INTRODUCTION

Potassium (K\(^+\)), the most abundant inorganic cation in plant cells, is essential for plant growth and thus strongly determines crop yield (Adams & Shin, 2014; Wang & Wu, 2017). The regulation of K\(^+\) in plant health is involved in many aspects of cell physiology and metabolism, including cell expansion, enzyme activation, stomatal opening, and turgor pressure maintenance (Anschütz et al., 2014). Considering the significant roles of K\(^+\) in crop yield and plant health, improving K\(^+\) use efficiency in plants is emerging as an important

Overexpression of OsHAK5 potassium transporter enhances virus resistance in rice (Oryza sativa)

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Abstract

Intracellular potassium (K\(^+\)) transported by plants under the action of a number of transport proteins is crucial for plant survival under distinct abiotic and biotic stresses. A correlation between K\(^+\) status and disease incidence has been found in many studies, but the roles of K\(^+\) in regulating disease resistance to viral diseases remain elusive. Here, we report that HIGH-AFFINITY K\(^+\) TRANSPORTER 5 (OsHAK5) regulates the infection of rice grassy stunt virus (RGSV), a negative-sense single-stranded bunyavirus, in rice (Oryza sativa). We found the K\(^+\) content in rice plants was significantly inhibited on RGSV infection. Meanwhile, a dramatic induction of OsHAK5 transcripts was observed in RGSV-infected rice plants and in rice plants with K\(^+\) deficiency. Genetic analysis indicated that disruption of OsHAK5 facilitated viral pathogenicity. In contrast, overexpression of OsHAK5 enhanced resistance to RGSV infection. Our analysis of reactive oxygen species (ROS) including H\(_2\)O\(_2\) and O\(^2-\), by DAB and NBT staining, respectively, indicated that RGSV infection as well as OsHAK5 overexpression increased ROS accumulation in rice leaves. The accumulation of ROS is perhaps involved in the induction of host resistance against RGSV infection in OsHAK5 transgenic overexpression rice plants. Furthermore, RGSV-encoded P3 induced OsHAK5 promoter activity, suggesting that RGSV P3 is probably an elicitor for the induction of OsHAK5 transcripts during RGSV infection. These findings indicate the crucial role of OsHAK5 in host resistance to virus infection. Our results may be exploited in the future to increase crop yield as well as improve host resistance via genetic manipulations.

KEYWORDS

host resistance, potassium ion, OsHAK5, reactive oxygen species, rice grassy stunt virus

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research topic (Shin, 2014). Much progress has been made on uncovering the functions of many key players that allow plants to sense K⁺ availability and absorb it (Gierth & Mäser, 2007; Hamamoto & Uozumi, 2014; Wang & Wu, 2013). In general, K⁺ channels mediate low-affinity K⁺ uptake and K⁺ transporters conduct high-affinity K⁺ uptake. The K⁺ transporter/high-affinity K⁺ transporter/K⁺ uptake protein (KT/HAK/KUP) family is among the major K⁺ acquisition systems in plants (Grabov, 2007). Thirteen genes encode KT/HAK/KUP transporters in the Arabidopsis genome (Mäser et al., 2001), and rice has 27 members in this family (Gupta et al., 2008). Functions of many high-affinity K⁺ transporters in potassium homeostasis and tolerance to abiotic stress have been elucidated (Li et al., 2018). For example, the K⁺ transporter HAK5 (High affinity K⁺ transporter 5) has been reported to mediate most of the K⁺ absorption in Arabidopsis (Wang & Wu, 2013). Zhao et al. (2016) demonstrated HAK5-mediated high-affinity K⁺ uptake was enhanced under K⁺-deficient conditions in Arabidopsis. They found that after low-K⁺ treatment, the inhibition on HAK5 transcription can be relieved by the phosphorylation of transcription factor ARF2 (Auxin Response Factor 2), which abolished its DNA-binding activity to the HAK5 promoter (Zhao et al., 2016). HAK5 in rice (OsHAK5) also plays a major role in K⁺ acquisition by roots faced with low external K⁺ and in K⁺ upward transport from roots to shoots in K⁺-deficient rice plants (Yang et al., 2014). Additionally, Yang et al. (2020) revealed that OsHAK5 regulates rice architecture such as plant height and tillering number via controlling the ATP-dependent transmembrane auxin fluxes (Yang et al., 2020). In rice, the roles of other OsHAKs, such as OsHAK16 and OsHAK21, have also been documented. Feng et al. (2019) reported that the transcriptional expression of OsHAK16 was up-regulated by K⁺ deficiency or salt stress. OsHAK16 overexpression improved K⁺ uptake and translocation from root to shoot, resulting in increased tolerance to salt stress (Feng et al., 2019). Shen et al. (2015) reported that oshak21 mutant rice plants accumulated less K⁺ and considerably more Na⁺ in both shoots and roots because of the lower K⁺ net uptake rate and higher Na⁺ uptake rate. Thus, the disruption of OsHAK21 rendered plants sensitive to salt stress (Shen et al., 2015). These findings demonstrate the crucial function of K⁺ transporters in K⁺ net uptake, by which the plant growth and abiotic stress responses are controlled.

