined following mechanical stimulation, while JA increased. Weighted Gene Co-expression Network Analysis (WGCNA) identified three modules significantly enriched in GO terms associated with cell wall biology, hormone signaling and general stress responses, which were highly correlated with mechanical stimulation and with biomechanical and geometrical traits documented in a separate study. Additionally, mechanical stimulation-triggered responses were dependent on the developmental stage of the internode and the duration of stimulation. This study provides insights into the underlying mechanisms of plant hormone-regulated thigmomorphogenesis in sorghum stems. The critical biological processes and hub genes described here may offer opportunities to improve lodging resistance in sorghum and other crops.

1. Introduction

Shoot structural integrity is vitally important to plant function, making possible an appropriate display of leaves for photosynthesis and supporting the reproductive structures for pollination and seed maturation. In the agricultural context, shoot structural integrity is essential for the successful harvesting of cereal grain and other products. Stem structural failure, or stem lodging, typically caused by severe weather or other factors, often results in massive yield losses due to the inability of mechanical harvesters to recover grain from lodged stems or the irreversible fatal breakage of stems (Berry et al., 2003, 2004). Stem lodging has been estimated to cause annual yield losses of 5–20% in maize (Tirado et al., 2021).

Lodging susceptibility or resistance can be attributed to a variety of parameters associated with stems. Geometry is important since shorter stems reduce the leverage that forces (such as wind) experienced by the top of the plant (panicle, flag leaf, etc.) exert on the lower parts of the stem where lodging occurs. Additionally, stem diameter has a major impact on load-bearing capacity, with larger diameters supporting higher loads (Feng et al., 2022). The stem is composed of internodes of various lengths and diameters arranged between nodes. Stem structural failure almost always occurs in the internodes, which are much weaker than the highly reinforced nodes (Gomez et al., 2017). The internode itself is composed of a variety of cells and tissues that can be generally

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categorized as a soft inner core of pith, and a stiffer outer layer of rind which includes the epidermis (Lee et al., 2020). Vascular bundles are present in both the pith and the rind and may contribute to structural support through thick-walled fibers and xylem cells. Cell wall components of these and other cells provide the base level of structure that is organized to produce a supportive internode. Both primary and secondary cell wall composition and organization are likely to contribute to the overall properties of the internode, including whether the internode can support the loads that it is exposed to, or whether it fails, leading to stem lodging.

In response to mechanical stimulation, plants may alter their growth and development through a process known as thigmomorphogenesis (Jaffe, 1973). Thigmomorphogenic responses generally occur slowly over time and may result in sustained changes in the growth and development of plants to support additional loading. Stem lodging is expected to occur when thigmomorphogenic acclimation is insufficient to meet the challenge of the mechanical forces experienced. Typical geometrical responses observed in herbaceous plants include a reduction in stem elongation and an increase in stem diameter (Chehab et al., 2009). These changes are the overt manifestation of events that occur at the cellular level, including a reduction in cell elongation (Biro et al., 1980; Wu et al., 2020). Mechanical stimulation can also influence the cell wall composition and organization that in turn contribute to the biomechanical properties of the stimulated structure (Gladala-Kostarz et al., 2020). Plants subjected to mechanical stimulation often show increased stem flexibility that may enable them to better withstand mechanical forces without failing (Jaffe et al., 1984; Telewski & Jaffe, 1986). Changes in cell expansion and cell wall properties are underpinned by changes in gene expression resulting from mechanical stimulation that have been studied in some detail (Xu, 1995; Purugganan et al., 1997; Lee et al., 2005). Furthermore, a variety of hormones have been implicated as modulators or coordinators of the thigmomorphogenic response, including JA (Chehab et al., 2012), GA (Lange & Lange, 2015), and ethylene (Wu et al., 2020).

There is increasing interest in sorghum as a bioenergy crop for its high productivity, widespread adaptability, genetic diversity, low input requirements and existing genetic improvement infrastructure (Rooney et al., 2007, Mullet et al., 2014). However, sorghum frequently suffers stem lodging in the field, especially in taller cultivars suitable for use as bioenergy feedstocks. Understanding the physiological and molecular mechanisms behind sorghum stem thigmomorphogenic responses is a necessary step in developing lodging-resistant lines. While there is a substantial amount of information available regarding thigmomorphogenesis in plants, there are very limited studies in sorghum with many gaps. For instance, how the stem's developmental stage and the duration of mechanical stimulation influence plant responses at the transcriptomic level have not been reported in sorghum. In this study, transcriptomic and hormonal responses of fully elongated (older) and elongating (younger) sorghum internodes to unidirectional mechanical stimulation under short-term (2 h After Initiating Mechanical Stimulation, AIMS) and long-term (26 h AIMS) treatment periods were assessed. It was hypothesized that the sorghum stem response to mechanical stimulation would be age- and dose-dependent, and that mechanical stimulation would alter stem hormone homeostasis and transcriptome expression. It was further hypothesized that transcriptome and hormonal changes would be associated with changes in morphology, anatomy, and mechanical properties elicited by mechanical stimulation.

2. Materials and methods

2.1. Plant material and growth conditions

Sorghum [*Sorghum bicolor* (L.) Moench] cv. Della, a sweet sorghum variety suitable for use as a bioenergy feedstock, was sown on May 29, 2020 in 14.88 L pots (27.6 cm D x 28.3 cm H) filled with a mix of natural soil (fine sandy loam) amended with 12.5% potting mix (Jolly Gardener C/20). Pots were provided with adequate water and nutrients (Osmocote 13–13–13 and soluble micronutrients) and grown in a greenhouse (located at 30.6° N) maintained at 26–30ºC day/21–26ºC night, under 14 h day/10 h night photoperiods with supplemental light provided by high-pressure sodium lamps (Lee et al., 2020).

2.2. Experimental treatments

Plants were subjected to mechanical stimulation 39 days after sowing, at a growth stage when the seventh internode was typically beginning to elongate and was less than 1 cm long. As previously described (Zargar et al., 2022), mechanical stimulation was provided using a motor-driven rigid frame that contacted plants 43 cm above the soil surface and moved the stems 20 cm in one direction (Fig. 1**a, Supplementary Video 1**). Stimulation was applied with a frequency of 3 cycles min^{-1} for a duration of 2 h, followed by a 6-hour resting period before starting the next stimulation cycle (Fig. 1**b**). An equal number of unstimulated plants were used as controls.

2.3. Sampling method

Rind tissue was sampled from internodes at two different developmental stages, actively elongating internodes (still flexible, typically internode 4 or 5), and nearly fully elongated internodes (typically internode 3 or 4). The numbering of internodes began from the most basal internode with at least 0.5 cm of elongation. When plants were stimulated, the plastic rod of the apparatus came into contact with the stem and the contact point was the point of stimulation as shown in Supplementary Figure 1. Since the force was applied unidirectionally, the side in contact with the point of stimulation experienced tension, while the opposite side experienced compression (Supplementary Figure 1). Rind samples were taken from the tension side of the stem near the middle of the internode by cutting a slice approximately 2 cm

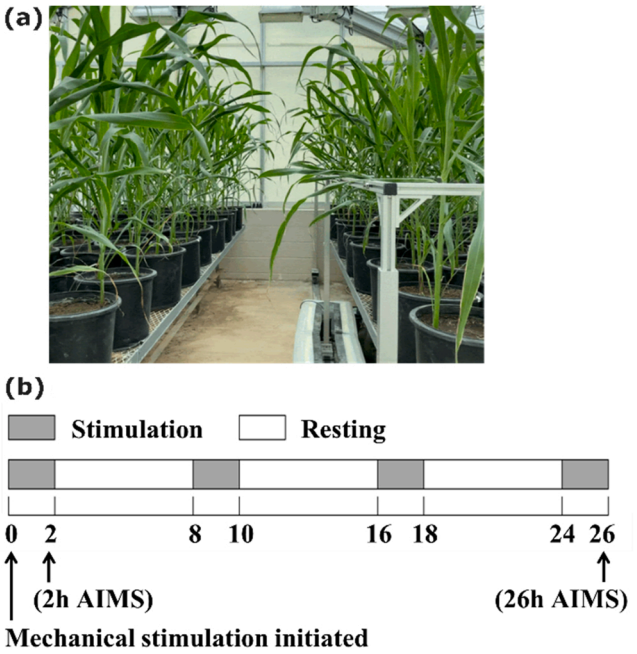

Fig. 1. Experimental design. (a) Control (left) and mechanically stimulated (right) groups of plants in the greenhouse when initiating mechanical stimulation. (b) Experimental scheme for the working period of the mechanical apparatus and sampling time points. The mechanical apparatus stimulated plants for 2 h, followed by a 6-hour resting period. The time points for harvesting internode samples are indicated by arrows, which was prior to mechanical stimulation, and then at 2 h and 26 h After Initiating Mechanical Stimulation (AIMS).

long by 5 mm wide by 2 mm deep from the outer edge of the internodes. Replicates were composed of a pool of rind tissues from 4 individual plants, with 4 replicates per treatment/internode/time point. Samples were harvested just prior to stimulation, and then at 2 and 26 h AIMS (Fig. 1**b**), and were immediately frozen in liquid N_2, ground to a fine powder and then stored at − 70°C.

