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A *myosin5* dsRNA that reduces the fungicide resistance and pathogenicity of *Fusarium asiaticum*

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ABSTRACT

Fungal resistance to fungicides is a serious challenge in crop protection. Although strategies have been found to prevent the development of fungicide resistance, rare strategy has been found to quickly reduce such resistance once it has occurred. We demonstrate that the application of dsRNAs, which inhibit the expression of the phenamacril (fungicide JS399-19) target gene – *Myosin 5* (*Myo5*) in *Fusarium*, decreased *F. asiaticum* resistance to phenamacril and infection. RNAi molecules derived from different regions of *Myo5* gene had different effects on phenamacril-resistance. Myo5-8 (one of *Myo5* segments) exhibited great and stable effect on phenamacril-resistant reduction both *in vivo* and *in vitro*. *Myo5* mRNA and protein were both reduced when mycelium was treated with Myo5-8 dsRNA. After a mixture of Myo5-8 dsRNA and phenamacril treatment, plants can highly control the infection of phenamacril-resistant strain. The antifungal activity of Myo5-8 dsRNA plus phenamacril effected longer than a single Myo5-8 dsRNA. In addition, no off-target sequences were found in wheat and/or other plant and animal species for Myo5-8 dsRNA sequence. Our findings suggest a new strategy for fungicide resistant reduction and for designing new fungicides to control pathogens which easily develop fungicide resistance.

1. Introduction

Fungicides valued at approximately 16 billion US dollars are used to constrain the estimated 20% loss of global crop production due to disease annually [1]. The effectiveness of fungicides, however, is limited by the development of fungicide-resistant populations of fungal pathogens [2]. Fungicide failure resulting from resistance has increased since the late 1960s, especially after the use of systemic and site-specific fungicides became widespread [3–6]. The development of resistance in a range of plant pathogens to specific site-of-action of fungicides, including benzimidazoles, 2-aminopyrimidine mildewicides, demethylation inhibitors, and quinone outside inhibitors, has been widely reported [7–11]. In addition, mutations resulting in fungicide resistance that originated in the field have appeared in clinical infections [12]. The chemistry can be removed and alternatives with different modes of action deployed to reduce the frequency of the resistance conferring alleles in the population, but ways to decrease resistance rapidly once it has developed have not been reported [4, 13].

Fusarium species infect a broad range of plants and cause various crop diseases including Fusarium head blight and seedling blight of wheat and barley, *Gibberella* ear rot and stalk rot of maize, bakanae of rice, and vascular diseases and wilts of tomato and cotton. *Fusarium* species also cause keratitis, onychomycosis, and cutaneous diseases in humans [14–17]. These fungi cause huge economic losses in crop production and also produce mycotoxins that threaten human and animal health that consume the crop products [18]. Natural resistance against *Fusarium* pathogens is inadequate and current protective measures against *Fusarium* species mainly rely on fungicides. As noted earlier, however, resistance limits fungicide effectiveness, and new fungicides are being developed much more slowly than *Fusarium* populations are developing resistance [5, 19].

A new single-site fungicide, phenamacril (experimental code JS399-19; a.i. 2-cyano-3-amino-3-phenylancryic acetate), reduces *Fusarium* infection and mycotoxin content in crops and has been widely used since 2015 [20, 21]. According to laboratory studies, however, *Fusarium* species have a high risk of developing phenamacril resistance [22, 23]. Although pathogens may acquire fungicide resistance *via* several mechanisms, the main mechanism causing resistance to single-site fungicides is an alteration of the target protein due to mutations in the encoding gene [10]. Thus, one approach to limit resistance buildup

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is using fungicides with different modes of action. This approach can delay the development of resistance but will result in multiple resistant problems [24, 25]. Phenamacril resistance in *Fusarium* spp. is caused by mutations in the *myosin-5* gene (*Myo5*) [21]. We hypothesize that changes in *Myo5* expression will alter fungal sensitivity to phenamacril.

