

Transcriptome analysis revealed mechanisms involved in improved germination and growth of sugarcane by ultrasonic treatment

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ABSTRACT

Ultrasonic treatment within a suitable range often has positive effects on seed germination and plant growth. In the present study, three sugarcane varieties, ROC22 (V1), LC05-136 (V2), and YT93-159 (V3), were treated with ultrasound waves with a mixed frequency of 20–40 kHz for 1, 2, and 5 min. Moreover, the changes in the transcriptome of the sugarcane variety ROC22 with and without ultrasonic treatment at 0, 6, and 12 days of germination were studied. The results showed that suitable ultrasonic treatment substantially increased the length, diameter, and germination rate of sugarcane buds and significantly improved the antioxidant enzyme activities of sugarcane to prevent oxidative burst of reactive oxygen species (ROS) from damaging cell membranes. A total of 2756 differentially expressed unigenes (DEGs) (1395 upregulated and 1361 downregulated) were identified in the day 0 comparison; a total of 831 DEGs (408 upregulated and 423 downregulated) were identified in the day 6 comparison, and 5987 DEGs (1862 upregulated and 4125 downregulated) were identified in the day 12 comparison. Furthermore, ultrasonic treatment significantly affected the hormone synthesis pathways, reduced the contents of auxin (IAA), abscisic acid (ABA), and jasmonic acid (JA), and increased the ratio of IAA/ABA to promote germination. The gene expression of gibberellin (GA) synthesis was upregulated, whereas the gene expression of ABA synthesis was downregulated, resulting in improved germination of sugarcane buds. Overall, the optimal exposure time for ultrasonic treatment differed among the varieties; however, an appropriate ultrasonic treatment could regulate the antioxidant system and hormone synthesis pathways, thereby promoting the germination of sugarcane. This study provides a theoretical basis and technical support for ultrasonic treatment employed to promote sugarcane germination and growth.

1. Introduction

All living things are capable of sensing and responding to physical stimuli (Telewski, 2006). Many creatures routinely use sound waves to locate or communicate (Gagliano et al., 2012a). Similarly, important processes such as plant growth, reproduction, and evolution are all affected by the physical environment (Gagliano et al., 2012b; Telewski, 2006). Studies have reported that plants can exchange information concerning root growth and development (Baluska et al., 2010; Ciszak et al., 2012). Therefore, the use of ultrasound to regulate plant

physiological and biochemical metabolism has received attention as a rapid and reagent-less physical method (Gallo et al., 2018; Rokhina et al., 2009). Research has shown that low-intensity ultrasound promotes cell proliferation in *Saccharomyces cerevisiae* (Wang et al., 2003), whereas ultrasonic treatment can effectively improve *Agrobacterium*-mediated transformation efficiency in flax (Beranova et al., 2008). Ultrasound was employed to regulate the differentiation of protocorm-like bodies during asexual reproduction of *Dendrobium* species (Wei et al., 2012). Additionally, Ananthakrishnan et al. (2007) showed that ultrasound can be used as a physical stimulus to manipulate

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the growth of plant cells and organs.

Moreover, ultrasonic seed treatment is a safe, inexpensive, convenient, and environmentally friendly physical technology that has had positive impacts on the germination and growth of dormant seeds as well as on plant development and metabolism (Huang et al., 2020; Yang et al., 2015). Nazari et al. (2014) reported that ultrasonic waves had significant potential to break seed dormancy; however, there was no linear correlation between the duration of ultrasonic treatment and the characteristics of seed growth and development. Liu et al. (2016) indicated that the germination of aged seeds and the growth of seedlings were promoted by ultrasonic treatment; this provided basic evidence for the treatment of aged grass seeds by ultrasound. Previous studies indicated that ultrasonic treatment affected the germination of the seeds of common beans by increasing the cotyledon cell area (Lahijanian and Nazari, 2017), whereas changing the frequency and time of ultrasonic treatment is able to regulate the dynamics of water uptake during barley germination (Abramov et al., 2019). Moreover, the germination rate of *Dioscorea* seeds was effectively improved under the action of ultrasonic waves, and the growth rate of seedlings was also increased (Andriamanparany and Buerkert, 2019). Recently, Wang et al. (2020a) demonstrated that ultrasonic treatment enhanced antioxidant enzyme activity as well as the contents of reducing sugars and flavonoids of *Tartary* buckwheat seeds, thereby promoting their germination and growth. Most of the previous studies have focused on the phenotypic and physiological effects of ultrasonic treatment in the seed germination process; nevertheless, the molecular mechanisms of ultrasonic treatment-induced improvements in seed germination have not been explored.

Sugarcane (*Saccharum* spp. hybrids), a tropical and subtropical crop, is the raw material for industrial sugar production and for the extraction of ethanol as an energy substitute (Cavalett et al., 2012; Liu et al., 2018). The sugarcane industry in China contributes to poverty alleviation, and more than 100 tropical countries rely on sugarcane to boost their economies (Deng et al., 2021; Ou et al., 2013). It has been reported before that ultrasonic treatment promotes the germination and growth of different crop seeds; however, the effects of ultrasonic treatment on the germination and growth of sugarcane have rarely been studied. Therefore, the present study aims to determine the changes in germination, growth, and physiological properties of different varieties of sugarcane under different ultrasound levels. Moreover, the molecular mechanism by which ultrasonic treatment promotes sugarcane germination is clarified through transcriptome sequencing.

2. Materials and methods

2.1. Experimental site and materials

The sugarcane germination test was conducted at the Sugarcane Laboratory of South China Agricultural University (SCAU), Guangzhou, Guangdong Province, China on January 30, 2021. Three sugarcane varieties (V), i.e., ROC22 (V1), LC05–136 (V2), and YT93–159 (V3), were obtained from the sugarcane test base of SCAU. To ensure the reliability of the test results, full, fresh, and uniform sugarcane buds (single buds) were selected as experimental materials before ultrasonic treatment, and the sugarcane buds were soaked and sterilized with carbendazim.

2.2. Experimental treatments

The ultrasonic (20–40 kHz mixed frequency) treatments were designed at four levels, namely no ultrasonic treatment (T0), ultrasonic treatment for 1 min (T1), ultrasonic treatment for 2 min (T2), and ultrasonic treatment for 5 min (T3), each processing 100 single sugarcane buds. The sugarcane buds were ultrasonically processed using a Plant Seed Production Increase Processor (JD-1 L, Guangzhou Golden Rice Agricultural Science & Technology Co., Ltd., Guangzhou, China). The processed sugarcane buds were placed into a plastic basin, and then, a

standard germination experiment was carried out in a lighting incubator (PGX-280A-3 H, Ningbo Life Instruments, China), lasting 12 days. The culture conditions were set as follows: 28 °C temperature, 75% relative humidity, 12:12 (L:D) photoperiod, and 3000 lx light intensity. In addition, an appropriate amount of water as per requirement was added to the plastic basin every day.

2.3. Plant sampling

For each treatment, 10 buds were randomly selected on days 0, 3, 6, 9, and 12 of the sugarcane germination, and the buds were placed in the light incubator was recorded as 0 days. The buds from the sugarcane were cut with an alcohol-sterilized knife, placed in a plastic bag, and stored at –80 °C until biochemical analysis (Deng et al., 2020).

2.4. Biochemical assays

Sugarcane buds were ground into powder with a pestle and mortar in an ice bath. Fresh samples (0.2 g) were added to 6 mL of 0.05 mol·L⁻¹ sodium phosphate buffer (PBS, pH 7.8), centrifuged at 8000 rpm for 20 min at 4 °C, and the supernatant was used to determine antioxidant enzyme activities, i.e., superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), as well as malondialdehyde (MDA) and soluble protein content using methods described previously (Ashraf et al., 2020; Ashraf and Tang, 2017; Li et al., 2021). The determination of superoxide anion (O²⁻) content was performed according to Mohammadi and Karr (2001).

In addition, the length (base to top) of 10 sugarcane buds was randomly measured at 3, 6, 9, and 12 days after germination. On the 12th day, the number of sugarcane buds in each treatment was counted to calculate the germination rate, and the diameter (middle) of the sugarcane buds in each treatment was measured at the same time.

2.5. Transcriptome sequencing and data processing

The transcriptome analysis comprised three groups, an untreated control (CK) and ultrasonic treatment of sugarcane variety ROC22 for 2 min at 0, 6, and 12 days, denoted as ROC22_CK_0, ROC22_2 min 0, ROC22_CK 6, ROC22_2 min 6, ROC22_CK 12, and ROC22_2 min 12. There were three biological replicates for each treatment, for a total of 18 samples, and each sample weighed about 0.5 g. Total RNA from sugarcane samples was extracted using a mirVana miRNA Isolation Kit (R4154, Magen, Guangzhou, China). The integrity of the RNA was then assessed by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The constructed library was sequenced using an Illumina HiSeqTM 2500 sequencer to generate 125 bp or 150 bp of paired-end data. The transcriptome sequencing and analysis were conducted by OE Biotech Co., Ltd. (Shanghai, China). The relevant steps included Unigene functional annotation, Unigene quantification, differential Unigene screening, functional enrichment, and cluster analysis.

