Phosphorylation of OsTGA5 by casein kinase II compromises its suppression of defense-related gene transcription in rice

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Abstract

Plants manage the high cost of immunity activation by suppressing the expression of defense genes during normal growth and rapidly switching them on upon pathogen invasion. TGAs are key transcription factors controlling the expression of defense genes. However, how TGAs function, especially in monocot plants like rice with continuously high levels of endogenous salicylic acid (SA) remains elusive. In this study, we characterized the role of OsTGA5 as a negative regulator of rice resistance against blast fungus by transcriptionally repressing the expression of various defense-related genes. Moreover, OsTGA5 repressed PTI responses and the accumulation of endogenous SA. Importantly, we showed that the nucleus-localized casein kinase II (CK2) complex interacts with and phosphorylates OsTGA5 on Ser-32, which reduces the affinity of OsTGA5 for the JIOsPR10 promoter, thereby alleviating the repression of JIOsPR10 transcription and increasing rice resistance. Furthermore, the in vivo phosphorylation of OsTGA5 Ser-32 was enhanced by blast fungus infection. The CK2 α subunit, depending on its kinase activity, positively regulated rice defense against blast fungus. Taken together, our results provide a mechanism for the role of OsTGA5 in negatively regulating the transcription of defense-related genes in rice and the repressive switch imposed by nuclear CK2-mediated phosphorylation during blast fungus invasion.
**In A NUTSHELL**

**Background:** Plants balance the high cost of immunity by suppressing the expression of defense genes during normal growth, while rapidly switching them on when perceiving pathogens. The regulation of the transactivation factors (TFs) controlling the transcription of defense genes plays an essential role in this switch. TGA-type TFs are key regulators of plant innate immunity. In Arabidopsis, pathogen attacks dramatically increase endogenous salicylic acid (SA) levels, which promote the nuclear enrichment of the NONEXPRESSER OF PR GENES 1 monomer, which helps dissociate the TGA2 oligomer into a dimer to induce the transcription of Pathogenesis-related (PR) genes. Although this signaling pathway plays a critical role in boosting immunity in Arabidopsis, how TGA activity is regulated in monocots is largely unknown.

**Question:** How do plant species such as rice, with constant high levels of SA even during pathogen infection, regulate TGA activity? This is a critical question, because rice TGA2.1 has been found to suppress the transcription of defense genes during normal growth and inhibit immunity against the bacterial pathogen.

**Findings:** OsTGA5, the closest rice homolog to Arabidopsis TGA2, negatively regulates rice resistance against blast fungus by blocking the transcription of various defense-related genes. We discovered that the nucleus-localized casein kinase II (CK2) complex interacts with and phosphorylates OsTGA5 at Ser-32, which is enhanced upon blast fungus infection. This phosphorylation decreases the binding of OsTGA5 to the promoter of the rice PR gene, thereby alleviating the transcriptional repression by OsTGA5 and promoting resistance against blast fungus. Thus, this CK2-based phosphorylation mechanism is an important molecular switch in rice involved in elevating the expression of defense genes normally suppressed by TGA upon pathogen invasion.

**Next steps:** We wish to explore the interplay, including functional redundancy or differentiation, and the formation of hetero- or homo-oligomers, between rice TGA members in controlling the transcription of defense genes. Moreover, determining the influence of CK2-mediated phosphorylation on rice TGAs interplay will help scientists to better understand how TGAs are regulated in rice.

**Introduction**

Plants deploy sophisticated defense systems to protect themselves from invasion by pathogenic microorganisms (Chisholm et al., 2006). In the early stage of infection, pathogen-associated molecular patterns (PAMPs) are perceived by pattern recognition receptors (PRRs) located at the cell membrane, which rapidly activate PAMP-triggered immunity (PTI) responses, including reactive oxygen species (ROS) bursts, mitogen-activated protein kinase (MAPK) cascade activation, calcium influx, and callose deposition (Dodd and Rathjen, 2010; Macho and Zipfel, 2014). To overcome this horizontal plant resistance, pathogens secrete effectors to disrupt PTI signaling pathways. Plant resistance I proteins, characterized by nucleotide-binding site (NBS) and leucine-rich repeat (LRR) domains, can recognize a subset of these effectors and induce a strong isolate-specific resistance, called effector-triggered immunity (ETI), which is often coupled with a hypersensitive response (HR), a form of programmed cell death (Alhoraibi et al., 2019). As a common feature of both PTI and ETI, transcriptional reprogramming, governed by transcription factors (TFs) and coregulatory proteins, is essential for increasing plant resistance by upregulating defense-related gene expression (Tsuda and Katagiri, 2010). Accumulating evidence indicates that certain plant TF families, such as ETHYLENE-RESPONSIVE FACTOR, basic leucine zipper (bZIP), basic helix-loop-helix, WRKY, NAM, ATAF, CUC (NAC), and MYB, control the activation of innate immunity (Tsuda and Somssich, 2015).

The TGA family of TFs is conserved in land plants and belongs to the bZIP super family of TFs, which contains 78 and 89 members in Arabidopsis (Arabidopsis thaliana) and rice (Oryza sativa), respectively (E et al., 2014; Dröge-Laser et al., 2018). Tobacco (Nicotiana tabacum) TGA1a was the first plant TF to be cloned, based on its ability to bind to the TGACGT sequence in the activation sequence-1 (as-1) element of the cauliflower mosaic virus 35S promoter (Katagiri et al., 1989). Since the discovery of this family, many studies have shown that TGA TFs function in plant defense responses against biotic and abiotic stresses (Gatz, 2013). The TGAs within the D subgroup of the Arabidopsis bZIP TF family are mainly involved in regulating pathogenesis-related (PR) gene expression through the salicylic acid (SA) signaling pathway, and they can be further classified into three clades: clade I (TGA1 and TGA4), clade II (TGA2, TGA5, and TGA6), and clade III (TGA3 and TGA7) (Jakoby et al., 2002). Members of clade II interact with the positive regulator NONEXPRESSER OF PR GENES 1 (NPR1) and are essential for SA-induced PR gene expression and systemic acquired resistance (SAR) (Desprès et al., 2000; Fan and Dong, 2002). Both SA-induced PR1 expression and SAR activation are abolished in the tga2 tga5 tga6 triple mutant (Zhang et al., 2003). However, clade II member TGA2 was shown to function as a negative regulator of PR transcription (Kesarwani et al., 2007). In rice, four TGA TFs, OsTGA2, rTGA2.1, OsTGA3 (also named rTGA2.2), and OsTGA5 (rTGA2.3), are grouped in the same clade as Arabidopsis.
TGA2/5/6 and interact with Arabidopsis NPR1 or its rice homologs NPR1 HOMOLOGs (NHs) (Moon et al., 2018). Of these, two TGAs have been functionally characterized in immunity; rTGA2.1 plays a negative role in rice basal defense against the bacterial pathogen Xanthomonas oryzae pv. oryzae (Xoo) (Fitzgerald et al., 2005), while OsTGA2 functions in the positive control of defense-related gene expression and resistance to Xoo (Moon et al., 2018). Therefore, TGA factors, despite being highly similar in sequence, exert distinct activities in regulating immune gene expression.

Casein kinase II (CK2) is a conserved serine/threonine kinase tetramer in eukaryotes that is composed of two catalytic α and two regulatory β subunits. CK2 in plants plays essential roles in regulating various physiological processes, such as light signaling, circadian rhythms, and phytohormone responses (Mulekar and Huq, 2014). There are four CK2α subunits in Arabidopsis, of which three localize to the nucleus and exhibit redundant functions in regulating circadian rhythms (Lu et al., 2011); the other CK2α functions in chloroplasts (Salinas et al., 2006). In rice, OsCK2α3, residing in the endoplasmic reticulum (ER) compartment, phosphorylates CKII phosphorylates OsTGA5 to switch defenses on.

