Agrobacterium-mediated transformation efficiency and grain phenotypes in six indica and japonica rice cultivars

Ke Chen[1#], Chanjuan Ye[1#], Jie Guo[1#], Dagang Chen[1], Tao Guo[2], Juan Liu[1], Chuanguang Liu[1*] and Xinqiao Zhou[1*]

[1] Rice Research Institute, Guangdong Academy of Agricultural Sciences; Key Laboratory of Genetics and Breeding of High Quality Rice in Southern China (Co-construction by Ministry and Province), Ministry of Agriculture and Rural Affairs; Guangdong Key Laboratory of New Technology in Rice Breeding; Guangdong Rice Engineering Laboratory, Guangzhou 510640, China

[2] State Key Laboratory of Crop Stress Biology for Arid Areas and Institute of Future Agriculture, Northwest A&F University, Yangling 712100, Shaanxi, China

[#] These authors contributed equally: Ke Chen, Chanjuan Ye, Jie Guo

[*] Corresponding authors, E-mail: guyliu@tom.com; 13428860986@139.com

Abstract

Plant transformation and regeneration have been continuously developed over the past four decades. In rice (Oryza sativa L.), Agrobacterium-mediated transformation has high efficiency in japonica and some indica cultivars using mature seeds and immature embryos. However, these protocols have low transformation efficiency in the latest indica cultivar that has been developed in South China since 2010. Here, we explored plant culture regeneration of the high-quality and high-yield indica cultivar Nanguizhan (NGZ) through CRISPR/Cas-based genome editing and traditional overexpression transformations. We compared transformation efficiency in this cultivar to four other widely-used indica cultivars and one japonica cultivar using mature seeds and the gene Grain Size and Number 1 (GSN1) as a case study. We observed universally smaller grain size in overexpression lines and bigger grain size in gene-editing lines among different cultivars. NGZ phenotypes make it an excellent model in which to investigate gene functions. We also examined the single nucleotide polymorphism (SNP) distribution and differences in expression levels of regeneration-related genes in calli, possibly revealing the source of NGZ's advantages in Agrobacterium-mediated transformation. These results shed light on the advanced application of NGZ in gene editing and overexpression transformation related to grain improvement, contributing to the 'rice breeding 4.0 era'.

INTRODUCTION

Plant tissue culture systems are fundamental tools for investigating gene functions in higher plant species. Plant tissue culture and Agrobacterium-mediated transformation have profoundly reshaped and promoted functional studies in crop research and breeding over the past three decades. Rice (Oryza sativa L.) is a staple food that feeds more than half of the world's population[1]. Since the end of the 20th century, protocols for tissue culture and Agrobacterium-mediated transformation in rice have been established[2–4]. Subsequently, scientists have expanded applications of these methods to allow use in more cultivars[5–8]. Several other transformation methods have also been applied to generate transgenic rice plants, including biolistic technology-based methods and nanotechnology gene delivery systems[9,10]. During the last century, particle acceleration, also known as microprojectile bombardment or gene gun technology, has been successfully applied to several commercial crops such as Roundup soybeans[11]. However, this technology has limitations; it induces cell damage and has low transformation efficiency and genome integration rates[12]. In recent years, nanoparticles and nanotechnologies have been widely applied in animal cells and organs. In this context, nanoparticles function as molecular vectors to harmlessly deliver bioactive materials into cells, and

have therefore been utilized as targeted drugs, cancer treatments, and genetic disease cures[13]. However, this novel technology is far from ready for widespread application in plants.

Agrobacterium tumefaciens-mediated transformation has been used to transform rice for over 20 years[2], but species- and genotype-specific difficulties have hindered broad application. Moreover, although there are classic japonica cultivars (such as ZH11 and Nipponbare) that can be used for transformation, there are no standard genetically tractable indica cultivars. Significant progress has been achieved in developing transformation methods; for instance, some indica cultivars have been recognized as easier to transform, tissue culture components have been improved[8], the essential genes that determine callus browning and regeneration rates have been identified[14], and specific enhancements have been made to transformation protocols[15]. However, a 'gold standard' protocol has yet to be established for functional studies and other applications of rice transformation, particularly in indica cultivars.

Grain weight is one of the key factors determining single plant yield production of rice and other cereal crops[16]. Reports on trade-off between grain number and grain size emerged and focused on the famous OsMKKK10-OsMKK4-OsMPK6 cascade[17,18]. Grain Size and Number 1 (GSN1) encodes a dual-

specificity phosphatase which negatively regulated the MAPK cascade and functioned as a developmental 'brake' in coordinating localized cell differentiation and proliferation[19]. Previously research confirmed that overexpression of *GSN1* reduced grain size, while knockout or knockdown of it would significantly enlarge the grain size[19,20]. This gene also provided huge potentials on grain size and yield production improvement.

We previously found that a novel elite *indica* cultivar, Nan Gui Zhan (NGZ)[21] (bred by the Rice Research Institute, Guangdong Academy of Agricultural Science), is suitable for rice transformation assays. To better evaluate the transformation regeneration rate, we here used optimized protocols appropriate for the *indica* cultivar Zhen Shan 97 (ZS97) and performed a comparison of overexpression and CRISPR-Cas9-mediated gene editing plasmid transformations. We compared five distinct elite *indica* rice cultivars and one *japonica* cultivar from South China, Central China, Southeast China, and India. We generated *GSN1* gene constructs; performed plant tissue culture experiments; measured callus induction and regeneration, root regeneration, and shoot regeneration rates; evaluated tissue culture performance; and assessed T_2 generation phenotypes and genotypes. Finally, we analyzed several genes related to callus regeneration and the associated haplotypes and expression patterns in these cultivars, which partially explained the differences in tissue culture outcomes. Our results indicated that a popular cultivar of rice with high yield production and high grain quality is sufficient for *Agrobacterium*-mediated transformation, indicating that this cultivar has a promising future in gene editing applications and functional studies associated with *indica* rice breeding.

MATERIALS AND METHODS

Plant materials

Five different *indica* rice cultivars (NGZ, ZS97, YD 6, HHZ, and Kasalath) and one rice *japonica* cultivar (ZH11) were obtained from BioRun Co. LTD. All grains were harvested in the same period in Hainan, 2020. All seeds were healthy and similar in size and appearance. All rice plants were cultivated in Guangzhou, China, under natural growth conditions.

### Plasmid construction and plant transformation

To generate the overexpression construct pCAMBIA1300-GSN1-FLAG, the full length of *GSN1* cDNA (*LOC_Os05g02500*) was cloned from rice elite cultivar FAZ1. The *GSN1* coding sequence was fused to a 3× FLAG tag and cloned into a plant binary vector with the Maize ubiquitin (UBI) promoter. To produce the CRISPR-Cas9 gene editing rice, we applied the pCAMBIA1300-sgRNA-Cas9 system for plasmid construction and sgRNA design[22,23]. We constructed the pYLCRISPR/Cas9 Pubi-GSN1-H plasmid for gene editing transformation. Two gRNAs (GSN1-U3F: 5'-GGCAGTTCTGGCGCTCCGCGTCG-3'; GSN1-U3R: 5'-AAACCGACGCGGAGCGCCAGAAC-3'; GSN1-U6AF: 5'-GCCGGCGGAGGGGGTGTCGGCGG-3'; GSN1-U6AR: 5'-AAACCCGCCGACACCCCCTCCGC-3') were utilized and introduced into the CRISPR-Cas9 main backbone by *Bsa*I accordingly[19]. These well-constructed plasmids were subsequently transformed into competent cells (*E. coli*, DH5α). *Agrobacterium tumefaciens*-mediated transformation of rice with strain EHA105 was applied as previously described methods[2].

Mature seed tissue culture and transformation

Mature seeds were harvested in Hainan, 2020. The whole tissue culture process was modified according to the method of Zhao et al.[24]. To dehull the mature rice seeds, the moldy surface and rotted embryo seeds were removed, then the healthy full seeds were left for tissue culture. The seeds were disinfected with 75% alcohol for 30 s, followed by washing three times (ddH$_2$O). The clorox chemical disinfectant was performed for 30 min, and seeds were washed using sterile water, three times. The seeds were then soaked in sterile water for an hour. After the disinfection process, the seeds were transferred to the callus induction medium to induce callus until the regeneration process. After the acquisition of a suitable size [2−2.5 mm (Kasalath), 3 mm (NGZ, HHZ), 3.5−4 mm (ZS97 and YD 6)] of callus, the plasmid-contained *Agrobacterium tumefaciens* (EHA105 strain) were cultivated by YEB medium supplemented with kanamycin (50 mg/L). For co-cultivation, the callus and strains were scraped into the suspension culture and shaken for 20 min at 180 rpm. OD$_{600}$ = 0.1−0.2. The infected callus was co-cultured 24 °C, 48 h under dark conditions. The selection was performed on a resting medium with 75 mg/L hygromycin. Two steps of selection process were applied. For ZH11, the pre-selection medium needs extra supplemented 35 mg/L hygromycin; the first stable selection medium contained 45 mg/L hygromycin; the second selection medium added 60 mg/L hygromycin. For *indica* rice selection process except YD 6, the pre-selection medium needs extra supplemented 15 mg/L hygromycin; the first stable selection medium contained 25 mg/L hygromycin; the second selection medium added 60 mg/L hygromycin. For YD 6, the pre-selection medium needs extra supplemented 5−10 mg/L hygromycin; the first stable selection medium contained 15−25 mg/L hygromycin; the second selection medium added 60 mg/L hygromycin. For the differentiation process, the dense, beige, and middle-size transformed callus were transferred to a differentiation medium (light 28 °C, 24 h) for a week. Green spots were observed in certain callus; 1 cm long young seedlings were grown in the medium for two weeks. The regeneration seedlings were transferred to the root induction medium. After ten days of cultivation (30 °C, 24 h), the regeneration root developed significantly. The young seedlings are now ready to be transferred to the natural environment. A simple diagram was exhibited the detailed steps and conditions for the plant tissue culture of rice in Fig. 1. The detailed parameters of all media through the tissue culture process were summarized in Supplemental Table S1. The total days of all cultivars transformation are listed in Supplemental Table S2.

