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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This manuscript details the further characterization of a Magnaporthe oryzae effector and delves into the specifics of components necessary for infection. MoHTR1 has previously been characterized as a fungal effector important for pathogenicity through its involvement in translocating to the host rice nucleus to alter rice gene transcription. This manuscript continues the characterization through identification of the NLS important for translocation. In addition, the authors have identified the import pathway for MoHTR1 into rice cells through the interaction between MoHTR1 and rice importins α. Finally, this manuscript details the crucial need for SUMOylation for MoHTR1 as this promotes the function of this fungal effector.

This manuscript is well written and is very detailed in its descriptions of methodologies and findings. Moving forward, this manuscript provides novel insights into nuclear effectors, their import pathways, and creates a relationship between SUMOylation and nuclear localization in fungi.

Comments:

The authors should include information on how PEG-mediated transfection was conducted with rice protoplast.

The authors should specify the type(s) of t-Test performed.

In Figure 4A, the authors should specify what was used as the control for the eGFP sample.

It would be ideal to specify what constructs were used for the 'positive' and 'negative' strains in the Y2H experiment.

The authors should include information on the total reads that were used to generated their RNA sequencing library.

In Figure 5, the authors should include units (kDa) for protein sizes.

In Line 326, the authors should specify/cite the Compute pI/Mw tool used.

Reviewer #2 (Remarks to the Author):

The manuscript presents a pioneering study identifying the core nuclear localization sequence (NLS) of MoHTR1, a nuclear effector from the rice blast pathogen Magnaporthe oryzae, responsible for its translocation into the host nucleus. The authors have further dissected the importin α- and SUMOylation-mediated transport mechanisms of this nuclear effector, elucidating its critical role in fungal virulence and the host immune response. These findings offer novel insights into the translocation mechanisms of nuclear effectors during host-pathogen interactions, underscoring their importance in the host's immune response. The discovery of the RxKK motif as an NLS is a significant contribution to the understanding of nuclear effector mechanisms in plant pathogens.

The manuscript is well-written and presents significant findings that could advance the field of plant pathology. However, addressing the major concerns listed above is essential for a clearer understanding of the underlying mechanisms and the manuscript's overall impact. Upon revision, this paper has the potential to be a strong candidate for consideration.

Major Concerns:

1. Line 216-219: In the SUMO deletion mutant (ΔMosmt3) infected rice cells, MoHTR1 was located in the BIC in 82% of inoculated cells, compared to wild type infected rice cells (98%). However, there was no significant difference in BIC localization proportion 218

between SUMOylation site-mutated MoHTR1 and the wild type infected rice cells (Supplementary Fig. 6). The manuscript should consider and discuss the possibility that SUMOylation may affect the secretion of MoHTR1 indirectly, for instance, by modifying other proteins that influence effector secretion, as previously discussed by Liu et al. (New Phytologist, 2018). The authors should avoid overemphasizing the conclusion that "SUMOylation regulates MoHTR1 secretion."

2. SUMOylation and Protein Stability: It would be valuable to include additional experiments that explore the relationship between SUMOylation and the stability or degradation pathways of the MoHTR1 effector.

3. Interaction with Importin α: The manuscript should investigate whether SUMOylation site mutations affect the interaction between MoHTR1 and importin α.

4. Mechanism of RxKK as NLS: Further clarification is needed on how the RxKK motif functions as an NLS and whether its absence impacts the interaction with importin α.

5. Novelty of RxKK NLS: The discussion should expand on the uniqueness of the RxKK NLS and its significance in the context of known NLSs, particularly within plant pathogens.

6. Conservation of NLS in other Effectors: An analysis of the conservation of the MoHTR1 NLS among other effectors or fungal effectors would strengthen the manuscript's claims regarding the uniqueness of the RxKK motif.

7. Line 145-148: In the wild type line, MoHTR1 was localized to the nucleus in 71 % of the protoplasts. However, in the osimpα1a-hetero line, nuclear localization of MoHTR1 was decreased to 51 % (Fig. 2C). The difference in this result does not appear to be very significant.

