OsGATA16, a GATA Transcription Factor, Confers Cold Tolerance by Repressing OsWRKY45-1 at the Seedling Stage in Rice

Hongjia Zhang
Jilin University
Tao Wu
Jilin University
Zhao Li
Jilin University
Kai Huang
Jilin University
Na-Eun Kim
Pusan National University
Ziming Ma
Jilin University
Soon-Wook Kwon
Pusan National University
Wenzhu Jiang (jwz1975@jlu.edu.cn)
Jilin University https://orcid.org/0000-0001-6359-0480
Xinglin Du
Jilin University

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Abstract

Background

Cold stress in rice is a major abiotic stress that adversely affects growth and substantially reduces rice yield. Identification of cold-related functional rice genes is important for breeding programs aimed at increasing resilience and yield in rice crops. GATA-family transcription factors involve diverse function in rice, however, their roles in the response to low-temperature stress remain unclear.

Results

A GATA-type zinc finger transcription factor, *OsGATA16*, that increases cold tolerance in rice. OsGATA16 is an OsGATA subfamily-II protein and contains eleven putative phosphorylation sites, NLS, and several conserved domains. Overexpression of *OsGATA16* increased tolerance to cold stress at seedling stage. Transcriptional analysis showed that *OsGATA16* was induced by cold and ABA treatments, but was repressed by drought, cytokinin, and JA. *OsGATA16* was expressed in all plant tissues, with highest expression in panicles. Subcellular localization and transcriptional analysis indicated that OsGATA16 acted as a nuclear-targeted transcriptional suppressor. Four cold-related genes (*OsWRKY45-1*, *OsSRFP1*, *OsCYL4*, and *OsMYB30*) were repressed in *OsGATA16*-overexpression lines compared with wild type after low-temperature exposure. Yeast one-hybrid and Dual-luciferase reporter assays showed that OsGATA16 bound to the promoter of *OsWRKY45-1* and repressed its expression. Eleven SNPs within *OsGATA16* were identified and haplotype analysis showed a polarization between Japonica and Indicia subspecies. A non-synonymous SNP was identified that explained differences in cold tolerance among the 137 rice accessions.

Conclusion

A novel GATA transcription factor, OsGATA16, plays a positive role in cold tolerance at the seedling stage in rice by direct repression of *OsWRKY45-1* expression. One SNP was identified that explained cold tolerance differences among rice accessions. These results support future breeding programs to improve cold tolerance in commercial rice crops.

Introduction

Rice (*Oryza sativa L.*) is an important staple food crop that provides sustenance for more than half the global population (Fairhurst and Dobermann 2002; Tang et al. 2019). Rice production is confined to certain cultivation regions due to its temperature sensitivity, and rice crops experience frequent environmental stresses, such as extremes of temperature, drought, and high salinity, which risk declines in the quality and abundance of rice production (Hussain et al. 2018; Kumar et al. 2014). Increasing global populations and the resulting increases in demand for food have prompted the expansion of rice production to less-suitable cultivation areas, increasing the probability that rice crops will be subject to severe environmental stresses (Zhang et al. 2017). For example, low temperatures in China reduced rice yield by 3–5 hundred million tons, with severe impacts on grain security (Zhang et al. 2017; Zhu et al. 2015). The optimal temperature for rice

growth is 26–30°C, and the impacts of exposure to cold temperatures vary according to growth stage. Low-temperature exposure at the seedling stage affects physiological metabolism (Zhang et al. 2014); exposure at the booting stage adversely affects the fructification percentage (Jiang et al. 2010); and exposure at the flowering and pollination stages affects pollination and fructification percentages (Shinada et al. 2013; Shakiba et al. 2017). The genetic and molecular basis of cold tolerance in rice is therefore an area of active research due to its practical relevance.

Plants respond to stresses, such as cold exposure, by activation of internal stress defense mechanisms that stimulate physiological responses. For example, overexpression of several stress-responsive genes, including *OsAPX1* and *OsiSAP8*, resulted in physiological changes that improved cold tolerance (Sato et al. 2011; Kanneganti and Gupta 2008). The rice cold signaling pathway is an area of active research, and one study identified COLD1 as a novel rice cold sensor. A SNP at this locus conferred cold tolerance in Japonica rice, and COLD1 was found to interact with a G-protein subunit and expedite GTPase activity (Ma et al. 2015). The plant hormone ABA was also found to be involved in the cold signaling pathway (Vishwakarma et al. 2017; Ma et al. 2009; Park et al. 2009; Fujii et al. 2009; Kim et al. 2012). Under cold stress, ABA levels were found to increase and stimulate binding of the ABA receptor PYR to PP2C, thus repressing PP2C binding to SnRK2. SnRK2 then phosphorylated other TFs, activating the expression of ABA response genes and increasing cold stress tolerance. Other diverse TFs involved in cold tolerance were also identified, such as bZIPs (Liu et al. 2012; Shimizu et al. 2005; Zou et al. 2008; Hossain et al. 2010), WRKYs (Kim et al. 2016; Yokotani et al. 2013), ZFPs (Liu et al. 2007; Huang et al. 2009; Zhang et al. 2012), and TCPs (Yang et al. 2013; Wang et al. 2014), which had positive or negative effects on cold tolerance in rice.

In plants, trans-acting factors interact with specific cis-acting elements in the promoters of target genes to activate or repress gene expression (Franco-Zorrilla et al. 2014) according to their diverse functional activities. One such TF family, the OsGATA family, contains highly conserved structures in the rice genome and is responsible for the regulation of a range of plant functions (Gupta et al. 2017). Members of the GATA family are DNA-binding proteins that bind to a specific sequence, XGATAY (X is T or A, Y is G or A), in the promoters of target genes (Reyes et al. 2004). GATA proteins contain a GATA-type zinc finger protein domain (C-X2-C-X(17–20)-C-X2-C) located close to the DNA-binding domain (Gupta et al. 2017). In previous studies, approximately 30 GATA genes were identified from rice and *Arabidopsis thaliana*, and were divided into four classes, A, B, C, and D, according to the numbers and locations of introns and exons (Reyes et al. 2004). A separate study identified 28 OsGATAs, divided into seven subfamilies (subfamily-I, II, III, IV, V, VI, VII) according to their gene structure and the number and positions of GATA domains. Subfamily-II was further subdivided into Class-A and Class-B according to the structural features (Gupta et al. 2017). A highly conserved HAN domain was identified at the N-terminal of three Class-A GATA-family proteins (*OsGATA9*, *OsGATA14*, and *OsGATA15*). Six Class-B proteins (*OsGATA8*, *OsGATA10*, *OsGATA11*, *OsGATA12*, *OsGATA13*, and *OsGATA16*) contained an LLM domain at the C-terminal. The specific functions of these proteins were explored in a separate study (Behringer and Schwechheimer 2015). GATA-family functions in plants have been explored only recently, in contrast to earlier research in fungi and animals (Tsai et al. 1994; Scazzocchio 2000; Tong et al. 2000; Zhang and He 2018). Several functions of GATA TFs have been identified in plants, including functions related to flowering, metabolism, leaf growth, organelle

development, and responses to plant hormones (Richter et al. 2010; Richter et al. 2013; Chiang et al. 2012; Hudson et al. 2013; Zhang et al. 2015; He et al. 2018).

In this study, OsGATA16, a GATA TF belonging to Class-B of OsGATA subfamily-II, was identified and characterized in rice. Overexpression transgenic lines were constructed, and OsGATA16 was shown to increase cold tolerance through repression of *OsWRKY45-1*, with no apparent adverse effects on multiple agronomic traits in rice.

Results

OsGATA16 Encodes a GATA Class-B TF

Several cold stress-related TFs were identified previously through bioinformatic screening, including OsGATA16 (LOC_Os06g37450), a novel GATA-type zinc finger TF containing eleven cold-related putative phosphorylation sites (Fig. 1a). The full-length coding region of *OsGATA16* contains 1173 nucleotides and encodes a protein containing 390 amino acid residues with a pI of 9.82 and MW of 41.1 KDa. The OsGATA16 protein structure includes a highly conserved GATA-type zinc finger protein domain, an LLM domain, and NLS (Fig. 1a). The provisional name for the protein in the National Center for Biotechnology Information (NCBI) database was OsGATA22; however, previous study named the protein as OsGATA16. OsGATA16 belongs to Class-B of GATA subfamily-II due to its LLM domain and exon and intron numbers (Behringer and Schwechheimer 2015). Comparison of *OsGATA16* homologous genes in diverse plant species (*Brachypodium distachyon*, *Setaria italic*, *Sorghum bicolor*, *Zea mays*, and *Arabidopsis thaliana*) revealed that the GATA zinc finger domains were highly conserved over substantial evolutionary time (Fig. 1b). Phylogenetic analysis of subfamily-II genes using MEGA7.0 showed that *OsGATA16* was most similar to *OsGATA11* (Fig. 1c).

