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E-mail: jzus@zju.edu.cn

Correspondence:

Physical interactions and mutational analysis of MoYpt7 in *Magnaporthe oryzae*[*#]

Lu-yao HUANG[1], Min WU[1], Xiao-yun YU[2], Lin LI[1], Fu-cheng LIN[1], Xiao-hong LIU[†‡1]

[1]*State Key Laboratory for Rice Biology, Biotechnology Institute, Zhejiang University, Hangzhou 310058, China*

[2]*Huangdao Entry-Exit Inspection and Quarantine Bureau, Huangdao 266555, China*

[†]E-mail: xhliu@zju.edu.cn

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In this study, we analyzed the physical interactions of the dominant negative isoform of MoYpt7. Our results show that MoYpt7 interacts with MoGdi1. The dominant negative isoform of MoYpt7 (dominant negative isoform, N125I) is essential for colony morphology, conidiation, and pathogenicity in the rice blast fungus. These results further demonstrate the biological functions of MoYpt7 in *Magnaporthe oryzae*.

Rice blast is caused by *M. oryzae*, which is one of the most destructive ascomycete pathogens of cultivated rice worldwide. Because of its well characterized genetic background, *M. oryzae* is the model of choice for studying the interactions between pathogens and plants (Ebbole, 2007). Small guanosine triphosphatase (GTPase) have been reported to play crucial roles in membrane trafficking, protein secretion, and development in *M. oryzae* (Zheng W et al., 2007; Chen et al., 2008; Ye et al., 2014; Zheng H et al., 2016). In our previous report, a small GTPase, MoYpt7, was found to influence the fusion of the vacuole and autophagosome and the development of *M. oryzae* (Liu et al., 2015).

Rab proteins play pivotal roles in converting guanosine triphosphate (GTP) and guanosine diphosphate (GDP) binding states. This leads to a reversible attachment of the membrane, which is critical for fusion in eukaryotic cells (Grosshans et al., 2006; Pylypenko et al., 2006; Hutagalung and Novick, 2011). GDP dissociation inhibitors (GDIs) are proteins that regulate the GDP–GTP exchange reaction of Rabs (Pylypenko et al., 2006; Wu et al., 2007). In *Saccharomyces cerevisiae*, mutations of the Rab protein, such as Ypt7 with a T22N mutation (GDP-bound form) and G68L mutation (GTP-bound form), have been found. These mutations modify the nucleotide-binding features of the gene products. The phenotypes of yeast, especially the morphology of the vacuole, were changed when Ypt7 was replaced by the GTP- or GDP-bound form (Wada et al., 1996). In *M. oryzae*, we have verified the effects of the constitutively active isoform of MoYpt7 (GTP-bound form, Q67L). Many functional single-septum conidia were obtained and the pathogenicity was reduced significantly in the transformants expressing MoYpt7-Q67L. In addition, the transformants exhibited normal vacuole morphology and autophagy (Liu et al., 2015). However, the biological functions of the dominant negative isoform (GDP-bound form) of MoYpt7 were overlooked.

In addition, to detect the interactions of MoYpt7 with other proteins, a green fluorescent protein (GFP)-trap-based pull-down assay was performed using whole cell lysates from a GFP-MoYpt7 strain verified by Liu et al. (2015). Using a GFP-TrapA and mass spectrometry (MS) analyses, MoGdi1 (MGG_ 07137) was identified to interact with MoYpt7 in *M. oryzae*.

‡ Corresponding author

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ORCID: Xiao-hong LIU, https://orcid.org/0000-0002-7466-1261

Further co-immunoprecipitation assays confirmed that MoGdi1 could indeed bind to MoYpt7. Reintroduction of the construction MoYpt7-3×Flag into the Δ*moypt7* null mutant could restore the normal phenotypic characteristics, including aerial hyphae, conidiation, pathogenicity, and the morphology of the vacuole. Total protein extracts were eluted from anti-Flag M2 beads of transformants co-expressing MoYpt7-3×Flag and MoGdi1-GFP. Using an anti-Flag antibody, we could detect a 27-kD band of the size expected of MoYpt7-3×Flag fusion (Fig. 1a). When using an anti-GFP antibody, we detected the 78-kD MoGdi1-GFP band in the eluent from anti-Flag M2 beads, which bind the proteins of the transformant co-expressing MoYpt7-3×Flag and MoGdi1-GFP. In the control experiment, no band was detected in the transformant expressing MoYpt7-3×Flag (Fig. 1a). These data indicated that MoGdi1 was co-immunoprecipitated with MoYpt7-3×Flag.