Although previous studies have been focused on the roles of high-affinity K⁺ transporters in plant growth and tolerance to abiotic stress, some recent studies have attached importance to the effect of K⁺ on plant susceptibility and sensitivity to distinct pathogens. The function of K⁺ in disease development in plants was initially indicated by the phenomenon that application of potassium fertilizer decreases the incidence of plant diseases (Amentmann et al., 2008; Wang et al., 2013). For example, foliar application of KCl reduces damage caused by Septoria tritici on wheat (Triticum aestivum) (Mann et al., 2010), and the application of KCl to K⁺-deficient soils increases rice resistance to stem rot and aggregate sheath spot (Williams & Smith, 2001). Genetic studies have also shown the effects of K⁺ functions on disease occurrence. For example, loss of the K⁺ channel OsAKT1 (rice ARABIDOPSIS K⁺ TRANSPORTER1) leads to decreased K⁺ content and reduced resistance against Magnaporthe oryzae (Shi et al., 2018). A further study indicated a role of suppression of host resistance by M. oryzae effector AvrPiz-t through interference with the association of OsAKT1 with its upstream regulator, the cytoplasmic kinase OsCIPK23, which also plays a positive role in K⁺ absorption and resistance to M. oryzae. As for viral diseases, the effect of K⁺ status on disease resistance is more complex. Perrenoud (1990) and Prabhū et al. (2007) reported that inhibition of viral infections is more frequent in plants with high K⁺ status. A correlation between plant K⁺ status and disease development has been established although there is variation in the data. The underlying mechanism of K⁺ in reducing pathogen virulence or enhancing host immunity remains enigmatic. In rice in particular, the functions of high-affinity K⁺ transporters that are involved in host resistance to viral pathogens need to be investigated.

Rice grassy stunt virus (RGSV) is a single-stranded, negative-sense RNA virus that causes severe disease symptoms including stunting, leaf yellowing, and reduced heading, thus resulting in large damage to rice yield. To understand how the virus damages rice growth and development, Satoh et al. (2013) examined gene expression responses associated with stunting, leaf yellowing, and excess tillering in RGSV-infected plants through transcriptome analysis. Zhang et al. (2020) demonstrated that RGSV-encoded P3 serves as a pathogenicity factor, by which the subunit of DNA methylation-associated RNA polymerase IV is degraded through the ubiquitin proteasome system and leads to the induction of disease symptoms. These studies may explain how RGSV infection influences plant growth and development, but the plant nutritional status, especially K⁺ status, during RGSV infection remains elusive, thus limiting the comprehensive understanding of the regulatory mechanism of RGSV infection.

In our study, we examined the function of K⁺ nutrition as well as the K⁺ transporter OsHAK5 on virus infection in rice. We determined that RGSV infection suppressed the K⁺ content in rice plants and, possibly as feedback, RGSV infection highly induced the transcriptional expression of OsHAK5. The disruption of OsHAK5 rendered plants sensitive to RGSV infection, while genetically overexpressing OsHAK5 enhanced disease resistance against RGSV. Rice plants grown in a low-K⁺ environment were hypersensitive to RGSV infection. We observed that reactive oxygen species (ROS) accumulation decreased in oshak5 rice plants, while it increased in OsHAK5 overexpression rice plants, suggesting that OsHAK5-mediated enhancement of rice resistance against RGSV may rely on ROS accumulation. We also examine whether RGSV-encoded P3 could be responsible for transcriptional up-regulation of OsHAK5 during RGSV infection. Our findings proved that K⁺ transporters are involved in positively modulating host resistance to virus infection in rice.
2 | RESULTS

2.1 | RGSV infection decreases K⁺ content in rice plants

RGSV infection induces typical disease symptoms on leaves such as leaf yellowing and rust spot (Figure 1a), which resemble symptoms induced by potassium deficiency. Thus, we compared K⁺ concentration in healthy and RGSV-infected rice plants to determine whether RGSV infection regulates K⁺ status in diseased rice plants. RGSV infection inhibited K⁺ accumulation in rice shoots, and the decrease of K⁺ accumulation by RGSV infection was intensified at 21 days postinoculation (dpi) (Figure 1b). To examine whether the decrease of K⁺ accumulation is because of the increase of virus accumulation, we performed reverse transcription-quantitative PCR (RT-qPCR) and western blotting assays to analyse the RGSV coat protein (CP) gene transcripts and CP levels, respectively. Both the protein level and transcripts level of RGSV CP in rice shoots significantly increased with time after inoculation (Figure 1c,d). RGSV infection inhibited rice growth, as evidenced by a significant decrease in plant height (Figure 1e) and dry weight of both leaves and roots (Figure 1f,g). These findings indicate that RGSV inhibits K⁺ accumulation in the diseased rice plants.

2.2 | RGSV infection significantly induces K⁺ transporter gene OsHAK5

Many high-affinity K⁺ transporters are essential for potassium ion homeostasis in plants. We performed RT-qPCR analysis to determine whether the expression levels of OsHAK genes (including OsHAK1, 4, 5, 7, and 17) were regulated by RGSV. We found that the transcripts of all the tested OsHAK genes increased on RGSV infection.
The differential expression of these OsHAK genes in healthy and RGSV-inoculated rice plants was consistent with the transcriptome data reported by Satoh et al. (2013). In this study, we focused on investigating the function of the OsHAK5 gene, as OsHAK5 undertakes most of the K⁺ uptake in plants (Wang & Wu, 2013). The expression of OsHAK5 in whole plants increased c.14-fold after RGSV inoculation for 30 days. We also determined the transcriptional change of OsHAK5 in different tissues: root, stem base, stem, sheath, and leaf. As shown in Figure 2b, the expression level of OsHAK5 was increased 1.5-fold in root, and was highly up-regulated in stem base, stem, and especially leaves on infection with RGSV.