Transcriptional Analysis of OsGATA16

The *OsGATA16* promoter region (2000 bp upstream of the initial ATG) was analyzed to gain insights into the biological function of OsGATA16. Using online tools (https://sogo.dna.affrc.go.jp), several putative *cis*-regulatory elements related to abiotic stress and hormones were found, including cold-responsive elements, dehydration responsive elements, ABA responsive elements, salt induced elements, and cytokinin responsive elements (Table 2).

The promoter analysis suggested that *OsGATA16* might be associated with abiotic stress and hormone responses via transcriptional regulation mechanisms. Therefore, we next examined *OsGATA16* transcriptional expression under conditions of abiotic stress (cold, drought, high salinity) and upon exposure to plant hormones (ABA, 6-BA, and JA). *OsGATA16* expression was induced by cold and ABA treatments, but suppressed by drought, 6-BA, and JA treatments (Fig. 2). Under cold and ABA treatments, *OsGATA16* expression increased within 3 h of exposure and then decreased gradually (Fig. 2a and 2c). Under drought and JA treatments, expression was rapidly and substantially repressed within 0.5 h, followed

by maintenance of low expression levels (Fig. 2b and 2e). Under BA treatment, *OsGATA16* expression was repressed more slowly, within 6 h (Fig. 2d). Expression after exposure to high NaCl levels was more complex, with an increase in expression following initial repression (Fig. 2f).

To further explore the temporal and spatial expression of *OsGATA16*, qRT-PCR was performed with diverse plant tissues from different rice growth stages. *OsGATA16* was expressed in all plant tissues tested, including young roots, stems, and leaves at the seedling stage, and stems, flag leaves, panicles, and leaf sheaths at the booting stage. Expression was most abundant in the panicles, followed by stems, leaf sheaths, young roots, and flag leaves (Fig. 3a).

OsGATA16 Overexpression Increases Cold Tolerance in Rice

Transcription of *OsGATA16* was induced by cold stress (Fig. 2a), and cold-responsive elements were identified in the promotor region (Table 1), suggesting the involvement of *OsGATA16* in the response to cold stress in rice. To assess this, transgenic rice lines were generated that overexpressed *OsGATA16*. The full coding region of *OsGATA16* under the control of the maize ubiquitin promoter was introduced into the Kitaake Japonica rice variety via Agrobacterium-mediated transformation (Fig. S1a). Two independent OE lines were obtained and named OE-1 and OE-2 (Fig. S1c). Expression analysis by qRT-PCR showed that *OsGATA16* expression in OE-1 and OE-2 lines was up-regulated substantially compared with WT plants under normal conditions (Fig. S1b).

Cold stress treatments were performed at the seedling stage in OE and WT rice plants. Plants were grown at a low temperature (8°C) for 7 days and then allowed to recover at a normal temperature (28°C) for 7 days. OE and WT seedlings displayed no apparent phenotypic differences before the cold treatment (Fig. 4a), but clear differences were observed after treatment (Fig. 4b). OE-1 and OE-2 plants showed significantly higher survival rates compared with WT plants (Fig. 4c). These results suggested that overexpression of *OsGATA16* in rice could improve cold tolerance at the seedling stage.

Additionally, rice agronomic traits were evaluated under normal field conditions, and no obvious differences for plant height and hundred-grain weight were observed between OE and WT plants (Fig. S2).

Nuclear Localization and Transcriptional Activity of OsGATA16

The OsGATA16 protein contains a basic NLS region (KVKKEKRADVDRSSLPFKKRC), suggesting that its activity lies within the nucleus. To determine the subcellular localization of OsGATA16, an OsGATA16-GFP fusion was constructed under the control of the ubiquitin promoter and was co-transformed into rice protoplast cells alongside a nuclear localization marker, D53-RFP (Zhou et al. 2013). As shown in Fig. 3b, GFP signal was observed in both the cytosol and nucleus of GFP-independent group, whereas the OsGATA16-GFP fusion protein localized predominantly at the nucleus. This indicates that OsGATA16 primarily functions within the rice cell nucleus.

Yeast two-hybrid and Dual-luciferase reporter assays were used to assess the transcriptional activity of OsGATA16. In two-hybrid analysis, the positive control exhibited growth on SD/-Trp and SD/-Trp-His media (Fig. 5a). The experimental target (BD-OsGATA16) results were consistent with the negative control (BD), with normal growth on SD/-Trp medium and inhibited growth on SD/-Trp-His medium, indicating transcriptional repression (Fig. 5a). In the dual-luciferase assay, effector and reporter plasmids were co-transformed into rice protoplast cells, with pPTRL (REN luciferase) used as an internal reference (Fig. 5b). As shown in Fig. 5c and 5d, LUC luciferase activities were significantly repressed in the experimental target (GAL4 BD-OsGATA16) compared with the control (GAL4 BD). Furthermore, when OsGATA16 was fused with the VP16 activation domain, the LUC luciferase activity of the target group (GAL4 BD-VP16-OsGATA16) was repressed > 2000-fold compared with the control (GAL4 BD-VP16). Taken together, these results indicate that OsGATA16 acts as a transcriptional suppressor, and this activity was intensified by the VP16 transactivation domain.

Overexpression of OsGATA16 Prompts Down-Regulation of Cold-Related Genes

Cold-sensitive genes were identified in the NCBI database, and a subset of genes was used to explore the cold-related signal transduction mechanism of OsGATA16 in rice. Expression of cold-related genes differed between OE and WT plants under normal and cold conditions, as determined using qRT-PCR with specific primers (Table 2). Four cold-sensitive genes were down-regulated in OE lines under normal and cold conditions: *OsWRKY45-1*, *OsSRFP1*, *OsCYL4*, and *OsMYB30* (Fig. S3). These results suggest that OsGATA16 acts as a cold-related functional factor and is involved in cold signaling pathways by direct or indirect regulation of these candidate genes.

OsGATA16 Suppresses the OsWRKY45-1 Promoter

GATA proteins are thought to bind to cis-regulatory elements containing GATA motifs (XGATAY). Several XGATAY elements were found in the promoter regions of four cold-responsive genes whose expression was repressed by *OsGATA16* overexpression (Table 3). This suggested that OsGATA16 might bind to the promoters of these candidate genes and regulate their transcription as part of the cold signaling pathway.

Yeast one-hybrid and Dual-luciferase reporter assays were used to assess OsGATA16 binding to the promoters of the candidate genes. Several reporter constructs were generated for the rice *in vivo* Dual-luciferase assay, with the LUC and REN luciferase genes under the control of candidate and *35S* promoters, respectively (Fig. 6a). Under the control of the *OsWRKY45-1* promoter, LUC luciferase activity was substantially repressed in OE lines compared with WT (Fig. 6b). By contrast, the *OsSRFP1* promoter exhibited slight activation (Fig. 6c), and the *OsMYB30* and *OsCYL4* promoters showed slight repression in the OE lines compared with WT (Fig. 6d and 6e). These results suggest that OsGATA16 represses the *OsWRKY45-1* promoter in rice. To further examine this interaction, effector and reporter constructs were generated for yeast one-hybrid analysis of the *OsWRKY45-1* promoter (Fig. 6f). Positive interactions in the experiment were indicated by a chromogenic reaction due to LacZ expression on growth medium containing X-Gal (SD/-Trp/-Ura/+X-Gal). As shown in Fig. 6g, independent expression of GAD or LacZ

(negative controls) resulted in no blue coloration, whereas the OsGATA16-*OsWRKY45-1*$_{pro}$ combination resulted in a strong chromogenic response, confirming their interaction (Fig. 6g).

Taken together, the qRT-PCR analysis, GATA motif screening, and Dual-luciferase reporter and yeast one-hybrid assays indicate that the OsGATA16 protein directly binds to the *OsWRKY45-1* promoter and represses its expression.

Haplotype and Functional SNP Analysis of OsGATA16

Re-sequencing data encompassing approximately two million high-quality SNPs from a collection of 137 rice accessions was used for haplotype analysis (Kim et al. 2016; Zhang et al. 2020). SNP positions and the structure of the *OsGATA16* gene are shown in Fig. 7a, with haplotype (Hap) information shown in Fig. 7b. In total, eleven SNPs were found in the promoter, UTR, intron, and exon regions of *OsGATA16* in five Haps. Linkage disequilibrium (LD) showed that the eleven SNPs in the 3 kb gene region showed strong LD relationships between the SNP pairs (Fig. 7c), as predicted by the degree of conservation of *OsGATA16*. Phenotype–haplotype relationships were analyzed, with a CT score (1–9 scale) used as the evaluation index. As shown in Fig. 7d, Hap2 and Hap3 exhibited significantly higher scores than the other Haps, indicating that these two Haps were associated with higher sensitivities to cold. Hap1 and Hap5 had CT scores that were lower than Hap2 and Hap3 but higher than Hap4, with Hap4 showing the highest tolerance for cold stress (Fig. 7d). The haplotype network and variation relationships of each Hap were assessed. Consistent with the phenotypic analysis, the five Haps divided into two groups. The first group comprised Hap1, Hap4, and Hap5 and contained most of the Japonica varieties. The second group comprised Hap2 and Hap3 and contained most of the Indica, Aus, and Aromatic varieties (Fig. 7e). Hap2 and Hap3 were closely related, as were Hap4 with Hap5, with only single SNP differences. However, Hap3 differed from Hap4 by five SNPs, and Hap4 also exhibited a distant relationship with Hap1 (Fig. 7e). These results indicate that the *OsGATA16* gene is polarized between the two major rice subspecies, Japonica and Indica.