2.4. Quantification of JA, ABA, IAA, GA_1 and GA_{20}

For the analysis of JA, ABA and IAA, approximately 70 mg of powdered rind sample was weighed, extracted, purified and quantified by LC-MS/MS using isotope dilution in multiple reaction monitoring mode as previously described (Liu & Finlayson, 2019). Stable isotope-labeled standards were included for each hormone quantified. Samples for GA_{20} and GA_1 measurements were extracted and purified in the same manner as the other hormones, but were subsequently derivatized with bromocholine as described in Kojima et al. (2009), prior to quantification by LC-MS/MS using the appropriate bromocholine derivative precursor/product transitions.

2.5. Total RNA extraction, RNA-Seq library construction and sequencing

For transcriptome profiling, RNA was extracted from 100 mg of powdered rind samples using TRIzol (Invitrogen) according to the manufacturer's directions. RNA samples were digested with DNase I and purified using RNA Monarch columns (NEB) according to the manufacturer's directions. RNA quality was evaluated by an Agilent 2100 Bioanalyzer. The three highest quality samples from each treatment/internode/time point were used to construct sequencing libraries using the NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina® (NEB) according to the manufacturer's directions. Libraries were examined by an Agilent 2100 Bioanalyzer prior to sequencing on an Illumina NovaSeq with 50 bp paired-end reads.

2.6. RNA-Seq data processing and differential gene expression analysis

RNA-Seq data was processed using the advanced computing resources provided by Texas A&M High Performance Research Computing. Sequencing produced approximately 20 million to 38 million paired-end reads per library (Supplementary Table 1). The quality of raw reads was assessed by FastQC (Andrews, 2010). After using Trimmomatic (Bolger et al., 2014) to remove adaptors and low-quality reads (slidingwindow:5:20, minlen:20), 83.7–92.1% of the clean reads were concordantly and uniquely mapped to the *Sorghum bicolor* reference genome (Phytozome v3.1.1) using HISAT2 (Kim et al., 2015) with SAMtools (Li et al., 2009) for filtering (Supplementary Table 1). The number of reads mapped to each gene was counted by featureCounts (Liao et al., 2014) and used to identify differentially expressed genes (DEGs) by edgeR (Robinson et al., 2010), with the criteria FDR < 0.05 and $|\log_2(\text{Fold Change, FC})| > 1$. Up-regulated and down-regulated DEGs were identified by $\log_2(\text{FC}) > 1$ and $\log_2(\text{FC}) < -1$, respectively.

2.7. GO and KEGG enrichment analysis

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were conducted using the R package "Clusterprofiler" (Wu et al., 2021), with customized GO and KO (KEGG orthology) annotation built in R (version 4.1.1). For genes without sorghum GO terms, the GO terms of the best rice match (*Oryza sativa*) were used. For genes lacking both sorghum and rice GO terms, GO terms of the best *Arabidopsis thaliana* match were used. In this way, 25,508 genes with GO terms (15,349 with sorghum GO terms, 1099 with rice GO terms and 9060 with *Arabidopsis* GO terms) were obtained. The same approach was used for customizing the KO annotation, and 10,331 genes with KO terms (8676 with sorghum KO terms, 880 with rice KO terms and 775 with Arabidopsis KO terms) were obtained and mapped to KEGG pathways using KEGGREST (Tenenbaum & Volkening, 2021). All best matches were obtained from the sorghum genome annotation (Phytozome v3.1.1). All GO and KO terms of best matches were obtained from Phytozome (version 12). Significantly enriched GO terms and KEGG pathways were identified with the criterion of q value < 0.05 and count > 1.

2.8. Weighted gene co-expression network analysis

Weighted gene co-expression network analysis (WGCNA) was performed in R using the WGCNA package (Langfelder & Horvath, 2008). The normalized counts ($\log_2$CPM, Counts Per Million) from edgeR were transformed to $\log_2(\text{CPM}+1)$ to be used as input. Low-variance genes were filtered out by relative variance > 0.01. The soft-thresholding power of 8 was selected and an unsigned network was constructed using the automatic network construction function blockwiseModules with default settings except power = 8 and mergeCutHeight = 0.35. The correlation of modules and traits was estimated by the correlation coefficient between the module eigengene (ME) and the trait, which was used to determine significant target modules for further analysis with $|\text{cor}| > 0.5$ and $p < 0.01$. Hub genes are genes with strong and high connectivity in target modules, which may be functionally important. In this study, candidate hub genes were defined by the absolute value of geneModuleMembership > 0.7 and geneTraitSignificance > 0.2. The network of candidate hub genes was visualized in Cytoscape_v3.8.2 (Shannon et al., 2003), and hub genes were identified by network analysis using a Cytoscape plugin, Network Analyzer.

2.9. Statistical analysis

Statistical comparisons of hormone abundances between control and stimulated groups were performed in R (version 4.1.1) using unpaired t-tests. p-value < 0.05 was set as the criterion for statistically significant differences.

3. Results

3.1. Summary of RNA-Seq data

In this study, over 95% of the clean reads for each sample were mapped to the reference genome. The percentage of uniquely mapped reads of all samples ranged from 83.7% to 92.1%. A total of 22,639–24,255 genes were expressed across the 24 samples representing two treatments (control and stimulated), two types of internodes (older: elongated and younger: elongating), two time points (2 and 26 h after initiating mechanical stimulation), and 3 replicates. A gene was considered to be expressed when its CPM (Counts per Million) was not less than 1 in at least 3 samples. A greater number of expressed genes were detected in the younger internodes compared to the older internodes. Detailed sequencing and mapping statistics are shown in Supplementary Table 1.

3.2. Identification and analysis of differentially expressed genes (DEGs)

Comparisons were conducted among sequencing samples to analyze differentially expressed genes (DEGs). According to the criteria FDR < 0.05 and $|\log_2(\text{Fold Change, FC})| > 1$, a total of 7557 DEGs were identified from all meaningful pairwise comparisons (Supplementary Table 2, 3, 4), most of which were differentially expressed by the internode developmental stage/position (younger versus older internodes). Mechanical stimulation specifically altered the expression of 918 genes, with most being induced rather than repressed (Fig. 2**a** and Supplementary Table 4). In the older internodes, more differentially expressed genes with up-regulation were detected at 2 h after initiating mechanical stimulation (AIMS) than at 26 h AIMS, but the reverse was