RNA interference (RNAi) has been used to control insect pests and virus diseases of plants and cellular diseases of human [26, 27]. Recent reports have shown that plants can be protected against *Fusarium graminearum* by host-induced gene silencing (HIGS) and by spray-induced gene silencing (SIGS) [28, 29]. However, the broad application of HIGS has been limited, by the lack of transformability of various crop plants, genetic instability and the issue of transgenic acceptance by the public presented. Some technical challenges also hinder the use of SIGS as a mainstream control strategy, such as the instability of dsRNA and the cost of RNA synthesis compared to conventional fungicides. A better option to improve SIGS was using dsRNA in combination with existing site-specific fungicide. Here, we describe an RNAi method to reduce *Fusarium* resistance to phenamacril. We identified and assayed the RNAi efficacy of different segments of the phenamacril target gene *Myo5* when such dsRNAs were applied to reduce fungicide resistance *in vitro*. RNAi molecules derived from different regions of *Myo5* gene had different effects on *F. asiaticum* resistance to phenamacril. Among eight segments, Myo5-8 exhibited great and stable effect on phenamacril-resistant reduction. The resistance of phenamacril-resistant strain FaJT041 decreased significantly when added Myo5-8 dsRNA into culture medium. Spraying Myo5-8 dsRNA on plant conferred high levels resistance to phenamacril-resistant *F. asiaticum*, with significant phenamacril-resistant reduction. Our findings provide a new strategy for reducing fungicide resistance after it has occurred.

2. Materials and methods

2.1. Strain constructions and culture conditions

Fusarium asiaticum (*Fa*) strain FaJT041 was isolated from a field in a region experiencing a Fusarium head blight epidemic in Jiangsu Province, China, and recovery of phenamacril-resistance in the lab [22]. *Fa*JT041 was used as the parental strain throughout this study. To construct FaJT041 Myo5RNAi transformants (FaJTMyo5RNAi), *Myo5* cDNA (3645 nt) from FaJT041 was divided into eight fragments (Myo5-1, 1–473 nt; Myo5-2, 452–945 nt; Myo5-3, 939–1454 nt; Myo5-4, 1381–1915 nt; Myo5-5, 1829–2314 nt; Myo5-6, 2254–2740 nt; Myo5-7, 2650–3207 nt and Myo5-8, 3149–3645 nt) and was constructed into eight RNAi vectors. Each vector carried a G418 resistance cassette and a transcriptional unit for hairpin RNA expression with a cutinase gene intron spacer from *Magnaporthe oryzae* [30], a glyceraldehyde 3 phosphate dehydrogenase promoter (gpd) and a trpC terminator from *Aspergillus nidulans* (Fig. 1A). The *PLS1* gene locus was used as an RNAi integration site [31]. The Myo5 protein was labeled with a GFP tag by inserting the coding region of the *GFP* gene downstream of the *Myo5* gene in FaJT041 strain, creating an in-frame fusion. PCRs were carried out in a TaKaRa PCR thermal cycler (TaKaRa TP600, Dalian, China) with gene-specific PCR primers. Southern blot analyses of FaJT-Myo5RNAi transformants were performed with the DIG labeling kit (Roche, Mannheim, Germany) [31]. All primers used in this study are listed in Table 1. Wheat variety Huaimai33 was maintained in our laboratory. Potato dextrose agar (PDA) and carboxymethyl cellulose (CMC) broth were used for mycelial growth and conidia production, respectively.

2.2. Fungicides susceptibility evaluation

For a fungicide-sensitivity assay in the laboratory, a 3-day-old mycelial plug (5 mm in diameter) was placed in the center of a PDA plate amended with phenamacril at 0, 50, 100, 200, 300, or 400 mg ml^{-1}. After 3 d at 25 °C, colony diameters were measured. Each combination of strain and concentration was represented by three biological replicates. The median effective concentration (EC_{50}) values were calculated with DPS software (version7.0, DPSInc., Cary, NC).

