Host- and virus-induced gene silencing of HOG1-MAPK cascade genes in *Rhizophagus irregularis* inhibit arbuscule development and reduce resistance of plants to drought stress

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Summary

Arbuscular mycorrhizal (AM) fungi can form beneficial associations with the most terrestrial vascular plant species. AM fungi not only facilitate plant nutrient acquisition but also enhance plant tolerance to various environmental stresses such as drought stress. However, the molecular mechanisms by which AM fungal mitogen-activated protein kinase (MAPK) cascades mediate the host adaptation to drought stimulus remains to be investigated. Recently, many studies have shown that virus-induced gene silencing (VIGS) and host-induced gene silencing (HIGS) strategies are used for functional studies of AM fungi. Here, we identify the three HOG1 (High Osmolarity Glycerol 1)-MAPK cascade genes *RiSte11*, *RiPbs2* and *RiHog1* from *Rhizophagus irregularis*. The expression levels of the three HOG1-MAPK genes are significantly increased in mycorrhizal roots of the plant *Astragalus sinicus* under severe drought stress. RiHog1 protein was predominantly localized in the nucleus of yeast in response to 1 M sorbitol treatment, and RiPbs2 interacts with RiSte11 or RiHog1 directly by pull-down assay. Importantly, VIGS or HIGS of *RiSte11*, *RiPbs2* or *RiHog1* hampers arbuscule development and decreases relative water content in plants during AM symbiosis. Moreover, silencing of HOG1-MAPK cascade genes led to the decreased expression of drought-resistant genes (*RiAQPs*, *RiTPSs*, *RiNTH1* and *Ri14-3-3*) in the AM fungal symbiont in response to drought stress. Taken together, this study demonstrates that VIGS or HIGS of AM fungal HOG1-MAPK cascade inhibits arbuscule development and expression of AM fungal drought-resistant genes under drought stress.

Introduction

In natural ecosystems, terrestrial vascular plants as sessile organisms are faced with highly variable environmental conditions, such as biotic and abiotic stresses (Zhu, 2016). Among the abiotic stresses, drought stress (DS) is harmful to plant growth and productivity. For example, drought inhibits photosynthesis, disrupts water balance and affects stomatal conductance, all of which lead to the decreased growth of plants (Chitarra *et al.*, 2016; Quiroga *et al.*, 2019). However, vascular plants have evolved many strategies to adapt to the DS. The establishment of arbuscular mycorrhizal (AM) symbiosis can improve plant nutrient uptake and other physiological alterations to cope with DS (Begum *et al.*, 2021; Püschel *et al.*, 2021).

Arbuscular mycorrhizal fungi are capable of forming beneficial associations with more than 72% of terrestrial vascular plants (Brundrett and Tedersoo, 2018; Genre *et al.*, 2020). The obligate biotrophic AM fungi need to consume sugars and lipids from host plants to complete their life cycles (An *et al.*, 2019; Bago *et al.*, 2003; Jiang *et al.*, 2017; Luginbuehl *et al.*, 2017), and in turn AM fungi facilitate plant growth and health not only by increasing mineral nutrient acquisition (particularly Pi) from surrounding soil but also protecting plants against multiple

abiotic stresses (Lenoir *et al.*, 2016; Wang *et al.*, 2017). Therefore, AM fungi are key endosymbionts of plant symbiosis and provide a friendly and sustainable way to agriculture development (Begum *et al.*, 2019; Gianinazzi *et al.*, 2010). AM symbiosis can improve the host plant's tolerance to drought by regulating plant physiological and molecular responses, such as the plant parameters: stomatal conductance, transpiration, photosynthesis, leaf or root hydration, root aquaporins (AQPs) and accentuating root uptake of mineral nutrients, in particular phosphate (Augé, 2001; Püschel *et al.*, 2021). Despite the great importance of AM symbiosis, the underlying molecular mechanisms by which AM fungi respond to DS remain poorly understood. However, arbuscular mycorrhizas have developed a common set of responsive mechanisms involved in resistance to DS to sense and relay this pressure signal, one of the most important signalling modules that transmit the perceived DS to the nucleus is the mitogen-activated protein kinase (MAPK) cascade (Liu *et al.*, 2015b).

Mitogen-activated protein kinase are serine/threonine protein kinases in all eukaryotes, which are evolutionarily conserved signalling hubs that regulate multiple stress responses in both plants and microorganisms (Lin *et al.*, 2021; Martínez-Soto and Ruiz-Herrera, 2017). MAPK cascades play critical roles in a series

of cellular signal transduction and cell growth regulation. A typical MAPK cascade reaction consists of three protein kinases: the MAPKKK-MAPKK-MAPK (Colcombet and Hirt, 2008). Among these MAPK cascades, the HOG (High Osmolarity Glycerol) pathway plays an important role in the plant responses to osmotic stress. In most filamentous fungi, HOG1-MAPK cascade is necessary for osmoregulation and hyphal growth or pathogenesis (Ji et al., 2012; Jiang et al., 2018; Zhou et al., 2019). In *Saccharomyces cerevisiae*, the Hog1 pathway is regulated by three MAPKKKs (Ste11, Ssk2 and Ssk22) in response to osmotic stress. Ste11 or Ssk2 phosphorylates the MAPKK Pbs2 and then Pbs2 activates MAPK kinase Hog1 (Murakami et al., 2008; Tatebayashi et al., 2020). Furthermore, the MAPK cascades of AM fungus or host plants show the potential correlation to improve the resistance to drought tolerance (Huang et al., 2020; Liu et al., 2015b). However, there is no evidence to prove the causal relations between AM fungal and plant MAPK enhancing mycorrhizal plants' drought tolerance.

Although recent studies have revealed that AM fungus *Rhizophagus irregularis* possess many MAPK genes (Chen et al., 2018; Tisserant et al., 2013), relatively little is known about their precise roles in AM symbiosis and responding to external stresses, largely due to the difficulty in genetic manipulation of these obligate biotrophic fungi. VIGS is widely used to identify gene function with the advantages of short period and high efficiency (Chantreau et al., 2015; Deng et al., 2012). Host-induced gene silencing (HIGS) is developed based on the well-known RNA interference, which is to express double-strand RNA (dsRNA) of target gene in host plants. Research on pathogenic fungi using silencing of genes to improve plant resistance has been reported (Cheng et al., 2015; Ghag et al., 2014; Xu et al., 2018). Moreover, VIGS and HIGS strategies were employed for functional studies of AM fungal genes during the *in planta* phase (Ezawa et al., 2020; Hartmann et al., 2020; Helber et al., 2011; Xie et al., 2016). Therefore, the powerful HIGS and VIGS techniques are two feasible strategies for the functional characterization of AM fungal genes during AM symbiosis.

Here, we report the positive responses of the plant *A. sinicus* to DS during AM symbiosis. We focus on the HOG1-MAPK cascade genes in the AM fungus *R. irregularis* DAOM197198. More importantly, we determine the biological functions of three HOG1-MAPK cascade genes (*RiSte11*, *RiPbs2* and *RiHog1*) of *R. irregularis* associated with host plant through both VIGS and HIGS strategies. Our findings reveal that AM fungus can improve host plant tolerance to drought through the HOG1-MAPK cascade of the fungal symbiont.

Results

Arbuscular mycorrhizal colonization enhances the *Astragalus sinicus* tolerance to drought stress

To estimate the role of arbuscular mycorrhiza in enhancing plant drought tolerance, the *A. sinicus* seedlings were colonized without (NM) or with (AM) *R. irregularis* under well water (WW) and DS conditions. The WW-treated pots contained 75% field water capacity (FWC), whereas the DS-treated pots contained 25% FWC. Our result showed that AM *A. sinicus* grew well, while the NM plants appeared with etiolated leaves (Figure S1a). It was found that *A. sinicus* roots formed arbuscular mycorrhiza after *R. irregularis* colonization (Figure S1b,c), while no AM fungal colonization was observed in NM roots (data not shown). And under the AM condition, there is no significant

difference in total colonization frequency (F%) and mycorrhization (M% and A%) between the WW and DS treatments (Figure S1d). Under both WW and DS conditions, the biomass of roots and shoots of AM *A. sinicus* was much higher than that of NM plants (Figure S1e,f). Particularly, under DS conditions, the fresh weight (FW) of AM roots is significantly higher than that of NM roots. Moreover, we found significant differences in the relative water content (RWC) and proline content of *A. sinicus* leaves between AM and NM plants under DS conditions. The data revealed that AM plants showed much higher RWC than NM plants, but significantly lower proline content than NM plants under DS conditions (Figure S1g,h). Proline is one of the most important physiological indicators for plants to cope with DS (Shrestha et al., 2021). Overall, the above results indicated that AM fungal colonization significantly improved the mycorrhizal *A. sinicus* drought resistance under DS.

Identification of HOG1-MAPK cascade genes in *Rhizophagus irregularis*

Fungal MAPKs are essential for fungus–plant interaction subjected to DS (Huang et al., 2020; Liu et al., 2015b). Because among them the HOG1-MAPK cascade plays a critical role in the fungal osmoregulation (Román et al., 2020; Wang et al., 2021), we initiated a search for the HOG1-MAPK cascade genes in *R. irregularis*, using the related MAPK cascade genes in *S. cerevisiae* and other filamentous fungi as reference sequences (Hamel et al., 2012). The *RiSte11*, *RiPbs2* and *RiHog1* from *R. irregularis* were identified by BLAST searches according to the released genome of *R. irregularis* strain DAOM 197198 (Chen et al., 2018; Tisserant et al., 2013). Results showed that the three candidate genes belong to the serine/threonine protein kinases MAPKKK, MAPKK and MAPK, respectively (Table S1). Further *in silico* analysis indicates that *RiSte11* contains 13 exons and 12 introns and encodes 690 amino acids, while *RiPbs2* contains 8 exons and 7 introns and encodes 351 amino acids; *RiHog1* contains 13 exons and 12 introns and encodes 359 amino acids (Figure S2a). The protein structure analysis reveals that RiSte11 contains a SAM_Ste11_fungal (Sterile alpha motif) domain, a Ras_bdg-2 domain (The binding region of several Ser/Thr protein kinases) and a Serine/Threonine protein kinases domain, while both RiPbs2 and RiHog1 contain only one Serine/Threonine protein kinases domain (Figure S2b). Taken together, these *in silico* data showed that the HOG1-MAPK cascade in AM fungus *R. irregularis* is composed of the protein kinases RiSte11, RiPbs2 and RiHog1.