MoGdi1 is highly homologous to Gdi1, a guanine nucleotide dissociation inhibitor in *S. cerevisiae* and *Homo sapiens* (Fig. 1b). MoGdi1 is 59% identical to *S. cerevisiae* Gdi1 (ScGdi1), 54% identical to HsGdi1β (the Rab GDI β in *H. sapiens*), and 53% to HsGdi1α (the Rab GDI α in *H. sapiens*). It has been reported that GDI can associate with many Rab

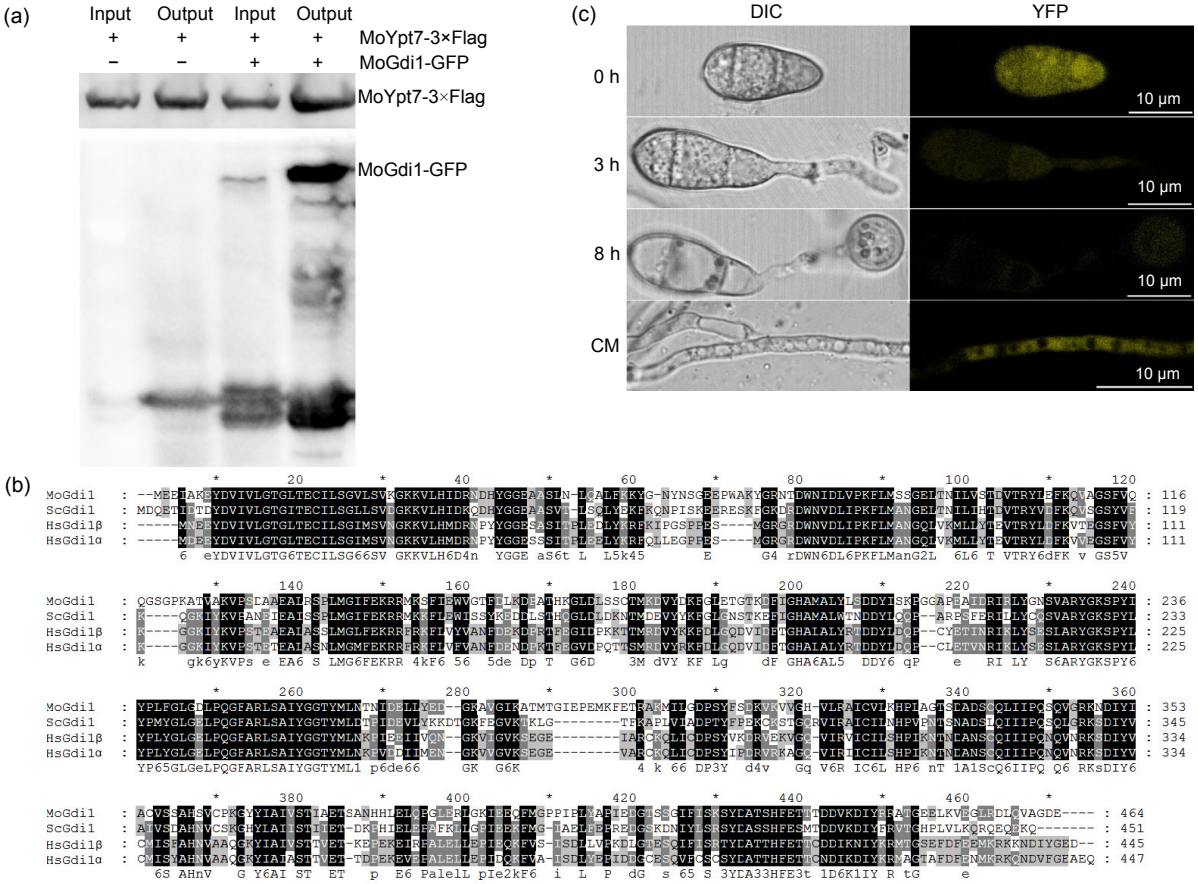

Fig. 1 Physical interactions of MoYpt7 with MoGdi1

(a) Co-immunoprecipitation assays for the interaction of MoYpt7 with MoGdi1. Total proteins were isolated from hyphae of a transformant expressing MoYpt7-3×Flag and a transformant co-expressing MoYpt7-3×Flag and MoGdi1-GFP. Proteins were then eluted from anti-Flag M2 beads. The presence of MoYpt7-3×Flag and MoGdi1-GFP was detected with anti-Flag and anti-GFP antibodies, respectively. The expected 78-kDa band of MoGdi1-GFP was detected in the eluted proteins of the transformant co-expressing MoYpt7-3×Flag and MoGdi1-GFP. A transformant expressing MoYpt7-3×Flag was used as a control. The same quantity of protein was used in Western blots. (b) Amino acid alignments of ScGdi1, HsGdi1, and MoGdi1 made with ClustalW2. ScGdi1, accession No. NP_011062; HsGdi1α, accession No. NP_001484; HsGdi1β, accession No. NP_001485. (c) MoGdi1 physically interacted with MoYpt7 during *M. oryzae* developmental phases examined. CM: complete medium; DIC: differential interference contrast; YFP: yellow fluorescent protein

proteins (Pfeffer and Aivazian, 2004). To check the interaction between MoYpt7 and MoGdi1 in vivo, a bimolecular fluorescence complementation (BiFC) assay was performed. Fusion constructs of YFPN-MoYpt7 and MoGdi1-YFPC were transformed into Guy11. In the transformants obtained, we observed yellow fluorescent protein (YFP) signals in the cytoplasm of conidia, appressoria, and hyphae in *M. oryzae* (Fig. 1c). Germinating conidia and appressoria showed slight fluorescence, and conidia and hyphae exhibited bright fluorescence. These results confirmed that MoYpt7 and MoGdi1 interact in vivo in the cytoplasm of the developmental stage, especially in conidia and hyphae.