Validation of transgenic plants

All transgenic plants were cultivated in natural conditions in Guangzhou (China). The DNA was extracted from the young leaves of each regenerated plant using the DNA extraction protocol as described[25]. For the CRISPR-Cas9 gene editing plants, special primers to amplify sequences with the target sites were used (GSN1-F: 5'-CTCCTCTCACCCGTGACCTC-3'; GSN1-R: 5'-TGGCCGAGCCGGCGCCTTCC-3'); *Hygromycin B phosphotransferase gene* (*HPT*) primers (HPTF: 5'-CGCATAACAGCG GTCATTGACTGGAGC-3'; HPTR: 5'-GCTGGGGCGTCGGTTTCCA CTATCCG-3'). For the transcriptional level evaluation, the gene-specific primers (GSN1-qRT-PCRF: 5'-CATCTAAAGGAGCTCG GCAATA-3'; GSN1-qRT-PCRR: 5'-CGAGCATTCAGCACTTCCA-3')
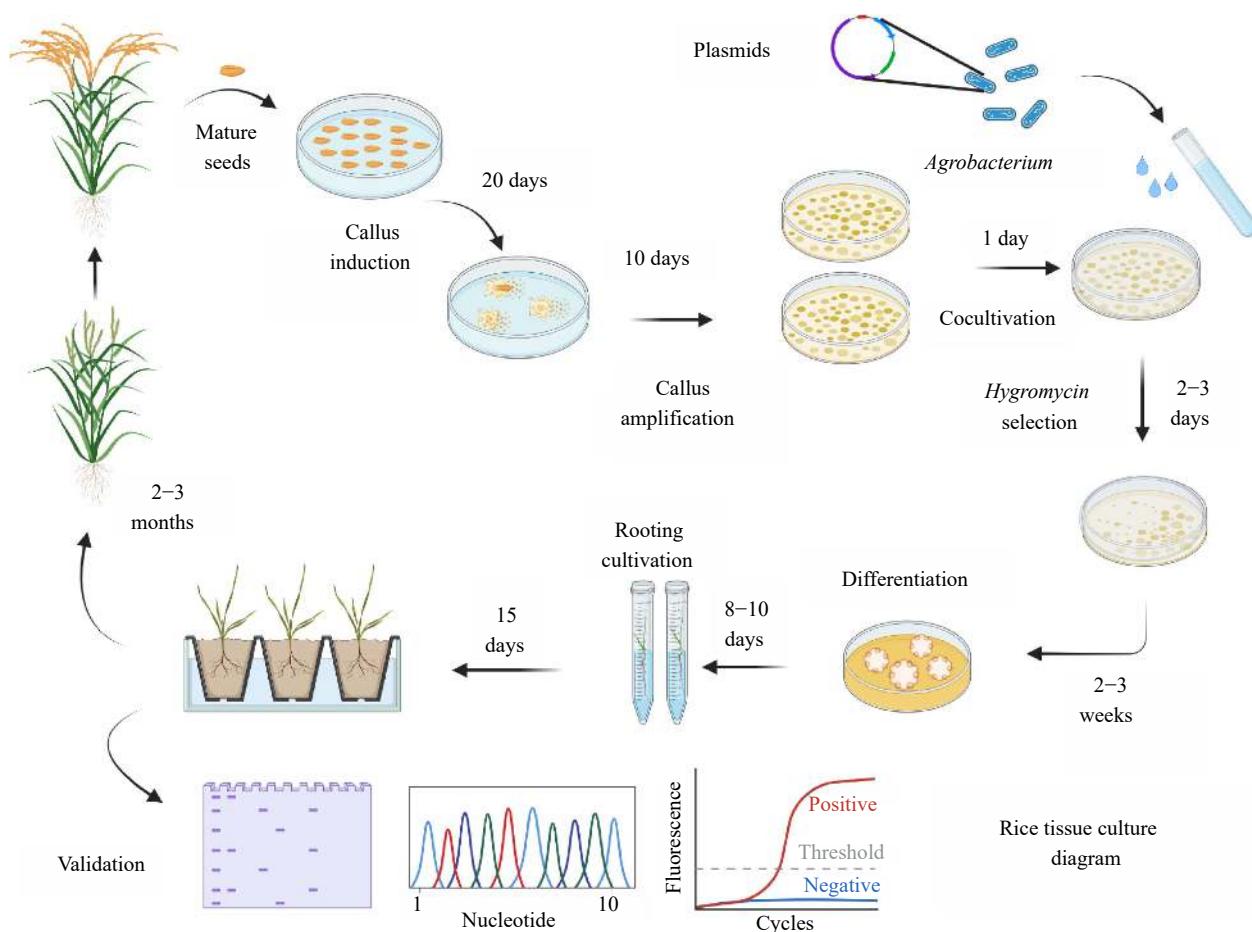
Fig. 1 Diagram showing how rice tissue culture is used together with *Agrobacterium*-mediated transformation.

were used. Total RNA was extracted from leave tissues using Trizol reagent (Invitrogen). A reverse transcription assay was performed using ReverTra Ace qPCR RT Master Mix with gDNA remover (TOYOBO). The quantitative RT-PCR analysis was performed on the CFX96 Touch Real-Time PCR Detection System (BioRad). The data were analyzed using the $2^{-\Delta\Delta Ct}$ method, and the qRT-PCR regents were Fast Start Universal SYBR Green Master Mix with ROX (Roche)[26]. The *Actin* gene was used as the internal control. All analyses were repeated three times. All the primers used in this study are listed in Supplemental Table S3.

Calculation of callus induction, regeneration, selection, and transformation efficiency

A thousand healthy dehulled seeds were selected for plasmid transformation and tissue culture assays from each rice cultivar. The callus induction efficiency was calculated as the induction calli divided the total seeds percentage on the callus induction medium. The resistant cellus induction efficiency was counted by the number of resistant callus induced divided by the total induced calli. The green spots emerging rate was counted by the number of green spots divided by the total resistant calli. The shoot formation rate is calculated by the same way. The transformation efficiency indicated the positive hygromycin selected samples. The gene editing transformation efficiency was based on sequence results, and overexpression lines were evaluated by qRT-PCR data.

Phenotypic and statistical data analysis

The whole plant phenotypic data and the seed observation were acquired by digital camera and Stereo microscope (Leica). Student's *t*-test was used in data analysis. Diagram figure was performed by the BioRender Software.

Sanger sequencing and sequence comparison

Based on MSU 7.0 version rice annotation database, we searched and designed primers to amplify callus-related gene fragments of six rice cultivars. All targeted sequences were performed by Sanger sequencing, and Lasergene Seqman software was used for DNA fragment comparison.

RESULTS

Tissue culture evaluation of six rice cultivars

Previous studies have indicated that transformation is much easier and has higher efficiency in *japonica* than *indica* rice cultivars[27,28]. Our group previously bred an ideal temperature-sensitive conventional *indica* cultivar, NGZ[21]. Here we used ZH11, the most popular and widely used *japonica* cultivar in transgenic rice research, for comparison[29]. ZH11 is a typical *japonica* cultivar with high native efficiency in *Agrobacterium*-mediated transformation[29−31]. We selected another five *indica* rice cultivars for tissue culture evaluation. These cultivars had a range of phenotypes and originated from various regions in South China, Central China, and India. All were mature cultivars

that have been consumed for a relatively long time (ranging from the 1960s to 2010s). We compared grain size between these six samples (Fig. 2), and found that they were highly diverse in critical grain-related agronomic traits (Supplemental Table S4). NGZ contained higher alkali spreading value and brown rice rate, lower milled width, chalky grain rate, chalkiness area, and amylose content among these five *indica* cultivars. Therefore, NGZ is a typical high grain quality rice cultivar that is suitable for functional studies and improvement experiments related to grain size and shape. It has a very low chalky grain rate (1.5%) and chalkiness area (0.5%), and these attributes, along with the shape and milled performance, are desirable to consumers. NGZ also has relatively low amylose content and protein content, giving it superior flavor (Supplemental Table S4)[32,33]. Based on the grain performance (Fig. 2), NGZ appears to be an excellent novel elite rice cultivar for studying grain size and panicles.

