Potassium Sulphate Induces Resistance of Rice against the Root-Knot Nematode *Meloidogyne graminicola*

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Abstract

Background Potassium (K), an important nutrient element, can improve the stress resistance/tolerance of crops. The application of K in resisting plant parasitic nematodes shows that the K treatment can effectively reduce the occurrence of nematode diseases and increase crop yield. However, data on K_2SO_4 induced rice resistance to *Meloidogyne graminicola* are still lacking. To evaluate rice resistance against *M. graminicola* induced by K2SO4 and to further clarify its mechanism is essential for the rational use of K fertilizer to ensure the safety of rice production.

Results In this work, K_2SO_4 treatment effectively reduced the numbers of both galls and nematodes in rice roots, and delayed the development of nematodes to the adult stage. Rather than by affecting the attractiveness of roots to nematodes and the morphological phenotype of giant cells at feeding sites, such effect was achieved by rapidly stimulating hydrogen peroxide (H_2O_2) accumulation, increasing callose deposition. Meanwhile, such induced resistance required the active participation of the potassium channel OsAKT1 and the potassium transporter OsHAK5. The numbers of both galls and nematodes were higher in both gene deficient plants than that in the wild-type plants, and the K_2SO_4-induced resistance showed weaker in the defective plants than in the wild-type plants.

Conclusions K_2SO_4 treatment effectively induces rice resistance to root-knot nematode *M. graminicola*. The mechanism of inducing resistance is to prime the basic defense of rice, up-regulating the expression of resistance-related genes and with the involvement of K^+ channel and transporter. These laid a foundation for further study on the mechanism of rice to defense against root-knot nematodes and the effective use of potassium fertilizer to improve rice resistance against nematodes in the field.

Background

Rice is one of the most widely grown cereal crops in the world and is related to the global food security. The root-knot nematode(RKN) *Meloidogyne graminicola* (MG) is an important pathogenic nematode in rice production, which roughly occurs in Asia, Africa and other regions [1, 2]. Chemical control is the primary means to control this nematode, but causing environment pollution. Instead, the environmentally friendly biological nematocides are sensitive to climatic, seasonal and geographical conditions, thus affecting the efficiency of field control. Zhan et al. (2018) identified only three varieties conferring resistance to RKNs from 136 commercial rice varieties in China [3]. Induced resistance (IR) has been attracted to reduce the damage of RKNs and ensure the safety of rice production [4, 5].

Under the stimulation of elicitors (biotic or abiotic agents), IR of plants was activated to avoid or reduce the harm of pathogens. The resulting resistance is usually broad-spectrum and persistent, so it is widely used to control plant diseases [6]. Spraying boron, zinc, and manganese on wheat leaves can improve the resistance to rust and greatly increase the enzymatic activities (i.e., peroxidase and poly phenoloxidase) [7]. Silicon treatment in rice roots can induce and improve the resistance of rice to *M. graminicola*, stimulate the basic defense response of host, and enhance the expression of defense-related genes in ET

pathway, resulting in slow development and reduced number of RKNs [4]. Soares et al. (2020) confirmed this result by foliar spraying of clay on rice [8]. The treatment of potassium nitrate (KNO3) in roots could induce the activities of resistance-related enzymes and the production of disease-resistant substances in tomato (*Solanum lycopersicum L.*), and significantly reduce the disease index of RKN *M. incognita* [9]. Xiang et al. (2018) demonstrated that potassium fertilizer application in soil could stimulate the secretion of phenic acid (cinnamic, ferulic and salicylic acids) in soybean (*Glycine max* (L.) Merr.) root system, and enhance the expression of disease-resistant genes such as phenylalanine ammoniase (PAL) and polyphenol oxidase (PPO), which effectively controlled soybean cyst nematode *Heterodera glycines* [10]. Application of K_2SO_4 in the field can reduce the damage of brown rot disease to potato [11], and induce the expression of defense-related genes in snap bean leaf tissue [12]. However, the data on the induction of host resistance to nematodes by K_2SO_4 is lacking.

In this work, the effect of K_2SO_4 on the resistance of rice to *M. graminicola* was evaluated by pot experiment in greenhouse, and the histopathological changes of rice treated with K_2SO_4 were observed by microscopy. After the effect was confirmed, its role in priming the basic defense reaction was determined. Then, the expression levels of disease resistance-related genes were quantified to preliminarily explore the molecular mechanism. Finally, the potassium (K^+) channel gene *OsAKT1* and transporter gene *OsHAK5* deficient lines were used to verify whether the OsAKT1 and OsHAK5 proteins are involved in the host resistance against *M. graminicola*.

Results

K_2SO_4 did not impact the mortality and infectivity of *M. graminicola* at low concentrations

The direct toxicity of K_2SO_4 to *M. graminicola* was determined by calculating the mortality of nematodes at different concentrations of K_2SO_4 solution 72 h after treatment. Although the mortality of nematodes increased along with the increase of K_2SO_4 concentrations ranging from 0.125 mM to 8 mM, there was no significant difference compared to the control water treatment (5.0 ± 0.7%), especially at 0.125 mM K_2SO_4 concentration (5.7 ± 1.4%) (Fig. 1a). These results indicated that the K_2SO_4 was not toxic to *M. graminicola* at low concentrations within 72 h.

To determine whether K_2SO_4 directly impact the infection and development of *M. graminicola*, the newly hatched second stage juveniles (J2s) were soaked in 0.5 mM K_2SO_4 solution for 48 h before inoculation. At 14 days post inoculation (14 dpi), no difference was observed in the total numbers of juveniles and adult females between the K_2SO_4-soaked and the water-soaked groups, which indicated that there was no direct effect of K_2SO_4 on the infection of *M. graminicola*. At the same time, most nematodes developed into adult females, and there was no significant difference in the numbers of third stage juvenile (J3), fourth stage juvenile (J4) or adult females between the two groups (Fig. 1b, c). These results indicated that K_2SO_4 did not suppress the development of *M. graminicola*.

K_2SO_4 treatment induced resistance of rice to *M. graminicola*

To evaluate the resistance of rice against *M. graminicola* induced by K_2SO_4,different concentrations of K_2SO_4 solution treated the rice roots 1 d before inoculation, and preliminary plant infection rate was assayed at 14 dpi. The results showed that compared to the water treatment, K_2SO_4 treatment could effectively reduce the numbers of both galls and nematodes (juveniles and adult females) at the concentration of 0.125 mM, and there was no significant difference among the K_2SO_4 treatments. After applying with K_2SO_4 solution at a concentration of 0.5 mM, the numbers of both root galls and nematodes were decreased significantly (57.2 ± 4.4%, 59.2 ± 6.6%) at 14 dpi (Fig. 2a, b). In addition, compared to the untreated control plants, the development of nematodes treated with K_2SO_4 was evidently delayed. At 14 dpi, the proportion of adult female in plants treated with K_2SO_4 (70.9 ± 5.6%) was significantly lower than that of plants in water control (90.7 ± 5.1%), and the proportion of juveniles (J3 + J4) (27.0 ± 6.3%) in plants treated with K_2SO_4 was higher than that of control plants (6.0 ± 3.2%) (Fig. 2c, e). These results indicated that the low concentrations of K_2SO_4 could effectively reduce the infection of nematodes and delay the development of nematodes. Therefore, the 0.5 mM K_2SO_4 solution was used in all the subsequent experiments. We also calculated the male/female ratio of nematodes, indicating that the ratio in rice roots treated with K_2SO_4 was lower than that in the untreated roots (Fig. 2d). Meanwhile, there was no significant difference in the fresh weight and height of rice shoots or roots between K_2SO_4 treatment and water treatment (Fig. 2f, g), suggesting that application of K_2SO_4 had no toxic effect on rice growth. All these results indicate that the application of K_2SO_4 can induce rice resistance against *M. graminicola*.