Of the eleven identified SNPs, SNP 8, is a non-synonymous SNP (AGT to AAT) in exon, and this results in an amino acid change from serine to aspartic acid. The SNP 8 haplotype was thus assessed for its association with cold tolerance. The SNP8 336^{A} and 336^{G} genotypes were found in both Japonica and Indica varieties, and the 336^{G} genotype was associated with higher cold tolerance in both subspecies. When considered together, Japonica and Indica cultivars with the 336^{A} genotype had an average CT score of 7.6, and those with the 336^{G} genotype had an average score of 4.11 (Fig. 7h). Independent consideration of the Japonica and Indica varieties yielded similar results, with the 336^{G} genotype having average CT scores of 4.21 and 3.71, and the 336^{A} genotype having average scores of 7.18 and 7.89, for Japonica and Indica varieties, respectively (Fig. 7f and Fig. 7g).

The haplotype grouping of eleven SNPs in *OsGATA16* suggested a possibility of different biological functions in Japonica and Indica varieties. However, SNP 8 was associated with cold tolerance in both rice subspecies, and the 336^{G} genotype may enhance the function of OsGATA16 during the cold response, thus conferring enhanced tolerance.

Table 1
Putative cis-acting elements in the *OsGATA16* promoter

Name	Sequence	Position	Annotation
LTRECOREATCOR15	CCGAC	17,153,960	cold response
EBOXBNNAPA	CANNTG	30,139,689,696,712,1386	cold response
ABRERATCAL	MACGYGB	688,734,1795	ABA response
ABRELATERD1	ACGTG	1731,1796,1821	dehydration response
MYBCORE	CNGTTR	889,972,1035	dehydration response
CPBCSPOR	TATTAG	1453,1667	cytokinin response
GT1GMSCAM4	GAAAAA	1250,1298,1655,1765	salt-induce

Table 2
Primers for qRT-PCR

Gene ID	Name	Forward primer	Reverse primer
LOC_Os12g44350	*Actin*	CCTGGCAGTATGAAGGTAGTTG	GAAGCACTTCATGTGGACGAT
LOC_Os06g37450	*OsGATA16*	TGCTTGAGCCCCAAAATATG	GCAGCTTCTCGGTATCGTAT
LOC_Os01g10840	*OsGSK1*	ACGGGTCACATCATCTCC	AGTTCCTACAACTCGCTCC
LOC_Os03g08570	*OsPDS*	ACTGGCTGCCTGTCATCT	TACTTGCGAAGCACCTAT
LOC_Os05g25770	*OsWRKY45-1*	GCAGCAATCGTCCGGGAATT	GCCTTTGGGTGCTTGGAGTTT
LOC_Os05g49890	*OsRAN2*	TGGTGGACTTAGGGATGG	GGAATGTGACCTGCTTGG
LOC_Os02g10920	*OsSRZ1*	ATGAACAGGAAGCCAGGAGACT	TCCACCGAAGGAGGAACCA
LOC_Os01g55940	*OsGH3-2*	GAAGATGAGCTGGACAGGAGGC	GGGCGGTGCTTGAAGTGAT
LOC_Os06g45140	*OsbZIP52*	GCGAATAAGAAGGATGGTGTC	GCTTGAAGAGGGATGAGTTTT
LOC_Os03g22680	*OsSRFP1*	ATTCGGCAGGATGGGATT	TCGTGGACTCGTTGTGGC
LOC_Os09g02270	*OsCYL4*	GACCTCGCCATCCTCAAC	AACTCGCCGAACTCCTTT
LOC_Os02g10200	*OsZFP185*	CCAAGTGCCACAAGGAGAT	CCCACCGTCACAACCATT
LOC_Os02g41510	*OsMYB30*	ACAACACCACGGACAGTTTCAC	CCGTCATTGCCAGCGTCT
LOC_Os07g05720	*OsTCP21*	CACGCGGAGATGACGCACTA	ACCCACAAGACCCGAGGACA
LOC_Os01g11550	*OsPCF5*	TCCAGAGCTACACGCCTGACC	ATGGCGATGTTGCTGGTGG
LOC_Os03g57190	*OsTCP8*	CATGTCCTCGGGTTTCTTGGG	GCTGCTGCTGATGGTGGTGG
LOC_Os10g28600	*OsTCD10*	GCCTGGTTTATTTCCTTG	GTCTCGACATCCCTCCTC
LOC_Os12g42190	*OsPCF8*	CCGTCGCTCGACTGCTCCTT	GGCTTGCTGCCGTTGGTGTT

Table 3
Putative XGATAY motifs in promoters of candidate genes

Name	Sequence	Position
OsWRKY45-1pro	XGATAY	134,517,660
OsSRFP1 pro	XGATAY	562,567,755,976,1006,1350
OsCYL4 pro	XGATAY	210
OsMYB30 pro	XGATAY	1241,1468

Discussion

TFs play important roles in plants and are involved in diverse stress signaling pathways through activation or inhibition of target gene expression. Recent research has explored the various functions of GATA TFs in rice, but their impact on cold tolerance has not been explored. Informatic and expression analysis of rice OsGATA family proteins demonstrated their involvement with abiotic stress responses, and several of the genes showed duplicated relationships and similar expression patterns during rice growth (Gupta et al. 2017). Similarly, GATA proteins in the *Chickpea* were shown to be involved in the response to ABA and Drought stress (Niu et al. 2020). These recent studies into GATA TFs suggest that members of the GATA gene family may be generally involved in responses to abiotic stress. In this study, a novel transcription factor, OsGATA16, was identified that increased cold tolerance in rice with no apparent impact on agronomic growth traits under field conditions. These results are consistent with previous assessments of GATA-family members and support the hypothesis of a broader role for this gene family in abiotic stress responses.

OsGATA16 was ubiquitously expressed in rice tissues, with the highest expression levels in panicles (Fig. 3a), indicating that OsGATA16 might be involved in the cold response as well as in the response to a range of abiotic stresses and phytohormones through association with other factors. Transcriptional analysis by qRT-PCR showed that *OsGATA16* expression was induced by cold and ABA treatments, and was suppressed by drought, BA, and JA treatments (Fig. 2). Several cis-acting elements were found in the *OsGATA16* promoter (Table 1), as well as a range of TF binding sites such as WRKY, MYB, ABRE, and bHLH. A transcriptional study of OsMyb4, a Myb TF involved in responses to stress, highlighted a regulatory network that facilitated the cold stress signaling pathway through mediator MYB, bZIP, NAC, ARF, ERF, and CCAAT-HAP TFs. *Osmyb4* overexpression also impacted panicle development, and *OsGATA16* expression increased 3.1-fold in overexpression lines (Park et al. 2010). OsRAN1, an evolutionarily conserved member of the small G-protein family, was found to have a significant role in improving cold tolerance in rice. Like *OsGATA16*, *OsRAN1* was also expressed ubiquitously in rice tissue and exhibited highest expression in panicles (Xu and Cai 2014). These studies are consistent with the findings that *OsGATA16* overexpression conferred improved cold tolerance, and that the highest *OsGATA16* expression was found in panicles. Together, this suggests that OsGATA16 may associate with other TFs in panicle tissues to mediate responses to cold exposure as well as to other abiotic stresses and phytohormones.

