Rice

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A Cyclin Gene *OsCYCB1;5* Regulates Seed Callus Induction in Rice Revealed by Genome Wide Association Study

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Abstract

Plant tissue culture is extensively employed in plant functional genomics research and crop genetic improvement breeding. The callus induction ability is critical for utilizing Agrobacterium-mediated genetic transformation. In this study, we conducted a genome-wide association study (GWAS) utilizing 368 rice accessions to identify traits associated with callus induction rate (CIR), resulting in the identification of a total of 104 significant SNP loci. Integrated with gene function annotation and transcriptome analysis, 13 high-confidence candidate genes involved in auxin-related, CYC cyclins, and histone H3K9-specific methyltransferase were identified in significant loci. Furthermore, we also verified a candidate gene, *Os05g0493500* (*OsCycB1;5*), and employed the CRISPR/Cas9 system to generate *OsCycB1;5* knockout mutants in rice (*Oryza sativa* L.). The *OscycB1;5* mutant displays significantly reduced callus induction and proliferation capacity, this result indicating *OsCycB1;5* is required for the callus formation in rice. Overall, this study provides several reliable loci and high-confidence candidate genes that may significantly affect callus formation in rice. This information will offer valuable insights into the mechanisms underlying callus formation not only in rice but also in other plants.

Keywords Rice, Callus induction rate, GWAS, Candidate gene, *OsCycB1*, *5*

Introduction

Rice is the most important food crop worldwide, it serves not only as a significant energy source for humans but also as a prime target for genetic manipulation (Liang et al. 2023). Currently, transgenic breeding has emerged as a pivotal strategy for cultivating elite rice varieties endowed with heightened stress resistance, increased yield, and superior quality. This approach facilitates the transfer of alien genes and enables the rapid introgression of novel traits into breeding programs. Furthermore, genetic transformation plays a foundational role in facilitating gene overexpression, RNA interference, and CRISPR/Cas9-mediated genome editing to elucidate gene function. However, the genetic transformation of plants, particularly in rice and other grass crops, heavily

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relies on the utilization of embryonic callus (EC) derived from embryos. Yet, the induction ability of EC exhibits significant genotype-dependent variation among different rice lines, with a majority of elite lines demonstrating inefficiency in EC formation (Yuan et al. 2001; Liang et al. 2023). Callus induction serves as the initial step in tissue culture, influencing subsequent differentiation and transformation efficiency (Zhang et al. 2019b). Therefore, efficient callus generation is pivotal for tissue culture propagation of plant species that are challenging to regenerate and for the genetic engineering process aimed at achieving desired phenotypic traits (Tuskan et al. 2018). Consequently, unraveling the genetic basis of EC induction holds promise for enhancing the efficiency of genetic transformation in rice (Liang et al. 2023).

Previous studies have indicated that the capability of rice embryos to induce EC is a quantitative trait controlled by nuclear genes and has a high heritability (Climenco et al. 2010; Liang et al. 2023). Numerous callus induction traits have been defined in rice, and quantitative trait loci (QTLs) associated with these traits have been successively identified (Table. S1). For instance, using 140 recombinant inbred lines (RILs) from the 93−11 × Nipponbare (NIP) cross and a high-resolution SNP-based genetic map, Li et al. (2013) identified two CIR-related QTLs, *qCIR-10* and *qCIR-12*. At present, several genes responsible for callus induction have been identified in numerous plant species. For instance, in *Arabidopsis*, genes such as *LBD* family genes (Fan et al. 2012) and *BBM* (Boutilier et al. 2002) are involved in callus formation through auxin induction (Ikeuchi et al. 2013). Studies in cotton have indicated that *WOX* genes play a pivotal role in callus induction (Muhammad Tajo et al. 2022). As everyone knows, the application of exogenous auxin and cytokinin can induce callus formation in various plant species. Typically, an intermediate ratio of auxin to cytokinin favors callus induction, whereas a high auxin-to-cytokinin ratio promotes root regeneration and a high cytokinin-to-auxin ratio promotes shoot regeneration (Skoog and Miller 1957; Ikeuchi et al. 2013). For example, auxin downregulates the KIP-RELATED PROTEIN (*KRP*) genes that encode cyclin-dependent kinase (*CDK*) inhibitors, thereby reacquiring cell proliferative competence during Arabidopsis EC formation (Anzola et al. 2010). Additionally, other hormones such as brassinosteroids, abscisic acid, and gibberellin can also induce callus formation and may, in some species, substitute for auxin or cytokinin in this process (Goren et al. 1979; Hu et al. 2000). For instance, the transcription factor ZmMYB138 promotes callus formation via gibberellin signal transduction in maize (Ge et al. 2016). During rice callus formation of the mature embryo, the high expression level of *OsARF18* disrupts the homeostasis of auxin response and represses the activation of PLETHORAs (PLT1 and PLT2) (Zhang et al. 2020). PLETHORA 1 (PLT1) and PLT2 are known to regulate stem cell maintenance, meristematic zone and cell proliferation in *Arabidopsis* (Kornet and Scheres 2009). Mutation of *OsGNOM1* resulted in a reduced rate of callus induction and changed the characteristics of callus in *indica*, but not in *japonica* background. It is likely that *OsGNOM1* may not function in precisely the same way in *indica* and japonica rice subspecies (Liu et al. 2009). RNA interference (RNAi) targeting *OsIAA10* resulted in the suppression of callus formation on tissue culture medium. However, embryo development in the *OsIAA10* RNAi line was not affected by synthetic auxin (2,4-D) treatment, indicating that *OsIAA10* might play a role in auxin perception and the subsequent activation of downstream genes, such as *CRL1*, which are involved in callus induction (Zhang et al. 2019b). Additionally, *OsHDA710* mutation (*hda710*) resulted in impaired callus formation of rice mature embryos and increased global histone H3 acetylation levels. *HDAC* inhibition decreased auxin response and cell proliferation in callus formation (Zhang et al. 2020). In plant tissue culture, the epidermal continuous cell division of the *Oslec1* mutant was lost, and the embryo-like structure could not be formed. The overexpression of *OsLEC1* also led to the formation of partially defective callus, indicating that the appropriate level of *OsLEC1* expression may be important for callus formation in scutellaria baicalensis (Guo et al. 2023).