HOG1-MAPK proteins from *Rhizophagus irregularis* are conserved across fungi species

To investigate the evolutionary relationships among RiSte11, RiPbs2, RiHog1 and other members of the MAPK cascade proteins in AM and other fungal species, we performed a phylogenetic analysis that included 94 well-sequenced MAPK genes from diverse fungi. Based on sequence similarity, the fungal MAPK proteins tested could be divided into three groups: MAPKKK, MAPKK and MAPK. An unrooted phylogenetic tree shows the close relationship between RiSte11, RiPbs2, RiHog1 and other AM fungal MAPK-related genes (Figure 1a). The MAPKKK group consists of Ste11-related proteins from different fungal species, while the RiPbs2 protein from *R. irregularis* belongs to the MAPKK group. RiSte11 and RiPbs2 proteins are closely related to the Ste11 or Pbs2 among other AM fungal species. Moreover, the RiHog1 protein belongs to the MAPK

group, and the RiHog1 is closely related to AM fungal proteins RdHog1, GcHog1, RcHog1, GigrHog1 and GigmHog1 from *Rhizophagus diaphanous*, *Glomus cerebriforme*, *Rhizophagus clarus*, *Gigaspora rosea* and *Gigaspora margarita*, respectively. This result indicates the conserved evolutionary origin of the RiSte11, RiPbs2 and RiHog1 proteins from *R. irregularis*, and the

Figure 1 The potential HOG1-MAPK cascade proteins in *Rhizophagus irregularis*. (a) Evolutionary relationships among *R. irregularis* RiSte11, RiPbs2, RiHog1 and related MAPK cascade proteins in other fungal species. The evolutionary history was inferred using MEGA7 software with the Neighbour-Joining method. Bootstrap values were calculated using 1000 replicates. RiSte11, RiPbs2 and RiHog1 proteins from *R. irregularis* are coloured in red. The bold branches represent the MAPK cascade proteins in other AM fungal species. (b–g) The predicted three-dimensional (3D) structures of *R. irregularis* RiSte11, RiPbs2 and RiHog1 proteins using the homologues derived from *Homo sapiens* ((b) 2xik.1.A; (d) 3w8q.1.A), *Arabidopsis thaliana* ((f) 5ci6.2.A) as templates, respectively. Arrows point out C-terminals and N-terminals. Homology modelling of the 3D structures of RiSte11, RiPbs2 and RiHog1 proteins were conducted with the Swiss Model program (https://swissmodel.expasy.org/).

three proteins may have the similarity in function with other HOG1-MAPK genes from other fungal species.

We further carried out homology modelling of RiSte11, RiPbs2 and RiHog1 proteins using *Homo sapiens* YSK1 (PDB-ID: 2xik.1.A), *Homo sapiens* MEK1 (3w8q.1.A) and *Arabidopsis thaliana* MPK6 (5ci6.2.A) as templates (Wang *et al.*, 2016), respectively. The predicted 3D models indicated that RiSte11, RiPbs2 and RiHog1 proteins were highly conserved compared with the MAPK-related proteins (templates) from their respective species (Figure 1b–g). Collectively, RiSte11, RiPbs2 and RiHog1 proteins are conserved across fungi species and closely relative to the AM fungi mitogen-activated protein kinases.

RiSte11, RiPbs2 and RiHog1 are expressed during the arbuscular mycorrhizal symbiosis

To determine whether the above functional predictions of RiSte11, RiPbs2 and RiHog1 have any physiological relevance, we first analysed the RNA-sequencing data of *RiSte11*, *RiPbs2* and *RiHog1* from *R. irregularis* transcriptomic databases (Morin *et al.*, 2019; Zeng *et al.*, 2018, 2020). The transcription of *RiSte11* in spores and mycorrhizal roots was higher than that in extraradical mycelium (ERM), while *RiPbs2* was uniformly expressed in spores, mycorrhizal roots and ERM. The transcription of *RiHog1* was slightly increased in spores and ERM compared with the mycorrhizal roots (Figure S3). Then the qRT-PCR was performed using cDNA from *A. sinicus* roots inoculated with *R. irregularis*. The results showed that transcripts of *RiSte11*, *RiPbs2* and *RiHog1* were also detectable in germinating spores, mycorrhizal *A. sinicus* roots (Myc-roots) and ERM. The transcription level of *RiSte11* in spores was almost twice that in ERM (Figure 2a). And as for *RiPbs2*, the expression difference among spores, Myc-roots and ERM were not obvious. While *RiHog1* was higher expressed in both spores and ERM than in Myc-roots (Figure 2b,c). The qRT-PCR results agree with the genomic analysis that these three genes are expressed in spores, mycorrhizal roots and ERM.

The results presented above prompted us to further determine the temporal expression of *RiSte11*, *RiPbs2* and *RiHog1* in mycorrhizal *A. sinicus* roots during different days post-inoculation (dpi). The transcripts of *RiSte11* and *RiPbs2* began to increase at 14 dpi, then kept increasing until 49 dpi (Figure 2d,e). While *RiHog1* transcripts showed a gradual increase trend from 14 to 56 dpi with AM fungal colonization (Figure 2f). We also analysed the expression levels of AM symbiotic marker genes *RiMST2* and *AsPT4* (Helber *et al.*, 2011; Xie *et al.*, 2013), which showed the expression of *RiMST2* and *AsPT4* kept increasing from 14 to 49 dpi (Figure 2g,h). During the later stage of symbiosis, higher expression levels of *RiSte11*, *RiPbs2* and *RiHog1* suggested that the three genes might play important roles in the physiological functions of mycorrhizal symbiosis. Taken together, our results from gene expression

analysis revealed that the HOG1-MAPK cascade genes in *R. irregularis* were induced during the *in planta* phase, suggesting that RiSte11, RiPbs2 and RiHog1 appear to be useful MAPKs that operate in the AM fungus *R. irregularis* crucial for the symbiotic relationship with plants.

RiSte11, RiPbs2 and RiHog1 are induced during the *in planta* phase in response to severe drought stress

To determine the effect of DS on the expression of *RiSte11*, *RiPbs2* and *RiHog1* during AM symbiosis, we performed a qRT-PCR analysis on the AM roots of *A. sinicus* plants exposed to different FWC. The results showed that DS caused weaker plant growth and plant leaves appeared etiolated (Figure S4a). Moreover, the severe DS led to the significantly decreased plant biomass, the lowered RWC and increased proline content in leaves (Figure S4b–d). Accordingly, the expression levels of HOG1-MAPK cascade genes in *R. irregularis* were also changed in response to different DSs. Both *RiSte11* and *RiHog1* showed elevated transcription levels in AM roots under severe DS (25% FWC) compared with the middle (50%FWC) and normal (75% FWC) water conditions, whereas *RiPbs2* exhibited an increased expression level in AM roots in the presence of the 25% FWC and 50% FWC (Figure 2i–k). The above results showed the transcription levels of *RiSte11*, *RiPbs2* and *RiHog1* increased significantly in mycorrhizal roots under severe DS, indicating that these three MAPKs may be manipulated by AM symbiosis for DS resistance.

Functional characterization of RiSte11, RiPbs2 and RiHog1 proteins in *Saccharomyces cerevisiae*

To determine whether the activity of RiSte11, RiPbs2 or RiHog1 modulates the growth of yeast cells in response to high osmotic stress, a complementation assay was performed with wild-type BY4741, Δ*ste11*, Δ*pbs2* and Δ*hog1* yeast mutant. The empty pFL61 was used in the complementation assay, which carries the phosphorglycerate kinase promoter (PGK) separated by the terminator at *Not* I site in *S. cerevisiae* (Minet *et al.*, 1992). Sorbitol is widely used for simulating drought environment to verify yeast function (Li *et al.*, 2013), so the growth of the positive transformants was measured on synthetic dropout (SD/-URA) medium and treated with different sorbitol concentrations. The result showed Δ*ste11-pFL61*, Δ*pbs2-pFL61*, Δ*hog1-pFL61*, Δ*ste11-pFL61-RiSte11*, Δ*pbs2-pFL61-RiPbs2* and Δ*hog1-pFL61-RiHog1* strains could grow as well as BY4741 strain with pFL61 on medium under standard condition. While under sorbitol condition, the Δ*ste11-pFL61-RiSte11*, Δ*pbs2-pFL61-RiPbs2* and Δ*hog1-pFL61-RiHog1* strains can complement the growth defect, but the Δ*ste11* strain expressing RiSte11 grow better than Δ*ste11* mutant under sorbitol treatment, and Δ*pbs2* and Δ*hog1* mutant could not survive well when given higher than 1 M sorbitol treatment (Figure 3a). The growth defects among Δ*ste11-pFL61-RiSte11*, Δ*pbs2-pFL61-RiPbs2*, Δ*hog1-pFL61-*
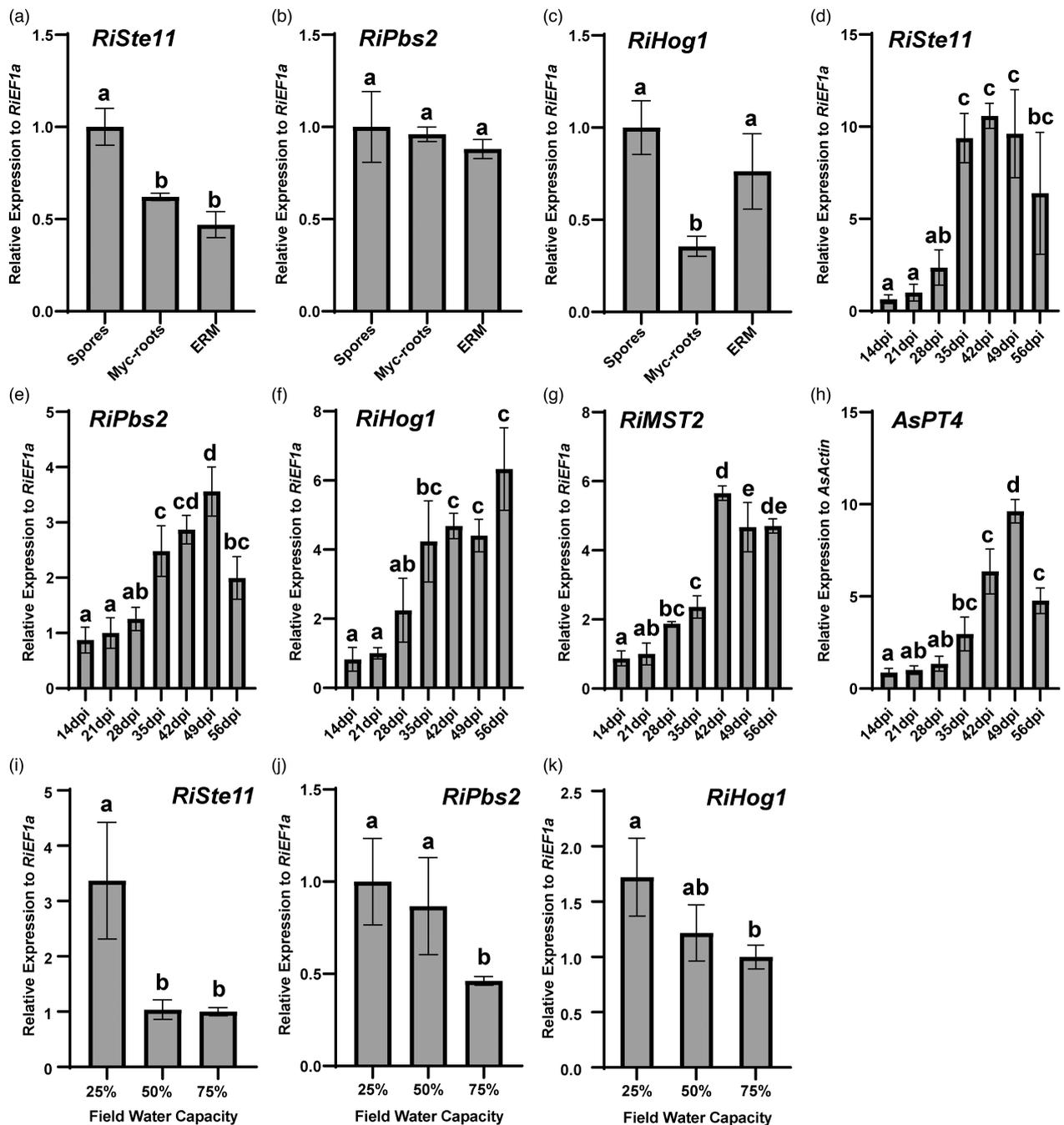
Figure 2 Expression patterns of *RiSte11*, *RiPbs2* and *RiHog1* from AM fungus *Rhizophagus irregularis*. (a–c) Expression levels of *RiSte11* (a), *RiPbs2* (b) and *RiHog1* (c) in different AM fungal tissues. Spores: germinating spores; Myc-roots: mycorrhizal *Astragalus sinicus* roots at 28 days post inoculation (dpi); ERM: extraradical mycelium. (d–h) Time-course analysis of the expression profiles of *RiSte11*, *RiPbs2*, *RiHog1*, *RiMST2* and *AsPT4* in *A. sinicus* roots at 14-, 21-, 28-, 35-, 42-, 49- and 56-days post inoculation (dpi) with *R. irregularis*. (i-k) Expression levels of *RiSte11*, *RiPbs2* and *RiHog1* from *R. irregularis* during the *in planta* phase at 42 dpi grown in 25%, 50% and 75% FWC. *RiEF1a* from *R. irregularis* and *A. sinicus AsActin* were used as reference genes for normalization. Error bars represent the means of three biological replicates with SE values, based on ANOVA-Tukey's test. The different letters indicate significant statistical differences between treatments at *P* < 0.05.