For fungicide sensitivity assays in the field, wheat cultivar Huaimai33 was grown according to normal agronomic practices at the experimental farm of Nanjing Agricultural University in 2016. When more than half of the wheat spikes reached anthesis [32], the field was divided into three plots (each plot was 4 × 5 m), each of which was sprayed with one of the following: (1) a control consisting of water; (2) 25% phenamacril at 375 g a.i. ha^{-1}; and (3) 25% phenamacril at 750 g a.i. ha^{-1}. The highly phenamacril resistant strain FaJT041 and the transformants FaJTMyo5RNAi-3, -4, -7, and -8 were each injected into 30 spikes (technical replicates) for each strain by single-floret injection at 24 h post spraying. Pathogenicity was assessed at 21 d post inoculation (dpi).

2.3. dsRNA silencing in vitro and application on living plant

dsRNAs were synthesized using MEGAscript RNAi Kit (Invitrogen, Carlsbad, CA, USA) following MEGAscript protocols. GFP dsRNA (derived from the GFP coding sequence, 560 nt, sequence ID: HF675000.1) was used as a control dsRNA. Fresh conidia were added to SNA media containing 0.1 pM Myo5-8 dsRNA with or without 10 ng μl^{-1} phenamacril at 1×10^3 ml^{-1} final concentration. These were cultured in a 96-well microtiter plate and observed with an inverted microscope.

For determination of dsRNA application on plant, 3-d-old coleoptiles were sprayed with 0.1 pM Myo5-8 dsRNA, 0.1 pM Myo5-8 dsRNA plus 10 ng μl^{-1} phenamacril or 0.1 pM GFP-specific 560-nt dsRNA (control). After 12 h, seedlings were inoculated with 3 µl of macroconidia suspension (5×10^5 spores/ml), and were carried out in growth chambers. Thirty seedlings (technical replicates) were inoculated in each treatment groups, and the brown lesions of diseased seedlings were measured and stained by calcofluor white (CFW) at 7 dpi.

2.4. Quantitative RT-PCR and western blotting hybridization

RNA samples were isolated with the RNA simple kit (TIANGEN Biotech, Beijing, China) from germ tubes grown for 48 h in SNA media containing 10 ng μl^{-1} phenamacril, 0.1 pM Myo5-8 dsRNA, or 0.1 pM Myo5-8 dsRNA with 10 ng μl^{-1} phenamacril, respectively. Quantitative RT-PCR (qRT-PCR) was performed with an ABI 7500 real-time detection system (Applied Biosystems, Foster City, CA, USA). The fungal housekeeping gene *ubiquitin C-terminal hydrolase UBH* (Gene ID: FGSG_01231) was used as an internal control. Data from three biological replicates were used to calculate the mean and standard deviation.

Mycelia harvested from 48 h cultures grown in SNA media containing 10 ng μl^{-1} phenamacril, 0.1 pM Myo5-8 dsRNA, or 0.1 pM Myo5-8 dsRNA with 10 ng μl^{-1} phenamacril were ground with liquid nitrogen. Proteins were isolated as described previously [33] and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to Immobilon-P Transfer membrane (Millipore, Billerica, MA, USA) in a Trans-Blot SD semi-dry transfer cell (Bio-Rad, Hercules, USA). The monoclonal anti-GFP ab32146 (Abcam, Cambridge, UK) and anti-GAPDH (as a reference) (Abbkine Scientific Co., Ltd) antibodies were used at a 1:5000 dilution for immunoblot analyses. The intensity of immunoblot bands was quantified using the ImageQuantTL software.

2.5. Microscopic analysis

Microscopic observation was performed using an inverted fluorescence microscope Olympus IX71 (Olympus Canada, Markham, ON). Images were captured and analyzed by Image-Pro Plus software (Media Cybernetics, Silver Spring, Maryland).