Currently, GWAS has been widely used in the genetic dissection of complex quantitative traits. This approach has been widely adopted in studying complex quantitative genetic traits in various plant species (Gudi et al. 2023). Previous studies have utilized GWAS to identify genetic factors associated with callus formation in various plant species (Htwe et al. 2024). For example, Liang et al. (2023) employed a combination of GWAS, WGCNA (Weighted Gene Co-expression Network Analysis), and gene-based association studies to delineate the co-expression network and hub genes responsible for embryogenic callus (EC) induction in maize. They identified that ZmARF23 binds to the ZmSAUR15 promoter, thereby regulating its expression and influencing EC induction. Nguyen et al. (2020) analyzed the variation in callus induction of 96 rose genotypes using GWAS and found 32 significantly associated SNPs with callus size. Htwe et al. (2024) explored the loci related to callus induction in oil palm using GWAS and identified 21 significant SNPs related to CIR. Luo et al. (2024) performed a GWAS for CIR in a population of 353 peanut accessions and detected a total of 53 significant SNPs. The first GWAS in rice was conducted in 2010 by Huang et al. (2010), who analyzed 14 agronomic traits in an *indica* population. These traits could be divided into five categories: morphological characteristics, yield components,

grain quality, coloration, and physiological features. More recently, Zhang et al. (2019b) conducted a GWAS on 529 sequenced rice accessions and revealed 88 loci correlated with the rate, speed, and time of callus induction, 21 of which were located in previously reported QTLs (Wu et al. 2022). Although these studies revealed some loci and candidate genes related to callus induction, However, the genetic regulation of callus induction in rice is till not fully elucidated. Therefore, it is necessary to further explore the genetic loci and candidate genes related to the induction ability in rice. This has guiding significance for further utilizing these genes for applications such as genetic transformation.

In this study, a natural population consisting of 368 rice accessions was used to evaluate the callus induction ability, and the CIR was measured ten days after plating on solid medium. Combining 994,188 SNPs, a GWAS was performed to identify the genetic loci and initial candidate genes associated with CIR. We then analyzed candidate genes using a database of transcriptome profiles from embryonic callus formation in rice scutellum. A total of 104 significant SNPs and candidate genes were identified. Moreover, a candidate gene *OsCycB1;5* was validated using CRISPR/Cas9 technology. Altogether, our results provide a basis for further elucidating the molecular mechanism behind CIR and callus induction ability of rice.

Results

Population Structure of the Association Panel

In this study, we collected 368 diverse rice accessions worldwide. To investigate the genetic relationships between these cultivars, a phylogenetic tree analysis was performed. The result of the phylogenetic tree showed that that these 368 rice accessions comprised three genetic groups (K=3) *japonica* cultivars and *indica* cultivars, Among them, *indica* cultivars are divided into two types of landrace and modern cultivar groups (Fig. 1A, C). Principal components analysis (PCA) showed the first two principal components explained 39.92% of the total genetic variation within these 368 rice accessions, and clearly divided into three parts. (Fig. 1B). The population-structure analysis also showed that there is some difference between landrace and modern cultivar. Simultaneously, these results also revealed that some resources are mixed type of *japonica* and *indica*. Based on the r^2 value, which declined to half of the maximum value, a Linkage disequilibrium (LD) decay rate of 44 kb was estimated for the 368 rice accessions (Fig. S1); this indicated the rice accessions studied here harbored a lower LD, and that the candidate genes are likely in 44 kb around the highlight marker.

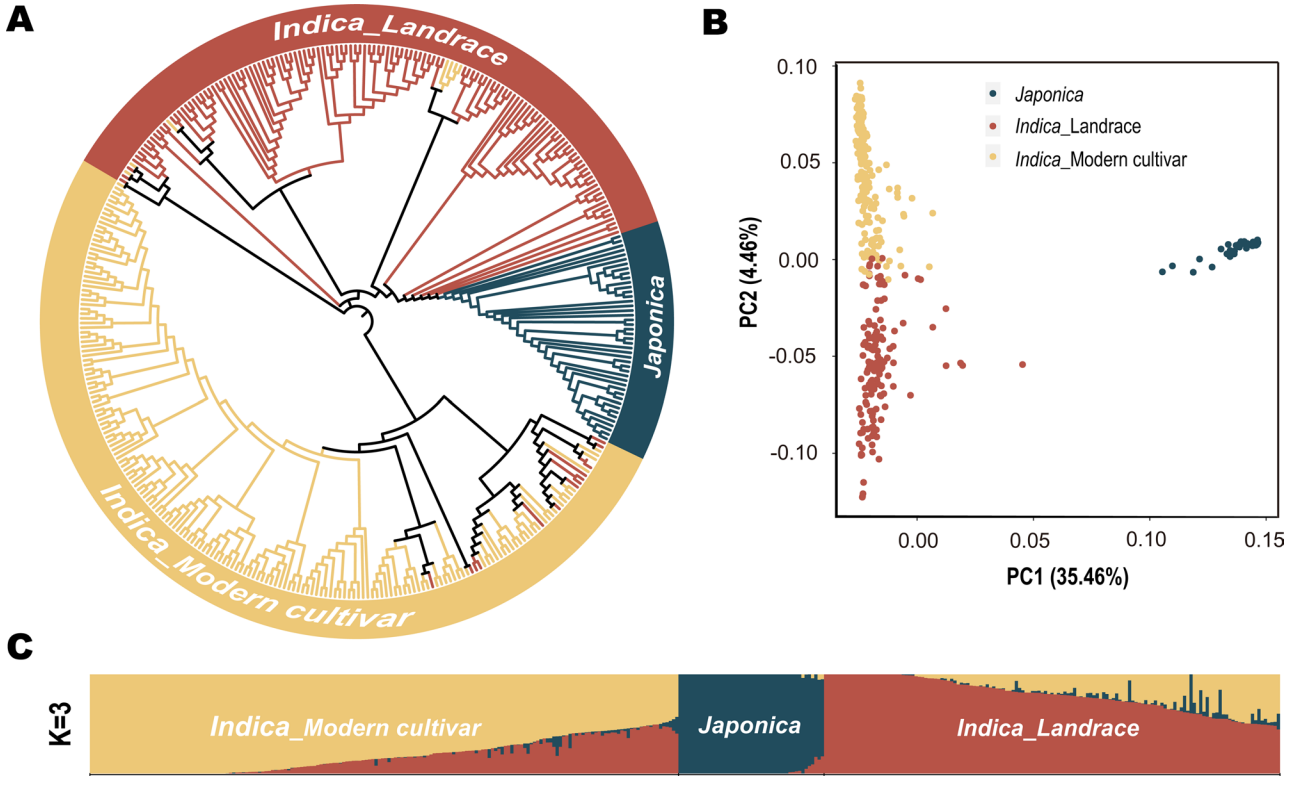

Fig. 1 Population structure of 368 diverse rice accessions. **A**, Phylogenetic tree of 368 rice accessions. **B**, Principal components analysis. **C**, Kinship analysis