RiHog1, and Δ*ste11-pFL61*, Δ*pbs2-pFL61*, Δ*hog1-pFL61* strains became gradually larger with sorbitol concentration increased (Figure 3b–g).

In yeast, trehalose is considered as a regulatory component to protect cells against various environmental stresses (Li *et al.*, 2009). To examine the physiological roles of RiSte11, RiPbs2 and RiHog1

in yeast cells under osmotic stress, we measured the trehalose content in the BY4741, Δ*ste11*, Δ*pbs2*, Δ*hog1* strains in response to 1 M sorbitol. As expected, the trehalose content in Δ*ste11-pFL61-RiSte11*, Δ*pbs2-pFL61-RiPbs2* and Δ*hog1-pFL61-RiHog1* strains were significantly higher than those in Δ*ste11*, Δ*pbs2*, Δ*hog1* carrying pFL61 under 1 M sorbitol condition. And the

trehalose content of the mutant strains expressing pFL61 was depressed in comparison with the control strain. While under standard conditions, the trehalose content of the Δste11-pFL61-RiSte11, Δpbs2-pFL61-RiPbs2 and Δhog1-pFL61-RiHog1 strains was not affected compared with the control (Figure 3h,i). The result indicated that Δste11 expressing RiSte11, Δpbs2 expressing RiPbs2 and Δhog1 expressing RiHog1 can complement the defect under sorbitol treatment and enhance the yeast cells' resistance to osmotic stress.

To further determine subcellular localization of the RiSte11, RiPbs2 and RiHog1 kinases in yeast cells, each of the HOG1-MAPK genes was fused in-frame with GFP were placed into the vector pUG36 under the control of the Adh-MET25 promoter. Confocal microscopy was conducted with the EY57 strain expressing each one of these three gene fusion constructs. The results showed that the GFP signals from all the three GFP-RiSte11, GFP-RiPbs2 and GFP-RiHog1 fusions displayed similar subcellular localization patterns that were in agreement with the free GFP cellular location under normal conditions. The result showed that the GFP signal from the pUG36-GFP free protein as a representative from this group was distributed throughout the cytoplasm and the nucleus of transformed cells (Figure 3j). Further subcellular localization analysis under osmotic stress (1 M sorbitol treatment) showed that GFP-RiSte11 and GFP-RiPbs2 were still localized throughout the cytoplasm, whereas the GFP-RiHog1 fusion was predominantly localized in the nucleus, indicating that RiHog1 may be localized both in the cytosol and the nucleus under standard conditions but predominately localized in the nucleus under osmotic stress. This result was further confirmed by the precise colocalization between GFP-RiHog1 and the DAPI-labelled nucleus (Figure 3k). These findings indicated that RiSte11 and RiPbs2 proteins were mainly localized in the cytosol, while RiHog1 protein was predominately localized to the nucleus in yeast cells during osmotic stress.

RiPbs2 interacts with RiSte11 or RiHog1 *in vitro*

It has been reported that the MAP kinase kinase Pbs2 interact with the MAP kinase kinase kinase Ste11 or MAP kinase Hog1 in yeast (Murakami et al., 2008; Tatebayashi et al., 2020). To investigate the potential interaction between RiSte11 and RiPbs2, RiPbs2 and RiHog1 from R. irregularis, we conducted the pull-down assay *in vitro* using a prokaryotic expression system. We purified the RiSte11, RiPbs2 and RiHog1 as a recombinant MBP-RiSte11, GST-RiPbs2, HIS-RiPbs2 and GST-RiHog1 fusion protein from Escherichia coli BL21 (DE3). As expected, the result showed that GST-RiPbs2 was pulled down by MBP-RiSte11 at about 65 kDa and HIS-RiPbs2 was pulled down by GST-RiHog1 at about 55 kDa (Figure 4). This finding confirmed that RiPbs2 interacts directly with RiSte11 or RiHog1 *in vitro*.

AM fungal HOG1-MAPK proteins (RiSte11, RiPbs2 and RiHog1) facilitate plant drought resistance during arbuscular mycorrhizal symbiosis

To further investigate the biological functions of R. irregularis HOG1-MAPK cascade genes, we generated RNA interference (RNAi) constructs using the HIGS strategy. The A. sinicus plants with hairy roots were chosen as the host to perform the plant transformation, and the positive hairy roots with red fluorescence were observed (Figure S5). Then we planted the A. sinicus seedlings with transformed roots containing the RiSte11-RNAi, RiPbs2-RNAi or RiHog1-RNAi construct. The results showed that the AM-colonized A. sinicus plants grew significantly better than

NM plants under DS, whereas the silencing of RiSte11, RiPbs2 or RiHog1 in R. irregularis negatively affected the growth performance of mycorrhizal A. sinicus to drought resistance (Figure S6). We next estimated the RWC and proline content in control and RNAi plants. Lower levels of RWC were found in the leaves of RiSte11-RNAi, RiPbs2-RNAi or RiHog1-RNAi lines than in that of control lines. By contrast, higher levels of proline content were detected in the leaves of RiSte11-RNAi, RiPbs2-RNAi and RiHog1-RNAi lines in response to DS compared with control lines (Figure S7). To conform and extend the HIGS data, we further investigated the roles of R. irregularis HOG1-MAPK cascade genes upon AM symbiosis by the VIGS assay. In the VIGS experiment, the results showed that the changes in RWC and proline content in these VIGS-mediated silenced lines had a similar trend with that in the HIGS experiment. The level of RWC was lower but the proline content is higher in the TRV2-RiSte11, TRV2-RiPbs2 or TRV2-RiHog1 plants than that in control plants under DS (Figure S8). Overall, both HIGS and VIGS results revealed that AM fungal RiSte11, RiPbs2 and RiHog1 enhanced plant drought resistance during AM symbiosis.

Arbuscular mycorrhizal fungal HOG1-MAPK proteins (RiSte11, RiPbs2 and RiHog1) are required for arbuscule development

To determine whether silencing of RiSte11, RiPbs2 or RiHog1 in R. irregularis affects the AM development under DS, we next estimated the role of RiSte11, RiPbs2 or RiHog1 in arbuscule development within plant roots. In the HIGS experiment, the expression levels of RiSte11, RiPbs2 and RiHog1 were decreased in RiSte11-RNAi, RiPbs2-RNAi or RiHog1-RNAi roots compared with in the control roots during DS (Figure 5a–c). The marker genes AsPT4 and RiMST2 also showed decreased expression in HIGS roots (Figure 5d,e). The total colonization frequency (F%) in most of RiSte11-RNAi, RiPbs2-RNAi or RiHog1-RNAi roots was slightly but not significantly lower than that in control lines, whereas the mycorrhiza intensity (M%) and arbuscule abundance (A%) of RiSte11-RNAi roots were reduced to average 32% and 15%, RiPbs2-RNAi roots were reduced to average 25% and 9%, RiHog1-RNAi roots were reduced to average 24% and 8%, respectively (Figure 5f–h). The arbuscules in control roots were mature with full branches. By contrast, most of the arbuscules in the RiSte11-RNAi, RiPbs2-RNAi and RiHog1-RNAi roots were severely collapsed or even dead (Figure 5i). The decreased abundance of arbuscules in the RiSte11-RNAi (or RiPbs2-RNAi, RiHog1-RNAi) lines was consistent with the expression levels of symbiotic marker genes AsPT4 and RiMST2 in A. sinicus and R. irregularis, respectively. Consistently, the expression levels and mycorrhizal colonization were also significantly reduced in the HIGS roots compared with the control roots under WW condition (Figure S9). This indicated that RiSte11, RiPbs2 and RiHog1 played a positive role in AM fungal root colonization.