K_2SO_4-induced resistance of rice to *M. graminicola* was achieved by neither impacting the attractiveness of rice roots to nematodes nor inhibiting the developmental phenotype of giant cells

In nature, attraction of the host roots to nematodes is of great importance in establishing infection [13]. Here, the nematodes in the range of 5 mm around the root tips treated with K_2SO_4 solution were counted. At 10 hours post inoculation (hpi), the numbers of nematodes attracted to the K_2SO_4 treated tips (26.2 ± 3.1) were not different from the water treated tips (25.7 ± 2.8) (Fig. 3a, b). The results indicated that K_2SO_4 treatment did not affect the attraction to *M. graminicola.*

The invaded J2 can induce the plant to form giant cell at the feeding site by secreting effectors from esophageal gland through stylet [14], and to acquire the nutritional needs of subsequent growth and development. Then the giant cells develop to form the root galls. In this experiment, the root galls of 7 dpi were collected and used for histological observation. The average cross-sectional area of giant cells in K_2SO_4 treated group was not significantly different from the untreated group (Fig. 3c, e). We also counted the numbers of giant cells in feeding sites; there was no significant difference between the two treatments (Fig. 3d). All these results indicated that the application of K_2SO_4 did not influence the development of giant cells.

K_2SO_4 induced resistance to *M. graminicola* in Rice by priming basal defense

Callose deposition is one of the markers of plant defense response, which can play a role in resistance to pathogen infection [15]. We observed the presence of callose in the gall tissue induced by RKNs after staining with aniline blue. The results showed that compared with the water treatment group, more callose was deposited in the K_2SO_4 treatment group, and the callose spots were larger and more dense (Fig. 4a, b), with 67.9% of increase in the average area of callose spots (Fig. 4a). These results suggest that increased callose deposition is one of the pathways of K_2SO_4-induced resistance in rice plants.

Hydrogen peroxide (H_2O_2) is a signaling molecule that mediates plant defense response to biological or abiotic adversity [16]. Our analyses indicated that the H_2O_2 response of rice roots could also be induced by K_2SO_4 alone, because the H_2O_2 level at 24 hpi was significantly higher than that of the control group. In the case of nematode challenge, compared with the rice without K_2SO_4 treatment, the level of H_2O_2 in treated rice was increased by 78.2% and 118.7% at 8 and 24 hpi, respectively, but it decreased at 72 hpi (Fig. 4c). In addition, the expression of *OsRbohB*, an H_2O_2 synthetic gene known to be involved in plant immune response [17], was increased in the K_2SO_4-treated rice at 8 and 24 hpi. Especially, at 8 hpi, the expression level of *OsRbohB* in the inoculated plant treated with K_2SO_4 was significantly higher than that in the non-treated ($P \leq 0.05$) (Fig. 4d), which was consistent with the results of H_2O_2 content (Fig. 4c). These results suggest that the application of K_2SO_4 can induce defense against *M. graminicola* by activating the rapid production of reactive oxygen species (ROS).

K_2SO_4 treatment promoted the expression of disease resistance-related genes in inducing rice resistance against *M. graminicola*

While the plant is attacked by the pathogens, it will activate the autoimmune response system to defend. Immune response is a complex process involving the transcription and expression of many disease resistance-related genes [18]. We analyzed the expression of several common disease resistance-related genes in different treatments and at different time points.

Firstly, we analyzed two salicylic acid (SA) pathway-related genes: SA transcription factor *OsWRKY45* and biosynthesis gene *OsICS1*. The expression of both *OsWRKY45* and *OsICS1* was significantly increased by K_2SO_4 treatment alone at 72 hpi, however, after inoculation, K_2SO_4 treatment only induced *OsWRKY45* at 24 hpi. *OsICS1* was not prominent at any time points (Fig. 5a). Therefore, SA pathway is not the main pathway in K_2SO_4-induced rice resistance against *M. graminicola*. Secondly, we analyzed two jasmonic acid (JA) pathway-related genes and two ethylene (ET) pathway-related genes. After treatment with K_2SO_4, JA transcription factor gene *OsJAMYB* was significantly increased in both inoculated and non-inoculated groups at 8 hpi (Fig. 5b). The expression pattern of JA biosynthesis gene *OsAOS2* was similar to that of *OsJAMYB*, but it was induced more than *OsJAMYB*, at 8 hpi (Fig. 5b). Although the ET signaling gene *OsEIN2* was not expressed much, it was expressed significantly higher in K_2SO_4-treated inoculated rice than in untreated. The expression of *OsEIN2* was slowly increased at all the

three time-points, while the expression of ET biosynthesis gene *OsACS1* was increased sharply in the K_2SO_4-treated and MG-inoculated group at 72 hpi, which was 6.98 times higher than that of untreated but MG-inoculated group (Fig. 5c). These results suggest that K_2SO_4 plays an active role in inducing the expressions of JA and ET pathways-related genes in rice resistance to RKNs. Thirdly, we analyzed the expression of pathogenesis-related (PR) genes. The expression of both *OsPR1a* and *OsPR1b* in the K_2SO_4-treated rice was significantly increased compared to the untreated group at 8 hpi. The expression of *OsPR1b* continued to be increased in the inoculated rice treated with K_2SO_4 after 8 hpi, and was still significantly higher than that in the untreated at 24 hpi (Fig. 5d). These results suggest that K_2SO_4 positively regulates the expression of these two genes in rice resistance to RKNs.

Finally, we analyzed the expression of two brassinolide (BR) pathway-related genes. The expression of BR receptor gene *OsBRI1* was significantly increased in both inoculated and uninoculated K_2SO_4-treated groups at 24 hpi and 72 hpi, while the expression level of BR biosynthesis gene *OsDwarf* in the inoculated group treated with K_2SO_4 was significantly higher than the untreated at 24 hpi (Fig. 5e). This indicates that BR pathway also plays an active role in the resistance process, and K_2SO_4 positively regulates the expression of BR pathway-related genes.

The resistance of rice against nematodes induced by K_2SO_4 required the participation of potassium absorption and transportation systems

To determine the role of potassium (K^+) absorption and transportation systems in disease resistance process, we first compared the expression of K^+ channel gene *OsAKT1* and K^+ transporter gene *OsHAK5* between K_2SO_4 application and non-application, and between nematode inoculation and non-inoculation. The expression of *OsAKT1* in the inoculated rice treated with K_2SO_4 was higher than that in both the untreated and in the non-inoculated within 72 h, especially at 8 h and 72 h (Fig. 6a). This suggests that *OsAKT1* expression is affected by K_2SO_4 and nematodes, which may be involved in the resistance process of rice against nematodes induced by K_2SO_4. Meanwhile, the expression of *OsHAK5* was up-regulated in all the treatments within 24 h after inoculation. Although there was no significant difference among the treatments, the expression of *OsHAK5* was slightly higher in the inoculated rice than in non-inoculated, and the expression of *OsHAK5* was slightly higher in the inoculated rice treated with K_2SO_4 than in the non-treated (Fig. 6a). This indicates that nematode infection and K_2SO_4 treatment have slight effects on *OsHAK5*.

Subsequently, we inoculated nematodes on the rice lines deficient in these two genes. Interestingly, in the presence of K_2SO4 at a concentration of 0.5 mM, both of the two defective plants (*OsAKT1*-RNAi *and OsHAK5*-Cr) became more susceptible than the wild-type plants. Specifically, compared with the two defective plants, the numbers of both galls and nematodes (juveniles and adult females) in the wild-type plants roots were reduced, and the proportion of juveniles (J3 + J4) were increased, while the proportion of adult females was reduced. (Fig. 6b, c). These results indicate that these two genes are associated with the inhibition of the infection and development of nematodes in rice. On the hand, in the case of K^+

starvation, the susceptibility of the two defect types was still higher than that of wild type (Fig. 6b, c). These results suggest that these two genes also play an independent role in nematode resistance. However, without K_2SO_4 treatment, the relative susceptibility of *OsAKT1* and *OsHAK5* deficient lines was weaker than that with the K_2SO_4 treatment (Fig. 6b, c). These results suggest that although both *OsAKT1* and *OsHAK5* have independent roles in the process of disease resistance, both were involved in K_2SO_4-induced resistance of rice against *M. graminicola*.