Results

Loss of OsTGA5 function confers enhanced rice resistance to blast fungus

To reach a better understanding of TGA-based regulation of rice immunity and identify the TGA member repressing rice defense against blast fungus, we functionally characterized the rice homolog of Arabidopsis TGA2. Through protein sequence alignment, we determined that among the rice TGA TFs within clade II, OsTGA5 is the most closely related to Arabidopsis TGA2 (Supplemental Figure S1). To explore the involvement of OsTGA5 in rice immunity, we used clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated nuclelease 9 (Cas9)-mediated genome editing to generate knockout mutants of OsTGA5 (Ostga5) in the rice japonica variety Nipponbare (Zhonghua11 (ZH11)). We obtained three homozygous Ostga5 mutants from independent primary transgenic plants. As shown in Figure 1A, the Ostga5-1 and Ostga5-2 mutants harbored a 1-bp insertion between nucleotides 65 and 66 of the OsTGA5 coding region, while Ostga5-3 carried a 5-bp deletion between nucleotides 61 and 65. All three alleles resulted in a frameshift in the Ostga5 coding region. When grown in a plot, these three Ostga5 mutants displayed no obvious defects in growth or development compared with ZH11 (Figure 1B and Supplemental
When challenged with conidial spores for *M. oryzae* isolate Guy11, which is virulent toward ZH11, we observed far fewer disease lesions on the leaves of *Ostga5* mutants compared with those on the wild-type (Figure 1C). To quantify the resistance of *Ostga5* mutants to blast fungus, we carried out punch inoculation assays with the leaves of *Ostga5-1*, *Ostga5-2*, *Ostga5-3*, and ZH11, and investigated the relative fungal biomass within the infected regions. The *Ostga5* mutants supported significantly less blast fungus growth than ZH11 (P < 0.01; Figure 1, D and E). We also performed a rice leaf sheath inoculation assay to monitor *M. oryzae* invasion in *Ostga5-1* and ZH11. The invasive hyphae (IH) of Guy11 extended to the neighboring cells of the first infected cell in ZH11 at 48-h postinoculation (hpi) and further extended to the adjacent cells at 72 hpi (Supplemental Figure S3). However, the IH in *Ostga5-1* leaf sheath cells was notably thinner than those in ZH11, and at 72 hpi, the extension of IH in *Ostga5-1* was markedly slower than that in ZH11 (Supplemental Figure S3). In addition, we challenged *Ostga5-1* with another blast fungal isolate, ZHONG1, which is virulent to ZH11 as well, and determined that *Ostga5-1* also displays increased resistance to ZHONG1 compared with the wild-type (Supplemental Figure S4). Taken together, our data indicate that OsTGA5 is a negative regulator of rice resistance against blast fungus.

The immune responses of *Ostga5-1* mutant are enhanced

To better understand the enhanced resistance against *M. oryzae* in the absence of OsTGA5, we investigated PTI responses of *Ostga5-1* and ZH11 induced by chitin. First, we measured the ROS burst from *Ostga5-1* and ZH11 leaves upon chitin treatment, which revealed higher ROS burst levels in *Ostga5-1* than in ZH11 at the tested time points (Figure 2A). Moreover, we observed a nearly 40% increase in callose deposition in *Ostga5-1* leaves compared with ZH11, as detected by the autofluorescence emitted by callose under ultraviolet (UV) light (Figure 2B). In addition, we determined the contents of the defense-related phytohormones salicylic acid (SA) and jasmonic acid (JA) in *Ostga5-1* and ZH11 plants with and without *M. oryzae* infection. The free SA levels were similar in *Ostga5-1* and ZH11 under mock treatment, whereas *M. oryzae* inoculation stimulated the accumulation of SA in the *Ostga5-1* mutant about two-fold relative to mock-treated plants, but not in ZH11 (Figure 2C). In contrast, the JA levels in *Ostga5-1* and ZH11 plants with or without *M. oryzae* infection were not notably different (Supplemental Figure S5). To verify the higher SA levels in the *Ostga5* mutant following *M. oryzae* invasion, we investigated the transcript levels of the genes *OsPR5*, *OsPR1a*, *Chitinase 1* (*OsCht1*), and *OsWRKY45* in the SA-mediated...
Figure 3  OsTGA5 suppresses JIOsPR10 transcription by binding to the TGACGT motif in its promoter. A, Relative transcript levels of JIOsPR10 in ZH11 and the Ostga5-1 mutant before and after spray-inoculation with Guy11 conidia, by RT-qPCR. UBQ served as the internal control. Data are means ± se (n = 3). Significant differences were determined by one-way ANOVA (**P < 0.01; *P < 0.05). This assay was performed in three independent replicates with similar results. B, OsTGA5 binds to the DNA fragment of the JIOsPR10 promoter containing the TGACGT motif, as determined by EMSA. Sequences of the probe and mutated probe are indicated at the top. Recombinant OsTGA5 protein (5 μg) was incubated with intact (lane 2) or mutated (lane 3) probe. C, OsTGA5 suppresses transcription from the JIOsPR10 promoter in ZH11 protoplasts when the TGACGT motif is present. Top, schematic diagrams of the constructs used in this assay. Bottom, relative LUC activity (normalized luminescence) of each indicated construct combination was calculated as luminescence/GUS activity. Data are means ± se (n = 10). Different lowercase letters indicate significant differences (P < 0.01, one-way ANOVA). This result is from one representative out of three independent experiments.

Defense pathway (Agrawal et al., 2000; Rakwal et al., 2001; Shimono et al., 2007; Hao et al., 2012) before and after M. oryzae infection. We established that the transcript levels of all these genes are significantly higher in Ostga5-1 plants after inoculation compared with those in ZH11 (P < 0.01; Figure 2D). Therefore, we conclude that loss of OsTGA5 function enhances the chitin-induced immune responses including ROS burst and callose deposition, and elevates endogenous SA levels upon blast fungus invasion.

Several lines of evidence support the idea that SA biosynthesis in rice mainly relies on the phenylalanine ammonia-lyase (PAL) pathway (Duan et al., 2014; Lefevere et al., 2020). Thus, to determine whether the increased resistance in the absence of OsTGA5 is dependent on the higher accumulation of SA, we treated ZH11 and Ostga5-1 with 2-aminoin-dane-2-phosphonic acid (AIP), an effective competitive inhibitor of PAL (Wang et al., 2018), for 24 h, and then inoculated the plants with Guy11 conidial spores by spraying. Compared with the mock treatment, AIP diminished the resistance of both ZH11 and Ostga5-1 plants (Supplemental Figure S6). Notably, Ostga5-1 plants still developed significantly fewer disease lesions than the wild-type after AIP treatment, suggesting that higher SA accumulation partially contributes to the enhanced resistance of Ostga5-1 against M. oryzae.

OsTGA5 binds to the TGACGT motif in the JIOsPR10 promoter and represses its transcription

To further characterize the negative regulation of rice immunity by OsTGA5, we analyzed the transcript levels of three known defense-related genes in ZH11 and the Ostga5-1 mutant before and after inoculation. JIOsPR10 was originally identified as a defense gene specifically induced by JA/SA and blast fungus infection (Jwa et al., 2001), and overexpression of JIOsPR10 can enhance rice resistance to blast fungus (Wu et al., 2016). OsPR10b is a PR gene that is upregulated upon M. oryzae infection (Mcgee et al., 2001). OsNAC4 encodes a plant-specific TF involved in plant hypersensitive cell death (Kaneda et al., 2009). As indicated by reverse transcription-quantitative PCR (RT-qPCR) assays, all three genes exhibited significantly higher resting and M. oryzae-induced transcript levels in Ostga5-1 than in ZH11 (Figure 3A and Supplemental Figure S7), indicating that OsTGA5 represses the transcription of multiple defense-related genes in rice.