In the VIGS experiment, the qRT-PCR was performed to estimate the transcripts of AM fungal RiSte11, RiPbs2, RiHog1 and AM-specific marker genes NbPT4 and RiMST2 (Helber et al., 2011; Wang et al., 2018). Under DS, transcript levels of RiSte11, RiPbs2 and RiHog1 were significantly reduced in the TRV2-RiSte11, TRV2-RiPbs2 and TRV2-RiHog1 roots, respectively, compared with the control roots (Figure 6a–c). Similarly, the expression levels of NbPT4 and RiMST2 were repressed in TRV2-RiSte11, TRV2-RiPbs2 and TRV2-RiHog1 roots, respectively, in relative to the control roots (Figure 6d,e). There was no significant difference in F% between the VIGS and control roots. However,
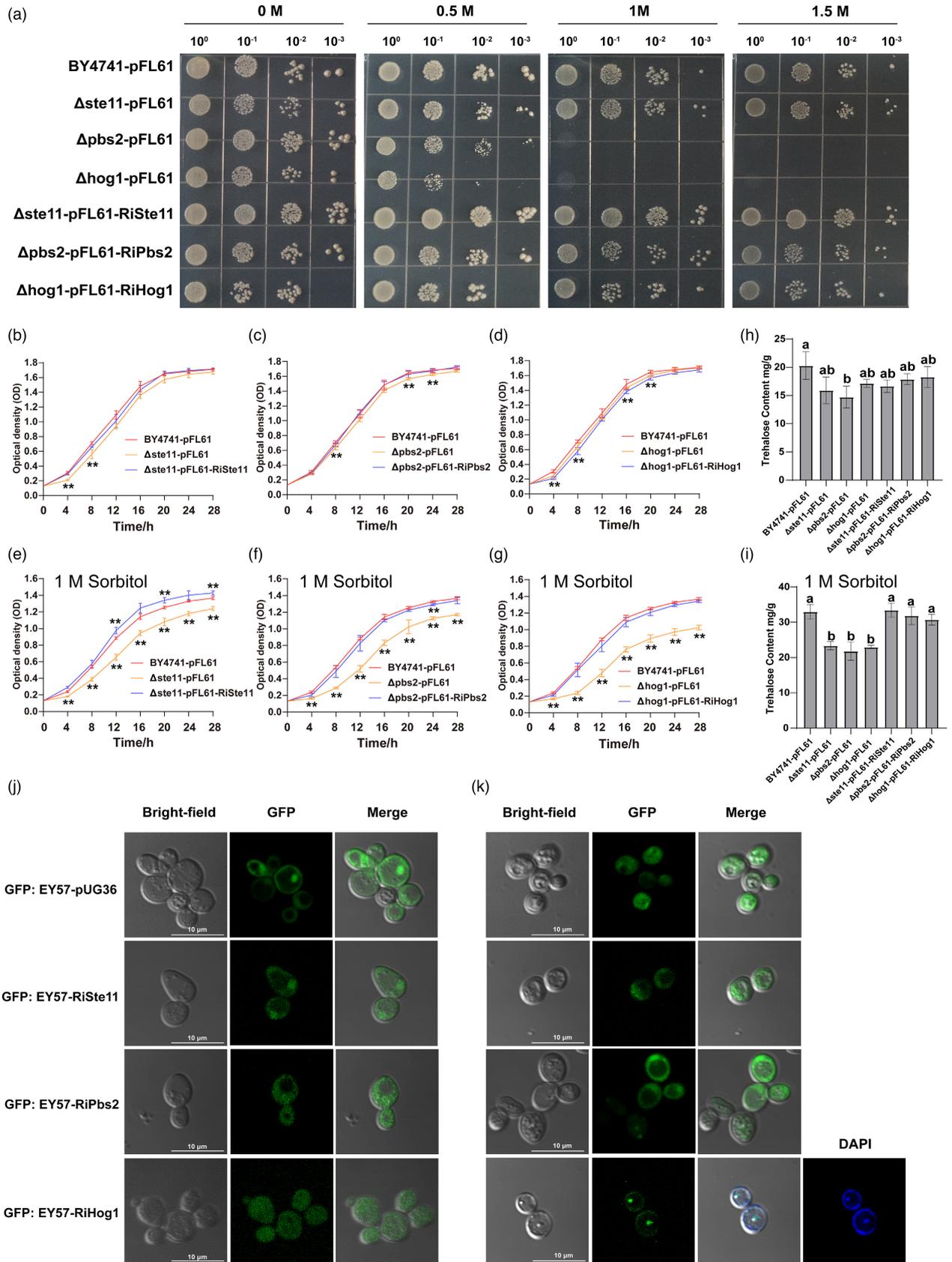
Figure 3 Functional characterization of RiSte11, RiPbs2 and RiHog1 in *Saccharomyces cerevisiae*. (a) Functional complementary analysis of yeast strains Δ*ste11*, Δ*pbs2*, Δ*hog1* expressing RiSte11, RiPbs2, RiHog1, respectively. They grow in the selective dropout (SD/-URA) medium containing different sorbitol concentrations. For the test, cells were grown to logarithmic phase and diluted to an OD_{600} of 0.2, then ten-fold serial dilutions of cells were plated on the SD/-URA solid medium containing 0, 0.5, 1 or 1.5 M sorbitol, respectively. (b–g) Growth performances of Δ*ste11-pFL61*, Δ*pbs2-pFL61*, Δ*hog1-pFL61*, Δ*ste11-pFL61-RiSte11*, Δ*pbs2-pFL61-RiPbs2*, Δ*hog1-pFL61-RiHog1* strains in the liquid medium containing 1 M sorbitol. The data represents the mean of three biological replicates ± SE, based on ANOVA-Tukey's test. Asterisks showed the difference compared with the BY4751-pFL61 strain, *$P < 0.05$, **$P < 0.01$. (h,i) Trehalose contents of transformed yeast BY4741 cells carrying pFL61, or complementary RiSte11, RiPbs2 or RiHog1 in response to non-sorbitol (h) or 1 M sorbitol (i). The data represents the mean of three biological replicates ± SE, based on ANOVA-Tukey's test. The different letters indicate significant statistical differences between treatments at $P < 0.05$. (j–k) Subcellular localization of the free GFP (EY57-pUG36), GFP-RiSte11, GFP-RiPbs2 and GFP-RiHog1 fusions in yeast cells in response to standard growth conditions (j) or medium containing 1 M sorbitol (k). The bright-field views are exhibited in the left panels, while the GFP fluorescence images of each GFP-protein fusions are present in the middle panels. The blue fluorescence marker DAPI shows the position of the nucleus. The right panels indicate the bright-field and GFP or DAPI merged images. Scale bars, 10 μm.

the *TRV2-RiSte11*, *TRV2-RiPbs2* and *TRV2-RiHog1* roots exhibited much lower M% and A% than the control roots (Figure 6f–h). The mature arbuscules were observed in the control roots, whereas the most arbuscules were severely impaired and collapsed (with many septa) in *TRV2-RiSte11*, *TRV2-RiPbs2* and *TRV2-RiHog1* roots (Figure 6i). And there was also a significant difference in AM development between the *TRV2-RiSte11*, *TRV2-RiPbs2*, *TRV2-RiHog1* and control lines under WW condition (Figure S10). Furthermore, based on the phylogenetic analysis of *A. thaliana* and other plants MAPKs (Chen et al., 2021; Lin et al., 1999), we found several MAPK-related genes in *Nicotiana benthamiana*, and they are named for *NbMAPKKK-1*, −2, *NbMKK1*, 3, 5, and *NbMPK3*, 6, 9 for expression analysis. We noticed that the knock-down of *RiSte11*, *RiPbs2* or *RiHog1* in *R. irregularis* did not affect the expression of MPAK-related genes in *N. benthamiana* (Figure S11). Taken together, these results revealed that these three HOG1-MAPK genes were required for arbuscule development.

Host-induced HOG1-MAPK genes silencing inhibits the expression of drought-resistant genes in arbuscular mycorrhizal fungal symbiont

To investigate the regulatory roles of *R. irregularis* HOG1-MAPK genes during AM symbiosis under DS, we analysed the expression profiles of drought-resistant genes mediated by the HOG1-MAPK

cascades in *R. irregularis*. According to the previous studies (Iturriaga et al., 2009; Li et al., 2013; Ocón et al., 2007; Sun et al., 2018), we essentially identified the AM fungal AQPs (RiAQP1 and RiAQP2), trehalose synthases (RiTPS1 and RiTPS2), neutral trehalase (RiNTH1) (Figure S3 and Table S1) and Ri14-3-3 protein-encoding genes, which are DS-inducible genes in *R. irregularis*. Accordingly, the transcript levels of *RiAQP1*, *RiAQP2*, *RiTPS1*, *RiTPS2 RiNTH1* and *Ri14-3-3* were significantly up-regulated in mycorrhizal roots under 25% FWC (Figure S12). Therefore, we further measured the expression levels of the above six drought-resistant genes in the *RiSte11-RNAi*, *RiPbs2-RNAi*, *RiHog1-RNAi* and control roots of HIGS experiment. The results showed that in relative to the control, the expression levels of *RiAQP1* and *Ri14-3-3* in HIGS roots were not affected significantly under WW condition (Figure 7a,f). While it showed significant reductions in gene expressions of *RiAQP2* and *RiNTH1* in both *RiPbs2-RNAi* and *RiHog1-RNAi* lines, *RiTPS1* and *RiTPS2* in *RiPbs2-RNAi* line under WW condition compared with their respective controls (Figure 7b–e). Furthermore, the expression levels of these all six genes were obviously down-regulated in the *RiSte11-RNAi*, *RiPbs2-RNAi* or *RiHog1-RNAi* lines compared with the control lines in response to drought (Figure 7g–l). In conclusion, the expression analysis indicates that AM fungal HOG1-MAPK cascade genes regulate the downstream drought-resistant genes.

Figure 4 RiPbs2 interacts with RiSte11 or RiHog1 *in vitro*. The pull-down assay shows a direct interaction between RiSte11 and RiPbs2, RiHog1 and RiPbs2 *in vitro*, respectively. Anti-MBP, anti-HIS and anti-GST antibodies were used in the immunoblotting analysis.

Figure 5 Host-induced gene silencing of HOG1-MAPK cascade genes in *Rhizophagus irregularis* inhibits arbuscule development in roots under drought stress. (a–h) Expression levels of *R. irregularis RiSte11* (a), *RiPbs2* (b), *RiHog1* (c), symbiosis-specific marker genes *AsPT4* (d) and *RiMST2* (e) in control and *RiSte11-RNAi*, *RiPbs2-RNAi* or *RiHog1-RNAi* lines. *A. sinicus AsActin* and *R. irregularis RiEF1a* genes were used as the reference genes for normalization, respectively. (f–h) Mycorrhizal colonization levels in control and *RiSte11-RNAi*, *RiPbs2-RNAi* or *RiHog1-RNAi* roots with *R. irregularis* after the WGA488 staining. F%, total frequency of colonization; M%, intensity of mycorrhiza; A%, arbuscule abundance. Error bars represent the means of five biological replicates with SE values. The different letters indicate significant statistical differences between treatments at $P < 0.05$, based on one-way ANOVA, Tukey's test. (I) Laser-scanning confocal microscopy images of *R. irregularis* in control and *RiSte11-RNAi*, *RiPbs2-RNAi* or *RiHog1-RNAi* roots after the WGA488 staining. ma, mature arbuscules; ca, collapsed arbuscule; da, degenerating arbuscules; ih, intraradical hyphae. Scale bars are shown in the images.