2.6. Validation of RNA-Seq data by real-time quantitative PCR

Real-time quantitative PCR (RT-qPCR) was performed to validate the RNA-Seq results for 14 gene transcripts (Table S1). Purified RNA was reverse transcribed to synthesize cDNA, according to the instructions of a HiScript III RT SuperMix for qPCR (+gDNA wiper) Nanjing Novartis Reverse Transcription Kit, and then diluted 10 times for later use. For the primer design, please refer Wang et al. (2020c). The *GAPDH* gene was used as the internal reference gene (Table S1). The primers were synthesized by Bioengineering (Shanghai) Co., Ltd. According to the reaction system from the Nanjing Novartis ChamQ Universal SYBR qPCR Master Mix manual, real-time fluorescence quantification (CFX96, Bio-Rad) was used for the amplification reaction. The amplification cycling program was as follows: 180 s at 95 °C, followed by 35 cycles of 95 °C for 10 s, 57 °C for 30 s, and finally 65–95 °C to prepare the melt

curve. Three replicates were set for each sample, and the relative expression level of 14 genes was determined using the instrument's analysis software.

2.7. Measurement of hormone content

The auxin (IAA), abscisic acid (ABA), and jasmonic acid (JA)

contents in fresh bud tissues of CK and after ultrasonic treatment for 2 min for sugarcane variety ROC22 at 0, 6, and 12 days were measured. The hormone extraction process was as follows: the sample (0.5 g) was put into a 10-mL centrifuge tube, and 5 mL of an isopropanol: water: formic acid (80:19:1, V/V/V) mixture was added, and the mixture was oscillated for 30 s with an ultrasonic cleaner (YJ25-12DT, Zhejiang, China) at room temperature and then sonicated for 15 min to assist

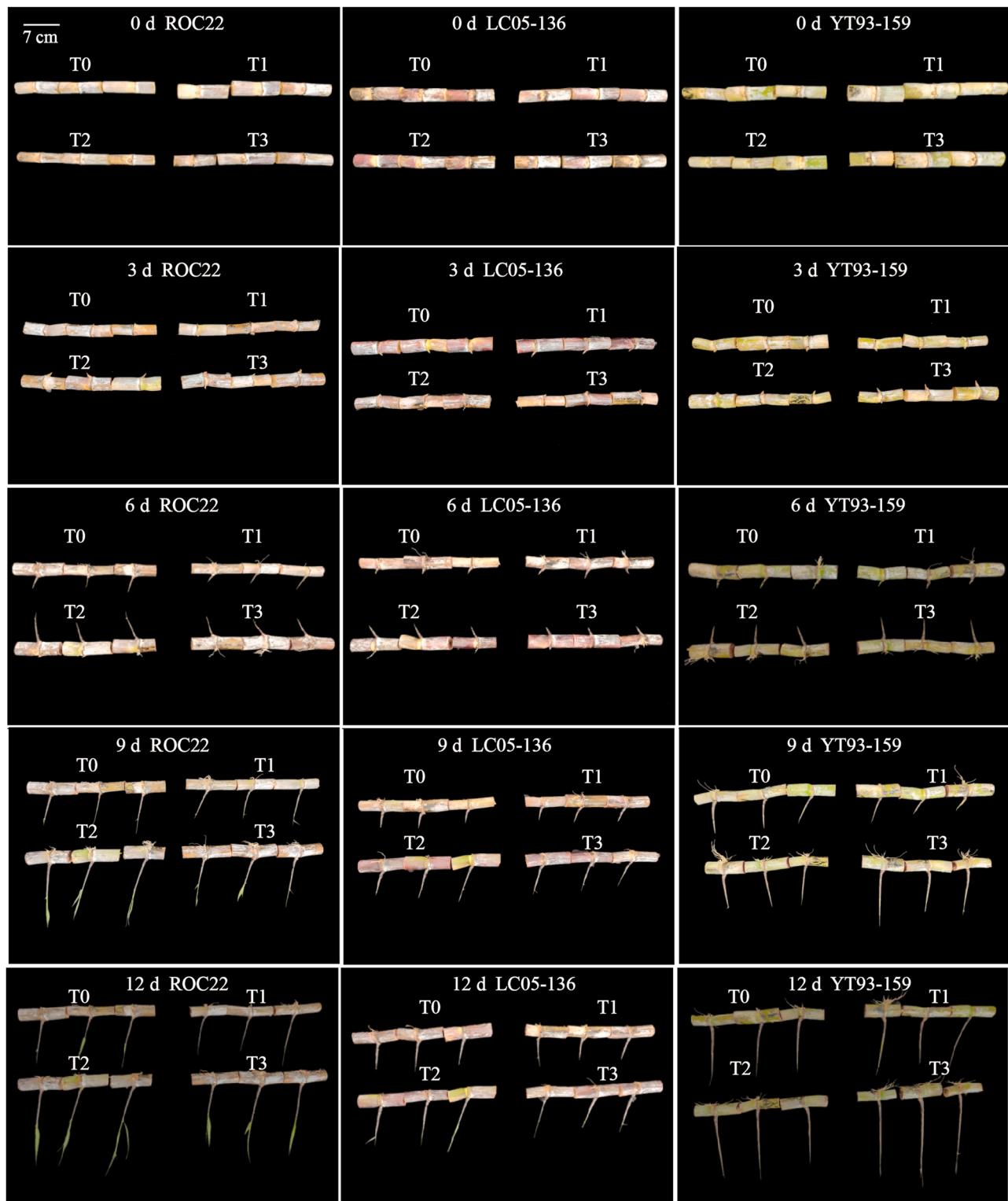


Fig. 1. Germination of sugarcane treated with and without ultrasound. The numbers 0, 3, 6, 9, and 12 represent the number of days after germination of sugarcane. T, treatment. The line segment is a scale bar.

extraction. In addition, dichloromethane (1 mL) was added to the centrifuge tube; the sample was again sonicated for 15 min under the same conditions and then centrifuged at 9000 rpm and 4 °C for 10 min, and the supernatant was transferred to a 10 mL centrifuge tube. Subsequently, the supernatant was concentrated by centrifugation with a vacuum centrifugal concentrator (6 K ES, VIRTIS, USA) under normal temperature conditions and reconstituted with 300 µL of methanol. Finally, the reconstituted solution was passed through a 0.22 µm nylon needle filter to obtain the hormone test solution. Enzyme-linked immunosorbent assay (ELISA) was used to determine the contents of IAA, ABA, and JA in sugarcane buds, and the kits used were purchased from Shanghai Enzyme-Linked Biotechnology Co., Ltd.

2.8. Sugarcane germination in the environment of exogenous hormones

To further understand the effects of f hormones on sugarcane germination, six treatments were designed: B0 (no hormone, as a control), B1 (low IAA/ABA ratio, i.e. 5 mg IAA and 15 mg ABA/L), B2 (high IAA/ABA ratio, i.e. 15 mg IAA and 5 mg ABA/L), B3 (appropriate concentration JA, i.e. 2 mg/L), B4 (high IAA/ABA ratio and appropriate JA concentration, i.e. 15 mg IAA, 5 mg ABA, and 2 mg JA/L), and B5 (ultrasonic treatment for 2 min, no hormone). IAA, ABA, and JA were dissolved in distilled water to produce hormone solutions. Three repetitions each treatment. Sugarcane buds (single buds) of ROC22 were germinated in plastic pots to which 500 mL of different hormone solutions were added (500 mL of distilled water for B0 and B5). The germination conditions of sugarcane were as described above.