To examine the induction of OsHAK5 by RGSV infection in planta, we analysed the transcription activity of the OsHAK5 promoter by applying a β-glucuronidase (GUS) reporter gene system. Under normal conditions, GUS staining was evident in the root (left image of Figure 2ca), root–shoot junction (left image of Figure 2cb), and leaf sheath (left image of Figure 2cc). On RGSV infection, OsHAK5 transcription in the stem base, stem, leaf sheath, and leaves were enhanced (Figure 2c), which is in agreement with the RT-qPCR data. We also performed RT-qPCR analysis of GUS transcripts and found that the GUS transcripts were dramatically up-regulated in the stem base, stem, and leaves by RGSV infection (Figure 2d).

(Figure 2a).
In addition to OsHAK genes, transcription changes of many other types of K⁺ transporters, including OsAKT1, OsHKT1;1, OsHKT1;5 and OsHKT2;1, were also analysed through RT-qPCR. We found that all the tested genes associated with K⁺ transport were up-regulated to varying degrees by RGSV (Figure S1). Considering that many KUP/HAK/KT family K⁺ transporters are induced during K⁺ deficiency (Ahmad et al., 2016; Alemán et al., 2009; Chen et al., 2015; Geiger et al., 2009; Li et al., 2014; Nieves-Cordones et al., 2010; Pyo et al., 2010; Rubio et al., 2014), it is probable that RGSV infection suppressed rice K⁺ content and, as a feedback, many K⁺ transporter genes were up-regulated by RGSV infection to ensure the K⁺ supply for rice normal growth.

2.3 Disruption of OsHAK5 inhibits rice growth and facilitates rice susceptibility to RGSV infection

To explore the biological roles of OsHAK5 in response to virus infection, we obtained a mutant line (PFG-3A0913B) from the SIGNAL database (http://signal.salk.edu/cgi-bin/RiceGE). The genetic background was cv. Dongjin. As shown in Figure 3a, oshak5 has a reduced tillering number and a slightly shorter plant height than wild-type rice plants. The genotype of the oshak5 mutant was confirmed through PCR (Figure S2). To determine whether the disruption of OsHAK5 regulates RGSV infection, virus inoculation assays were conducted using RGSV-coarying brown planthoppers (Nilaparvata lugens). After RGSV inoculation, the growth status of oshak5 mutant lines was worse than wild-type rice plants, indicating RGSV infection affected the growth of oshak5 mutant lines even more (Figure 3a). Western blotting as well as RT-qPCR analysis indicated that the oshak5 mutant lines accumulated more RGSV, as seen by a significant increase in RGSV CP (Figure 3b) and CP transcripts (Figure 3c) in oshak5 mutant lines. The expression levels of OsHAK5 in mock- and RGSV-inoculated wild-type as well as oshak5 mutant rice plants were compared by RT-qPCR. The results indicated that in the wild-type rice plants, RGSV infection highly induced OsHAK5 expression, whereas no induction of OsHAK5 expression was found in oshak5 mutant rice plants (Figure 3d). The plant height and tillering number of mock- and RGSV-inoculated wild-type rice plants as well as oshak5 mutant rice plants were also analysed. As shown in Figure 3e,f, RGSV-inoculated oshak5 plants showed decreased height as well as tillering number, indicating enhanced disease severity. These results indicate that disruption of OsHAK5 enhances RGSV infection.

2.4 Overexpression of OsHAK5 results in enhanced resistance to RGSV infection and a disease-like phenotype

To determine whether OsHAK5 overexpression improves rice resistance to RGSV infection, we inoculated OsHAK5 overexpression lines with RGSV. As described previously, OsHAK5 overexpression resulted in a slight reduction in plant height but generated more tillering when compared to wild-type rice plants (Figure 4a). OsHAK5 transgenic overexpression lines showed enhanced resistance to RGSV infection, as evidenced by the good growth of OsHAK5 transgenic overexpression lines (Figure 4a). Western blotting showed that RGSV CP accumulated less in OsHAK5 overexpression rice plants than in wild-type rice plants (Figure 4b). RGSV CP transcripts in RGSV-inoculated wild-type and OsHAK5 overexpression rice plants were compared by RT-qPCR. The accumulation of RGSV CP transcripts decreased in OsHAK5 overexpression rice plants (Figure 4c). The expression levels of OsHAK5 in mock- and RGSV-inoculated wild-type rice plants as well OsHAK5 overexpression rice plants were compared by RT-qPCR. The OsHAK5 transcripts in RGSV-inoculated OsHAK5 overexpression rice plants were the highest, which is approximately 12 times that in mock-inoculated wild-type rice plants (Figure 4d). The plant height and tillering number of mock- and RGSV-inoculated wild-type and OsHAK5 overexpression rice plants were analysed. A significant decrease in plant height and an increase in tillering number were observed in the OsHAK5 overexpression lines when compared with wild-type rice plants (Figure 4e,f). These findings demonstrate that OsHAK5 has the ability to improve host resistance to RGSV infection but induces disease-like symptoms.