Rice TGA subclade II members are known to selectively bind to the DNA sequence TGACGT (Moon et al., 2018). We identified one copy of this sequence in the JIOsPR10 promoter region (274–279 bp upstream from the transcription start site, TSS). To investigate whether OsTGA5 can physically bind to the JIOsPR10 promoter, we synthesized a 50-bp fragment of the JIOsPR10 promoter containing the TGACGT motif (JIOsPR10 probe) to perform an electrophoretic mobility shift assay (EMSA). We also produced a variant fragment carrying the sequence GTCTTC instead of TGACGT (JIOsPR10 probe-m) to determine whether TGACGT is required for binding. We observed that recombinant purified maltose-binding protein (MBP)-OsTGA5 protein can bind to the intact JIOsPR10 probe, causing a shift in the mobility of the oligonucleotide probe, but not to the mutated probe (Figure 3B). This result demonstrates that OsTGA5 can bind to the JIOsPR10 promoter by targeting the TGACGT motif.

To assess whether OsTGA5 suppresses JIOsPR10 transcription in vivo, we designed a construct expressing the firefly
luciferase (LUC) reporter gene under the control of the JIoSPR10 promoter (a 2,022-bp fragment upstream of the TSS), which we named Reporter 1. We also generated a mutated version of Reporter 1 harboring the same mutation in the TGACGT motif as in the JIoSPR10 probe-m used for EMSA (named Reporter 2). As a control, we used the β-GLUCURONIDASE (GUS) reporter gene under the control of an Arabidopsis UBIQUITIN (UBQ) promoter. We then co-transfected the Reporter 1 construct and a plasmid expressing OsTGA5-HA into rice protoplasts: relative LUC activity was significantly lower than when Reporter 1 was co-transfected with the empty HA vector control (EV) (Figure 3C). Notably, relative LUC activity from Reporter 2 (with the mutated JIoSPR10 promoter) co-transfected with EV increased to over three-fold over that from Reporter 1 with EV. We also noticed that the repressing effect imposed by OsTGA5 on relative LUC activity from Reporter 2 was abolished (Figure 3C). Taken together, our data indicate that OsTGA5 suppresses JIoSPR10 transcription by binding to the TGACGT motif within its promoter.

OsTGA5 suppresses the transcription of multiple genes involved in various defense pathways

To identify the complement of defense-related genes under the control of OsTGA5 in the rice genome, we performed transcriptome deep sequencing (RNA-seq) of ZH11 and Ostga5-1 leaves harvested before (0 h) and 12, 24, and 48 h after Guy11 inoculation. We conducted a pairwise comparison to identify differentially expressed genes (DEGs; upregulated or downregulated genes with fold-change >2) between Ostga5-1 and ZH11, which revealed more upregulated genes in Ostga5-1 relative to ZH11 at the different time points compared with the number of downregulated genes (Supplemental Table S1). Two and 29 genes were consistently downregulated and upregulated, respectively, in Ostga5-1 compared with ZH11 at all time points, suggesting a repressive role for OsTGA5 in transcription during the activation of rice innate immunity (Figure 4A). Furthermore, we selected all DEGs containing an OsTGA5-binding motif in their promoters (defined as 2,000 bp upstream from the TSSs, Supplemental Table S1), and used them to conduct a Gene Ontology (GO) enrichment analysis. The upregulated genes in Ostga5-1, but not the downregulated genes, were enriched for terms related to various biological processes and molecular functions (Figure 4B). In particular, the two biological processes, response to stress and stimulus (highlighted by red dots in Figure 4B), were enriched with eight genes, including one PR gene, three genes encoding NBS-LRR proteins and two genes encoding peroxidases (Supplemental Table S2), suggesting that OsTGA5 directly suppresses the transcription of these genes involved in immunity.

Considering the drastic changes in the local growth environment, such as continuous darkness and high humidity during the first 24 hpi, we focused on with the DEGs in Ostga5-1 and ZH11 at 48 hpi to exclude effects arising from the inoculation conditions and to identify OsTGA5-regulated genes specifically involved in defense pathways. Accordingly, we plotted the transcript profiles of the DEGs in Ostga5-1 and ZH11 at 48 hpi relative to those at 0 hpi (48 versus 0 h; Figure 4C). Interestingly, in contrast to DEGs in ZH11 48 versus 0 h, none of the downregulated genes in

Figure 4 Identification of DEGs in Ostga5-1 and ZH11 by RNA-seq. A, Venn diagrams showing the number of downregulated (left) or upregulated (right) genes in Ostga5-1 relative to ZH11 before and at different time points after M. oryzae inoculation. B, GO term enrichment analyses of all upregulated genes in Ostga5-1 containing the TGACGT motif in their promoters. Red circles indicate the terms where putative defense-related genes are enriched. BP, biological process; MF, molecular function. C, GO enrichment analyses using the DEGs identified in Ostga5-1 and ZH11 at 48 hpi relative to 0 hpi. The term of nucleotide binding (indicated by green circle) and the terms of monooxygenase activity, catalytic activity and electron carrier activity (indicated by blue circles) are specifically enriched in Ostga5-1 and ZH11, respectively.
Ostga5-1 at 48 versus 0 h appeared to be enriched into a single GO term (Figure 4C). Moreover, the upregulated genes in ZH11 were specifically enriched in pathways related to regulation of catalysis, monooxygenase, and electron carrier activities (Figure 4C, highlighted by blue dots). In contrast, the upregulated genes in Ostga5-1 at 48 versus 0 h were specifically enriched in one GO term related to nucleotide binding (Figure 4C, highlighted by green dot), which contains 288 genes, including 26 genes encoding NBS-LRR proteins, 117 receptor-like kinase and seven MAPK genes, and 16 and 6 genes potentially involved in reduction–oxidation and calcium signaling pathways, respectively (Supplemental Data Set S1). The same 288 genes were also enriched for the biological processes of cell death and response to stress, as indicated by GO enrichment analyses (Supplemental Figure S8).

Figure 5 Ostga5 interacts with the nuclear CK2 kinase complex. A, OsCK2β1 interacts with Ostga5 and Osck2α2, as shown by Y2H. The combinations with the empty vectors AD (pGADT7) or BD (pGBK7) were used as negative controls. B, Determining the in vivo interaction between OsCK2β1 and Ostga5, and OsCK2β1 and Osck2α2 by co-IP assays via Agrobacterium-mediated transient expression in N. benthamiana leaves. The combinations of OsCK2β1-GFP and Ostga5-HA, or OsCK2β1-GFP and Osck2α2-HA, constructs were co-infiltrated in N. benthamiana; Ostga5-HA or Osck2α2-HA co-infiltrated with GFP served as negative controls. Proteins were extracted 3 d after infiltration, and IP was carried out with anti-GFP beads. Immunoblotting was conducted with anti-GFP and anti-HA antibodies. C–E, Ostga5 and Osck2α2 interact in vivo, as determined by co-IP, split-LUC complementation, and BiFC assays. C, Osck2α2-GFP and Ostga5-HA constructs were co-infiltrated in N. benthamiana; Ostga5-HA co-infiltrated with GFP served as the negative control. D, Constructs encoding Ostga5-nLUC (N-terminal half of LUC) and cLUC-Osck2α2 (C-terminal half of LUC) were co-infiltrated in N. benthamiana leaves. Three days later, infiltrated leaves were detached and sprayed with 1 mM luciferin, and the bioluminescence images were captured by a CCD camera. The combinations cLUC-GUS + Ostga5-nLUC, and cLUC-Osck2α2 + GUS-nLUC were co-infiltrated as the negative controls. E, Constructs encoding nYFP-Ostga5 and cYFP-Osck2α2 were co-infiltrated in N. benthamiana leaves. The combinations of constructs nYFP-Ostga5 + cYFP-GUS, and nYFP-GUS + cYFP-Osck2α2 were used as the negative controls. Three days after infiltration, YFP signals were observed using a confocal microscope. Scale bars = 20 μm.