Table 1 Statistics of seed CIR in rice populations (10d)

Population	Accessions	Average value	SD	Min	Median	Max
All	368	0.67	0.19	0.04	0.73	0.95
Japonica	45	0.44	0.23	0.04	0.4	0.92
Indica	320	0.71	0.16	0.11	0.75	0.95
Mixed	3	0.6	*0.11*	0.5	0.57	0.72

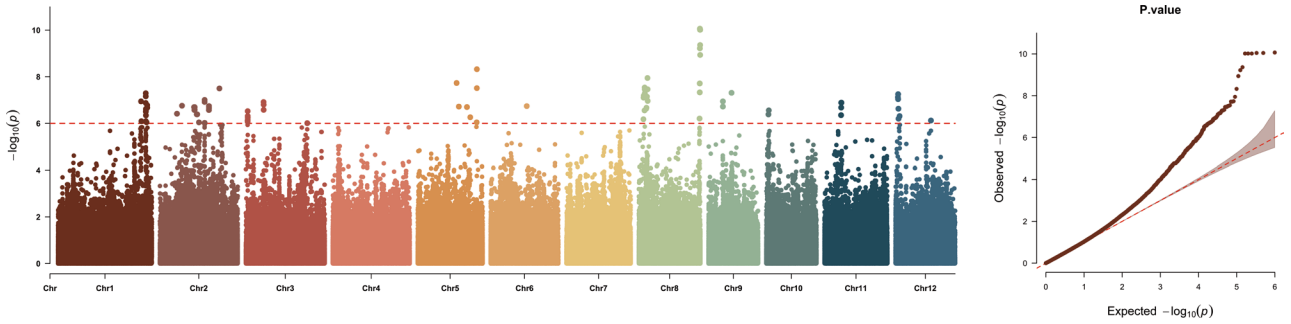

Fig. 2 Manhattan plots and QQ plots for the CIR by MLM. The horizontal red line indicates the genome-wide significance threshold (-lg10(P) = 6)

Callus Induction Ability in Diverse Rice Germplasms

According to the previous research study, the CIR was the most intuitive trait representing callus induction capacity. Therefore, the CIR on the 10th day of inoculation has been used as an index to test the seed tissue culture ability of rice accession in this study. The average values and standard deviations of the CIR for the entire rice population and sub populations are shown in Table 1. The average CIR across all 368 accessions was 67%. In the two main groups (Ind and Jap), the CIR of *indica* was slightly higher, ranging from 0.11 to 0.95, while CIR of *japonica* ranged from 0.04 to 0.92, with a higher standard deviation between accessions. These results suggesting that callus formation and induction capacity is genotype dependent and genetically controlled by multiple genes.

Significant Loci and Candidate Genes Affecting CIR Revealed by GWAS

To identify and localize genetic factors associated with CIR, we used the mixed linear model (MLM) to conduct a GWAS with the data for the average of CIR of 368 rice accessions, analyzed using 994,188 high-confidence SNPs screened via whole-genome resequencing. The QQ plots for the SNPs revealed that the distribution of observed association P-values was similar to the distribution of expected associations, indicating that the false positives in the GWAS in this study were effectively controlled (Fig. 2). When the *P* value was less than 10^{-6}, 104 significant SNPs ($P<0.000001$) were identified with CIR (Fig. 2 and Table. S3). These SNP markers were distributed widely on 10 chromosomes in the rice genome, except for chromosome 4 and 7. Base on the LD, significant SNPs within ~44 kb up and down genomic regions has been considered to be the same QTL. These significant SNPs were colocalized and formed large conspicuous cluster, and delineated into a same QTL for CIR. such as QTL *qCIR1.3*, *qCIR2.9*, *qCIR5.5*, *qCIR8.11*, *qCIR8.12* and *qCIR11.1* contained 10, 8, 3, 3, 4 and 3 SNPs, respectively.

According to the LD decay (44 kb) of this association panel, genes located 44 kb upstream or downstream of the lead SNPs of the significant loci associated with CIR were considered as candidate genes involved in rice callus induction. Based on gene annotation information and known functional homologs, we identified 13 reasonable candidate genes that might be involved in the processes of callus formation, including those related to auxin, CYC cyclins, and histone H3K9-specific methyltransferase (Table 2). Among these genes, *OsLSD1* encodes a rice zinc finger protein that has been reported to play an important role in callus differentiation in rice (Wang et al. 2005). This finding indicates that the variations in *OsLSD1* are likely related to CIR in our study.

To assess the responses of the candidate genes to callus formation, the candidate genes were analyzed using a database of transcriptome profiles from embryonic callus formation in rice scutellum. The expression of 13 candidate genes was analyzed at six EC-induction stages (Table. S4). Among these genes, *OsSDG715* and *OsCycB1;5* were down-regulated at all of the stages in *OEMIR393b* line compared to wild-type NIP. These indicated that *OsSDG715* and *OsCycB1;5* may involved in the process of callus formation in rice, because overexpression of *MIR393b* resulted in the loss of scutellum-derived callus formation on CIM. However, the expression level of *OsLSD1* and LC4 in *OEMIR393b* line were higher than that in NIP at each of the stages. These results preliminarily revealed that the difference of expression patterns of candidate genes in different lines could be an important factor which influenced the callus induction capacity in rice (Fig. 3).