OsGATA16 localized to the cell nucleus and acted as a transcription repressor. Transcriptional analysis of cold-sensitive genes in *OsGATA16*-overexpressing (OE) and WT lines by qRT-PCR revealed that *OsWRKY45-1*, *OsSRFP1*, *OsCYL4*, and *OsMYB30* transcription was repressed in OE lines compared with WT. Yeast one-hybrid and Dual-luciferase reporter assays confirmed that OsGATA16 bound to and suppressed the activity of the *OsWRKY45-1* promoter. Previous research reported the involvement of OsWRKY45-1 in the response to low temperatures (Tao et al. 2011). OsWRKY45-1 and OsWRKY45-2 (alleles of OsWRKY45) played different roles in the response to ABA and salt stress, but showed similar sensitivities to cold and drought stress (Tao et al. 2011). This suggests that OsGATA16 improves cold tolerance in rice by repressing the expression of the cold-sensitive gene *OsWRKY45-1*. Recent research identified several novel functions for OsWRKY45, and it is thus possible that OsGATA16 repression of *OsWRKY45-1* expression is involved in other biological functions in addition to the cold response. The *OsWRKY45-1* and *OsWRKY45-2* alleles

encode proteins that differ by ten amino acids, and several reports associate OsWRKY45 (OsWRKY45-1 or OsWRKY45-2) with disease in rice (Tao et al. 2009). The two alleles exhibited contrasting roles in resistance to bacterial blight caused by *Xoo* and bacterial leaf streak caused by *Xoc*. Overexpression of *OsWRKY45-1* reduced resistance to *Xoo* and *Xoc* but increased resistance to rice blast disease, caused by the fungus *Magnaporthe grisea*. The response to *Xoo* infection was accompanied by increased accumulation of SA and JA (Tao et al. 2009). In this study, expression of *OsGATA16* was repressed by JA exposure, suggesting that OsGATA16 may act as a positive regulator of disease resistance: upon infection, elevated JA levels would suppress *OsGATA16* expression and lead to de-repression of *OsWRKY45-1*, increasing resistance to *M. grisea* but decreasing resistance to *Xoo* and *Xoc*. Another regulatory factor, OsNPR1, also affected disease resistance in rice. Overexpression of *OsNPR1* conferred disease resistance to bacterial blight (Yuan et al. 2007), and OsNPR1 was found to repress the accumulation of *OsbHLH6* in the cell nucleus, thereby repressing JA signaling (Meng et al. 2020). Microarray analysis of rice transcription during *Xoo* infection showed that *OsGATA16* expression decreased upon infection (Kong et al. 2020). Taken together, these results suggest that OsGATA16 might act as a regulator of *Xoo*, *Xoc*, and *M. grisea* resistance. We propose that the bacterial blight resistance associated with *OsNPR1* overexpression (Yuan et al. 2007) and *OsbHLH6* consumption in the cell nucleus (Meng et al. 2020) occurred as a result of increased expression of *OsGATA16* due to repression of JA signaling. The decrease in *OsGATA16* expression after *Xoo* expression (Kong et al. 2020) further suggests that OsGATA16 plays an antagonistic role against *Xoo*. Furthermore, *OsGATA16* was induced by ABA treatment (Fig. 2c), and ABA signaling was negatively regulated by OsWRKY45-1 and positively regulated by OsWRKY45-2 (Tao et al. 2011), suggesting that repression of the *OsWRKY45-1* promoter by OsGATA16 may involve the ABA signaling pathway. Further analysis is needed to confirm the mechanisms by which OsGATA16 mediates disease resilience.

GATA-family TF proteins are highly conserved. Most family members retain a GATA-type zinc finger protein domain proximal to the DNA-binding domain, with a zinc finger protein domain also involved in identifying GATA TF recognition sequences (Behringer and Schwechheimer 2015). Several functions of GATA-family TFs have been identified, including functions associated with cytokinin, nitrate, and light responses, and with chloroplast development and plant growth. Responses of the family are diverse, as illustrated by *Cga1* (OsGATA11), which was induced by cytokinin (Hudson et al. 2013), in contrast to the repression of *OsGATA16* by cytokinin. This may indicate antagonistic functions for GATA-family members in cytokinin mechanisms, or may suggest the involvement of different GATA-family members in the transcriptional regulation of different signaling pathways.

Rice subspecies Japonica and Indica exhibit polarization for many agronomic traits, including adaptation and resilience to low temperatures (Ma et al. 2015). Japonica varieties generally display better tolerance to cold stress than Indica varieties, due to evolutionary adaptations to growth in regions with different climates (Wang et al. 2014). Some cold-related genes may have retained their ancestral functions in older varieties, but environmental adaptations may have supported the persistence of novel alleles with different functions in cultivated rice varieties that have been further selected and preserved by breeding processes (Kim et al. 2016). For example, *OsbZIP73*, which is involved in the ABA-dependent cold signaling pathway, harbors a single SNP between Japonica and Indica varieties. The SNP is located in an exon and leads to an

amino acid disparity that partially explains differences in cold tolerance between subspecies (Liu et al. 2018). In another study, an SNP (SNP2) in *COLD1*, a novel cold sensor in rice, was highly variable among diverse subspecies, but was conserved in Japonica varieties and was associated with cold tolerance in cultivated rice (Ma et al. 2015). In this study, haplotype analysis of the *OsGATA16* gene detected novel alleles associated with different subspecies. Eleven SNPs were identified within a strong LD block, five Haps were distinguished according to SNP variation, and Japonica and Indica varieties were clearly defined in two separate groups. Phenotypic analysis showed that the Indica group was significantly more cold-sensitive than the Japonica group. A non-synonymous functional SNP (SNP 8, $336^{A/G}$) was significantly associated with cold tolerance in both Japonica and Indica varieties when considered separately or together. As with *OsbZIP73* and *COLD1*, OsGATA16 showed clear differentiation between rice subspecies and conferred cold tolerance in rice. Furthermore, the 336^{G} allele was significantly associated with cold tolerance in both Indica and Japonica varieties, and has potential as a novel functional allele for improving cold resilience in rice breeding programs.

Conclusion

A novel GATA transcription factor, OsGATA16, response to abiotic stress and highest expression in panicle, acted as a transcriptional suppressor performed function in nuclear, showed positive role in cold tolerance at the seedling stage in rice by direct repression of *OsWRKY45-1* expression. One candidate-functional SNP was identified that explained cold tolerance differences among diverse rice varieties. These results support future breeding programs to improve cold tolerance in commercial rice crops.

Materials And Methods

Bioinformatics Evaluation of OsGATA16

For bioinformatics analysis, protein sequences were obtained from the NCBI and analyzed using NCBI protein BLAST. Putative phosphorylation sites were predicted using an online tool at http://gps.biocuckoo.org, and promoter sequences were assessed using an online tool at https://sogo.dna.affrc.go.jp. Alignment of *OsGATA16* homologous genes from diverse species was performed using DNAMAN, and an evolutionary tree was constructed using software MEGA7.0, NT-tree function was used with repeats of 1000-bootstraps.

Growth Conditions, Stress Treatments, and Expression Patterns

WT seeds and T3 seeds of *OsGATA16* transgenic overexpression lines were germinated for 3 days at room temperature. Geminated seeds were sown in soil in pots and cultivated under a 16 h light/8 h dark cycle at 26°C/28°C with 65% humidity. To test the induction of *OsGATA16* expression by stress treatments, rice seedlings at the 3-leaf stage were treated with cold (4°C), drought (dehydrated at 28°C with 65% humidity), 200 mM NaCl, 100 μM ABA, 100 μM BA, or 100 μM JA. Young roots, stems, seedling-stage leaves and

stems, flag leaves, young panicles, and booting stage leaf sheaths were collected for expression pattern analysis.

RNA Isolation and Quantitative Real-Time PCR

Total RNA was isolated from stress-treated rice seedlings using an RNA Prep Pure Plant Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. RNA (2 μg) was reverse transcribed to cDNA using a RevertAid RT Reverse Transcription Kit (Thermo Scientific, Waltham, USA), and qRT-PCR was conducted using an Agilent Stratagene Mx3005P Quantitative Real-Time PCR system with SGExcel FastSYBR qPCR Mixture (Sangon Biotech, Shanghai, China). The *β-actin* gene was used as an internal qRT-PCR control. Sequences for gene-specific primers are shown in Table 2. Three replicate experiments were performed for each sample. The relative quantitation method (*ΔΔCT*) was used to evaluate quantitative variation among replicates.

Subcellular Localization

The *OsGATA16* coding region lacking the stop codon was inserted into the 1305-Ubi-GFP vector between the *Kpn*I and *BamH*I restriction sites, in-frame with GFP under the control of the ubiquitin promoter. D53-RFP, which targets to the nucleus, was used as a co-localization marker (Zhou et al. 2013). The expression construct and localization marker (10μg) were transiently co-transformed into rice protoplasts and incubated overnight in darkness at 28°C, as described previously (Chen et al. 2006). Fluorescence was observed with a Zeiss LSM780 confocal laser microscope (Carl Zeiss, Germany).

Construction of Pubi:OsGATA16 Plasmid and Generation of Transgenic Overexpression Rice Lines

Full-length *OsGATA16* cDNA was amplified from total RNA from *Oryza sativa* subsp. *japonica* cv. Kitaake and inserted into the modified vector pCAMBIA1301. The resulting overexpression vector, *Pubi*:OsGATA16, was introduced into WT Kitaake by *Agrobacterium*-mediated transformation (Hiei et al. 1994). T3 homozygotic OE and WT Kitaake lines were selected for analysis.

Dual-Luciferase Reporter Assay

Recombinant effectors GAL4 DB-OsGATA16 and GAL4 DB-VP16-OsGATA16 were constructed and co-transformed into rice protoplasts alongside reporter 35S-GAL4-LUC, with pPTRL (*Renilla reniformis* luciferase) as a reference control. For protein-DNA-binding analysis, the promoters of each candidate gene were inserted into the pGreenII vector, and the constructed *promoter::LUC* vectors were transformed into WT and OE lines. After incubation overnight at 28°C in darkness, protoplasts were examined using the Dual-Luciferase® Reporter Assay System (Promega), according to the manufacturer's instructions. Luminescence was detected using a GloMax® Discover Multimode Microplate Reader (Promega).

