Rice blast caused by *Magnaporthe oryzae* is a severe rice disease resulting in yield and economic losses in rice production (Wilson & Talbot, 2009). Controlling rice blast disease development is vital for rice cultivation. Thus, we need a better understanding of the details of the rice blast fungus\(^1\)'s pathogenic mechanism.

\(M\). *oryzae\) penetrates the rice leaves using an infection structure called the appressorium (Ryder & Talbot, 2015). There is a well-defined sequence of events from the germination of the conidium forming a germ tube, followed by appressorium formation and the subsequent penetration peg penetration of the leaf epidermis.
The apex of the germ tube swells to generate an appressorium. A relatively recent article showed that the appressorial growth, maturation, and even infection were accompanied by changes in appressorium intracellular polarity (Patkar et al., 2010). The polarity disappears in the early stage of appressorial expansion, including the appressorium’s early maturation. Later, the polarity is restored at the base of the appressorium in contact with the rice leaf, and a penetration peg is generated to penetrate and infect the rice cell with the help of turgor-generated mechanical pressure (Howard & Valent, 1996). Thus, there is a signal regulating the reestablishment of polar growth in the appressorium and orchestrating penetration peg formation.

Before penetration peg formation, appressorium turgor pressure increases to about 8.0 MPa (Veneault-Fourrey et al., 2006). A turgor-sensing histidine-aspartate kinase, Sln1, can detect the pressure change, then organize the septin protein localizing around what is to become the penetration pore part of the appressorium facing the leaf surface (Dagdas et al., 2012; Ryder et al., 2019). Chitinases and chitin synthetases also need to be mobilized to support the penetration peg expansion with a new cell wall (Kong et al., 2012).

**FIGURE 1** (a) Localization of MoSep3-mCherry (top) and GFP-MoCK2 (middle) in the appressorium visualized, using a GFP-MoCKa construct. CK2-holoenzyme ring structure (HRS) shows the three-dimensional structure of the MoCK2 holoenzyme in the appressorium. MoSep3-mCherry forms a ring structure at the penetration pore. The streak in the image of GFP-MoCK2 seen through the penetration peg (middle set of images) is the GFP-MoCK2 ring structure positioned perpendicular to the MoSep3-mCherry ring at the bottom of the appressorium. Video S1 shows a 3D scan visualizing the GFP-MoCKa (CK2-HRS) ring structure. All bars = 10 μm. (b) GFP-MoCKa localization in an appressorium change with germination time (0 hr). Only conidia: GFP-MoCK2 is localized in the nuclei and around the conidial septa (12 hr). Germinated conidia with germ tube and developing appressorium: GFP-MoCK2 is mainly localized in the appressorium nuclei and at germ tube to appressorium septa. After 16 hr of germination, GFP-MoCK2 has assembled as a ring structure in the appressorial cytoplasm. We selected 0, 12, and 16 hr to highlight the changes in the localization of GFP-MoCK2. The streak across the penetration peg is the ring structure of GFP-MoCK2 in the appressorium. A three-dimensional image of this structure is shown in Video S2. All size bars = 10 μm.
the correct localization of septin proteins as a ring, the septin ring recruits the exocyst complex to participate in the polarity reestablishment process (Gupta et al., 2015). The exocyst complex plays an essential role in the cell secretion pathway and is required for vesicle docking to the plasma membrane to make a new cell wall (Chavez-Dozal et al., 2015). The exocyst complex contains eight subunits: Sec3, Sec5, Sec6, Sec8, Sec10, and Sec15, and exocyst complex proteins Exo70 and Exo84 (He & Guo, 2009). Gupta et al. (2015) demonstrated that the septin proteins recruit the exocyst complex to localize at the base of the appressorium to regulate the penetration peg formation, thus taking part in the reestablishment of polar growth. Interestingly, the Exo70-GFP accumulates at the forming appressorial pore about 11 hr after conidium germination (Gupta et al., 2015). Other proteins also localize around the penetration pore to regulate the penetration peg formation (Ryder et al., 2013), but Exo70 seems essential.

Our previous study (Zhang et al., 2019) showed that protein kinase CK2 assembles a ring structure in the appressorium perpendicular to the septin protein ring. In another study, we further showed that CK2 probably has a chaperoning effect on intrinsically disordered proteins. In the latter study, we also got data indicating that CK2 directly interacts with septins, explaining why it accumulates at septal and appressorial pores (Zhang et al., 2020). The CK2 holoenzyme consists of three proteins, the kinase CKa, and two regulatory subunits CKb1 and CKb2 (Zhang et al., 2019). We show ring assembly again in a repeat experiment, including the septin ring’s visualization (Figure 1a and Video S1). Deleting any of the CK2 regulatory subunits reduces the penetration peg formation affecting pathogenicity (Zhang et al., 2019), indicating that CK2 may reestablish the necessary appressorial polarity for the formation of the penetration peg.

