RNAi-MEDIATED RESISTANCE TO RICE BLAST FUNGUS: TRANSFORMATIVE AND NON-TRANSFORMATIVE APPROACHES

A Thesis Submitted

In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

Atrayee Sarkar

Registration No. NITD/PhD/BT/2017/00972

Under the Supervision

of

Dr. Subhankar Roy-Barman



DEPARTMENT OF BIOTECHNOLOGY

National Institute of Technology Durgapur

West Bengal -713209

India

October, 2021

ABSTRACT

Rice is as important staple crop, widely consumed in several countries. *Magnaporthe oryzae*, a hemibiotrophic fungus is one of the top ten deadliest pathogens of rice, that annually destroys enough global produce to feed 60 million people. Especially with the UN's goal of 'food for sustainable future', global food security is of utmost importance. Developing more disease-resistant and climate-resilient cultivars is the only way of safeguarding our crops of the future. Disease resistance is that survival state where the plant's potential of fighting the pathogen is higher than the pathogenesis potential of the pathogen. The strategies of fungal blast control mostly rely on use of chemical fungicides and breeding for generation of resistant cultivars. However, *M. oryzae* has a very fast-evolving genome, which often renders such cultivars susceptible, in a few years time.

In this study, I aimed to develop a durable resistance against *M. oryzae*, utilising the RNA interference (RNAi) technology. Over the past decade, RNAi has been used in crop protection, whereby pathogenicity genes or pathogen survival–associated genes have been selectively targeted for post – transcriptional gene silencing, via precursor double-stranded RNAs (dsRNAs). Previously, gene knockdown has been achieved in *M. oryzae* either by Host Induced Gene Silencing (HIGS) via expression of fungal gene–specific hairpin RNA (hpRNA) in the host genome, or, via Bromovirus-mediated gene silencing (VIGS)(Guo et al., 2019; Zhu et al., 2017). A recent non-transformative strategy of gene silencing that involves exogenous spray of naked or nanocarrier-bound precursor dsRNA has emerged to be promising in the past few years. This RNA-based crop protection strategy, also known as Spray Induced Gene Silencing (SIGS) has not been explored in the rice-fungal blast pathosystem. Although in the last few years, environmental RNAi and a possible cross kingdom RNA exchange model has been established behind the functioning of SIGS, a lot of mechanistic details are yet unknown. This study was intended to validate and optimise the application of exogenous silencing RNAs in protecting rice against *Magnaporthe*, and silence one or more pathogenicity genes that are either directly or indirectly involved in attenuation of PTI, with and without any significant genetic modification of rice. Rice initially generates reactive oxygen species (ROS) as a part of PTI response, to hinder the growth and colonisation of *Magnaporthe*. There is a ROS scavenging mechanism in place that *Magnaporthe* uses to counter this host-derived ROS. Two fungal genes that are involved in ROS-neutralisation and effector delivery, respectively, in rice cells were selected for silencing based on their lack of homology with the host and other genes of *Magnaporthe* itself. A detailed assay for uptake of exogenously supplied, *in vitro* synthesised dsRNA and