Yeast One-Hybrid Assay

The full-length coding region of *OsGATA16* was inserted into the pJG4-5 vector between the *EcoR*I and *Xho*I restriction sites and named GAD-OsGATA16, with Trp1 within the vector acting as a selection marker. The *OsWRKY45-1* promoter was inserted into the pLacZi2μ vector between the *EcoR*I and *Sal*I sites and named as *Promoter*::*LacZ*, with Ura3 and *LacZ* in the vector acting as selection markers. The two recombinant plasmids were co-transformed into the EGY48 yeast strain and cultivated on SD medium lacking Trp and Ura and containing X-Gal (SD/-Trp-Ura + X-Gal) at 30°C for 2 days. The blue color of the chromogenic reaction was indicative of protein-DNA binding.

Yeast Two-Hybrid Assay

The OsGATA16 coding sequence was purified by PCR and cloned into the pBridge (BD) vector to yield BD-OsGATA16, which was then transformed into the Y2HGold yeast strain and cultivated on yeast medium. Empty BD vector was used as a negative control. Transformed strains were initially cultivated on SD medium without Trp (SD/-Trp) to select transformants, and then transferred to SD medium without Trp and His (SD/-Trp-His) to assess the transcriptional activity of OsGATA16.

Haplotype Analysis

Genotype and phenotype data were collected for haplotype analysis. Genotype data were collected and filtered as described previously (Kim et al. 2016; Zhang et al. 2020). Phenotype data comprised evaluation scores for cold tolerance during the seedling stage. Rice was cultivated under field conditions with inundation with cold water (13°C) up to 10 days, followed by normal temperature recovery for 1 week. Plants were scored 1–9 according to their sensitivity to cold stress, with 9 = most cold sensitive. For haplotype analysis, SNPs within the *OsGATA16* genic and promoter regions (upstream 2000 bp) were identified, and their position relative to the start of the 5' UTR was recorded. Haplotype grouping followed SNP variation in each haplotype (Hap). Only samples without missing data and without heterozygosis were used for haplotype analysis. Visualization of haplotype variation analysis was performed using PopArt software, with the number of transverse lines between each Hap representing nucleotide variation. LD block analysis was performed using Haploview software, with D' as the evaluation criterion for LD level.

Abbreviations

LLM: Leucine-Leucine-Methionine

HAN: Hanaba Taranu

NLS: Nuclear localization signal

OE: Overexpression transgenic line

WT: Wild type

CT: Cold tolerance

qRT-PCR: Quantitative real-time PCR

ABA: Abscisic acid

BA: Cytokinin

JA: Jasmonic acid

GFP: Green fluorescent protein

RFP: Red fluorescent protein

LUC: Firefly luciferase

REN: Renilla luciferase

TF: transcription factor

Xoo: Xanthomonas oryzae pv. oryzae

Xoc: Xanthomonas oryzae pv. Oryzicola

M.grisea: Magnaporthe grisea

Trp: Tryptophane

Leu: Leucine

His: Histidine

Ura: Uracil

X-gal: 5-Bromo-4-chloro-3-indolyl β-D-galactoside

Hap: Haplotype

LD: Linkage disequilibrium

Declarations

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing Interests

The authors declare that they have no competing interests.

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Authors' Contributions

HZ, TW, SWK, WJ, and XD participated in the experimental design. HZ, ZL, KH, NEK, and ZM performed the research. HZ, TW, SWK, WJ, and XD participated in the paper writing and manuscript amending.

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Figures

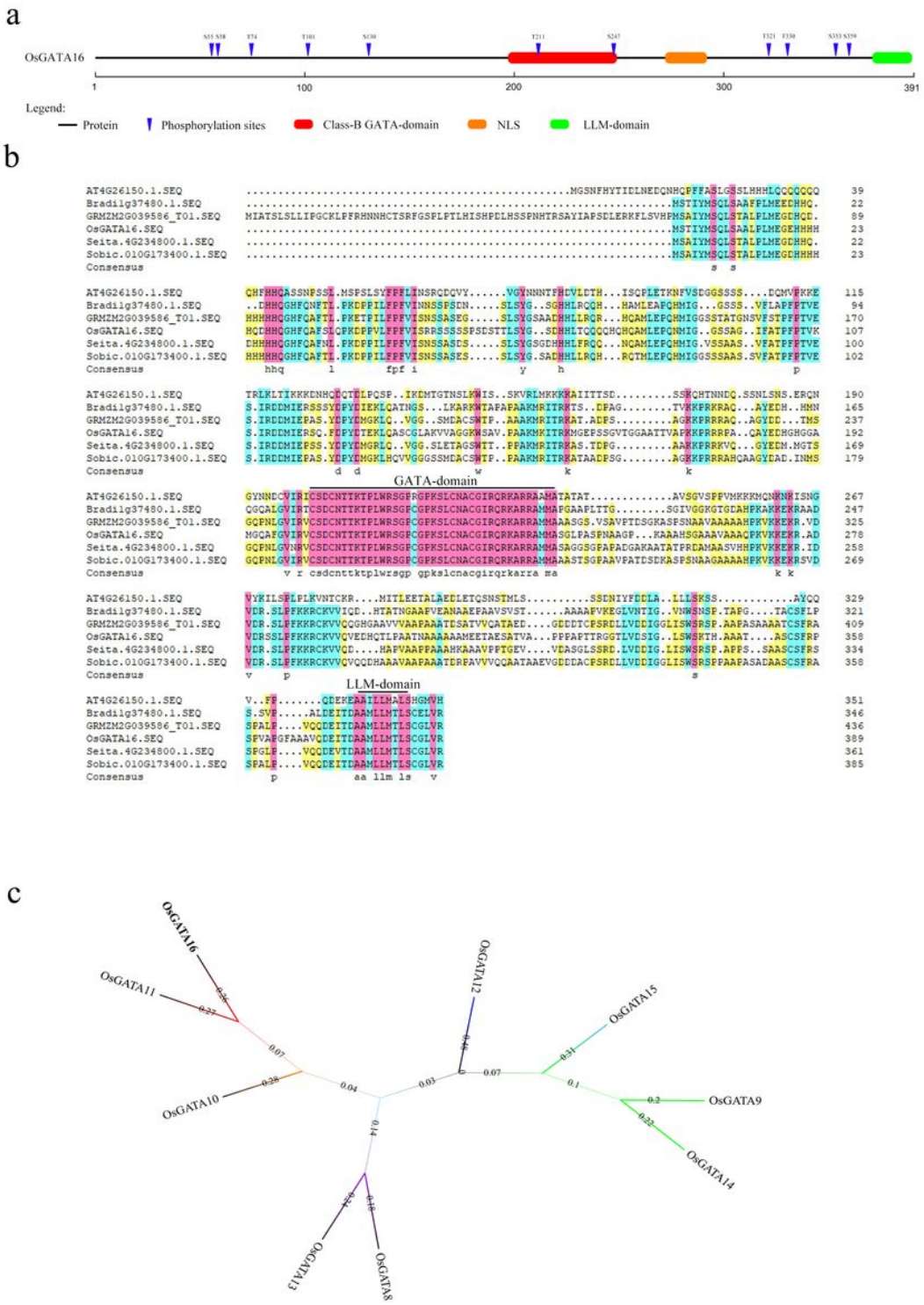


Figure 1

Bioinformatic analysis of OsGATA16 protein. (a) Structure of OsGATA16 protein. Numbers correspond to locations within the full-length protein. (b) Comparison of OsGATA16 homologous genes in various species. Black lines represent diverse domain, pink represents identical amino acid residues, and similar amino acid residues are highlighted in blue and yellow. (c) Phylogenetic tree of OsGATA subfamily-II proteins. Numbers represent level of approximation.