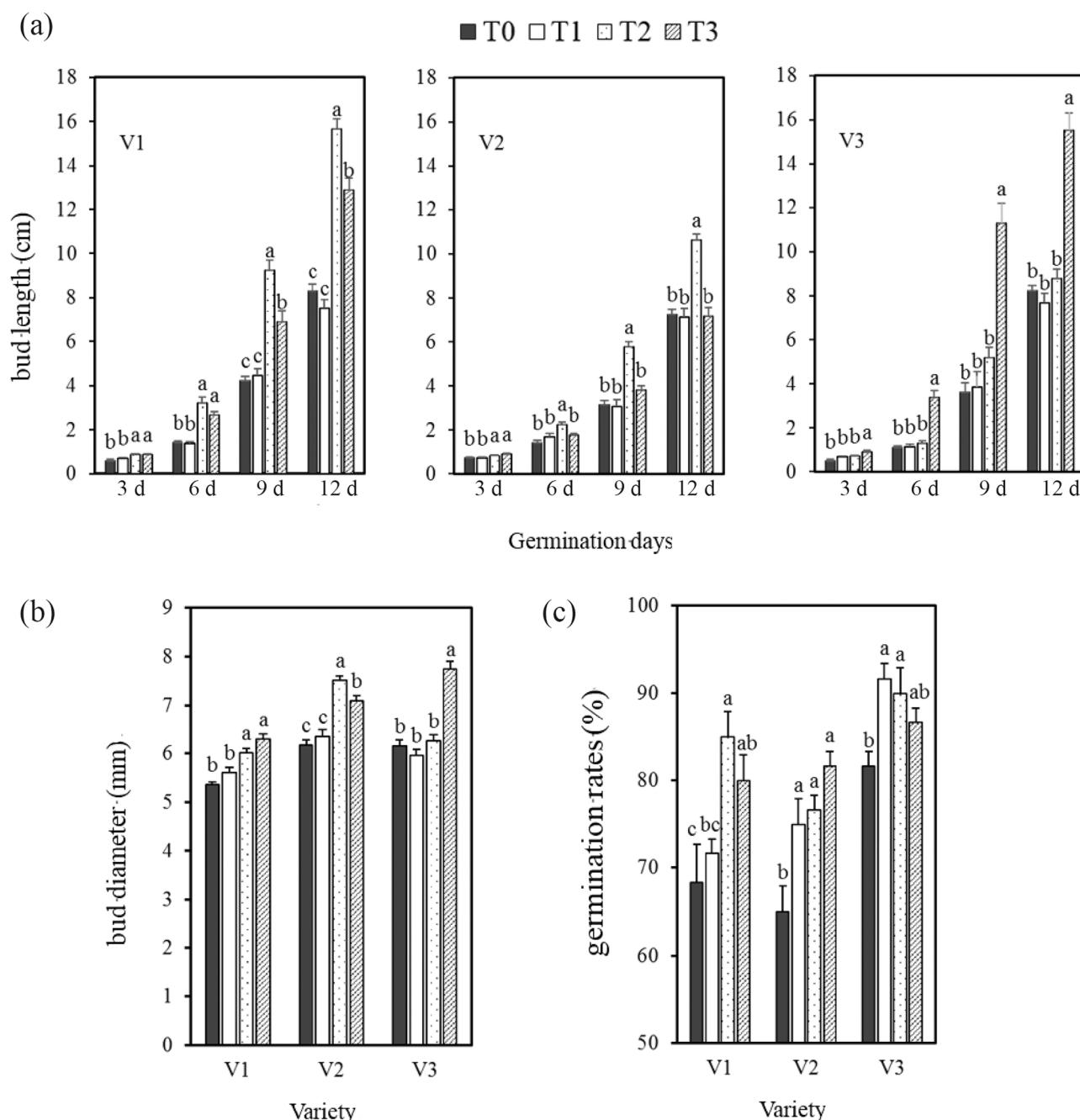


Fig. 2. Agronomic traits of sugarcane germination. (a) Bud length. (b) Bud diameter. (c) Germination rate. Different lowercase letters within a column indicate a difference at the 0.05 level. V, variety. T, treatment.

Moreover, the bud lengths were measured at 12 days.

2.9. Statistical analysis

Analysis of variance (ANOVA) was performed by using SPSS software (version 21.0; SPSS Inc., Chicago, USA), whereas differences between specific treatments were analyzed using least significant difference (LSD) tests at the 0.05 probability level. Moreover, paired-sample t-tests were performed using SPSS software (95% confidence intervals), and Pearson's correlation was used to examine the association between T0 and T2 of V1.

3. Results

3.1. Effects of ultrasonic treatment on germination and growth of sugarcane buds

The sugarcane began to germinate on the second day after being put into the lighting incubator, and the growth of different varieties of sugarcane under the various treatment times was significantly different (Fig. 1). Compared with T0, suitable ultrasonic treatment significantly increased the sugarcane shoot length of the three cultivars during germination; specifically, the bud lengths of T2 and T3 increased significantly in V1, while the bud lengths of V2 and V3 increased significantly at T2 and T3, respectively (Fig. 2a). Similarly, suitable ultrasonic treatment significantly affected the diameter and germination rate of sugarcane buds at 12 days. The results showed that compared with T0, both T2 and T3 increased the diameters of sugarcane buds of V1 and V2, while T3 promoted the growth of sugarcane buds of V3 (Fig. 2b). Furthermore, we found that the germination rate of sugarcane buds was

significantly improved after ultrasonic treatment. Compared with T0, the germination rates of T2 and T3 increased significantly for V1, whereas the germination rates of T1, T2, and T3 increased significantly for V2 and V3 at 12 days (Fig. 2c). In general, ultrasonic treatment can promote the germination and growth of sugarcane buds, although the appropriate treatment duration required by different varieties may be different. For these three varieties, ultrasonic treatment at this mix frequency (20–40 kHz) for 2–5 min is appropriate.

3.2. Effect of ultrasonic treatment on antioxidant system

For V1, compared with T0, T2 significantly increased SOD and POD activities during sugarcane germination, but T2 significantly increased CAT activity only at 6 and 12 days (Table 1). For V2, compared with T0, T2 tended to significantly increase SOD, CAT, and POD activities in the later stages (6, 9, and 12 days) of sugarcane germination (Table 1). Unlike V1 and V2, V3 required longer ultrasonic treatment time to increase the activities of antioxidant enzymes. Compared with T0, T3 significantly increased SOD, CAT, and POD activities on days 3, 6, 9, and 12 (Table 1). In general, these results suggest that the protective enzyme system of sugarcane buds after ultrasonic treatment can operate effectively to avoid the massive accumulation of reactive oxygen species (ROS), protect the cell membrane, and thus promote the germination of sugarcane buds.

3.3. Oxidative damage and soluble protein content

Compared with T0, the T2 and T3 significantly decreased the O²⁻ content of V1 at 6, 9, and 12 days, whereas T2 led to a significant decrease in O²⁻ content at 6 and 9 days, and T3 at 3, 6, 9, and 12 days for

Table 1
Effects of ultrasonic treatment on antioxidant enzyme activity of sugarcane during budding.

Antioxidant enzyme	Variety	Treatment	0 d	3 d	6 d	9 d	12 d
SOD (U·g ⁻¹ FW)	V1	T0	515.72 ± 0.94b	588.39 ± 10.16b	526.97 ± 9.75b	511.55 ± 4.49c	553.17 ± 8.35c
		T1	682.01 ± 2.75a	621.39 ± 10.15b	471.55 ± 9.87c	655.81 ± 2.06b	613.89 ± 45.73 BCE
		T2	663.55 ± 6.08a	726.64 ± 17.27a	687.27 ± 19.31a	709.64 ± 17.31a	699.00 ± 1.78a
		T3	658.98 ± 14.11a	629.50 ± 27.85b	454.90 ± 10.20c	660.45 ± 4.39b	631.61 ± 3.42ab
	V2	T0	478.16 ± 5.41c	416.14 ± 15.52c	570.81 ± 5.27b	576.13 ± 11.22b	525.31 ± 5.14d
		T1	591.82 ± 11.73b	550.76 ± 15.74ab	562.08 ± 16.14b	619.75 ± 4.94b	666.49 ± 10.03b
		T2	672.60 ± 20.14a	606.13 ± 15.83a	571.53 ± 8.26b	712.29 ± 31.44a	613.35 ± 2.28c
		T3	641.57 ± 12.94a	520.31 ± 28.68b	686.35 ± 28.61a	621.54 ± 24.73b	734.68 ± 14.07a
	V3	T0	626.20 ± 3.36d	586.01 ± 13.36b	578.76 ± 2.12d	608.27 ± 19.58b	529.40 ± 4.74b
		T1	637.99 ± 3.28c	494.61 ± 10.52d	592.67 ± 4.44c	634.24 ± 5.12b	521.43 ± 5.00b
		T2	767.78 ± 0.64b	540.68 ± 3.84c	605.53 ± 1.96b	607.33 ± 16.27b	615.09 ± 9.75a
		T3	823.31 ± 5.24a	663.04 ± 17.32a	748.67 ± 5.38a	795.73 ± 18.38a	602.12 ± 13.13a
CAT (U·g ⁻¹ ·min ⁻¹ FW)	V1	T0	88.22 ± 4.39a	69.58 ± 4.64c	88.62 ± 0.01b	109.60 ± 4.52b	156.78 ± 8.73b
		T1	69.29 ± 4.43a	110.16 ± 12.33b	88.82 ± 4.40b	99.23 ± 0.08b	108.72 ± 4.25c
		T2	29.79 ± 4.44b	68.84 ± 4.48c	191.54 ± 0.07a	177.73 ± 0.18ab	208.60 ± 3.98a
		T3	78.65 ± 8.80a	207.78 ± 4.93a	68.85 ± 4.41c	256.64 ± 52.16a	119.23 ± 8.89c
	V2	T0	49.20 ± 4.35ab	159.56 ± 9.06a	79.38 ± 0.01b	198.08 ± 8.92b	78.51 ± 0.03d
		T1	69.50 ± 13.33a	117.53 ± 8.52b	108.46 ± 4.42b	99.21 ± 0.15c	99.18 ± 0.16b
		T2	39.65 ± 8.86ab	109.03 ± 4.48b	275.79 ± 0.03a	257.81 ± 0.06a	157.87 ± 0.14a
		T3	29.80 ± 4.45b	108.58 ± 4.73b	304.46 ± 21.99a	98.77 ± 0.17c	79.07 ± 0.13c
	V3	T0	68.82 ± 4.40ab	39.74 ± 0.04d	119.97 ± 8.93c	59.42 ± 0.07d	79.15 ± 8.58b
		T1	49.30 ± 4.41 BCE	256.71 ± 8.32a	259.93 ± 8.91a	178.44 ± 0.02a	79.25 ± 0.09b
		T2	78.92 ± 8.83a	215.43 ± 8.78b	178.52 ± 0.02b	108.69 ± 4.76c	89.15 ± 4.43b
		T3	29.78 ± 4.44c	119.55 ± 0.01c	158.38 ± 9.27b	118.32 ± 0.25b	118.11 ± 0.03a
POD (U·g ⁻¹ ·min ⁻¹ FW)	V1	T0	535.16 ± 4.36d	550.77 ± 2.01b	476.36 ± 2.68c	345.38 ± 5.63b	329.30 ± 12.66b
		T1	692.90 ± 12.37b	638.81 ± 29.89ab	365.21 ± 7.38d	271.83 ± 7.14c	321.65 ± 4.10b
		T2	1010.67 ± 4.48a	737.00 ± 35.69a	893.87 ± 8.93a	430.65 ± 4.47a	504.33 ± 5.97a
		T3	564.92 ± 5.91c	641.25 ± 34.57ab	750.30 ± 6.57b	443.72 ± 18.80a	329.86 ± 3.55b
	V2	T0	609.05 ± 5.58d	517.30 ± 19.16b	498.15 ± 13.45c	372.10 ± 10.48b	426.55 ± 6.99b
		T1	734.68 ± 19.67b	503.85 ± 7.68b	550.16 ± 3.07ab	181.80 ± 14.23c	428.21 ± 6.68b
		T2	660.23 ± 15.22c	728.47 ± 16.64a	583.10 ± 5.42a	487.95 ± 27.80a	518.49 ± 15.21a
		T3	801.26 ± 6.86a	667.53 ± 36.34a	555.85 ± 12.99ab	381.27 ± 45.71b	447.40 ± 11.82b
	V3	T0	381.42 ± 7.22b	495.48 ± 1.35b	404.56 ± 1.87b	131.19 ± 11.93b	181.61 ± 3.88b
		T1	550.18 ± 34.48a	493.81 ± 8.44b	229.50 ± 52.99c	76.93 ± 2.37b	267.45 ± 5.38a
		T2	205.18 ± 2.30c	458.26 ± 7.50c	285.63 ± 1.13c	201.56 ± 32.30a	198.80 ± 15.13b
		T3	419.62 ± 1.67b	531.90 ± 7.23a	501.88 ± 11.77a	214.13 ± 1.77a	240.07 ± 9.81a