Table 2 Summary of significantly associated loci and candidate genes identified by genome-wide association studies

QTL	Chr.	Lead SNP	*P*-value	Gene Locus	Gene Annotation (Gene Name)
qCIR1.3	1	1_40488340	6.89E-08	*Os01g0923700*	Histidine kinase (*OHK2*)
	1	1_40488340	6.89E-08	*Os01g0923900*	LEAF INCLINATION 4 (*LC4*)
qCIR1.6	1	1_41173465	2.11E-07	*Os01g0936000*	Glutaredoxin (*OsGRX7*)
qCIR2.9	2	2_20643333	9.86E-08	*Os02g0550600*	Alkaline/neutral invertase gene; short-root5 gene (*OsCYT-INV1*; *SRT5*)
qCIR5.5	5	5_24247048	5.44E-07	*Os05g0493500*	Rice cyclin gene (*CycB1; 5*)
	5	5_27297062	4.73E-09	*Os05g0547850*	programmed cell death 5 (*OsPDCD5*)
qCIR8.4	8	8_2781649	5.10E-08	*Os08g0148267*	Oxidoreductase family, NAD-binding Rossmann fold containing protein
qCIR8.5	8	8_3412934	2.35E-07	*Os08g0159500*	Rice Zinc Finger Protein (*OsLSD1*)
qCIR8.11	8	8_28255770	9.64E-11	*Os08g0561500*	Auxin-induced protein 5NG4
qCIR8.12	8	8_28313290	9.64E-11	*Os08g0565700*	Histone H3K9 Methyltransferase Gene (*SDG715*)
qCIR11.1	11	11_7593068	4.44E-07	*Os11g0242500*	Cyclin-dependent kinase
qCIR12.2	12	12_1183174	9.12E-08	*Os12g0124700*	Putative DNA replication initiation protein (CDC45A)
qCIR12.5	12	12_1886384	4.88E-07	*Os12g0139400*	A-type response regulator gene (*OsRR10*)

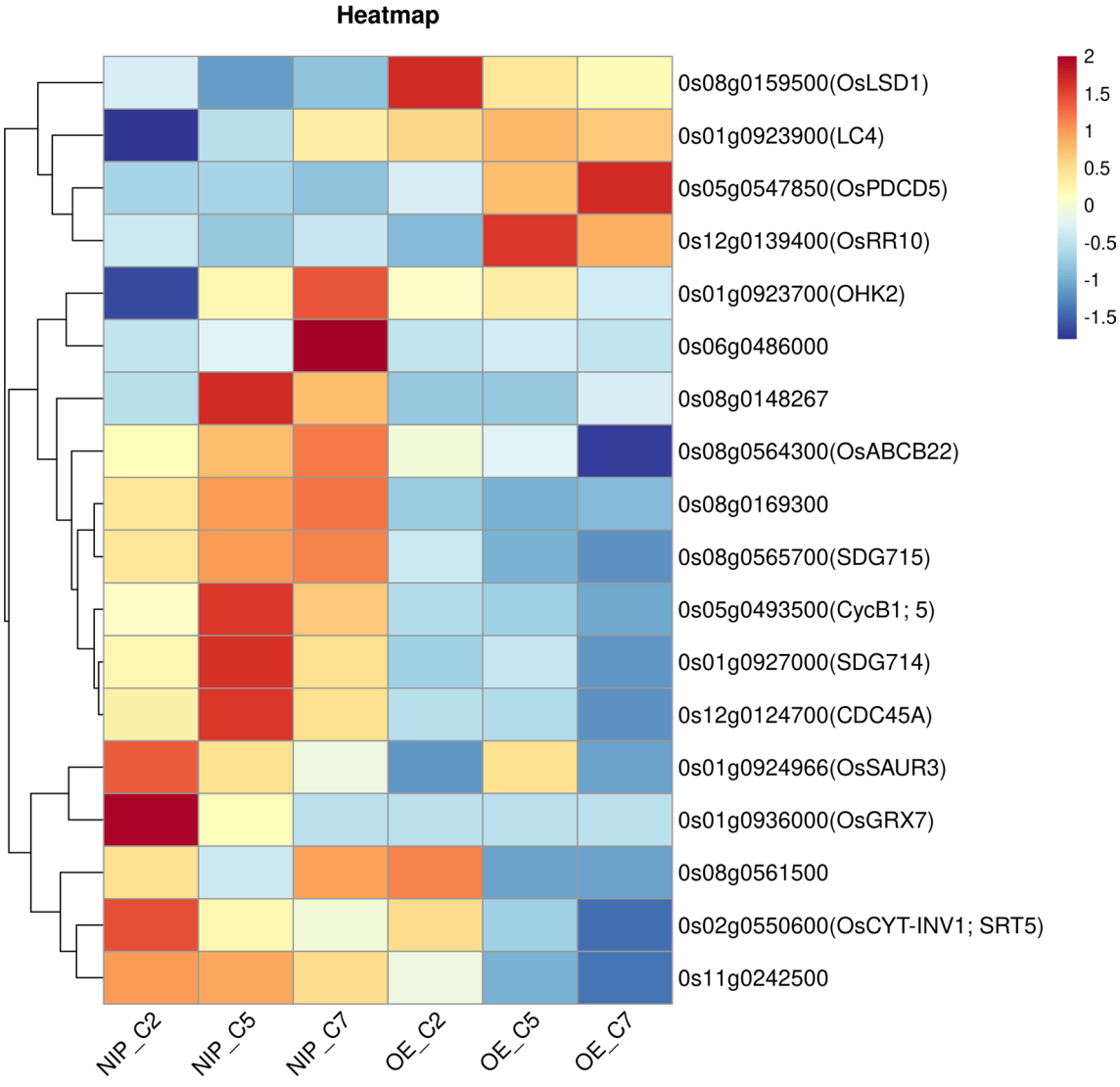

Fig. 3 Expression level heat map of 13 candidate callus induction genes expressed. Candidate genes relative expression levels from wild-type and *35Spro:OsMIR393b* at 2, 5, and 7 d cultured on CIM

OsCycB1;5, A Rice Cyclin Gene, Regulates CIR in Rice

Based on the GWAS analysis of CIR, we selected a significant associated locus (Chr05_ 24247048, P=5.44E-07) on Chromosome 5 for further analyze (Fig. 4A). This locus was located in a 30 kb LD region (Chr05:24220000–24280000) and covered 12 genes. Among them, *Os05g0493500* (*OsCycB1;5*) encoded a cyclin gene was identified as the candidate gene. Haplotype analysis showed that *OsCycB1;5* contained a non-synonymous SNP (Chr.5_24242729; T/G) on exon, which formed two major haplotypes (Fig. 4B). Accessions carrying the GG (alternate) allele showed a significantly higher CIR than those carrying the TT (reference) allele (Fig. 4B). Moreover, the expression level of *OsCycB1;5* in NIP was higher than that in *OEMIR393b* line indicated that *OsCycB1;5* may be involved in rice callus induction. Therefore, *OsCycB1;5* was selected as the candidate gene. *OsCycB1;5* is a rice cyclin gene homologous to *CYCB*1-1 in *Zea mays* (Fig. S3).