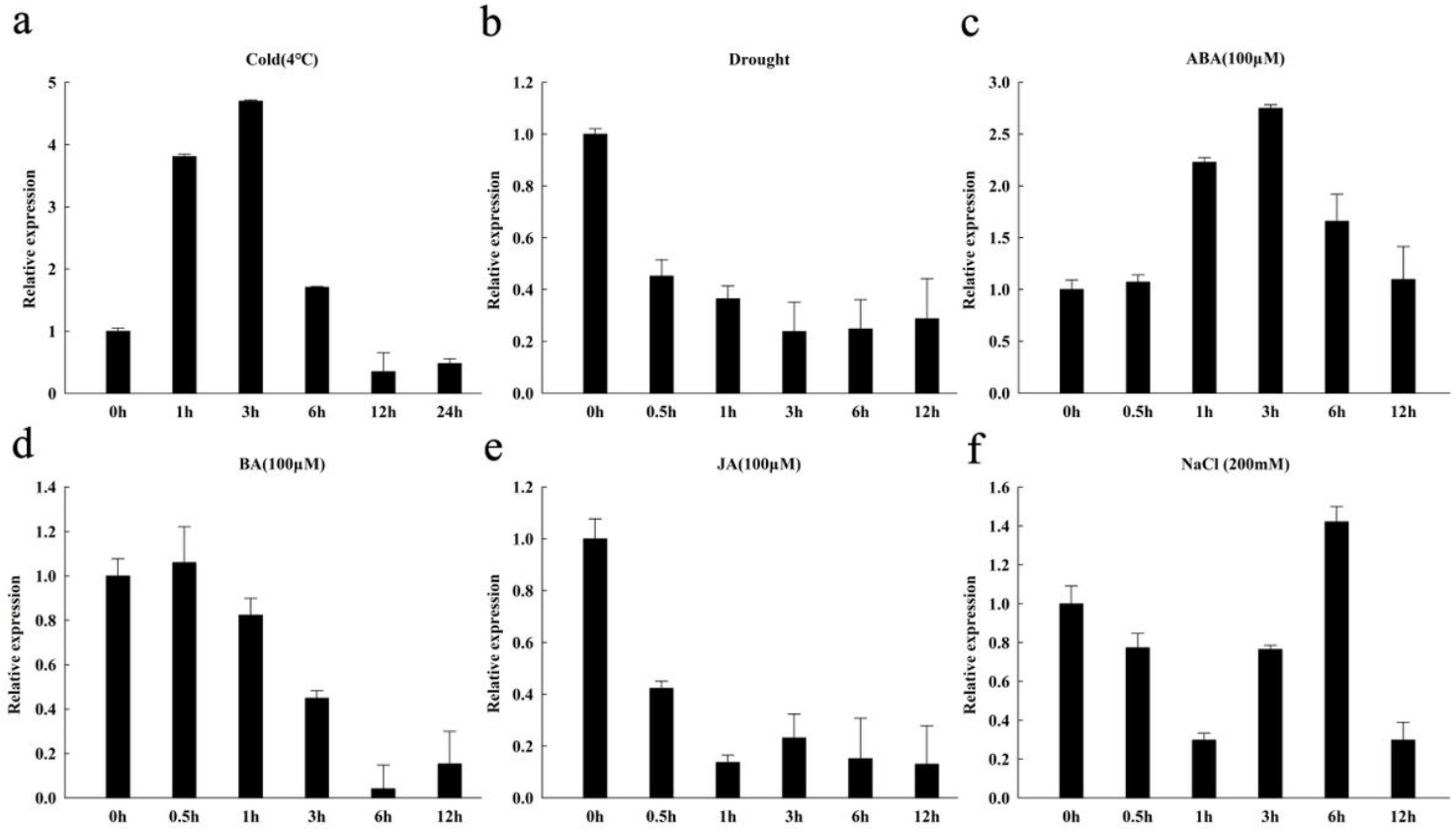


Figure 2

Time-course expression analysis of OsGATA16 after exposure to abiotic stress or phytohormones. (a) Cold (4°C), (b) Drought, (c) Abscisic acid (ABA), (d) 6-benzylaminopurine (BA), (e) Jasmonic acid (JA), and (f) NaCl. Data represent the mean ±SE from three replicates.

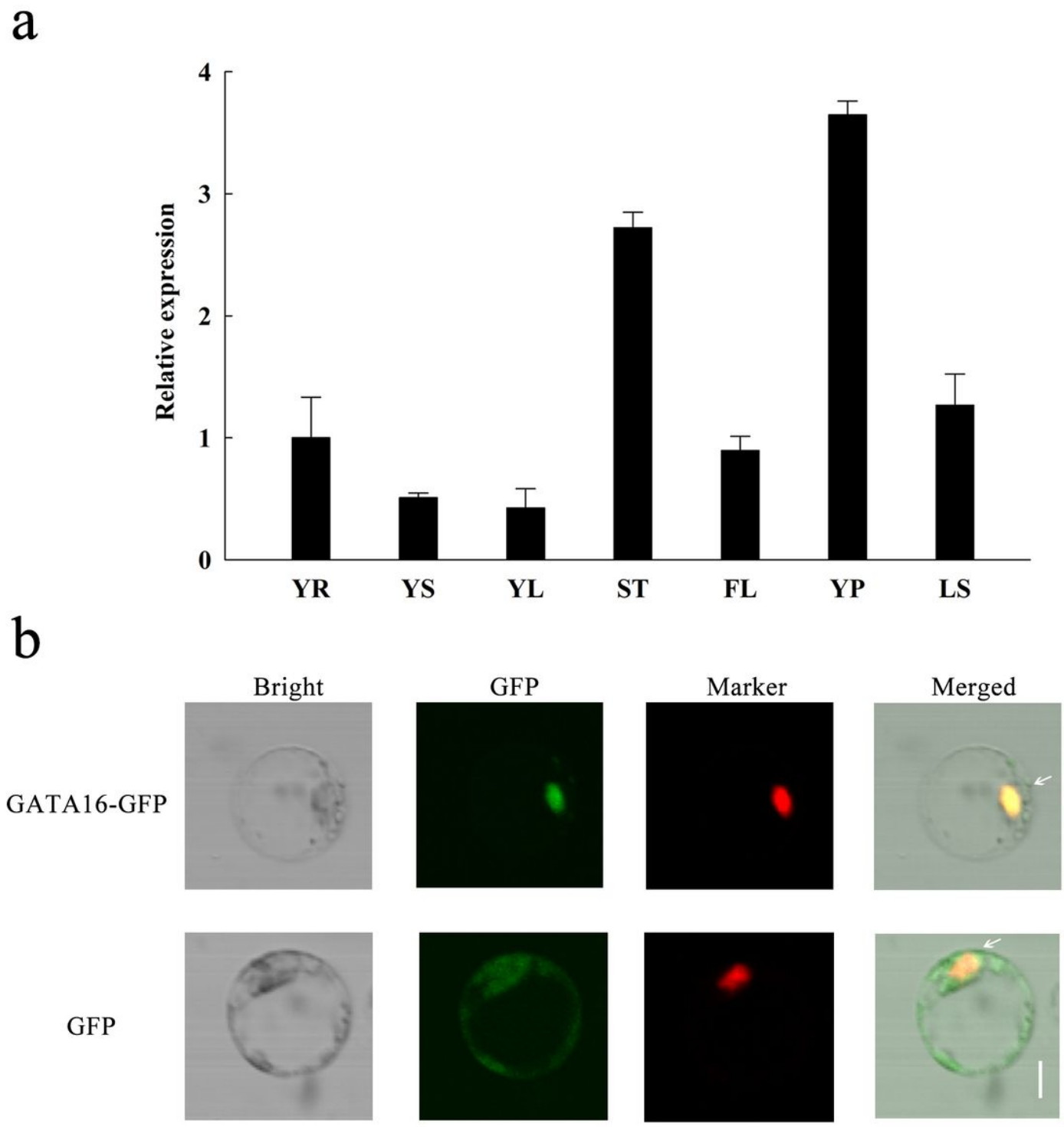


Figure 3

Tissue-specific expression and subcellular localization of OsGATA16 in rice. (a) OsGATA16 expression in different plant tissues. Root (YR), stem (YS), and leaves (YL) at the seedling stage, and stem (ST), flag leaves (FL), panicles (YP), and leaf sheaths (LS). Data represent the mean ±SE from three replicates. (b) Subcellular localization of OsGATA16 in rice. GATA16-GFP: GFP fusion with OsGATA16 protein; D53-RFP: nuclear marker. Arrows indicate nuclei. Bar = 10 μm.