Note: Data presented are means ± standard error (SE). Different lowercase letters within a column indicate a difference at the 0.05 level. V, variety. T, treatment.

V2, T3 significantly decreased the O²⁻ content of V3 at 6, 9, and 12 days (Table 2). Furthermore, the T2 and T3 substantially decreased the MDA content during the later stages (9 and 12 days) of budding of the three sugarcane varieties (Table 2). In addition, the soluble protein content of sugarcane buds was significantly increased after appropriate ultrasonic treatment. In general, appropriate ultrasonic treatment can significantly reduce the content of O²⁻ and MDA and increase the content of soluble protein during the germination of sugarcane buds.

3.4. RNA-Seq data analysis and DEGs analysis

In this analysis, the transcriptome sequencing of six processes (18 samples) was completed, and a total of 121.57 G of clean data were obtained. The effective data volume of each sample was distributed between 6.49 G and 7.09 G; the Q30 base distribution was between 93.43% and 93.97%, and the average GC content was 52.75%. There were 81918 Unigene strips that were spliced together, with a total length of 92140986 bp and an average length of 1124.8 bp. Table S2 shows an overview of the sequencing and alignment results of the transcriptome analysis. We used diamond software to compare Unigenes to NR, KOG, GO, Swissprot, eggNOG, and KEGG databases, and we used HMMER software with the Pfam database to perform Unigene functional analysis. The numbers of Unigenes annotated by KEGG, KOG, GO, Pfam, Swissprot, eggNOG, and NR databases were 9383, 24058, 28427, 28583, 31451, 45343, and 50874, respectively (Fig. S1).

The principal component analysis (PCA) indicated that the first two principal components explained 90.08% of the variance of the transcriptome data across the six treatments (Fig. S2a). Except for one sample in ROC22_2 min 6, the other samples had greater biological reproducibility, confirming the credibility of the results obtained from

the transcriptome analysis. Moreover, the correlation test between samples agreed with the above results (Fig. S2b).

In addition, a total of 2756 DEGs (1395 upregulated and 1361 downregulated) were identified in the day 0 comparison; a total of 831 DEGs (408 upregulated and 423 downregulated) were observed in the day 6 comparison, and 5987 DEGs (1862 upregulated and 4125 downregulated) were identified in the day 12 comparison (Fig. 3a). Further analysis revealed significant differences in transcriptomes among the three comparisons, as Venn diagrams indicated that only nine DEGs were common to the three comparison groups (Fig. 3b). The overall DEGs distributions on days 0, 6, and 12 are shown in Fig. 3c–e. Cluster analysis of the changes in DEGs expression between the three comparisons is shown in Fig. S3a–b.

To gain an in-depth understanding of the functions of the DEGs identified on days 0, 6, and 12, after obtaining the DEGs, GO (biological process, cellular composition, and molecular function) functional enrichment analysis was performed (Ashburner et al., 2000). This experiment focused on ROS that were significantly enriched in biological processes, including “defense response” and “regulation of defense response” (Fig. S4a–c).

Moreover, pathway analysis was performed on the three contrasting differentially expressed protein-coding genes, and the significance of their differential gene enrichment was determined. Thus, the top 20 bubble charts from the KEGG enrichment analysis were obtained (Fig. 4a–c). The results showed that the pathways “plant hormone signal transduction” and “MAPK signal” were significantly enriched in comparison groups. For the functional annotation based on DEGs, we screened 40 candidate genes related to sugarcane germination and growth from the KEGG pathways, including IAA, ABA, gibberellin (GA), JA, and other hormone synthesis related genes as well as adenosine

Table 2
Effects of ultrasonic treatment on O²⁻, MDA, and soluble protein content of sugarcane during budding.