Plasmid constructs and transgenic plant development

We previously reported that *Grain Size and Number 1* (*GSN1*), which encodes the MAPK phosphatase OsMKP1, negatively regulates the MAPK cascade and controls trade-offs between grain size and number[19]. We here used two plasmids to generate transgenic plants and evaluate tissue culture
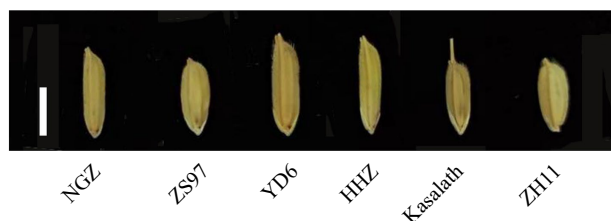
Fig. 2 Grain shapes of rice cultivars used in this study. Scale bar = 0.5 cm.

performance (Supplemental Fig. S1): one for *GSN1* overexpression and the other for *GSN1* knockout *via* CRISPR-Cas9 gene editing (Supplemental Fig. S1). A functional gene editing system together with an overexpression system would enable most molecular mechanistic studies in rice. The components of the callus induction medium, resuspension medium, regeneration medium, selection medium, differentiation medium, and root induction medium have been optimized for *japonica* and *indica* rice cultivars (Supplemental Table S1).

Callus induction evaluation

We put all *indica* rice cultivars into some aspect of callus induction medium (CIM) to perform callus induction assays. The CIM used for ZH11 was slightly modified with respect to the ratio of sucrose, glucose, and phytohormone (Supplemental Table S1). One hundred healthy, intact grains were selected from each rice cultivar for each transformation event, and there were five biological replicates (500 seeds in total per cultivar). When seeds were cultured on CIM, more than 65% of total seeds were induced into embryogenic calli (Tables 1 & 2). We performed statistical analysis to display the survival rates of all plant tissue culture stages among six cultivars (Fig. 3). After growing on CIM for 14 d, we removed the endosperm and roots to obtain pure callus tissue for the next stage of tissue culture. The *japonica* cultivar ZH11 had the highest callus induction rate at 88.7%. In comparison, NGZ and ZS97 had similarly high calls induction rates (82%, NGZ; 88.3%, ZS97) (Tables 1 & 2). The Kasalath rice cultivar had the lowest callus induction rate (64.2%). We observed yellowish, compressed callus shapes in most *indica* cultivars and ZH11. Only very small calli were generated from Kasalath (Supplemental Fig. S2). In general, most *indica* cultivars formed calli earlier than ZH11 (Supplemental Table S5). Based on the phenotypical and statistical analysis as well as the callus induction rate, we

Table 1. Number of samples and survival rate percentage at each transformation step with the pYLCRISPR/Cas9Pubi-GSN1-H (*GSN1*-KO) plasmid in six rice cultivars.

Sample name	Sample quantity					
	Mature seeds	Callus induction	Resistant callus	Green spot	Shoot induction	Transformation
NGZ	1000 (100%)	820 (82%)**	111 (26.7%)**	34 (30.6%)n.s	24 (70.5%)n.s	24 (4.8%)n.s
ZS97	1000 (100%)	833 (83.3%)*	271 (62.0%)*	41 (15.1%)n.s	26 (63.4%)n.s	26 (5.2%)n.s
YD 6	1000 (100%)	776 (77.6%)***	54 (14.5%)***	12 (22.2%)*	6 (50%)*	6 (1.2%)*
HHZ	1000 (100%)	727 (72.7%)***	261 (71.1%)*	36 (13.7%)n.s	22 (61.1%)n.s	22 (4.4%)n.s
Kasalath	1000 (100%)	642 (64.2%)***	196 (62.2%)n.s	140 (71.4%)*	17 (12.1%)n.s	17 (3.4%)n.s
ZH11	1000 (100%)	887 (88.7%)	189 (41.6%)	55 (29.1%)	24 (43.6%)	24 (4.8%)

Data are given as the mean. n.s, no significance, * *P* < 0.05; ** *P* < 0.01; *** *P* < 0.001 compared with the corresponding ZH11 using Student's *t*-test. The callus induction rate was caculated from the overall induced callus which was used for the whole studies.

Table 2. Number of samples and survival rate percentage at each transformation step with the pCAMBIA1300-GSN1-FLAG (*GSN1*-OX) plasmid in six rice cultivars.

Sample name	Sample quantity					
	Mature seeds	Callus induction	Resistant callus	Green spot	Shoot induction	Transformation
NGZ	1000 (100%)	820 (82%)**	217 (53.5%)*	69 (31.8%)*	39 (56.5%)n.s	39 (7.8%)n.s
ZS97	1000 (100%)	833 (83.3%)*	283 (71.5%)***	56 (19.8%)*	52 (92.9%)n.s	52 (10.4%)n.s
YD 6	1000 (100%)	776 (77.6%)***	243 (60.14%)***	60 (24.7%)*	17 (28.3%)n.s	17 (3.4%)n.s
HHZ	1000 (100%)	727 (72.7%)***	146 (40.6%)n.s	62 (42.5%)*	23 (37.1%)n.s	23 (4.6%)n.s
Kasalath	1000 (100%)	642 (64.2%)***	229 (45.8%)*	64 (27.9%)*	13 (20.3%)*	13 (2.6%)*
ZH11	1000 (100%)	887 (88.7%)	160 (36.9%)	206 (161.5%)	37 (17.9%)	37 (7.4%)

Data are given as the mean. n.s, no significance, * *P* < 0.05; ** *P* < 0.01; *** *P* < 0.001 compared with the corresponding ZH11 using Student's *t*-test. The callus induction rate was caculated from the overall induced callus which was used for the whole studies.
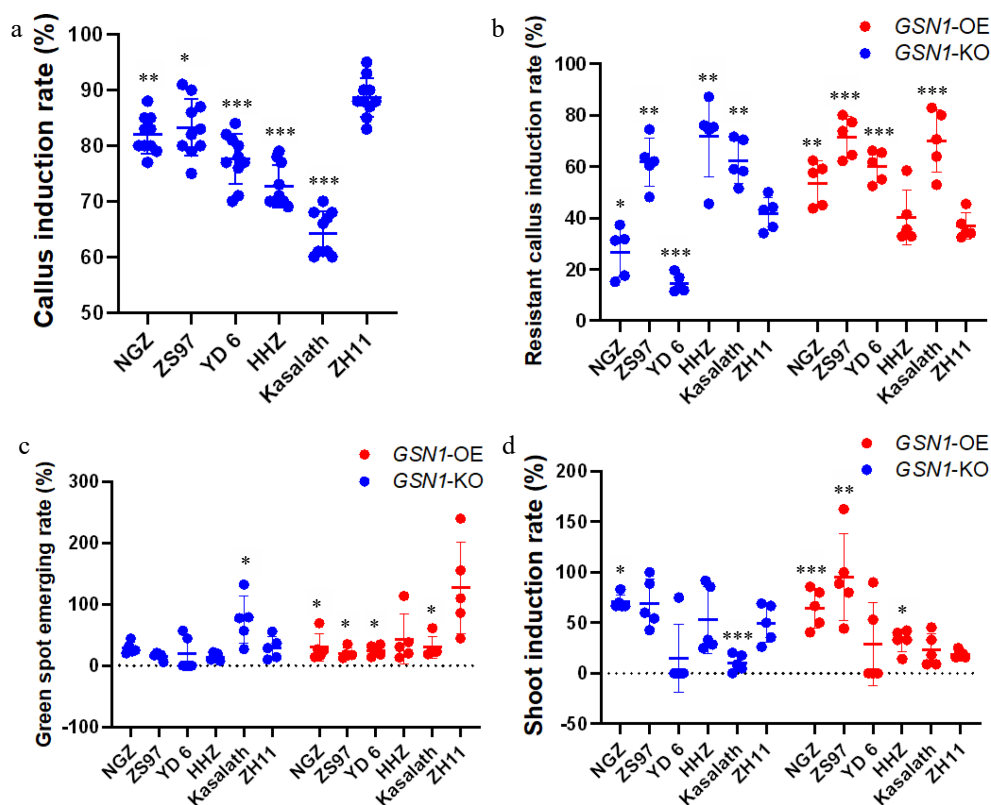
Fig. 3 Statistical analysis of plant tissue culture among six cultivars for the pYLCRISPR/Cas9Pubi-GSN1-H (*GSN1*-KO) plasmid and the pCAMBIA1300-GSN1-FLAG (*GSN1*-OX) plasmid. (a) The callus induction rate of six rice cultivars; (b) The resistant callus induction rate of six cultivars; (c) The green spot emerging rate of six cultivars; (d) The shoot induction rate of six cultivars. For callus induction process, ten independent biological repeats were performed (n = 10). For specific plasmid transformation process, five independent biological repeats were carried (n = 5), each performance utilized 100 healthy intact seeds from every cultivar for plant tissue culture process. Data are presented as the mean ± standard deviation. * < 0.05, ** < 0.01, *** < 0.001 (Student's *t*-test).

admitted that the six cultivars ranked as follows: ZH11 (88.7%) > ZS97 (83.3%) > NGZ (82%) > YD 6 (77.6%) > HHZ (72.7%) > Kasalath (64.2%) (Fig. 3, Supplemental Fig. S2, Tables 1 & 2). These results confirmed that the *indica* cultivar NGZ had excellent callus induction capacity under commercial CIM conditions.