To validate the function of *OsCycB1;5* in callus formation, we generated transgenic lines with *OsCycB1;5* knockout via CRISPR/Cas9 technology in the cultivar NIP. DNA sequencing showed that a T/A base was inserted into the gRNA knockout site and resulted in a frameshift mutation (Fig. 4C). First, we cultured mature seeds of wild-type rice on callus-inducing medium with a high level of auxin. The embryo of the *OsCycB1;5* mutant was similar to the wild-type before callus induction. After 5 days of callus induction on MS media, we observed callus formation in the wild-type, but it markedly impaired in the *OsCycB1;5* mutant. After 10 days, many calli were visible to the naked eye in wild-type, besides, we also compared the CIR between the wild-type and two independent mutant lines. The results showed that the CIR of *OsCycB1;5* mutant lines were significantly lower than that of the wild-type (Fig. 4D, E, Fig. S4).

To further measure the proliferation rate of callus, a certain quantity of embryogenic callus was suspended in liquid medium. At 0–15 days, there was barely difference of the callus between the wild-type and the *OsCycB1;5* mutant lines. At 20 days, mutant lines showed slow growth and stagnant differentiation, with only partially translucent granular protrusions appearing on the surface of the callus, while the wild-type callus continued to grow and formed translucent, smooth-surfaced spheres. At 30 days, we poured off the medium, and measured the

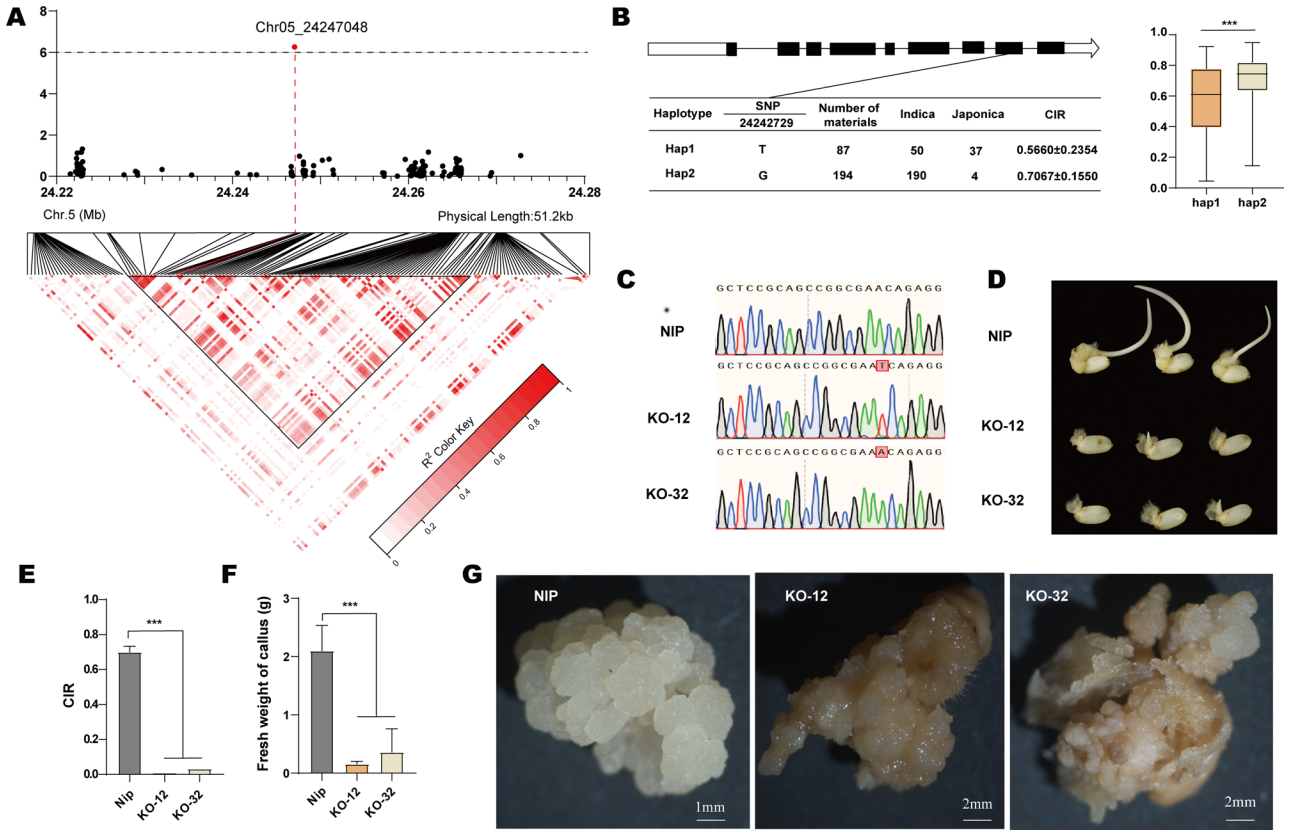

Fig. 4 The callus induction and proliferation capacity were reduced in *OsCycB1;5* mutant. **A**, Manhattan plot (top) and linkage disequilibrium heat map (bottom) of the *qCIR5.4* region, with the red arrow indicating the lead SNP position. **B**, Gene structure and haplotype analysis of *OsCycB1;5*.**C**, Homozygous gene editing line of *OsCycB1;5*.**D**, Wild-type and *OsCycB1;5* mutant on MS at 10 d. **E**, Induction rate of wild-type and *OsCycB1;5* mutant at 10d. **F**, Statistical analysis of freash weight of callus in wild-type and *OsCycB1;5* mutant on MS at 30 d. **G**, Wild-type and *OsCycB1;5* mutant on MS liquid medium at 30 d

fresh weight of the callus. The results showed that the growth rate of the wild-type callus was faster than that in *OsCycB1;5* mutant lines (Fig. 4F, G). Thus, we conclude that *OsCycB1;5* can regulates callus formation and CIR in rice.

Cytological Observation

To further observe cell morphology, callus from both wild-type and *OsCyCB1;5* mutant lines cultured for 10 days was examined using scanning electron microscopy. Cytological analysis of the 10-day-old callus showed that the cells of the wild-type were full and spherical, with closely packed arrangements, and groups of embryonic cells were clearly observable. In contrast, the callus cells of the *OsCyCB1;5* mutants were irregularly shaped, wrinkled, shriveled, disordered, and did not form distinct, enriched cell clusters (Fig. 5A-F).