To better sort out the timing of events, we performed detailed temporal observations of green fluorescent protein (GFP) labelled-MoCK2 fluorescence cellular localization during appressorium development. The experimental procedures used are given in File S1. The GFP-MoCK2 accumulated in nuclei and septa in conidia, where septins are localized. It was also detected at the septum between the tube germ and the appressorium. After 12 hr, GFP-MoCK2 mainly concentrated in appressorium nuclei and the appressorium septal pore

**FIGURE 2** The localization of GFP-MoCK2 in hyphae of GFP-CKa strains (a) and GFP-CKa ΔMosep3 strains (b). White arrows indicate the nucleolar localization of MoCK2 in both GFP-CKa and GFP-CKa ΔMosep3 strains (a, b). The red arrows indicate the presence of GFP-MoCK2 at the septal pores of the GFP-CKa strains (a) and the absence of GFP-MoCK2 from the septal pore of GFP-CKa ΔMosep3 strains (b). All bars = 10 μm. The localization of MoCK2 in the appressorium of GFP-CKa strains (c) and GFP-CKa ΔMosep3 strains (d). All bars = 10 μm. Three-dimensional images for the ring structure localization of MoCK2 in the appressorium of GFP-CKa strains (two rings in this case) (e) and GFP-CKa ΔMosep3 strains without ring structures (f)
due to the loss of conidial nuclei (Veneault-Fourrey et al., 2006). We found that the GFP-MoCK2 assembled a ring structure in the appressorium’s cytoplasm (Figure 1b and Video S2) only after 16 hr of germination. The late occurrence of the MoCK2 ring structure indicates that the localization and ring formation of the MoCK2 holoenzyme in the appressorium are affected by some protein to occur at the right stage of the appressorial maturation and affect penetration peg formation.

Previous research has shown that the Sln1-septin-exocyst complex localized at the base of the appressorium in contact with the leaf surface forms a ring structure that influences growth polarity and affects penetration peg formation necessary for pathogenicity (Gupta et al., 2015). We hypothesize that septin ring and exocyst complex proteins could affect the correct localization of MoCK2. Removal of septin ring components or exocyst complex components should result in MoCK2 mislocalization that could affect both penetration peg formation and the pathogenicity of *M. oryzae*.

To test the hypothesized relationship between the septin proteins and MoCK2 in *M. oryzae*, we first investigated the localization of GFP-MoCK2 in a ΔMosep3 deletion mutant. We chose MoSep3 because deleting any one of the septin proteins, MoSep3, MoSep4, or MoSep5, destroys the septin-ring structure at the base of the appressorium, and the MoSep3 deletion resulted in the least pathogenesis on rice (Dagdas et al., 2012). Thus, we expressed GFP-MoCKa, GFP-MoCKb1, and GFP-MoCKb2 in the ΔMosep3 deletion mutant. The positive transformants were verified using quantitative reverse transcription PCR (Figure S3).

We detected the cellular localization of GFP-MoCKa, GFP-MoCKb1, and GFP-MoCKb2 components in the background strain Ku80 and the ΔMosep3 deletion mutant, and found that compared with the background strains, the deletion of Sep3 proteins changed the localization of MoCK2 in hyphae and appressoria (Figure 2). In GFP-MoCKa ΔMosep3 strains, we found that the GFP signal was absent from the septal pores but was present in the GFP-MoCKa background strains, despite similar nuclear localization (Figure 2a,b). The appressorial localization disappeared, and there was no CK2-holoenzyme ring structure (HRS) formed in the GFP-MoCKa ΔMosep3 deletion mutant (Figure 2d,f), as could be seen in the GFP-MoCKa strains (Figure 2c,e and Video S3a,b).

Figures S1 and S2 show the changed localization of GFP-MoCKb1 in GFP-MoCKb1 ΔMosep3 compared with GFP-MoCKb1 strains (Figure S1) and GFP-MoCKb2 compared to the GFP-MoCKb2 ΔMosep3 strains (Figure S2), respectively. The localization of both regulatory subunits was affected by the ΔMosep3 deletion in the same way as found for the catalytic subunit in GFP-MoCKa ΔMosep3 (Figure 2). Both regulatory subunits were absent from the septal pore and nucleolus in ΔMosep3 deletion mutants (Figures S1a,b and S2a,b). The CK2-HRS typically found in the appressorium was not produced by the ΔMosep3 deletion mutants (Figures S1c–f and S2c–f).