Discussion

Potassium can enhance plant resistance to biological stresses [19]. The present study showed that K_2SO_4 could effectively induce the resistance of rice, reduce the numbers of both galls and nematodes and delay the development of *M. graminicola*. Similar to the effects of silicon and biochar [4, 5], K_2SO_4 treatment had no direct effect on the survival, infection and development of *M. graminicola*. Similar to the results observed in *M. incognita* and *H. glycines* [9, 10], application of high concentration of K^+ was not helpful to the control of *M. graminicola*. The application of K_2SO_4 at low concentrations at 24 h before inoculation can effectively reduce the susceptibility of rice to *M. graminicola*, but has no negative impact on the growth of rice. The reproduction mode of *M. graminicola* is combining cross-fertilization and facultative meiotic parthenogenesis [20]. Sexual reproduction is conducive to the release of higher genetic diversity [21]. The male/female ratio of nematodes in rice roots treated with K_2SO_4 was lower than that in the untreated control, indicating that K_2SO_4 treatment inhibited the sex differentiation of RKNs. The depletion of homoglutathione content increased the proportion of males [22], and the similar result can be obtained without K_2SO_4 treatment, which is consistent with the view that sex determination of parthenogenetic RKN species is strongly influenced by environmental factors [23].

Plant parasitic nematodes can be attracted by hosts and promote their infections [13]. Similar to the results observed in β-Aminobutyric acid (BABA) and thiamine treated rice plants, K_2SO_4 has no significant effect on attraction of nematodes [24, 25]. However, treatment with metabolites of *Aspergillus welwitschiae* can significantly affect the attractiveness of rice roots to nematodes [26, 27]. When the nematode moved and invaded the host plant, the infective J2s migrated to the vascular column cells, and induced the root cells to differentiate into specialized giant cells (GCs) for feeding sites [14]. Previous researches observed that the morphology or size of giant cells may be changed by the treatments of BABA or αβ-Dehydrocurvularin [24, 27]. However, K_2SO_4 treatment had no significant effect on the phenotype of giant cells, which is similar to the infection of *M. javanica* in potassium-treated tomato roots [28].

In most cases, host plant resistance is characterized by local necrosis of plant cells at the site of infection hypersensitive reaction (HR), which is typically characterized by the outbreak of ROS [29]. In the induced resistance to root-knot nematode *M. javanica*, the production of H_2O_2 may be helpful for tomato and cucumber plants to defense against RKNs infection [30, 31]. Ascorbate oxidation activated systemic defense against *M. graminicola*, which was also related to the accumulation of H_2O_2 [32]. Overexpression

of respiratory burst oxidase homolog B (*RbohB*), a gene related to ROS generation, will enhance the resistance of *Arabidopsis* to nematodes. [33]. On the contrary, the interference or silencing of *RBOH1* gene, which encoding NADPH oxidase for the production of apoplastic H_2O_2, increased the susceptibility of tomato to *M. incognita* [34]. In the process of rice resistance to nematodes, K_2SO_4 treatment significantly increased the expression of *OsRbohB* after inoculation, and stimulated the accumulation of H_2O_2 within 24 h, indicating that K_2SO_4 primed the basic defense of rice against *M. graminicola* by stimulating hydrogen peroxide accumulation.

Callose deposition plays a vital role in the defense of plants against various biological stresses [15]. Ellinger et al. [35] observed that spraying pathogen elicitor flg22 can induce callose deposition in plant leaves, and increase the resistance of *Arabidopsis* to powdery mildew in the early stage. Bian et al. [36] found that validamycin A treatment significantly enhanced the deposition of callose in *Arabidopsis* leaves to defense against different fungi. Similarly, K_2SO_4 treatment can also enhance the deposition of callose in rice root galls, so it is possible that this way is also used to prevent nematode infection. Validamycin A induced broad-spectrum resistance was involved in SA and JA/ET signaling pathways. However, further research is needed to elucidate the pathway involved in K_2SO_4-induced resistance.

The complex defense signaling pathways involved in plant response to biological stress are regulated by different hormones [37]. The deletion of genes related to defense signaling pathways (e.g., JA, ET and BR) will result in the reduction of plant defense against pathogens (e.g., *M. graminicola*, *Botrytis cinerea* and *M. incognita*) [34, 36, 37]. Previous studies have shown that after the JA pathway is antagonized by gibberellin and monocrotaline, rice is more susceptible to RKNs [38, 39]. The resistance of rice to *M. graminicola* induced by BABA or ascorbate oxidation depends on JA and ET pathways [24, 32]. The enhancement of rice resistance to *M. graminicola* induced by biochar and silicon is related to the increased transcription levels of ET pathway-related genes [4, 5]. Our results showed that the expressions of JA pathway-related genes were up-regulation after the K_2SO_4 treatment in the early stage; meanwhile the expressions of ET pathway-related genes were very low and were not significantly expressed until the JA biosynthesis gene *OsAOS2* was highly expressed. This was consistent with the view that the ET pathway requires JA biosynthesis [37]. As an important part of the plant defense system, PR protein can cause HR when it is accumulated in the infected site [40]. *OsPR1a* and *OsPR1b* can be induced by exogenous plant hormones JA, SA and ET, respectively, play an important role in rice disease resistance [37, 41]. Our research shows the expression of these two genes were significantly up-regulated in inoculated or non-inoculated rice after treated with K_2SO_4 in a short period of time, while H_2O_2 was also accumulated, both of which were involved in HR [29, 40]. Brassinosteroids (BRs) play an active role in plant innate immunity and enhance the level of H_2O_2 in the apoplast [42, 43]. Nahar et al. [44] observed that high concentration of exogenous epibrassinolide significantly reduced the susceptibility of rice to *M. graminicola*. In our study, at 24 hpi when H_2O_2 accumulated significantly, K_2SO_4 treatment significantly increased the expression of these two BR pathway-related genes in rice. These results were consistent with the view that BRs enhance H_2O_2 accumulation [43]. K_2SO_4 positively regulates the expression of resistance-related genes during rice defense against *M. graminicola* infection, and its process may be

related to the accumulation of H_2O_2 and HR. However, further research is needed to elucidate the role of different pathways in K_2SO_4-induced hormone metabolisms.

Potassium plays key role in maintaining the activity of cytoplasmic enzymes [45]. If potassium deficiency occurs, the normal physiological and biochemical reactions of plants will be correspondingly inhibited [46]. However, most K^+ in soil is dehydrated and cannot be used by plants [47]. Venkatesan et al. [48] observed that the content of K^+ in rice was decreased after *M. graminicolais* infection. In order to improve the utilization efficiency of K^+, plants ensure the absorption and transport of K^+ *in vitro* and *in vivo* through K^+ transporters and channels [49]. Shi et al. [50] observed that the effector protein AvrPiz-t from *Magnaporthe oryzae*, a pathogen of rice blast, targets the K^+ channel OsAKT1 to destroy plant immunity, and the deletion of *OsAKT1* resulted in the decrease of K^+ content and the resistance to *Magnaporthe oryzae*. The high affinity K^+ transporter HAK5 could interact with Raf-like Kinase ILK1 which is helpful to plant defense against bacterial pathogens, and is necessary for innate immunity [51]. Our data showed that, compared with the wild-type plants, the susceptibility of the OsAKT1 and OsHAK5 defective plants was significantly increased without K_2SO_4 treatment, indicating that these two genes play an independent and positive role in the resistance of rice to *M. graminicola.* However, the control effects of K_2SO_4 treatment on the two defective lines were lower than that on the wild-type, indicating that the resistance induced by K_2SO_4 requires the participation of these two genes. The decrease of resistance to nematodes led by the deletion of OsAKT1 may be similar to how *M. oryzae* destroys rice systemic immunity [50, 52]. The positive response to K_2SO_4 treatment and nematode infection also showed that *OsAKT1* had great initiative in regulating the loss of K^+ and maintaining the dynamic balance of K^+, so as to reduce the internal competition of pathogens for nutritional resources, protect the activity of plant enzymes, and improve the synthesis of defense compounds [19, 53]. OsHAK5 is active in mobilization of potassium in rice, not only mediates the absorption of K^+ in the external environment, but also mediates the transport of K^+ from root to shoot or from source to sink in plants, and also plays an important role in the control of potassium/sodium concentration ratio [54], but its expression is inhibited when the external K^+ is sufficient [55]. Therefore, in our experiment, the expression level of *OsHAK5* in K_2SO_4 alone treatment was slightly lower than other inoculated treatments within 24 h. These results indicate that the contribution of *OsAKT1* and *OsHAK5* in the host's resistance to RKNs infection is positive and K^+ plays a major role in this process.