Discussion

It has been affirmed that AM fungi can alleviate the negative effects of drought by regulating plant physiological and molecular responses (Püschel *et al.*, 2021; Zhang *et al.*, 2020). Our results also confirmed that AM symbiosis has the ability to improve host performance under DS. As a result, mycorrhizal plants might acquire more nutrition and promote the transport of water exposed to DS (Gholamhoseini *et al.*, 2013; Hazzoumi *et al.*, 2015; Hu *et al.*, 2020; Leyva-Morales *et al.*, 2019). Fungal MAPK cascades serve as protein kinases, which have significant importance in connecting external stress signals with cellular responses and downstream gene expression (Kou and Naqvi, 2016). In this study, we investigated the biological functions of HOG1-MAPK cascade genes that operated in *R. irregularis* during AM symbiosis under DS.

Many studies have shown that plant MAPK cascade improves plant drought resistance (Zhu *et al.*, 2020, 2021), but the mechanism of AM fungal MAPK cascade in promoting plant drought resistance is rarely reported. Although recent studies show the potential correlation between AM fungal and plants MAPK cascades (Huang *et al.*, 2020; Liu *et al.*, 2015b), there is no evidence to prove the causal relations between AM fungal and plant MAPK enhancing mycorrhizal plants' drought tolerance. These allowed us to propose that AM fungi might regulate the plant drought-stress response through the activation of MAPK proteins. The HOG1-MAPK cascade is conserved in fungi including yeast and other pathogenic fungi to activate responses to
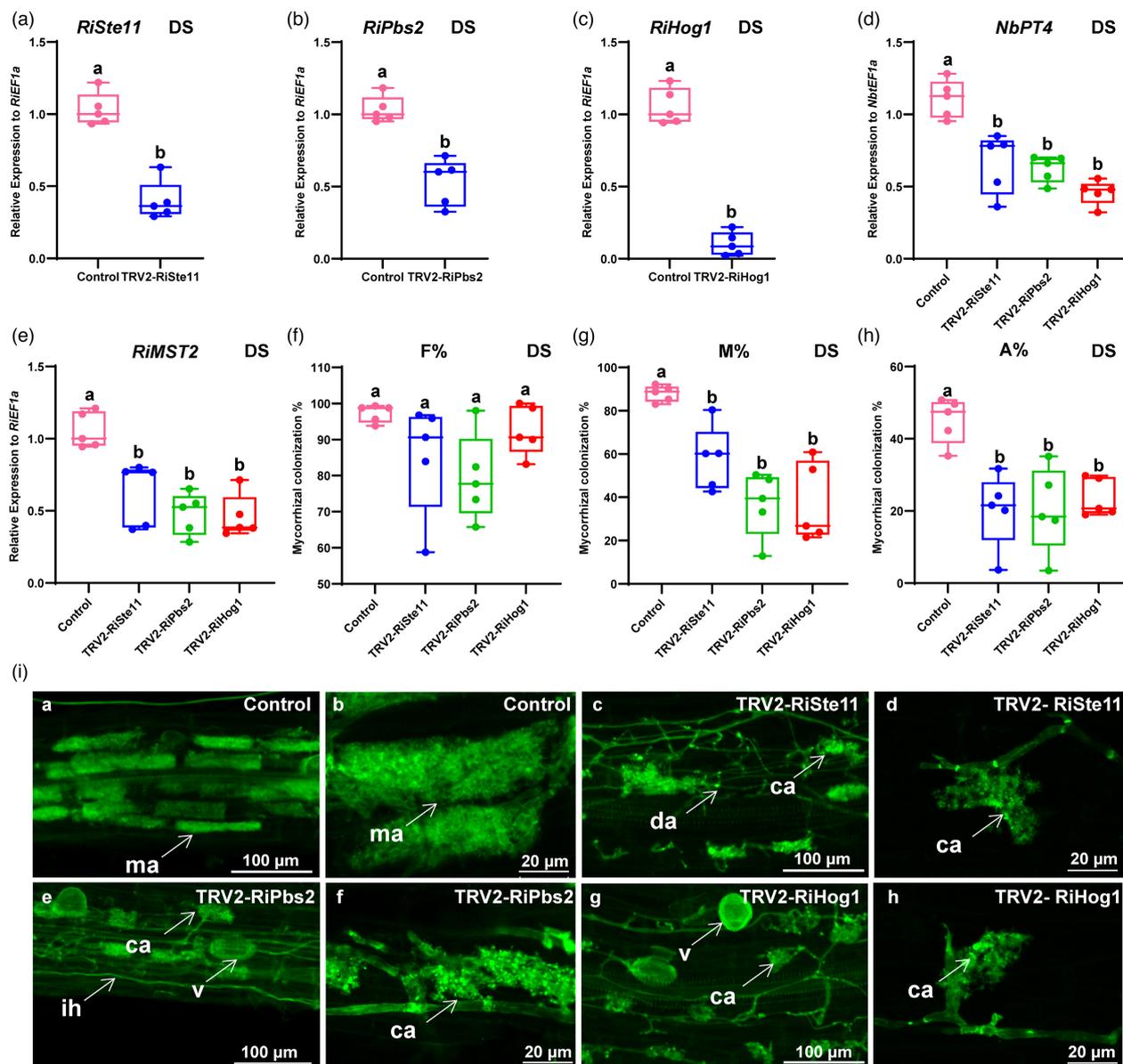
Figure 6 Molecular and arbuscular mycorrhizal phenotypes of virus-induced gene silencing of *RiSte11*, *RiPbs2* or *RiHog1* in *Nicotiana benthamiana* under drought stress. Expression levels of *RiSte11* (a), *RiPbs2* (b), *RiHog1* (c), symbiosis-specific marker genes *NbPT4* (d) and *RiMST2* (e) in control and *TRV2-RiSte11*, *TRV2-RiPbs2* or *TRV2-RiHog1* roots with *Rhizophagus irregularis*. The *N. benthamiana NbtEF1a* and *R. irregularis RiEF1a* genes were used as the reference genes for normalization, respectively. (f–h) Mycorrhization colonization levels of the control and *RiSte11*-silenced (*TRV2-RiSte11*), *RiPbs2*-silenced (*TRV2-RiPbs2*) or *RiHog1*-silenced (*TRV2-RiHog1*) *N. benthamiana* roots with *R. irregularis*. F%, total frequency of colonization; M%, intensity of mycorrhiza; A%, arbuscule abundance. Error bars represent the means of five biological replicates ± SE. The different letters indicate significant statistical differences between treatments at $P < 0.05$, based on one-way ANOVA, Tukey's test. I. Arbuscule phenotype of *R. irregularis* in control and *TRV2-RiSte11*, *TRV2-RiPbs2* or *TRV2-RiHog1* roots. Laser-scanning confocal microscopy images of *R. irregularis* in control and *TRV2-RiSte11*, *TRV2-RiPbs2*, *TRV2-RiHog1* roots after the WGA488 staining. ca, collapsed arbuscule; da, degenerating arbuscules; ih, intraradical hyphae; ma, mature arbuscules; v, vesicle. Scale bars are as shown in the images.

different stress signals (Hamel *et al.*, 2012). We found the three HOG1-MAPK cascades (*RiSte11*, *RiPbs2* and *RiHog1*) had similarities with other known HOG1-MAPK homologues from closely related fungal species. These indicated the HOG1-MAPK cascade may have a similar function across fungal species that respond to abiotic stress (Martínez-Soto and Ruiz-Herrera, 2017). The development of AM symbiosis consists of different stages through the progression of AM fungal hyphae during root colonization