Ostga5 interacts with the nucleus-localized CK2 kinase complex

To further investigate the regulatory mechanism behind Ostga5-mediated rice immunity, we turned to Ostga5-binding proteins by screening a rice cDNA library via yeast two-hybrid (Y2H) assays. We thus identified the regulatory subunit of the nucleus-localized CK2 kinase complex OsCK2β1 (Chen et al., 2015) as a candidate partner of Ostga5 (Figure 5A and Supplemental Figure S9). We validated the interaction between OsCK2β1 and Ostga5 in vivo by co-immunoprecipitation (co-IP) assays with an antibody against the green fluorescent protein (GFP) in total protein extracts from Nicotiana benthamiana leaves transectly co-expressing Ostga5-HA and Osck2α2-GFP (Figure 5B, left). Sequencing revealed that ZH11, like Nipponbare, carries a premature stop codon in Hd6. Thus, Osck2α2 is the unique catalytic subunit specifically localizing to the nucleus (Supplemental Figure S9). We performed Y2H and co-IP assays to determine whether Osck2α2 and
OsCK2α2 phosphorylates OsTGA5 at Ser32 and compromises its DNA binding ability and suppression on JIoSPR10 transcription. A, Identification of the OsTGA5 residue phosphorylated by OsCK2α2 via mass spectrometry analysis. The in vitro kinase assays were performed with recombinant GST-OsCK2α2 and MBP-OsTGA5; the kinase-deficient form GST-OsCK2α2kd was used as a negative control. Annotated spectra for the phosphorylated peptide of OsTGA5 are shown at the bottom with "p" indicating the phosphorylation site (indicated by an arrow). B, OsCK2α2 phosphorylates OsTGA5 at S32 in vitro. The kinase assays were carried out with the indicated recombinant proteins, followed by immunoblotting with an antibody specifically recognizing phosphorylated S32 of OsTGA5 (OsTGA5 S32p). The S32A mutation (nonphosphorylatable, OsTGA5S32A) was introduced to verify the specificity of the antibody against OsTGA5 S32p. The amount of recombinant proteins are shown by Coomassie blue staining. C, S32 of OsTGA5 can be phosphorylated by OsCK2α2 in planta. Total proteins were extracted from N. benthamiana (Continued)
OsCK2β1 can form a complex. Indeed, OsCK2α2 is associated with OsCK2β1 both in yeast and in planta (Figure 5, A and B). We also tested whether OsCK2α2 can interact with OsTGA5, and found no direct binding between them in yeast (Figure 5A). However, we observed a positive in vivo interaction between OsCK2α2 and OsTGA5 via co-IP, split-LUC complementation and bimolecular fluorescence complementation (BiFC) assays; in particular, BiFC revealed that OsCK2α2 and OsTGA5 interact in the nucleus (Figure 5, C–E). Therefore, OsTGA5 associates with the nucleus-localized CK2 complex by directly binding to the β subunit.

OsCK2α2-mediated phosphorylation of OsTGA5 at Serine 32 reduces its DNA-binding ability and suppression of JIosPR10 transcription

The β subunit of the CK2 kinase complex usually mediates substrate recognition of the CK2 complex (Mulekar and Huq, 2014), which prompted us to investigate whether OsCK2α2 can phosphorylate OsTGA5. Lysine 63 (K63) in OsCK2α2 is the conserved amino acid essential for ATP binding and for kinase activity. To test whether the CK2 complex phosphorylates OsTGA5, we generated a kinase-deficient variant of OsCK2α2 (OsCK2α2kd) with K63 mutated to arginine (R) as a negative control for the kinase assay. We incubated purified recombinant MBP-OsTGA5 for an in vitro kinase assay together with glutathione S-transferase (GST)-OsCK2α2 or GST-OsCK2α2kd, followed by mass spectrometry analysis of MBP-TGA5 to identify phosphorylation site(s). We established that serine (S) 32 of OsTGA5 is the unique site being phosphorylated by OsCK2α2 (with 100% confidence), but not by OsCK2α2kd (Figure 6A). The S32 residue is conserved among TGA members within clade II of the D subgroup in rice and Arabidopsis (Supplemental Figure S10).

To confirm the phosphorylation of OsTGA5 by OsCK2α2, we produced a polyclonal antibody specifically recognizing OsTGA5 phosphorylated at S32 (OsTGA5S32p). Immunoblotting with the anti-OsTGA5S32p antibody following in vitro kinase assays with the recombinant proteins showed that the S32 phosphorylation signal could be detected when recombinant OsTGA5 was incubated with OsCK2α2, but not with OsCK2α2kd (Figure 6B). Similarly, we failed to detect any phosphorylation signal when recombinant OsTGA5S32A, in which S32 was mutated to a non-phosphorylatable alanine (A) residue, was incubated with OsCK2α2 (Figure 6B). These data demonstrate that OsCK2α2 can phosphorylate OsTGA5 at S32 in vitro, which can be specifically recognized by the anti-OsTGA5S32p antibody. Furthermore, to determine the in vivo phosphorylation of OsTGA5 by OsCK2α2, we transiently co-expressed OsTGA5-HA and OsCK2α2-GFP or OsCK2α2kd-GFP in N. benthamiana leaves. As a negative control, OsTGA5S32A-HA and OsCK2α2-GFP were also co-expressed. We extracted total proteins from N. benthamiana leaves and subjected them to IP with an anti-HA antibody, followed by immunoblotting using the anti-OsTGA5S32p antibody. We observed a strong S32 phosphorylation signal when OsTGA5 was co-expressed with OsCK2α2, whereas no signal was detected in the sample co-expressing OsTGA5 and OsCK2α2kd or the negative control (Figure 6C). Thus, OsTGA5 is phosphorylated at S32 by OsCK2α2 both in vitro and in vivo.

To further investigate the regulation of OsTGA5 activity by CK2 phosphorylation, we introduced the S32D mutation in OsTGA5 to generate a phosphomimic variant of the protein. We determined that as with OsTGA5, both OsTGA5S32D and OsTGA5S32A localize to the nucleus when their encoding constructs were transfected in rice protoplasts (Supplemental Figure S11A), indicating that the phosphorylation status at S32 does not affect OsTGA5 localization. Moreover, S32 phosphorylation had little influence on the interaction between OsTGA5 and NH1 (Supplemental Figure S11B). We then turned to EMSAs to investigate whether the phosphorylation status of OsTGA5 (OsTGA5, OsTGA5S32D, and OsTGA5S32A) affected its affinity for the binding motif within the JIosPR10 promoter. To remain in the linear range of the assay, we decreased the amount of recombinant OsTGA5 protein mixed with the probes from 5 (used for Figure 3B) to 0.1 μg, which was the smallest amount necessary to detect a clear electrophoresis mobility shift in this assay, and 0.25 μg. With both lower amounts of recombinant protein, we detected a clear mobility shift with OsTGA5; the intensity of the band decreased with recombinant OsTGA5S32D, whereas OsTGA5S32A exhibited the highest affinity for the DNA probe (Figure 6D). Therefore, phosphorylation of OsTGA5 at S32 decreases its affinity for DNA.