2.5 ROS-mediated disease resistance is activated by OsHAK5

Considering that ROS production is an important signal of HAK5 transcriptional up-regulation under low K⁺ status in Arabidopsis, we first evaluated the accumulation of ROSH₂O₂ and O₂⁻ in wild-type and OsHAK5 overexpression rice plants to determine whether OsHAK5 mediates the enhancement of host resistance to RGSV infection by regulating ROS accumulation. 3,3′-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) staining indicated that the accumulation of H₂O₂ and O₂⁻ decreased in oshak5 mutant rice leaves when compared with the wild-type cv. Dongjin rice plants (Figure 5a). By contrast, OsHAK5 overexpression rice plants accumulated more H₂O₂ and O₂⁻ compared to wild-type Nipponbare rice plants, and RGSV infection also increased the accumulation of H₂O₂ and O₂⁻ (Figure 5b). We also analysed the H₂O₂ content extracted from the leaves of mock- and RGSV-inoculated rice seedlings. In oshak5 knockout rice leaves, less H₂O₂ was detected (Figure 5c), whereas rice seedlings overexpressing OsHAK5 accumulated more H₂O₂ than the wild type (Figure 5d). The activities of peroxidase (POD) and catalase (CAT) in wild-type, oshak5 mutant, and OsHAK5 overexpression rice plants were explored. POD activity decreased slightly in oshak5 mutant rice plants (Figure 5e) but increased slightly in OsHAK5 overexpression rice plants (Figure 5f). CAT activity was suppressed in oshak5 mutant rice plants (Figure 5g) but strongly induced in OsHAK5 overexpression rice plants (Figure 5h).
FIGURE 3  The phenotype analysis of oshak5 mutant transgenic rice plants. (a) Phenotypes of oshak5 mutant rice plants inoculated with or without RGSV. (b) Detection of RGSV coat protein (CP) in RGSV-inoculated wild-type (WT) rice and oshak5 mutant lines by western blotting assay. (c) Detection of RGSV CP transcripts in RGSV-inoculated WT, empty vector (EV) transgenic, and oshak5 mutant lines by reverse transcription-quantitative PCR (RT-qPCR). (d) Relative expression of OsHAK5 in mock- and RGSV-inoculated WT, EV transgenic, and oshak5 mutant lines through RT-qPCR. (e) Plant height of mock- and RGSV-inoculated WT, EV transgenic, and oshak5 mutant lines. Rice Actin1 was used as an internal reference in RT-qPCR. Rice actin was used as loading control in western blotting. WT, Dongjin rice plants; EV, empty vector transgenic rice plants; oshak5, oshak5 mutant rice plants; RGSV, RGSV-inoculated rice plants; **p < 0.01 determined using Student's t test; values followed by a different letter within the same column are significantly different at α = 0.05; error bars, standard deviations; scale bars, 15 cm
We also analysed the transcription changes of genes associated with ROS production and degradation in OsHAK5 overexpression and oshak5 mutant rice plants through RT-qPCR. The transcripts of CuZnSOD1 and MnSOD1 were expressed more in the leaves of OsHAK5 overexpression rice plants than in the wild-type rice leaves (Figure S3a). However, the expression level of CuZnSOD1 was dramatically reduced in oshak5 mutant rice plants (Figure S3b). In contrast, OsHAK5 overexpression rice plants expressed less CATB and CATB in leaves than in wild-type rice leaves whereas the expression level of CATA and CATB was slightly increased in oshak5 mutant rice plants. MnSOD1 was not changed in OsHAK5 overexpression and oshak5 mutant rice plants. These findings suggest that ROS signalling is activated by the induction of OsHAK5 expression either by RGSV infection or transgenically.

### 2.6 K⁺ deficiency enhances RGSV pathogenicity

Considering that OsHAK5 overexpression significantly increases K⁺ concentration in rice (Yang et al., 2014, 2020), we speculated that the positive role of OsHAK5 in host resistance against RGSV might be achieved through promoting K⁺ transport and increasing endogenous K⁺, and, conversely, whether low K⁺ levels promoted viral infection. To determine whether K⁺ deficiency promotes RGSV infection, we grew rice seedlings under low K⁺ (5 μM) conditions through the hydroculture method; the normal concentration of K⁺ (1 mM) served as controls. RGSV inoculation was performed to examine the response of the two sets of rice seedlings. Rice seedlings under low K⁺ treatment showed enhanced disease symptoms compared to those under normal conditions (Figure 6a). We also observed the effect of RGSV infection as well as low K⁺ status on root morphology. We found that roots of rice seedlings growing under low K⁺ treatment produced fewer lateral roots and weaker axial roots compared to those growing under normal conditions (Figure 6b). RGSV CP accumulation increased in the roots and leaves of rice seedlings growing under low K⁺ treatment (Figure 6c). RGSV CP transcripts significantly increased in roots and leaves of rice seedlings growing under low K⁺ treatment compared to the controls (Figure 6d). Rice plants with low K⁺ status had a higher disease incidence rate than those growing under normal conditions (Figure 6e). To further clarify the effect of low K⁺ status on RGSV pathogenicity, we used a range of K⁺ concentrations (0.3 mM, 0.1 mM, 5 μM) for the treatment of rice seedlings by the hydroculture method. As shown in Figure S4a, rice seedlings showed gradually enhanced disease symptoms as the K⁺ concentration decreased. Rice seedlings treated with 5 μM K⁺ showed the most severe disease symptoms, including the shortest plant height and the most rust spots on leaves (Figure S4a). RGSV CP accumulated more in the rice seedlings than in the controls as the K⁺ concentration decreased (Figure S4b). The length of roots and shoots also gradually decreased as the K⁺ concentration decreased (Figure S4c,d). The total length, surface area, average diameter, and volume of root were also calculated. Both RGSV infection and low K⁺ status severely affected root growth, as seen by a significant decrease in total length, surface area, average diameter, and volume (Figure S5a-d). To evaluate the effect of RGSV infection and low K⁺ treatment on plant biomass, we compared the root dry weight and shoot dry weight. We found that both RGSV infection and low K⁺ treatment significantly reduced the root dry weight as well as shoot dry weight; RGSV-inoculated root seedlings treated by low K⁺ had the lowest dry weight in root and shoot (Figure S6a,b). Additionally, RGSV and low K⁺ treatment significantly influenced the root length (Figure S6c), while the shoot length was dramatically decreased by RGSV infection but not by low K⁺ (Figure S6d). Thus, RGSV infection and low K⁺ treatment seriously affected the growth and development of roots, and low K⁺ treatment made rice more sensitive to RGSV infection.