Conclusion

For the outstanding performance of potassium in plant resistance to biological stress, we further expanded the data of potassium in helping understand rice defense against RKNs in this study. Treatment of K_2SO_4 at low concentrations could effectively improve the defense of rice against *M. graminicola*. K_2SO_4-induced resistance was achieved by priming the basic defense of rice, inducing the expression of resistance-related genes against plant diseases, with the involvement of K^+ channel and transporter. These results laid a foundation for further study on the mechanism of rice to defense against

root-knot nematodes. However, the mechanisms of resistance-related genes, the role of K^+ channel and transporter will be further explored to better understand IR in rice. Thereafter, K_2SO_4-induced resistance will provide a theoretical guidance for the integrative control of nematodes in rice.

Materials And Methods

Plant materials and K_2SO_4

Wild-type (WT) Rice seeds (*Oryza sativa* cv. Nipponbare) were friendly offered by the United States Department of Agriculture (GSOR-100) and cultivated at Hunan Agricultural University in Changsha, Hunan Province, China. Transgenic *OsAKT1*-RNAi lines deficient in K^+ channel OsAKT1 were collected from the State Key Laboratory for Biology of Plant Diseases and Insect Pests as gifts by Wang et al. [56] and identified by qRT-PCR (Supplementary Fig. 1). Transgenic *OsHAK5*-Cr lines deficient in K^+ transporter OsHAK5 were generated from NPB by the CRISPR/Cas9 [57] and identified by PCR (Supplementary Fig. 2). In the experiment, rice seeds were soaked in 4–5% sodium hypochlorite (NaOCl) for 5–8 minutes to disinfect, then washed with distilled water for three times, and then germinated at 28 ℃ in the incubator. Budding seeds are seeded in each polyvinyl chloride (PVC) tube containing Super Absorbent Polymer (SAP) substrates (1:400 (w: v) mixture of sand and SAP) [58]. Rice seedlings were maintained in a greenhouse at 28°C with 75% relative humidity and 16 h / 8 h light / dark photoperiod, and irrigated with 10 ml of K_2SO_4-free nutrient solution (NH_4NO_3 914 g, $NaH_2PO_4{\cdot}2H_2O$ 403 g, $CaCl_2$ 886 g, $MnCl_2{\cdot}4H_2O$ 15.0 g, $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ 0.74 g, H_3BO_3 9.34 g, $FeCl_3{\cdot}6H_2O$ 77.0 g, and $C_6H_8O_7{\cdot}H_2O$ 119 g in 10 liter water) every three days according to the manual of Cock et al. [59] and Hogland solution. K_2SO_4 (CAS 7778-80-5, purity not less than 99%, pH 5–8 at 25 ℃) was provided by Nanjing Chemical Reagent Co., Ltd, China. At first, K_2SO_4 stock solution (400mM) was prepared, and then prepared into dilution of different concentrations (0.125, 0.5, 2, and 8 mM).

Collection, culture and hatching of *M. graminicola*

RKNs collected from Pingjiang County, Yueyang City, Hunan Province, were identified with morphological and molecular methods as Golden and Birchfield [60] and Htay et al. [61], respectively, and subcultured on Nipponbare in a glass greenhouse at about 28 ℃. After the nematode was fully developed and matured, the eggs were isolated from the root galls and incubated at 28 ℃ for 72 h. The hatched J2 was filtered with a 25-μm sieve and diluted with distilled water into a suspension of about 100 nematodes per ml for inoculation [62].

Direct effects of K_2SO_4 on *M. graminicola*

Place J2 s (about 100 pieces) in a 6-well culture plate (φ 3.5 cm) containing 1 ml of diluted K_2SO_4 solution or distilled water. After culturing for 72 h, a few drops of 1 M NaOH were added in the solution. Nematodes that change their shape are considered to be alive, while stiff nematodes that do not respond are classified as dead [63]. The experiment was carried out three times with four replicates each time.

In order to determine the direct effect of K_2SO_4 on the infection and development of *M. graminicola*, recently hatched J2s were soaked in K_2SO_4 solution or distilled water for 48 h before inoculation. Then each two-weeks-old rice plant planted in PVC tubes containing SAP substrates was inoculated with approximately 100 J2s and maintained in a greenhouse at 28°C with 75% relative humidity. At 14 dpi, the root samples were carefully cleaned, wrapped with microcloth, bleached with 5% NaOCl for 5–10 minutes, then rinsed with distilled water at least three times to remove residual NaOCl, and then dyed in acid fuchsin staining [37]. After the dye on the root surface was washed with distilled water, the roots samples were placed in glycerin to observe the number of nematodes in the root at each development stage under the stereomicroscope. Microsoft Excel 6.0 (Redmond, Washington, USA) was used to calculate the proportion of nematodes of different ages (adult females or J3 / J4). The experiment was carried out twice with 6 replicates each time.

Evaluation of rice resistance against *M. graminicola* induced by K_2SO_4

In order to test whether K_2SO_4 treatment induced rice resistance to *M. graminicola*, two-week-old rice roots were irrigated with 10 ml K_2SO_4 dilution one day before inoculation (approximately 100 J2s per plant). After measuring the height and fresh weight of each plant at 14 dpi, the roots were dyed according to the method described previously, and then the number of nematodes per developmental stage and sex and the galls number were counted. Galls of each treatment were randomly selected to take photos with Dual-Sensor Monochrome and Color Camera (Olympus DP80) under the microscope BX53 research microscope (Olympus Optical Company, Tokyo, Japan) at 4 magnifications. The test was carried out three times with six replicates each time.

Attraction of rice roots to *M. graminicola* and giant cells phenotype after K_2SO_4 treatment

According to the method described by Wang et al. [64], the trapping experiment of nematode was carried out. First, 11.5 g of Pluronic F-127 powder (sigma Aldrich, Brussels, Belgium) was added into 50 ml sterile water, stirred on ice and placed in a refrigerator at 4 ℃ to dissolve it completely. The roots of 2-week-old rice were soaked in K_2SO_4 dilution or water. After 24 h, the root tip of about 1 cm was cut and placed in the center of the culture hole (φ 3.5 cm) containing 1 ml Pluronic gel and approximately 100 J2s. After 10 hours at room temperature, under microscope BX53 research microscope, the nematodes near the root were photographed with Dual-Sensor Monochrome and Color Camera, and then the numbers of nematodes within 5 mm from the root tip were counted. The experiment was carried out three times with six replicates each time.

Giant cell phenotypes were observed according to the method described by Ji et al. [65]. The galls of rice at 7 dpi was fixed in 1× PIPES buffer overnight, then dehydrated with ethanol diluents step by step, and then embedded according to the instructions of Technovit 7100 kit. CryoStar NX50 Cryostat (Thermo Fisher Scientific, MA, USA) was used to cut gall tissue into 10 μm slices and placed on the slides. After stained with 0.5% toluidine blue for 5 min, the tissue was observed by microscope BX53 research microscope. The experiment was repeated twice with 10 galls per replicate.

Microscopic observation of callose deposition and quantification of H_2O_2

Callose deposition was detected according to Millet et al. [66]. Rice was inoculated with approximately 100 J2s and maintained in a greenhouse at 28°C. At 7 dpi, 20 root galls were randomly taken from each treatment and fixed overnight in ethanol acetic acid solution, and then diluted with ethanol for dehydration. The galls were stained with 1% aniline blue. The deposition of callose was detected by Olympus IX83 inverted microscope (Olympus Optical Company, Tokyo, Japan) under ultraviolet light. Image J software was used to quantify. The experiment was repeated three times.

Four treatments were used for 2-week-old rice plants, including K_2SO_4 treatment alone, water treatment or K_2SO_4 treatment before inoculation, and water treatment alone as control. Root samples were collected for quantitative analysis of H_2O_2 at 8, 24 and 72 hpi. Fresh root sample (100 mg) collected from each group of six rices was treated according to the method described by Ji et al. [24], and then the H_2O_2 accumulation was detected by the trichloroacetic acid (TCA) method described by Velikova et al. [67]. The experiment was repeated three times.

Relative quantitative analysis of expression levels of disease resistance-related genes

In order to analyze the effect of K_2SO_4 treatment on the expression level of defense related genes in rice, four treatment groups as described in the section of quantification of H_2O_2 were set up. Total RNA was extracted from root samples of 6 plants using RNeasy plant Mini Kit, and cDNA was synthesized using PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (TAKARA, Japan). The primer pairs for qRT PCR analysis of resistance-related genes and internal reference genes are listed in Table 1. All qRT-PCR analyses were performed using a 7500-fast real-time PCR system (Thermo Fisher technologies, Beijing, China) with two independent biological repeats and three technical replicates. The relative expression level of resistance-related gene was characterized by the fold change compared with the control. The relative expression of genes was calculated by $2^{-\Delta\Delta Ct}$ method [68].