(Parniske, 2008). During arbuscule formation, the hypha enters the root and grows into the cortex cells where arbuscules was established (Gutjahr and Parniske, 2013; Harrison, 2012). Arbuscules are considered to be the primary sites for nutrient exchange between the host and AM fungi (Bonfante and Genre, 2010). Our study also showed that *RiSte11*, *RiPbs2* and *RiHog1* genes were expressed during the *in planta* phase. Based on these findings, we propose that HOG1-MAPK cascade may regulate plants'
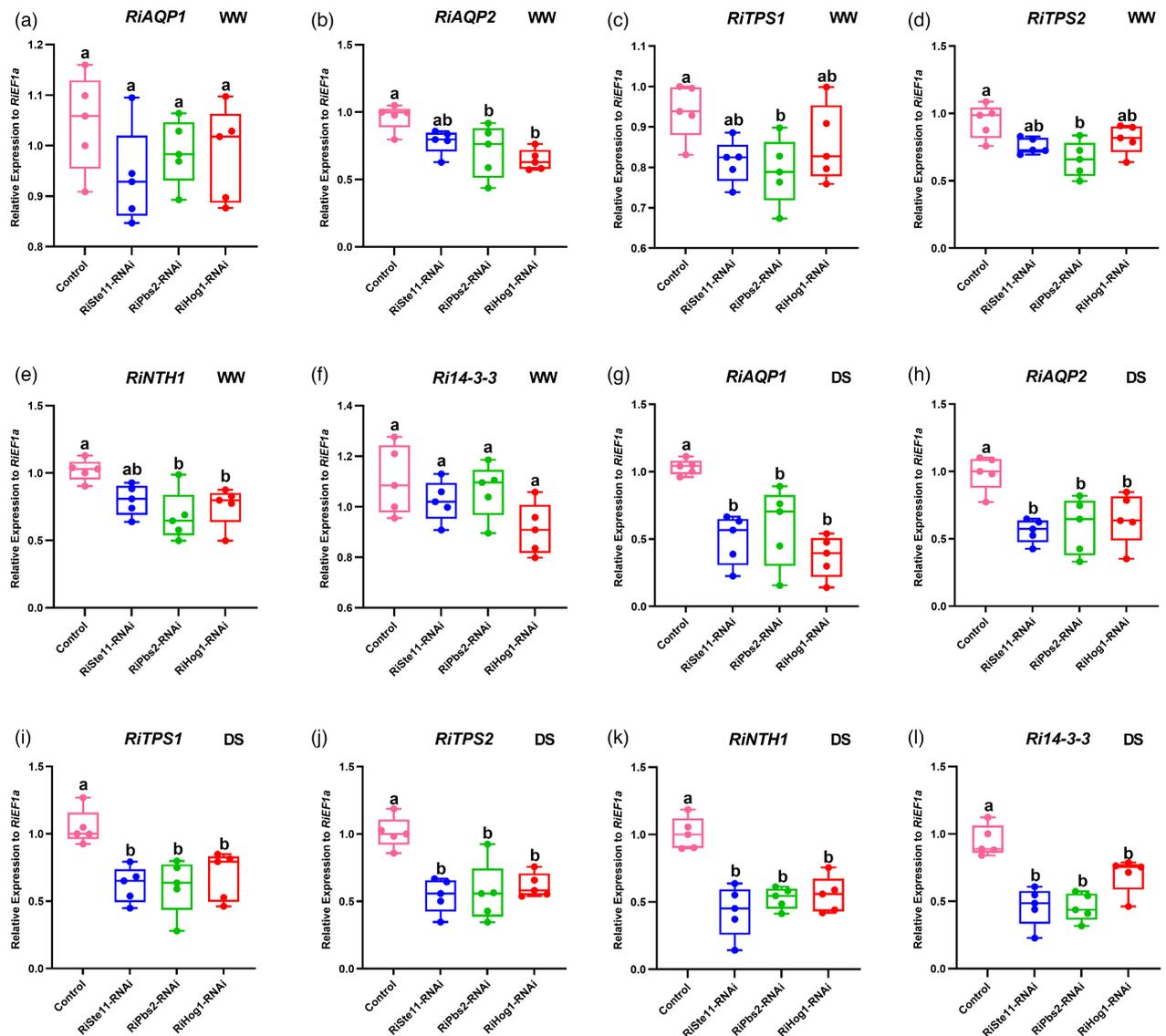
Figure 7 Knock-down of *RiSte11*, *RiPbs2* or *RiHog1* via host-induced gene silencing affects the expression levels of downstream genes *RiAQP1/2*, *RiTPS1/2*, *RiNTH1*, *Ri14-3-3*. The downstream genes of HOG1-MAPK cascade are identified to be *AQP* (encoding Aquaporin), *TPS* (encoding Trehalose-6-phosphate synthase), *NTH* (encoding Neutral trehalase) and *Ri14-3-3* (Sun et al., 2018). (a–f) Expression levels of *RiAQP1*, *RiAQP2*, *RiTPS1*, *RiTPS2*, *RiNTH1* and *Ri14-3-3* in control, *RiSte11-RNAi*, *RiPbs2-RNAi* or *RiHog1-RNAi* roots of *Astragalus sinicus* with *Rhizophagus irregularis* under well water condition. (g–l) Expression levels of *RiAQP1*, *RiAQP2*, *RiTPS1*, *RiTPS2*, *RiNTH1* and *Ri14-3-3* in control, *RiSte11-RNAi*, *RiPbs2-RNAi* or *RiHog1-RNAi* roots of *A. sinicus* with *R. irregularis* under drought stress. *R. irregularis* RiEF1a was used as the reference gene for normalization. Error bars represent the means of five biological replicates ± SE. The different letters indicate significantly statistical differences between treatments at $P < 0.05$, based on one-way ANOVA, Tukey's test.

drought tolerance at the symbiotic interface of arbuscular mycorrhizas. Previous research showed DS up-regulated the levels of AM fungal MAPK transcripts in mycorrhizal plant roots (Liu et al., 2015b) and, thus, supported our results (Figure 2i–k). Therefore, we hypothesized that the AM fungal HOG1-MAPK cascade is necessary in response to plant-fungus AM symbiosis to drought.

Furthermore, EhHOG1 from fungus *Eurotium herbariorum* and PiHOG1 from *Piriformospora indica* can complement the growth defect of the yeast mutant Δ*Hog1* strain under high osmotic stress conditions (Jin et al., 2005; Jogawat et al., 2016). Mutants of Ste11, Pbs2 and Hog1 also exhibited elevated sensitivity to osmotic stress in yeast (Alonso-Monge et al., 2001). Considering

our results, it can, thus, be said RiSte11, RiPbs2 and RiHog1 of *R. irregularis* play important roles in response to drought. ScHog1 in *S. cerevisiae* and PpHog1 in *Pichia pastoris* can translocate between the cytoplasm and nucleus in response to osmolarity environments, which is an important mechanism of activating or repressing their target genes (Reiser et al., 1999; Wang et al., 2021). Our findings imply the importance of nucleus-localized RiHog1 protein in AM fungal symbiont in response to DS. Moreover, the pull-down result in our study was in accordance with the interaction of Ste11 with Pbs2, Pbs2 with Hog1 in yeast (Murakami et al., 2008; Tatebayashi et al., 2020). The importance of Pbs2 and Hog1 kinases in regulating stress tolerance has been demonstrated in different kinds of fungi

(Arana *et al.*, 2005; Day *et al.*, 2018; Jogawat *et al.*, 2016; Liu *et al.*, 2017). The results showed the potential role of RiHog1 in receiving drought signal from RiSte11 to RiPbs2 and transmitting the signal into the nucleus. According to previous studies, RiSte11 also could receive the signal from DS sensor sho1, then rely on it to the MAP Kinase Kinase Pbs2, which could transmit the signal to Hog1 in yeast cells (Hamel *et al.*, 2012; Zarrinpar *et al.*, 2004). In addition, whether the phosphorylation mechanism exists among these three HOG1-MAPK proteins needed to be further studied.

The importance of the HOG1-MAPK cascade in regulating plant root colonization/infection, and stress tolerance has been demonstrated in beneficial and pathogenic fungi (Jogawat *et al.*, 2016; Tian *et al.*, 2016). Nevertheless, the functions of *RiSte11*, *RiPbs2* and *RiHog1* during AM development are still unclear. In this study, the VIGS or HIGS of *RiSte11*, *RiPbs2* or *RiHog1* within roots colonized with *R. irregularis* showed collapsed arbuscules and hyphae with septa under DS (Figures 5 and 6), indicating the significance of *RiSte11*, *RiPbs2* and *RiHog1* for AM development. It is proposed the osmosis stress transmit by HOG1-MAPK cascade acts as a signal to the AM fungal symbiont developing within plant roots. This view is consistent with the previous research that AM fungal 14–3-3 protein is involved in arbuscule formation under abiotic stress (Sun *et al.*, 2018). The HOG1-MAPK cascade may affect the arbuscule development by regulating *Ri14-3-3* gene. Moreover, the expression levels of MAPK-related genes in *N. benthamiana* were not affected in the RNAi lines (Figure S11), coupled with that Xie *et al.* (2022) confirmed that the non-target gene (similar to the targeted gene) expression was not suppressed in the targeted RNAi lines. Therefore, it is reasonable to believe that silencing of *RiSte11*, *RiPbs2* and *RiHog1* did not affect the non-target genes of plants and other microorganisms. Knock-down of HOG1-MAPK cascade genes in *R. irregularis* also affected plants' physiological parameters, indicating that silencing of *RiSte11*, *RiPbs2* or *RiHog1* influenced the coordinating regulation of *R. irregularis*-plants symbiosis in response to DS.

It has been demonstrated that AQPs, trehalose-6-phosphate synthase (TPS) and neutral trehalase (NTH) genes are important factors in response to osmotic stress in plant–microorganism interactions (Iturriaga *et al.*, 2009; Li *et al.*, 2013, 2017; Ocón *et al.*, 2007). For example, TPS and neutral trehalase (NTH1) genes could improve plant resistance to osmotic stress (Acosta-Pérez *et al.*, 2020; Liu *et al.*, 2015a; Ocón *et al.*, 2007). Li *et al.* (2013) cloned two functional AQP genes from *G. intraradices* and proposed MAPK cascade may initiate gene expression to cope with osmotic stress. Our study confirmed that *RiSte11*, *RiPbs2* and *RiHog1* genes could regulate the expressions of drought-resistant genes in *R. irregularis* in response to DS (Figure 7), suggesting AM fungal HOG1-MAPK cascade genes improve plants' drought tolerance by regulating drought-resistant genes. Although the expression levels of *RiAQP2*, *RiTPS1/2* and *RiNTH1* were also decreased in some *RiSte11-RNAi* or *RiPbs2-RNAi* lines compared with the control under WW condition, it can be explained that the regulation of HOG1-MAPK cascade and downstream drought-resistant gene may incompletely depend on DS. However, further research about this phenomenon is required.

Based on our findings, we propose a working model in which *R. irregularis* HOG1-MAPK cascade regulates the drought-resistant genes in response to DS (Figure 8) to maintain A< development. Under DS, *A. sinicus* and AM fungus *R. irregularis*

can establish symbiosis. Higher expression levels of *RiSte11* (MAPKKK), *RiPbs2* (MAPKK) and *RiHog1* (MAPK) were detected during AM symbiosis under DS, indicating RiSte11 may be activated by stimulated receptor (or related kinase) and then relays signal to RiPbs2, leading to the activation of RiHog1 through direct protein–protein interaction (Hamel *et al.*, 2012). As a result, MAP kinase RiHog1 enters the nucleus and further transmits the signal to the putative transcription factor (TF) to activate the expression of downstream drought-resistant genes (such as *RiAQP1*, *RiAQP2*, *RiTPS1*, *RiTPS2*, *RiNTH1* and *Ri14-3-3*). The knock-down of *RiSte11*, *RiPbs2* and *RiHog1* leads to decreased expressions of drought-resistant-related genes and collapse arbuscules, suggesting that *R. irregularis* HOG1-MAPK cascade proteins are essential for arbuscule development under DS. In future studies, functions of the potential sensor RiSho1 and its putative interactions RiCDC42/RiSte20 in response to DS should be characterized (Hamel *et al.*, 2012). It is important to confirm how the three HOG1-MAPK proteins transmit stress signals, and whether the RiHog1 directly interacts with the potential TF (such as Msn2) to control downstream drought-resistant-related genes (Sun *et al.*, 2018).

In conclusion, we identified three HOG1-MAPK cascade genes, *RiSte11*, *RiPbs2* and *RiHog1* from AM fungus *R. irregularis*. The three HOG1-MAPK cascade genes showed high expression levels during mycorrhizal symbiosis and they were induced by DS. Based on the VIGS and HIGS results, it was affirmed that AM fungal HOG1-MAPK cascade genes were essential for arbuscule development and contributed to plant tolerance to DS. Importantly, the knock-down of HOG1-MAPK genes resulted in the decreased expression of drought-resistant genes in AM fungus. Our results provide physiological and molecular evidence for AM fungus improving plant drought tolerance as well as insights into AM fungal HOG1-MAPK cascade regulating arbuscule development under DS.

Experimental procedures

Biological materials and growth conditions

The AM fungus used in this study is *R. irregularis* DAOM197198, which was kindly provided by Dr. Jianyong An (Huazhong Agricultural University, Wuhan, China). Spores of *R. irregularis* are propagated on *Trifolium repens* L.