Immunoassay and fluorescence detection verified that MoYpt7 interacts with MoGdi1, a homologue of the GDI in yeast. MoGdi1 could bind MoYpt7 to the cytoplasm from the vacuolar membrane. Numerous Rab proteins have been verified to interact with GDIs. Only one GDI gene exists in yeast. Two isoforms of GDI are found in humans: GDIα (enriched in the brain) and GDIβ (expressed ubiquitously). The GDIs bind to the GDP-bound form of Rab small GTPases and not only prevent exchange (maintaining the small GTPases in an off-state), but also prevent the small GTPases from localizing at the membrane, their place of action. Rab-GDI complexes are active and recycle Rab proteins that can deliver Rabs to specific and distinct membrane-bound compartments (Pfeffer and Aivazian, 2004). In our study, BiFC and immunology assays showed that MoYpt7 interacts with MoGdi1, homologous to yeast Gdi1, both in vitro and in vivo. The uniform distribution of enhanced YFP fluorescence in the cytoplasm suggests that MoGdi1 can pull MoYpt7 from the vacuole membrane into the cytoplasm. The Rab-GDI complexes are conserved in *M. oryzae*. Unfortunately, we could not obtain the null mutants for MoGdi1, even after screening more than 500 transformants from five independent transformations. We deduced that *MoGDI1* is an essential gene in *M. oryzae*.

In addition, we obtained the dominant negative isoform of MpYpt7 which remains bound to GDP. The asparagine at position 125 of MoYpt7 was changed to isoleucine (N125I). Four putative dominant negative transformants (MoYpt7-DN3, 4, 5, 6) were obtained. Quantitative polymerase chain reaction (PCR) analysis showed that the expression level of MoYpt7 in the MoYpt7-DN5 transformant was 30-fold higher than that in the wild-type strain Guy11. The expression of the other transformants (MoYpt7-DN3, 4, 6) was about 10-fold higher than that of MoYpt7 (Fig. 2a). These results suggest that the dominant negative isoform of MoYpt7 was expressed successfully in the MoYpt7-DN transformants.