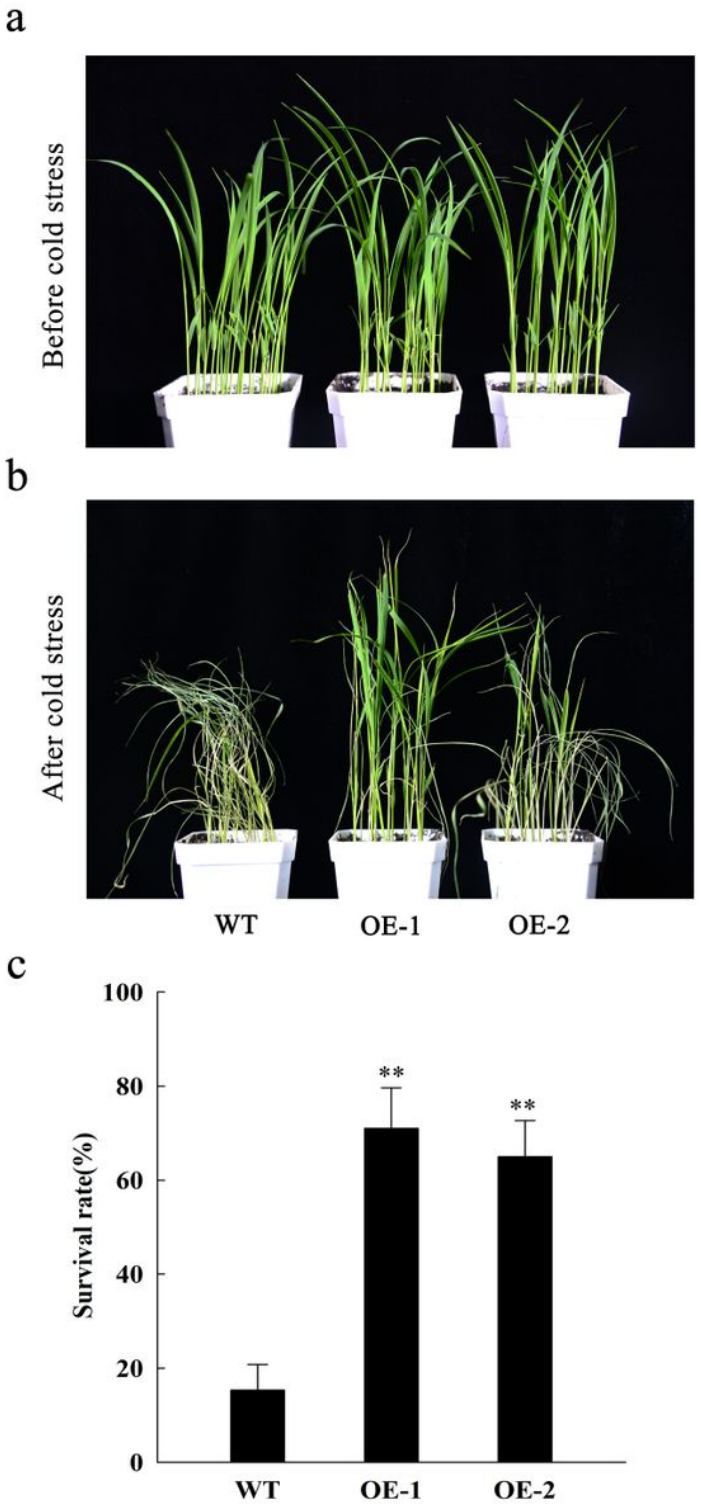


Figure 4

OsGATA16 overexpression phenotype and survival rate after exposure to cold stress. (a) Wild-type (WT) and OsGATA16-overexpression (OE) lines at the 3-leaf seedling stage, prior to exposure to cold stress (8°C). (b) WT and OE seedlings after cold stress exposure and recovery at room temperature. (c) Survival of OE and WT plants after exposure to cold stress. Data represent the mean ± SE from five replicates. Asterisks indicate significant differences in survival rate (Student's t-test, **$p<0.01$).

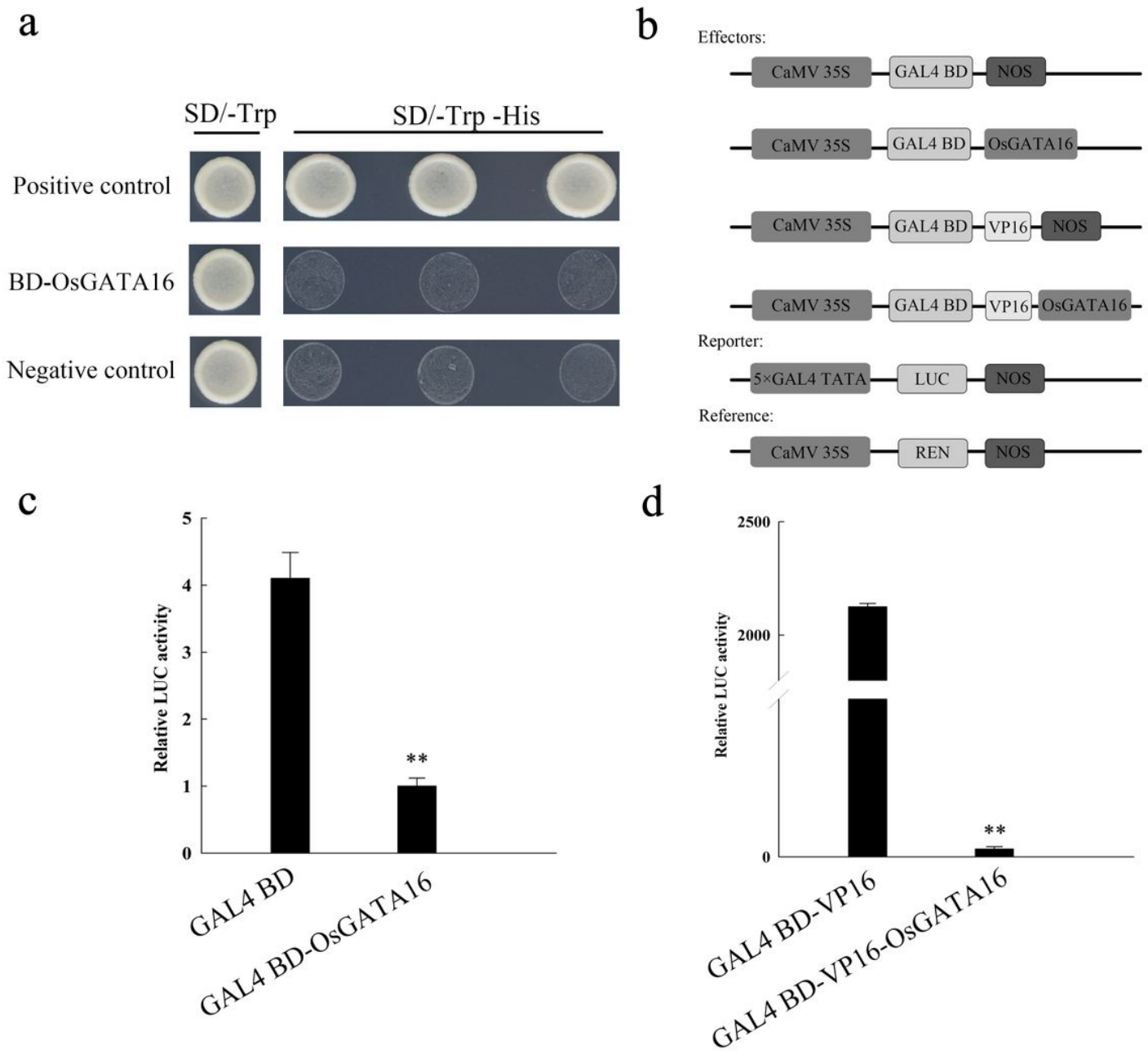


Figure 5

Transcriptional activity of OsGATA16 in rice. (a) Yeast two-hybrid analysis of OsGATA16 transcriptional activity. (b) Schematic representation of recombinant effector, reporter, and reference (pPTRL) plasmids for Dual-luciferase reporter analysis. (c-d) Relative LUC activity with control and OsGATA16 effector constructs. Data represent the mean ± SE from three replicates. Asterisks indicate significant differences in relative LUC activity (Student's t-test, **p<0.01).