**FIGURE 3** The localization of MoCK2 in ΔMoexo70 deletion mutant. The white arrows indicate that nucleolar localization of MoCKa is found in GFP-Cka ΔMoexo70 strains, but absent in GFP-Ckb1 ΔMoexo70 and GFP-Ckb2 ΔMoexo70 strains expressing GFP-Ckb1 and GFP-Ckb2, respectively. The red arrows indicate the absence of GFP-MoCK2 from the septal pore for all three MoCK2 subunits in the ΔMoexo70 strains. All bars = 10 μm. The CK2 holoenzyme ring structure was absent in appressoria at 16 hr of germination when these are generally present, and the three-dimensional images showed no ring formation of any of the MoCK2 three subunits in ΔMoexo70 deletion mutants.
S2c-f, and Videos S4a,b and S5a,b). These results indicate that CK2 localization in hyphae and especially appressoria was affected by the Sep3 protein. The absence of the septin ring structure caused a non-assembly of the CK2-HRS, perpendicular to the septin ring.

To test the hypothesized relationship between the MoCK2 proteins and the exocyst complex, we needed to delete a gene belonging to the exocyst complex. The localization of MoExo70 at the base occurs at about 11 hr of germination. This assembly of MoExo70 into a ring is thus earlier (Gupta et al., 2015) than the CK2-HRS formation. We made a knockout strain, ΔMoexo70, to test the effect of MoExo70 on the localization of MoCK2.

GFP fusion proteins of all three MoCK2 components were separately expressed in the ΔMoexo70 deletion mutant (Figure S4) to detect the deletion's effects on their localization. The results showed that the localization of all MoCK2 three subunits, MoCKa, MoCKb1, and MoCKb2, was different in the ΔMoexo70 deletion mutant compared to their localization in the background strains (Figure 3 and Videos S6a,b,c). The GFP fluorescence of all three subunits usually found at the septa was absent in the ΔMoexo70 deletion mutants. In addition, there was no fluorescence in the nucleolus for any of the two regulatory subunits in the ΔMoexo70 deletion mutants. The deletion of MoExo70 characteristically inhibited the CK2-HRS formation in the appressorium as hypothesized. Thus, we can conclude that the assembled exocyst complex at the base of the appressorium is crucial for the correct localization and assembly of the MoCK2 as a ring perpendicular to the penetration pore opening.

An interesting observation for the ΔMosep3 mutant was that the hyphae appeared swollen (Figures 2b, S1b, and S2b). We speculate that this could be caused by changes in cell wall structure or, maybe more plausible, that increased turgor pressure in hyphal compartments due to the transport of assimilated nutrients from older hyphae to the growing tip through septa could be hindered in some way. Support for this observation is that such swollen hyphae can be seen in the ΔMosep3 mutant in the original paper describing the septin proteins for M. oryzae (Figure S3) (Dagdas et al., 2012), although the authors did not comment on this. Support for our speculation is that we found significant but not large differences in colony diameter after growing wild type and the ΔMosep3 mutant on complete medium (CM) at 25 °C for 9 days. The wild type and ΔMosep3 colony diameters were 43.5 ± 0.42 mm (SEM) and 40.44 ± 0.32 mm (SEM), respectively (p for the null hypothesis having the same mean = 0.005). How the septal pore proteins mechanistically influence flow dynamics through septa and appressorium pores should be studied in future experiments.

We have now gained a better understanding of the timing and the events needed for appressorial polarity reestablishment and penetration peg formation, and these are as follows: Sln1 (turgor-sensing histidine-aspartate kinase) senses turgor pressure caused by glycerol accumulation in the appressorium, causing the septin proteins to recruit the exocyst complex and septin ring, and exocyst complex to colocalize at the base of the appressorium (Ryder et al., 2019). After this, the protein kinase MoCK2 begins assembling the CK2-HRS, and the formation of the penetration peg is prompted (Figure 4).

**FIGURE 4** The MoCK2 ring structure formation that appears to be part of the appressorial polarity reestablishment is dependent on the earlier formed septin and exocyst rings at the base of the appressorium.
Finally, we can conclude that the polarity growth reestablishment of appressoria is a highly complex process and MoCK2 assembling a ring structure takes part in this process. However, CK2-HRS has several possible functions that need to be clarified concerning its roles for appressorium and penetration peg function, including cytoplasmic content migration into the host through the penetration pore (Zhang et al., 2020). In future research, we will focus on the formation, development, and fate of the CK2-HRS to further elucidate its roles in the infection mechanism of M. oryzae.

ACKNOWLEDGEMENTS
This work was supported by the National Natural Science Foundation of China (grant 32060597).