Compared to the wild type strain Guy11, the MoYpt7-DN transformants had slightly reduced growth, sparse hyphae, and significantly reduced conidiation on complete medium (CM) (Fig. 2b). There was a 20-fold decrease in the MoYpt7-DN transformants (Fig. 2c). Small amounts of conidia were observed on the conidiophores (Fig. 2d). These results show that conidiation is related to the proper states of MoYpt7. The MoYpt7-DN transformants produced very few conidia, so mycelial plugs of the MoYpt7-DN transformants were inoculated onto barley leaves to assess the function of MoYpt7-DN in pathogenicity. Plugs of the wild-type strain Guy11 caused severe lesions on the barley leaves, but plugs of the MoYpt7-DN transformants failed to produce observable lesions (Fig. 2e). The change at position 125 of MoYpt7 impaired the pathogenicity of the fungus. In our previous study, GFP-MoYpt7 localized to the vacuolar membrane (Liu et al., 2015). To verify the localization of the dominant negative isoform of MoYpt7, MoYpt7-DN transformants were checked using fluorescent microscopy. We observed that GFP-MoYpt7^{N125I} localized to the cytoplasm (Fig. 2f). The differences between GFP-MoYpt7 and GFP-MoYpt7^{N125I} showed that the change at position 125 of MoYpt7 impaired the cellular sub-location of MoYpt7.

Rab GTPases perform their function by converting between GTP-bound and GDP-bound states. In *S. cerevisiae*, mutational analysis of Ypt7 function revealed that Q68L was in the GTP-bound state and T22N was in the GDP-bound state. Ypt7^{Q68L} played key roles in vacuolar morphogenesis, and Ypt7^{T22N} caused loss of function in vacuolar morphogenesis (Wada et al., 1996). In *Aspergillus nidulans*, *avaA* with a T22N mutation (GDP-bound form) showed fragmentation of vacuoles. Overexpression of the G67L (GTP-bound form) of *avaA* caused harmful effects on hyphal growth and vacuolated abnormal swellings (Ohsumi et al., 2002). In *M. oryzae*, the MoYpt7^{Q67L} mutant exhibited normal vacuole morphology.

