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Research Article

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Effects of gibberellin priming on seedling emergence and transcripts involved in mesocotyl elongation in rice under deep direct-seeding conditions

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Abstract: Mesocotyl elongation is a key trait influencing seedling emergence and establishment in direct-seeding rice cultivation. The phytohormone gibberellin (GA) has positive effects on mesocotyl elongation in rice. However, the physiological and molecular basis underlying the regulation of mesocotyl elongation mediated by GA priming under deep-sowing conditions remains largely unclear. In the present study, we performed a physiological and comprehensive transcriptomic analysis of the function of GA priming in mesocotyl elongation and seedling emergence using a direct-seeding *japonica* rice cultivar ZH10 at a 5-cm sowing depth. Physiological experiments indicated that GA priming significantly improved rice seedling emergence by increasing the activity of starch-metabolizing enzymes and compatible solute content to supply the energy essential for subsequent development. Transcriptomic analysis revealed 7074 differentially expressed genes (false discovery rate of <0.05, $|\log_2(\text{fold change})|$ of ⩾1) after GA priming. Furthermore, gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) enrichment analyses revealed that genes associated with transcriptional regulation, plant hormone biosynthesis or signaling, and starch and sucrose metabolism were critical for GA-mediated promotion of rice mesocotyl elongation. Further analyses showed that the expression of the transcription factor (TF) genes (v-myb avian myeloblastosis viral oncogene homolog (MYB) alternative splicing 1 (*MYBAS1*), phytochrome-interacting factors 1 (*PIF1*), *Oryza sativa* teosinte branched 1/cycloidea/proliferating cell factor 5 (*OsTCP5*), slender 1 (*SLN1*), and mini zinc finger 1 (*MIF1*)), plant hormone biosynthesis or signaling genes (brassinazole-resistant 1 (*BZR1*), ent-kaurenoic acid oxidase-like (*KAO*), GRETCHEN HAGEN 3.2 (*GH3.2*), and small auxin up RNA 36 (*SAUR36*)), and starch and sucrose metabolism genes (α-amylases (*AMY2A* and *AMY1.4*)) was highly correlated with the mesocotyl elongation and deep-sowing tolerance response. These results enhance our understanding of how nutrient metabolism-related substances and genes regulate rice mesocotyl elongation. This may facilitate future studies on related genes and the development of novel rice varieties tolerant to deep sowing.

Key words: Mesocotyl; Rice (*Oryza sativa* L.); Direct-seeding; Gibberellin; Deep sowing; Transcriptomic analysis

1 Introduction

In the wake of increasingly severe land, labor, and water resource shortages in recent years, dry direct-seeding has become a popular cultivation method in rice-planting areas. The method is relatively simple, convenient, and highly efficient (Zhao et al., 2018; Zhan et al., 2020). Deep sowing is a common practice in dry direct-seeding rice, since shallow sowing usually results in drought stress, bird damage, and lodging after heading. Besides avoiding these risks, deep seeding improves nutrient and water absorption from deeper soil layers in dry direct-seeding cultivation (Lee et al., 2017; Zhan et al., 2020). However, poor seedling establishment can lead to reduced yields in dry direct-seeding cultivation systems when seeds are sown deep in the soil, and this has limited the adoption of this technique. To take full advantage of dry direct-seeding in rice production, both agronomic and genetic solutions are required.

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Previous studies have reported that rice coleoptiles and mesocotyls are primarily responsible for seedling emergence because they are elongated during germination and push the plumule above the soil surface when seeds are sown deeply (Dilday et al., 1990). In soil-sand culture, mesocotyl elongation is greater than coleoptile elongation, whereas in submerged conditions, the reverse is true. This highlights the positive role of mesocotyl elongation in promoting faster and uniform rice seedling emergence from deeper soil layers (Alibu et al., 2012). Genotypes with long mesocotyls usually emerge more quickly and uniformly (Zhang et al., 2005; Zhan et al., 2020). Therefore, mesocotyl elongation is an important trait for deep-sowing tolerance, which is critical in dry direct-seeding rice production (Wu et al., 2015; Lu et al., 2016; Zhao et al., 2018).

Mesocotyl elongation in rice is regulated by both genetic and environmental factors and varies considerably among varieties and genotypes (Lee et al., 2012). A previous genetic analysis revealed that mesocotyl length is controlled by quantitative trait loci (QTL) (Redoña and Mackill, 1996). With rapid advancements in molecular marker technologies, QTLs associated with mesocotyl elongation have been identified using various segregating populations including recombinant inbred lines (RILs), backcross recombinant inbred lines (BILs), chromosome segment substitution lines (CSSLs), and doubled haploids (DHs) (Redoña and Mackill, 1996; Cai and Morishima, 2002; Cao et al., 2005; Huang et al., 2010; Lee et al., 2012, 2017). In recent years, genome-wide association study (GWAS) was adopted to identify QTLs and candidate genes for mesocotyl length in rice. By an integrated analysis of GWAS, linkage mapping, differences in allelic frequency between phenotypic pools, and gene expression, thirteen QTLs and two new major-effect genes (*Oryza sativa* mesocotyl length 1 (*OsML1*) and *OsML2*) were finally identified (Zhao et al., 2018).

Besides genetic factors, mesocotyl elongation can be influenced by environmental and physiological factors, such as sowing depth, light, and phytohormones. In general, deep-seeding can, to a certain extent, promote mesocotyl growth in rice seedlings. However, mesocotyl elongation can be inhibited by light exposure (Osterlund et al., 2000; Feng et al., 2017; Lee et al., 2017). Light can increase the expression of a polyamine oxidase (*OsPAO5*), which negatively regulates mesocotyl elongation and seedling emergence in rice (Lv et al., 2021). Furthermore, mesocotyl elongation in rice can be promoted by moderate abscisic acid (ABA), brassinosteroids (BRs), ethylene (ETH), gibberellin (GA), and indole-3-acetic acid (IAA), but inhibited by jasmonate (JA), karrikin, and strigolactones (SLs) (Watanabe et al., 2001; Cao et al., 2005; Xiong et al., 2017; Sun et al., 2018; Zhan et al., 2020; Zheng et al., 2020). For example, ABA promotes mesocotyl elongation by stimulating cell division in the meristem (Watanabe et al., 2001). Conversely, SLs inhibit mesocotyl elongation by controlling cell division during seedling emergence in the dark. Cytokinins (CKs) regulate mesocotyl elongation by antagonizing SLs. SLs together with CKs regulate the expression of *O. sativa* teosinte branched 1/cycloidea/proliferating cell factor 5 (*OsTCP5*), a transcription factor (TF) belonging to the cell division-regulating TCP family whose expression levels are negatively correlated with mesocotyl length (Hu et al., 2014). In *Arabidopsis*, GAs play key roles as central nodes in hypocotyl elongation regulatory networks by controlling the abundance of DELLA proteins, which promote the degradation of phytochrome-interacting factor 3 (PIF3) and PIF4 which can activate the transcription of expansion-promoting genes during the growth of etiolated seedlings (Claeys et al., 2014). Studies indicated that the application of exogenous GA promotes mesocotyl elongation by participating in phytochrome regulation and GA-mediated changes in cortical microtubule arrays in rice (Liang et al., 2016). In addition, ETH signaling mediated by *O. sativa* ethylene-insensitive 3-like 2 (*OsEIL2*) and ethylene-insensitive protein 2 (*OsEIN2*) suppresses the expression of glycinin G1 (*GY1*) and other genes controlling JA biosynthesis to reduce JA levels and accelerate mesocotyl growth by increasing cell elongation in etiolated rice seedlings (Xiong et al., 2017). BR-promoted mesocotyl elongation works by inhibiting the phosphorylation of a U-type cyclin, CYC U2, by *O. sativa* glycogen synthase kinase 2 (OsGSK2). Notably, SL can suppress mesocotyl growth by degrading the OsGSK2-phosphorylated CYC U2 via the F-box protein D3, which plays a critical role in the SL signaling pathway. Therefore, OsGSK2 is thought to regulate mesocotyl elongation by coordinating SL and BR signaling during domestication (Sun et al., 2018). A recent study showed that karrikin and SL signaling act in parallel and additively to modulate the expression of downstream

genes and negatively regulate rice mesocotyl elongation in the dark (Zheng et al., 2020).

Seed priming can be defined as a cost-effective and simple technique that controls the hydration levels within seeds and induces metabolic activities to facilitate germination and prevent imbibition damage (Salah et al., 2015). The positive effects of priming treatments on germination and seedling emergence are related mainly to the metabolic processes activated including DNA repair and antioxidant mechanisms, syntheses of nucleic acids and proteins, and adenosine triphosphate (ATP) production (Paparella et al., 2015). Seed priming also promotes the synthesis of amino acids related to GA biosynthesis in germinating seeds. This results in the enhanced biosynthesis of soluble sugars as the primary energy source and α-amylase as a catalyst in starch hydrolysis for glycolysis. Sugars and starch are associated with energy production during seed germination and vigorous seedling growth (Ding et al., 2012; He et al., 2019). Previous studies have revealed that seed priming by hormones or other chemicals can improve seed germination, seedling emergence, and seedling establishment in rice under stress conditions (Hussain et al., 2015). GA priming could enhance rice seedling emergence and establishment, mainly by promoting mesocotyl elongation, and could broaden the choice of varieties suitable for deep direct-seeding (our unpublished data).

Although the key roles of GA in rice mesocotyl elongation and seedling emergence have been acknowledged, the mechanisms underlying regulation of mesocotyl elongation by GA priming under deep-seeding conditions remain largely unclear. Here, we investigated the physiological and transcriptomic changes in rice mesocotyls in response to priming treatments with different concentrations of GA using the direct-seeding *japonica* rice variety ZH10. The seedling emergence and establishment of ZH10 were found to be poor under non-priming treatment conditions at a sowing depth of 5 cm. Our aim was to comprehend the biological processes and molecular basis underlying regulation of mesocotyl elongation mediated by GA priming under deep-sowing conditions to facilitate the identification of genes associated with mesocotyl elongation and deep-sowing tolerance for genetic improvement. This study enhanced our understanding of the potential mechanisms underlying deep-seeding tolerance in rice via GA signaling.