Table 1
Primer pairs used in this study

Genes	GenBank accession or locus number	Primer sequences (5'→3')	Function
OsRbohB	LOC4326027	F: CTGGACAGGACCAAGAGCAG	H_2O_2 biosynthesis
		R: ATCTTGAACGGAGCAGCACA	
OsWRKY45	AK066255	F: AATTCGGTGGTCGTCAAGAA	SA transcription factor
		R: AAGTAGGCCTTTGGGTGCTT	
OsICS1	LOC9268489	F: TGTCCCCACAAAGGCATCCTGG	SA biosynthesis
		R: TGGCCCTCAACCTTTAAACATGCC	
OsJAMYB	AY026332	F: GAGGACCAGAGTGCAAAAGC	JA transcription factor
		R: CATGGCATCCTTGAACCTCT	
OsAOS2	NM_001055971.1	F: CAATACGTGTACTGGTCGAATGG	JA biosynthesis
		R: AAGGTGTCGTACCGGAGGAA	
OsEIN2	LOC107278000	F: TAGGGGGACTTTGACCATTG	ET signaling
		R: TGGAAGGGACCAGAAGTGTT	
OsACS1	AK071011	F: GATGGTCTCGGATGATCACA	ET biosynthesis
		R: GTCGGGGGAAAACTGAAAAT	
OsPR1a	AJ278436	F: GTATGCTATGCTACGTGTTTATGC	pathogenesis-related protein
		R: GCAAATACGGCTGACAGTACAG	
OsPR1b	AK107926	F: ACGCCTTCACGGTCCATAC	pathogenesis-related protein
		R: AAACAGAAAGAAACAGAGGGAGTAC	
OsBRI1	LOC4324691	F: CTTTCTCGGCACTTTCCTTG	BR receptor
		R: AGGGAACCAAGCTCTGGAAT	
OsDwarf	AB084385	F: ATGGCCTTGCTATCTGCACT	BR biosynthesis
		R: GGGTGTAGAGCTCTGCCTTG	
OsAKT1	LOC4326245	F: CTTACCAGAAAACCGAATCACC	K^+ channel
		R: CCTCTCTCGGGTTTAATGGAG	
OsHAK5	LOC_Os01g70490	F: ACCGATCAGCAAGATAGAAACA	K^+ transporter

		R: TAAGCTCTCAAATTCTGCTGGA	
OsEXP	LOC_Os03g27010	F: TGTGAGCAGCTTCTCGTTTG	Reference gene
		R: TGTTGTTGCCTGTGAGATCG	
OsEXPnarsai	LOC_Os07g02340.1	F: AGGAACATGGAGAAGAACAAGG	Reference gene
		R: CAGAGGTGGTGCAGATGAAA	
OsEif5C	SM00515	F: CACGTTACGGTGACACCTTTT	Reference gene
		R: GACGCTCTCCTTCTTCCTCAG	

Effects of potassium ion transporters on resistance of rice to *M. graminicola*

To determine whether the K^+ channel OsAKT1 and transporter OsHAK5 respond to K_2SO_4 treatment or nematode infection, the expression levels of *OsAKT1* and *OsHAK5* were determined by the same method in the previous section. The primer sequences for qRT-PCR analysis of *OsAKT1*, *OsHAK5* are listed in Table 1. K_2SO_4 solutions at a concentration of 0.5 mM were treated and approximately 100 J2s were inoculated on rice roots.

In order to determine whether OsAKT1 and OsHAK5 are involved in the resistance of rice to RKNs induced by K_2SO_4, K_2SO_4 solutions were irrigated on 2-week-old OsAKT1-RNAi lines and OsHAK5-Cr transgenic lines, and approximately 100 J2s were inoculated on plant roots. The wild-type Nipponbare was used as control. At 14 dpi, the numbers of galls and nematodes were counted using the method described previously. The whole experiment was carried out three times with 6 plants in each repeat.

Statistical analysis of data

SPSS version 25 (IBM, Armonk, NY, USA) was used to calculate the mean and standard errors of the data. Statistical significance ($P \leq 0.05$) was obtained by Duncan´s multiple range tests.

Abbreviations

MG: Meloidogyne graminicola; hpi: hours post inoculation; dpi: days post inoculation. J2: second stage juvenile.

Declarations

Ethics approval and consent to participate

The collection of wild-type rice seeds (*Oryza sativa* cv. Nipponbare) was permitted by the United States Department of Agriculture and complied with relevant institutional, national, and international guidelines

and legislation.

Consent for publication

Not Applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no conflict of interest.

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Author Contributions

WKH and JL conceived the project and designed the experiments. MYL, WS, CX, JZJ, JZ performed the experiments. MYL, WKH, JL, CX, HP, SML and LAK analyzed the data. DLP, JL, WKH, and LYD provided the experimental materials. DLP coordinated the project. MYL, WKH and SML wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Figures

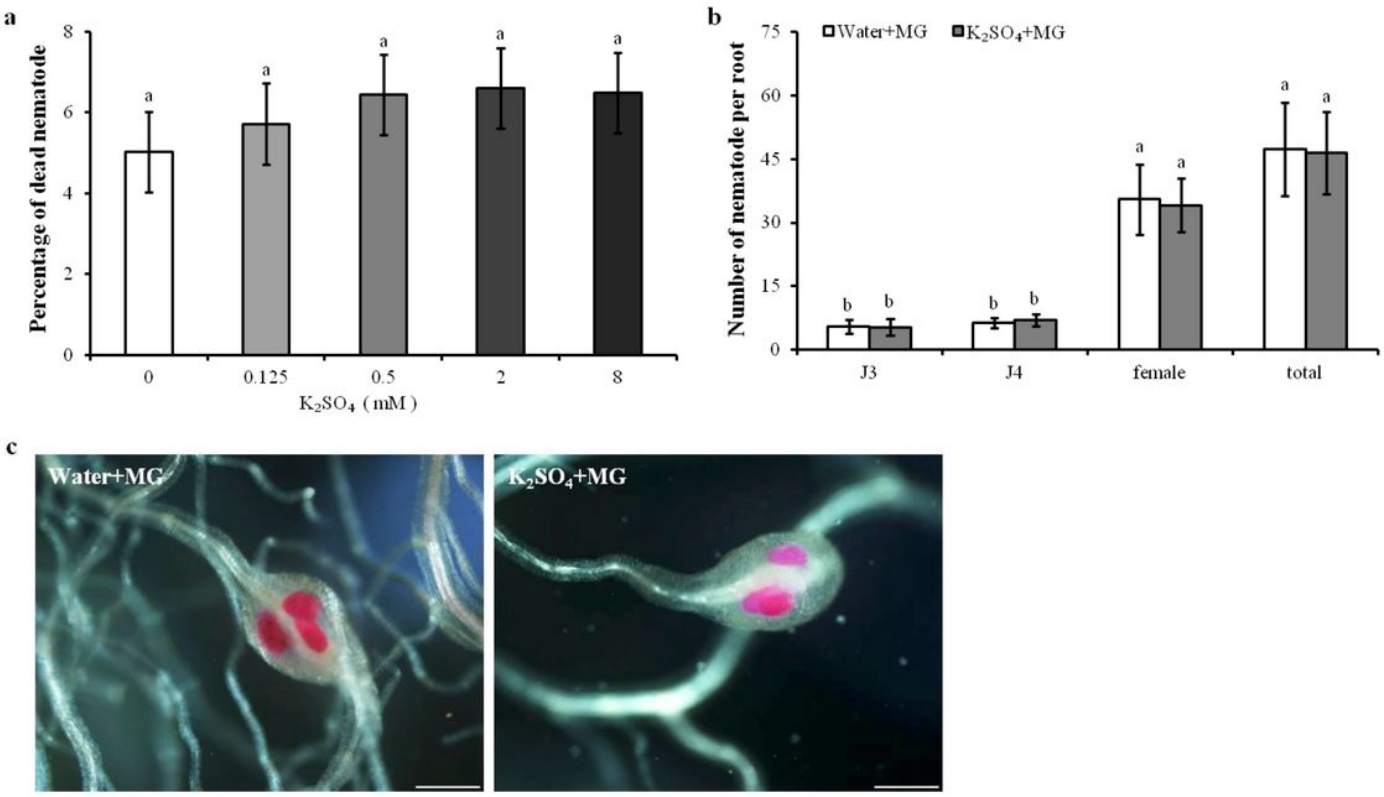

Figure 1

Direct effects of K2SO4 on M. graminicola (MG). a Mortality of J2s after incubated at different concentrations of K2SO4 for 72 h. The experiment was repeated three times with four replicates each time. Data are presented as the mean ± SE of 4 replicates. b Infectivity and development of K2SO4-incubated and water-incubated MG in rice roots. c K2SO4-incubated and water-incubated MG in plants photographed at 14 dpi. Bar = 500 μm. The experiment was repeated three times, with 6 individual plants in each replicate. Data are presented as the mean ± SE of 6 replicates.