To observe differentiation at the cellular level, callus samples of similar age were isolated for transmission electron microscopy (TEM) analysis (see experimental procedures). The results showed that the cells of wild-type were highly vacuolated, with less starch and fewer subcellular organelles. In contrast, the cells of *OsCyCB1;5* mutant exhibited abundant organelles, more small vacuoles, and a larger number of mitochondria and starch granules compared to the wild-type (Fig. 5G-L). In plants, a high degree of vacuolation and decreased cytoplasm are indicative of well-differentiated cells (Hu et al. 2011). These results suggest that the callus of *OsCyCB1;5* mutant initiated differentiation competence later and progressed more slowly than the wild-type.

Discussion

Importance of Dissecting Genetic Control of Rice Callus Induction Ability

Callus induction is the initial step for plant regeneration through somatic embryogenesis or organogenesis in many crops, including rice, maize, soybean, and wheat. To enhance callus induction ability, it is essential to understand the genetic basis of this trait by identifying candidate QTLs that significantly influence callus induction. However, most previous studies have employed methods based on QTL mapping through biparental populations, whereas studies utilizing GWAS remain relatively scarce. In this study, 368 rice accessions were collected from across wordwide to dissect the genetic architecture controlling callus induction ability. Through GWAS with high-density SNP markers, 104 SNPs were found to be significantly associated with CIR. Among these, four significant SNPs 5_18981147, 9_6656721, 9_6656721, and 12_16359371 identified in this study were similar to previously described by Zhang et al. (2019b). Moreover, we also identified several known callus-related genes located near the significant SNPs. For instance, *OsLSD1* is located near the significant SNP 8_3412934 and has been reported to regulate callus differentiation in rice (Wang et al. 2005). These findings verify the credibility of using this association panel to detect the genetic control of callus induction ability in rice. However, the significant loci and candidate genes identified in this study differ substantially from those in Zhang et al. (2019b), indicating that our findings possess a certain degree of novelty. Therefore, the results of this study will contribute to a deeper understanding of rice callus tissue formation and facilitate subsequent cloning and functional studies of related genes.

Candidate Genes and Underlying Pathways Involved in Rice Callus Formation

In the current study, we identified candidate genes by integrating GWAS, transcriptome analysis, functional annotation, and the pathways involved for homologous genes within QTLs that have smaller LD decay regions and higher -Log10 P values. Using this strategy, we efficiently identified 13 promising genes located within the 11 QTLs associated with CIR (Fig. S6).

First, it is widely known that the Phytohormone espesicially the auxins and cytokinins were critical for the establishment of the callus in various plants (Jiménez V. M. 2005). Interestingly, we identified four genes related to auxins and two related to cytokinins within four QTLs. *Os01g0923900* encodes an F-box protein named *LEAF INCLINATION 4* (*LC4*) (Qu et al. 2019), which has been proven to mediate auxin effects in regulating rice leaf inclination and architecture. *Os01g0924966* encodes an auxin-responsive gene family member, *OsSAUR3*. Additionally, *Os08g0561500* encodes an auxin-induced protein, 5NG4.

In addition, cytokinins are involved in key developmental processes in rice (*Oryza sativa*), including the regulation of cell proliferation and callus induction (Azizi et al. 2015). *Os01g0923700* encodes a histidine kinase (*OHK2*), which has been functionally identified as a homotypic cytokinin receptor in rice (Choi et al. 2012). The other candidate gene, *Os12g0139400*, encodes an A-type response regulator gene (*OsRR10*) (Jain et al. 2006), which has been characterized as responsive to cytokinin.

Many studies have confirmed that cell cycle regulation plays a pivotal role in plant callus development (Qu et al. 2022). Notably, we also identified three cell cycle-related genes within three QTLs. *Os05g0493500* encodes the rice cyclin gene *CycB1;5*, *Os11g0242500* encodes a cyclin-dependent kinase, and *Os12g0124700* encodes a putative DNA replication initiation protein (CDC45A) (Elmaghrabi et al. 2017; Yang et al. 2024).

Histone lysine methylation plays a pivotal role in heterochromatin formation and the modulation of gene expression (Dambacher et al. 2010). In plants, the *SDG*