8. Line 297: This RxKK is unique and not present in NLS regions of other fungal nuclear effectors. The statement that the RxKK motif is unique should be supported by an analysis of predicted effectors from other species. Without this, the claim of uniqueness may be premature.

9. Specificity of Interaction with Importin α: The manuscript should provide a rationale for why MoHTR1 interacts with OsImpα but not with MoImpα. Structural analysis of the proteins might offer clues to this specificity.

Minor Concerns:

Figure 2B would benefit from the inclusion of images showing both white light and fluorescence merge, which would enhance the visualization of the results.

Reviewer #3 (Remarks to the Author):

This study focused on the further characterization of a previously identified host nuclear-localized fungal effector, MoHTR1. Rice blast disease caused by Magnaporthe oryzae is the most serious disease of cultivated rice. Secreted effectors mediate the pathogen-rice interaction. Fungal secreted effectors that target the host nucleus to reprogram the host response are less well known that apoplectic or cytoplasmic effectors. Previously, the authors identified MoHTR1 as being secreted into host cells, where they targeted the nucleus. However, the mechanism was unclear. Here, the authors show that an NLS signal is recognized by rice a-importin, and is sufficient to traffic effectors into host nuclei. Although the work is important, however, it does not reveal anything new about the importation of proteins into nuclei, which would be expected to involve a NLS and a rice importin. Thus, it is hard to determine how much of the presented work is truly novel compared to what was already presented in the earlier investigation (Kim et al. 2020). Furthermore, the authors conclude that the MoHTR1 NLS is not recognized by the fungus, but this wasn't sufficiently demonstrated (see below for details). For example, the NLS would be masked form detection by the fungus due to the folding of the protein in the ER (If the nuclear effectors carry a signal peptide, this wasn't clear). This does not mean it is an NLS incapable of recognition by fungal a-importin, as the authors claim. Also, a strain lacking the NLS is still fully virulent to my eyes, therefore the necessity of the NLS on MoHTR1 is unclear, and why the loss of Htr1 was more detrimental to infection than the loss of NLS alone was not discussed. In general, the manuscript is poorly written and often confusing, which may be impeding the ability of the authors to convey their findings. Therefore, I find the current version of the work difficult to follow and the results more suitable to a specialized journal.

Main:

The abstract is poorly written and often confusing, eg "..will provide unprecedented insights into the significance of plant-specific NLS on nuclear effector and its role.." which nuclear effector?

The introduction also suffers from poor writing, for example line 47: "Effector of plant pathogens is a virulence determinant factor", which effector?

Line 92: "These two nuclear effectors are localized to the host nucleus without additional NLS but not in the fungal nucleus". Do these protein have an SP? If so, this would direct the translating protein to the ER for secretion and the NLS would not be recognized in the fungal cytoplasm.

Line 93: "However, it is unknown how these nuclear effectors migrate to the host nucleus and what roles of NLS in the modulation of host immunity"- poorly written, what roles of NLS are you thinking about?

Line 104: "..on the role of NLS in the nuclear effector..", which nuclear effector, all of them or just MoHTR1?

I am confused. Line 92 states "These two nuclear effectors are localized to the host nucleus without additional NLS", but Line 109 states : "MoHTR1, a nuclear effector, was predicted to possess a single nuclear NLS, PGRSKKE, using the WoLF PSORT program". Did the authors not look for an NLS in the first report? Why not? It seems a logical analysis to have done in the first paper.

Line 115: "We cloned the coding sequences of MoHTR1, MoHTR1ΔNLS, NLS_K-R and NLS_K, R-A into eGFP expression plasmid" do the author's mean they fused the coding sequences to eGFP? It is not clear.

Line 118: "the accumulation rate of MoHTR1ΔNLS decreased to 14 %"- what accounts for the remaining 14 % accumulation in nuclei?