2 Materials and methods

2.1 Plant materials and growth conditions

The rice variety ZH10 (*O. sativa* L. ssp. *japonica*) was bred by the Henan Academy of Agricultural Sciences, China and approved by the National Crop Variety Approval Committee in 2012 (approval No. 2012043). Healthy and full ZH10 seeds were surface-sterilized with 75% (volume fraction) alcohol for 1 min and soaked in 15% (0.15 g/mL) NaClO solution for 20 min, followed by rinsing with sterilized distilled water five times. For the seed priming treatment, the sterilized seeds were primed with 0, 10, 50, or 100 mg/L gibberellin A3 (GA_3, CAS: 76738-62-0, Sigma-Aldrich, St. Louis, MO, USA) at 20 ℃ in the dark for 24 h. Subsequently, the seeds were slowly air-dried at 25 ℃ until they reached their original moisture content. After priming, 20 seeds per treatment were sown in plastic germination boxes (19 cm long, 13 cm wide, and 8 cm high). The base inside each box was covered with a mixture (100 g) of paddy soil and seedling substrate (Pindstrup) at the ratio of 1:1 (mass ratio). Two or three treatments were sown in each box. After seeding, a 5-cm soil layer (1200 g of mixture) was placed over the seeds followed by sprinkling with distilled water (385 g). The boxes were weighed and placed in a growth chamber at 70% relative humidity for seed germination, and the seedlings were grown at 28 ℃ under a 14-h light/10-h dark cycle. The boxes were replenished with distilled water every 24 h to maintain total weight. Unprimed seeds were used as control (CK). On the 7th and 10th days, the seedling emergence ratio and seedling height above the soil were measured. On the 10th day after sowing, the mesocotyl and coleoptile lengths of plants in all treatments were measured using a ruler.

2.2 Amylase activity and soluble sugar content

To measure amylase activity and soluble sugar content, rice seedlings without husks (0.1 g) were obtained from each treatment at five time points (1, 3, 5, 7, and 10 d after sowing). The α-amylase and β-amylase activity and soluble sugar content were assayed separately using α-amylase, β-amylase, and soluble sugar assay kits (Comin Biotechnology Co., Ltd., Suzhou, China) according to the manufacturer's instructions. For preparation of amylase stock solution, 0.1 g of plant samples in 1 mL of distilled water were

ground into homogenate, which was then poured into a centrifuge tube (2 mL) and incubated at 25 ℃ for 15 min, with shaking every 5 min. After centrifugation at 3000*g* for 10 min, the supernatant was collected for further determination. Using colorimetric determination of 3,5-dinitro-salicylic acid, the activity of α-amylase and β-amylase was assayed spectrophotometrically based on monitoring absorbance at 540 nm, according to previous reports (He et al., 2019; Yang et al., 2019; Huang et al., 2020). Amylase activity was expressed as "mg/(min·g fresh weight (FW))." For measurement of soluble sugar content, the prepared homogenate in a centrifuge tube (2 mL) with a lid was incubated at 95 ℃ for 10 min, and then quickly cooled. After centrifugation at 8000*g* for 10 min at 25 ℃, the supernatant was used to determine the soluble sugar content spectrophotometrically at 620 nm by the anthrone colorimetry method (Yang et al., 2017). The soluble sugar content was calculated in terms of "mg/g FW."

2.3 Construction of cDNA libraries and RNA sequencing

On the 10th day after sowing, mesocotyls of treated seedlings (10 and 50 mg/L GA_3) and control seedlings (0 mg/L GA_3) at the 5-cm sowing depth were collected (with three biological replicates) and immediately frozen in liquid nitrogen for RNA sampling. Total RNA was isolated from mesocotyls using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The quantity and purity of total RNA were measured using a NanoDrop2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and an Agilent Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA), using an RNA integrity number of >7 as cut-off. Poly(A) messenger RNAs (mRNAs) were enriched from 1 μg of total RNA using magnetic oligo (dT) beads (Invitrogen) and broken into short fragments of about 300 bp. The cleaved RNA fragments were then reversely transcribed into complementary DNA (cDNA) for library preparation according to the protocol provided along with the TruSeq RNA sample preparation kit (Illumina, San Diego, CA, USA). The constructed RNA-Seq libraries (including three GAPT (50) libraries (priming treatment with 50 mg/L GA_3), three GAPT (10) libraries (priming treatment with 10 mg/L GA_3), and three GAPT (0) libraries (priming treatment with 0 mg/L GA_3)) were sequenced using an Illumina Hiseq4000 sequencer at Majorbio Co., Ltd. (Shanghai, China). The raw reads were submitted to the Sequence Read Archive of the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/Traces/sra) with accession No. PRJNA681386.

2.4 RNA-Seq data analysis

The raw reads were processed by filtering the adaptor sequences and removing low-quality sequences to obtain clean reads. All clean reads were then mapped to the reference genome (Michigan State University (MSU) Rice Genome Annotation Project Release 7, available at http://rice.plantbiology.msu.edu). Only uniquely matched reads were selected for the following analysis. Levels of gene expression were estimated by calculating fragments per kilobase of exon per million mapped read (FPKM) values using the RNA-Seq by Expectation-Maximization (RSEM) software package (http://deweylab.github.io/RSEM), and genes with FPKM≥0.2 were retained. A false discovery rate (FDR) value of <0.05 and a $|\log_2(\text{fold change})|$ value of ≥1 were applied as the criteria for identifying significant differentially expressed genes (DEGs). Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) enrichment analyses of the DEGs were conducted using GOATOOLS and KOBAS software, respectively (Feng et al., 2017). Statistical significance was tested using Fisher's exact test. Significant enrichment was detected with a corrected *P* value or FDR of <0.05. TFs were identified using the plant TF database (http://planttfdb.cbi.pku.edu.cn).

2.5 Quantitative real-time polymerase chain reaction analysis

On the 10th day after sowing, mesocotyls were collected (three biological replicates) and immediately frozen in liquid nitrogen. Total RNA was extracted according to the procedure described in Section 2.3. First-strand cDNA synthesis was performed using the PrimeScript RT Master Mix (Perfect Real Time) kit (RR036A; TaKaRa, Dalian, China). For quantitative real-time polymerase chain reaction (qRT-PCR), the TB Green Premix Ex Taq II (Tli RNaseH Plus) kit (RR820; TaKaRa, Dalian, China) was used with primers against target genes. qRT-PCR was performed in a 25-mL reaction mixture containing 1× SYBR Premix Ex Taq II and 0.4 mmol/L of each primer using

a Bio-Rad CFX96 Touch thermal cycler (Hercules, CA, USA) with the following thermocycler conditions: pre-denaturing at 95 ℃ for 30 s, 40 cycles of 5 s at 95 ℃, and 30 s at 60 ℃. Rice actin I gene was selected as the reference gene. The relative expression levels of all target genes under different conditions (different priming treatments or different sowing depths) were calculated using the $2^{-\Delta\Delta C_T}$ method, as described by Wang et al. (2012). The gene-specific primers designed using Primer Premier 5.0 (Premier Biosoft International, Palo Alto, CA, USA) are listed in Table S1.

2.6 Statistical analysis

Values described in the results section are expressed as mean±standard deviation (SD). Statistical analysis was conducted using one-way analysis of variance (ANOVA) and Duncan's multiple range test using SPSS Statistics 16.0 (SPSS Inc., Armonk, NY, USA) to determine significant differences at $P<0.05$. All analyses were performed using three biological replicates.

3 Results

3.1 Effects of GAs on the seedling emergence and establishment of dry direct-seeded rice under deep-sowing conditions

Compared with the control seeds with no priming treatment (CK), ZH10 seeds with GAPT (10), GAPT (50), and GAPT (100) showed significantly increased germination speeds and seedling emergence rates. However, the promoting effect of GAPT (0) was weak. As the GA_3 concentration increased within a certain range, mesocotyl length increased significantly, whereas the change in coleoptile length was not so obvious (Table 1), confirming that mesocotyl elongation is the primary driver of germination in dry direct-seeding rice under deep-sowing conditions. GA priming promoted seedling emergence and establishment mainly via enhanced mesocotyl elongation under deep-sowing conditions.

Although GAPT (100) markedly improved the germination speed and seedling emergence rate as well as the mesocotyl and coleoptile lengths in ZH10, its promotional effect on mesocotyl elongation was not stronger than that of GAPT (50), implying that in the present study, GAPT (50) was the optimum priming concentration for regulating seedling emergence in dry direct-seeding rice under deep-sowing conditions.

The physiological properties of the samples in different treatments were examined 1, 3, 5, 7, and 10 d after sowing at the 5-cm sowing depth. The mesocotyls of ZH10 seedlings with GAPT (0) started to elongate 1 d after sowing, and elongated markedly from 5 to 7 d after sowing, after which the length of the mesocotyl stopped increasing. Compared with GAPT (0), GAPT (10) and GAPT (50) increased the speed of mesocotyl elongation in the ZH10 seedlings, and mesocotyls continued to elongate gradually from 5 to 10 d after sowing. Furthermore, mesocotyls subjected to GA priming were always longer than those with no priming at all measurement time points, and the promotion effect seemed to be dose-dependent (Figs. 1a and 1b). Further studies showed that compared with GAPT (0), GAPT (10) and GAPT (50) significantly increased the α-amylase and β-amylase activity (Figs. 1c and 1d). The α-amylase activity of samples subjected to GAPT (50) was significantly higher than that of samples subjected to GAPT (10) 1, 3, 5, and 10 d after sowing,

Table 1 Effects of gibberellin A3 (GA_3) priming of seeds of ZH10 on seedling emergence and mesocotyl and coleoptile growth at a 5-cm sowing depth

Treatment	Seedling emergence rate (%)		Mesocotyl length at 10 d (cm)	Coleoptile length at 10 d (cm)
	7 d	10 d		
CK	0[c]	62.00±2.89[b]	0.59±0.31[c]	3.16±0.37[b]
GAPT (0)	22.00±2.89[b]	73.00±5.77[b]	0.61±0.27[c]	3.32±0.41[b]
GAPT (10)	40.00±0.00[a]	88.00±2.89[a]	0.87±0.25[b]	3.81±0.36[a]
GAPT (50)	42.00±2.89[a]	95.00±5.00[a]	1.15±0.32[a]	3.79±0.28[a]
GAPT (100)	45.00±5.00[a]	95.00±0.00[a]	1.09±0.38[a]	3.89±0.43[a]

CK, control; GAPT (0), priming treatment with 0 mg/L GA_3; GAPT (10), priming treatment with 10 mg/L GA_3; GAPT (50), priming treatment with 50 mg/L GA_3; GAPT (100), priming treatment with 100 mg/L GA_3. Values are shown as mean±standard deviation (SD) of three replicates (20 seeds primed with different GA_3 concentrations were considered a replicate). Different letters in the same column indicate significant differences in samples with different treatments at $P<0.05$, according to Duncan's test.