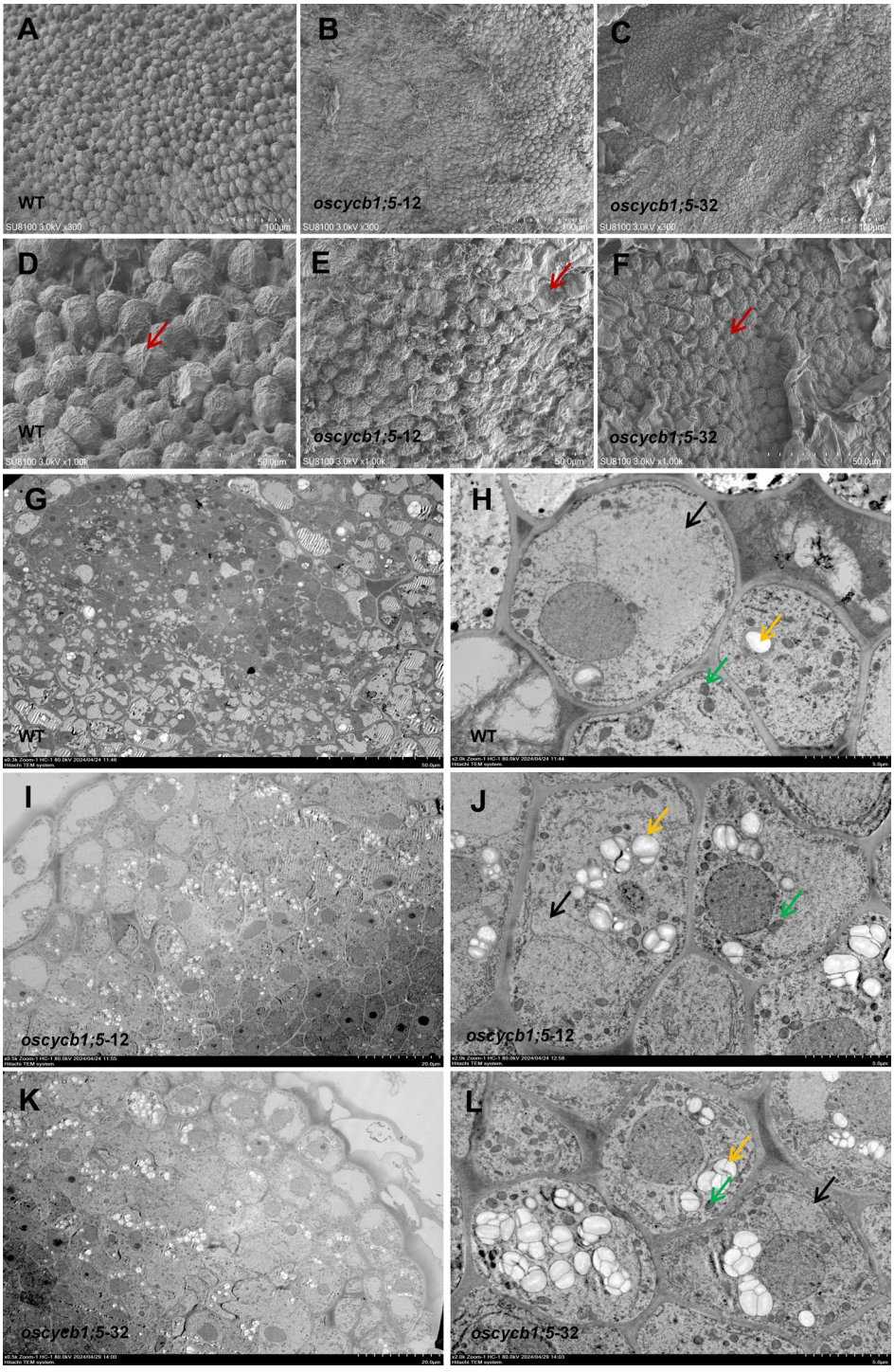

Fig. 5 Cytological observation of callus formation. The calli scanned by transmission electron microscopy, WT (**A**, **D**) and *oscycb1;5* mutant (**B**, **C**, **E**, **F**). Red arrows point to the callus cells, Scale bars, 100 μm (**A**, **D**), 50 μm (**B**, **C**, **E**, **F**). Transmission electron microscopy SEM analysis of callus formation, WT (**G**, **H**) and *oscycb1;5* mutant (**I**-**L**). Yellow arrows point to the starch grain; green arrows point to the mitochondria, black arrows point to the vacuole; Scale bars, 100 μm (**A**, **D**); 50 μm (**B**, **C**, **E**, **F**)

family regulates the methylation of histone lysine residues, thereby influencing various biological processes, such as flowering transition, hormone regulation, and callus formation (Xu et al. 2015). For instance, a recent study revealed that *SDG8*-mediated H3K36m3 plays a role in auxin signaling, facilitating callus induction in plants (Qu et al. 2022). Furthermore, in this study, we were pleased to discover one candidate gene encoding *SDG* family members: *SDG715*.

Collectively, these high-confidence candidate genes, including those related to auxin, CYC cyclins, and histone H3K9-specific methyltransferase, provide new targets for studying the formation of rice callus tissue.

OsCycB1;5 is Involved in Callus Growth and Development

The cell cycle plays a critical role in regulating the activities of plants, including seed germination, growth, and organ formation (Scofield et al. 2014). Different regeneration methods, such as tissue repair, de novo organogenesis, and somatic embryogenesis, all involve the formation of callus (Lee and Seo 2018; Xu et al. 2012). Callus cells will re-enter the cell cycle and proliferate, eventually producing various organs containing new plants, which is achieved through the reactivation of core cell cycle regulators CYCLIN (CYC) and CYCLIN-DEPENDENT KINASES (Inzé and De Veylder 2006; Ikeuchi et al. 2013, 2017). Moreover, many signaling cascades activated in callus are indispensable for cell division. During callus induction, genes encoding cell cycle regulatory proteins, chromatin structural proteins, and DNA synthesis-related proteins are up-regulated (Xu et al. 2012). Some studies have shown that cell cycle related genes are involved in the callus formation. For example, in *Arabidopsis*, phytohormone-stimulated callus formation and cell cycle reactivation in hypocotyls are associated with the capacity to express *CDKA* and *CYCB1;1* (Huang et al. 2003; Ohtani and Sugiyama 2005). Furthermore, overexpression of *ZmCYCB1-1* significantly accelerated the induction of green tissues and the growth of embryos in *Zea mays*. The improvement of histological characteristics and growth rate of plant embryos can be used to shorten the explant culture cycle (Zhao et al. 2022). In this study, we validated the function of *OsCycB1;5* using CRISPR/Cas9 technology. Indeed, in vitro inoculation culture of embryogenic callus demonstrated that *OsCycB1;5* significantly reduced callus induction and inhibited callus proliferation. Subsequent cytology revealed that the callus cells of *OsCycB1;5* mutant lines shriveled and contained small mitochondria, starch granules, protein bodies, and other cytoplasmic components. These results indicate that the absence of *OsCycB1;5* leads to lower levels of cell differentiation. Therefore, we speculated that knocking out *OsCycB1;5* may lead to changes in the expression of genes related to DNA replication, thereby affecting the normal cell cycle activity and resulting in a reduced growth rate of callus tissue. These results are similar to earlier reports in the B-Type Cyclin CYCB1-1 regulates embryonic callus development in Maize (Zhao et al. 2022). Taken together, our findings provide further evidence that *OsCycB1;5* plays an important role in callus formation by regulating cell development in rice.

Conclusion

In this study, we identified 104 significant SNPs and 13 candidate genes for CIR using GWAS and transcriptomic datasets. Moreover, we conducted functional analysis of a candidate gene, OsCyCB1;5, and validated it in rice callus formation. Our research not only enhances comprehension of the molecular mechanism underlying callus induction but also bears substantial significance for enhancing the CIR in rice.

Materials and Methods

Plant Materials and Callus Induction Medium

A total of 368 rice accessions are listed with detailed information in Table 1. They can be classified into three subgroups: *japonica* ($n=45$), *indica* ($n=320$), and the mixed group ($n=3$). These accessions were collected for observations of traits related to callus induction. MS was used as the main callus induction medium, and 2.5 mg/L 2,4-D, 600 mg/L proline, and 800 mg/L Casein Enzymatic Hydrolysate were added. Additionally, 30 g/L sucrose was added, and the pH of the medium was adjusted to 5.8. Before boiling, 3.0 g/L phytagel was added to the medium, and it was autoclaved at 121 °C for 15 min. The liquid medium does not require the addition of phytagel.