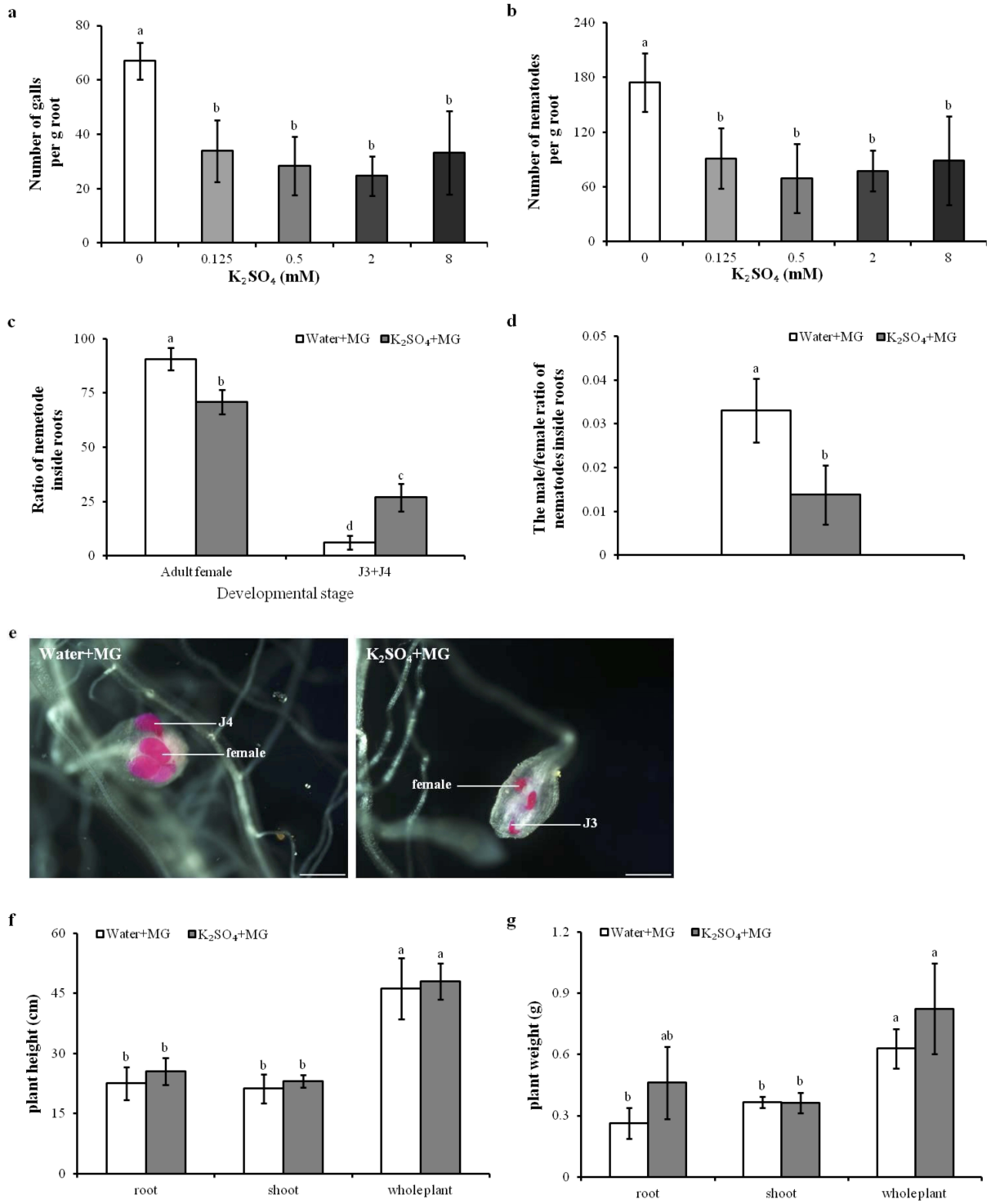

Figure 2

Effects of K2SO4 on nematode infection and plant growth. a The numbers of galls on rice roots treated with different concentrations of K2SO4 solution at 14 dpi. b The numbers of nematodes on rice roots treated with different concentrations of K2SO4 solution at 14 dpi. c The ratio of different developmental stage nematodes in roots at 14 dpi. d The male/female ratio of nematodes in roots at 14 dpi. e Photograph of different developmental stage nematodes in roots at 14 dpi. Bar = 500μm. f Plant height

at 14 dpi. (G) Plant weight at 14 dpi. The bars in the different graphs represent the mean ± SE of data from three independent biological replicates, each containing 6 individual plants. Different letters indicate statistically significant differences (Duncan's multiple range test at $P \leq 0.05$)