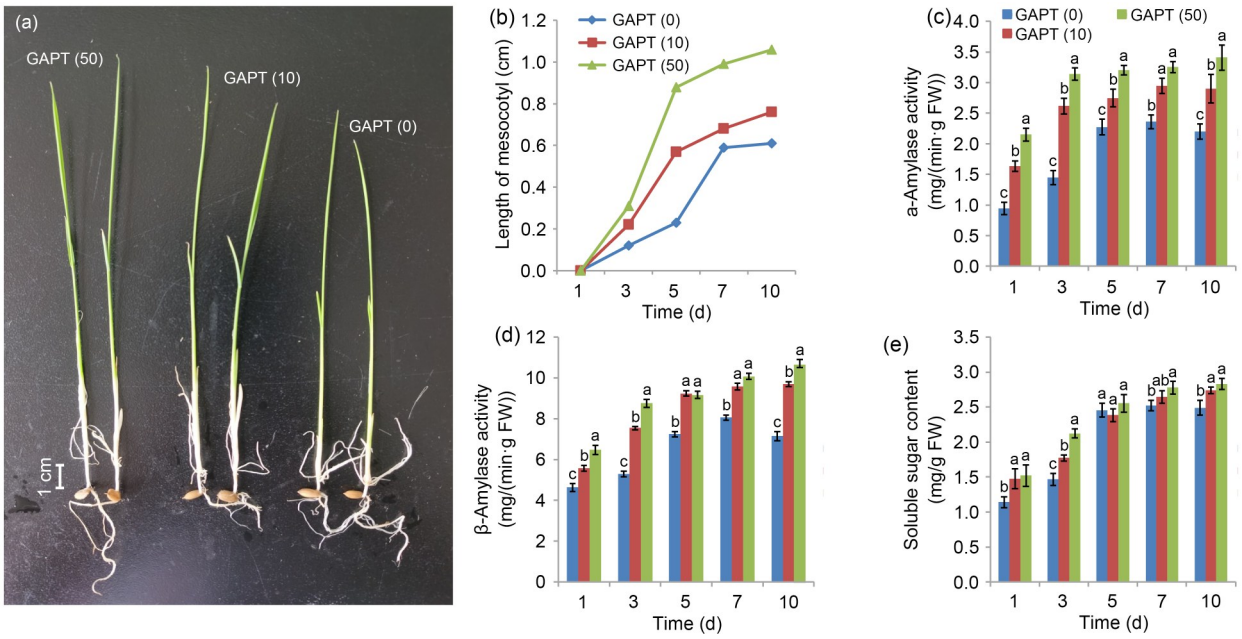


Fig. 1 Phenotypic and physiological assays of ZH10 seedlings under conditions of GAPT (0), GAPT (10), and GAPT (50). (a) Mesocotyl length of ZH10 seedlings under conditions of GAPT (0), GAPT (10), and GAPT (50) 10 d after sowing at the 5-cm sowing depth. (b–e) Mesocotyl length (b), α-amylase activity (c), β-amylase activity (d), and soluble sugar content (e) of ZH10 materials under conditions of GAPT (0), GAPT (10), and GAPT (50) 1, 3, 5, 7, and 10 d after sowing at the 5-cm sowing depth. GAPT (0), priming treatment with 0 mg/L GA_3; GAPT (10), priming treatment with 10 mg/L GA_3; GAPT (50), priming treatment with 50 mg/L GA_3. Values are shown as mean±standard deviation (SD) of three biological replicates. Different letters on columns indicate significant differences among samples with different treatments at the same time point at $P<0.05$, according to Duncan's test. GA_3, gibberellin A3; FW, fresh weight.

but there were no notable differences between the two treatments on the 7th day after sowing (Fig. 1c). Under conditions of GAPT (50), α-amylase activity increased with an increase in culture time, although changes from 3 d after sowing were not significant. The α-amylase activity of ZH10 seedlings under the other two treatment conditions stopped increasing 7 d after sowing, and under conditions of GAPT (0), they even began to decline. The change in β-amylase activity showed a trend similar to that of α-amylase activity (Fig. 1d). The soluble sugar content did not differ significantly between GAPT (0) and GAPT (10) samples 5 and 7 d after sowing. However, compared with GAPT (0), GAPT (50) showed considerably improved soluble sugar content at all measurement time points, except 5 d after sowing. The soluble sugar content also increased with the increase in culture time under conditions of GAPT (10) and GAPT (50), which was different from the trend observed under conditions of GAPT (0) (Fig. 1e).

Overall, GAPT (50) promoted seedling emergence in dry direct-seeding rice under deep-sowing conditions by increasing the activity of starch-metabolizing enzymes and the content of compatible solutes to supply the energy required for subsequent mesocotyl elongation.

3.2 Transcriptome analysis of rice mesocotyl responses to GA priming under deep-sowing conditions

To understand the molecular mechanisms underlying the regulation of deep-sowing responses by GA priming and to identify genes related to mesocotyl elongation affected by GA priming, we constructed and sequenced three cDNA libraries derived from the rice mesocotyls of GAPT (0), GAPT (10), and GAPT (50) treatments, with biological triplicates. For GAPT (0), GAPT (10), and GAPT (50), we obtained 45.8, 58.9, and 59.8 million clean reads on a HiSeq 2500 platform, consisting of 6.8, 8.9, and 8.8 Gb clean bases (Q30%>93.34), respectively (Table S2). On average, about 96.73% of the reads could be mapped into the rice genome (*O. sativa* L. ssp. *japonica*), and between 86.72% and 93.48% were uniquely aligned, suggesting that the RNA-Seq results were reliable and suitable

for the following analyses (Table S2). Gene expression trends in mesocotyls under conditions of GAPT (0), GAPT (10), and GAPT (50) were illustrated using a Venn diagram (Fig. S1a). Among the genes (average FPKM values >0.2), 25 952 were expressed under all three treatments. Furthermore, 437, 425, and 789 were co-expressed in GAPT (0) and GAPT (10), GAPT (0) and GAPT (50), and GAPT (10) and GAPT (50) mesocotyls, respectively, whereas 1047, 462, and 431 genes were uniquely expressed in GAPT (0), GAPT (10), and GAPT (50) mesocotyls, respectively. The correlation coefficient range was 0.94–0.96 among different samples under all three treatments (Table S3), indicating that gene expression correlations among the three biological replicates were high.

According to the criterion for identifying significant DEGs (FDR value <0.05 and $|\log_2(\text{fold change})| \geq 1$), a total of 7074 DEGs were detected in at least one of the three different comparisons, namely GAPT (50)/GAPT (0), GAPT (10)/GAPT (0), and GAPT (50)/GAPT (10). There were 5466 DEGs (2324 up- and 3142 down-regulated) in GAPT (50)/GAPT (0), 5111 (2360 up- and 2751 down-regulated) in GAPT (10)/GAPT (0), and 671 (201 up- and 470 down-regulated) in GAPT (50)/GAPT (10) comparisons. The number of DEGs in the GAPT (50)/GAPT (10) comparison was the lowest among the three comparisons, indicating that the difference in gene expression patterns between GAPT (50) and GAPT (10) was much lower than those of the other comparisons (Fig. 2a). As illustrated in the Venn diagram, a total of 210 DEGs were found to be co-expressed in all three comparisons (Fig. 2b). Based on the transcript abundance, the co-expressed DEGs were divided into five major clades using a hierarchical clustering analysis, implying their divergent functions in the GA-regulated deep-seeding response (Fig. S1b). For example, the DEGs in Clade I had a significantly lower transcript abundance in GAPT (50), whereas their expression levels increased in GAPT (10) and reached maximum values in GAPT (0). Considering the function of GA in promoting mesocotyl elongation, such expression patterns highlight the negative roles of Clade I genes in rice mesocotyl elongation under deep-sowing conditions. Meanwhile, the DEGs in Clade III and Clade V were considered to be positive regulators because their transcript abundance was significantly higher under conditions of GAPT (50) than under conditions of GAPT (10) or GAPT (0). Furthermore, the co-expression patterns of DEGs in the same clade implied their involvement in similar pathways of deep-seeding responses in rice mesocotyls.

3.3 GO and KEGG enrichment analyses

To understand the function of all DEGs after GAPTs, GO enrichment analysis was conducted using GOATOOLS. With respect to “biological processes,”

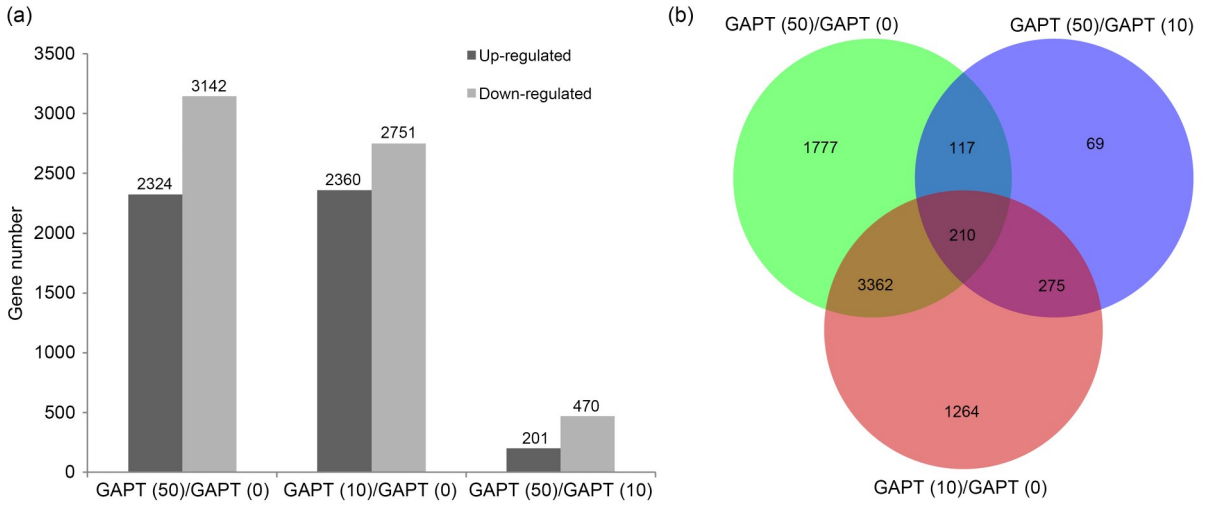


Fig. 2 Comparative analysis of transcriptional changes in rice mesocotyls in response to different GAPT concentrations. (a) Number of DEGs in three different comparisons. (b) Venn diagram showing the distribution of the identified DEGs with more than two-fold changes as well as FDR values less than 0.05 for at least one out of three different comparisons. GAPT (0), priming treatment with 0 mg/L GA_3; GAPT (10), priming treatment with 10 mg/L GA_3; GAPT (50), priming treatment with 50 mg/L GA_3. GA_3, gibberellin A3; FDR, false discovery rate; DEG, differentially expressed gene.

