Effects of Mutations in the Phenamacril-Binding Site of Fusarium Myosin-1 on Its Motor Function and Phenamacril Sensitivity

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ABSTRACT: Phenamacril is a Fusarium-specific fungicide used for Fusarium head blight management. The target of phenamacril is FgMyo1, the sole class I myosin in Fusarium graminearum. The point mutation S217 in FgMyo1 is responsible for the high resistance of F. graminearum to phenamacril. Recent structural studies have shown that phenamacril binds to the 50 kDa cleft of the FgMyo1 motor domain, forming extensive interactions, including a hydrogen bond between the cyano group of phenamacril and the hydroxyl group of S217. Here, we produced S217T or S217L each increased the IC50 of FgMyo1IQ2, a truncated FgMyo1 composed of the motor domain and two IQ motifs complexed with the F. graminearum calmodulin calmodulin in insect S9 cells. Phenamacril potently inhibited both the basal and the actin-activated ATPase activities of FgMyo1IQ2, with an IC50 in a micromolar range. S217 mutations of FgMyo1IQ2 substantially increased the IC50 of phenamacril. S217T or S217L each increased the IC50 of phenamacril for ~60-fold, while S217A only increased the IC50 for ~4-fold. These results indicate that the hydroxyl group of S217 plays an important, but nonessential role in phenamacril binding and that the bulky side chain at the position 217 sterically hinders phenamacril binding. On the other hand, S217P, which might alter the local conformation of the phenamacril-binding site, completely abolished the phenamacril inhibition. Because the cyano group of phenamacril does not form discernible interactions with FgMyo1 other than the nonessential hydrogen bond with the S217 hydroxyl group, we propose the cyano group of phenamacril as a key modification site for the development of novel fungicides.

INTRODUCTION

Fusarium graminearum is a major pathogen responsible for the outbreak of Fusarium head blight, a worldwide fungi disease in major cereal crops. The cyanoacrylate compound phenamacril (2-cyano-3-amino-3-phenylacryic acetate, JS399-19) is a Fusarium-specific fungicide, which potently inhibits the mycelia growth of a subset of Fusarium species, including F. graminearum, F. moniliforme, F. oxysporum, and F. avenaceum, but not the other related fungal pathogens. We recently identified the sole class I myosin in F. graminearum (FgMyo1) as the target of phenamacril. FgMyo1 belongs to the long-tailed class I myosin, composed of three distinct domains, i.e., the motor domain, the neck region or the light chain-binding domain (LCBD), and the characteristic C-terminal long tail domain. The motor domain contains the ATP-binding site and the actin-binding site and is able to convert the energy from ATP hydrolysis into a conformational change. The LCBD of FgMyo1 contains two IQ motifs, which serve as the binding sites for calmodulin (CaM) or CaM-like light chain. The α-helical structure of myosin IQ motifs are stabilized by the bound light chains, allowing the LCBD to function as a rigid lever arm to amplify the conformational change of the motor domain during the ATP hydrolysis cycle. Moreover, the LCBD might also play a role in regulating the motor function. While CaM is a common light chain for many unconventional myosins, including class I myosin, CaM-like light chain can also serve as the light chain for several class I myosins. FgMyo1 and FgCaM (F. graminearum CaM) co-expressed in insect S9 cells could be co-purified and crystallized as an FgMyo1/FgCaM complex. However, the authentic light chain of FgMyo1 remains to be determined. The long tail domain of FgMyo1 comprises two conserved tail homology (TH1 and TH2) domains, an Src homology 3 (SH3) domain and a C-terminal acidic A-domain (CA domain). The long tail domain of class I myosin directly or indirectly influences the actin assembly.

Phenamacril is a specific inhibitor of FgMyo1. We and others have shown that the motor activity of FgMyo1 was strongly inhibited by phenamacril. In contrast, two FgMyo1 single-residue mutants (S217L and E420K), identified from the phenamacril-resistant strains of F. graminearum, were only slightly inhibited by phenamacril. Because both S217 and E420 are located in the 50 kDa cleft of the FgMyo1 motor domain, we and others proposed that phenamacril binds to the...
50 kDa cleft of FgMyo1, thus inhibiting its motor function.\(^5,\!^6,\!^9\) The crystal structure of phenamacril-bound FgMyo1 has recently been solved, validating that phenamacril indeed binds to the 50 kDa cleft of the FgMyo1 motor domain (Figure 1).\(^10\)