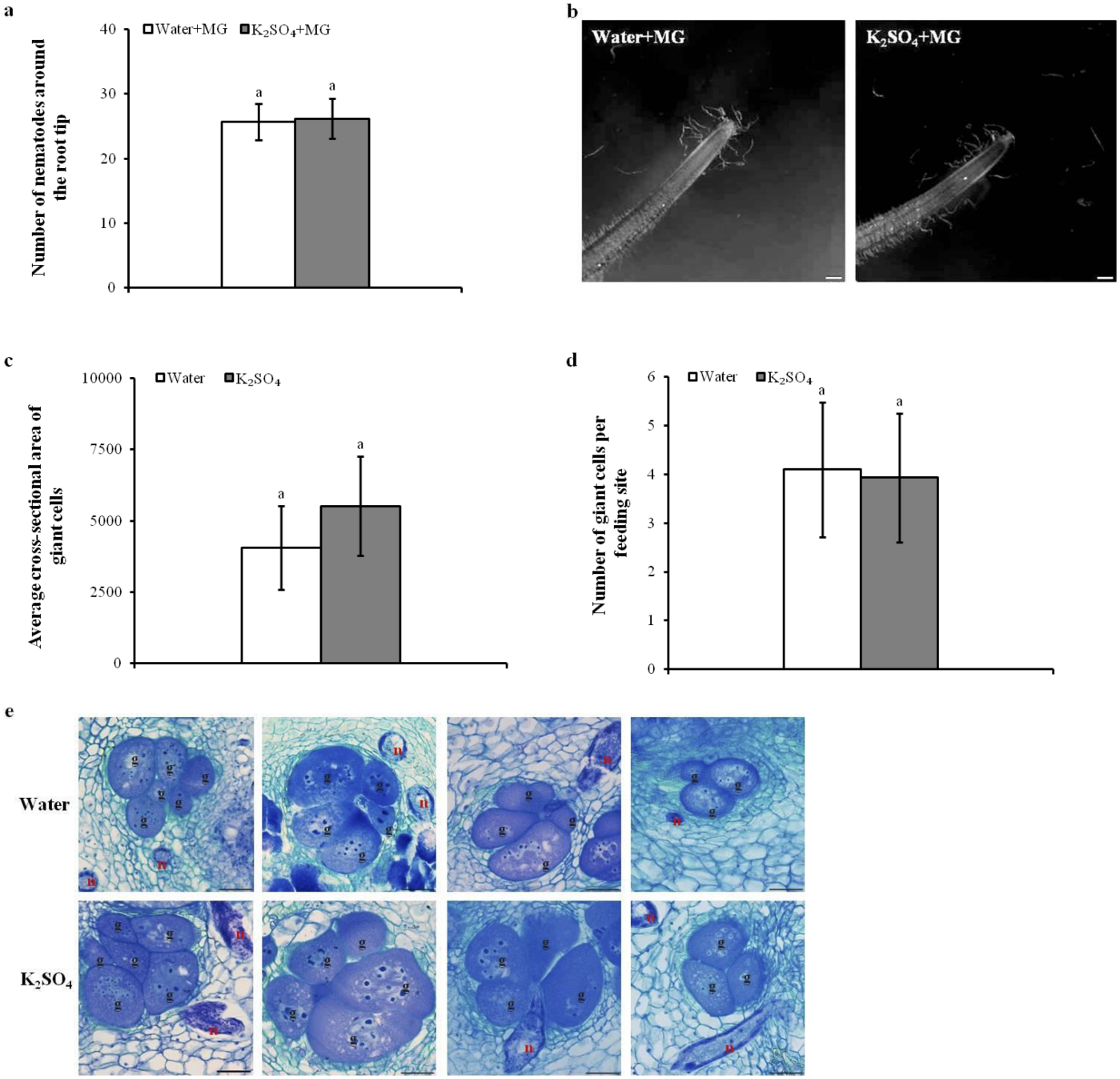

Figure 3

Effects of K2SO4 on the attractiveness of rice roots to M. graminicola and the phenotype of giant cells. a Nematodes attracted within 5 mm from the root tip were counted at 10 h after inoculation. The experiment was repeated three times, with 6 individual roots in each replicate. Data are presented as the mean ± SE of 6 replicates. b Attraction of nematodes towards the rice root tip after treated with K2SO4 or

water. Bar = 200 μm. c Measuring of the size of giant cells at 7 dpi. d Number of giant cells per feeding site at 7 dpi. e Light micrographs of M. graminicola feeding sites. Water: water control. K2SO4: K2SO4 treated group. g: giant cell. n: nematode. Bar = 50 μm. Data of c and d presented are the mean ± SE of two independent experiments, each performed using ten galls.

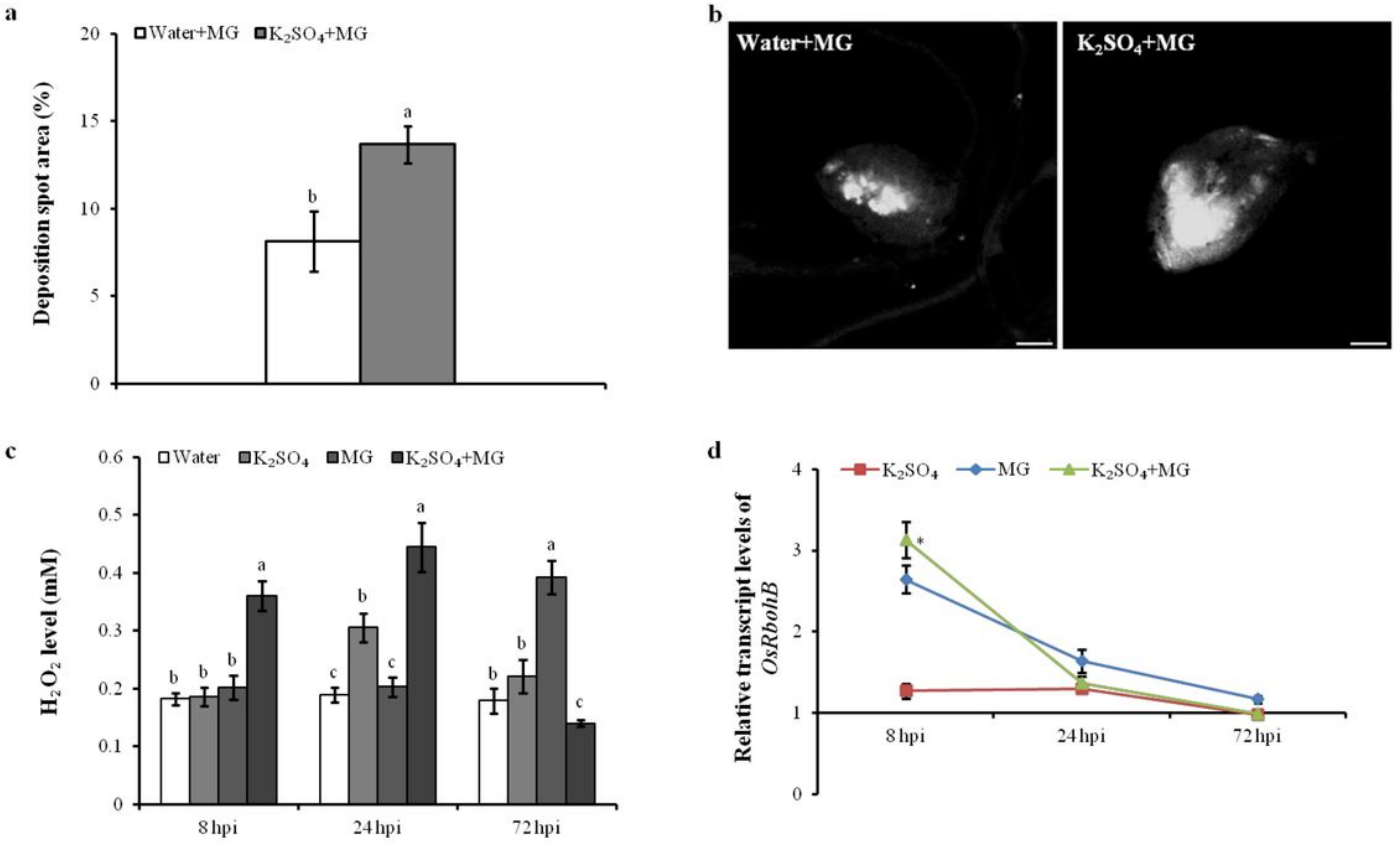

Figure 4

Direct effects of K2SO4 on M. graminicola (MG). a Mortality of J2s after incubated at different concentrations of K2SO4 for 72 h. The experiment was repeated three times with four replicates each time. Data are presented as the mean ± SE of 4 replicates. b Infectivity and development of K2SO4-incubated and water-incubated MG in rice roots. c K2SO4-incubated and water-incubated MG in plants photographed at 14 dpi. Bar = 500 μm. The experiment was repeated three times, with 6 individual plants in each replicate. Data are presented as the mean ± SE of 6 replicates.