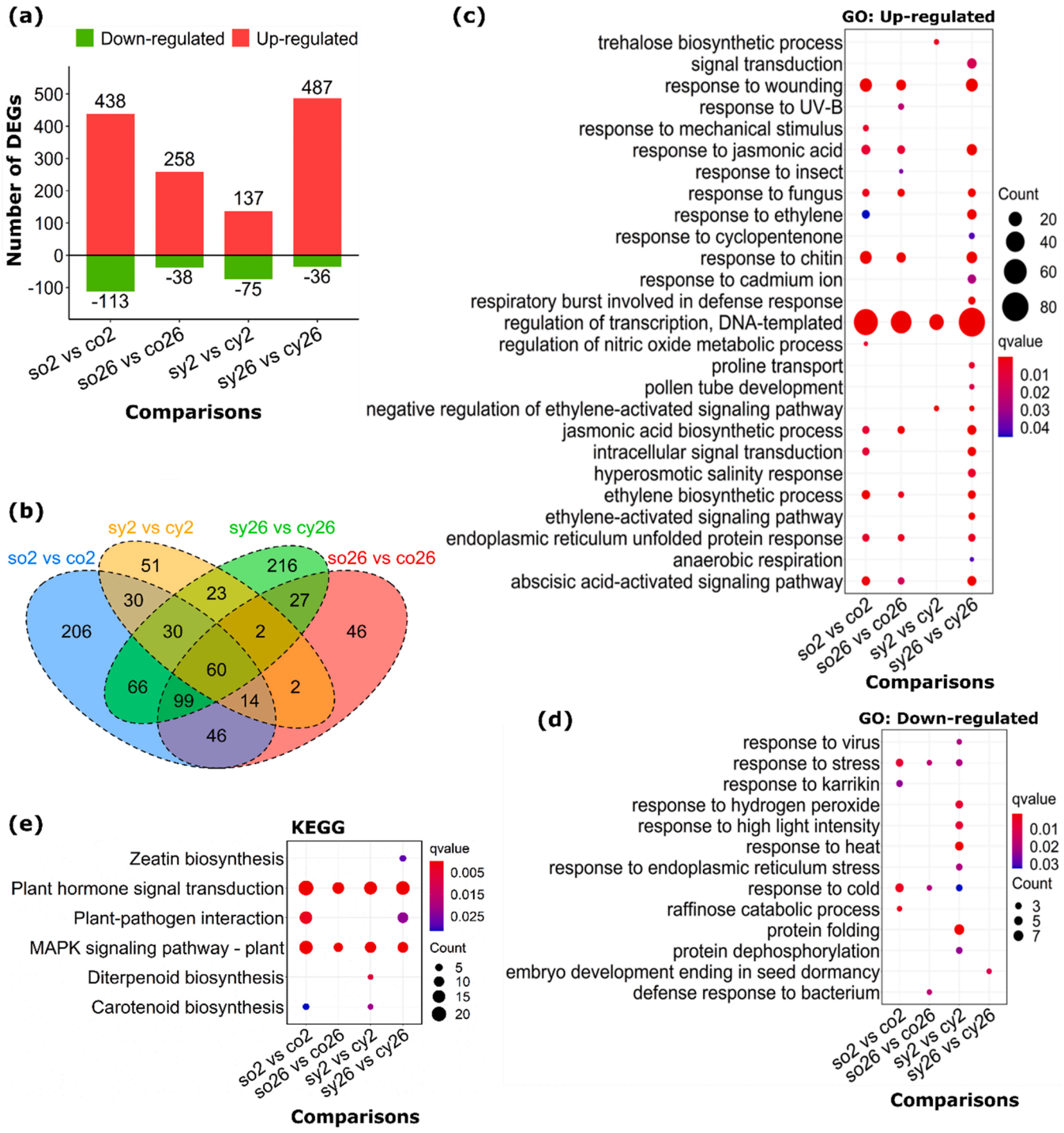

Fig. 2. Identification and analysis of differentially expressed genes (DEGs) under 4 comparisons (so2 vs co2: stimulated vs control older internodes at 2 h AIMS (after initiating mechanical stimulation); so26 vs co26: stimulated vs control older internodes at 26 h AIMS; sy2 vs cy2: stimulated vs control younger internodes at 2 h AIMS; sy26 vs cy26: stimulated vs control younger internodes at 26 h AIMS). (a) DEG statistics for all 4 comparisons. Red and green indicate up- and down-regulated genes, respectively. (b) Venn diagram displaying the distribution and overlaps of DEGs responsive to mechanical stimulation in the 4 comparisons. (c and d) Significantly enriched GO terms (biological process) of up- and down-regulated DEGs, respectively, in each comparison. (e) Significantly enriched KEGG pathways of DEGs in each comparison. High and low q values are represented by blue and red, respectively.

observed in younger internodes. Venn analysis showed that 206 DEGs, 51 DEGs, 46 DEGs and 216 DEGs were exclusively responsive to mechanical stimulation in older-2 h, younger-2 h, older-26 h and younger-26 h comparisons, respectively (Fig. 2**b**). A set of 60 common genes, which may play central roles in response to mechanical stimulation, were detected across all 4 comparisons (Fig. 2**b**, Supplementary Figure 2).

To understand and classify the function of mechanically induced DEGs in each comparison, GO (Gene Ontology) analysis was conducted for the three GO categories including Biological Process (BP), Molecular Function (MF) and Cellular Component (CC) (Supplementary Table 5), and all significantly enriched GO-BP terms were plotted (Fig. 2c, d). For up-regulated genes, the largest number of enriched GO terms were observed in younger internodes at 26 h AIMS while the smallest number

of terms were in the younger internode at 2 h AIMS (Fig. 2c). One GO term ("regulation of transcription, DNA-templated") was significantly enriched in all 4 comparisons, while 8 other terms were enriched in 3 out of 4 comparisons. Half of these terms were associated with plant hormones including JA, ethylene and ABA. In contrast, fewer numbers of enriched GO terms were detected for down-regulated genes, and there was little sharing of terms between each comparison. Only 2 terms (related to stress responses) were shared by 3 comparisons (Fig. 2d). To further identify the metabolic processes associated with mechanical stimulation of the stem, KEGG pathway analysis was also performed (Supplementary Table 5). A total of 6 significantly enriched pathways were observed. Two pathways ("plant hormone signal transduction" and "MAPK signaling pathway-plant") were commonly enriched in all 4 comparisons (Fig. 2e). Terms including "carotenoid biosynthesis", "zeatin biosynthesis" and "diterpenoid biosynthesis", which are associated with biosynthesis of ABA, cytokinins and GAs respectively, were also enriched. A large proportion of DEGs were associated with plant hormone-related GO terms and KEGG pathways, which implicates the involvement and importance of hormone signaling in regulating stem responses to mechanical stimulation. Terms and genes associated with transcriptional regulation were also prominent, emphasizing the widespread genetic reprogramming that occurred in response to the treatment.

3.3. *Analysis of common and unique DEGs across combinations of internode stages and time points*

In total, 60 common DEGs were identified in all 4 comparisons (Fig. 2b), which may represent a basic defense network in response to mechanical stimulation in both older and younger internodes experiencing both short-term and long-term stimulation. GO enrichment analysis (Fig. 3a) revealed that the common DEGs were significantly enriched in a single BP term ("regulation of transcription, DNA-templated") that was observed previously (Fig. 2c), which indicated transcriptional regulation was central to the response to mechanical stimulation. In addition, the molecular function term "calcium ion binding" was also significantly enriched (Supplementary Table 6), which implied that the calcium signaling pathway was fundamental to the thigmomorphogenesis program as has been reported previously (Chehab et al., 2009). KEGG analysis showed the enrichment of "plant-pathogen interaction" and "MAPK signaling pathway-plant" (Fig. 3b), which was consistent with the results of the individual 4 comparisons (Fig. 2e). Stimulated plants in this study did not show signs of infection by pathogens. The enrichment of genes in "plant-pathogen interaction" may indicate that they have roles in regulating thigmomorphogenesis that have not been annotated yet.

In addition to the common DEGs, each comparison had its own unique DEGs whose expression was not significantly altered in the other 3 comparisons, which may indicate specific developmental stage/time responses. Among the 4 comparisons, 206, 51, 46 and 216 DEGs were uniquely detected in older-2 h, younger-2 h, older-26 h and younger-26 h combinations, respectively (Fig. 2b). Most of the enriched GO terms of the unique DEGs were identified in the previous analyses, except for 4 new ones identified in the older internodes at 2 h AIMS related to catabolic processes of salicylic acid, lignin and phenylalanine ("salicylic acid catabolic process", "phenylpropanoid biosynthetic process", "L-phenylalanine catabolic process", "lignin catabolic process") (Fig. 3c). Similar results were observed for KEGG analysis, with only one new pathway enriched in the younger internodes at 2 h AIMS correlated with metabolism of arginine and proline (Fig. 3d and Supplementary Table 6).

3.4. *Phytohormone responses to mechanical stimulation*

The abundances of several phytohormones in the rind of older and younger internodes were modified by mechanical stimulation (Fig. 4).

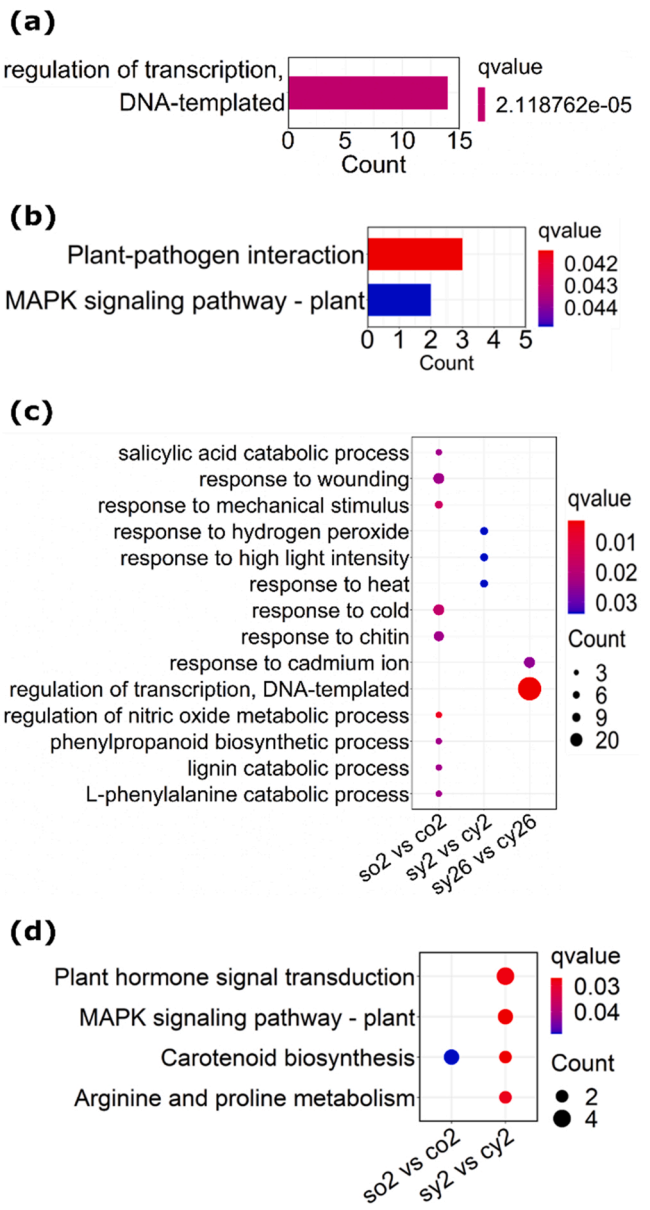

Fig. 3. Analysis of common and unique DEGs in 4 comparisons. Significantly enriched GO terms (biological process) (a) and KEGG pathways (b) of common DEGs. Significantly enriched GO terms (biological process) (c) and KEGG pathways (d) of unique DEGs in each comparison.