Content	Variety	Treatment	0 d	3 d	6 d	9 d	12 d
O ²⁻ ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ FW)	V1	T0	33.69 ± 0.27c	34.56 ± 0.12c	43.62 ± 0.23a	33.59 ± 1.11a	35.73 ± 0.43a
		T1	33.53 ± 0.10c	37.46 ± 0.29a	33.27 ± 0.10c	28.91 ± 1.08b	32.59 ± 0.90b
		T2	37.60 ± 0.21a	35.68 ± 0.65 BCE	39.74 ± 0.23b	28.77 ± 0.46b	28.54 ± 0.49c
	V2	T0	36.09 ± 0.27b	36.73 ± 0.16ab	32.17 ± 0.10d	32.57 ± 1.08a	31.18 ± 0.62b
		T1	28.83 ± 1.06b	37.77 ± 0.40a	41.22 ± 0.79a	34.27 ± 0.38a	36.13 ± 0.46a
		T2	29.83 ± 0.18ab	29.46 ± 0.56c	36.35 ± 0.35b	30.42 ± 0.71b	33.94 ± 0.34a
	V3	T0	31.27 ± 1.48ab	38.37 ± 1.22a	28.28 ± 0.42d	29.71 ± 0.20b	34.50 ± 1.16a
		T1	32.49 ± 0.28a	32.68 ± 0.29b	31.63 ± 0.37c	21.69 ± 1.11c	30.96 ± 0.27b
		T2	30.19 ± 1.53b	31.26 ± 0.35ab	25.20 ± 0.21a	26.55 ± 0.22a	28.88 ± 0.20a
	MDA ($\mu\text{mol}\cdot\text{g}^{-1}$ FW)	T1	24.86 ± 0.37c	31.67 ± 1.65ab	23.54 ± 0.32b	22.11 ± 0.11b	23.25 ± 0.26b
		T2	25.03 ± 0.42c	28.93 ± 0.18b	24.78 ± 0.12ab	20.85 ± 0.49c	24.40 ± 0.56b
		T3	33.13 ± 0.28a	34.42 ± 0.21a	19.84 ± 0.68c	19.19 ± 0.31d	23.26 ± 0.99b
Soluble Protein (mg·g ⁻¹ FW)	V1	T0	12.47 ± 0.16b	12.35 ± 0.19a	14.51 ± 0.17a	9.98 ± 0.40a	9.27 ± 0.46a
		T1	11.24 ± 0.11c	10.85 ± 0.21b	9.78 ± 0.07b	6.94 ± 0.10b	5.56 ± 0.03c
		T2	13.48 ± 0.22a	9.14 ± 0.02c	10.17 ± 0.12b	6.92 ± 0.35b	4.59 ± 0.24d
	V2	T3	12.97 ± 0.11ab	11.07 ± 0.25b	9.03 ± 0.11c	7.42 ± 0.44b	7.22 ± 0.14b
		T0	7.54 ± 0.08b	14.91 ± 0.40a	12.58 ± 0.12a	10.48 ± 0.29b	9.52 ± 0.11a
		T1	7.27 ± 0.12b	14.31 ± 0.22ab	11.96 ± 0.50a	12.74 ± 0.29a	9.30 ± 0.41a
	V3	T2	7.43 ± 0.05b	11.35 ± 0.49c	12.02 ± 0.17a	8.38 ± 0.22c	7.41 ± 0.13b
		T3	9.49 ± 0.11a	13.33 ± 0.10b	11.33 ± 0.48a	6.76 ± 0.027d	7.22 ± 0.18b
		T0	6.54 ± 0.03c	11.63 ± 0.07a	8.78 ± 0.09a	11.15 ± 0.22a	8.03 ± 0.15a
	V1	T1	6.93 ± 0.13b	8.34 ± 0.20b	6.12 ± 0.08b	7.47 ± 0.04b	6.81 ± 0.19b
		T2	6.56 ± 0.10c	6.71 ± 0.14c	9.21 ± 0.55a	6.62 ± 0.30c	6.52 ± 0.07b
		T3	7.82 ± 0.08a	11.14 ± 0.22a	8.27 ± 0.20a	5.75 ± 0.04d	4.54 ± 0.18c
	V2	T0	5.47 ± 0.58c	8.22 ± 0.43b	8.84 ± 0.16d	8.69 ± 0.40b	6.09 ± 0.48b
		T1	4.40 ± 0.50c	7.66 ± 0.09b	9.94 ± 0.36c	12.40 ± 0.60a	5.27 ± 0.36b
		T2	10.51 ± 0.18a	8.52 ± 0.39b	11.87 ± 0.31a	11.32 ± 0.60a	8.95 ± 0.23a
		T3	7.00 ± 0.24b	11.37 ± 0.79a	10.92 ± 0.10b	11.89 ± 1.59a	4.84 ± 0.28b
		T0	2.03 ± 0.60c	10.71 ± 0.21a	9.99 ± 0.31c	10.17 ± 1.12a	4.90 ± 0.14b
		T1	3.81 ± 0.37b	8.29 ± 1.38a	13.81 ± 0.34a	8.18 ± 1.12a	6.04 ± 0.80ab
		T2	6.29 ± 0.14a	9.79 ± 0.47a	13.41 ± 0.18a	9.02 ± 0.53a	6.97 ± 0.13a
		T3	6.17 ± 0.22a	9.04 ± 0.41a	11.25 ± 0.31b	7.21 ± 0.44a	4.26 ± 0.65b
		T0	1.90 ± 0.19c	6.20 ± 0.58b	9.80 ± 0.41b	5.88 ± 1.50b	7.64 ± 0.29d
	V3	T1	2.59 ± 0.20b	7.01 ± 0.70b	6.13 ± 0.49c	5.65 ± 0.23b	8.37 ± 0.17c
		T2	1.01 ± 0.18d	10.75 ± 0.43a	6.60 ± 0.044c	9.71 ± 0.12a	10.60 ± 0.15a
		T3	3.29 ± 0.12a	10.70 ± 0.38a	11.83 ± 0.58a	10.70 ± 0.13a	9.55 ± 0.16b

Note: Data presented are means ± standard error (SE). Different lowercase letters within a column indicate a difference at the 0.05 level. V, variety. T, treatment.

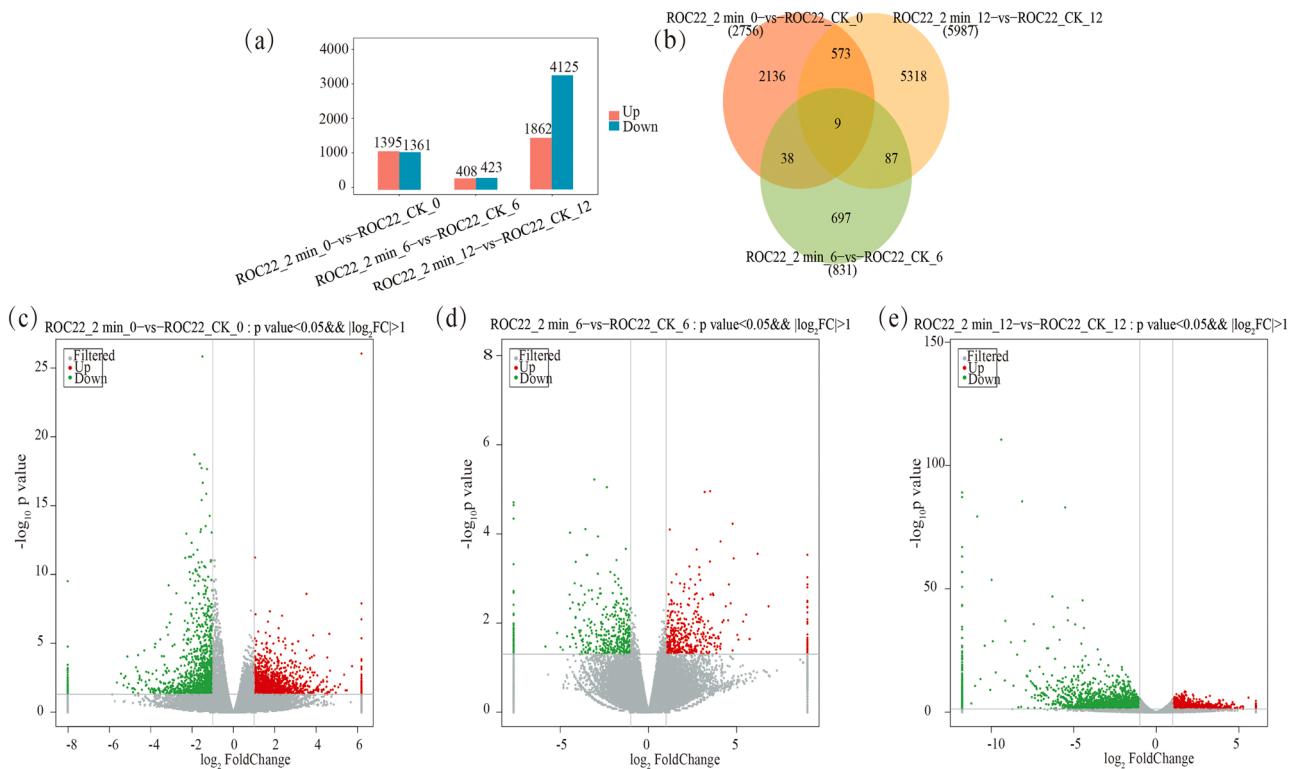


Fig. 3. Numbers of DEGs identified from the three comparisons (a). Venn diagram for DEGs identified in different comparisons (b). Differential expression volcano map for 0 (c), 6 (d), and 12 days (e). Gray represents Unigenes with no difference; red represents upregulated Unigenes, and green represents downregulated Unigenes (p value < 0.05 , $|\log_2 \text{FC}| > 1$). ROC22 represents the sugarcane variety; CK and 2 min represent without ultrasonic treatment and ultrasonic treatment 2 min, respectively, and 0, 6, and 12 are the days of sugarcane germination.

triphosphate (ATP) and antioxidant enzyme synthesis related genes (Fig. 4d-f).

3.5. Real-time quantitative PCR validation

To verify the transcriptome results and the gene expression related to hormone synthesis, 11 genes (eight of which were related to hormone synthesis in sugarcane bud germination) were selected from the KEGG pathways for RT-qPCR analysis (Table S1). Furthermore, three genes (*CRY1*, *HY5* and *RVE*) (Table S1) related to light signaling pathways were validated. Correlation analysis and linear fitting of the gene expression quantity (FPKM) obtained by transcriptome sequencing and the relative quantitative results of RT-qPCR of the corresponding genes were performed. The RT-qPCR results for 14 genes were significantly correlated with the RNA-Seq results ($r = 0.836$, $p < 0.001$), indicating that the transcriptome sequencing results were reliable (Fig. 5a and b). In addition, the results showed that the relative expression levels of genes related to hormone synthesis in sugarcane buds would be altered after ultrasonic treatment, and the trend was basically the same as that of RNA-Seq (Fig. 5b). Moreover, the expression of four differentially expressed genes involved in light signaling, *CRY1*, *HY5*, *PIF*, and *RVE*, were validated by RT-qPCR, and the results showed that ultrasonic treatment affected the expression of the above genes. Therefore, it can be inferred that ultrasound treatment may affect the light signal transduction during the germination of sugarcane buds (Fig. 5b).