Given the effects of phosphorylation at the S32 residue in vitro, we wished to analyze whether effector constructs expressing OsTGA5, OsTGA5S32D, or OsTGA5S32A would show distinct regulation of the JIosPR10 transcription. We thus

Figure 6 (Continued)
transfected rice protoplasts with each effector construct and the LUC reporter construct (Reporter from Figure 3C). Similar to OsTGA5, the transient overexpression of OsTGA5S32A significantly decreased relative LUC activity compared with that obtained with the empty vector control (P < 0.01; Figure 6E). In contrast, protoplasts transfected with OsTGA5S32D exhibited significantly higher relative LUC activity than those expressing OsTGA5 or OsTGA5S32A, indicating that repression of JIosPR10 transcription by OsTGA5 is compromised by phosphorylation at S32. Taken together, our results indicate that the phosphorylation at S32 by OsCK2α2 reduces OsTGA5 affinity to the JIosPR10 promoter, thereby alleviating the suppression of JIosPR10 transcription.

Ser32 phosphorylation alleviates the negative regulation of OsTGA5 over rice defense

To evaluate the contribution of OsTGA5 phosphorylation at S32 in rice resistance against blast fungus, we created transgenic lines overexpressing OsTGA5, OsTGA5S32A, or OsTGA5S32D with an N-terminal FLAG tag in the Ostga5-1 mutant background. We selected two lines for each construct from the T1 generation (Ostga5-1 FLAG-OsTGA5-OE lines #1 and #16; Ostga5-1 FLAG-OsTGA5S32A_OE lines #3 and #5; Ostga5-1 FLAG-OsTGA5S32D_OE lines #18 and #20) with comparable and higher levels of OsTGA5, OsTGA5S32A, or OsTGA5S32D transcripts, respectively, for further analysis (Figure 7A). We first measured relative JIosPR10 transcript levels in ZH11, Ostga5-1, and the transgenic plants after M. oryzae inoculation. We observed that OsTGA5 overexpression in the Ostga5-1 background reduces JIosPR10 transcripts to levels lower than in ZH11 at 24 hpi, with OsTGA5S32A overexpression resulting in even lower relative JIosPR10 transcript levels. However, relative JIosPR10 transcript levels were notably higher in the Ostga5-1 FLAG-OsTGA5S32D-OE plants compared with Ostga5-1 OsTGA5-OE and Ostga5-1 OsTGA5S32A_OE lines (Figure 7B), suggesting that in vivo phosphorylation of OsTGA5 at S32 alleviates its repression of JIosPR10 transcription.

We then challenged these transgenic plants with punch inoculations with Guy11 conidial spores. The transgenic plants overexpressing OsTGA5 were more susceptible to the fungus than the wild-type ZH11 (Figure 7C). In agreement with the JIosPR10 transcript levels above, the Ostga5-1 FLAG-OsTGA5S32A_OE lines showed increased susceptibility, compared with the Ostga5-1 FLAG-OsTGA5-OE plants, whereas the Ostga5-1 FLAG-OsTGA5S32D_OE lines were significantly less susceptible than the Ostga5-1 OsTGA5-OE or Ostga5-1 OsTGA5S32A_OE lines. Taken together, our data indicate that phosphorylation of OsTGA5 at S32 compromises OsTGA5 function as a negative regulator of JIosPR10 transcription and rice defense against blast fungus.

Furthermore, to determine the phosphorylation levels of OsTGA5 S32 before and after M. oryzae infection, we challenged the Ostga5-1 FLAG-OsTGA5-OE lines with Guy11 conidial spores by spraying and harvested the leaves before (0 hpi) and after (24 and 48 hpi) M. oryzae inoculation. We then performed immunoblotting assays with the anti-OsTGA5S32p antibody after IP with an anti-FLAG antibody. We observed that both Ostga5-1 FLAG-OsTGA5-OE lines show higher phosphorylation of OsTGA5 at S32 at 48 hpi relative to 0 hpi (Figure 7D). Moreover, to investigate the influence of PTI activation on OsTGA5 phosphorylation at S32, we transiently expressed OsTGA5-HA in ZH11 protoplasts, which were then treated with chitin. As shown in Figure 7E, phosphorylation of OsTGA5 S32 was markedly enhanced 20 min after chitin treatment but returned to basal levels within 60 min. Thus, these results indicate that blast fungus invasion can promote the in vivo phosphorylation of OsTGA5 at S32.

Knockout of OsCK2α2 compromises rice resistance against blast fungus

Consistent with the enhancement of OsTGA5 S32 phosphorylation by blast fungus infection, relative OsCK2α1 transcript levels were dramatically induced by M. oryzae inoculation in ZH11, rising to over 100 times higher levels than in mock samples, whereas OsCK2α2 transcripts levels only slightly increased upon inoculation (Figure 8A). Considering the possibility that the CK2α subunit may function without β subunit-mediated substrate recognition (Mulekar and Huq, 2014), we generated knockout mutants of OsCK2α2 in ZH11 by CRISPR/Cas9 genome editing. We obtained three homozygous Osck2α2 mutants from independent T0 transgenic plants, all carrying single but different 1-bp insertions at the same position, which were named Osck2α2-1, Osck2α2-2, and Osck2α2-3, respectively (Figure 8B). When challenged with Guy11 by punch inoculation, all Osck2α2 mutants displayed increased susceptibility compared with ZH11 (Figures 8C). The Osck2α2-1 mutant was more susceptible to the M. oryzae isolate ZHONG1 as well (Supplemental Figure S4). In addition, we determined that both resting and M. oryzae-induced JIosPR10 transcript levels are significantly lower in the Osck2α2 mutants than in ZH11 (Figure 8D); chitin-induced ROS burst and callose deposition were also attenuated in the absence of OsCK2α2 (Supplemental Figure S12). Furthermore, we assessed the in vivo phosphorylation of OsTGA5 S32 by transfecting the OsTGA5-HA construct in ZH11 and Osck2α2-1 protoplasts. After treatment with chitin for 20 min, we detected OsTGA5 phosphorylation at S32 in ZH11, but not in Osck2α2-1 protoplasts (Figure 8E), indicating that OsCK2α2 is required for the phosphorylation of OsTGA5 in rice.

To investigate whether the kinase activity of OsCK2α2 is essential for inducing JIosPR10 transcription and enhancing resistance against blast fungus, we overexpressed OsCK2α2 and OsCK2α2kd in ZH11. As shown in Supplemental Figure S13, A and B, the T1 transgenic plants overexpressing OsCK2α2 displayed a dramatic upregulation of JIosPR10 transcript levels, but not those overexpressing OsCK2α2kd. Upon inoculation with M. oryzae, the OsCK2α2-OE plants showed greater resistance compared with ZH11 (Supplemental Figure S13C).
Interestingly, the plants overexpressing OsCK2α2kd were more susceptible than ZH11 (Supplemental Figure S13C), which may be attributed to the alteration of endogenous OsCK2α function by overexpressing its kinase-deficient form. Taken together, our data demonstrate that OsCK2α2, depending on its kinase activity, functions as a positive regulator of JIOsPR10 transcription and immune responses against blast fungus.
At different time points after spray-inoculation with Guy11 conidia. Water fungus. A, Relative transcript levels of OsTGA5 in ZH11 at different time points after spray-inoculation with Guy11 conidia. Water containing 0.02% Tween-20 was used as the mock control. UBQ served as the internal control. Data are means ± SE (n = 3). Significant differences were determined by one-way ANOVA (**P < 0.01). These data are from one representative out of three independent experiments. B, Mutation sites of homozygous Osck2 mutants generated via CRISPR/Cas9 gene editing in ZH11. C, Four-week-old plants of ZH11 and Osck2 mutants were punch-inoculated with Guy11 conidia. The diseased leaves were photographed at 8 dpi (left). Fungal biomass of punch-inoculated leaves was determined (right). Data are means ± SE from three biological replicates. D, Relative transcript levels of JI OsPR10 in ZH11 and Osck2 mutants before and after inoculation with M. oryzae. UBQ was used as the internal control. Data are means ± SE (n = 3). Significant differences were determined by one-way ANOVA (**P < 0.01). The data are from one representative out of three independent experiments. E, OsTGA5 is not phosphorylated upon chitin treatment in the Osck2 mutants. OsTGA5-HA was transiently transfected in ZH11 and Osck2 mutants protoplasts. Following treatment with 1 μM chitin for 20 min, OsTGA5 S22 phosphorylation was analyzed by immunoblotting with the anti-OsTGA5S32p antibody.