Control JA levels declined over the first two hours of the experiment, possibly as a result of an endogenous rhythm. Mechanical stimulation elevated JA levels relative to controls at both 2 and 26 h after initiating mechanical stimulation (AIMS), though this effect was only significant in the older internodes. The abundance of ABA was significantly reduced relative to controls following mechanical stimulation at both time points, and in both older and younger internodes. IAA levels were suppressed by mechanical stimulation in both internodes, with significant differences observed at 26 h AIMS. The abundance of bioactive GA_1 in mechanically stimulated internode rinds was similar to controls at 2 h AIMS but was reduced at 26 h AIMS in both internodes, though the reduction was only significant in the older internode. A similar trend was observed for GA_{20}, the immediate precursor to GA_1, which also declined at 26 h AIMS in both internodes. Taken together, the hormone analyses showed that JA, ABA, IAA and GA levels were altered by mechanical stimulation and could potentially be involved in thigmomorphogenic responses.

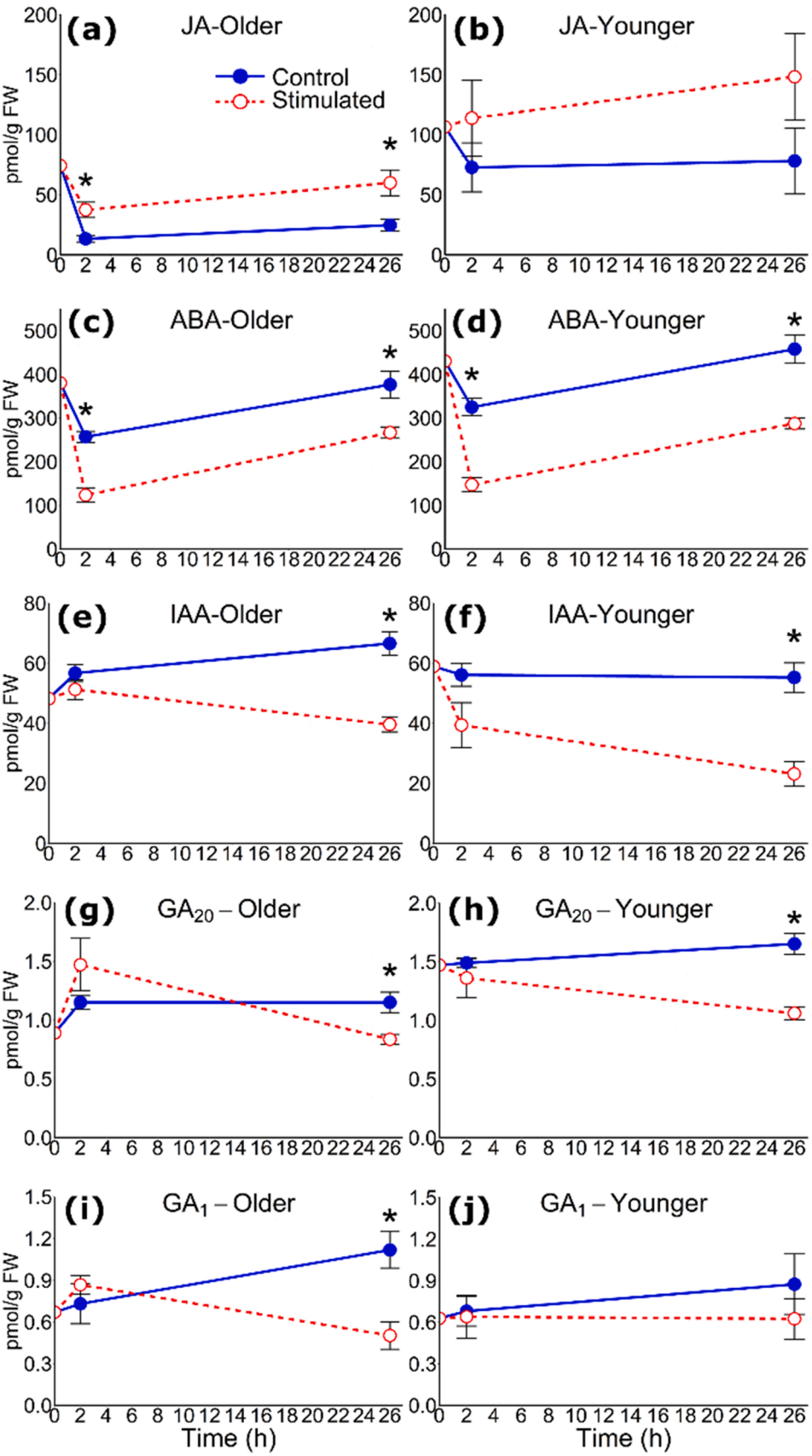

Fig. 4. Plant hormone abundances in older and younger internodes before mechanical stimulation and at 2 h and 26 h after initiating mechanical stimulation. (a) Jasmonic acid (JA), older internode; (b) JA, younger internode; (c) Abscisic acid (ABA), older internode; (d) ABA, younger internode; (e) Indole-3-acetic acid (IAA), older internode; (f) IAA, younger internode; (g) Gibberellin A_{20} (GA_{20}), older internode; (h) GA_{20}, younger internode; (i) Gibberellin A_1 (GA_1), older internode; (j) GA_1, younger internode. Data are means ± SE with n = 4. Asterisks mark significant differences (p-value < 0.05) between control and stimulated internodes at the same time point.

3.5. Construction of weighted gene co-expression network and detection of associated modules

Weighted gene co-expression network analysis (WGCNA) (Langfelder & Horvath, 2008) is a data mining method used to generate gene co-expression networks and detect clusters of genes with similar expression patterns (modules). A set of 13,866 high-variance genes from the transcriptome sequencing data, including 918 DEGs, were used as input for WGCNA. A total of 9 distinct co-expression modules (excluding the grey module containing all unassigned genes) were identified by WGCNA, each consisting of 51–5712 correlated genes (Supplementary Table 7). To identify modules significantly associated with traits of interest, module-trait relationships were also constructed (Fig. 5). The traits included treatment conditions (control/stimulation), internode developmental stages (older/younger) and hormone levels (JA, ABA, IAA, GA_{20} and GA_1) presented in this study as well as geometrical traits (length, diameter) and biomechanical properties of internodes (elastic modulus, strength and flexural stiffness) reported in a companion study (Zargar et al., 2022). All 9 modules showed significant correlation with the various traits ($|cor| > 0.5$, $p < 0.01$). Three modules were significantly associated with mechanical stimulation. The black module was positively correlated with the stimulation, which was also negatively correlated with GA_1 levels. The yellow module showed the strongest positive correlation with mechanical stimulation and was also significantly associated with many other traits including JA, ABA, IAA, length, diameter, modulus, strength and stiffness, which may indicate the critical role of the yellow module in regulating sorghum stem thigmomorphogenesis. In contrast, the red module was negatively associated with the stimulation as well as with JA abundances.

The overall gene expression trends of each module are represented by the module eigengene expression provided in Fig. 6. Among the three modules (black, yellow and red) significantly associated with stimulation, genes in the black and yellow modules showed increased expression due to mechanical stimulation, while those in the red module showed decreased expression. Therefore, the black, yellow and red modules were selected as target modules for further analysis of the mechanical stimulation response of sorghum stems.