3.6. Effects and verification of ultrasound on hormone content and hormone ratio in sugarcane bud germination

The transcriptome analysis showed that the DEGs were enriched in the plant hormone signaling pathways of KEGG, especially the biosynthesis of IAA, ABA, and JA. During the germination of sugarcane buds, the hormone determination results showed that the contents of all three

hormones (IAA, ABA, and JA) were decreased with the progress of sugarcane germination, and there were significant differences compared with CK. Compared with CK, the IAA content under ultrasonic treatment for 2 min was significantly increased at 12 days (Fig. 6a), whereas ABA content was substantially reduced at 6 and 12 days (Fig. 6b). In contrast, the content of JA was significantly increased at 6 and 12 days (Fig. 6c). Furthermore, the IAA/ABA ratio under the ultrasonic treatment for 2 min was higher than that of CK at 6 and 12 days, but at 0 days, the IAA/ABA ratio was lower than that of CK (Fig. 6d). The above analysis indicated that ultrasound promoted the increase of IAA/ABA ratio and JA content during the process of sugarcane bud germination, thus promoting the germination of sugarcane. In order to verify this fact, we conducted an experiment on the effect of exogenous hormones on the germination of sugarcane buds. The results showed that exogenous hormones IAA, ABA, and JA affected the germination of sugarcane (Fig. 7). Compared with B0 (Control), the germination of sugarcane was inhibited under low IAA/ABA ratio and promoted under high IAA/ABA ratio or appropriate concentration JA solutions. Under the mixed solution of high IAA/ABA ratio and appropriate concentration of JA (B4), the germination speed of sugarcane was basically the same as that in the ultrasonic treatment (B5) (Fig. 7).

4. Discussion

4.1. Effects of ultrasonic treatment on ROS and osmotic regulators

Ultrasonic treatment promoted cell division and accelerated seed germination, whereas its effectiveness was associated with the frequency and time of exposure to the ultrasonic waves (Nazari et al., 2014). In this study, we found that ultrasonic treatment of 20–40 kHz mixed frequency for 2–5 min could promote the germination and growth of sugarcane buds. There is evidence that suitable ultrasonic treatment can increase the activity of antioxidant enzymes to maintain

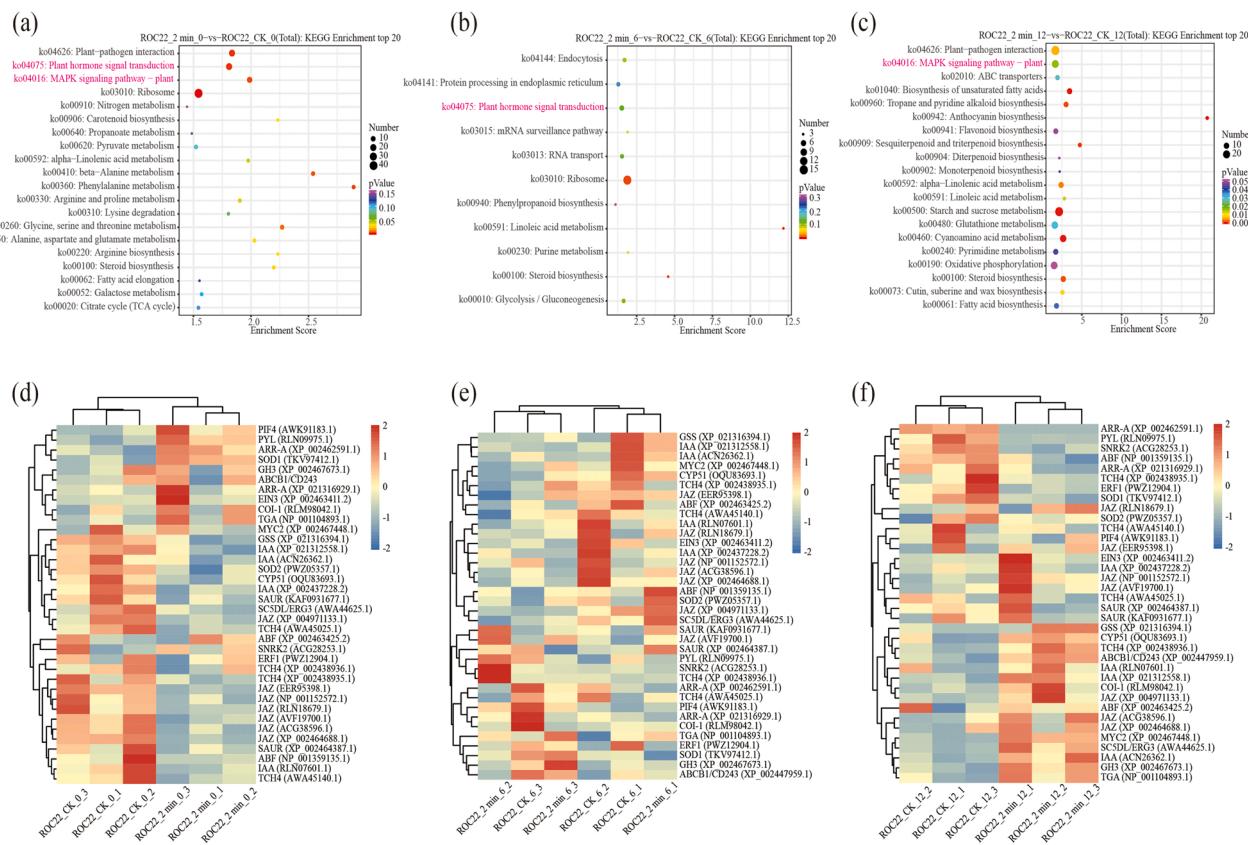


Fig. 4. KEGG enrichment analysis top 20 bubble chart for 0 (a), 6 (b) and 12 days (c) comparison. The Enrichment Score on the horizontal axis is the enrichment score. The larger the bubble, the greater the number of differential protein encoding genes, and the color of the bubble changes from purple-blue-green-red. The smaller the enrichment p value, the greater the degree of significance. The heat map of DEGs related to the promotion of the germination of sugarcane by ultrasonic treatment. (d) day 0 comparison, (e) day 6 comparison, (f) day 12 comparison. Relative transcript level was indicated on a color scale from red (high) to blue (low). DEGs names are on the right side of the figure, Outside the brackets is KEGG gene name, inside the brackets is NR annotation. ROC22 represents the sugarcane variety, CK and 2 min represent without ultrasonic treatment and ultrasonic treatment 2 min, respectively, and 0, 6 and 12 are the days of sugarcane germination. The numbers 1, 2 and 3 are sample repetitions.

the normal ability of plants to scavenge free radicals and ROS, thereby promoting seedling growth (Chen et al., 2013; da Silva and Dobranszki, 2014). Our results showed that the activities of antioxidant-related enzymes (SOD, POD, and CAT) were significantly increased by the ultrasonic treatment, as this contributed to the reduction of superoxide anion content and enhanced the antioxidant properties of sugarcane buds and promoted germination of sugarcane buds. In plants, free radicals cause lipid peroxidation and result in MDA accumulation and the associated cytotoxic effects (Hafez et al., 2020). Previous studies have shown that suitable ultrasonic treatment can scavenge free radicals from plant cells, protect cell membranes, and increase the rate of intracellular transport (Huang et al., 2020). In this study, ultrasonic treatment for 2 min reduced the MDA content significantly in sugarcane buds, which indicates that appropriate ultrasonic treatment can effectively remove free radicals, thereby reducing the production of ROS and protecting cell membranes.

Lipid metabolism is essential for plant seed germination, whereas the synthesis of JA and its derivatives is involved in lipid metabolism that can regulate various processes in plants (Chini et al., 2018). For the JA signal transduction pathway in plants, *JAR1* catalyzes the binding of JA and isoleucine (Ile) to form a JA-Ile complex that then specifically binds to the JA receptor *COI1* and then the transcription repressor protein *JAZ* protein that acts to release the signal transmission transcription factor *MYC2* and activate the transcription of JA early response genes (Kazan and Manners, 2013; Major et al., 2017; Ruan et al., 2019). Previous studies showed that JA synthesis related genes were up-regulated during seed germination to promote JA synthesis, thereby reducing ROS,

improving resistance, and promoting seed germination and growth (Hu et al., 2017; Wang et al., 2020b). In this study, the transcriptome results revealed that there were differences in the three comparative lipid synthesis-related pathways (Fig. S4a–c) as well as in the key genes responsible for JA synthesis (Figs. S5–S7). Specifically, *COI1* and *MYC2* gene expression levels were significantly up-regulated on the 12th day, and *JAZ* gene expression was also mainly up-regulated. This indicates that appropriate ultrasonic treatment may promote JA synthesis, reduce ROS, and thus promote the germination of sugarcane buds.