**Figure 8** Knockout of OsCK2a2 compromises rice resistance against blast fungus. A, Relative transcript levels of OsCK2a2 and OsCK2β1 in ZH11 at different time points after spray-inoculation with Guy11 conidia. Water containing 0.02% Tween-20 was used as the mock control. UBQ served as the internal control. Data are means ± SE (n = 3). Significant differences were determined by one-way ANOVA (**P < 0.01). These data are from one representative out of three independent experiments. B, Mutation sites of homozygous Osck2 mutants generated via CRISPR/Cas9 gene editing in ZH11. C, Four-week-old plants of ZH11 and Osck2 mutants were punch-inoculated with Guy11 conidia. The diseased leaves were photographed at 8 dpi (left). Fungal biomass of punch-inoculated leaves was determined (right). Data are means ± SE from three biological replicates. D, Relative transcript levels of JI OsPR10 in ZH11 and Osck2 mutants before and after inoculation with M. oryzae. UBQ was used as the internal control. Data are means ± SE (n = 3). Significant differences were determined by one-way ANOVA (**P < 0.01). The data are from one representative out of three independent experiments. E, OsTGA5 is not phosphorylated upon chitin treatment in the Osck2 mutants. OsTGA5-HA was transiently transfected in ZH11 and Osck2 mutants protoplasts. Following treatment with 1 μM chitin for 20 min, OsTGA5 S22 phosphorylation was analyzed by immunoblotting with the anti-OsTGA5S32p antibody.

**Discussion**

In this study, we demonstrated that OsTGA5 plays a negative role in rice immune responses against blast fungus.

Following M. oryzae invasion, the loss of OsTGA5 led to greater accumulation of SA. Moreover, chitin-induced ROS burst and callose deposition were also enhanced in the Ostga5 mutant. Both OsTGA5 and rTGA2.1 negatively regulate rice immunity. However, perturbing the function of rTGA2.1 results in increased rice resistance to Xoo with a developmental cost, such as dwarfing and reduced tiller numbers (Fitzgerald et al., 2005). It is worth noting here that the Ostga5 mutants generated in this study displayed increased resistance to blast fungus with no obvious growth or development penalty, suggesting the potential of utilizing OsTGA5 alteration in rice resistance breeding.

OsTGA5 binds to the TGACGT motif in the JI OsPR10 promoter and suppresses its transcription. Furthermore, our RNA-seq analyses shed light on the repression imposed by OsTGA5 on the transcription of multiple defense-related genes in various signaling pathways. TGA2 in Arabidopsis possesses a parallel basic function in repressing PR1 expression, whereas it acts as a positive regulator of PR1 in the tga5 tga6 double mutant (Kesarwani et al., 2007), implying that the specific regulatory function of At TGA2 is complex and can vary due to its interplay with other TGA members. AtTGA2 suppresses PR1 transcription by forming an oligomer that sits on its cognate binding site within the PR1 promoter and hinders transcription (Boyle et al., 2009). Whether OsTGA5 functions in a similar way to repress its target genes still needs to be explored.

Although previous studies have indicated that CK2 controls plant virus propagation (Hung et al., 2014; Hu et al., 2015), its role in plant immune responses against fungal pathogens is still ambiguous. In tobacco, SA increases the activity of nuclear CK2, and treatment with a CK2 inhibitor impairs transcription of a GUS reporter gene driven by the as-1 element (Hidalgo et al., 2001), suggesting a role for nuclear CK2α in inducing the transcription of TGA-targeted genes. A later study with elaborate biochemical assays showed that Ser-11, Thr-12, and Thr-16 of Arabidopsis TGA2 are possible phosphorylation sites by CK2, with the phosphorylation being enhanced by SA, although a truncated form of TGA2 lacking the first 20 amino acids can still be phosphorylated by CK2 in Arabidopsis leaf extracts (Kang and Klessig, 2005). Moreover, the DNA affinity of Arabidopsis TGA2 was attenuated by CK2, but the phosphorylation of Ser-11, Thr-12, and Thr-16 did not contribute to this regulation (Kang and Klessig, 2005), implying that additional CK2-phosphorylating site(s) exist and are involved in impairing TGA2 binding to DNA. Our study reveals a mechanism by which TGA activity is regulated by CK2 in rice: the unique nuclear CK2α subunit in typical japonica rice cultivars phosphorylates OsTGA5 at S32 and alleviates its transcriptional suppression of downstream defense genes by decreasing OsTGA5 affinity to its binding DNA sequence. Our results add a positive role for CK2 in mediating plant defense against fungal pathogens to the multiple biological processes it participates in.
Upon nuclear enrichment of NPR1 promoted by higher endogenous SA levels in Arabidopsis, the BTB/POZ domain of NPR1 interacts with the N-terminal repression domain of TGA2, which in turn disassembles the TGA2 oligomer and attenuates its repression of target genes (Boyle et al., 2009). The S32 residue is located in the homologous repression domain of OsTGA5, but its phosphorylation did not affect the interaction between OsTGA5 and NH1. In addition, in contrast to Arabidopsis, rice plants accumulate continuously high SA levels during normal growth, which exhibit little change after pathogen infection (Yang et al., 2013). Thus, SA does not appear to be the initial signal to impair the activity of OsTGA5 as a transcriptional repressor. In this case, phosphorylation of OsTGA5 by CK2 may act as a critical switch to elicit the expression of defense-related genes upon blast fungus invasion. In agreement, our results indicate that the in vivo phosphorylation of OsTGA5 at S32 is indeed enhanced by blast fungus inoculation and chitin treatment. Different from the overexpression of OsTGA5 or OsTGA5S32A, Ostga5-1 OsTGA5S32D-OE lines were not more susceptible than the wild-type, which further suggests that CK2-dependent phosphorylation of OsTGA5 S32 compromises the suppressing role of OsTGA5 in rice immunity.

Taken together, we propose a working model to illustrate the molecular switch controlling OsTGA5 activity by nuclear CK2 during the activation of rice defense against blast fungus (Figure 9). Under normal growth conditions, OsTGA5 binds to the promoters of defense-related genes and suppresses their transcription to maintain a proper energy balance, so that nuclear CK2 is mainly engaged in regulating rice growth and development. Upon blast fungus invasion, increased expression of the CK2β subunit gene promotes the association between CK2 and OsTGA5, leading to enhanced phosphorylation of OsTGA5 at S32. Subsequently, phosphorylated OsTGA5 is released from the promoters of its target genes, and the transcription of defense-related genes is induced. Nevertheless, during the interaction between rice and blast fungus, other signaling pathway(s) may also exist to enhance the phosphorylation of OsTGA5 by CK2, which awaits to be determined through further studies.