**Figure 1.** Structure of phenamacril-bound FgMyo1. (A) Chemical structure of fungicide phenamacril. (B) Three-dimensional structure of the phenamacril-bound FgMyo1 (PDB code 6U14). This structure contains the motor domain and the first IQ motif in complex with FgCaM. The upper panel shows the whole structure, and the lower panel shows an enlarged view of the boxed region in the upper panel. The four subdomains of the motor domain are shown in different colors as follows: the 25 kDa subdomain (light green), the upper 50 kDa subdomain (light orange), the lower 50 kDa subdomain (light blue), and the 20 kDa subdomain (gray). FgCaM is colored in magenta. Phenamacril is shown as spheres. ATP is shown as sticks, and Mg\(^{2+}\) is shown as a red sphere. S217 and E420 are shown as green sticks, and other residues studied in this work are shown as cyan sticks.

With the exception of hydrophilic interactions with S217 and D540, the binding of phenamacril to the 50-kDa cleft is driven largely by hydrophobic interactions. The hydroxyl (OH) group of S217 forms a hydrogen bond with the cyano group of phenamacril, consistent with the phenamacril resistance observed in FgMyo1 S217L mutant.

In this study, we identified FgCaM as the light chain of FgMyo1 and characterized the phenamacril inhibition on the ATPase activity of FgMyo1IQ2, a truncated FgMyo1 composed of the motor domain and two IQ motifs in complex with FgCaM. By mutagenesis of S217, we found that the OH group of S217 plays an important but nonessential role in the interaction with phenamacril.

## RESULTS

### Identification of FgCaM as the Light Chain of FgMyo1

We first identified the light chain of FgMyo1, FgMyo1IQ2, a truncated FgMyo1 containing the motor domain, two IQ motifs, and an N-terminal FLAG-tag, was expressed in insect Sf9 cells (Figure 2A). To identify the light chain binding to FgMyo1IQ2, we took an approach previously used in the identification of the light chains of vertebrate myosin-19.\(^14\) It is known that the light chains associated with myosin IQ motifs are CaM or CaM-like light chains. Unlike most other proteins, CaM or CaM-light chains are able to refold into the native state after removing the denaturant. Therefore, we first extracted myosin light chain from the mycelium of *F. graminearum* with a solution containing denaturant urea, which denatures most proteins including myosin and the light chain. The extract was then subjected to the renaturation process. We expected that only a minority of proteins, including CaM and CaM-like light chain, will be recovered. The crude extract of myosin light chains from the mycelium of *F. graminearum* was incubated with the FgMyo1IQ2 pre-bound to Anti-FLAG Sepharose. The bound proteins were then eluted and subjected to SDS-PAGE separation and mass spectra analysis. SDS-PAGE of the eluted proteins showed an ~90 kDa band of FgMyo1IQ2 and an ~20 kDa band of an unknown protein (Figure 2B).

Mass spectrometry (MS) analysis of the ~20 kDa band revealed eight peptide sequences matching FgCaM, which is 85.2% identical in sequence to vertebrate CaM (Figure 2C,D). This result suggests that FgCaM is the light chain of FgMyo1. We therefore co-expressed FgMyo1IQ2 and FgCaM in insect Sf9 cells and obtained the purified FgMyo1IQ2 in complex with FgCaM. We quantified the amount of the FgMyo1IQ2-bound FgCaM based on the band density in a Coomassie Brilliant Blue stained SDS gel with a known concentration of FgCaM as a standard. The estimated stoichiometry of FgCaM versus FgMyo1IQ2 was about 2, indicating strong interactions between the two IQ motifs of FgMyo1 and FgCaM.