Previous studies have shown that the synthesis and decomposition of ATP were accelerated after ultrasonic treatment; the cell membrane transport speed was increased, and the synthesis of various metabolites was promoted (Hwang et al., 2016). In this study, transcriptome results showed that related genes of the ATP-binding cassette were also significantly upregulated at 12 days, which is conducive to the synthesis of metabolites. Therefore, in the present study, the sugarcane buds had increased contents of osmotic regulators (soluble proteins) after ultrasonic treatment, which is conducive to maintaining the stability of cell membrane.

4.2. Effects of ultrasonic treatment on the regulation of IAA, ABA and GA

Hormones play indispensable roles in plant germination, growth, development, maturation, and senescence. IAA and ABA affect the basic processes of plant cell elongation and division, thereby regulating plant germination and growth (Kende and Zeevaart, 1997). Ultrasonic treatment is a special physical stimulation method with multiple complex

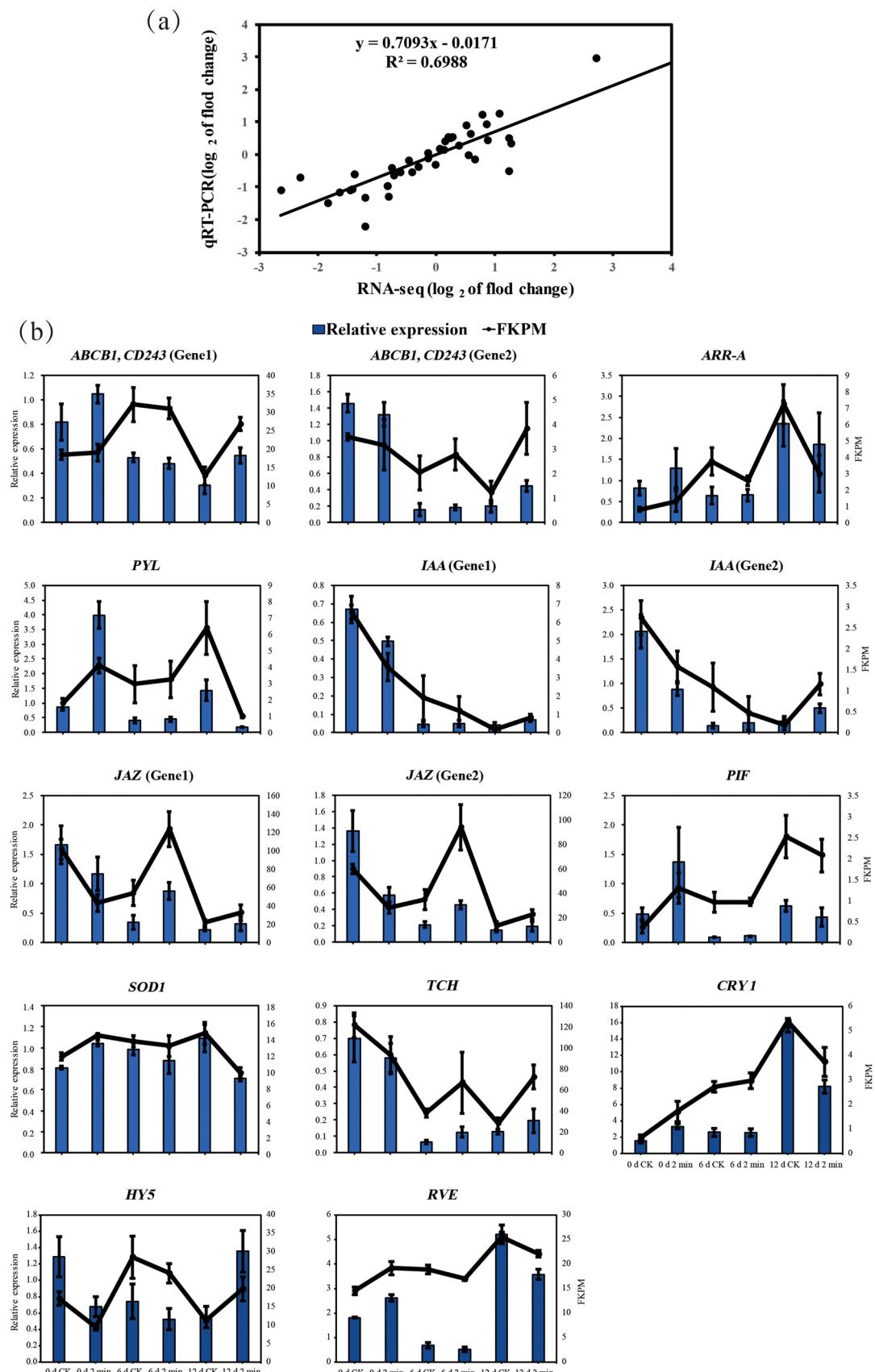


Fig. 5. Coefficient analysis of gene expression levels obtained from RNA-Seq and RT-qPCR data (a). Expression of the selected 14 genes revealed by RNA-Seq and RT-qPCR (b). Log₂ fold change: log₂ fold change in gene expression between ultrasonic treatment and non-ultrasonic treatment samples. ROC22 represents the sugarcane variety; CK and 2 min represent without ultrasonic treatment and ultrasonic treatment for 2 min, respectively, and 0, 6, and 12 are the days after sugarcane germination. Eight genes, including *IAA* (Gene 1), *IAA* (Gene 2), *PYL*, *ARR-A*, *PIF4*, *JAZ* (Gene 1), *JAZ* (Gene 2) and *TCH4*, are related to hormone pathway.

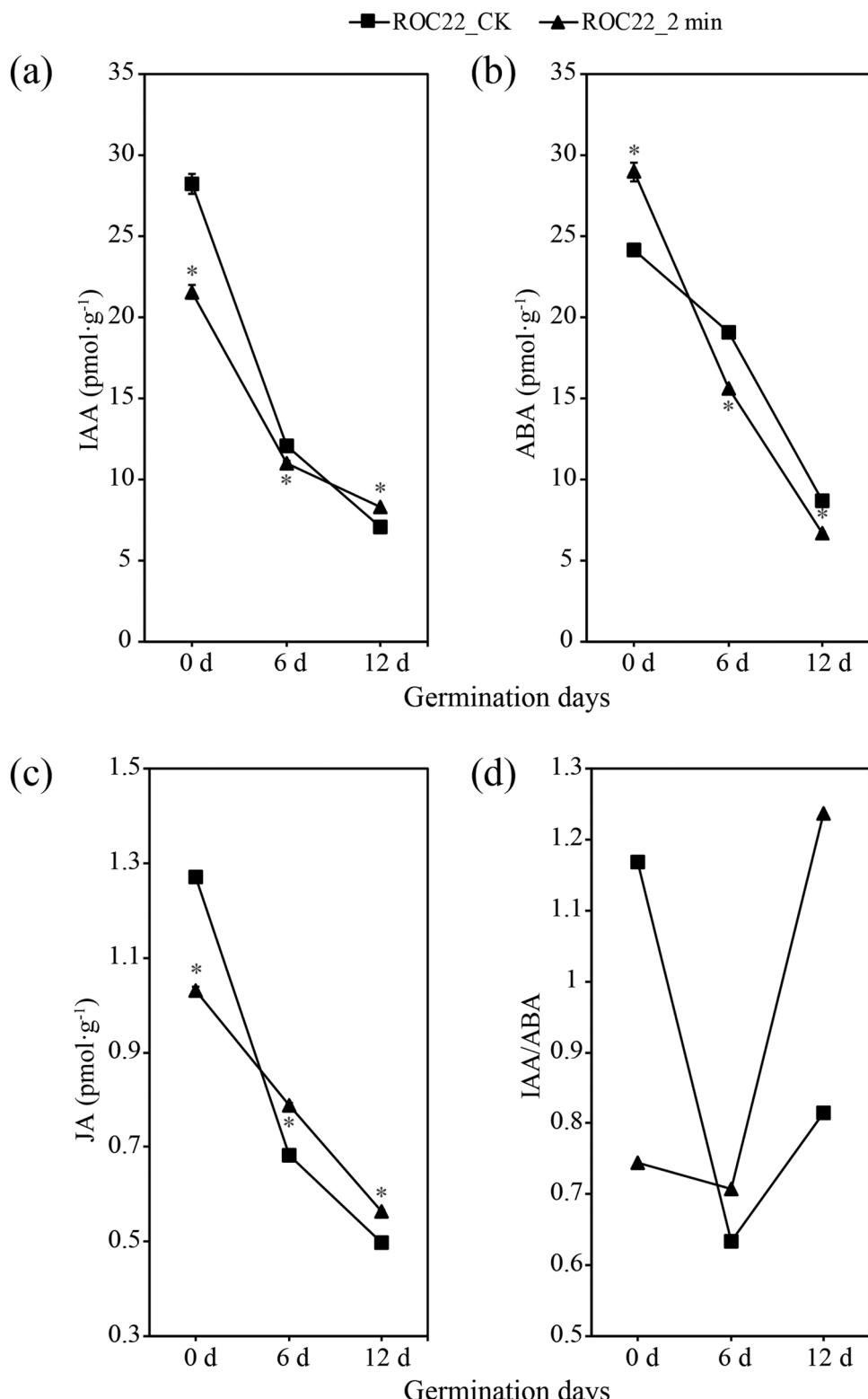


Fig. 6. Hormone determination of sugarcane buds with and without ultrasonic treatment. (a) IAA content, (b) ABA content, (c) JA content, (d) IAA/ABA. Data presented are means \pm standard error (SE) of three replicates. Asterisks denote a significant difference between treatments at the same time-point (Student's *t*-test, $p < 0.05$).