### 2.7 RGSV-encoded P3 can induce the promoter activity of OsHAK5 in vitro

We have shown the effect of OsHAK5 on improvement of rice disease resistance to RGSV infection. However, how RGSV infection results in the transcriptional induction of OsHAK5 remains unknown. We investigated whether RGSV-encoded proteins promote OsHAK5 expression through enhancing the activity of the OsHAK5 promoter. This was examined by applying the GUS reporter gene system. As shown in Figure 7a, the infiltrated region of Nicotiana benthamiana leaves co-expressing HA-tagged RGSV P3 and OsHAK5 promoter exhibited more GUS signal than the infiltrated region co-expressing empty vector with the OsHAK5 promoter (Figure 7a). Western blotting assays conducted with anti-HA antibody indicated the expression of RGSV P3 (Figure 7b). The up-regulated GUS expression in the infiltrated region of N. benthamiana leaves co-expressing RGSV P3 and OsHAK5 promoter was verified by RT-qPCR analysis (Figure 7c). Other myc-tagged RGSV-encoded proteins, including P2, P4, PC3, and PC5, had no visible effect on the activity of the OsHAK5 promoter. RGSV P5 slightly increased the activity of the OsHAK5 promoter, while RGSV P1 dramatically inhibited the activity of OsHAK5 promoter. These findings were evidenced by the GUS staining assays (Figure 7a). The expression of tested RGSV-encoded proteins was verified by western blotting assays with anti-myc antibody (Figure 7b). GUS transcripts in N. benthamiana leaves co-inoculated with the OsHAK5 promoter and selected RGSV-encoded proteins were analysed by RT-qPCR. The data showed that GUS transcripts in the N. benthamiana leaves co-inoculated with the OsHAK5 promoter and RGSV P5 were slightly higher than in the controls, whereas in N. benthamiana leaves co-inoculated with the OsHAK5 promoter and RGSV P1, the GUS transcripts were significantly suppressed (Figure 7c). To confirm that RGSV P3 can induce OsHAK5 expression, we compared the expression levels of OsHAK5 in wild-type
and RGSV P3 overexpression rice plants by RT-qPCR. We found that the transcripts of OsHAK5 in P3 transgenic overexpression plants were approximately 6-fold greater than those of wild-type plants, suggesting that the up-regulated expression of OsHAK5 is probably induced by RGSV P3 in vivo (Figure 7d). These data suggested that RGSV P3 may directly or indirectly interact with the
promoter region of OsHAK5, thus leading to the up-regulation of OsHAK5 expression after RGSV invades rice plants.

3 | DISCUSSION

3.1 | Potassium is an emerging factor that is involved in plant–microbe interactions

In recent years, it has been reported that potassium nutrition plays a critical role in disease resistance to plant pathogens, including fungi, bacteria, and viruses (Amtmann et al., 2008). For example, during Sarocladium oryzae infection, potassium deficiency aggravates yield loss in rice through restricting the translocation of non-structural carbohydrates (Zhang et al., 2019). In some cases, plant pathogens like M. oryzae counter host immune defences through modulating a host potassium channel (Shi et al., 2018). Viral diseases are also affected by potassium. Many studies have determined positive roles of potassium in host resistance to viruses. For example, in soybean (Glycine max), overexpression of the GmAKT2 potassium channel gene enhances resistance to soybean mosaic virus (Zhou et al., 2014). All the known information indicates potassium is a crucial factor that is involved in plant–microbe interactions. Additionally, physiological changes of the host plant under conditions of infection by different pathogens always involve potassium homeostasis. For example, K\textsuperscript{+} deficiency induces malonaldehyde (MDA) accumulation, thus resulting in membrane lipid peroxidation (Hu et al., 2015). We found that, under RGSV infection and low K\textsuperscript{+} conditions, MDA accumulation was significantly increased (Figure S8a and Table S2). K\textsuperscript{+} deficiency also induces accumulation of low-molecular-weight compounds in host plants, such as soluble sugars and amino acids (Hu et al., 2017). The increase in total soluble sugar was also monitored in rice leaves under RGSV infection and low K\textsuperscript{+} treatment (Figure S8b and Table S3). Either RGSV infection or K\textsuperscript{+} deficiency reduced the accumulation of total soluble protein (Figure S8c and Table S4), suggesting that soluble amino acids may be induced under RGSV infection and K deficiency. These findings also indicated that RGSV infection influences the potassium content in diseased rice plants, followed by MDA accumulation and generation of low-molecular-weight compounds. These primary metabolites of carbon are readily accessible to pathogens (Lecompte et al., 2017) and are probably easily hijacked by pathogens. How does K\textsuperscript{+} concentration in the diseased plants reduce during RGSV infection? One possible answer is that the activities of virus infection such as replication, and cell-to-cell and long-distance movement might occupy or disturb the K\textsuperscript{+} transport process. This possibility is supported by the phenomenon that most viral pathogen invasion results in nutritional imbalance. For example, the C4 protein from tomato yellow leaf curl virus interacts with the intracellular domain of BARELY ANY MERISTEM 1 (BAM1) and BAM2 at the plasma membrane and plasmodesmata, the cytoplasmic connections between plant cells, interfering with the function of these RLKs in cell-to-cell movement to promote efficient spread and accumulation of the virus during the initial stages of infection (Rosas-Díaz et al., 2018; Tran & Citovsky, 2021). Another possibility is that the K\textsuperscript{+} transport process may be affected by direct interactions between viral proteins and K\textsuperscript{+} transport proteins. For example, effector protein AvrPiz-t interacts with the rice K\textsuperscript{+} channel protein OsAKT1 to specifically suppress OsAKT1-mediated K\textsuperscript{+} transport (Shi et al., 2018). Direct evidence is needed to answer this crucial question in the future.