Line 132: "However, MoHTR1 did not interact with fungal importin α", As noted above, for the native protein, the NLS would not be recognized by fungal a-importin if the protein has an SP because it would be hidden from fungal a-importin in the ER. The yeast-two hybrid analysis suggests the NLS is not recognized by fungal a-importin, but this needs to be better determined in vivo. One way would be to attach the MoHTR1 NLS to a fungal cytoplasmic protein (perhaps hyphal-expressed eGFP is sufficient) and monitor whether or not it is taken up into the fungal nucleus.

Line 147: "nuclear localization of MoHTR1 was decreased to 51 %", again, what accounts for the remaining 51 % nuclear accumulation in the absence of rice a-importin?

Line 152: "The Simian virus 40 T antigen NLS (SV NLS), a most well-known classical NLS, has been

153 widely used as a nuclear positioning marker in various previous studies". Citations missing.

Line 161-line 173. The authors state that rice nuclear localization of Avr-Pita and Pwl2 increased from 20 % and 37 % of nuclei in infected cells to 73 % and 83 %, respectively, when the MoHTR1 NLS was added. However, what accounts for the background rate of nuclear accumulation of AVR-PITA and Pwl2 lacking the NLS? These are not normally shown to accumulate in rice nuclei unless (in the case of Pwl2) the rice NLS is added. I worry, then, that the background rate of nuclear accumulation is artifactual, which jeopardizes the interpretation of the localization data when the

NLS is added. Also, suggesting MoHTR1 NLS “escorts” cytoplasmic effectors is misleading as it infers that MoHTR1 plays this role during infection, acting something like a chaperone, when really the authors mean that adding the NLS increases nuclear localization. Therefore, remove statements of “escorting”.

Line 192: “Since MoHTR1 NLS is a plant-specific NLS”, I do not think this has been satisfactorily determined, see above regarding recognition by the fungus.

Figure 8a: In the image, MoHTR1 lacking the NLS is spreading cell to cell like WT and thus the nuclear localization signal is not apparently needed for infection. Although the authors provide some statistical analysis of the differences in cell-to-cell spread of the strains in planta at 60 hpi, which appears to show a reduction in virulence of MoHTR1 lacking NLS compared to WT, 60 hpi is a poor time to take data because infected cells are already dying and the fungus is entering necrosis; thus in my experience, it is not possible to quantify infection at this late time point using leaf sheaths. To be more accurate, I suggest the authors instead monitor the fungal mass of the three strains in Fig. 8, using for example qPCR of fungal tubulin genes compared to rice tubulin genes, in order to determine if there is indeed a reduction in virulence by the MoHTR1 strain lacking the NLS. As it stands, the evidence presented that the NLS is required for virulence is exceptionally weak.

Figure 8b, the infection is poor for WT and it is hard to distinguish differences in lesion size between WT, delmohtr1 and Mohtr1 lacking the NLS.

Responses to reviewer's comments and suggestions

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Thank you for your comments. Our point-by-point responses to your comments/suggestions are shown below.

1. The authors should include information on how PEG-mediated transfection was conducted with rice protoplast.

A. We added in more detail methods for PEG-mediated transfection in the Methods part (Line 459-463).

2. The authors should specify the type(s) of t-Test performed.

A. We performed unpaired two-tailed Student's *t*-test. We additionally described the type of statistical analysis in the figure legends.

3. In Figure 4A, the authors should specify what was used as the control for the eGFP sample.

A. Thank you for your comment. We used an eGFP expression plasmid as the negative control. As your comment, we revised all Figures, including Figure 4A, to label eGFP instead of control.

4. It would be ideal to specify what constructs were used for the 'positive' and 'negative' strains in the Y2H experiment.

A. We additionally described the information about the prey and bait plasmids used to generate

the positive and negative control strains for the Y2H assay in the Methods part (Line 436-438).