after GAPT (10), these DEGs were enriched mainly in categories such as transcription (GO: 0006375), cell wall polysaccharide metabolic process (GO: 0010383), and cell cycle process (GO: 0022402) (Table S4). The results of GO analysis after GAPT (50) revealed trends similar to those observed after GAPT (10) (Table S4), implying the importance of such biological processes in the responses of rice mesocotyls to GA. In addition, GO terms for response to auxin (GO: 0009733), regulation of hormone levels (GO: 0010817), cell division (GO: 0051301), and brassinosteroid-mediated signaling pathway (GO: 0010817) were significantly enriched after GAPT (50) (Table S4). This is consistent with a study indicating that GA supports auxin-mediated hypocotyl elongation in *Arabidopsis* (Feng et al., 2017), and another study showing that BR-promoted cell division and mesocotyl elongation occur via the suppression of phosphorylation of a U-type cyclin in rice (Sun et al., 2018). This indicates that hormones play a major role in mesocotyl elongation under deep-sowing conditions.

With respect to "molecular functions," GO terms for nucleic acid-binding TF activity (GO: 0001071) were significantly enriched in both GAPT (10)/GAPT (0) and GAPT (50)/GAPT (0) comparisons (Table S4), highlighting the involvement of the TFs in the deep-seeding responses of rice mesocotyls. Our GO enrichment analyses provided valuable insights into the specific biological processes and molecular functions of transcriptome alterations in rice mesocotyls after GAPT under deep-sowing conditions.

To gain a thorough understanding of the pathways altered in rice mesocotyls at the transcriptional level after GAPT under deep-sowing conditions, we subjected the DEGs to pathway enrichment analysis using KEGG pathway database. The plant hormone signal transduction, glyoxylate and dicarboxylate metabolism, diterpenoid biosynthesis, α-linolenic acid metabolism, and phenylpropanoid biosynthesis pathways were significantly enriched in GAPT (10)/GAPT (0) and GAPT (50)/GAPT (0) comparisons (Fig. 3). After GAPT (50), plant hormone signal transduction (map04075) was the most significantly enriched pathway (Fig. 3b). Both GO and KEGG enrichment analyses revealed that hormone signaling was significantly enriched after GAPT, which showed that plant hormones play a critical role in the regulation of rice mesocotyl elongation mediated by GAPT under deep-sowing conditions.

3.4 qRT-PCR validation of differentially expressed genes

To confirm the transcription profiles generated by RNA-Seq and DESeq, we selected 12 genes, including four encoding TFs (*Os01g0646300*, *Os01g0763200*, *Os04g0618600*, and *Os11g0700500*), one related to the GA biosynthetic process (*Os06g0110000*), one related to protein phosphorylation (*Os01g0206700*), three related to the BR-mediated signaling pathway (*Os01g0864000*, *Os07g0580500*, and *Os09g0293500*), and three related to auxin (*Os01g0190300*, *OS01g0764800*, and *Os02g0643800*), to evaluate their expression by qRT-PCR. The results of this analysis were in agreement with those of RNA-Seq (Fig. S2), indicating that our transcriptomic data were highly reliable.

3.5 Expression profiles of transcription factor in mesocotyls after GAPT

Among the DEGs, a total of 210 TFs (77 up- and 133 down-regulated) were identified in GAPT (10)/GAPT (0) comparison, and 218 (59 up- and 159 down-regulated) in GAPT (50)/GAPT (0) comparison (Fig. 4a). The number of down-regulated TFs was always higher than that of up-regulated TFs in the two comparisons. The TFs belonged to the following families: AP2/ERF (ETH responsive factor), C2H2 (Cys2/His2), NAC (NAM, ATAF1/2, CUC2), MYB (v-myb avian myeloblastosis viral oncogene homolog)-related, WRKY (containing WRKYGQK domain), bZIP (basic region/leucine zipper), GRAS (containing conserved C-terminal GRAS domain), HB (homeobox), MYB, TCP, ZF-HD (zinc finger-homeodomain), bHLH (basic helix-loop-helix), B3, and LBD (lateral organ boundaries domain). Compared with those in the control, all members of the TCP (10 genes), ZF-HD (10 genes), B3 (7 genes), and LBD (6 genes) TF families showed down-regulation patterns in response to GAPT (Fig. S3). Most members of the AP2/ERF, bHLH, C2H2, GRAS, and HB TF families were also down-regulated after GAPT (Fig. S3). In contrast, most members of the NAC and MYB-related TF families were up-regulated after GAPT compared with those in the control (Fig. S3). Overall, the number of up-regulated TFs was equivalent to the number of down-regulated TFs in the bZIP and MYB families. Among the down-regulated genes, the largest family was AP2/ERF (31 genes), followed by C2H2 (16 genes), TCP (10 genes), and ZF-HD (10 genes) (Fig. S3). About 152 differentially expressed

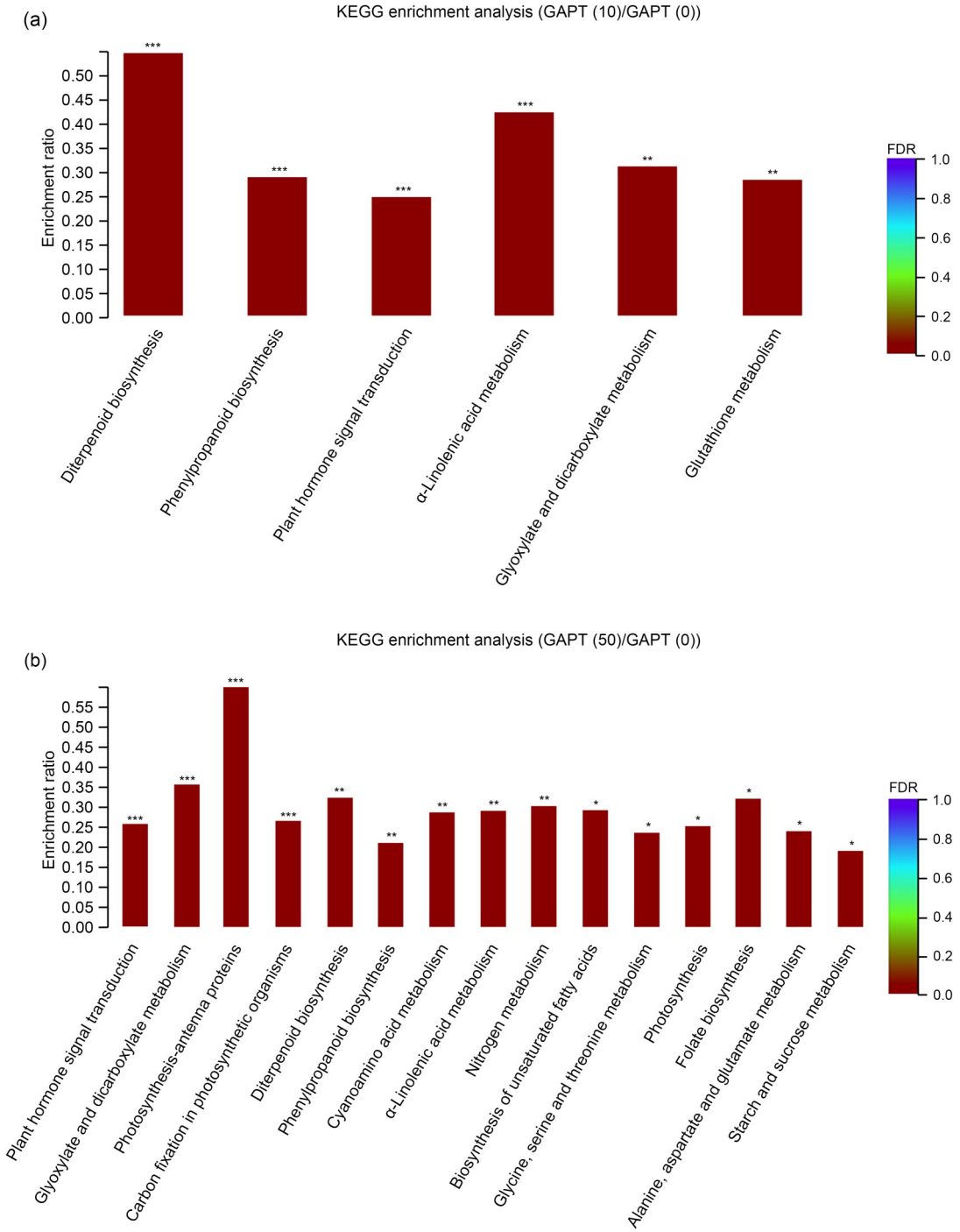


Fig. 3 KEGG pathway enrichment analysis of the effect of GAPT (10) (a) and GAPT (50) (b) on rice mesocotyls under deep-sowing conditions. GAPT (0), priming treatment with 0 mg/L GA_3; GAPT (10), priming treatment with 10 mg/L GA_3; GAPT (50), priming treatment with 50 mg/L GA_3. Pathways with an FDR value less than 0.05 are shown. *, **, and * indicate FDR values lower than 0.001, 0.01, and 0.05, respectively. KEGG, Kyoto encyclopedia of genes and genomes; GA_3, gibberellin A3; FDR, false discovery rate.**

TFs were found to be commonly present under both comparisons (Fig. 4b). One of the co-expressed genes, MYB alternative splicing 1 (*MYBAS1*; *Os11g0700500*), which positively regulates mesocotyl elongation in rice (Jiang, 2018), exhibited increased expression after GAPT (Fig. 4c). *OsTCP5* (*Os01g0763200*), a member

of cell division-regulating TCP family and whose expression is inversely correlated with mesocotyl elongation (Hu et al., 2014), was markedly down-regulated by GAPT (Fig. 4c). The GRAS TF slender 1 (*SLN1*) (*Os01g0646300*), which encodes a DELLA protein, showed a down-regulation pattern after GAPT (Fig. 4c). In *Arabidopsis*, exogenous GA regulates the formation of the functional GA–GID1 (GIBBERELLIN-INSENSITIVE DWARF1)–DELLA complex, leading to the ubiquitination and subsequent degradation of DELLA proteins, and in turn, the release of PIFs promote hypocotyl elongation (Li et al., 2016). Notably, we also observed significantly higher expression of bHLH TF, *PIF1* (*Os04g0618600*) after GAPT compared with the control (Fig. 4c). Nevertheless, there was no solid genetic evidence indicating that the TFs participated in the direct regulation of mesocotyl elongation via GA in the present study.