3.2 | OsHAK5 can improve K\textsuperscript{+} absorbance and ROS production

In our study, RGSV infection and K\textsuperscript{+} deficiency accelerated leaf chlorosis. This symptom is probably due to leaf senescence. Wang et al. (2012) suggested that superoxide radicals (O\textsuperscript{2-}) and hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) (Xu et al., 2011) increase under K\textsuperscript{+} deficiency, which is probably responsible for the acceleration of leaf senescence. Chlorophyll degradation may be another reason for leaf yellowing (Fang et al., 1998), possibly due to oxidative stress developing in the chloroplast because chloroplasts are one of the main generators of ROS, which are highly reactive and can cause severe damage to cell structure and cellular components (Djanaguiraman et al., 2009). Therefore, leaf senescence could be correlated closely with oxidative stress (Kukavica & Jovanovic, 2010) and involve ROS (Saed et al., 2003). Previous studies have demonstrated the physiological functions of OsHAK5 in K\textsuperscript{+} acquisition and transport from roots to shoots under low K\textsuperscript{+} supply conditions (Yang et al., 2014). Shin and Schachtman (2004) showed that ROS increased after K\textsuperscript{+} deprivation in a discrete region of the root and H\textsubscript{2}O\textsubscript{2} was required, but was not sufficient, for the

FIGURE 4 The phenotype analysis of OsHAK5 overexpression transgenic rice plants. (a) Phenotypes of OsHAK5 overexpression transgenic rice plants inoculated with or without RGSV. (b) Detection of RGSV coat protein (CP) expression in RGSV-inoculated healthy rice and OsHAK5 overexpression lines by western blotting assay. (c) Detection of RGSV CP transcripts in RGSV-inoculated wild-type (WT), empty vector (EV) transgenic, and OsHAK5 overexpression lines by reverse transcription-quantitative PCR (RT-qPCR). (d) Relative transcripts in RGSV- inoculated wild-type rice and OsHAK5 overexpression lines by western blotting assay. (e) Detection of RGSV multiplication and generation of low-molecular-weight compounds in host plants, such as soluble sugars and amino acids (Hu et al., 2017). The increase in total soluble sugar was also monitored in rice leaves under RGSV infection and low K\textsuperscript{+} treatment (Figure S8b and Table S3). Either RGSV infection or K\textsuperscript{+} deficiency reduced the accumulation of total soluble protein (Figure S8c and Table S4), suggesting that soluble amino acids may be induced under RGSV infection and K deficiency. These findings also indicated that RGSV infection influences the potassium content in diseased rice plants, followed by MDA accumulation and generation of low-molecular-weight compounds. These primary metabolites of carbon are readily accessible to pathogens (Lecompte et al., 2017) and are probably easily hijacked by pathogens. How does K\textsuperscript{+} concentration in the diseased plants reduce during RGSV infection? One possible answer is that the activities of virus infection such as replication, and cell-to-cell and long-distance movement might occupy or disturb the K\textsuperscript{+} transport process. This possibility is supported by the phenomenon that most viral pathogen invasion results in nutritional imbalance. For example, the C4 protein from tomato yellow leaf curl virus interacts with the intracellular domain of BARELY ANY MERISTEM 1 (BAM1) and BAM2 at the plasma membrane and plasmodesmata, the cytoplasmic connections between plant cells, interfering with the function of these RLKs in cell-to-cell movement to promote efficient spread and accumulation of the virus during the initial stages of infection (Rosas-Díaz et al., 2018; Tran & Citovsky, 2021). Another possibility is that the K\textsuperscript{+} transport process may be affected by direct interactions between viral proteins and K\textsuperscript{+} transport proteins. For example, effector protein AvrPiz-t interacts with the rice K\textsuperscript{+} channel protein OsAKT1 to specifically suppress OsAKT1-mediated K\textsuperscript{+} transport (Shi et al., 2018). Direct evidence is needed to answer this crucial question in the future.