Astragalus sinicus L. was used in the gene expression analysis and the HIGS experiment. For the time-course experiment, the plant roots colonized with *R. irregularis* were harvested at 14-, 21-, 28-, 35-, 42-, 49- and 56-dpi (days post inoculation). Tobacco (*N. benthamiana*) was used in this study for the VIGS experiment. The seedlings of *A. sinicus* or *N. benthamiana* were inoculated with ~500 germinating spores/seedlings. The pot cultures contained a vermiculite and sand mix (v/v, 1:3). Plants were grown in controlled conditions, with a temperature of 24°C/18°C Day/night, 16/8-h light/dark cycle and relative humidity of 65%. All plants were watered twice per week with a modified Long-Ashton (mLA) nutrient solution containing 30 µM NaH_2PO_4 (Hewitt, 1966).

Yeast strains, wild-type BY4741 (Euroscarf acc. num. Y00000: MATα; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*); Δste11 (Euroscarf acc. num. Y05271: BY4741; MATα; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*; YLR362w::kanMX4); Δpbs2 (Euroscarf acc. num.Y07101: BY4741; MATα; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*; YJL128c::kanMX4); Δhog1 (Euroscarf acc. num. Y02724: BY4741: MATα; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*; YLR113w::kanMX4).
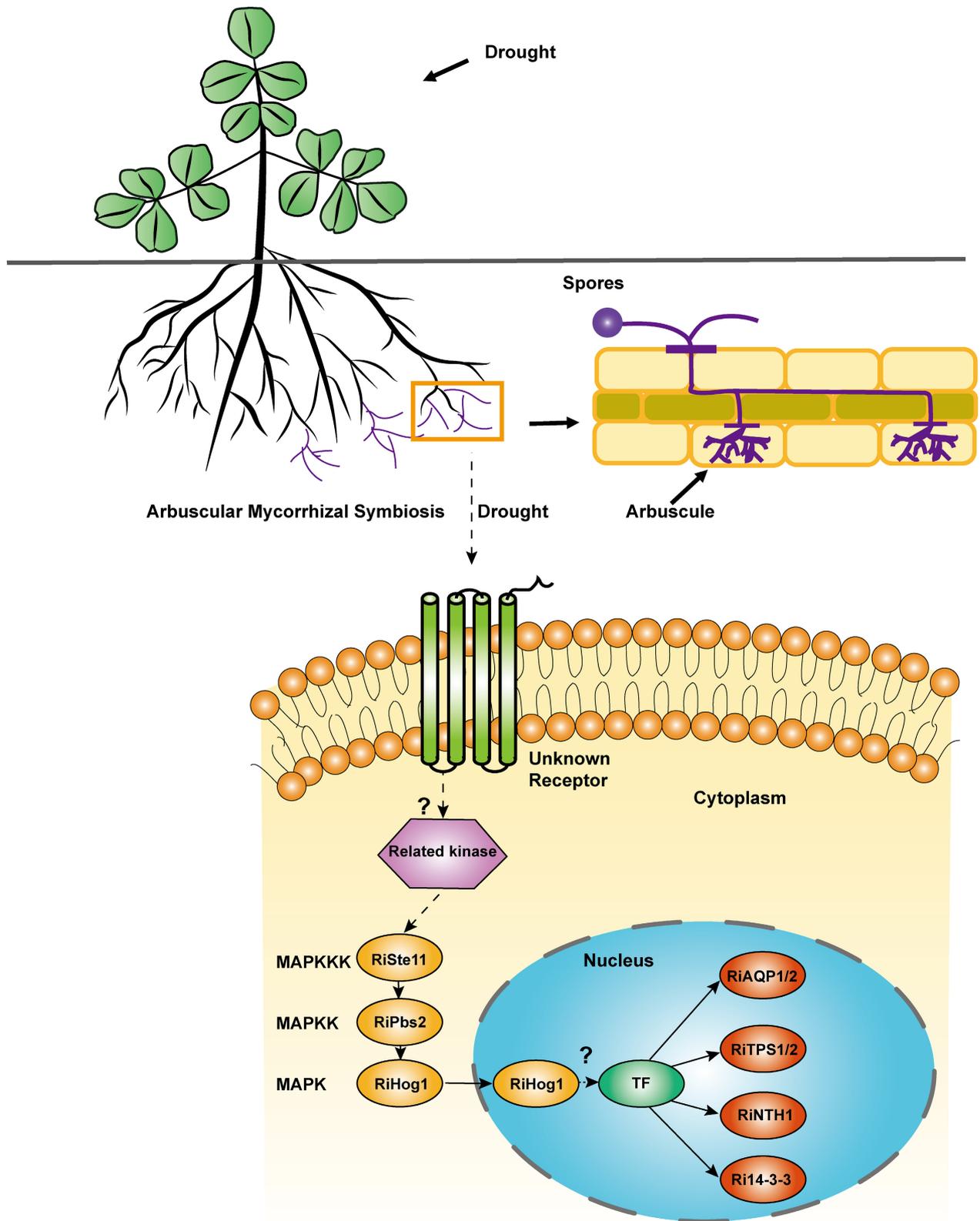
Figure 8 Proposed working model in which the *Rhizophagus irregularis* HOG1-MAPK cascade regulates arbuscule development and drought-resistant genes during AM symbiosis in response to drought stress. The picture shows that *Astragalus sinicus* is colonized with *R. irregularis* to establish a symbiosis relationship. In *R. irregularis*, the perception of the drought stress signal via the unidentified receptor, which may transmit the signal to the MAP kinase cascade through related kinases (homologues Cdc42 GTPase and Ste20 PAK-like kinase in yeasts) (Hamel *et al*., 2012). Higher expression levels of *RiSte11* (MAPKKK), *RiPbs2* (MAPKK) and *RiHog1* (MAPK) were detected during AM symbiosis under drought stress, indicating RiSte11 may be activated by stimulated receptor (or related kinase) and then relays the signal to RiPbs2, leading to the activation of RiHog1 through direct protein–protein interaction. As a result, MAP kinase RiHog1 enters the nucleus and further transmits the signal to the putative transcription factor (TF) to activate the expression of downstream drought-resistant genes (such as *RiAQP1*, *RiAQP2*, *RiTPS1*, *RiTPS2*, *RiNTH1* and *Ri14-3-3*).

Water treatment

In the pot system, the *A. sinicus* seedlings were colonized with *R. irregularis* and grown under normal water conditions for 28 days. Then plants were treated by 75% FWC, 50% FWC and 25% FWC for additional 14 days, respectively. In the HIGS and VIGS experiments, the plants were inoculated without (NM) or with *R. irregularis* (AM) for 28 days, then treated by WW (75% FWC) or DS (25%FWC) for additional 14 days. All plants were collected at 42 dpi and then stored at −80°C for subsequent analyses.

Genomic and transcriptomic data analyses of *Rhizophagus irregularis*

The *R. irregularis* HOG1-MAPK cascade gene sequences *RiSte11*, *RiPbs2* and *RiHog1* were obtained from the DDBJ and GEO databases (Morin *et al*., 2019; Zeng *et al*., 2018, 2020). DESeq2-normalized expression data of *RiSte11*, *RiPbs2*, *RiHog1*, *RiAQP1/2*, *RiTPS1/2*, *RiNTH1*, at six different developmental stages of *R. irregularis*, were plotted in heatmaps for comparison. Heatmaps were generated with the CLC Genomics Workbench (v20) software. Expression values (FPKM) calculation and expressed gene analysis (FDR-corrected $P < 0.05$, Benjamini–Hochberg test) were performed as described in Morin *et al*. (2019). The details are described in supporting methods S1.

Gene expression analysis

Total RNA was extracted from different tissues of *R. irregularis* and mycorrhizal roots using Fungal RNA Kit (OMEGA-R6840) according to the manufacturers' instructions, and the first-strand cDNA synthesis was initiated with the HiScript III reverse transcriptase (Vazyme-R323-01, Nanjing, China). Real-time qRT-PCR reactions were performed in a 96-well Real-time PCR system (Bio-Rad). All the reactions were performed with three technical replicates of three biological replicates. The *R. irregularis* *RiEF1a*, *A. sinicus* *AsActin*, *N. benthamiana* *NbtEF1a* were used as the endogenous reference gene for normalization, respectively. Relative expression levels of target genes were computed by the $2^{-\Delta\Delta Ct}$ method of relative quantification. A list of gene-specific primers used for real-time qRT-PCR is given in Table S2.

Heterologous expression in yeast

The open reading frames of *RiSte11*, *RiPbs2* or *RiHog1* were inserted into the yeast expression vector pFL61 (Minet *et al*., 1992). The resulting plasmid *pFL61-RiSte11*, *pFL61-RiPbs2* or *pFL61-RiHog1* was transformed into yeast mutant Δ*ste11*, Δ*pbs2*, Δ*hog1* strains via PEG/LiAc-mediated transformation, respectively (Gietz and Schiestl, 2007). The transformants adjusted to an OD₆₀₀ of 0.2. Subsequently, serial dilutions (×10, ×100, ×1000 and ×10 000) were plated on SD/-URA

solid medium containing 0.0, 0.5, 1.0 or 1.5 M sorbitol, and 2% glucose was added as the carbon source. Growth was recorded after 3 days at 30°C.

For yeast growth analysis under osmotic stress, overnight cultures of different transformed cells in the SD/-URA liquid medium. The starting inoculation was adjusted to an OD₆₀₀ of 0.12. The cells were incubated in SD/-URA liquid medium containing 1 M sorbitol at 30°C in a shaking incubator (200 rpm), during which samples were taken every 4 h. Each growth assay was replicated independently three times.

The subcellular localization in yeast was performed by fusing the N-terminus of RiStell, RiPbs2 or RiHog1 to a green fluorescent protein (GFP). The ORFs of *RiSte11*, *RiPbs2* and *RiHog1* were inserted into the yeast expression vector pUG36 carrying the GFP reporter gene (Niedenthal *et al*., 1996). The resulting plasmid *pUG36-RiStel1*, *pUG36-RiPbs2* or *pUG36-RiHog1* was transformed into EY57 cells as described above.