### Characterization of FgMyo1 ATPase Activity and Its Inhibition by Phenamacril

Similar to the most characterized myosins, FgMyo1IQ2 displayed a very low basal ATPase activity (ATPase activity in the absence of actin), which was strongly activated by actin (Figure 3A). FgMyo1 ATPase activities at various actin concentrations were fitted to the Michaelis–Menten equation, defining $V_{\text{max}}$ of $10.71 \pm 1.31$ s\(^{-1}\) and $K_{\text{actin}}$ of $15.4 \pm 2.2$ μM (Figure 3A and Table 1). As a control, the ATPase activities of mouse myosin-5a HMM at various actin concentrations were measured under similar conditions. We obtained a $V_{\text{max}}$ of $11.68 \pm 1.43$ s\(^{-1}\) and a $K_{\text{actin}}$ of $16.54 \pm 1.74$ μM for myosin-5a HMM, both of which are very similar to the reported values.\(^15\) It is known that the most characterized class I myosins from vertebrates have a very low actin-activated ATPase activity ($V_{\text{max}}$ less than 1 s\(^{-1}\))\(^16,\!^{18}\) and the long-tailed class I myosins from lower eukaryotes have a much higher ATPase activity ($V_{\text{max}}$ of 5-15 s\(^{-1}\))\(^19,\!^{22}\) FgMyo1 can therefore be considered as a typical long-tailed class I myosin from lower eukaryotes in terms of the actin-activated ATPase activity. We previously reported ~3 s\(^{-1}\) of the actin-activated ATPase activity of FgMyo1IQ2 in complex with vertebrate CaM, which was measured in the presence of 40 μM actin and 150 mM NaCl.\(^3\) Under similar conditions, the actin-activated ATPase activity of FgMyo1IQ2 in complex with FgCaM was 3.2 s\(^{-1}\). These results indicate that substitution of FgCaM with vertebrate CaM in FgMyo1IQ2 does not greatly alter FgMyo1IQ2 ATPase activity.

We previously demonstrated that phenamacril strongly inhibits the actin-activated ATPase activity of FgMyo1IQ2 in complex with vertebrate CaM.\(^3\) Here, we analyzed the phenamacril inhibition on the ATPase activity of FgMyo1IQ2 in complex with FgCaM. The basal ATPase activity of
FgMyo1IQ2 was inhibited by phenamacril to a large extent (95.3 ± 5.1% maximal inhibition) with an IC50 of 0.605 ± 0.113 μM phenamacril (Figure 3B). The actin-activated ATPase activity (in the presence of 40 μM actin) of FgMyo1 was almost completely inhibited by phenamacril with an IC50 of 1.10 ± 0.06 μM phenamacril (Figure 3C). Therefore, phenamacril inhibited both the basal and the actin-activated ATPase activities of FgMyo1.
phenamacril. In the presence of 1 mM phenamacril, the ATP-dependent interaction between FgMyo1 and F-actin was largely unaffected by phenamacril. Notably, phenamacril reduced the amount of the actin-pelleted FgMyo1IQ2 in the presence of ATP slightly (comparing lane 4 and lane 8 in Figure 3D,E), similar to the effect of blebbistatin on the actin sedimentation of myosin II in the presence of ATP.23

Effects of FgMyo1 S217 Mutations on Phenamacril Inhibition. We previously showed that the S217L mutant of FgMyo1 resists phenamacril inhibition,5 consistent with the direct interaction between the side chain of S217 and phenamacril.10 To further determine the role of the S217 side chain on the phenamacril inhibition of FgMyo1, we produced four more FgMyo1IQ2 S217 mutants, i.e., S217A, S217T, S217G, and S217P, and examined their sensitivities to phenamacril. All five FgMyo1 S217 mutants were expressed in S9 cells and purified using Anti-FLAG Sepharose chromatography as described for the wild-type. The representative preparations of the purified FgMyo1IQ2 S217 mutants are shown in Figure 4A. With the exception of S217L, all FgMyo1 S217 mutants displayed relatively normal basal ATPase activities and actin-activated ATPase activities (Figure 4B). The actin-activated ATPase activity of FgMyo1-S217L was about 30% as that of the wild-type (Figure 4B).

Next, we examined the effect of FgMyo1 S217 mutations on the phenamacril inhibition. The actin-activated ATPase activities of all S217 mutants except S217P were inhibited by phenamacril, although their IC\textsubscript{50} values were higher than that for the wild-type (Figure 5 and Table 1). The IC\textsubscript{50} for S217A and S217G were 5–10 times as that for the wild-type, indicating that the OH group of S217 plays a role in the interaction with phenamacril but is not essential for the interaction. On the other hand, the IC\textsubscript{50} for S217T and S217L were about 60 times of that for the wild-type, suggesting that the bulky side chain might sterically hinder the binding of phenamacril to the motor domain. Most strikingly, S217P was not inhibited by phenamacril even at the 500 \( \mu M \) concentration.