5. The authors should include information on the total reads that were used to generated their RNA sequencing library.

A. As your suggestion, we added the summary of the RNA sequencing library to Supplementary Table 6.

6. In Figure 5, the authors should include units (kDa) for protein sizes.

A. We added molecular weight unit in the Figure 5.

7. In Line 326, the authors should specify/cite the Compute pI/Mw tool used.

A. We added information on the website of Compute pI/Mw tool (Line 353).

Reviewer #2 (Remarks to the Author):

The manuscript presents a pioneering study identifying the core nuclear localization sequence (NLS) of MoHTR1, a nuclear effector from the rice blast pathogen Magnaporthe oryzae, responsible for its translocation into the host nucleus. The authors have further dissected the importin α- and SUMOylation-mediated transport mechanisms of this nuclear effector, elucidating its critical role in fungal virulence and the host immune response. These findings offer novel insights into the translocation mechanisms of nuclear effectors during host-pathogen interactions, underscoring their importance in the host's immune response. The discovery of the RxKK motif as an NLS is a significant contribution to the understanding of nuclear effector mechanisms in plant pathogens.

The manuscript is well-written and presents significant findings that could advance the field of plant pathology. However, addressing the major concerns listed above is essential for a clearer understanding of the underlying mechanisms and the manuscript's overall impact. Upon revision, this paper has the potential to be a strong candidate for consideration.

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Major Concerns:

1. Line 216-219: In the SUMO deletion mutant (ΔMosmt3) infected rice cells, MoHTR1 was located in the BIC in 82% of inoculated cells, compared to wild type infected rice cells (98%). However, there was no significant difference in BIC localization proportion 218 between SUMOylation site-mutated MoHTR1 and the wild type infected rice cells (Supplementary Fig. 6). The manuscript should consider and discuss the possibility that SUMOylation may affect the secretion of MoHTR1 indirectly, for instance, by modifying other proteins that influence effector secretion, as previously discussed by Liu et al. (New Phytologist, 2018). The authors should avoid overemphasizing the conclusion that "SUMOylation regulates MoHTR1 secretion."

A. Thank you for this comment. We agree that the sentences we described in the Discussion part could be overinterpreted as "SUMOylation regulates MoHTR1 secretion." We modified the Discussion part to reflect that SUMOylation may affect the secretion of MoHTR1 indirectly (Line 378-380).

2. SUMOylation and Protein Stability: It would be valuable to include additional experiments that explore the relationship between SUMOylation and the stability or degradation pathways of the MoHTR1 effector.

A. Thank you for the suggestion. According to your comment, we have confirmed that the stability of MoHTR1 depends on SUMOylation. The protein levels of both MoHTR1 and SUMOylation site-mutated MoHTR1 were maintained when treated with a 26S proteasome inhibitor. However, the SUMOylation site-mutated MoHTR1 was degraded rapidly than MoHTR1 without inhibitor treatment. These additional results are described in line 241-248 and Fig. 6D.

3. Interaction with Importin α: The manuscript should investigate whether SUMOylation site mutations affect the interaction between MoHTR1 and importin α.

A. Thank you for the suggestion. Three SUMOylation site-mutated MoHTR1 variants were interacted with OsImpα1a and OsImpα1b, but did not interact with OsImpα in BiFC assay. Therefore, we speculate that the decreased nuclear localization of SUMOylation site-mutated MoHTR1 is due to reduced protein stability and impaired interaction with OsImpα. We added these results in line 245-251 and Fig. 6C.

4. Mechanism of RxKK as NLS: Further clarification is needed on how the RxKK motif functions as an NLS and whether its absence impacts the interaction with importin α.

A. We appreciate this suggestion. When we conducted BiFC assay, MoHTR1 without NLS did not interact with three rice importin αs. These additional results are described in line 141-147 and Fig. 2B.

5. Novelty of RxKK NLS: The discussion should expand on the uniqueness of the RxKK NLS and its significance in the context of known NLSs, particularly within plant pathogens.

A. Please see our response to major concern Number 6 below.

6. Conservation of NLS in other Effectors: An analysis of the conservation of the MoHTR1 NLS among other effectors or fungal effectors would strengthen the manuscript's claims regarding the uniqueness of the RxKK motif.