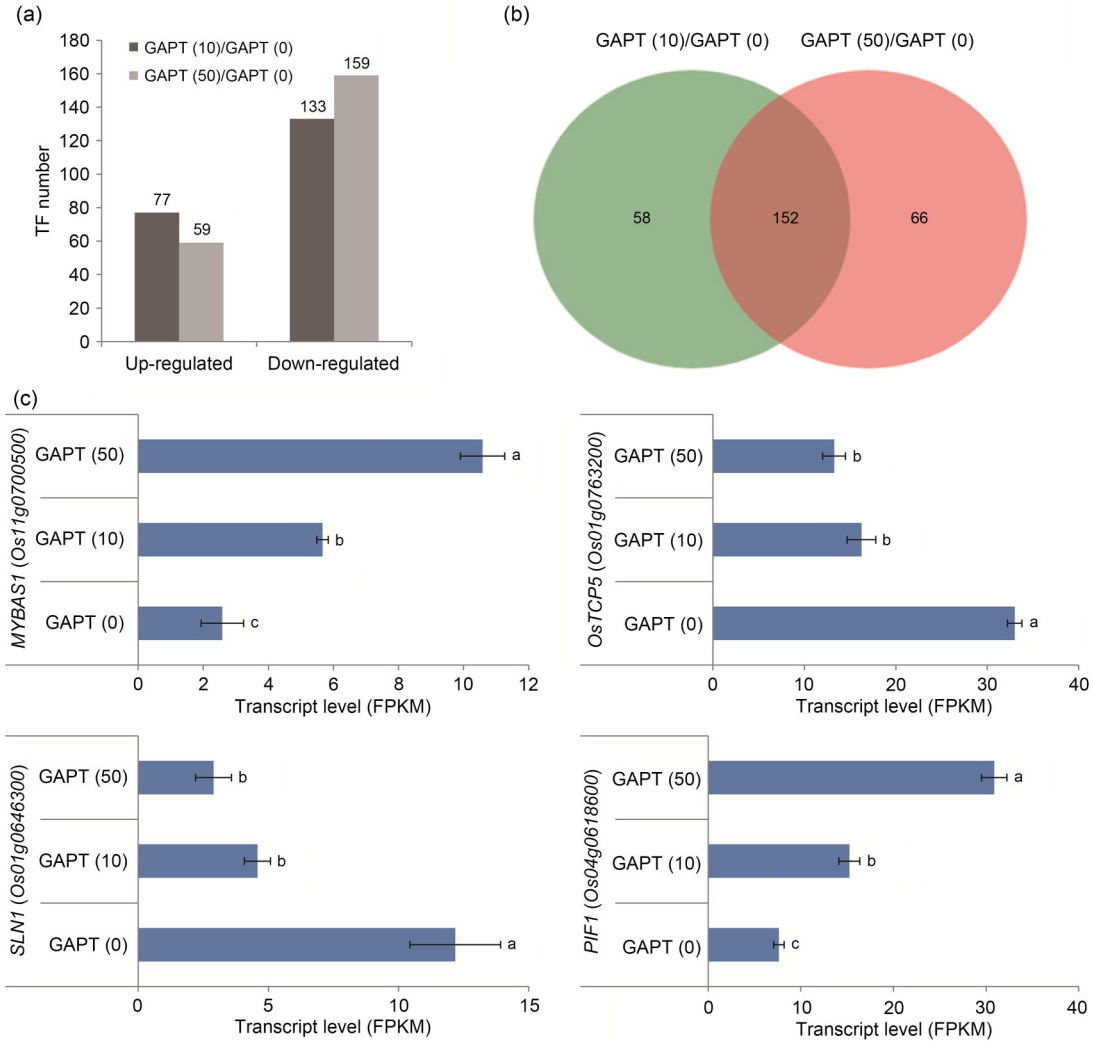
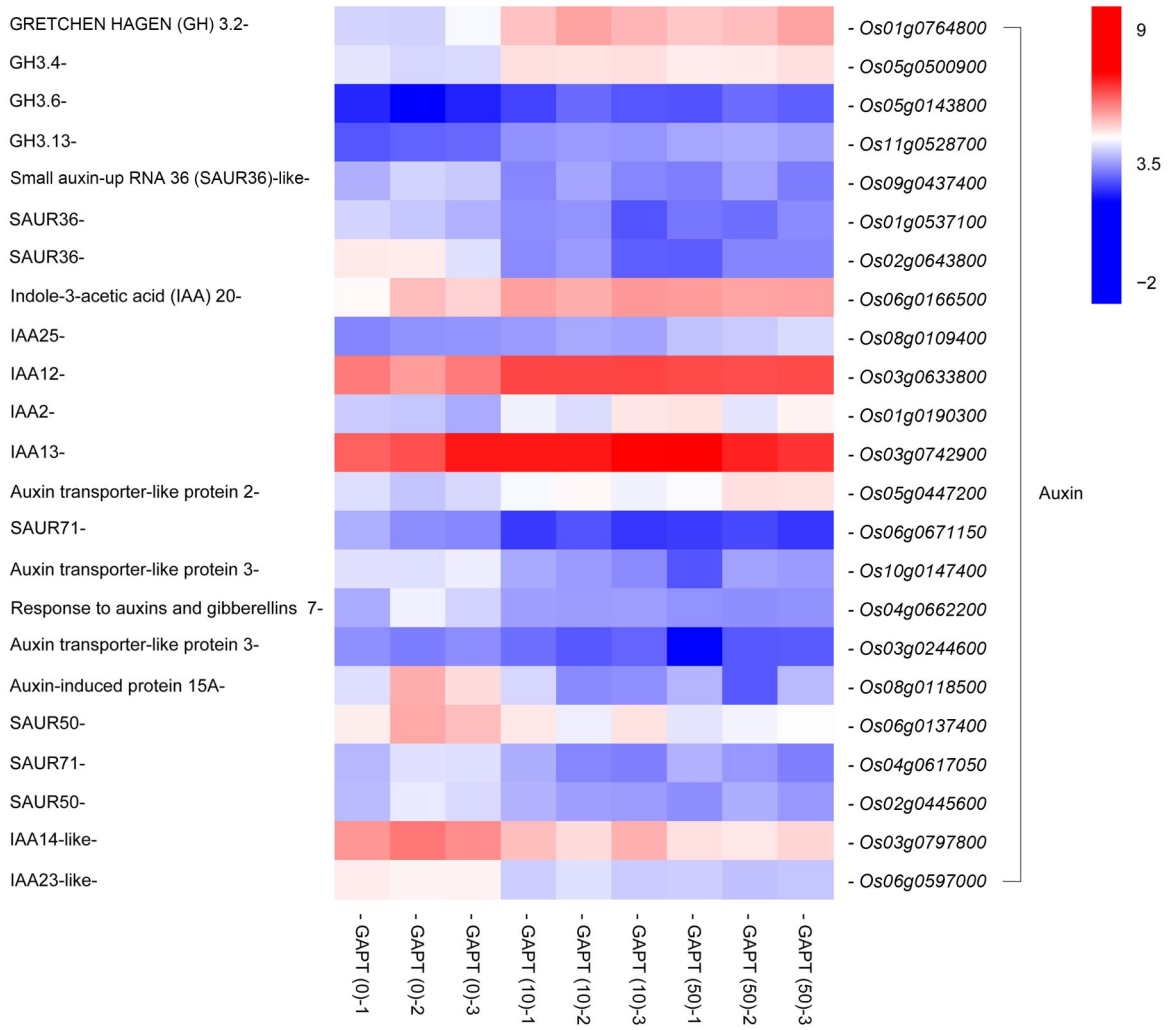


Fig. 4 Expression analyses of transcription factors in mesocotyls after GAPT. (a) Number of transcription factors (TFs) that were differentially expressed in two comparisons; (b) Venn diagram showing the differentially expressed TFs that were commonly detected in the two comparisons; (c) Expression trends of four TFs (*MYBAS1*, *OsTCP5*, *SLN1*, and *PIF1*) in rice mesocotyls under different GAPT concentrations. GAPT (0), priming treatment with 0 mg/L GA_3; GAPT (10), priming treatment with 10 mg/L GA_3; GAPT (50), priming treatment with 50 mg/L GA_3. For each treatment, the gene expression levels are shown as FPKM values by mean±standard deviation (SD) of three biological replicates. Different lowercase letters indicate significant differences among samples under different treatments at $P<0.05$, according to Duncan's test. *MYBAS1*, v-myb avian myeloblastosis viral oncogene homolog (MYB) alternative splicing 1; *OsTCP5*, *Oryza sativa* teosinte branched 1/cycloidea/proliferating cell factor 5; *SLN1*, slender 1; *PIF1*, phytochrome-interacting factor 1; GA_3, gibberellin A3; FPKM, fragments per kilobase of exon per million mapped read.