3.2 | OsHAK5 can improve K\textsuperscript{+} absorbance and ROS production

In our study, RGSV infection and K\textsuperscript{+} deficiency accelerated leaf chlorosis. This symptom is probably due to leaf senescence. Wang et al. (2012) suggested that superoxide radicals (O\textsuperscript{2-}) and hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) (Xu et al., 2011) increase under K\textsuperscript{+} deficiency, which is probably responsible for the acceleration of leaf senescence. Chlorophyll degradation may be another reason for leaf yellowing (Fang et al., 1998), possibly due to oxidative stress developing in the chloroplast because chloroplasts are one of the main generators of ROS, which are highly reactive and can cause severe damage to cell structure and cellular components (Djanaguiraman et al., 2009). Therefore, leaf senescence could be correlated closely with oxidative stress (Kukavica & Jovanovic, 2010) and involve ROS (Saed et al., 2003). Previous studies have demonstrated the physiological functions of OsHAK5 in K\textsuperscript{+} acquisition and transport from roots to shoots under low K\textsuperscript{+} supply conditions (Yang et al., 2014). Shin and Schachtman (2004) showed that ROS increased after K\textsuperscript{+} deprivation in a discrete region of the root and H\textsubscript{2}O\textsubscript{2} was required, but was not sufficient, for the
FIGURE 5 3,3'-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) staining and detection of peroxidase (POD), catalase (CAT), and H$_2$O$_2$ content in oshak5 mutant lines, OsHAK5 overexpression transgenic lines and the corresponding wild-type rice plants. (a, b) DAB and NBT staining in oshak5 mutant lines, OsHAK5 overexpression transgenic lines, and the corresponding wild-type rice plants (Dongjin and Nipponbare [Nip], respectively). (c, d) Detection of H$_2$O$_2$ in oshak5 mutant lines, OsHAK5 overexpression transgenic lines, and the corresponding wild-type rice plants. (e, f) Detection of POD in oshak5 mutant lines, OsHAK5 overexpression transgenic lines, and the corresponding wild-type rice plants. (g, h) Detection of CAT in oshak5 mutant lines, OsHAK5 overexpression transgenic lines, and the corresponding wild-type rice plants. Mock, healthy Nipponbare rice sample; RGSV, RGSV-infected Nipponbare rice sample. Values followed by a different letter within the same column are significantly different at $\alpha = 0.05$; error bars, standard deviations; scale bars, 1 cm.
induction of a peroxidase, HAK5, and an unknown gene at 6 and 30 h after deprivation. In Arabidopsis, the production of ROS is an important signal for transcriptional up-regulation of AtHAK5. From our studies, the positive role of OsHAK5 in K⁺ absorbance and ROS production in rice plants has also been shown.

3.3 | RGSV P3 may be required for the transcription activation of OsHAK5

It seems that the virulence protein P3 encoded by RGSV improved the transcriptional expression of OsHAK5 during RGSV infection. We consider that RGSV infection initially inhibited the K⁺ absorbance ability, and thus resulted in low K⁺ content in RGSV-diseased rice plants. To improve the K⁺ supply for the survival of diseased rice plants, the K⁺ transport channels were activated as a feedback response to low K⁺ conditions. Our data indicated that RGSV P3 was able to induce OsHAK5 expression through enhancing the activity of the OsHAK5 promoter. Whether there is direct binding between RGSV P3 and the OsHAK5 promoter needs to be investigated in further studies. The promoter-binding activity of effectors exploited by distinct pathogens has already been shown in many cases, for example, the transcription factor ARF2 (Auxin Response Factor 2) directly binds to the AtHAK5 promoter and represses AtHAK5 expression under K⁺-sufficient conditions in Arabidopsis thaliana (Zhao et al., 2016).

In this study, we determined the essential roles of K⁺ supply in the responses to RGSV infection in rice (Figure 8). First, RGSV infection reduced the K⁺ content in diseased rice plants, and to remedy the K⁺ supply for rice growth demand, OsHAK5 expression was highly induced. Two aspects were affected when OsHAK5 was over-expressed through the transgenic method. On the one hand, over-expression of OsHAK5 induced certain symptom-like phenotypes, including a slight dwarfism and excessive tillering; on the other hand, OsHAK5 activated a ROS-mediated antiviral defence signal through regulating ROS-associated gene expression. We also examined
if RGSV-encoded P3 may be responsible for the up-regulation of OsHAK5 in RGSV-inoculated rice plants by enhancing the promoter activity of OsHAK5. Our study clearly verified that potassium is involved in host-virus interactions and provided insights into the nutrition environment when viral infection happens, thus helping us to understand the process of virus infection. Our study demonstrated that the K⁺ signal plays an essential role in host resistance, which may serve as a direction for both molecular breeding of resistant rice varieties and disease control.

4 | EXPERIMENTAL PROCEDURES

4.1 | Plant materials, growth conditions, and virus infection

Rice (Oryza sativa ‘Dongjin’) was used as the wild-type control. Seeds of the osak5 mutant line (accession number PFG-3A09138) with a Dongjin background were obtained from the SIGNAL database (http://signal.salk.edu/cgi-bin/RiceGE) (Miyao et al., 2003). Homozygous mutant plants were identified by PCR procedures and used for further analyses. All plant materials used in this study were grown in a phytotron or in an experimental field at the Fujian Agricultural and Forestry University (Fuzhou, China).

Rice seeds were sterilized in 2.5% (vol/vol) sodium hypochlorite for 20 min, rinsed thoroughly with deionized water and held under wet gauze for 2 days at 30°C in darkness. Germinated seeds were then sown into 96-well plates for hydroponic cultivation and maintained in the phytotron under the following photoperiod/temperature combination: 16 h light at 28°C/8 h dark at 28°C each day. Our hydroponic procedure used 1/4 Kimura nutrient solution with the pH adjusted to 5.0–5.5 with KOH (final K⁺ concentration was 0.8–1 mM). The hydroponic medium was changed every 3 days.


**4.2 | K⁺ content determination and K⁺ starvation treatment**

Rice seedlings were grown in hydroponic medium solution with or without viral challenge. The shoots and roots were excised from intact seedlings, rinsed twice with tap water followed by deionized water, and dried in an oven at 110°C for 2 days, then 80°C for 3–5 h until constant weight. Dry samples were weighed and extracted with sulphuric acid and hydrogen peroxide at 200–300°C. K⁺ content in the extractions was measured using an atomic absorption flame spectrometer.

In the K⁺ starvation treatment, final K⁺ concentration was changed to 5 μM. Under K⁺ starvation treatment, 3-day-old rice seedlings were transferred into low K⁺ hydroponic medium (containing 0.3 mM, 0.1 mM, and 5 μM K⁺) with the pH adjusted to 5.0–5.5 with NaOH for 20 days.