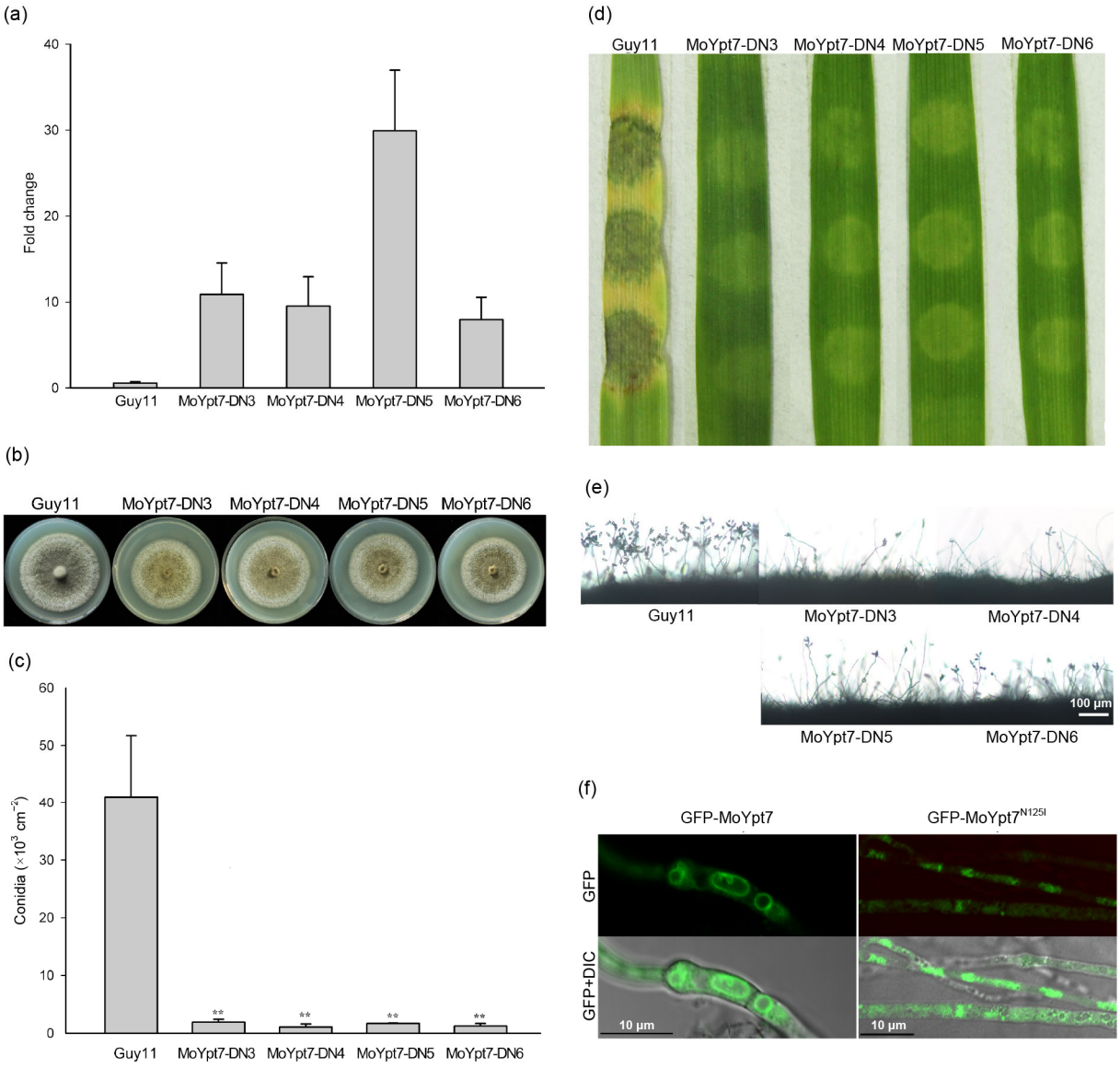

Fig. 2 Colony morphology, conidiation, and pathogenicity

(a) Relative transcript abundance of MoYpt7 in the MoYpt7-DN mutants. Data were presented by mean±standard deviation (SD); all $P<0.01$ vs. Guy11. (b) Characteristics of the MoYpt7-DN mutants. Guy11 and the MoYpt7-DN mutants were grown on CM medium for 7 d. (c) Conidiation of Guy11 and MoYpt7-DN mutants. Data were presented by mean±SD. ** $P<0.01$ vs. Guy11. (d) Development of conidia on conidiophores observed using a light microscope under cover slips 24 h after induction of conidiation. Fewer conidia developed in the MoYpt7-DN mutants. (e) Pathogenicity of the MoYpt7-DN mutants. (f) Localizations of GFP-MoYpt7 and GFP-MoYpt7^{N125I}. DIC: differential interference contrast

In addition, overexpression of the T22N of MoYpt7 did not cause any phenotypic change in *M. oryzae* (data not shown). Therefore, the mutation of N125I of MoYpt7 was introduced into *M. oryzae*. MoYpt7^{N125I} caused severe defects in conidiation and pathogenicity. Our results support the reported biological functions of MoYpt7 in *M. oryzae*.

Materials and methods

GFP-trap assay

Protein manipulation and immunoblot analysis were performed as described by Liu et al. (2015). For the GFP-trap-based pull-down assays, total protein extracts of transformants expressing GFP-MoYpt7

(Liu et al., 2015) were prepared to pull down binding proteins using a GFP-TrapA kit (Chromotek, Germany). Bound proteins were analyzed by MS. Detailed methods of MS performed by BGI of China are available in supplementary information Data S1.