3.6 Expression patterns of genes associated with plant hormones

The GO and KEGG enrichment analyses revealed that plant hormones play an important role in GA-dependent promotion of mesocotyl elongation in rice (Fig. 3 and Table S4). The expression profiles of genes associated with synthesis or signaling of phytohormones, including auxin, ABA, BRs, GA, and JA, were investigated. Of the 23 DEGs associated with auxin, four GRETCHEN HAGEN 3 (GH3) genes (*Os01g0764800*, *Os05g0500900*, *Os05g0143800*, and *Os11g0528700*) encoding IAA-amido synthetase were up-regulated, whereas three small auxin-up RNA 36 (SAUR36) genes (*Os04g0537100*, *Os04g0537100*, and *Os02g0643800*) encoding auxin-responsive protein were down-regulated after GAPT (10) or GAPT (50). Repressed expression of *SAUR36* by GA was also observed in *Arabidopsis thaliana* (Stamm and Kumar, 2013). The expression of other auxin signaling genes was enhanced or reduced by GAPT (Fig. 5 and Table S5). Of the nine DEGs associated with the BR-mediated signaling pathway identified in GAPT (50)/GAPT (0), four were up-regulated and five were down-regulated. One up-regulated gene (*Os09g0293500*) encoded BR insensitive 1-like receptor kinase (BRL1), another (*Os08g0342300*) encoded BRL3, and the other two (*Os07g0580500* and *Os02g0233200*) encoded brassinazole-resistant 1 (BZR1) and BZR4, respectively. The five down-regulated genes included one transcription repressor ovate family protein 8-like (*Os01g0864000*), one serine carboxypeptidase 24 (*Os02g0794500*), one TF antagonist of positive regulator of grain length 1 (APG1; *Os05g0139100*), one protein increased leaf inclination 6 (ILI6; *Os03g0171300*), and one protein dwarf and low-tillering-like (DLT; *Os06g0127800*) (Fig. 5 and



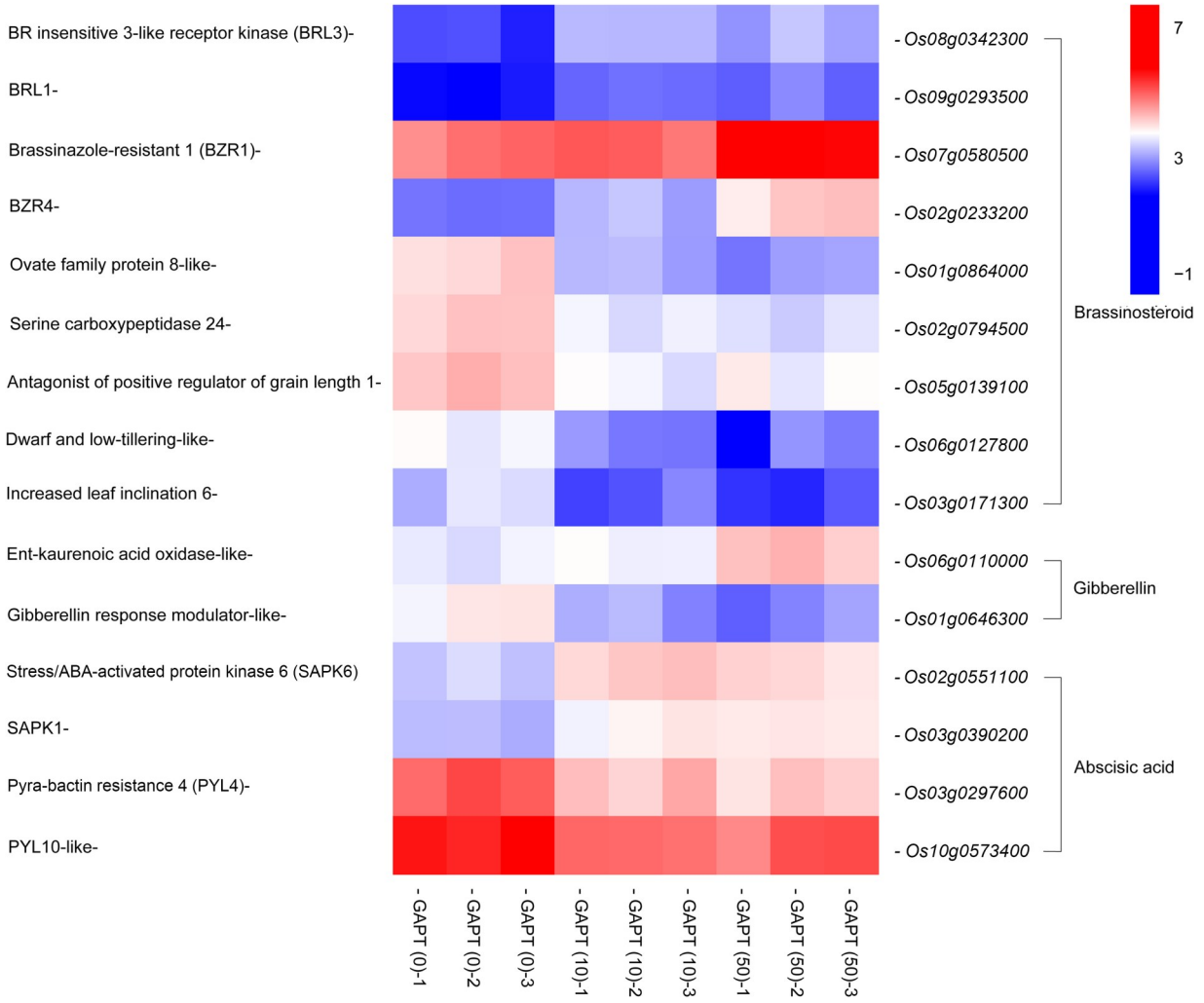


Fig. 5 Expression profiles of some focused DEGs related to phytohormone signaling or synthesis in response to GAPT under deep sowing conditions. The DEGs used here met three criteria: FDR<0.05, $|\log_2(\text{fold change})| \geq 1$, and average FPKM>0.2, as this figure shows. Red and blue colors depict up- and down-regulation, respectively. The scale shows $\log_2$(fold change). ABA, abscisic acid; DEG, differentially expressed gene; GAPT, priming treatment with gibberellin A3 (GA_3); FDR, false discovery rate; FPKM, fragments per kilobase of exon per million mapped read.

Table S5). We found that one gene (*Os06g0110000*) encoding an ent-kaurenoic acid oxidase-like (KAO) involved in GA biosynthesis was up-regulated, and one gene (*Os01g0646300*) encoding a GA response modulator was down-regulated after GAPT (Fig. 5 and Table S5). Of the four ABA signaling genes, the expression of two genes (*Os03g0390200* and *Os02g0551100*) encoding stress/ABA-activated protein kinases (SAPK1 and SAPK6) was induced, and that of two ABA receptor (pyra-bactin resistance 4 (PYL4) and PYL10) genes (*Os03g0297600* and *Os10g0573400*) was repressed by GAPT (Fig. 5 and Table S5). Fifteen genes associated with the JA signaling pathway were identified, including five (four up- and one down-regulated) encoding ZIM domain-containing proteins and ten zinc-finger protein genes (eight up- and two down-regulated) (Table S5).

3.7 Expression patterns of genes associated with starch and sucrose metabolisms

KEGG pathway enrichment analysis revealed that 23 genes (19 up- and 4 down-regulated) related to starch and sucrose metabolisms were differentially expressed in response to GAPT. Of the 12 DEGs encoding α- or β-glucosidase, which belong to the glycoside hydrolase family, 9 exhibited up-regulated

expression patterns (Fig. 6 and Table S6). Among the 19 up-regulated genes, 2 genes (*Os06g0713800* and *Os08g0473600*) encoded α-amylases (AMY2A and AMY1.4) belonging to the Amy1 and Amy2 subfamilies, respectively (Fig. 6 and Table S6). All genes of the Amy1 and Amy2 subfamilies of rice have been shown to be under phytohormonal control because the *cis*-acting GA response element is conserved in their promoters (Park et al., 2010). Two adenosine diphosphate-glucose pyrophosphorylase (ADP-Glc PPase) genes (*Os01g0633100* and *Os03g0735000*) were up-regulated (Fig. 6 and Table S6). One gene (*Os01g0190400*) encoding a hexokinase (HXK) exhibited increased expression after GAPT (50) (Fig. 6 and Table S6). Three sucrose metabolism-related genes, including two sucrose synthase (SUS) genes (*Os03g0340500* and *Os04g0249500*) and one sucrose phosphate synthase (SPS) gene (*Os01g0919400*), showed significantly higher expression after GAPT (Fig. 6 and Table S6). In general, changes in the transcript levels of genes associated with starch and sucrose metabolisms were consistent with the increases in amylase activity and soluble sugar content of dry direct-seeding rice after GAPT under deep-sowing conditions.