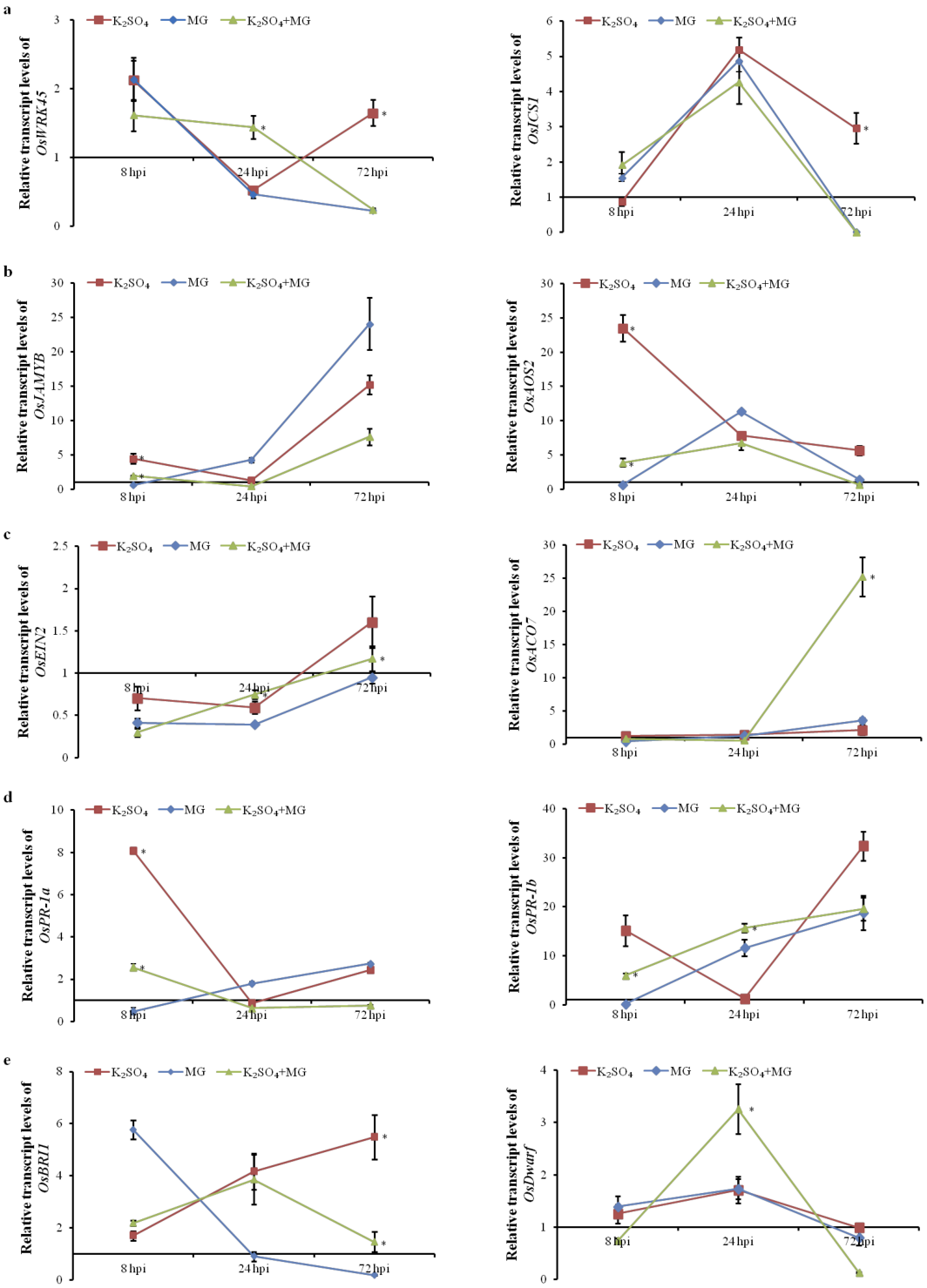

Figure 5

Expression of the disease resistence-related genes in rice treated with K2SO4 and inoculated with nematodes. a Salicylic acid (SA) signal pathway-related genes (SA transcription factor WRKY45 and signal biosynthesis gene OsICS1). b Jasmonic acid (JA) signal pathway-related gene (JA transcription factor OsJAMYB and biosynthesis gene OsAOS2). c Ethylene (ET) signal pathway-related gene (ET signaling gene OsEIN2 and biosynthesis gene OsACS1). d Pathogenesis-related genes (OsPR1a and

OsPR1b). e Brassinolide (BR) signal pathway-related genes (BR receptor gene OsBRI1 and biosynthesis gene OsDwarf). Gene expression was analyzed at 8, 24 and 72 hpi and normalized with three internal reference genes, OsEXP, OsEif5C and OsEXPnarsai. All data are shown as relative transcript levels in comparison to the control (expression level set at 1). The bars represent the mean expression levels ± SE from two independent biological replicates and three technical replicates, each containing a pool of 6 plants. Asterisks indicate significant differential expressions (Duncan's multiple range test with $P \leq 0.05$).

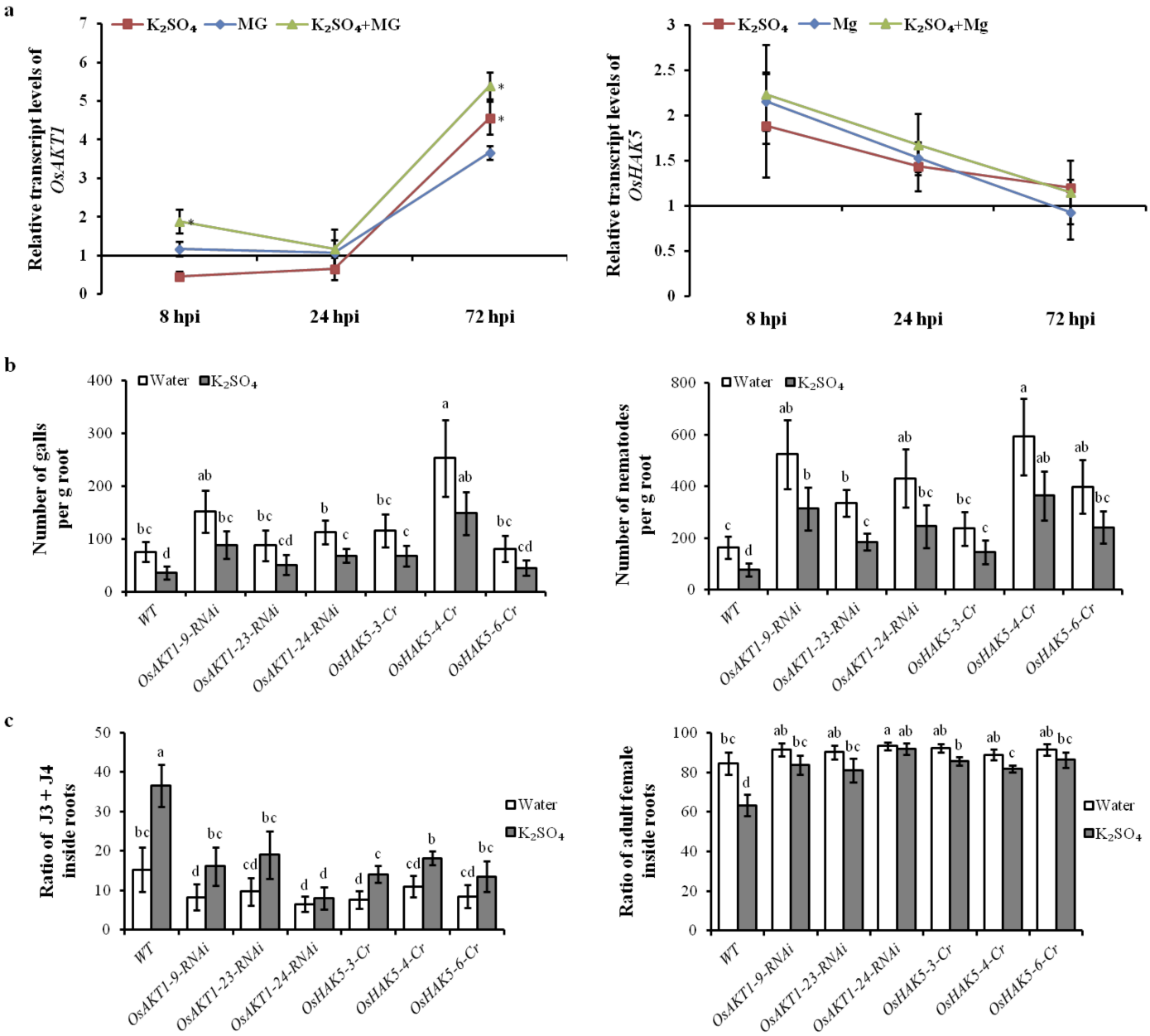

Figure 6

Effects of potassium channel OsAKT1 and transporter OsHAK5 on host resistance to M. graminicola. a Expression analyses of the potassium channel gene OsAKT1 and transporter gene OsHAK5. Gene expression was analyzed at 8, 24 and 72 hpi and normalized with three internal reference genes, OsEXP,

OsEif5C and OsEXPnarsai. All data are shown as relative transcript levels in comparison with the control (expression level set at 1). The bars represent the mean expression levels ± SE from two independent biological replicates and three technical replicates, each containing a pool of 6 plants. Asterisks indicate significant differential expression (Duncan's multiple range test with $P \leq 0.05$). b The numbers of galls and nematodes in OsAKT1-RNAi lines (deficient in K + channel OsAKT1) and OsHAK5-Cr lines (deficient in K+ transporter OsHAK5) roots treated with or without K2SO4 at 14 dpi. c The ratio of juveniles (J3 + J4) and adult females in OsAKT1-RNAi lines and OsHAK5-Cr lines roots treated with or without K2SO4 at 14 dpi. The bars in the different graphs represent the mean ± SE of data from three independent biological replicates, each containing 6 individual plants. Different letters indicate statistically significant differences (Duncan's multiple range test at $P \leq 0.05$).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigure1.jpg
- SupplementaryFigure2.jpg