A. Thank you for your comment. RxKK is the first characterized core NLS sequence in plant pathogenic fungi. Among the reported fungal nuclear effectors, neither predicted nor characterized NLS contain the RxKK sequence. Furthermore, RxKK sequence has not been found in any known characterized effectors of *M. oryzae*. To clarify, we have revised the statement to "This RxKK is unique and not present in NLS regions of other reported fungal nuclear effectors" in Discussion part, line 321-326. We summarized previously reported NLS of plant fungal nuclear effectors in the Supplementary Table 4.

7. Line 145-148: In the wild type line, MoHTR1 was localized to the nucleus in 71 % of the protoplasts. However, in the osimpα1a-hetero line, nuclear localization of MoHTR1 was decreased to 51 % (Fig. 2C). The difference in this result does not appear to be very significant.

A. Thank you for this comment. One of reasons for the nuclear localization of MoHTR1 not being significantly decreased in *osimpα1a* might be that the *osimpα1a* T-DNA insertion mutant is a

heterozygous mutant, not a homozygous mutant. Furthermore, rice has two other importin αs in addition to Osimpα1a, and we confirmed that they also interact with MoHTR1 *in vivo*. Therefore, we speculate that other importin αs might be also involved in the nuclear localization of MoHTR1. These results are described in Result part, page 6.

8. Line 297: This RxKK is unique and not present in NLS regions of other fungal nuclear effectors. The statement that the RxKK motif is unique should be supported by an analysis of predicted effectors from other species. Without this, the claim of uniqueness may be premature.

A. Please see our above response to major concern Number 6 above.

9. Specificity of Interaction with Importin α: The manuscript should provide a rationale for why MoHTR1 interacts with OsImpα but not with MoImpα. Structural analysis of the proteins might offer clues to this specificity.

A. Thank you for your suggestion. We performed a structural analysis of the interactions between MoHTR1 and Importin αs using AlphaFold program. The ipTM scores, which indicate the accuracy of protein-protein interaction predictions, between MoHTR1 NLS and OsImpα, OsImpα1a, and OsImpα1b were 0.51, 0.49, and 0.49, respectively. When analyzing MoHTR1$^{\Delta NLS}$, the ipTM scores were decreased to 0.23, 0.23, and 0.22 with OsImpα, OsImpα1a, and OsImpα1b, respectively. Moreover, the ipTM scores for MoHTR1 NLS with MoImpα was 0.6, which decreased to 0.29 for MoHTR1$^{\Delta NLS}$ with MoImpα. Despite this structural analysis, the results did not provide insights into interaction specificity. Therefore, we additionally observed localization of eGFP by tagging the MoHTR1 NLS in the fungal conidia. The MoHTR1 NLS:eGFP was localized in cytoplasm. Therefore, these results with Y2H results in this study indicate that MoHTR1 NLS does not contribute to nuclear localization in fungal cells. We added these results in line 127-140, Supplementary Table 1, and Supplementary Figure 1 and 2.

Minor Concerns:

1. Figure 2B would benefit from the inclusion of images showing both white light and fluorescence merge, which would enhance the visualization of the results.

A. We modified Figure 2B by merging the DIC and fluorescence images.

Reviewer #3 (Remarks to the Author):