biological effects (Liu et al., 2003). Previous study has shown that ultrasonic treatment can effectively change the IAA and ABA contents of *Chrysanthemum* callus to regulate its differentiation and growth (Wang et al., 2004). Wei et al. (2012) indicated that endogenous hormone changes may be caused by the influence of ultrasound during the

differentiation of protocorm-like bodies. Similar findings were found in our study, suitable ultrasonic treatment clearly enhanced the germination and growth of sugarcane buds, and this may have been due to changes in hormonal content in sugarcane bud cells (Fig. 6a and b). In addition, this study found that during the process of sugarcane

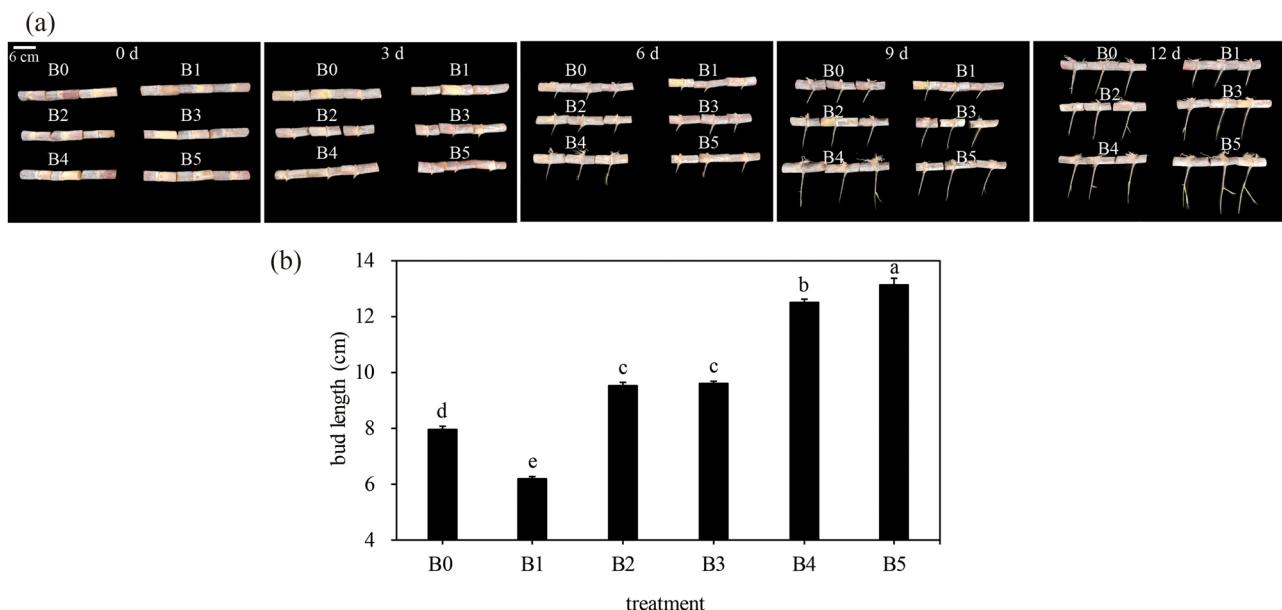


Fig. 7. Effects of exogenous hormones (IAA, ABA, and JA) on sugarcane (ROC22) germination. (a) Phenotype of sugarcane germination. (b) Bud length of sugarcane at 12 days. 0, 3, 6, 9, and 12 days represent the germination days of sugarcane. B, treatment. The line segment is a scale bar.

germination, the contents of IAA and ABA showed significant downward trends, indicating that high content of IAA and ABA help sugarcane buds to stay dormant, and low content of IAA and ABA are stimuli for sugarcane buds to germinate, which is basically consistent with the research findings of Carrera-Castano et al. (2020) and Kucera et al. (2005). It was also observed that after ultrasonic treatment, the IAA/ABA ratio in the sugarcane buds increased significantly, and this change promoted the germination of sugarcane. The subsequent exogenous hormone model test results also verified that high IAA/ABA ratio can promote sugarcane germination. In other crops, da Silva and Dobranszki (2014) and Wang et al. (2004) have similar findings. In the transcriptome results and RT-qPCR verification, the expression of the three comparative IAA and ABA related genes also showed a downward trend, which is basically consistent with the change trend of the hormone content (IAA and ABA) during the germination of sugarcane buds (Figs. S5–S7; Fig. 5b). Therefore, we infer that during the germination of sugarcane buds, ultrasound regulates the expression of genes related to IAA and ABA, reduces IAA and ABA content, increases IAA/ABA ratio, and promotes sugarcane germination and growth.

Gibberellin (GA) plays an important role in seed germination and growth. In the GA signal transduction pathway, the *DELLA* protein has the function of binding to other regulatory proteins in protein-protein interactions (Daviere and Achard, 2013; Lilley et al., 2013). The DNA-binding ability of transcription factors (*TFs*) is inhibited by interacting with *DELLA*, thereby negatively regulating the transcription of GA (Daviere and Achard, 2016; Yoshida et al., 2014). In this study, the *TF* gene expression was upregulated at 0 days, and *DELLA* gene expression was downregulated at 12 days (Fig. S5 and Fig. S7), demonstrating that ultrasonic treatment could promote GA synthesis to promote the germination and growth of sugarcane buds. This is similar to the research results of Bentsink and Koornneef (2008).

Our transcriptome results also indicated that a small number of differentially expressed genes were enriched in the cytokinin (CTK) and salicylic acid (SA) synthesis pathways, but there were very few differentially expressed genes in this regard, and these are not discussed in this article (Figs. S5–S7).

4.3. Ultrasonic treatment-mediated germination involves other mechanisms

The phenomenon of light-regulated seed growth and development is not only a physiological process but also a process of light signal transduction and light-regulated gene expression (Heschel et al., 2008; Kircher et al., 2002). In this study, the KEGG enrichment pathway results (top 20) showed that no enrichment pathway related to the direction of light signal regulation were found (Fig. 4a–c), although we obtained four differentially expressed genes related to light signals through screening, namely *CRY1*, *HY5*, *PIF*, and *RVE*. RNA-Seq and RT-qPCR confirmed that the expression of the four genes mentioned above was affected by ultrasonic treatment. However, due to too few relevant genes, the mechanism of light signal transduction during sugarcane germination mediated by ultrasonic treatment needs to be further explored.

5. Conclusions

Overall, the current study revealed that suitable ultrasonic treatment (20–40 kHz mixed frequency, 2–5 min) promotes the germination and growth of sugarcane buds. In addition, ultrasonic treatment affected the regulation of many DEGs involved in sugarcane hormone synthesis, hormone ratio, signal transduction, and anti-oxidation. Ultrasonic treatment could promote the germination of sugarcane buds owing to improvement in antioxidant capacity and prevention of the damage from ROS and through regulation of IAA, ABA, and JA and other hormone signaling pathways involved in the sugarcane bud germination processes. The mechanism of regulation of ROS and hormone pathway by DEGs explains how suitable ultrasonic treatment can promote sugarcane germination and growth, and thus, the results provide a theoretical basis for ultrasonic application in sugarcane production.

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CRediT authorship contribution statement

Zeng Zhen: Conceptualization, Investigation, Methodology, Formal analysis, software, Writing – original draft, Writing – review & editing. **Xiangli Liu:** Conceptualization, Investigation, Writing – review & editing. **Quanqing Deng:** Conceptualization, Writing – review & editing. **Umair Ashraf:** Supervision, Writing – review & editing. **Jianwen Chen:** Conceptualization, Funding acquisition, Resources, Writing – review & editing. **Wankuan Shen:** Conceptualization, Funding acquisition, Resources, Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests, Wankuan Shen reports financial support was provided by National Natural Science Foundation of China. Wankuan Shen reports financial support was provided by Guangdong Provincial Team of Technical Systalk Innovation for Sugarcane Sisal Hemp Industry.

Data Availability

Data will be made available on request.

Acknowledgments

We thank the NCBI official website for storing the transcriptome raw data. RNA-Seq repository can be found online at: <https://www.ncbi.nlm.nih.gov/sra/PRJNA793161>.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.indcrop.2022.116104](https://doi.org/10.1016/j.indcrop.2022.116104).

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