3.6. Functional enrichment analysis for target modules

To reveal the major functions associated with the target co-expressed modules (black, red and yellow), gene ontology and KEGG pathway enrichment analyses were performed for genes in each module (Supplementary Table 8). In the black module, which was positively associated with mechanical stimulation, 8 out of 11 significantly enriched GO terms were associated with cell wall biology, including "cell wall biogenesis", "xylan biosynthetic process" and "cellulose biosynthetic process" (Fig. 7**a**), which implied an effect of mechanical stimulation on cell wall composition and organization. KEGG analysis revealed an enrichment in betalain biosynthesis (Fig. 7**a**). In the red module, which was negatively correlated with mechanical stimulation, 6 out of 9 significantly enriched GO terms with the highest fold enrichment were directly or indirectly associated with lignin metabolism, such as "lignin catabolic process", "L-phenylalanine catabolic process", "phenylpropanoid metabolic process" and "coumarin biosynthetic process" (Fig. 7**b**), which suggested an important role of lignin in response to mechanical stimulation. The red module was also enriched in two KEGG terms, "Plant hormone signal transduction" and "MAPK signaling pathway – plant" (Fig. 7**b**). These terms were also found to be conserved across the 4 comparisons in DEGs induced by mechanical stimulation (Fig. 2**e**). In the yellow module, which showed the strongest correlation with mechanical stimulation, a total of 20 significantly enriched GO

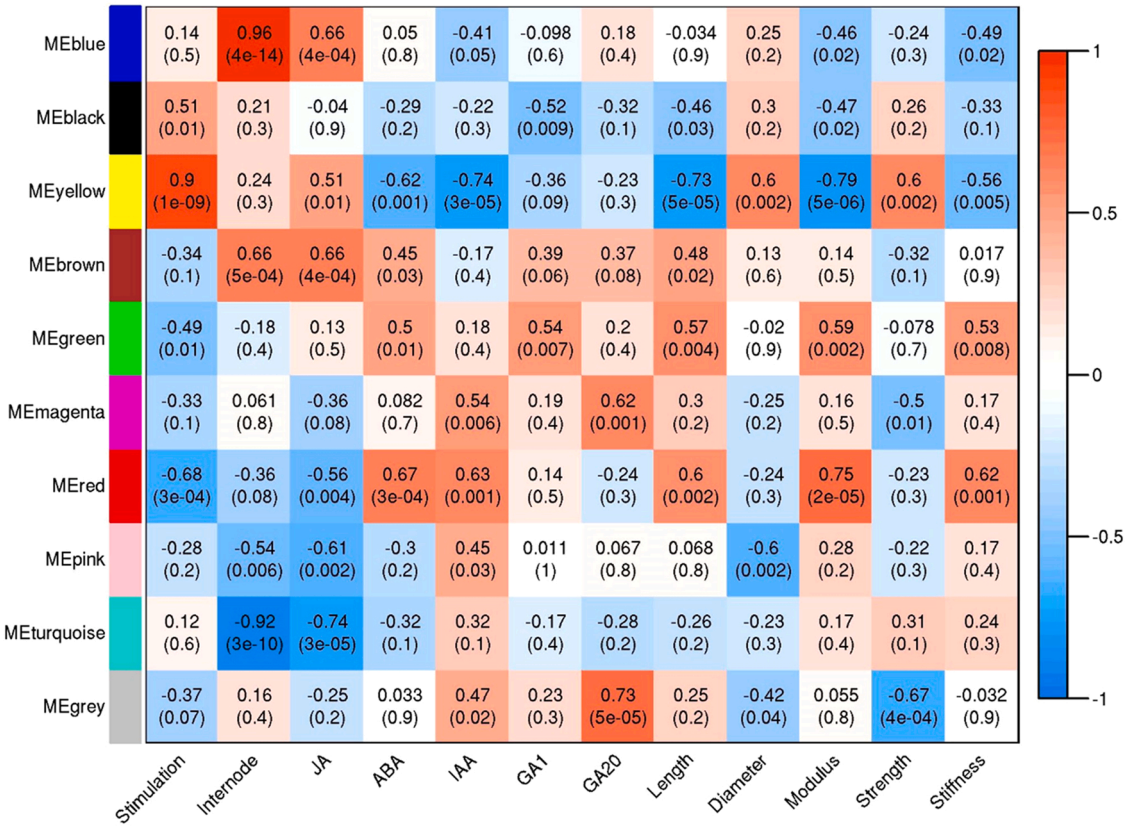

Fig. 5. The correlation of Weighted Gene Co-expression Network Analysis (WGCNA) modules with various traits. Each cell contains the corresponding Pearson correlation value and (p-value). The heat map is color-coded by correlation values according to the color legend: red represents a positive correlation, while blue represents a negative correlation.

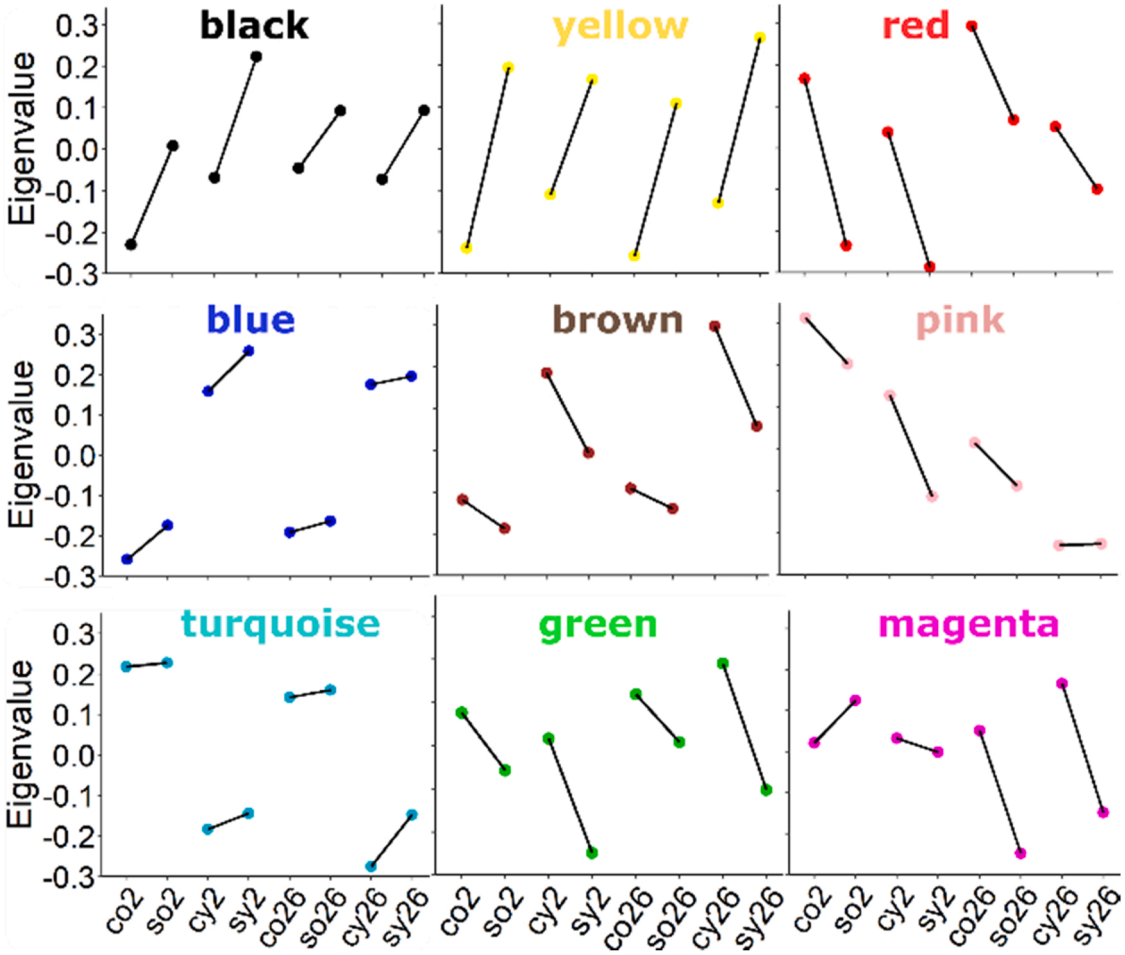

Fig. 6. The eigengene expression patterns for identified modules. c/s: control/stimulated; o/y: older/younger internode; 2/26: 2 h/26 h after initiating mechanical stimulation.

terms were identified, which were generally associated with processes related to hormones and responses to various stresses, such as "response to mechanical stimulus", "response to wounding", "jasmonic acid biosynthetic process" and "abscisic acid-activated signaling pathway" (Fig. 7**c**). In addition, the term "cell wall macromolecule catabolic process" was also detected. KEGG analysis discovered 5 pathways that were significantly enriched in the yellow module (Fig. 7**c**), including "Zeatin biosynthesis", "Plant hormone signal transduction", "plant-pathogen interaction" and "MAPK signaling pathway – plant". Both enriched GO terms and KEGG pathways in the yellow module showed considerable overlap with terms found in the DEG set (Fig. 2**c, e**), which verified the robust relationship between the yellow module and the mechanical stimulation treatment.