This study focused on the further characterization of a previously identified host nuclear-localized fungal effector, MoHTR1. Rice blast disease caused by *Magnaporthe oryzae* is the most serious disease of cultivated rice. Secreted effectors mediate the pathogen-rice interaction. Fungal secreted effectors that target the host nucleus to reprogram the host response are less well known that apoplectic or cytoplasmic effectors. Previously, the authors identified MoHTR1 as being secreted into host cells, where they targeted the nucleus. However, the mechanism was unclear. Here, the authors show that an NLS signal is recognized by rice a-importin, and is sufficient to traffic effectors into host nuclei. Although the work is important, however, it does not reveal anything new about the importation of proteins into nuclei, which would be expected to involve a NLS and a rice importin. Thus, it is hard to determine how much of the presented work is truly novel compared to what was already presented in the earlier investigation (Kim et al. 2020). Furthermore, the authors conclude that the MoHTR1 NLS is not recognized by the fungus, but this wasn't sufficiently demonstrated (see below for details). For example, the NLS would be masked form detection by the fungus due to the folding of the protein in the ER (If the nuclear effectors carry a signal peptide, this wasn't clear). This does not mean it is an NLS incapable of recognition by fungal a-importin, as the authors claim. Also, a strain lacking the NLS is still fully virulent to my eyes, therefore the necessity of the NLS on MoHTR1 is unclear, and why the loss of Htr1 was more detrimental to infection than the loss of NLS alone was not discussed. In general, the manuscript is poorly written and often confusing, which may be impeding the ability of the authors to convey their findings. Therefore, I find the current version of the work difficult to follow and the results more suitable to a specialized journal.

Thank you for your critical comments. Our point-by-point responses to your comments/suggestions are shown below.

Main:

1. The abstract is poorly written and often confusing, eg "..will provide unprecedented insights into the significance of plant-specific NLS on nuclear effector and its role.." which nuclear effector?

A. As your comment, it seemed to be an ambiguous expression. Therefore, we revised it specifically to " fungal nuclear effectors".

2. The introduction also suffers from poor writing, for example line 47: "Effector of plant pathogens is a virulence determinant factor", which effector?

A. Many reference papers on plant pathogen's effectors define an effector as a secreted protein that improves the pathogen's colonization in host plants. We believe the sentence written in line 40, provides a broad definition of effectors.

Giraldo, M. C., & Valent, B. Filamentous plant pathogen effectors in action. *Nature Reviews*

Microbiology, **11**, 800-814 (2013).

Toruño, T. Y., Stergiopoulos, I., & Coaker, G. Plant-pathogen effectors: cellular probes interfering with plant defenses in spatial and temporal manners. *Annual review of phytopathology*, **54**, 419-441 (2016).

Todd, J. N. A., Carreón-Anguiano, K. G., Islas-Flores, I., & Canto-Canché, B. Microbial Effectors: Key determinants in plant health and disease. *Microorganisms*, **10**, 1980 (2022).

3. Line 92: "These two nuclear effectors are localized to the host nucleus without additional NLS but not in the fungal nucleus". Do these protein have an SP? If so, this would direct the translating protein to the ER for secretion and the NLS would not be recognized in the fungal cytoplasm.

A. Thank you for the comment. We used the MoHTR1 coding sequence without the signal peptide to observe localization in both rice protoplasts and fungal conidia. We additionally described these in the Method part.

4. Line 93: "However, it is unknown how these nuclear effectors migrate to the host nucleus and what roles of NLS in the modulation of host immunity"- poorly written, what roles of NLS are you thinking about?

A. In our previous study, MoHTR1 regulates the host immune response. Therefore, we speculated that the nuclear localization of MoHTR1 via its NLS is important for modulating host immunity. we revised this sentence to clarify for the readers. (Line 86-88)

5. Line 104: "..on the role of NLS in the nuclear effector..", which nuclear effector, all of them or just MoHTR1?

A. We revised this sentence to "role of NLS in nuclear effectors of plant pathogenic fungi".

6. I am confused. Line 92 states "These two nuclear effectors are localized to the host nucleus without additional NLS", but Line 109 states : "MoHTR1, a nuclear effector, was predicted to possess a single nuclear NLS, PGRSKKE, using the WoLF PSORT program". Did the authors not look for an NLS in the first report? Why not? It seems a logical analysis to have done in the first paper.

A. Thank you for this comment. Since the previous study focused on verifying the function of MoHTRs as transcription factors in the host, we only confirmed the presence of putative NLS

without characterization of its functionality. Therefore, this study aims to delve into the nuclear localization mechanism of MoHTR1.