Inoculation and Callus Culture

Mature dehulled seeds of the 368 rice accessions were sterilized in 75% ethanol for 30 s. Ethanol was washed off with sterile water, followed by gentle shaking in 2% NaClO for 15 min. The seeds were then washed several times with sterilized water. A total of 90 seeds in good condition, without pathogen infection, were selected. Fifteen seeds were placed in petri dishes containing callus induction solid medium, and six petri dishes were prepared for each accession. The petri dishes were then cultured at 26 °C in total darkness. To ensure accuracy, the mature seeds of the 368 rice accessions were conducted in triplicate.

Phenotypic Data Collection and Statistical Analysis

Observations began 5 days after seeds were plated on the solid medium. The rate of seed CIR was recorded on the 10th day. The CIR was calculated as the CIR = the number of mature seeds forming embryonic callus / total number of cultured seeds (Kwon et al. 2001) (Table. S2).

Differences in phenotypic values between subpopulations were examined using a T-test: two-sample heteroscedasticity hypothesis if the F-test result is significant ($P<0.05$).

Genome-Wide Association Study

A total of 994,188 high-quality SNPs were used to identify the significant SNP loci. These SNPs were filtered using a missing rate of ≤0.01 and a minor allele frequency of ≥0.05. The variants calling was performed with gatk-package-4.5.0.0. The variants hard-filter was done with VCFtools 0.1.16 version. GWAS was performed using GAPIT (version 3.4) software with MLM models (Wang et al. 2021). *P*-value$<1.0\times10^{-6}$ were considered significant SNP-trait associations. VCF2D is v1.54 was used to calculated the evolutionary tree. Adimixture 1.3.0 was used to do analysis of population structure. R package CMplot was used to build Manhattan plot and QQ plot. The LD decay for different subgroups and the entire association panel was calculated using the parameter r^2 with PopLDdecay software (v3.41) (Zhang et al. 2019a). R package geneHapR was used to do the haploid types analysis. R package LDheatmap was used to build the LD heatmap.

Candidate Gene Expression Analysis

Based on the results of LD analysis of 368 rice germplasms in this study, the candidate gene region was determined by the LD decay distance of the chromosome where the significantly associated SNP marker is located. The corresponding LD interval upstream and downstream of the SNP marker was used to define the candidate gene region. Within this interval, the Rice Genome Annotation Project database was utilized to identify all potential candidate genes. An RNA-seq dataset, which aids in analyzing these candidate genes, is available in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE179594 and can be accessed at http://xulinlab.cemps.ac.cn/. This dataset provides gene expression data for rice mature seeds of wild-type NIP and *35Spro: MIR393b* (*OEMIR393b*) grown on callus-inducing medium for 2, 5, and 7 days. The expression of candidate genes was compared between wild-type and overexpressed lines.

Vector Construction and Rice Transformation

A CRISPR/Cas9 vector targeting *Os05g0493500* (*OsCycB1;5*) was constructed as previously described. A 20-bp DNA sequence harboring a protospacer adjacent motif within the exon of *Os05g0493500* was combined with a U3-gRNA box. The resulting constructs of U3-*Os05g0493500*-gRNA were cleaved with *BsaI* and inserted into the pRGEB32 vector. The pRGEB32-*Os05g0493500* plasmid was then delivered into the Agrobacterium tumefaciens EHA105 strain for infecting NIP callus. Regeneration of transgenic plants from the callus was achieved on selection medium containing 50 mg L^{-1} hygromycin and 250 mg L^{-1} cefotaxime. The CRISPR-GE database (http://skl.scau.edu.cn/home/) and the RIGW database (http:/rice.hzau.edu.cnhice rs1/) were used to predict potential off-target sites by BLAST analysis of the rice genome using our designed single guide RNA (gRNA) (20 bp). This search identified 14 genes as potential off-target sites. Examination revealed that Cas9-Grna OsCycB1;5 mismatch splice sites were not present in the predicted sequences of off-target genes in the OsCycB1:5-ko lines. The primers used are listed in Table S5 and results in Fig S5.

Cloning and Clustering Analysis of the OsCycB1;5 Gene

The reference sequence of *OsCycB1;5* was obtained from the gene database of the National Center for Biotechnology Information (NCBI). Genomic DNA was extracted from callus of the NIP using a plant DNA extraction kit (TIANGEN, Beijing, China). Primers used to amplify the full-length genomic sequence of *OsCycB1;5* were designed using CE design software (F: 5'-TTCACACGG TAATCACAGAGATAGAGC-3'; R: 5'-TTATGGAAAAA GAATACTTTTCAT-3'). The full-length genomic DNA sequence of *OsCycB1;5* was amplified using Phanta Uc Super-Fidelity DNA Polymerase for Library Amplification (Vazyme, P507-01). For B-type cyclin cluster analysis, an unrooted neighbor-joining phylogenetic tree of nine genes in *Arabidopsis*, five genes in rice, and eight genes in maize was generated by MEGA11.0 using full-length protein sequences. MEME was used to analyze the conserved domains of B-type cyclin cluster protein, and TBtools v2.083 generates pictures.

Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM)

Fresh calluses of wild-type and transgenic plants cultured for 10 days were taken and placed in 2mL EP tubes for labeling. The calluses were fixed overnight at 4 °C in 0.1 M phosphate buffer (pH 7.0) containing 2.5% glutaraldehyde and sent to Wuhan Sevier Biotechnology Company for electron microscope scanning.

Abbreviations

GWAS	Genome Wide Association Study
CIR	Callus Induction Rate
SNP	Single Nucleotide Polymorphism
EC	Embryonic Callus
QTL	Quantitative Trait Loci
RIL	Recombinant Inbred Lines
RNAi	RNA Interference
WGCNA	Weighted Gene Co-expression Network Analysis
LD	Linkage Disequilibrium
GEO	Gene Expression Omnibus
SEM	Scanning Electron Microscope
TEM	Transmission Electron Microscope

MLM	Mixed linear model
NIP	Nipponbare
PCA	Principal Components Analysis

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12284-024-00742-8.

Supplementary Material 1

Supplementary Material 2

Author Contributions

WS performed most of the experiments and wrote the manuscript. JZ analyzed the data and corrected the manuscript. WL performed GWAS analysis and prepared figures. WS, SL, HC, YG, and SC investigated the phenotype data. JW provided some constructive suggestions for the experiments. TG, HL, and DR conceptualized the project and designed the study. All authors read and approved the final manuscript.

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Data Availability

No datasets were generated or analysed during the current study.

Declarations

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Competing Interests

The authors declare no competing interests.

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