Materials and methods

Plant materials and growth conditions

The generation of the knockout mutants for OsTGA5 and OsCK2α2 in the ZH11 background was conducted via CRISPR/Cas9 gene editing as described (Wang et al., 2015). The sgRNA target sequences for OsTGA5 and OsCK2α2 were 5'-ccactgtgctgaaggccaca-3' and 5'-ccctcaccgtccaatgggggtg-3', respectively. To obtain transgenic plants overexpressing OsTGA5/OsTGA5S32A/OsTGA5S32D and OsCK2α2/OsCK2α2kd, their respective coding sequences were cloned into a binary vector with the maize UBQ promoter and the Neomycin phosphotransferase II selection marker and pCXUN-HA vector with the maize UBQ promoter and the Hygromycin phosphotransferase selection marker. The resulting constructs were transformed into Ostga5-1 and ZH11, respectively. Primer sequences used for cloning the coding sequences by PCR can be found in Supplemental Table S3. The germinated rice seeds were grown in a chamber (Conviron, Winnipeg, Canada; PGC20) at 28°C with a 12-h light (600–800 μmol/m²/s)/12-h dark cycle and 70% humidity.
Blast fungus inoculation
The rice blast fungus isolates, Guy11 and ZHONG1, pre-cultured on CMII medium, were grown on rice bran medium for 10 days in the dark at 25°C. After the aerial hyphae were flattened off, the growth plates were incubated under light (200–400 µmol/m²/s, 12-h light/12-h dark cycle) for sporulation. The conidial spores were collected in water with 0.02% (v/v) Tween-20. Spraying and punch inoculation were carried out with 3- and 5-week-old rice plants, respectively, using the method previously described (Tian et al., 2020). Measuring the fungal biomass in the punch-inoculated leaf tissues was performed as described previously (Park et al., 2012). Two punched leaves detached from different plants were collected as one biological replicate. Leaf sheath inoculation was performed with about 30-day-old plants following the method described previously (Tian et al., 2020).

ROS assay and callose deposition
The ROS burst assay was performed as described previously (Tian et al., 2020). In brief, rice leaf discs of 7-day-old seedlings were cut and suspended in 100 µL water in a 96-well plate overnight. To detect ROS production, water was replaced with 100 µL reaction solution (20 µM luminol and 2.5 µg/mL peroxidase) containing 400 nM chitin (hexa-N-acetylchitohexaose). Time-dependent quantification of ROS production was recorded on a Mithras luminometer (Berthold) every 2 min for 1 h. Sixteen replicates were performed for each sample. For observing and quantifying chitin-induced callose deposition, the leaves of 7-day-old seedlings were detached and incubated with 400 nM chitin. The assay was performed as described previously (Yang et al., 2019). The callose deposits were observed using UV light (excitation 405 nm, emission 498 nm; Zeiss LSM880). The numbers of deposits were counted according to all fields of vision using ImageJ version 1.43U software (Schneider et al., 2012).

Measurement of SA and JA contents
For measuring endogenous SA and JA levels, 3-week-old seedlings of ZH11 and Ostgas-1 were sprayed with Guy11 conidial spore suspension (2 × 10⁸ spores/mL), with water spraying as the mock treatment. The leaf tissues from six plants were harvested as one biological replicate. The extraction and quantification of SA and JA were carried out according to the manufacturer’s instructions (Wuhan Metware Biotechnology Co., Ltd., Wuhan, China), using the method previously described (He et al., 2020).

SA inhibitor AIP treatment
Three-week-old rice seedlings were treated with 30 µM AIP (BIOBOMEI, Hefei, China; AT6923) by spraying or with water as a mock control 24 h before inoculation with Guy11 conidial spores. At 4 dpi, the average number of disease lesions per leaf was calculated by counting the lesions on the most seriously diseased leaves of three individual plants.

RT-qPCR
Total RNA was extracted using the TRIzol reagent (CWBio, Beijing, China; CW05805) from leaf tissues, and 2 µg RNA was subjected to first-strand cDNA synthesis using EasyScript One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen, Beijing, China; AE311). qPCR was then performed using the PerfectStart Green qPCR SuperMix (TransGen, AQ601) on a CFX connect Real-time system (Bio-Rad, Hercules, California, USA). UBQ was used as the internal control. Sequences of the primers for amplifying the plant defense-related genes are provided in Supplemental Table S3.

Transcriptome deep sequencing and data analysis
Seven to eight 3-week-old ZH11 or Ostgas-1 plants were collected at each time point as one biological replicate, and the RNA-seq analysis was performed on two biological replicates. Total RNA was extracted from the leaf tissues of ZH11 and the Ostgas-1 mutant before and after Guy11 inoculation. The preparation and sequencing of the libraries were carried out at the Novogene Bioinformatics Institute (Beijing, China) as described previously (Zhang et al., 2017) with minor modifications. Briefly, total RNA was treated with DNase I to completely remove genomic DNA contamination, then purified using oligo poly(T) conjugated to magnetic beads. cDNA was synthesized and selected by size (370–420 bp) using AMPure XP system (Beckman Coulter, Beverly, California, USA) for mRNA-seq library construction. The final library was quality-controlled and paired-end sequenced on an Illumina platform (NovaSeq 6000) with a read length of 150 bp.

For data analysis, the raw reads were trimmed using Trimmomatic (version 0.39) to remove primer/adapter contamination and reads with poor quality. Paired-end clean reads were aligned to the japonica Nipponbare reference genome (MSU 7.0) using Hisat2. The mapped reads from each sample were assembled by StringTie (version 1.3.4d). FeatureCounts (version 1.6.4) was used to count the read number mapped to each gene. The Fragments Per Kilobase of transcript per Million mapped reads values of each gene were then calculated based on the length of the gene and read counts mapped to the corresponding gene. DEGs between two conditions/groups (two biological replicates per condition) were determined using the DESeq2 R package (version 1.30.1). Genes with a P-adj < 0.05 and |Log2(fold-change)| > 1 were defined as differentially expressed. Biological processes, cellular components, and molecular functions of DEGs were determined by GO term enrichment analysis Toolkit and Database for Agricultural Community (AgriGo version 2.0, http://systemsbiology.cpol.cn/agriGOv2/). Go terms with P-value < 0.05 were considered as significant GO terms.

Y2H assay
The coding sequence of OstGAS was cloned into the bait vector pGBK7T. The Matchmaker Gold Y2H system (Clontech, Mountain View, California, USA, 630489) carrying a rice cDNA library was used to screen for OsTGA5-
interacting proteins. Yeast strain AH109 was transformed and screened for positive clones, which were then sequenced to identify the putative interactors. For Y2H assay, the coding sequences of OsTGA5, OsCK2α2, and OsCK2β1 were amplified by PCR using gene-specific primers (Supplemental Table S3) and cloned into pGADT7 or pGBK7 (Clontech) as indicated. The appropriate pairs of constructs were transformed into yeast strain AH109. The yeast clones were grown on synthetic defined (SD) medium lacking Trp and Leu (SD –Trp –Leu) at 30°C for 2 days and then spotted onto SD medium lacking Trp, Leu, and His (SD –Trp –Leu –His) or SD –Trp –Leu –His –Ade to detect interactions.

**Split-LUC complementation assay**

The coding sequences of OsTGA5, OsCK2α2, and OsCK2β1 were cloned into nLUC or cLUC vector (Chen et al., 2008) as indicated. The resulting constructs were then transiently co-expressed in *N. benthamiana* leaves via Agrobacterium (*Agrobacterium tumefaciens*, strain GV3101)-mediated infiltration, with agrobacteria resuspended (final OD600 = 0.6) in infiltration buffer (10 mM MgCl₂, 10 mM MES, 200 mM acetasoryngone, pH 5.6). Three days postinfiltration, 1 mM D-luciferin was sprayed onto detached leaves, which were then kept in the dark for 5–10 min. LUC activity was detected using the LB985 NightSHADE plant imaging system (Berthold, Germany).

**BiFC assay**

The coding sequences of OsTGA5 and OsCK2α2 were cloned into pSY736 (with N-terminal nYFP) and pSY735 vectors (with N-terminal cYFP), respectively. The resulting expression cassettes were then individually transferred to the vector pMDC32. The final constructs were introduced in *N. benthamiana* leaves. The YFP signals were detected using a confocal microscope (Zeiss LSM880) with excitation 488 nm, emission 546 nm. For the IP and immunoblotting assays with protein samples extracted from rice protoplasts, the extraction buffer and washing buffer were the same as those used for the above co-IP assay.