Survival rates of transformed resistant calli

We next transformed *Agrobacterium tumefaciens* strain EHA105 with either the overexpression plasmid pCAMBIA1300-GSN1-FLAG (*GSN1*-OX) or the gene editing knockout plasmid pYLCRISPR/Cas9Pubi-*GSN1*-H (*GSN1*-KO). All calli from each rice cultivar were incubated with transformed *Agrobacterium* in a co-cultivation medium under dark conditions at 28°C for one day. Calli were then transferred to resistant calli selection medium and grown for two weeks under dark conditions for the first round of selection. We observed that fresh calli of different cultivars differed in appearance and behavior. ZH11 calli were more yellow and compressed, had longer induction times, and generated multiple small fresh calli. Calli of the five *indica* cultivars were generally a lighter yellow (although some were white) and larger. The *indica* cultivars calli displayed severe browning or even death over the course of the cultivation time. We observed that in comparison to the other rice cultivars, NGZ was more sensitive to *Agrobacterium* infection (Tables 1 & 2). Statistical analysis showed that except HHZ, the overall resistant calli induction rate was lower for those transformed with the CRISPR plasmid than the overexpression plasmid

(Fig. 3). NGZ and YD 6 had the two lowest resistant calli induction rates in *GSN1*-KO transformation event (Fig. 3). Many NGZ calli had *Agrobacterium* contamination; kanamycin was insufficient to inhibit agrobacterial growth. Successfully induced NGZ resistant calli were dry and clean (Supplemental Fig. S3). A similar phenomenon was observed in YD 6, but the percentage of contaminated calli was lower than the percentage of brown calli. ZS97 had the best performance, with a higher resistant calli induction rate than even ZH11 (Tables 1 & 2). Kasalath had similar responses to transformation with the two plasmids, whereas NGZ, HHZ and YD 6 had different responses to transformation with the gene editing and overexpression plasmids (Fig. 3). With respect to regeneration rate, the six cultivars ranked as follows: ZS97 (62%, 71.5%) > Kasalath (62.2%, 45.8%) > HHZ (71.1%, 40.6%) > ZH11 (41.6%, 36.9%) > NGZ (26.7%, 53.5%) > YD 6 (14.5%, 60.14%) (Tables 1&2). Overall, these results suggested that transformation with the gene editing plasmid led to a lower resistant calli induction rate, and that NGZ calli were sensitive to *Agrobacterium* infection.

Differentiation evaluation

After 2−3 weeks of regeneration growth, calli were transferred to differentiation medium and incubated at 28 °C under full-light conditions. Interestingly, *indica* cultivar Kasalath calli transformed with the *GSN1*-KO construct and *japonica* cultivar ZH11 calli transformed with the *GSN1*-OX construct showed large number of green spots (Supplemental Fig. S4). Consistent

with previous reports, *indica* cultivars had lower greening numbers than *japonica* cultivars[34]. Although a considerable number of regenerated NGZ calli transformed with the *GSN1*-KO construct died, more than 30% of the calli exhibited their greening area. Kasalath had the highest greening rate at 71.4%. ZS97 and HHZ had the lowest percentage of greening (15.1% and 13.7%). With respect to greening rate, the six cultivars were ranked as follows: Kasalath (71.4%) > NGZ (30.6%) > ZH11 (29.1%) > YD 6 (22.2%) > ZS97 (15.1%) > HHZ (13.7%) (Table 1).

Calli transformed with the *GSN1*-OX construct had higher greening rates compared to those transformed with *GSN1*-KO. Surprisingly, ZH11 had a higher greening rate than calli regeneration rate. This phenomenon may be due to the high regeneration capacity of calli and the induction rate in *japonica* cultivars (Table 2). Furthermore, NGZ had the highest greening amount among all *indica* cultivars tested, indicating that NGZ had robust viability in plants and calli. Although the resistant callus induction rate was relatively low in HHZ, the greening rate was 42.5% for *GSN1*-OX transformants, suggesting that HHZ also had a strong capacity for greening (Table 2). We further investigated the size and shape of calli for all six cultivars. NGZ and ZS97 had larger calli and greening areas, whereas YD 6, HHZ, and Kasalath had green spots on smaller calli. Moreover, in comparison to ZH11, all *indica* cultivars showed darker calli and few were newly generated (Supplemental Fig. S4). With respect to differentiation, the six cultivars ranked as follows: ZH11 (161.5%) > HHZ (42.5%) > NGZ (31.8%) > Kasalath (27.9%) > YD 6 (24.9%) > ZS97 (19.8%) (Table 2).

Shoot and root growth evaluation

After 2−3 weeks of growth in differentiation medium, young plants were transferred into shoot induction medium (SIM). During this stage, there was a difference in shoot formation between *GSN1*-KO and *GSN1*-OX transformants (Tables 1 & 2). There were fewer *GSN1*-KO than *GSN1*-OX lines, and the shoot formation time was longer for *GSN1*-KO transformants. Although Kasalath plants showed more than 140 greening spots in total, only 17 transformed plants formed shoots (12.1%). Aside from Kasalath, the *indica* cultivars had higher shoot formation rates than the *japonica* cultivar ZH11 (Table 2). Similarly, in the *GSN1*-OX transformation assay, Kasalath had the lowest shoot induction rate (20.3%). Surprisingly, the ZH11 shoot induction rate in *GSN1*-OX (17.9%) was lower than the shoot induction rate with the *GSN1*-KO plasmids (43.6%) (Tables 1 & 2). ZS97 had the highest shoot formation rate (92.9%). Moreover, shoot and root lengths were longer in *GSN1*-OX than *GSN1*-KO transformants across all cultivars (Supplemental Fig. S5). Hyperhidrosis was not observed at any stage of the plant tissue transformation process. Plants that formed shoots and roots were selected with Hygromycin B. All shoot-forming plants counted in Tables 1 & 2 were confirmed as transformants. Notably, quite amount of transformed YD 6 calli and plants were killed by Hygromycin B in the shoot formation medium, consistent with previous research[35].

T$_1$ transgenic plant phenotyping

The T$_0$ seedlings of the six cultivars bearing the *GSN1*-OX and *GSN1*-KO plasmids were grown in Hainan (China). T$_1$ seeds were harvested from T$_0$ plants. Leaf pieces (2 cm) from each transformed plant were collected for Hygromycin B verification and PCR product amplification (Supplemental Fig. S6a and S6b).

The results showed that all samples had been successfully transformed and revealed alterations at the molecular level. Sanger sequencing was conducted to verify the *GSN1*-KO constructs in all six cultivars (Supplemental Fig. S7); the results showed that all transformed lines had been successfully modified, functionally disabling *GSN1* in those lines. Seed phenotypes were measured and analyzed in all wild-type and transgenic lines in the six cultivars (Fig. 4). The positive transgenic plants' phenotypes are well associated with genotypical alteration. We also performed qRT-PCR and confirmed that the *GSN1*-OX transformed plants in six cultivars have successfully increased the expression level of *GSN1* (Supplemental Fig. S8). Two independent lines derived from each cultivar were selected for each construct to further investigate *GSN1* function. Grain length was similar in all rice cultivars; the *GSN1*-OX and *GSN1*-KO lines had reduced and increased grain length, respectively. However, Kasalath and HHZ did not show significant grain elongation because the transgenic lines were heterozygous. ZS97, YD 6, and Kasalath showed decreased grain width in *GSN1*-OX lines, whereas *GSN1*-KO lines had wider grains. Interestingly, two high grain quality rice cultivars, NGZ and HHZ, showed different trends in seed width in *GSN1*-OX lines compared to the other cultivars. However, the *GSN1*-KO lines had wider grains in these cultivars, similar to the other cultivars. Grain thickness was similar between cultivars, except HHZ, for each type of transformant. In general, the *GSN1*-OX lines had thinner grains than the wild-type lines, whereas *GSN1*-KO lines had thicker grains. In contrast, HHZ showed the opposite effects in the OX and KO lines. In summary, transforming rice plants with a *GSN1* gene editing knockout or overexpression construct led to changes in seed shape across all six rice cultivars, as previously reported[19].