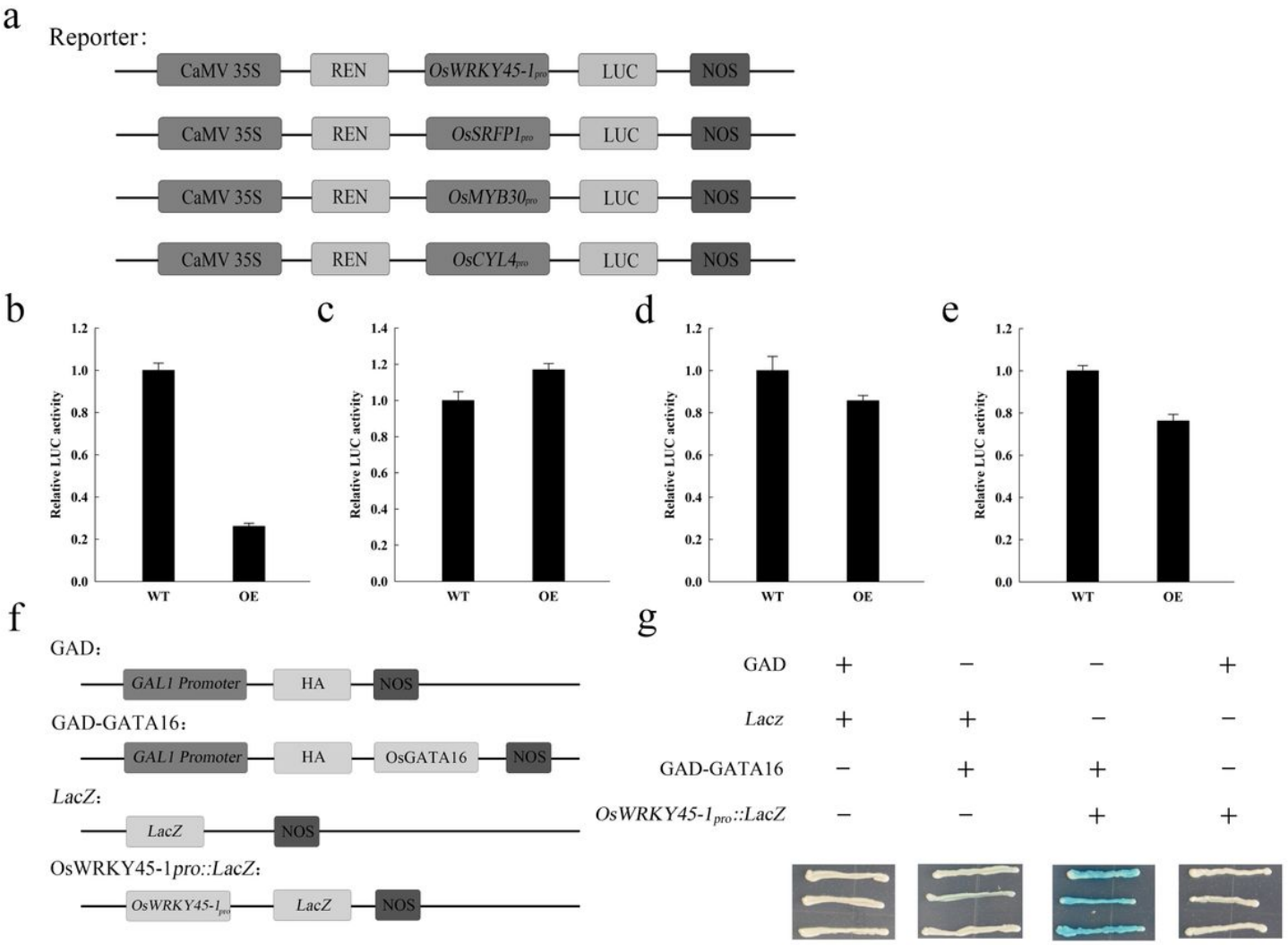
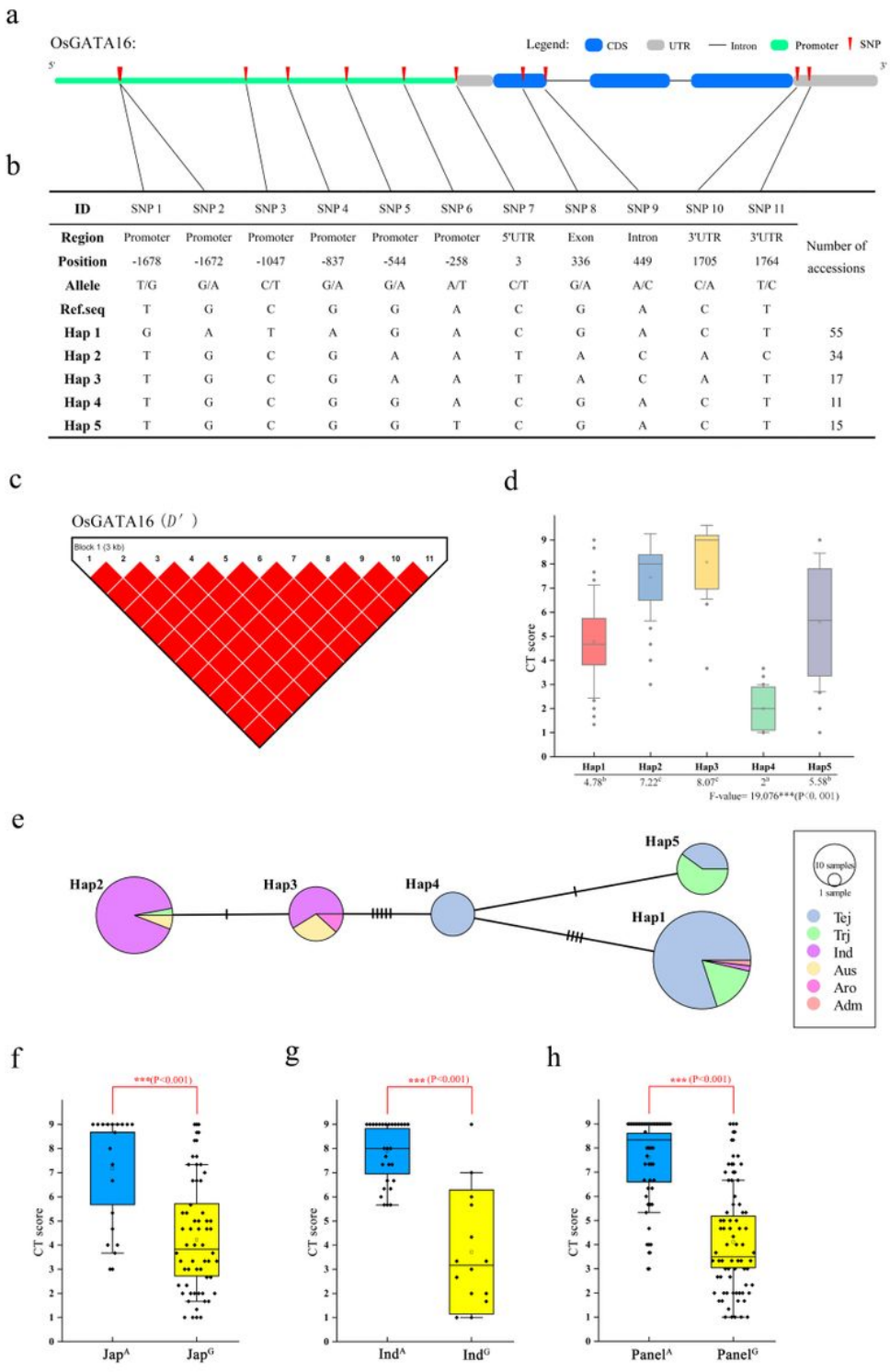


Figure 6

OsGATA16 interaction with the OsWRKY45-1 promoter in rice. (a) Schematic representation of reporter plasmids for Dual-luciferase reporter analysis, with REN luciferase as an internal control (b-e) Relative LUC activity in wild-type and OsGATA16-overexpression (OE) lines with (b) OsWRKY45-1, (c) OsSRFP1, (d) OsCYL4, and (e) OsMYB30 reporter constructs. Data represent the mean ± SE from three replicates. (f) Schematic representation of recombinant plasmids for yeast one-hybrid analysis of the OsWRKY45-1 promoter. (g) Yeast one-hybrid assay results. Blue coloration is indicative of protein–promoter interaction.



ID	SNP 1	SNP 2	SNP 3	SNP 4	SNP 5	SNP 6	SNP 7	SNP 8	SNP 9	SNP 10	SNP 11	
Region	Promoter	Promoter	Promoter	Promoter	Promoter	Promoter	5'UTR	Exon	Intron	3'UTR	3'UTR	Number of accessions
Position	-1678	-1672	-1047	-837	-544	-258	3	336	449	1705	1764	
Allele	T/G	G/A	C/T	G/A	G/A	A/T	C/T	G/A	A/C	C/A	T/C	
Ref.seq	T	G	C	G	G	A	C	G	A	C	T	
Hap 1	G	A	T	A	G	A	C	G	A	C	T	55
Hap 2	T	G	C	G	A	A	T	A	C	A	C	34
Hap 3	T	G	C	G	A	A	T	A	C	A	T	17
Hap 4	T	G	C	G	G	A	C	G	A	C	T	11
Hap 5	T	G	C	G	G	T	C	G	A	C	T	15

Figure 7

Haplotype analysis of OsGATA16. (a) Structural representation of OsGATA16 and upstream promoter region. (b) OsGATA16 SNPs and haplotype groups in 137 rice accessions. SNP positions are given relative to the start of the 5'UTR. Hap: haplotype (c) Linkage disequilibrium (LD) analysis of OsGATA16. The eleven SNPs shown in (b) were used for LD block assessments, with SNP numbers as in (b). D was used for evaluation of LD. Red indicates complete linkage equilibrium between each SNP. (d) Relationship of cold-tolerant phenotype with haplotype. Cold-tolerance (CT) score is on a 1–9 scale, with 1 representing highest

CT. Different letters indicate significant CT differences among haplotypes (ANOVA, Duncan test) (e) Haplotype network variation. Circle size represents the number of accessions in each Hap, and the number of transverse lines between each Hap represents the number of nucleotide variations. Tej: Temperate Japonica; Trj: Tropical Japonica; Ind: Indica; Aus: Aus; Aro: Aromatic; and Adm: Admixture rice varieties. (f-h) SNP 8 haplotype relationship with CT. Superscript A and G indicate the A and G genotypes in Japonica (f), Indica (g), and Japonica plus Indica (h) accessions. Asterisks indicate significant differences in CT between genotypes (Student's t-test, ***p<0.001).

Supplementary Files

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