**4.3 | RNA extraction and transcript analysis**

Total RNA was extracted from treated seedlings with RNAiso plus reagent (TaKaRa) following the manufacturer’s instructions. First-strand cDNA was synthesized using a PrimeScript RT reagent kit with gDNA eraser (TaKaRa). The primer sequences for RT-PCR analysis are listed in Table S1. Relative quantitative results were normalized to OsActin and are presented here as mean normalized transcript levels obtained by the comparative cycle threshold method (2^ΔΔCt). Quantitative PCR assays were performed in 20-μl reaction volumes using the SYBR Premix ExTaq kit (TaKaRa) on a 7500 fast real-time PCR system (Applied Biosystems).

**4.4 | Protein extraction and western blotting**

Thirty days after virus inoculation, leaves of RGSV-inoculated rice plants were collected for western blotting assays. The leaves were crushed in a lysis buffer (50 mM Tris pH 7.5, 300 mM NaCl, 1 mM PMSF, 1% protease inhibitor cocktail) and then boiled at 99°C for 10 min. The plant extract was then centrifuged for 10 min at 12,000 × g. The supernatant was fractionated by 12% SDS-PAGE under reducing conditions and transferred to a polyvinylidene fluoride membrane using a transfer apparatus according to the manufacturer’s protocols (Bio-Rad). After incubation with 5% nonfat milk in Tris-buffered saline with Tween 20 (TBST; 137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 2 mM KH₂PO₄, 0.3% Tween 20) for 30 min, the membrane was incubated with an antibody against RGSV CP (1:2000 dilution) for 2 h at room temperature or overnight at 4°C. The membrane was rinsed three times (10 min each) with TBST by using a rocker. The rinsed membrane was incubated with goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (http://www.miltenyibiotech.com). For a loading control, the membrane was also incubated with an antibody specific to the plant actin (1:10,000 dilution) for 2 h at room temperature and then with a horseradish peroxidase-conjugated goat anti-mouse antibody (Bio-Rad). Blots were washed with TBST three times for 10 min each and developed with an enhanced chemiluminescence system according to the manufacturer (Bio-Rad).

**4.5 | Detection of POD activity, CAT activity, and H₂O₂ content**

The POD and CAT enzymes were extracted as described previously (Djanaguiraman et al., 2009). Fresh leaf tissue (0.1 g) was crushed in 1 ml of precooled 50 mM phosphate buffer at pH 7.8. The homogenate was centrifuged at 12,000 × g for 20 min at 4°C. The clear supernatant was used for measuring enzyme activities. POD activity was estimated as decomposition rate of H₂O₂ by POD, with guaiacol as hydrogen donor, by measuring the rate of change in absorbance at 704 nm (Lei et al., 2006). CAT was assayed according to Djanaguiraman et al. (2009) by potassium permanganate titration and expressed as μM H₂O₂ g⁻¹ fresh weight min⁻¹. The H₂O₂ content was extracted as described previously (Okuda et al., 1991). The reaction mixture contained 1 ml of the extraction, 400 μl of peroxidase (0.25 U), and 80 μl of MBTH in a total volume of 1.5 ml. The reaction was initiated by adding the peroxidase at 25°C. The increase of absorbance at 590 nm was monitored.

**4.6 | DAB and NBT staining**

H₂O₂ and O²⁻ were detected with DAB and NBT staining, respectively. For DAB staining, rice leaves were infiltrated with 1 mg/ml DAB solution (pH 5.7) and then incubated overnight (about 8 h) at room temperature in darkness. Leaves were then destained three times with 95% ethanol and heated in a water bath at 95°C for 15 min. For NBT staining, rice leaves were infiltrated with 0.5 mg/ml NBT solution (pH 7.5) after which the leaves were maintained overnight at room temperature. The staining was drained off three times by immersing the leaves in absolute ethanol and heating in a boiling water bath for 10 min, cooling for 30 min at room temperature, transferring the leaves on a paper and then photographing the stained leaves against a contrast background for documentation.
4.7 | GUS staining

The promoter region of OsHAK5 was inserted into the pMDC163 vector. The coding sequence of RGSV P3 was cloned into the plant transient expression vector pEarleyGate202. The resulting plasmids were introduced into Agrobacterium tumefaciens GV3101 by heat shock. The leaves were used for GUS histochemical staining 2 days after inoculation according to Lagarde et al. (1996).

4.8 | Detection of MDA, total soluble sugar, and total soluble protein content

Measurements of the content of MDA, total soluble sugar, and total soluble protein were performed according to the operation manual of Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

4.9 | Root morphology observation

The 3-day-old rice seedlings were transferred into normal and low K⁺ (5 μM K⁺) hydroponic medium, with or without RGSV infection for 30 days. A root image analysis system was used to analyse root total length, average length, surface area and volume.

4.10 | Statistical analysis

The statistical analyses were performed using IBM SPSS Statistics 19 and data were analysed by Student’s t tests or a one-way analysis of variance with Fisher’s least significant difference test; p ≤ 0.05 was used as a threshold for identifying significant differences. All experiments were repeated at least three times. For phenotypic analysis, each line had at least 30 plants to collect the data. For localization experiments, at least two plants were used for each treatment and three repetitions were performed.

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AUTHOR CONTRIBUTIONS
Z.W., C.Z., L.Y., and G.L. designed the experiments. X.J., X.S., S.C., and P.W. performed the experiments. Z.W., C.Z., L.Y., and X.J. analysed the data. X.J., C.Z., and Z.W. wrote the manuscript.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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