**Recombinant protein production and purification**

The coding sequences of OsTGA5, OsTGA5S32D, and OsTGA5S32A were cloned into the pMAL-c2G vector (adding a MBP tag at the N terminus), and the OsCK2α2 coding sequence was cloned into pGEX-4T-1 vector (adding a GST tag at the N terminus). The fusion proteins were produced in *Escherichia coli* strain BL21(DE3) (induced with 0.5 mM IPTG at OD600 = 0.6 and grown at 18°C overnight). The recombinant proteins were purified according to the manufacturer’s instructions. Amylose resin (BioLabs, Ipswich, Massachusetts, USA; E8021S) and glutathione resin (GE Healthcare, Chicago, Illinois, USA; 17075605) were used for purifying the recombinant MBP- and GST-tagged proteins, respectively.

**In vitro kinase activity and mass spectrometry analysis**

The in vitro kinase assay was performed as previously described (Kang and Klessig, 2005) with minor modifications. Briefly, 4 μg of purified GST-OsCK2α2 or GST-OsCK2α2kd combined with 2 μg of purified MBP-OsTGA5 was incubated in a 40-μL reaction mixture (50 mM Tris–HCl, pH 7.5, 10 mM MgCl₂, 50 mM KCl, 1 mM DTT, and 100 μM ATP) for 30 min at 30°C. The reaction components were separated by SDS-PAGE. After Coomassie blue staining, the MBP-OsTGA5 bands were excised and digested with trypsin. The phosphopeptides were enriched with solvent A (water with 0.1% [v/v] formic acid) and subjected to liquid chromatography-tandem mass spectrometry (LC–MS/MS) analysis as described before (Liu et al., 2017).

**Detection of in vivo phosphorylation**

A polyclonal antibody specifically recognizing phosphorylated Serine 32 of OsTGA5 (OsTGA5S32p) was produced by China; A21010, 1:10,000 dilution). The primers used for the constructions are shown in Supplemental Table S3.
Abra (Shanghai, China) using the phosphopeptide ALAAAaPSdSDRS as antigen. The phosphospecific antibody was further purified using an affinity column conjugated with the phosphorylated and nonphosphorylated peptides. To detect the phosphorylation of OsTGAS S32 by OsCK2a2 in planta, the combinations of constructs OsTGAS-HA + OsCK2a2-GFP, OsTGAS-HA + OsCK2a2kd-GFP, and OsTGAS- S32A-HA + OsCK2a2-GFP were co-infiltrated in N. benthamiana leaves by Agrobacterium-mediated infiltration. Total proteins from each sample were extracted as above for co-IPs with N. benthamiana samples and immunoprecipitated with 10 µL anti-HA agarose beads (Abmart, M20013M). Immunoblotting was then performed with anti-HA and anti-OsTGAS S32p antibodies (1:1,000 dilution). Anti-rabbit secondary antibody (Abbkine, A21020, 1:10,000 dilution) was used for anti-OsTGAS S32p antibody.

Electrophoretic mobility shift assay
A 39-bp DNA fragment of the JIOsPR10 promoter containing the TGACGT sequence or its mutated version was synthesized with Cy5 end-label as probes; sequences are provided in Supplemental Table S3. The probes were incubated with the indicated amount of recombinant OsTGAS protein in a 20-µL reaction (100 mM Tris–HCl, pH 7.5, 100 mM KCl, 50-mM MgCl2, 1-mM DTT, 0.05-mg/mL poly[dI-dC]) at 4°C for 30 min. For detecting the shift in mobility, a native polyacrylamide gel containing 3.5% (w/v) acrylamide for 2 and OsCK2a1. Plants were co-infiltrated in N. benthamiana leaves by Agrobacterium-mediated infiltration. Total proteins from each sample were extracted as above for co-IPs with N. benthamiana samples and immunoprecipitated with 10 µL anti-HA agarose beads (Abmart, M20013M). Immunoblotting was then performed with anti-HA and anti-OsTGAS S32p antibodies (1:1,000 dilution). Anti-rabbit secondary antibody (Abbkine, A21020, 1:10,000 dilution) was used for anti-OsTGAS S32p antibody.

Rice protein extraction and IP assays
Total protein was extracted from rice leaves in the same extraction buffer as for N. benthamiana protein. The samples were incubated with 10 µL FLAG beads (Sigma-Aldrich, St. Louis, Missouri, USA; A2220-5ML) at 4°C for 2 h with gentle shaking, then immunoblotting was performed with anti-FLAG (Sigma-Aldrich; F1804, 1:5,000 dilution with antimouse secondary antibody) and anti-OsTGAS S32p antibodies.

Statistical analyses
All statistical analyses were performed with one-way ANOVA and are provided in Supplemental Data Set S2.

Accession numbers
Sequence data from this article can be found in the EMBL/GenBank data libraries under accession numbers: OsTGAS, LOC_Os01g17260; OsCK2a2, LOC_Os07g02350; OsCK2b1, LOC_Os10g41520; JIOsPR10, LOC_Os03g18850; OsPR1a, LOC_Os07g03710; OsWRKY45, LOC_Os05g25770; OsPR5, LOC_Os12g43380; OsCht1, LOC_Os06g51060; OsPR10b, LOC_Os12g36850; OsNAC4, LOC_Os01g60020; and UBQ, LOC_Os03g13170. The RNA sequencing data have been deposited at the NCBI SRA database, under accession number PRJNA741871, which are publicly accessible at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA741871.

Supplemental data
The following materials are available in the online version of this article.

Supplemental Figure S1. Phylogenetic tree showing the homology among the rice TGA members within clade II of the D subgroup and Arabidopsis TGA2.
Supplemental Figure S2. Mature panicles of ZH11 and the Ostga5 mutants.
Supplemental Figure S3. Leaf sheath inoculation of ZH11 and Ostga5-1 with Guy11 conidia.
Supplemental Figure S4. Punch inoculation of ZH11, Ostga5-1, and Osck2a2-1 plants with M. oryzae isolate ZHONG1.
Supplemental Figure S5. JA levels in ZH11 and Ostga5-1 with or without M. oryzae inoculation.
Supplemental Figure S6. The SA inhibitor AIP partially compromises the resistance of Ostga5-1 against blast fungus.
Supplemental Figure S7. The transcriptional levels of OsPR10b and OsNAC4 in ZH11 and Ostga5-1 with or without M. oryzae inoculation.
Supplemental Figure S8. GO enrichment analyses of the specific upregulated genes in Ostga5-1 at 48 hpi relative to 0 hpi.
Supplemental Figure S9. Subcellular localization of OsCK2a2 and OsCK2b1.
Supplemental Figure S10. Sequence alignment of the TGA members within clade II of the D subgroup in Arabidopsis and rice.
Supplemental Figure S11. Mutation of OsTGA5 S32 to D or A does not affect its nuclear localization or interaction with NH1.

Supplemental Figure S12. Chitin-induced PTI responses are compromised in the Osck2x2-1 mutant.

Supplemental Figure S13. Overexpression of OsCK2x2, but not its kinase-deficient form, enhances JIOsPR10 transcription and resistance against blast fungus.

Supplemental Table S1. Summary of the DEGs in Ostga5-1 before and after Guy11 inoculation compared with ZH11.

Supplemental Table S2. List of the upregulated genes in Ostga5-1 containing TGACGT in their promoters potentially involved in response to stress and stimulus.

Supplemental Table S3. Sequence of the primers used in this study.

Supplemental Data Set S1. List of the specifically upregulated genes in Ostga5-1 48 versus 0 h, compared with ZH11 48 versus 0h.

Supplemental Data Set S2. ANOVA results.

Supplemental File S1. Text file of the alignment used to generate the phylogenetic tree in Supplemental Figure S1.

Supplemental File S2. The Newick format file of the tree in Supplemental Figure S1.

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Conflict of interest statement. The authors declare no conflict of interest.

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