Expression patterns and single nucleotide polymorphisms (SNPs) in potentially essential genes contributed to rice callus transformation efficiency

To further explore the molecular basis of differences in transformation efficiency between the selected *indica* and *japonica* cultivars, we performed quantitative reverse transcription PCR (qRT-PCR) to verify expression of callus formation-related genes and to establish the associations between gene expression patterns and cultivars (Fig. 5). The expression of our callus-related genes, including *OsCRL*, *OsBOC1*, *OsBBM3*, and *OsSET1*, were evaluated[14,36−38] (Fig. 5). The expression of *OsBBM3* was significantly higher in callus samples from Kasalath compared to the other cultivars. Also, Kasalath owned the highest expression among all six cultivars of these four genes. The rest callus samples showed relatively lower expression levels (Fig. 5). We also found that HHZ has different expression patterns in *OsBOC1*, *OsCRL* and *OsSET1* (Higher expression in *OsCRL*, but lower expression in *OsBBM3*, *OsBOC1* and *OsSET1*). Except the *OsBOC1*, the expression of other three genes in YD 6 exhibited significantly lower than that in other cultivars. To further investigate the associations of haplotypes, expression levels and phenotypes, we performed Sanger sequencing to identify insertion/deletion mutations (InDels) and SNPs in these genes (Fig. 6). We cloned the promoter regions, untranslated regions (UTRs), coding regions, and introns of these genes. The sequencing results showed that two *OsCRL* SNPs were non-synonymous. N143D seemed to exist in *japonica* and *indica*, position 143 Asparagine (D) seems to be existed in *japonica*,
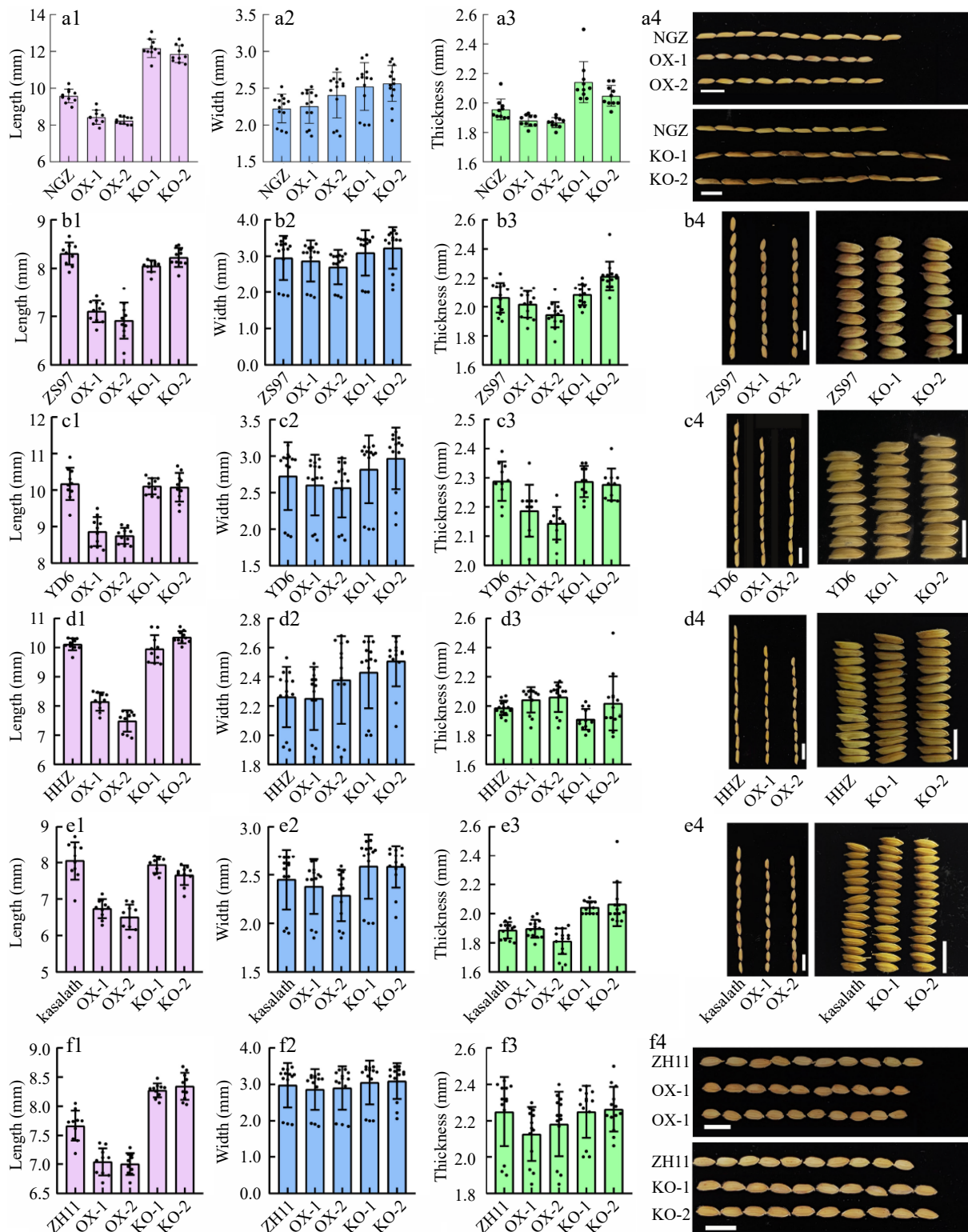
Fig. 4 Phenotypes of T$_2$ seeds of the six cultivars used in this study. For each cultivar, the average grain length (at left in pink, a1–f1), grain width (at middle left in blue, a2–f2), and grain thickness (at middle right in green, a3–f3) is shown for the wild-type, two *GSN1*-overexpression lines (OX-1 and OX-2), and two *GSN1*-knockout lines (KO-1 and KO-2). On the right (a4–f4) are grain phenotypes in the wild-type and mutant lines for each cultivar. Scale bar = 1 cm, n = 10.

and position 143 *Aspartic acid* (D) are shown in *indica* cultivars. In this gene, H114N is also another SNP that appeared in different cultivars. Position 114 Aspartic acid appeared only in NGZ and Kasalath (Fig. 6a). *OsBOC1* encodes a SIMILAR TO

RADICAL-INDUCED CELL DEATH ONE (SRO) protein that contributes to callus browning[14]. We identified two SNPs in coding regions, which is R32S and P377L. In our sequencing results, the R32S and P337L only existed in NGZ and Kasalath
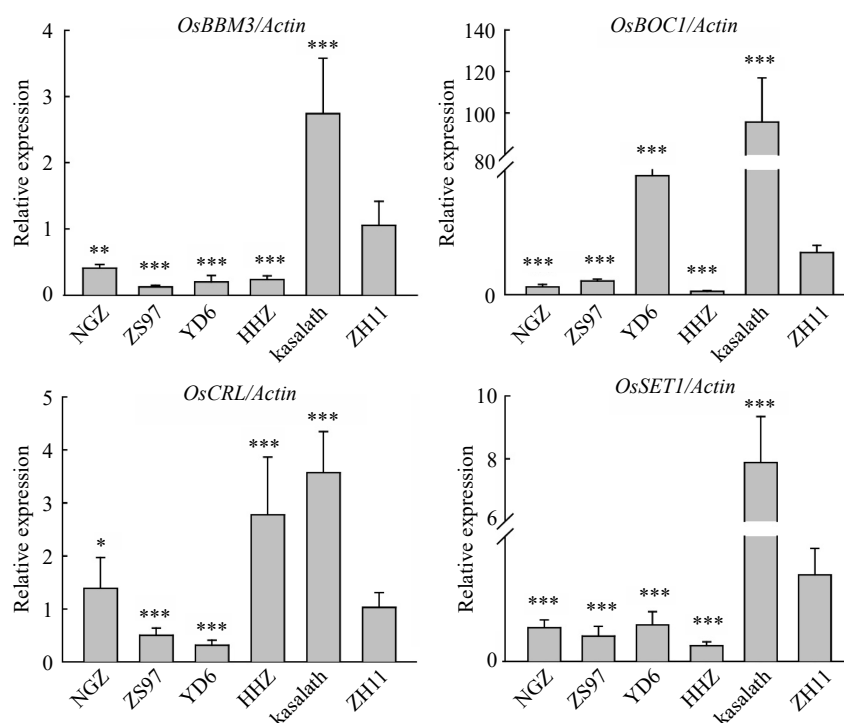
Fig. 5 Quantitative reverse transcription PCR (qRT-PCR) analysis of the callus related genes. Samples are harvested from calli after 7 d in CIM. The *Actin* gene was used as internal control to normalize gene expression data. Values are given as the mean ± SD. * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$ compared with the ZH11 control using Student's *t*-test. Standard deviations were calculated from three biological replicates.