7. Line 115: "We cloned the coding sequences of MoHTR1, MoHTR1ΔNLS, NLS_K-R and NLS_K, R-A into eGFP expression plasmid" do the author's mean they fused the coding sequences to eGFP? It is not clear.

A. Thank you for your question. We used gateway system. To fuse the eGFP to the coding sequences, the amplified CDS was first cloned into a donor vector, then reacted with destination vector (p2GWF7, eGFP expression vector under CaMV 35S promoter). We added detail information about the cloning process in Methods part, line 477-480.

8. Line 118: "the accumulation rate of MoHTR1ΔNLS decreased to 14 %"- what accounts for the remaining 14 % accumulation in nuclei?

A. Thank you for your question. In this study, the nuclear localization rate of MoHTR1 decreased from 66 % to 14 % upon deletion of the NLS. These results suggest that the MoHTR1 NLS plays a major role in the nuclear translocation of MoHTR1. However, small molecules less than 40 kDa could diffuse into the nucleus without the NLS-mediated importing pathway. Therefore, we cannot completely exclude other nuclear transport systems. Similar observations were reported in other studies. NLS-deleted nuclear effectors of *Verticillium dahliae*, VdSCP7 and Vd424Y, still accumulated in the nucleus, although it is not completely understood.

Zhang, L. *et al*. The Verticillium-specific protein VdSCP7 localizes to the plant nucleus and modulates immunity to fungal infections. *New Phytologist* ***215***, 368-381 (2017).

Liu, L. *et al*. Verticillium dahliae secreted protein Vd424Y is required for full virulence, targets the nucleus of plant cells, and induces cell death. *Molecular Plant Pathology* **22**, 1109-1120 (2021).

9. Line 132: "However, MoHTR1 did not interact with fungal importin α", As noted above, for the native protein, the NLS would not be recognized by fungal a-importin if the protein has an SP because it would be hidden from fungal a-importin in the ER. The yeast-two hybrid analysis suggests the NLS is not recognized by fungal a-importin, but this needs to be better determined in vivo. One way would be to attach the MoHTR1 NLS to a fungal cytoplasmic protein (perhaps hyphal-expressed eGFP is sufficient) and monitor whether or not it is taken up into the fungal nucleus.

A. Thank you for the suggestion. We observed localization of eGFP by tagging the MoHTR1 NLS

in fungal conidia. When we observed localization of SV NLS:eGFP, a positive control, eGFP was localized in the nucleus. However, both eGFP and MoHTR1 NLS: eGFP are localized in the cytoplasm. These results and Y2H results indicate that MoHTR1 NLS does not contribute to nuclear localization in fungal cells. We added these results in line 135-140 and Supplementary Figure 2.

10. Line 147: "nuclear localization of MoHTR1 was decreased to 51 %", again, what accounts for the remaining 51 % nuclear accumulation in the absence of rice a-importin?

A. As we described in Reviewer 2's Number 7 above, one of reasons for the nuclear localization of MoHTR1 not being significantly decreased in osimpα1a might be that the osimpα1a T-DNA insertion mutant is a heterozygous mutant, not a homozygous mutant. Furthermore, rice has two other importin αs in addition to Osimpα1a, and we confirmed that they also interact with MoHTR1 in vivo. Therefore, we speculate that other importin αs might be also involved in the nuclear localization of MoHTR1. These results are described in Result part, page 6.

11. Line 152: "The Simian virus 40 T antigen NLS (SV NLS), a most well-known classical NLS, has been 153 widely used as a nuclear positioning marker in various previous studies". Citations missing.

A. Thank you for finding the citation missing. We added the reference of You et al., 2019.

12. Line 161-line 173. The authors state that rice nuclear localization of Avr-Pita and Pwl2 increased from 20 % and 37 % of nuclei in infected cells to 73 % and 83 %, respectively, when the MoHTR1 NLS was added. However, what accounts for the background rate of nuclear accumulation of AVR-PITA and Pwl2 lacking the NLS? These are not normally shown to accumulate in rice nuclei unless (in the case of Pwl2) the rice NLS is added. I worry, then, that the background rate of nuclear accumulation is artifactual, which jeopardizes the interpretation of the localization data when the NLS is added. Also, suggesting MoHTR1 NLS "escorts" cytoplasmic effectors is misleading as it infers that MoHTR1 plays this role during infection, acting something like a chaperone, when really the authors mean that adding the NLS increases nuclear localization. Therefore, remove statements of "escorting".