3.7. Identification and visualization of hub genes

Hub genes are highly and strongly interconnected nodes in a module and are considered as potential candidates for master regulatory functions associated with the module. Thus, hub genes identified in the three target modules significantly associated with mechanical stimulation are candidates for key roles in the sorghum stem response to mechanical stimulation. In the black module, 14 hub genes with the highest degree values (49−57) were detected, and their subnetwork is illustrated in Fig. 8a. The expression of all 14 genes was up-regulated in sorghum stems following mechanical stimulation (Supplementary Figure 3a). In the red module, 11 genes were determined as hub genes with degree values between 79 and 116. Two of them were annotated as bZIP transcription factors (Sobic.003G332200, Sobic.002G162800) (Fig. 8b). Except for an unknown gene (Sobic.002G248100), the rest of the 10 hub genes were down-regulated in response to mechanical stimulation (Supplementary Figure 3b). In the yellow module, 20 genes with the highest degree values (255−274) were identified as hub genes (Fig. 8c). Seven of them corresponded to known or putative transcription factors from the ERF (Sobic.002G269400, Sobic.004G283201, Sobic.002G225700, Sobic.007G077200, Sobic.007G077300), WRKY (Sobic.004G065900) and C2H2 (Sobic.001G035000) families, which were also major transcription factor families detected in the DEG analysis (Supplementary Figure 2) and have been reported to be involved in various plant stress responses (Erpen et al., 2018). All 20 hub genes were DEGs, and their expression levels were all elevated due to mechanical stimulation (Supplementary Figure 3c).

4. Discussion

4.1. Thigmomorphogenesis resulting from mechanical stimulation is associated with transcriptome reprogramming and altered hormone abundances

Mechanical stimulation of sorghum resulted in considerable transcriptional reprogramming of internode rind tissue, with biological processes connected to transcription, cell wall modification and hormone biosynthesis and signaling showing significant over-representation. These biological processes were associated with concurrent changes in the abundances of several hormones, including JA, ABA, IAA and GA_1, and with modifications in the geometrical and biomechanical properties of the internodes following mechanical

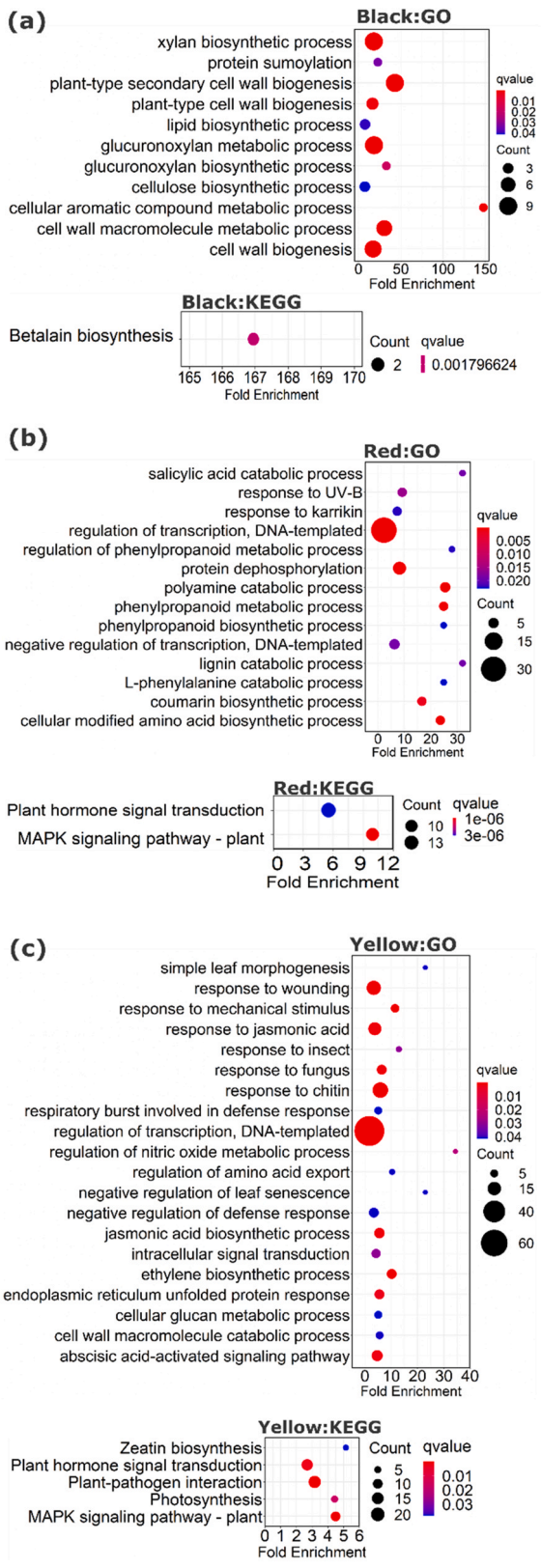

Fig. 7. Significantly enriched GO terms (biological process) and KEGG pathways in black (a), red (b) and yellow modules (c). High and low q values are represented by blue and red colors, respectively. Fold enrichment was calculated by GeneRatio (ratio of input genes that are annotated in a term) / BgRatio (ratio of all genes that are annotated in this term).

stimulation. The hormonal and transcriptional changes that arise in response to mechanical stimulation are likely the underlying drivers of these thigmomorphogenic alterations that produce plants able to withstand stronger mechanical forces (such as wind) that would otherwise cause stem lodging in unstimulated plants (Zargar et al., 2022; Lee et al., 2005; Pomiès et al., 2017; Chehab et al., 2012; Lange & Lange, 2015; Wu et al., 2020; Nam et al., 2020).

A companion study using plants from the same experiment described here investigated the geometrical and biomechanical changes in sorghum internodes resulting from 7 weeks of mechanical stimulation (Zargar et al., 2022). Mechanical stimulation reduced internode elongation, and this effect was greater in the younger (elongating) internodes than that in the older internodes which had nearly fully elongated when the treatment was begun (Zargar et al., 2022). Aside from the obvious reduction in height, plants that were mechanically stimulated for 7 weeks appeared healthy, matured normally, and did not display signs of damage in thin sections prepared from the internodes (Zargar et al., 2022). Mechanical stimulation also reduced the elastic modulus and increased the strength in both internodes and reduced flexural stiffness in the younger internodes (Zargar et al., 2022). Lower elastic modulus with higher strength indicates the tissue is easier to bend (softer) but larger force is required to break it. Thus, the thigmomorphogenic responses of sorghum to mechanical stimulation produced plants that were shorter, stronger, and more flexible. These features were previously shown to be associated with resistance to stem lodging in a diverse range of bioenergy sorghum cultivars (Gomez et al., 2018). The present study extends those findings by providing evidence for hormonal and transcriptional changes induced by mechanical stimulation that produces stem modifications expected to improve lodging resistance in dynamic environments.

4.2. *Mechanical stimulation alters hormone abundances and signaling*

DEGs were enriched in GO terms associated with plant hormones including JA, ABA and ethylene, and KEGG analysis showed that the term “Plant hormone signal transduction pathway” was enriched in all four comparisons. Mechanical stimulation increased JA levels in sorghum internode rind tissue, as has been reported in many other species including *Medicago truncatula* and Arabidopsis (Tretner et al., 2008; Chehab et al., 2012). Homologs of several genes involved in JA biosynthesis, including *AOS* (Sobic.004G093200, Sobic.001G077400), *LOX1* (Sobic.003G385900) and *OPR2* (Sobic.010G084300) were induced by mechanical stimulation, providing a potential mechanism for the elevated JA levels observed. JA is necessary for the reduced growth of Arabidopsis following wounding, since the growth of JA deficient Arabidopsis mutants was not impacted by wounding (Zhang & Turner, 2008). Arabidopsis mutants deficient in JA biosynthesis or signaling were also used to show that JA is required for typical touch-induced thigmomorphogenesis, while transgenic JA overproducers displayed a constitutive thigmomorphogenic phenotype (Chehab et al., 2012). The JA-induced reduction of growth has been investigated in some detail. Arabidopsis *CYCB* reporter expression was inhibited by both wounding and JA, suggesting that JA acts to inhibit mitosis (Zhang & Turner, 2008), and JA also promotes investment in defense responses at the expense of growth (Guo et al., 2018). Thus, JA appears to act as a central organizing molecule to prepare plants receiving mechanical stimulation for future stresses.