(Fig. 6b), the other rice cultivars did not show significant SNPs or InDels at that locus. We did not identify nonsynonymous SNPs or InDels in the coding regions of *OsBBM3* or *OsSET1* among the tested cultivars (Fig. 6c & d). In summary, our results revealed that the callus induction-related genes might contribute to the callus browning and induction rate variation in *japonica* and *indica* cultivars.
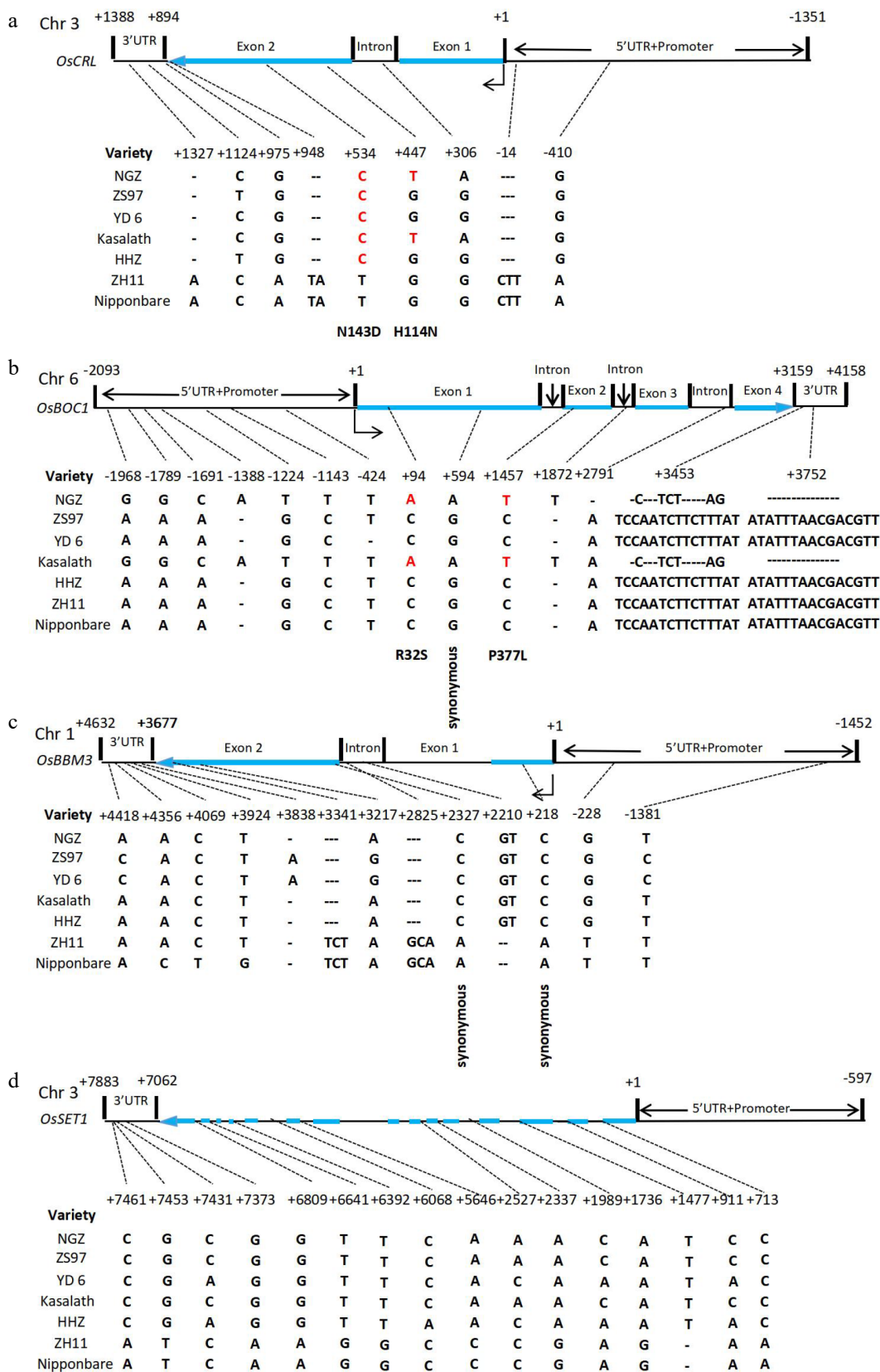
DISCUSSION

This study compared transformation efficiency of several rice cultivars and identified genetic variations that may explain variations in transformation efficiency between cultivars. Transformation has been a challenge in *indica* cultivars for decades. Previous transformation methods have functioned well in *japonica* rice cultivars[5] such as ZH11 and Nipponbare. However, the cost and low transformation rate have hindered functional studies and application of *indica* cultivars. At present, optimized protocols have been developed only for *japonica* and a few *indica* cultivars[8,15,39]. Several strategies can be used to advance rice transformation methods and address this issue. First, more elite rice cultivar transformation optimization protocols should be invested in and developed. There is currently little incentive for biotechnology companies to invest in optimizing protocols without support and/or funding from research institutes, universities, or the government. Universities and companies are likely to only apply fully developed methods to novel rice transformation assays, which would not improve the transformation rate. Therefore, developing a specific project or increasing support for new method development would promote optimization of novel rice cultivar transformation methods. In addition, researchers should be encouraged to apply novel and advanced transformation

methods. The field of plant biotechnology engineering currently uses biolistic technology and *Agrobacterium*-mediated transformation as the primary foreign material delivery methods in plants[9,40]. Within more than half a century of development, few species-specific transformation protocols have been established. The uncertainty of the integrated positions of foreign DNA fragments and *Agrobacterium* DNA contamination have decreased public trust in transgenic food safety[15]. However, the nanoparticle-mediated plasmid DNA delivery method has been successfully developed and applied to rice leaves and seeds[41,42]. This novel technology could aid in improving rice transformation methods.

NGZ, an elite *indica* cultivar, was developed and validated in 2016. Its grain quality is level I based on the Chinese Ministry of Agriculture and Rural Affairs Grain Quality Standard (GB/T17891-2017). The average grain yield of NGZ is over 8.25 t/hm². This rice cultivar has been a main recommended cultivar for growth in South China since 2020. Plant tissue culture experiments in the present study showed that this cultivar has a high induction rate compared to other common *indica* species. Our data also showed that during the callus induction phase, 82% of calli had been generated by 14 d in NGZ, which was close to the rates of ZS97 and ZH11 (Tables 1 & 2 and Supplemental Fig. S2). However, NGZ had unexpectedly low resistant callus induction rate in both the *GSN1*-OX and *GSN1*-KO transgenic lines compared to the equivalent transformants in ZS97 and ZH11 (Tables 1, 2, Fig. 3, and Supplemental Fig. S3). In the latter stages, including the green spot, shoot, and root formation stages, NGZ performed as well as the ZS97 rice cultivar (Tables 1 & 2). Except for the *Agrobacterium* infection stage, NGZ generally had superior transformation and induction rates compared to the other *indica* cultivars. Interestingly, NGZ and the other *indica* subspecies used the same chemical reagents

a Chr 3 — OsCRL

Positions: +1388, +894 (3'UTR); Exon 2; Intron; Exon 1; +1; 5'UTR+Promoter; −1351

Variety	+1327	+1124	+975	+948	+534	+447	+306	−14	−410
NGZ	-	C	G	--	C	T	A	---	G
ZS97	-	T	G	--	C	G	G	---	G
YD 6	-	C	G	--	C	G	G	---	G
Kasalath	-	C	G	--	C	T	A	---	G
HHZ	-	T	G	--	C	G	G	---	G
ZH11	A	C	A	TA	T	G	G	CTT	A
Nipponbare	A	C	A	TA	T	G	G	CTT	A

N143D   H114N

b Chr 6 — OsBOC1

Positions: −2093 (5'UTR+Promoter); +1; Exon 1; Intron; Exon 2; Intron; Exon 3; Intron; Exon 4; 3'UTR; +3159, +4158

Variety	−1968	−1789	−1691	−1388	−1224	−1143	−424	+94	+594	+1457	+1872	+2791	+3453	+3752
NGZ	G	G	C	A	T	T	T	A	A	T	T	-	-C---TCT-----AG	---------------
ZS97	A	A	A	-	G	C	T	C	G	C	-	A	TCCAATCTTCTTTAT	ATATTTAACGACGTT
YD 6	A	A	A	-	G	C	-	C	G	C	-	A	TCCAATCTTCTTTAT	ATATTTAACGACGTT
Kasalath	G	G	C	A	T	T	T	A	A	T	T	A	-C---TCT-----AG	---------------
HHZ	A	A	A	-	G	C	T	C	G	C	-	A	TCCAATCTTCTTTAT	ATATTTAACGACGTT
ZH11	A	A	A	-	G	C	T	C	G	C	-	A	TCCAATCTTCTTTAT	ATATTTAACGACGTT
Nipponbare	A	A	A	-	G	C	T	C	G	C	-	A	TCCAATCTTCTTTAT	ATATTTAACGACGTT

R32S   synonymous   P377L

c Chr 1 — OsBBM3

Positions: +4632, +3677 (3'UTR); Exon 2; Intron; Exon 1; +1; 5'UTR+Promoter; −1452

Variety	+4418	+4356	+4069	+3924	+3838	+3341	+3217	+2825	+2327	+2210	+218	−228	−1381
NGZ	A	A	C	T	-	---	A	---	C	GT	C	G	T
ZS97	C	A	C	T	A	---	G	---	C	GT	C	G	C
YD 6	C	A	C	T	A	---	G	---	C	GT	C	G	C
Kasalath	A	A	C	T	-	---	A	---	C	GT	C	G	T
HHZ	A	A	C	T	-	---	A	---	C	GT	C	G	T
ZH11	A	A	C	T	-	TCT	A	GCA	A	--	A	T	T
Nipponbare	A	C	T	G	-	TCT	A	GCA	A	--	A	T	T

synonymous   synonymous

d Chr 3 — OsSET1

Positions: +7883, +7062 (3'UTR); +1; 5'UTR+Promoter; −597

Variety	+7461	+7453	+7431	+7373	+6809	+6641	+6392	+6068	+5646	+2527	+2337	+1989	+1736	+1477	+911	+713
NGZ	C	G	C	G	G	T	T	C	A	A	A	C	A	T	C	C
ZS97	C	G	C	G	G	T	T	C	A	A	A	C	A	T	C	C
YD 6	C	G	A	G	G	T	T	C	A	C	A	A	A	T	A	C
Kasalath	C	G	C	G	G	T	T	C	A	A	A	C	A	T	C	C
HHZ	C	G	A	G	G	T	T	A	A	C	A	A	A	T	A	C
ZH11	A	T	C	A	A	G	G	C	C	C	G	A	G	-	A	A
Nipponbare	A	T	C	A	A	G	G	C	C	C	G	A	G	-	A	A

Fig. 6 Single nucleotide polymorphisms (SNPs) and insertion/deletion mutations (InDels) in four genes related to plant tissue culture in seven cultivars. The key and main SNPs and InDels are shown in the promoter regions, untranslated regions (UTRs), coding regions, and introns. Genes were analyzed for the six cultivars included in the experimental section of this study using Nipponbare as the reference genome. Results are shown for (a) *OsCRL*, (b) *OsBOC1*, (c) *OsBBM3*, and (d) *OsSET1*. Bases shown in red in (a) and (b) indicate potential functional SNPs that may contribute to callus induction and browning.

and protocols while ZS97 is well optimized (Supplemental Table S1). These results indicated that there could be considerable improvement in NGZ transformation efficiency.