A. Thank you for your comment. When we observed more than 100 infection sites, Avr-Pita and PWL2 without additional NLS was clearly localized in the 20 % and 37 % of nuclei. A similar observation was already reported by Khang et al., 2010. In that study, it was described that PWL2 localizes in presumed rice nuclei even under optimal pinhole settings of the microscope. However, further studies are needed to understand why both cytoplasmic effectors, Avr-Pita and PWL2, localize in the host nucleus despite lacking a predicted NLS. In

addition, we changed “escort” to “translocate” as your comment.

Khang, C. H. *et al.*, Translocation of *Magnaporthe oryzae* effectors into rice cells and their subsequent cell-to-cell movement. *The Plant Cell* **22**, 1388-1403 (2010).

13. Line 192: “Since MoHTR1 NLS is a plant-specific NLS”, I do not think this has been satisfactorily determined, see above regarding recognition by the fungus.

A. Please see our response to main concern Number 9 above.

14. Figure 8a: In the image, MoHTR1 lacking the NLS is spreading cell to cell like WT and thus the nuclear localization signal is not apparently needed for infection. Although the authors provide some statistical analysis of the differences in cell-to-cell spread of the strains in planta at 60 hpi, which appears to show a reduction in virulence of MoHTR1 lacking NLS compared to WT, 60 hpi is a poor time to take data because infected cells are already dying and the fungus is entering necrosis; thus in my experience, it is not possible to quantify infection at this late time point using leaf sheaths. To be more accurate, I suggest the authors instead monitor the fungal mass of the three strains in Fig. 8, using for example qPCR of fungal tubulin genes compared to rice tubulin genes, in order to determine if there is indeed a reduction in virulence by the MoHTR1 strain lacking the NLS. As it stands, the evidence presented that the NLS is required for virulence is exceptionally weak.

A. Thank you for insightful suggestion. When we inoculated into the sheath with less number of conidia, the types of invasive growth could be distinguished at 60 hpi. However, as your advice, we performed qPCR of fungal reference gene compared to rice reference gene for a more accurate comparison of virulence. To measure the fungal mass in the infected rice leaves, we collected infected rice leaves at 4 dpi and quantified the *OsUbi* and *MoPot2* gene levels by DNA-based qPCR. As a result, the fungal mass of WT-infected rice was 33, whereas the fungal mass was decreased in the Δ*Mohtr1*, and Δ*Mohtr1*::MoHTR1$^{\Delta NLS}$ to 7 and 12, respectively. These results indicate that the NLS is required for full virulence of *M. oryzae*. We have included these new data in Fig. 8B and described in line 276-282.

15. Figure 8b, the infection is poor for WT and it is hard to distinguish differences in lesion size between WT, delmohtr1 and Mohtr1 lacking the NLS.

A. We are confident that compared to WT, the virulence of Δ*Mohtr1*, and Δ*Mohtr1*::MoHTR1$^{\Delta NLS}$ was reduced. These results have confirmed through repeated experiments. We additionally attached the replicated experimental results in the Source data.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors did a great job at incorporating the suggestions for improvement of the manuscript. The revisions made are appropriate, improving the manuscript in its ability to be reproduced along with further validation of their stated claims. The revised manuscript allows for a more comprehensive illustration of MoHTR1 and its NLS, in role in M. oryzae infection.

Reviewer #2 (Remarks to the Author):

I believe the authors have thoroughly addressed all my concerns.

Reviewer #3 (Remarks to the Author):

The author's have done a good job of addressing my concerns and the paper is greatly improved.