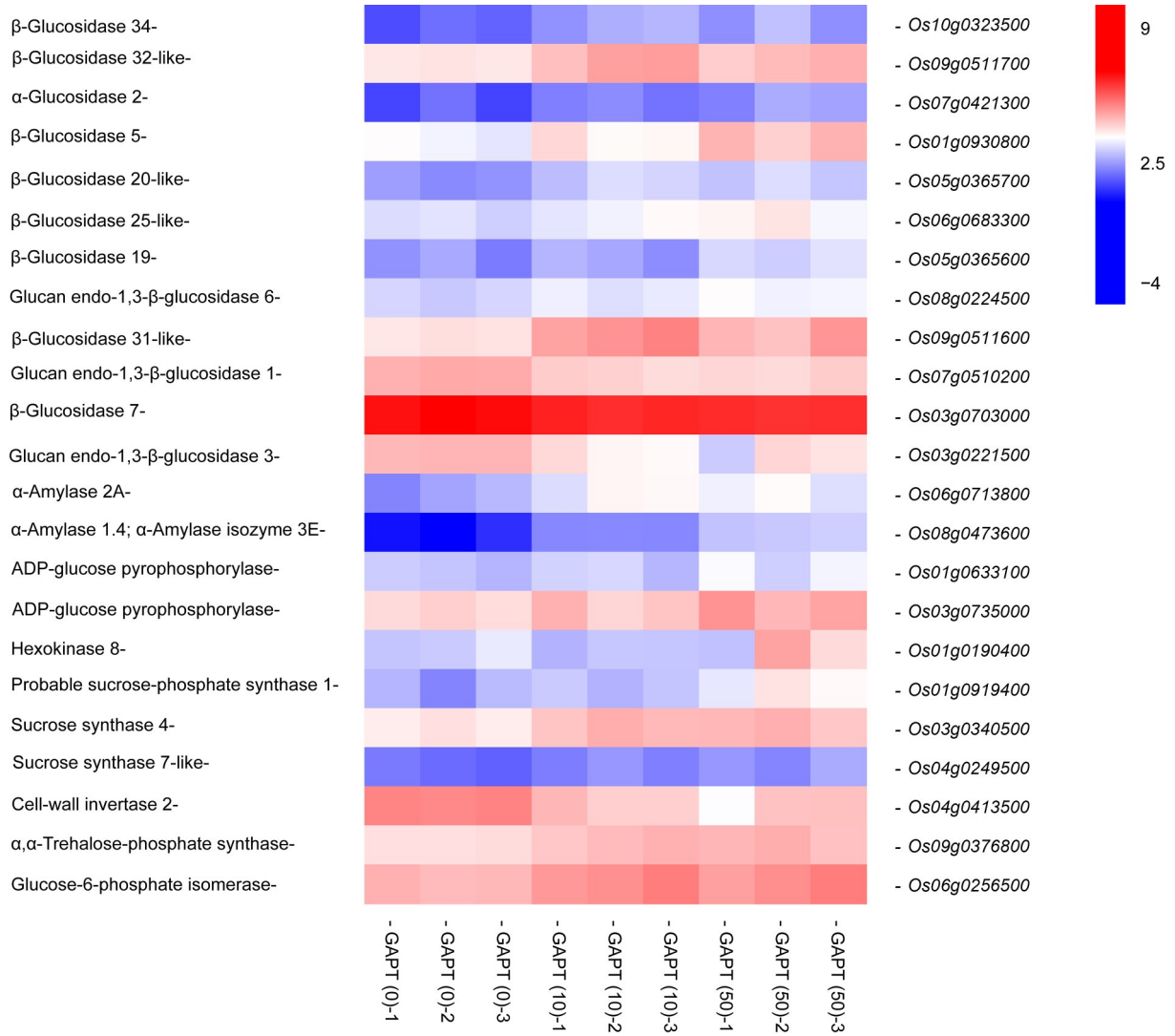


Fig. 6 Expression profiles of some focused DEGs related to starch and sucrose metabolisms in response to GAPT under deep-sowing conditions. The DEGs used here met three criteria: FDR<0.05, $|\log_2(\text{fold change})|\geq 1$, and average FPKM>0.2, as this figure shows. Red and blue colors depict up- and down-regulation, respectively. The scale shows $\log_2$(fold change). DEG, differentially expressed gene; GAPT, priming treatment with gibberellin A3 (GA_3); FDR, false discovery rate; FPKM, fragments per kilobase of exon per million mapped read.

3.8 Expression patterns of candidate DEGs under different sowing depths

To identify potential genes associated with deep-sowing tolerance via GA signaling for genetic improvement, 21 DEGs potentially associated with mesocotyl elongation were selected, and their expression patterns in rice mesocotyls under different sowing depths were studied. The qRT-PCR results showed that 11 genes were relevant to deep-sowing tolerance. With increasing sowing depth, the expression of *MYBAS1*, *PIF1*, *BZR1*, *KAO*, *GH3.2*, *AMY2A*, and *AMY1.4* increased, although the changes in the expression of *KAO* and *AMY2A* were not obvious at the 5-cm sowing depth compared with at the 3-cm sowing depth (Fig. 7a). Conversely, the expression of *OsTCP5*, *SLN1*, *SAUR36*, and mini zinc finger 1 (*MIF1*) was gradually down-regulated along with increasing sowing depth (Fig. 7b). There were no obvious correlations between the sowing depth and the expression of the other ten genes (Fig. S4). These results imply that *MYBAS1*, *PIF1*, *BZR1*, *KAO*, *GH3.2*, *AMY2A*, and *AMY1.4* are positive regulators, whereas *OsTCP5*, *SLN1*, *SAUR36*, and *MIF1* are negative regulators potentially associated with deep-sowing tolerance.

4 Discussion

Seedling emergence under deep-sowing conditions is largely determined by mesocotyl elongation, not by coleoptile elongation (Alibu et al., 2012; Lee et al., 2017). Similarly, in the present study, seed priming with GA improved seedling emergence and the establishment of dry direct-seeding rice mainly via the promotion of mesocotyl elongation (Table 1). As the main factor influencing the germination dynamics of monocotyledonous crops, mesocotyls have been extensively used as experimental systems to study the mechanisms underlying phytohormone regulation of plant growth. However, detailed studies of the biological processes and molecular mechanisms underlying the mediation of mesocotyl elongation by GA priming under deep-sowing conditions have not been reported. To identify the underlying regulatory networks and potential genes involved in GA-regulated mesocotyl elongation and deep-sowing tolerance responses, we analyzed the transcriptomic changes in rice mesocotyls along a 0–10–50-mg/L GA priming gradient for the first time. We identified 7074 DEGs (including 3427 induced genes and 3647 repressed genes) in rice mesocotyl transcriptomes (Fig. 2), indicating that the priming of seeds with GA can induce changes in the expression of numerous genes in rice mesocotyls. GO and KEGG enrichment analyses revealed that the genes whose expression changed after GAPT were largely related to TFs and plant hormone biosynthesis or signaling (Fig. 3 and Table S4). This result was expected, as TFs and plant hormones play important roles in the promotion of mesocotyl elongation by GA.

Phytohormones regulate the expression of TFs (Feng et al., 2017), and transcriptional regulation plays a very important role in regulating plant growth and

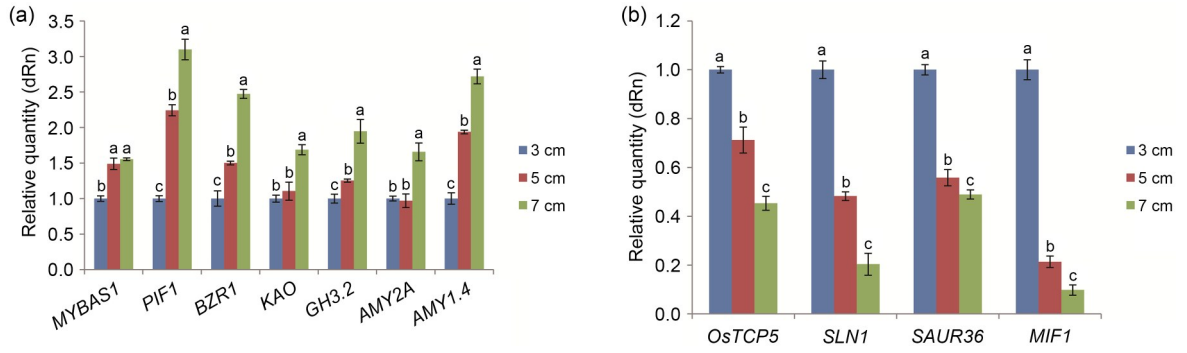


Fig. 7 Expression trends of positive (a) and negative (b) regulators potentially associated with mesocotyl elongation at different sowing depths. Values are shown as mean±standard deviation (SD) of three biological replicates. Different letters on columns represent significant differences among samples under different sowing depths at $P<0.05$ based on Duncan's test. dRn, delta normalized reporter; *MYBAS1*, v-myb avian myeloblastosis viral oncogene homolog (MYB) alternative splicing 1; *PIF1*, phytochrome-interacting factors 1; *BZR1*, brassinazole-resistant 1; *KAO*, ent-kaurenoic acid oxidase-like; *GH3.2*, GRETCHEN HAGEN 3.2; *AMY*, amylase; *OsTCP5*, *Oryza sativa* teosinte branched 1/cycloidea/proliferating cell factor 5; *SLN1*, slender 1; *SAUR36*, small auxin-up RNA 36; *MIF1*, mini zinc finger 1.

development. In the present study, 428 TF genes belonging to 14 TF families were found to respond to GAPT (10) or GAPT (50) (Fig. 4a). These numerous TF genes suggest that in rice mesocotyls the transcriptional regulation in response to GA priming treatments is complicated. Among the 292 down-regulated TFs, TCP, ZF-HD, and LBD family genes, whose members were all down-regulated, could be considered as important candidate genes (Fig. S3). TCP TFs, including 13 class I and 11 class II TCPs, are involved in regulating a variety of biological processes such as cell division, cell differentiation, plant hormone signaling, and organogenesis (Zhang et al., 2019). SL- and CK-regulated OsTCP5, a member of class II TCPs which repress cell growth and proliferation, negatively controls cell division during rice mesocotyl elongation (Hu et al., 2014). Overexpression of *MIF1*, which is a member of the ZF-HD family, induced a reduction in hypocotyl length via multiple hormonal (GA, auxin, and brassinosteroid) regulation processes in *Arabidopsis* (Hu and Ma, 2006). In transgenic rice plants overexpressing *LBD12-1*, an LBD family TF, symptoms of abnormal growth and development, such as stunted growth and reduced shoot apical meristem (SAM), were observed (Ma et al., 2017). The results of the present study indicated that the TCP, ZF-HD, and LBD TFs play important roles as negative regulators in GA-mediated regulation of mesocotyl elongation.