We tested a reduced *Agrobacterium* co-culture time period to alleviate NGZ callus browning and contamination problems without much success (data not shown). We concluded that it is possible that the regeneration-related genes, such as *OsBOC1* and *OsSET1*, expression mode is not suitable in NGZ when co-cultured with *Agrobacterium*. A new reports focusing on *WOX11* and *CRL1 (OsCRL)* positively regulated root hair development, and it have shown that the importance of *CRL* genes in root development[43]. It is possible that the low regeneration efficiency in YD 6 is due to the low expression rate of *CRL* in it (Figs 5 & 6, Tables 1 & 2). Previous reports indicated that the upregulation of *OsBOC1* is capable of reducing the browning rate in cultivated rice and wild rice[14]. In the present study, we found that the SNPs from Kasalath and NGZ are identical, indicating that the high callus transformation efficiency of *OsBOC1* in NGZ is associated with Kasalath (Fig. 6). Interestingly, YD 6 callus is easier to be browned, but the qRT-PCR result of *OsBOC1* in YD 6 sample is the highest among *indica* cultivars (Fig. 5), these results suggest that other factors might also be involved in YD 6 callus browning regulation. The expression of *OsBOC1*, *OsCRL* and *OsSET1* are extremely high in Kasalath (Fig, 5, Tables 1 & 2), also hinted that the high transgenic successful rate in Kasalath. Recently, the maize GARP transcription factor gene *GOLDEN2* was overexpressed in rice calli; this improved callus regeneration by activating chloroplast development[44]. In wheat, GROWTH-REGULATING FACTOR 4 (GRF4) and its cofactor GRF-INTERACTING FACTOR 1 (GIF1) function as a regulatory module and increase regeneration efficiency in transgenic plants[45]. Integrating the *ZmBBM* and *GRF4-GIF1* modules into the CRISPR-Cas9 plasmid significantly enhanced the regeneration rate in maize[46], suggesting that these genes may function through a different pathway to regulate plant regeneration. It is possible that the *GOLDEN2*, *OsBBMs*, and *GRF4-GIF1* modules would also increase the survival and regeneration rate in NGZ and other *indica* cultivars.

Except genetic factors, other physiological factors, such as *Agrobacterium* strains, phytohormone contents, carbohydrates, calli quality etc., also largely determined the plant tissue culture process and transformation efficiency[47]. Earlier studies focusing on improving specific rice variety transformation efficiency through plant tissue culture process. Optimizing this experiment by adjusting medium components as well as temperature, light density, *agrobacterium* concentration have successfully enhanced the transformation efficiency and resistant calli amount in certain rice cultivars[48]. This is also one of our future working directions to attempt linking physiological phenotypes to genetic basis, eventually we can optimize the rice transformation efficiency from the bottom.

In rice, *GSN1* has been reported to control grain size and spikelet trade-offs[19], and *ERECTA* (*ER*) functions as an upstream regulator of the MAPK cascade to activate cytokinin signals during spikelet differentiation[49]. These regulatory networks contribute to spikelet formation and seed maturation. In this study, we used *GSN1* as a case study to evaluate rice transformation efficiency (Fig. 4). Our data showed that overexpressing or knocking out *GSN1* caused the largest phenotype changes in the *indica* cultivars HHZ and NGZ (Fig. 4). These typical high grain quality rice cultivars grow well in South China,

and compared to other *indica* types, they had longer and thinner grains. However, overall yield production should still be increased. Our study provides potential strategies to improve gene editing and transgenic methods to achieve high yield in high grain quality rice.

Our work compared NGZ to the other four *indica* cultivars and a *japonica* cultivar in plant tissue culture regeneration efficiency and transformed efficiency. Although NGZ's performance is not as excellent as ZS97 in *indica* cultivars (Tables 1 & 2), its good performance in plant tissue culture regeneration efficiency is recommendable. All media used in our experiments are called LY media, which is provided by BioRun Co. LTD. We listed all the ingredients and usages in Supplemental Table S1. According to technicians from BioRun Company, these media for rice *indica* cultivar plant tissue culture is well optimized, especially worked well in ZS97. However, ZS97 is an *indica* bred more than 40 years ago, its agronomical traits are not the best options for current rice studies. Although this is a well-optimized *indica* cultivar and performance the best in our experiment, NGZ, a newly bred *indica* cultivar in 2016, its grain quality, grain shape, abiotic stress, biotic resistance, and other essential agronomical traits are better than ZS97 and more suitable for phenotypical observation and studies in rice science. Moreover, NGZ's plant tissue culture optimization and plant tissue culture potentials will be our effort in the following research aim. Thus, we hope that NGZ would be applied in gene editing research and molecular basis studies.

CONCLUSIONS

In summary, we here used *GSN1* as a case study to compare the regeneration efficiency of one *japonica* and five *indica* rice cultivars. Each cultivar was transformed with a *GSN1*-overexpression plasmid and a CRISPR-Cas9 *GSN1*-knockout plasmid. Plants transformed with the gene editing plasmid had lower overall regeneration rates than those with the overexpression plasmid. NGZ and ZS97 had the highest transformation efficiency of the *indica* cultivars, but both required longer culture times than the *japonica* cultivar. We also verified expression patterns, SNPs and InDels of selected genes related to callus regeneration among these cultivars and provided possible explanations for differences in transformation efficiency between *indica* cultivars. Finally, we discussed the disadvantages of NGZ callus browning. We also discussed the advantages and possibility to apply NGZ in the future gene editing and functional studies. Our findings related to NGZ callus regeneration and reducing callus browning are expected to increase the application of NGZ in rice transformation assays.

ACKNOWLEDGMENTS

Conflict of interest

The authors declare that they have no conflict of interest.

Dates

REFERENCES

1. Fukagawa NK, Ziska LH. 2019. Rice: Importance for Global Nutrition. *Journal of Nutritional Science and Vitaminology* 65:S2−S3

2. Hiei Y, Ohta S, Komari T, Kumashiro T. 1994. Efficient transformation of rice (*Oryza sativa* L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. *The Plant Journal* 6:271−82

3. Lin YJ, Zhang Q. 2005. Optimising the tissue culture conditions for high efficiency transformation of indica rice. *Plant Cell Reports* 23:540−47

4. Ge X, Chu Z, Lin Y, Wang S. 2006. A tissue culture system for different germplasms of *indica* rice. *Plant Cell Reports* 25:392−402

5. Hiei Y, Komari T. 2008. *Agrobacterium*-mediated transformation of rice using immature embryos or calli induced from mature seed. *Nature Protocols* 3:824−34

6. Ozawa K. 2009. Establishment of a high efficiency Agrobacterium-mediated transformation system of rice (*Oryza sativa* L.). *Plant Science* 176:522−27

7. Susanto FA, Wijayanti P, Fauzia AN, Komalasari RD, Nuringtyas TR, et al. 2020. Establishment of a plant tissue culture system and genetic transformation for agronomic improvement of Indonesian black rice (*Oryza sativa* L.). *Plant Cell, Tissue and Organ Culture (PCTOC)* 141:605−17

8. Molina-Risco M, Ibarra O, Faion-Molina M, Kim B, Septiningsih EM, et al. 2021. Optimizing *Agrobacterium*-mediated transformation and CRISPR-Cas9 gene editing in the tropical *Japonica* rice variety presidio. *International Journal of Molecular Sciences* 22:10909

9. Matsumoto TK, Gonsalves D. 2012. Biolistic and other non-Agrobacterium technologies of plant transformation. In *Plant Biotechnology and Agriculture*, eds. Altman A, Hasegawa PM. Academic Press. pp. 117−29. https://doi.org/10.1016/B978-0-12-381466-1.00008-0

10. Kwak SY, Lew TTS, Sweeney CJ, Koman VB, Wong MH, et al. 2019. Chloroplast-selective gene delivery and expression in planta using chitosan-complexed single-walled carbon nanotube carriers. *Nature Nanotechnology* 14:447−55

11. Njiti VN, Myers O Jr, Schroeder D, Lightfoot DA. 2003. Roundup Ready Soybean: Glyphosate Effects on Fusarium solani Root Colonization and Sudden Death Syndrome. *Agronomy Journal* 95:1140−45