Virus-induced gene silencing

Tobacco rattle virus (TRV)-based expression vectors pTRV1 and pTRV2 were used in the experiment as described previously (Zhang and Liu, 2014). A 228 bp of *RiSte11* at 5′ end or a 235 bp of *RiSte11* at 3′ end, a 262 bp fragment of *RiPbs2* at 5′ coding region or 287 bp of *RiPbs2* at 3′ end, a 338 bp of the *RiHog1* cDNA at 5′ end or a 241 bp fragment of *RiHog1* cDNA at 3′ end was, respectively, PCR-amplified using primers (Table S2), the specific regions are shown in Figure S13. The three amplified target fragments were inserted into the pTRV2. The resulting plasmids, *pTRV2-RiSte11*, *pTRV2-RiPbs2*, *pTRV2-RiHog1* and pTRV1 were transformed into *Agrobacterium tumefaciens* GV3101, respectively. The 6-leaf-stage *N. benthamiana* plants were selected to infiltrate the re-suspended *Agrobacterium* culture into leaves. After 2 weeks of inoculation, plant roots and leaves were harvested to conduct subsequent experiments.

Host-induced gene silencing

The gateway vectors (Invitrogen) were used for the HIGS experiment. Constructs were created as described by Sun *et al*. (2018). The specific region of *RiSte11*, *RiPbs2* and *RiHog1* are designed as the VIGS experiment. The amplified fragments were recombined into the entry vector pDONR221 (Invitrogen), then the LR reaction was recombined the target sequence into the destination vector pK7GWIWG2D(II)-RootRed (Limpens *et al*., 2004) according to the instructions in Gateway protocol. The corresponding Cheap-RNAi empty vector was used as the control. The *Agrobacterium rhizogenes* K599 carrying the resulting plasmids was used for the generation of *A. sinicus* hairy roots. Red fluorescence from the DsRed reporter was detected in the transgenic hairy roots using a fluorescence stereomicroscope

(Nikon, SMZ18). The hairy roots with strong red fluorescence were selected for subsequent experiments.

In vitro pull-down experiment

For the pull-down assay, the full-length CDS of *RiSte11* was fused to pETMALc-H, *RiPbs2* was fused to pET-32a and pGEX, RiHog1 was fused to pGEX to generate MBP-RiSte11, His-RiPbs2, GST-RiPbs2 and GST-RiHog1 constructs. Recombinant proteins were separately expressed in *Escherichia coli* DE3 cells (Transgen, China) by growing cultures to OD_{600} = 0.6 at 20°C. And recombinant proteins were induced with 0.2 mM IPTG for 12 h. Purified MBP-RiSte11, MBP (negative control) proteins were incubated with MBP amylose beads (New England Biolabs, E8035) and GST-RiHog1, GST (negative control) proteins were incubated with GST amylose agarose beads (Thermo Scientific, 78 601) at 4°C for 1 h. After washing the beads five times with PBS buffer, GST-RiPbs2 with MBP-RiSte11 or MBP, His-RiPbs2 with GST-RiHog1 or GST were pulled down at 4°C for 2 h. Then the bound proteins were boiled in 5× SDS sample buffer. Samples were subjected to the subsequent separation on an SDS–PAGE gel and immunoblotting analysis using anti-MBP (Abmart, M20051), anti-His (Abmart, M20001) and anti-GST (Abmart, M20007) antibodies as described in Situ *et al*. (2020).

Quantification of mycorrhizal colonization

AM roots were digested in 10% KOH (w/v) at 90°C for 30 min, washed in 2% HCl (v/v) for 10 min and then washed three times with sterile water. Roots were stained with 5.0 μg/mL wheat germ agglutinin (WGA) Alexa Fluor 488 conjugates (Invitrogen) for 30 min at 37°C and washed in 1 × HBSS (Hanks' Balanced Salt Solution). Mycorrhizal colonization was quantified according to the MYCOCALC program (http://www2.dijon.inra.fr/mychintec/Mycocalc-prg/download.html) as described previously (Trouvelot *et al*., 1986).

Microscopy

A NiKon Eclipse Ni-U fluorescence microscope was used to examine the mycorrhizal colonization of plant roots. The *A. sinicus* transgenic roots with DsRed fluorescence were selected using a NiKon SMZ18 fluorescence stereomicroscope. Zeiss 780 laser scanning confocal microscope was used to observe the subcellular localization of GFP-fusion constructs in *S. cerevisiae* and arbuscule phenotype in the VIGS and HIGS experiments. GFP signal was detected using an excitation wavelength of 488 nm and emission between 500 and 550 nm, while DAPI signal was detected using an excitation wavelength of 345 nm and emission at 455 nm.

Trehalose content analysis

A Trehalose Extraction Kit (Solarbio, BC0330, Beijing, China) was used to examine the trehalose content. Transformed yeast cells were cultured in 100 mL SD/-URA medium without or with 1 M sorbitol. The starting inoculation was adjusted to an OD_{600} of 0.1. And cultured samples were taken at 24 h after sorbitol treatment. Then the trehalose content was measured at 620 nm with a UV/vis spectrophotometer (Mapada, Shanghai, China). Each growth assay was replicated independently three times.

Proline content analysis

The extraction and determination of proline in plants were performed according to Bates *et al*. (1973). Briefly, plant leaves were frozen with liquid N_2 and grounded into powder. Then the proline content was measured at 520 nm with a UV/vis spectrophotometer (Mapada, Shanghai, China). Proline content was calculated on the basis of a proline standard curve. Data were expressed as μg g^{-1} FW.

Field water capacity and relative water content measurements

The FWC was measured as described previously (Janeczko *et al*., 2016). The sand in the pot was placed into a pallet with water for 24 h. Then the pot was left to allow the excess water to drip off. The mass of sand in the pot was defined as M1. Subsequently, the sand was dried and weighed to yield M2. The maximum (100%) field soil water capacity was calculated from the M1-M2 subtraction. The obtained data allowed us to estimate the amount of water that is necessary to hydrate the soil at 75%, 50% and 25% of the maximum water capacity.

To determine the RWC in plants, fresh plants leaves were weighed immediately to get the FW, these leaves were then soaked in deionized water for 4 h and saturated weight (SW) was determined, these leaves were dried for 48 h at 75°C to determine the dry weight (DW). RWC in leaves was calculated as: RWC = (FW − DW)/(SW − DW) × 100%.

Bioinformatics

Molecular weight and isoelectric point (IP) are predicted by the Protparam (http://www.expasy.org/tools/protparam). Homology modelling of the AM fungal MAPK cascade proteins was predicted by the program Swiss Model (http://swissmodel.expasy.org/). The ORFs of AM fungal MAPK genes are predicted by the ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/). The domains are indicated through the SMART program (http://smart.embl-heidelberg.de/).

Phylogenetic analysis

Analysis of the gene sequences was carried out using the NCBI Blast Server for the homology searches (www.ncbi.nlm.nih.gov). MEGA7 software (Kumar *et al*., 2016) was used to perform phylogenetic analysis. The multiple sequence alignment was progressed by ClustalW, and then the aligned sequences were loaded into MEGA7 for phylogenetic analysis. A phylogenetic tree was constructed using 1000 bootstrap replicates. The evolutionary history was inferred using the Neighbour-Joining method. Accession numbers of the predicted proteins are given in Table S3.

Statistical analyses

The one-way ANOVA with Tukey's test was used to make multiple comparisons. Averages with different letters indicate a significant difference at $P < 0.05$. Differences are described as statistical significance marked with asterisks for values of *$P < 0.05$ and **$P < 0.01$.

Accession numbers

Sequence data from this article can be found in the AM fungal Genome databases and GenBank libraries under the following accession numbers for HOG1-MAPK proteins: *R. irregularis* RiSte11 (XP_025172837.1), RiPbs2 (XP_025178881.1), RiHog1 (XP_025185210.1), *G. rosea* GigrHog1 (RIB04295.1), *R. clarus* RcHog1 (GET01709.1), *G. margarita* GigmHog1 (KAF0432463.1), *G. cerebriforme* GcHog1 (RIA92778.1), *R. diaphanous* RdHog1

(RGB42528.1), *S. cerevisiae* ScHog1 (NP_013214.1). Other accession numbers of the predicted proteins are given in Table S3.

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Conflicts of interest

The authors declare no conflicts of interest.

Author contributions

H.C., M.T. and X.X. designed the study and experiments. S.W. performed experiments. S.W., X.X. and X.C. performed data analysis. S.W., Y.R. and W.L. performed the experiment of plant management. X.F. and H.W. assisted with the interpretation of the results. S.W., X.X., H.C. and M.T. wrote the article. M.T. and H.C. acquired the funding, administrated the project and supervised it.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1
Figure S1 Effects of arbuscular mycorrhiza and non-mycorrhiza on the growth, relative water and proline contents of *Astragalus sinicus* in response to well water and drought stress.
Figure S2 Characteristics of *RiSte11*, *RiPbs2* and *RiHog1* in *Rhizophagus irregularis*.
Figure S3 Heatmap analysis of HOG1-MAPK cascade (*RiSte11*, *RiPbs2*, *RiHog1*) and drought-resistant genes (*RiAQP1/2*, *RiTPS1/2*, *RiNTH1*) in *Rhizophagus irregularis* under different developmental conditions.
Figure S4 Expressions of *RiSte11, RiPbs2* and *RiHog1* in *Rhizophagus irregularis* colonized roots of *Astragalus sinicus* at 42 days post inoculation (dpi) in response to drought stress.
Figure S5 Microscopy images of the composite *Astragalus sinicus* hairy roots with control, *RiSte11*, *RiPbs2* or *RiHog1* gene mediated by host-induced gene silencing.
Figure S6 Overall growth performance of the composite *Astragalus sinicus* with hairy roots carrying the empty vector (control), *RiSte11-*, *RiPbs2-* or *RiHog1-RNAi* construct mediated by host-induced gene silencing.
Figure S7 Host-induced gene silencing of HOG1-MAPK cascade genes in *Rhizophagus irregularis* affects the RWC and proline content in *Astragalus sinicus* leaves.
Figure S8 Virus-induced gene silencing of HOG1-MAPK cascade genes in *Rhizophagus irregularis* affects the RWC and proline content in *Nicotiana benthamiana* leaves.
Figure S9 Host-induced gene silencing of HOG1-MAPK cascade genes in *Rhizophagus irregularis* inhibits arbuscule development in roots under well water condition.
Figure S10 Molecular and arbuscular mycorrhizal phenotypes of virus-induced gene silencing of *RiSte11*, *RiPbs2* or *RiHog1* in *Nicotiana benthamiana* under well water condition.
Figure S11 Virus-induced gene silencing of *RiSte11*, *RiPbs2* or *RiHog1* in *Rhizophagus irregularis* does not affect expression levels of MAPK cascade genes in *Nicotiana benthamiana*.
Figure S12 The expression of *RiAQP1/2*, *RiTPS1/2*, *RiNTH1* and *Ri14-3-3* in response to drought stress.
Table S1 Characteristics of HOG1-MAPK cascade and drought-resistant genes in *Rhizophagus irregularis*.
Table S2 PCR primers used for HOG1-MAPK cascade genes in *Rhizophagus irregularis*.
Table S3 The accession numbers of fungal MAPK proteins used in this study.