A MoYpt7-3×Flag fusion construct was generated by cloning a MoYpt7 coding domain sequence (CDS) fragment amplified with the MoYpt7-3×Flag-F and MoYpt7-3×Flag-R primers and ligated into the pKD5-3×Flag vector using an In-Fusion HD Cloning Kit (Clontech, USA). MoGdi-GFP fusion construct was generated by cloning a MoGdi1 CDS fragment amplified with MoGdi-GFP-F and MoGdi-GFP-R primers and ligated into the pKD7-GFP vector using an In-Fusion HD Cloning Kit (Clontech, USA). For co-immunoprecipitation assays, we used PCR to identify the true transformants expressing both the MoYpt7-3×Flag and MoGdi-GFP fusion constructs, and used Western blots to confirm them. Then, total protein extracts were isolated from hyphae, and anti-Flag M2 beads (Sigma, UK) were incubated with the protein. Proteins eluted from the M2 beads were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and analyzed for the presence of MoYpt7-3×Flag and MoGdi-GFP fusions by Western blotting using anti-Flag and anti-GFP antibodies.

Bimolecular fluorescence complementation

$YFP^{1–156}$ and $YFP^{156–239}$ were amplified from pEYFP and cloned into the *Xba*I/*Sal*I sites of pKD5 and pKD6, respectively, to generate pKD5-YFPN and pKD6-YFPC. The full-length CDS of MoYpt7 was cloned into pKD5-YFPN and tagged at the C-terminus to YFP1-156 to generate YFPN-MoYpt7. The full-length CDS of MoGdi1 was cloned into pKD6-YFPC and tagged at the N-terminus to $YFP^{156–239}$ to generate MoGdi1-YFPC. The two constructs were co-transformed in pairs into wild-type Guy11. The selected strains were confirmed on defined complex medium (DCM) plates supplemented with both 100 μg/ml chlorimuron-ethyl and 800 μg/ml geneticin, with appropriate controls. The primer pairs used are listed in Table S1.

Generation of the dominant negative mutant

The dominant negative MoYpt7 mutant ($MoYpt7^{N125I}$) was generated by changing asparagine at position 125 to isoleucine via fusion. The two fragments were amplified from MoYpt7 cDNA using the MoYpt7DN-F1/R1 and MoYpt7DN-F2/R2 primers. The two fragments were fused and then cloned to the *Sal*I site of the pKD5GFP vector to generate $MoYpt7^{N125I}$. After DNA sequencing, the pKD5-$MoYpt7^{N125I}$ construct was reintroduced into Guy11 via *Agrobacterium tumefaciens*-mediated transformation (ATMT). DNA sequencing and quantitative real-time PCR (qRT-PCR) were used to examine the positive transformants using the MoYpt7qRT-F/R primers. The primers are listed in Table S1.

Phenotypic assays

Conidia and conidiophore formation and pathogenicity assays were performed as reported previously (Liu et al., 2015). Light and epifluorescence microscopic examinations were carried out with an Eclipse 80i microscope (Nikon, Japan) and a ZEISS LSM780 inverted confocal microscope (Carl Zeiss Inc., Germany).

Compliance with ethics guidelines

Lu-yao HUANG, Min WU, Xiao-yun YU, Lin LI, Fu-cheng LIN, and Xiao-hong LIU declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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List of electronic supplementary materials

Data S1 Materials and methods of mass spectrometry (MS)
Table S1 Primers used in this study

中文概要

题　目：稻瘟病菌 MoYpt7 互作蛋白和点突变的研究

目　的：研究 MoYpt7 与 MoGdi1 的互作关系以及定点突变体 $MoYpt7^{N125I}$ 的生物学功能。

创新点：补充了 MoYpt7 在稻瘟病菌致病过程中的生物学功能。

方　法：采用双分子荧光互补和免疫共沉淀的方法检测 MoYpt7 与 MoGdi1 的相互作用。利用点突变的方法，分析 MoYpt7 的鸟嘌呤二核苷酸磷酸（GDP）结合形式的生物学功能。

结　论：MoYpt7 与 Rab-GDP 解离抑制因子的一个同源物 MoGdi1 相互作用，获得了 MoYpt7 的 GDP 结合形式的过表达突变体。该突变体菌落形态发生变化，产孢能力和致病性显著下降。同时，稻瘟病菌 $MoYpt7^{N125I}$ 的亚细胞定位发生变化。

关键词：双分子荧光互补；免疫沉淀；MoGdi1；MoYpt7