12. Vuorinen AL, Nieminen A, Gaba V, Sikorskaite S, Valkonen JP. 2013. Biolistic DNA delivery to leaf tissue of plants with the non-vacuum gene gun (HandyGun). In *Biolistic DNA Delivery. Methods in Molecular Biology*, eds. Sudowe S, Reske-Kunz AB. 940: XIV,415. New Jersey: Humana Totowa. pp. 45−51. https://doi.org/10.1007/978-1-62703-110-3_4

13. Gu Z, Biswas A, Zhao M, Tang Y. 2011. Tailoring nanocarriers for intracellular protein delivery. *Chemical Society Reviews* 40:3638−55

14. Zhang K, Su J, Xu M, Zhou Z, Zhu X, et al. 2020. A common wild rice-derived *BOC1* allele reduces callus browning in *indica* rice transformation. *Nature Communications* 11:443

15. Elakhdar A, Fukuda M, Kubo T. 2021. *Agrobacterium*-mediated transformation of *Japonica* rice using mature embryos and regenerated transgenic plants. *Bio-protocol* 11:e4143

16. Chen K, Łyskowski A, Jaremko Ł, Jaremko M. 2021. Genetic and molecular factors determining grain weight in rice. *Frontiers in Plant Science* 12:605799

17. Xu R, Duan P, Yu H, Zhou Z, Zhang B, et al. 2018. Control of Grain Size and Weight by the OsMKKK10-OsMKK4-OsMAPK6 Signaling Pathway in Rice. *Molecular Plant* 11:860−73

18. Xu R, Yu H, Wang J, Duan P, Zhang B, et al. 2018. A mitogen-activated protein kinase phosphatase influences grain size and weight in rice. *The Plant Journal* 95:937−46

19. Guo T, Chen K, Dong N, Shi C, Ye W, et al. 2018. *GRAIN SIZE AND NUMBER1* Negatively Regulates the OsMKKK10-OsMKK4-OsMPK6 Cascade to Coordinate the Trade-off between Grain Number per Panicle and Grain Size in Rice. *The Plant Cell* 30:871−88

20. Wang T, Zou T, He Z, Yuan G, Luo T, et al. 2019. GRAIN LENGTH AND AWN 1 negatively regulates grain size in rice. *Journal of Integrative Plant Biology* 61:1036−42

21. Ministry of Agriculture and Rural Affairs of the People's Republic of China. 2020. *Ministry of Agriculture and Rural Affairs of the People's Republic of China Announcement No. 360*. www.zzj.moa.gov.cn/gsgg/202012/t20201202_6357482.htm

22. Ma X, Zhang Q, Zhu Q, Liu W, Chen Y, et al. 2015. A robust CRISPR/Cas9 system for convenient, high-efficiency multiplex genome editing in monocot and dicot plants. *Molecular Plant* 8:1274−84

23. Xie X, Ma X, Zhu Q, Zeng D, Li G, et al. 2017. CRISPR-GE: A convenient software toolkit for CRISPR-based genome editing. *Molecular Plant* 10:1246−49

24. Zhao W, Zheng S, Ling H. 2011. An efficient regeneration system and Agrobacterium-mediated transformation of Chinese upland rice cultivar Handao297. *Plant Cell, Tissue and Organ Culture (PCTOC)* 106:475−83

25. Duan YB, Zhao FL, Chen HD, Li H, Ni DH, et al. 2015. A simplified genomic DNA extraction protocol for pre-germination genotyping in rice. *Genet Mol Res* 14:6369−75

26. Rao X, Huang X, Zhou Z, Lin X. 2013. An improvement of the $2^{-\Delta\Delta Cт}$ method for quantitative real-time polymerase chain reaction data analysis. *Biostatistics, Bioinformatics and Biomathematics* 3:71−85

27. Toki S, Hara N, Ono K, Onodera H, Tagiri A, et al. 2006. Early infection of scutellum tissue with Agrobacterium allows high-speed transformation of rice. *The Plant Journal* 47:969−76

28. Sahoo KK, Tripathi AK, Pareek A, Sopory SK, Singla-Pareek SL. 2011. An improved protocol for efficient transformation and regeneration of diverse indica rice cultivars. *Plant Methods* 7:49

29. Lin H, Xia P, Wing R, Zhang Q, Luo M. 2012. Dynamic intra-japonica subspecies variation and resource application. *Molecular Plant* 5:218−30

30. Zhang J, Li C, Wu C, Xiong L, Chen G, et al. 2006. RMD: a rice mutant database for functional analysis of the rice genome. *Nucleic Acids Research* 34:D745−D748

31. Chen S, Wang A, Li W, Wang Z, Cai X. 2008. Establishing a gene trap system mediated by T-DNA(GUS) in rice. *10.1111/j.1744-7909.2007.00611.x* 50:742−51

32. Saika H, Toki S. 2010. Mature seed-derived callus of the model indica rice variety Kasalath is highly competent in *Agrobacterium*-mediated transformation. *Plant Cell Reports* 29:1351−64

33. Zhou X, Chen D, Guo J, Chen P, Li L, et al. 2021. Genetic improvement of grain quality traits in indica inbred rice cultivars developed in South China during 1956−2020. *Euphytica* 218:8

34. Zhang W, Dewey RE, Boss W, Phillippy BQ, Qu R. 2013. Enhanced *Agrobacterium*-mediated transformation efficiencies in monocot cells is associated with attenuated defense responses. *Plant Molecular Biology* 81:273−86

35. Li S, Yan S, Wang A, Zou G, Huang X, et al. 2013. Identification of QTLs associated with tissue culture response through sequencing-based genotyping of RILs derived from 93-11 × Nipponbare in rice (*Oryza sativa*). *Plant Cell Reports* 32:103−16

36. Liu X, Zhou C, Zhao Y, Zhou S, Wang W, Zhou DX. 2014. The rice enhancer of zeste [E(z)] genes *SDG711* and *SDG718* are respectively involved in long day and short day signaling to mediate the accurate photoperiod control of flowering time. *Frontiers in Plant Science* 5:591

37. Coudert Y, Le VAT, Adam H, Bès M, Vignols F, et al. 2015. Identification of CROWN ROOTLESS1-regulated genes in rice reveals specific and conserved elements of postembryonic root formation. *New Phytologist* 206:243−54

38. Khanday I, Skinner D, Yang B, Mercier R, Sundaresan V. 2019. A male-expressed rice embryogenic trigger redirected for asexual propagation through seeds. *Nature* 565:91−95

39. Liang Y, Biswas S, Kim B, Bailey-Serres J, Septiningsih EM. 2021. Improved transformation and regeneration of *Indica* rice: Disruption of *SUB1A* as a test case via CRISPR-Cas9. *International Journal of Molecular Sciences* 22:6989

40. Luu VT, Stiebner M, Maldonado PE, Valdés S, Marín D, et al. 2020. Efficient *Agrobacterium*-mediated transformation of the Elite-*Indica* rice variety Komboka. *Bio-protocol* 10:e3739

41. Demirer GS, Zhang H, Matos JL, Goh NS, Cunningham FJ, et al. 2019. High aspect ratio nanomaterials enable delivery of functional genetic material without DNA integration in mature plants. *Nature Nanotechnology* 14:456−64

42. Dunbar T, Tsakirpaloglou N, Septiningsih EM, Thomson MJ. 2022. Carbon Nanotube-Mediated Plasmid DNA Delivery in Rice Leaves and Seeds. *International Journal of Molecular Sciences* 23:4081

43. Geng L, Li Q, Jiao L, Xiang Y, Deng Q, et al. 2023. WOX11 and CRL1 act synergistically to promote crown root development by maintaining cytokinin homeostasis in rice. *New Phytologist* 237:204−16

44. Luo W, Tan J, Li T, Feng Z, Ding Z, et al. 2023. Overexpression of maize GOLDEN2 in rice and maize calli improves regeneration by activating chloroplast development. *Science China Life Sciences* 66:340−49

45. Debernardi JM, Tricoli DM, Ercoli MF, Hayta S, Ronald P, et al. 2020. A GRF-GIF chimeric protein improves the regeneration efficiency of transgenic plants. *Nature Biotechnology* 38:1274−79

46. Chen Z, Debernardi JM, Dubcovsky J, Gallavotti A. 2022. The combination of morphogenic regulators BABY BOOM and GRF-GIF improves maize transformation efficiency. *BioRxiv* Preprint

47. Chen E, Zhang P, Zuo S, Li A, Zhang Y, et al. 2004. Factors affecting *Agrobacterium*-mediated transformation efficiency in rice. *Rice Science* 11:181−85

48. Li S, Wang S, Yin F, Zou L, Qi D, et al. 2005. Some factors affecting *Agrobacterium*-mediated transformation frequency in rice. *Chinese Journal of Rice Science* 19:231−37

49. Guo T, Lu Z, Shan J, Ye W, Dong N, et al. 2020. *ERECTA1* Acts Upstream of the OsMKKK10-OsMKK4-OsMPK6 Cascade to Control Spikelet Number by Regulating Cytokinin Metabolism in Rice. *The Plant Cell* 32:2763−79