In plants, bHLH TFs are involved in the regulation of cell elongation in response to GA (Ikeda et al., 2012). The GRAS TF family is unique, and its members are abundantly expressed in higher plants, with diverse functions during plant growth and development, e.g., GA signal transduction (Yang et al., 2018). PIF proteins, which can be induced in dark conditions, belong to the bHLH-type TF subfamily and are directly involved in the regulation of numerous target genes, whereas DELLA proteins are a group of plant-specific GRAS TFs that negatively regulate GA signaling (Hirano et al., 2012; Soy et al., 2014; Paik et al., 2017). In *Arabidopsis*, the functions of PIFs and DELLAs in regulating hypocotyl elongation have been intensively studied. DELLA proteins directly bind to the GA receptor GID1 to form a functional GA–GID1–DELLA complex in the presence of GA. This leads to the ubiquitination and subsequent degradation of DELLAs via the ubiquitin–proteasome system, which in turn releases PIF proteins to accelerate hypocotyl growth. Conversely, in the absence of GA, the accumulated DELLAs sequester PIFs away from their target promoters and promote their degradation by the 26S proteasome, which can result in short hypocotyls (Li et al., 2016). Loss of four major PIFs (PIF1, PIF3, PIF4, and PIF5) leads to the inhibition of hypocotyl elongation even when plants are grown in the dark, whereas plants with overexpressed individual PIF genes show elongated hypocotyls under light exposure (Zheng et al., 2016). In rice, elongated mesocotyls were detected in seedlings overexpressing *OsPIL14* (PIF-like14) or those missing the DELLA protein slender rice 1 (SLR1) in the dark (Mo et al., 2020). Consistently, in the present study, the expression of one gene encoding bHLH TF PIF1 was up-regulated, and that of one DELLA protein gene (*SLN1*) was down-regulated after treatment with exogenous GA (Fig. 4c). MYB TFs have been found to be involved in the regulation of cell cycle progression, cell differentiation, and other developmental processes in plants. Overexpression of MYB hypocotyl elongation-related (*MYBH*) markedly promoted hypocotyl elongation by increasing PIF accumulation and subsequently enhancing auxin biosynthesis in *A. thaliana* (Kwon et al., 2013). In rice, a knock-out mutant of *MYBAS1*, a rice MYB TF, exhibited a shorter mesocotyl at a 4-cm sowing depth, indicating that MYBAS1 is a positive regulator of rice mesocotyl elongation (Jiang, 2018). Similarly, in the present study, *MYBAS1* expression increased significantly with the elongation of the mesocotyl following GAPT (Fig. 4c).

Plant hormones and other associated factors influence mesocotyl and coleoptile growth (Watanabe et al., 2001; Hu et al., 2014). Here, drastic changes in the expression of genes related to phytohormones were detected in response to GAPT (Fig. 5 and Table S5). Generally, plant hormones such as GA and auxins are believed to have "growth-promoting" effects, and they regulate various growth and developmental processes throughout plant life cycles. The crosstalk between GA and auxin pathways has been discussed for many years. Each of these phytohormones promotes the biosynthesis of the other (Frigerio et al., 2006). The *GH3* genes, which are early auxin-responsive genes, encode IAA-amido synthetases, which positively modulate active auxin homeostasis by binding IAA with excess amino acids to form amino acid conjugates (Staswick et al., 2005). It has been reported that the down-regulated

GH3 genes inhibit hypocotyl elongation under exposure to light (Feng et al., 2017). The present study showed that all four *GH3* genes were significantly up-regulated in response to GAPT (Fig. 5 and Table S5). GAPT also induced the expression of KAO (Fig. 5 and Table S5), which led to the conversion of ent-kaurenoic acid to GA_{12}, the precursor of all GAs. Only one *KAO*-like gene has been identified in rice. A previous study showed that mutation of *OsKAO* resulted in the generation of a severely dwarfed phenotype, and active GA levels in the *OsKAO* mutant decreased drastically relative to those in the wild type (Sakamoto et al., 2004). The enhanced expression of *KAO* after GAPT implied that exogenous GA promoted mesocotyl elongation and seedling emergence of dry direct-seeding rice under deep-sowing conditions by regulating the activity of enzymes involved in GA metabolism. However, GAPT repressed a gibberellin response modulator-like gene encoding SLN1 (Fig. 5 and Table S5), which is also a GRAS TF and negatively regulates GA responses, mainly by sequestering TFs. These studies suggest that GAPT modulates mesocotyl growth by regulating auxin and GA signaling in combination to control critical plant developmental processes. Furthermore, we found that GAPT induced the expression of *BRL1*, *BRL3*, and *BZR1* (Fig. 5 and Table S5). The BR signaling pathway is essential for GA-mediated promotion of hypocotyl elongation. As functional BR receptors, only BRL1 and BRL3 have the ability to bind the most active BR. BZR1 is a BR-activated TF required for GA-mediated promotion of hypocotyl elongation together with PIFs (Caño-Delgado et al., 2004; Bai et al., 2012; Oh et al., 2012). GAs promote cell elongation and plant development, at least in part, through releasing DELLA-mediated inhibition of BZR1 (Li and He, 2013). BRs enhance GA accumulation by regulating the expression of GA metabolic genes to promote cell elongation in rice (Tong et al., 2014). Furthermore, GAs regulate shade-induced hypocotyl elongation downstream of mutual promotion of auxin and BRs in soybean (Jiang et al., 2020). The upregulation of *BRL1*, *BRL3*, and *BZR1* revealed the importance of BR signaling in the promotion of rice mesocotyl elongation mediated by GA priming.

Mesocotyl elongation length was found to be significantly correlated with α-amylase activity, β-amylase activity, and soluble sugar content during the germination of weedy rice. Enhanced amylase activity at the beginning of weedy rice germination promoted starch degradation, supplying materials and energy required for mesocotyl elongation (Ma et al., 2014). GAs substantially impact carbohydrate (including starch and soluble sugars) contents during seed germination. In higher plants, starch granules composed of amorphous amylose and semi-crystalline amylopectin provide plants with energy for seed germination and seedling growth in the dark (Carciofi et al., 2012). While ADP-Glc PPase is an essential starch biosynthetic enzyme that produces ADP-Glc for starch biosynthesis (Stewart et al., 2011), HXK plays a major role in the regulation of sugar sensing and signaling as a dual-function enzyme involved in the formation of hexose-6-phosphate, which is essential for starch synthesis. HXK1, the first Glc sensor identified in plants, has a positive function in Glc promotion of *Arabidopsis* hypocotyl elongation in the dark (Zhang and He, 2015). Our transcriptomic data showed increased expression of two ADP-Glc PPase genes and an HXK gene in rice mesocotyls after GAPT (50) (Fig. 6 and Table S6). GA-induced α-amylase has also been reported to play a very important role in the degradation of starch to provide substrates and energy for seed germination and seedling establishment (Kaneko et al., 2002; Kato-Noguchi and Macías, 2005; He et al., 2019). Increased α-amylase activity in the samples with GAPT was detected (Fig. 1c). Correspondingly, we observed significantly higher expression of two α-amylase genes, *AMY2A* and *AMY1.4*, in rice mesocotyls in response to GAPT (Fig. 6 and Table S6).

The expression of other genes related to sucrose biosynthesis and metabolism, such as two SUS genes and one SPS gene, was also enhanced in the present study (Fig. 6 and Table S6). Previous studies have shown that sugars (including fructose, sucrose, and glucose) can stimulate *Arabidopsis* hypocotyl elongation in the dark when GAs are present (Zhang et al., 2010). SUS is known to catalyze the reversible reaction of sucrose with uridine diphosphate (UDP) to form UDP-glucose and UDP-fructose, whereas SPS is responsible for sucrose biosynthesis (Jiang et al., 2015), indicating their essential functions in the promotion of mesocotyl growth and seedling emergence by GAPT. The enhanced expression of SUS and SPS genes was consistent with observed increases in the soluble sugar content of dry direct-seeding rice after GAPT under deep-sowing conditions (Fig. 1e). It is possible that

GAPT promoted mesocotyl elongation and seedling emergence of dry direct-seeding rice from the deep soil layer by altering the expression of important sucrose and starch metabolism regulator genes to increase amylase activity and soluble sugar content to supply the essential energy.

In summary, we identified 7074 DEGs across GAPT (0), GAPT (10), and GAPT (50) in rice seedlings under deep-sowing conditions. Physiological and transcriptomic analyses suggested that compared with the control, GAPT significantly enhanced mesocotyl elongation and seedling emergence of dry direct-seeding rice under deep-sowing conditions by increasing the expression of essential sucrose and starch metabolism regulator genes, including two *SUS*, one *SPS*, three starch biosynthetic enzyme genes (two *ADP-Glc PPase* and one *HXK*), and two α-amylase genes (*AMY2A* and *AMY1.4*), which in turn increased the amylase activity and soluble sugar content required to supply the energy essential for subsequent developmental processes. The GA priming treatments also enhanced the expression of several genes related to mesocotyl elongation, such as TF genes *PIF1* and *MYBAS1*, as well as plant hormone biosynthesis genes, *GH3.2*, *BZR1*, and *KAO*, while inhibiting the expression of genes negatively correlated with mesocotyl length, such as *SLN1*, *OsTCP5*, *SAUR36*, and *MIF1*, to activate the corresponding pathways, which in turn enhanced deep-seeding tolerance. The expression trends of 11 genes at different sowing depths (Fig. 7) further revealed that *MYBAS1*, *PIF1*, *BZR1*, *KAO*, *GH3.2*, *AMY2A*, and *AMY1.4*, as positive regulators, and *OsTCP5*, *SLN1*, *SAUR36*, and *MIF1*, as negative regulators, are probably involved in the response to deep-sowing tolerance. Therefore, suitable molecular manipulations of these critical genes could facilitate the genetic improvement of deep-sowing tolerance of dry direct-seeding rice.

5 Conclusions

Mesocotyl elongation is the main factor responsible for the emergence and establishment of dry direct-seeding rice seedling under deep-sowing conditions. The data presented in the present study revealed that amylase activity and soluble sugar content were related to mesocotyl elongation and seedling emergence of dry direct-seeding rice mediated by GA priming under deep-sowing conditions. Additionally, transcriptomic analyses indicated that DEGs related to transcription regulation, plant hormone biosynthesis or signaling, and starch and sucrose metabolisms were probably involved in the promotion of rice mesocotyl elongation mediated by GA priming. The gene expression trends under different sowing depths further confirmed that the TF genes *MYBAS1*, *PIF1*, *OsTCP5*, *SLN1*, and *MIF1*, plant hormone biosynthesis or signaling genes *BZR1*, *KAO*, *GH3.2*, and *SAUR36*, and starch and sucrose metabolism genes *AMY2A* and *AMY1.4*, were highly related to deep-sowing tolerance of dry direct-seeding rice.

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Author contributions

Ya WANG contributed to data analysis and writing of the manuscript. Yuetao WANG, Ruifang YANG, Fuhua WANG, Jing FU, Wenbo YANG, Tao BAI, and Shengxuan WANG performed the experimental research and data analysis. Haiqing YIN contributed to the study design, and writing and editing of the manuscript. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Ya WANG, Yuetao WANG, Ruifang YANG, Fuhua WANG, Jing FU, Wenbo YANG, Tao BAI, Shengxuan WANG, and Haiqing YIN declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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Supplementary information

Tables S1–S6; Figs. S1–S4