Although both FgMyo1 S217T and S217L were inhibited by phenamacril with similar IC\textsubscript{50} values, the extents of phenamacril inhibition were quite different (Figure 5). For S217T, ~84% of the actin-activated ATPase activity was inhibited by 500 \( \mu M \) phenamacril, and the curve fitting shows that ~96% activity could be inhibited by the saturated concentration of phenamacril. In contrast, ~47% of the actin-activated ATPase activity of S217L was inhibited by 500 \( \mu M \) phenamacril, and the curve fitting shows that only ~52% of activity could be inhibited by the saturated concentration of phenamacril. At least two possibilities may account for the partial inhibition of S217L by phenamacril. First is the heterogeneity of the purified S217L protein. About half of S217L was able to bind to phenamacril while the other half was not. Another possibility is that the phenamacril-bound S217L retained half the level of the actin-activated ATPase activity. At present, we are unable to distinguish these two possibilities.

Effects of the Two Newly Identified FgMyo1 Mutations on Phenamacril Inhibition. Besides F. graminearum, several additional Fusarium fungi have been tested for susceptibility to phenamacril.4,24 The K216Q/N380K double mutation in Fusariumavenaceum (Fa) myosin-I results in a moderate resistance to phenamacril in Fa strains.4 Another double mutation, V1S1A/S418T, in Fusarium oxysporum (Fo)
myosin-1, was proposed to be responsible for the low resistance to phenamacril in the plant pathogenic Fo strains.24

To determine whether these two double mutations affect the phenamacril inhibition of the FgMyo1 motor function, we introduced these mutations into FgMyo1IQ2 and examined their sensitivity to phenamacril inhibition. Both K216Q/N380K and V151A/S418T mutants of FgMyo1IQ2 were expressed in insect Sf9 cells and purified as for the wild-type (Figure 6A). Both mutants showed normal levels of basal and actin-activated ATPase activities as the wild-type (Figure 6B). Although the actin-activated ATPase activities of both mutants were strongly inhibited by phenamacril, their IC_{50} were substantially higher than that of the wild-type (Figure 6C and Table 1). The IC_{50} of phenamacril for K216Q/N380K and V151A/S418T mutants were 18.96 ± 0.64 μM and 9.06 ± 0.33 μM, respectively, about 17- and 8-fold as that of the wild-type, i.e., about 17- and 8-fold as that of the wild-type (Table 1).

Table 1. ATPase Activities of FgMyo1IQ2 Mutants and Their Sensitivities to Phenamacril Inhibition

| FgMyo1IQ2     | V_{0} (s^{-1}) | V_{40μM} (s^{-1}) | maximal inhibition (%) | IC_{50} (μM) | RF value |
|---------------|----------------|-------------------|------------------------|--------------|----------|
| WT            | 0.21 ± 0.02    | 8.14 ± 1.23       | 100.7 ± 1.8            | 1.10 ± 0.06  | 1        |
| S217A         | 0.31 ± 0.07    | 9.18 ± 0.30       | 97.0 ± 0.3             | 5.78 ± 0.11  | 5.2      |
| S217G         | 0.29 ± 0.01    | 6.64 ± 0.15       | 95.1 ± 2.3             | 14.74 ± 1.01 | 13.4     |
| S217T         | 0.54 ± 0.02    | 15.18 ± 0.31      | 95.9 ± 2.0             | 69.32 ± 15.54| 62.4     |
| S217L         | 0.33 ± 0.07    | 2.26 ± 0.59       | 52.2 ± 3.6             | 68.03 ± 15.92| 61.8     |
| S217P         | 0.55 ± 0.06    | 8.51 ± 0.22       | 95.1 ± 0.8             | 18.96 ± 0.64 | 17.2     |
| K216Q/N380K   | 0.41 ± 0.01    | 11.47 ± 0.48      | 97.1 ± 0.8             | 9.06 ± 0.33  | 8.2      |
| V151A/S418T   | 0.27 ± 0.02    | 11.51 ± 0.41      | 95.1 ± 0.8             | 18.96 ± 0.64 | 17.2     |

Note: V_{0}, the basal ATPase activity, i.e., the ATPase activity in the absence of actin; V_{40μM}, the ATPase activity in the presence of 40 μM actin; and RF value, IC_{50} of FgMyo1IQ2 mutant /IC_{50} of FgMyo1IQ2 WT.

V151 is located at the motor domain surface opposite to the S0 kDa cleft. The slight increase in IC_{50} from V151A/S418T is likely due to the allostery of the myosin motor domain.