siRNA, and its efficiency in specific silencing of target gene(s) was conducted in *M. oryzae*, using a GFP-reporter system . A therapeutic dosage of dsRNA was optimised over a time-course for effective silencing of target gene, both in case of *in vitro* feeding and *in planta* spray applications. Our studies showed that 300 nM dsRNA when sprayed on abraded leaves, can assert almost 70% silencing of the target gene, easily upto 7 d. Besides, the sprayed dsRNA can move to systemic, unsprayed plant parts and induce silencing, although upto 4 d post treatment (dpt). While in liquid media, as low as 25 ng/µl can induce silencing of reporter *GFP* in a cytoplasmic GFP transformant of *M. oryzae* from as early as 2 dpt, with a maximum of almost 80% reduction in GFP fluorescence on 4dpt. The wild type strain on being fed for 50 nM dsRNA for two consecutive generations, could exhibit silencing-associated phenotypic changes. *MoDES1* which is a defense suppressor of rice when silenced, not only interfered with the ROS scavenging and host colonisation potential of *Magnaporthe*, but also reduced its extracellular laccase and peroxidase activity, and virulence potential. Effective silencing of *MoDES1* via foliar spray application of ds*DES1* in rice, conferred partial resistance against blast disease via maintenance of PTI and complementing to its natural immunity. *MoEXO70* which is a crucial component of the exocyst complex transporting and delivering cytoplasmic effectors into rice cells, was used as the second target for HIGS. As a proof-of-concept, the ds*EXO70*-treated mycelia was found to be compromised in exocytosis–driven secretion of pathogenicity determinants. The treated spores showed defect in Spore Tip Mucilage (STM)-mediated appressorium penetration peg, consequently reducing their infection potential. The *in vivo* efficacy of the designed ds*EXO70* was evaluated by pretreating the rice leaves and sheaths with it, 24 h prior to wild-type spore infection. While rice sheath assay indicated less proliferative Invasive hyphae (IH) and impeded host colonisation, 3,3'-diaminobenzidine (DAB) staining revealed that it could be possibly due to the interference in effector- secretion leading to host-derived ROS generation. The ds*EXO70*-treated leaves showed markedly reduced disease severity and lesser fungal invasive growth. The distal leaf parts showed an upregulation of *OsPR1a,* the marker of SA-mediated defense. Besides, there was an upregulation of a blast resistance indicator and K^+ channel transporter *OsAKT1*, in infected ds*EXO70*-treated plants with respect to the infected untreated plants, which indicated low levels of a major cytoplasmic effector Avr-Piz-t in the infected host cells. A drastic reduction in *MoEXO70* transcripts coupled with reduced fungal biomass marker *28S* rDNA was documented from semi-quantitative PCR analysis of RNA extracted from the treated and infected leaves. Building upon the *in vitro* and *in vivo* validation of dsRNA- mediated silencing of *MoEXO70*, an *Agrobacterium*-pRNAi-*EXO70*

construct harbouring the *MoEXO70*- specific hpRNA was used for *in planta* piercing-based transformation of the susceptible variety CO-39. The putative T_0 lines carrying empty pRNAi-GG and pRNAi-*EXO70* were screened by Geneticin (G418) leaf painting and PCR analysis. Further the plants were grown under green-house conditions to set seeds upto T2 generation. Although the empty vector transformants showed disease severity similar to the untransformed wild type CO-43, some of the putative hpRNA construct carrying plants showed significantly diminished disease symptoms on being challenged with *Magnaporthe* spores. A significant 67-86% downregulation of *MoEXO70* was observed in some of these lines, aligned with the other disease resistance parameters like impaired intracellular growth of *M. oryzae* across infected rice cells, higher levels of host-derived ROS, HR response and reduction in fungal growth indicator *Mo28Sr*DNA. The resistance phenotypes resultant of the successful silencing of *MoEXO70* in the transgenic lines, were attributed to the stable expression of the ds*EXO70* in their genomes. The pRNAi-*EXO70* expressing transgenic plants did not show any trade-offs in their agronomic traits, emanated from putative off–target effects. As a conclusion, this work implemented the phenomena of dsRNA and siRNA uptake by *M. oryzae*, cross-kingdom RNA exchange between rice and blast fungal cells, and the HIGS and SIGS technology to impart disease resistance in rice, against *Magnaporthe*. It also shed some light on the effectiveness of naked dsRNAs in selective silencing of target genes, under *in vitro* and *in vivo* conditions. Besides the B157 strain, the transgenic line also exhibited significant reduction in disease symptoms, when challenged with few other Indian strains of *M. oryzae*. The stable resistance imparted by *in planta* silencing of *MoEXO70* indicated that it is possible to reduce disease severity by targeting the delivery of cytoplasmic effectors into host cells. The successful development of partial resistance merely by spraying naked ds*DES1* showed not only that uptake of ds*DES1* by the fungus via the plant cells can lead to better reinforcement of PTI, but also that silencing of a *M. oryzae* pathogenicity gene is possible in rice, even without genetic modification. This work also optimised the efficient use of a more novel and environment-friendly SIGS, as an alternative crop protection strategy to HIGS, in context of the model Rice–*M. oryzae* pathosystem.