AUTHOR CONTRIBUTIONS
L.Z., S.O., and Z.W. conceived and designed the experiments. L.Z. and Y.C. performed the experiments. L.Z., S.O., and Z.W. analysed the data and wrote the manuscript.

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

ORCID
Lianhu Zhang https://orcid.org/0000-0001-7427-3720
Guodong Lu https://orcid.org/0000-0001-6325-9165
Stefan Olsson https://orcid.org/0000-0003-1931-9081
Zonghua Wang https://orcid.org/0000-0002-0869-9683

REFERENCES
Chavez-Dozal, A.A., Bernardo, S.M., Rane, H.S., Herrera, G., Kulkarny, V., Wagener, J. et al. (2015) The Candida albicans exocyst subunit Sec6 contributes to cell wall integrity and is a determinant of hyphal branching. Eukaryotic Cell, 14, 684–697.

Dagdas, Y.F., Yoshino, K., Dagdas, G., Ryder, L.S., Bielska, E., Steinberg, G. et al. (2012) Septin-mediated plant cell invasion by the rice blast fungus, Magnaporthe oryzae. Science, 336, 1590–1595.

Gupta, Y.K., Dagdas, Y.F., Martinez-Rocha, A.L., Kershaw, M.J., Littlejohn, G.R., Ryder, L.S. et al. (2015) Septin-dependent assembly of the exocyst is essential for plant infection by Magnaporthe oryzae. The Plant Cell, 27, 3277–3289.

Hamer, J.E., Howard, R.J., Chumley, F.G. & Valent, B. (1988) A mechanism for surface attachment in spores of a plant pathogenic fungus. Science, 239, 288–290.

He, B. & Guo, W. (2009) The exocyst complex in polarized exocytosis. Current Opinion in Cell Biology, 21, 537–542.

Howard, R.J. & Valent, B. (1996) Breaking and entering: host penetration by the fungal rice blast pathogen Magnaporthe grisea. Annual Review of Microbiology, 50, 491–512.

Kong, L.-A., Yang, J., Li, G.-T., Qi, L.-L., Zhang, Y.-J., Wang, C.-F. et al. (2012) Different chitin synthase genes are required for various developmental and plant infection processes in the rice blast fungus Magnaporthe oryzae. PLoS Pathogens, 8, e1002526.

Patkar, R.N., Suresh, A. & Naqvi, N.I. (2010) MoTea4-mediated polarized growth is essential for proper asexual development and pathogenesis in Magnaporthe oryzae. Eukaryotic Cell, 9, 1029–1038.

Ryder, L.S., Dagdas, Y.F., Kershaw, M.J., Venkataraman, C., Madzvamuse, A., Yan, X. et al. (2019) A sensor kinase controls turgor-driven plant infection by the rice blast fungus. Nature, 574, 423–427.

Ryder, L.S., Dagdas, Y.F., Mentlak, T.A., Kershaw, M.J., Thornton, C.R., Schuster, M. et al. (2013) NADPH oxidases regulate septin-mediated cytoskeletal remodeling during plant infection by the rice blast fungus. Proceedings of the National Academy of Sciences of the United States of America, 110, 3179–3184.

Ryder, L.S. & Talbot, N.J. (2015) Regulation of appressorium development in pathogenic fungi. Current Opinion in Plant Biology, 26, 8–13.

Veneault-Fourrey, C., Barooah, M., Egan, M., Wakley, G. & Talbot, N.J. (2006) Autophagic fungal cell death is necessary for infection by the rice blast fungus. Science, 312, 580–583.

Wilson, R.A. & Talbot, N.J. (2009) Under pressure: investigating the biology of plant infection by Magnaporthe oryzae. Nature Reviews Microbiology, 7, 185–195.

Zhang, L., Zhang, D., Chen, Y., Ye, W., Lin, Q., Guo, W. et al. (2019) Magnaporthe oryzae CK2 accumulates in nuclei, nucleoli, at septal pores and forms a large ring structure in appressoria, and is involved in rice blast pathogenesis. Frontiers in Cellular and Infection Microbiology, 9, 113.

Zhang, L., Zhang, D., Liu, D., Li, Y., Li, H., Xie, Y. et al. (2020) Conserved eukaryotic kinase CK2 chaperone intrinsically disordered protein interactions. Applied and Environmental Microbiology, 86, e02191-19.

SUPPORTING INFORMATION
Additional Supporting Information may be found online in the Supporting Information section.

How to cite this article: Zhang L, Cai Y, Li Y, et al. MoSep3 and MoExo70 are needed for MoCK2 ring assembly essential for appressorium function in the rice blast fungus, Magnaporthe oryzae. Mol Plant Pathol. 2021:00:1–6. https://doi.org/10.1111/mpp.13092