The abundance of the natural auxin IAA declined in internodes of mechanically stimulated sorghum. Sorghum internode elongation is dependent on the sufficient supply of auxin in the polar auxin transport stream, as evidenced by the dwarf phenotype of *dw3* mutants deficient in a MDR auxin transporter (Multani et al., 2003). Therefore, a reduction in IAA levels may result in decreased internode lengths reported previously (Zargar et al., 2022). There is little reliable prior information concerning the potential role of auxin in thigmomorphogenesis. Although adventitious root formation induced by wind-driven mechanical stimulation

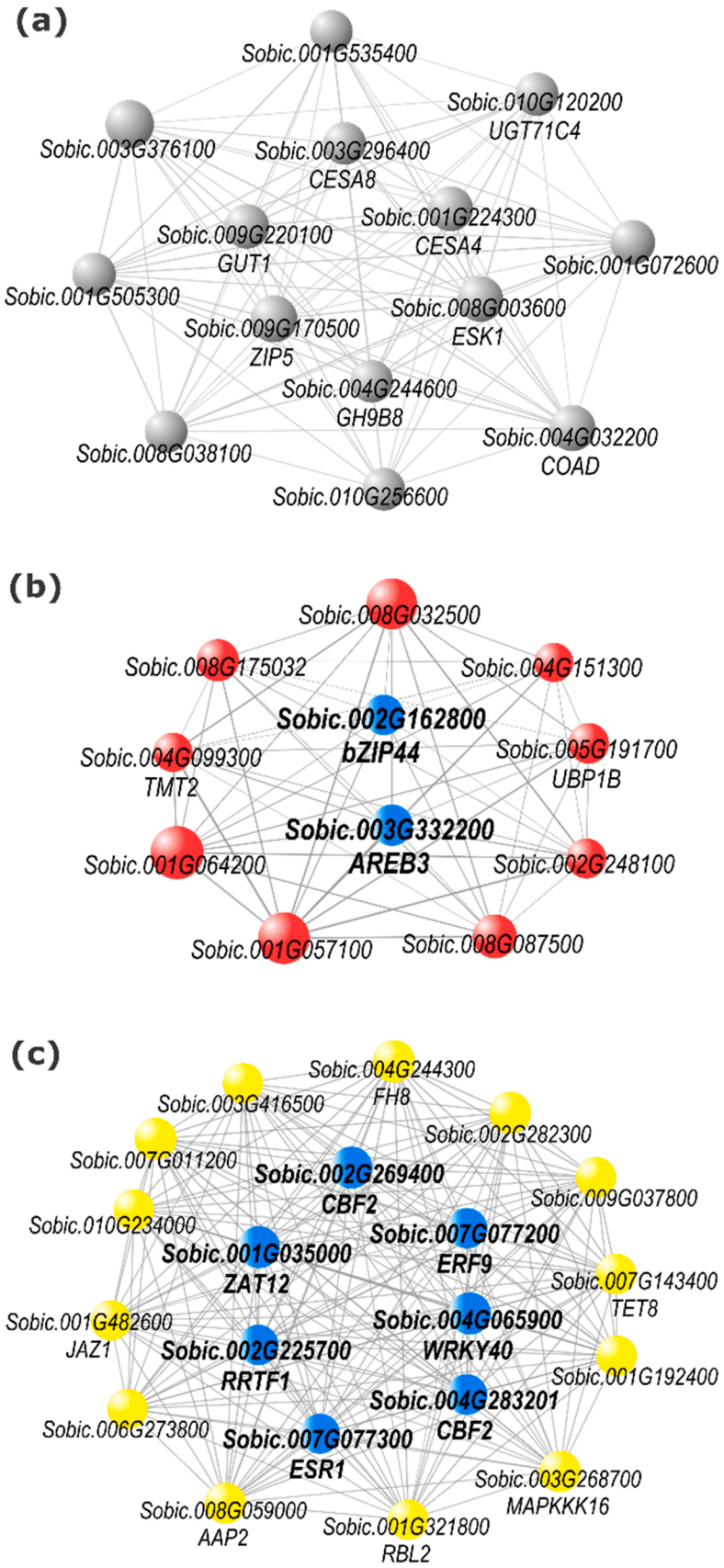

Fig. 8. Network visualization and expression profiles of hub genes in black (a), red (b) and yellow (c) modules. Hub genes encoding transcription factors are shown in bold. The gene symbols of Arabidopsis homologs of hub genes are annotated under the corresponding sorghum gene ID, if available.

was associated with auxin signaling in *Brachypodium distachyon*, the effect was attributed to soil contact with the stem but not stem bending stresses (Nam et al., 2020). In addition to altering auxin abundance, mechanical stimulation also induced the expression of the homolog (Sobic.003G319200) of Arabidopsis *PBP1* more than 8-fold in younger internodes at 26 h AIMS. The Arabidopsis PBP1 protein interacts with PINOID to negatively regulate auxin signaling (Christensen et al., 2000; Benjamins et al., 2003), and thus could contribute to the reduced elongation observed with bending. In view of the general importance of auxin in regulating plant development, further investigation of the role of IAA in thigmomorphogenesis seems warranted.

Mechanical stimulation of sorghum resulted in significantly decreased accumulation of bioactive GA_1 in older internode rind tissue, and of its immediate precursor, GA_{20} in both internodes. Both IAA and GAs are well known to play important roles in regulating stem elongation (Kou et al., 2021). An early study provided evidence that mechanical perturbation of *Phaseolus vulgaris* decreased stem elongation and bioactive GA levels (Suge, 1978). More recent work defined a role for GAs in the touch response of Arabidopsis (Lange & Lange, 2015). Repetitive touching reduced the height of the Arabidopsis bolt and also reduced the abundances of several GAs, including bioactive GA_4. The authors suggested that GA catabolism via the *GA2OX7* gene was involved, since its expression was induced by touch, and loss of function prevented touch-induced reductions in bolt length and the decline in GA_4 abundance (Lange & Lange, 2015). In the present study, two homologs of Arabidopsis *GA2OX* (Sobic.009G077500, Sobic.003G300800) were induced by mechanical stimulation, potentially providing a similar mechanism for reduced bioactive GA levels. The reduced total height of sorghum plants resulting from mechanical stimulation reported previously (Zargar et al., 2022) might be mediated by decreased IAA and GA_1 levels, in addition to elevated JA.

ABA levels declined in both younger and older internodes at both time points following mechanical stimulation. The expression of a homolog of the ABA catabolism gene *CYP707A1* (Sobic.004G268700) was induced in older internodes at 2 h after initiating the bending treatment, potentially accounting for the decreased abundance. Additionally, homologs of *PYL5* (Sobic.009G170700, Sobic.001G403300), putative ABA receptors, were up-regulated following mechanical stimulation. Increased expression of these potential receptors could reflect a feedback response to the observed reduction in ABA levels after mechanical stimulation. Although there are previous reports of altered ABA levels in plants in response to mechanical stimulation (Jeong & Ota, 1980; Erner & Jaffe, 1982), these studies reported increased ABA accumulation following mechanical stimulation, whereas in the current case ABA levels declined. The discrepancy may be related to the duration of stimulation, which continued for 10–30 days in the earlier reports. A study in rice suggested that inhibition of ABA-biosynthesis led to increased JA levels, indicating an antagonism between ABA and JA, which may account for the reduction of ABA to some extent (Kyndt et al., 2017).

Ethylene levels were not measured in the current study due to logistical issues; however, the transcriptome data provided evidence for an association between ethylene and mechanical stimulation, as has been reported previously (Brown & Leopold, 1973; Biro & Jaffe, 1984; Botella et al., 1995). A recent report provided evidence that ethylene signaling induced by mechanical stimulation in Arabidopsis suppressed expression of the pectin modifying gene *PGX3*, resulting in increased cell wall modulus (Wu et al., 2020). While a sorghum homolog of *PGX3* did show a numerical decline with mechanical stimulation, the effect did not meet the significance criteria of the FDR, and the potential role of this pathway remains unresolved.

4.3. Key biological processes and hub genes associated with thigmomorphogenesis

WGCNA identified a total of 9 modules, 3 of which (black, red, yellow) were significantly correlated with mechanical stimulation and may be involved in regulating thigmomorphogenesis of the sorghum stem (Fig. 5). A deeper understanding of how mechanical stimulation produces thigmomorphogenic outcomes may be realized by investigating the composition of these modules.

The black module was enriched in GO terms mostly associated with the cell wall, such as “cell wall biogenesis”, and terms associated with hemicellulose (xylan, glucuronoxylan) and cellulose. Eigengene expression of the black module tended to increase following mechanical stimulation, possibly indicating cell-wall remodeling based on the enhancement of cell wall macromolecules in response to mechanical stimulation. Hub genes detected in the black module included cell-wall related genes such as homologs of *CESA4* (Sobic.001G224300), *CESA8* (Sobic.003G296400), *GUT1/IRX10* (Sobic.009G220100) and *ESK1* (Sobic.008G003600) (Fig. 8a). CESA4 and CESA8 are cellulose synthases, while GUT1 is likely involved in hemicellulose (glucuronoxylan) biosynthesis. A previous study reported that loss of CESA4 function in rice decreased the levels of cellulose, hemicellulose and lignin and resulted in weaker culms (Ma et al., 2021). Arabidopsis GUT1 is required to maintain normal xylan content and normal secondary cell wall structure (Brown et al., 2009; Wu et al., 2009), and together with ESK1 functions to form acetylated xylan backbones (Urbanowicz et al., 2014).

Eigengene expression in the red module was suppressed by mechanical stimulation. The red module was positively correlated with elastic modulus and stiffness indicating potential roles for module members in regulating these biomechanical traits. Several GO terms related to lignin were overrepresented in the red module, including “lignin catabolic process” and “L-phenylalanine catabolic process”. Down-regulation of genes including these terms could reflect the suppression of processes that would otherwise reduce lignin accumulation, which was consistent with the observation of increased lignin content in mechanically stimulated internodes of sorghum (Zargar et al., 2022). The increased lignin is likely to impact internode biomechanical properties, perhaps including the correlated elastic modulus and flexural stiffness traits. Although no hub genes related to the lignin metabolic process were identified in the red module, hub genes known to be associated with stress responses were detected, such as *UBP1B* (Sobic.005G191700) and *bZIP44* (Sobic.002G162800) (Nguyen et al., 2016; Weltmeier et al., 2009). The red module was also positively correlated with internode length and IAA abundance, but negatively correlated with JA levels. Genes in this module may function to promote internode elongation in response to IAA in the absence of mechanical stimulation and to repress elongation as IAA levels decline and JA levels rise following the onset of mechanical stimulation.

Among all modules, the yellow module had the strongest positive correlation with mechanical stimulation, showed increased eigengene expression with mechanical stimulation, and contained the most DEGs. This module was positively correlated with internode diameter and strength, and negatively correlated with internode length, elastic modulus and stiffness, indicating that genes in this module might influence these traits. Three DEGs annotated with the term “Cellular glucan metabolic process” encoding xyloglucan endo-transglycosylases were members of the yellow module, and might play a role in modifying existing cell wall hemicelluloses (Stratilová et al., 2020). Hormone-related terms including ethylene, JA and ABA were overrepresented in the yellow module, and the yellow module was positively correlated with JA abundance and negatively correlated with IAA, supporting a critical role of hormones in thigmomorphogenesis. Both mechanical stimulation and wounding terms were enriched in the yellow module. The plants did not show obvious signs of damage from the bending treatment, which suggests that the distinction between the two terms is not clear cut. Among the 20 hub genes in the yellow module, seven encoded transcription factors which were also highly differentially expressed following mechanical stimulation, including *ZAT12* (Sobic.001G035000), *WRKY40* (Sobic.004G065900), *CBF2* (Sobic.002G269400, Sobic.004G283201), *RRTF1* (Sobic.002G225700),

ERF9 (Sobic.007G077200) and *ESR1* (Sobic.007G077300). Some of these genes have been associated with mechanical stimulation in other species, including *WRKY40*, *CBF2*, and *ZAT12* (Lee et al., 2005, Martin et al., 2014, Cazzonelli et al., 2014). Interestingly, a close homolog of *ZAT12* in poplar functions in repressing stem elongation and regulating biomechanical properties of the poplar stem (*PtaZFP2*, Martin et al., 2014). Given the previous reports and discoveries reported here, the seven transcription factor-encoding genes might play roles in regulating sorghum stem thigmomorphogenesis, which could be useful for future studies in thigmomorphogenesis and stem lodging in sorghum and other crops.

4.4. The sorghum stem response to mechanical stimulation is age- and dose-dependent

Various studies have shown that plant responses to mechanical stimulation can be quite diverse (Braam, 2005). Even within the same species, the effect of mechanical stimulation may vary depending on the developmental stage of the tissue when the stimulus is initiated. In the case of mechanically stimulated sorghum, at maturity the younger internode showed a larger reduction in length than the older internode, as well as a more pronounced increase in lignin content (Zargar et al., 2022). These differences likely reflect the differential and cumulative effects of the transcriptional and hormonal changes affecting internode development that occurred over the long duration of the treatment (7 weeks). Mechanical stimulation also reduced the elastic modulus of the younger internode by ~53%, while the reduction in the older internode was only ~24%. Younger tissues usually show greater sensitivity to mechanical stimulation than older tissues, in some cases even if they were not stimulated directly. In beans, the length reduction of the 4th internode due to rubbing the 1st internode was much larger in younger plants than that in older plants (Jaffe, 1976), an example of an age-dependent effect. The youngest leaf of cauliflower showed the greatest decrease in leaf weight following mechanical stimulation by brushing (Biddington et al, 1985). Two-week-old wheat plants also displayed a more pronounced response to mechanical stimulation than 4- and 6-week-old plants, including a larger reduction in plant height (Hindhaugh et al., 2021). It is possible that younger tissues are more tender and susceptible to external stresses and thus must respond robustly to survive harsh environmental perturbations (Chehab et al., 2009). Another non-exclusive possibility is that the younger, actively growing tissues show an enhanced response (Zargar et al., 2022) because their cells are less differentiated at the time of stimulation, providing additional developmental plasticity. The detection of 298 uniquely responsive genes in the older internode and 290 uniquely responsive genes in the younger internode indicated developmental stage-specific responses that could underly the differences in the geometrical and biomechanical outcomes in these tissues (Fig. 2b).

The impact of mechanical stimulation is known to vary with the duration of mechanical stimulation, which may be considered as a dose-dependent response. Short-term brushing treatment (18 days) did not affect the total fruit number or weight in 7 varieties of tomato, however long-term treatment (28 days) reduced the total weight of fruits in 2 out of the 7 lines tested (Johjima et al., 1992). In tobacco, mechanical stress applied within a 30-minute window led to substantial deformation of young leaves, while the same amount of stress applied over several hours produced less deformation (Sahaf & Sharon, 2016). In the current study, internode rind samples harvested at 2 h AIMS had experienced a single 2-hour repetitive bending treatment (short-term) while samples harvested at 26 h AIMS had experienced a total of 8 h of repetitive bending (long-term) (Fig. 1b). Two hours of mechanical stimulation caused IAA levels to decrease by ~20%, while IAA declined to ~48% at 26 h AIMS. Similarly, GA_1 and GA_{20} levels decreased more at 26 h AIMS than at the early time point. More differentially expressed genes were detected at 2 h AIMS compared with 26 h AIMS in the older internode, suggesting a reduction of the transcriptional response with additional bending, but the reverse was observed in the younger internode (Fig. 2a). The geometry of the applied stimulation provided greater stresses to the lower, older internodes compared to the higher, younger internodes, which might explain the greater number of DEGs in the older internodes at 2 h. The reduced number of DEGs in the older internodes compared to the younger internodes at 26 h could reflect feedback inhibition resulting from the elevated response at 2 h, and/or might result from an enhanced systemic signal contribution to the younger internodes from more basal parts of the plant. Additional research is required to test these hypotheses.

A total of 287 unique DEGs were detected at 2 h AIMS, while 289 unique DEGs were observed at 26 h AIMS. Although the numbers of unique DEGs were remarkably similar, there were major differences in the enriched GO terms between them (Supplementary Table 9). Unique DEGs at 26 h AIMS were only enriched in 2 GO terms with "regulation of transcription, DNA-templated" most prominent, while unique DEGs at 2 h AIMS were enriched in 17 GO terms that were mostly related to response to various stresses, supporting dose-dependent transcriptional reprogramming of the sorghum stem with mechanical stimulation. In summary, the developmental stage- and dose-dependent changes in the stem likely reflect the complexity of the plastic response of sorghum to adapt to a wide range of mechanical stresses.

5. Conclusions

Exposure of the sorghum stem to repetitive mechanical stimulation altered JA, IAA, ABA, and GA hormone homeostasis and transcriptome expression. In accordance with previously discovered roles of IAA and GA_1 in promoting stem elongation and JA in promoting thigmomorphogenesis, the reduction in IAA and GA_1 levels and the increase in JA may account for the shorter height of mechanically stimulated sorghum plants. Unexpectedly, ABA declined following mechanical stimulation. Antagonism between ABA and JA homeostasis has been reported before, which may also function in sorghum stem thigmomorphogenesis. The transcriptome data provided evidence for the enrichment of transcriptional regulation and hormone signaling-related biological processes associated with mechanical stimulation and also discovered terms related to cell wall remodeling, including terms associated with lignins, cellulose and xylans. Modifications in these processes at the gene expression level may alter cell wall properties that in turn modify stem biomechanical properties to better adapt to mechanical forces without failing. Further compositional analysis might help elucidate the roles of lignins, cellulose and xylans in this process. Developing a more thorough understanding of the function of responsive transcription factors could provide insights into how manipulation of the underlying networks may promote lodging resistance.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

RNA-Seq data have been deposited with the link to BioProject accession number PRJNA842061 in the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA842061). Other data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plantsci.2022.111555.

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