**DISCUSSION**

FgMyo1, the sole class I myosin in *F. graminearum*, is essential for fungus growth.5 Here, we characterized the motor property of FgMyo1 and its inhibition by phenamacril, a small molecule for *F. graminearum* control. We demonstrated that FgMyo1 has robust actin-activated ATPase activity and interacts with actin in an ATP-dependent manner. Phenamacril potently inhibited both the basal and the actin-activated ATPase activities of FgMyo1 but did not greatly affect the interaction between actin and FgMyo1. Mutagenesis of S217, a key phenamacril-interacting residue in FgMyo1, showed that the side chain of S217 is not essential for phenamacril inhibition.
In the crystal structure of phenamacril-bound FgMyo1, phenamacril binds to the motor domain by interacting with 17 residues in the 50 kDa cleft. Most of those interactions are hydrophobic, except for two hydrophilic interactions, i.e., the hydrogen bond between the cyano group of phenamacril and the OH group of the side chain of S217 and a putative charged interaction between the amino group of phenamacril and the OH group of the side chain of D540.

Our current work suggests that the hydrogen bond between the cyano group of phenamacril and the OH group of S217 is not essential for phenamacril binding. Substitution of S217 with alanine, a residue structurally identical to serine except for the lack of the OH group, only increased the IC50 by ~4-fold, indicating that the OH group of S217 plays a limited role in the interaction with phenamacril and is nonessential for the interaction. Similarly, substitution of S217 with glycine, which has no side chain, only increased the IC50 by ~12-fold. On the other hand, introducing a bulky side chain at this position (such as S217T and S217L) increased the IC50 of phenamacril by ~60-fold, suggesting that the bulky side chain at 217 might sterically hinder phenamacril from binding to the 50 kDa cleft. Most strikingly, S217P mutation completely abolished the phenamacril inhibition. Given the exceptional conformational rigidity of proline, S217P mutation likely altered the local conformation of the 50 kDa cleft and disrupted multiple interactions with phenamacril. Taken together, we propose that the high resistance of S217 mutants is due to steric obstruction or local conformational change, rather than the loss of the hydrogen bond between the cyano group of phenamacril and the OH group of S217.

Phenamacril is a potent fungicide specific to a subset of Fusarium species, including F. graminearum. However, the fungus frequently develops resistance due to point mutations in the motor domain of FgMyo1 under selection pressure from phenamacril. As such, there is an urgent need for the development of novel fungicides. The recently determined structure of phenamacril-bound FgMyo1 sets a strong foundation for the development of novel FgMyo1 inhibitors for Fusarium management. The high affinity of phenamacril to FgMyo1 can be attributed to its extensive interactions with multiple residues in the 50 kDa cleft. Because the cyano group of phenamacril does not form any discernible interaction with FgMyo1 except for the nonessential hydrogen bond with the S217 OH group, we propose the cyano group of phenamacril as a key modification site for the development of novel fungicides. Wollenberg et al. recently showed that substitution of the cyano group of phenamacril with an ethyl ester completely abolishes the fungicidal activity and the inhibition of FgMyo1 ATPase activity. This is likely caused by the steric hindrance of the ethyl ester, which is much bulkier than the cyano group. We expect that phenamacril analogues with smaller substituents in place of the cyano group might retain the fungicidal activity.

The actin-activated ATPase activity of FgMyo1-S217L was about 30% as that of the wild-type. This is consistent with the observation that S217L mutation decreased mycelial growth, sporulation, and pathogenicity of F. fujikuroi. In contrast, FgMyo1-S217P had a similar level of the actin-activated ATPase activity as the wild-type, consistent with the normal fitness of the F. fujikuroi strain containing S217L mutation. Intriguingly, the F. graminearum strain containing S217L mutation did not show detectable changes in hyphal growth and conidiation when compared with the wild-type, suggesting that the relative low activity of FgMyo1-S217L is not the rate-limiting step for F. graminearum growth. On the other hand, the F. graminearum strain expressing FgMyo1-E420K mutant, which retained less than 5% of the actin-activated ATPase activity of the wild-type, was vital but grew much slower than the wild-type. Those results suggest that maintaining a substantial level of FgMyo1 motor activity is essential for the normal growth of F. graminearum.

## EXPERIMENTAL PROCEDURES

### Materials

Restriction enzymes and modifying enzymes were purchased from New England Biolabs (Beverly, MA), unless indicated otherwise. AccuScript Reverse Transcriptase and Pfu Ultra DNA polymerase were from Stratagene. Anti-FLAG M2 affinity agarose, phosphoenol pyruvate, 2,4-dinitrophenylhydrazine, glucose oxidase, and pyruvate kinase were from Sigma-Aldrich (St. Louis, MO). ATP, dithiothreitol (DTT), and dimethyl sulfoxide (DMSO) were from Ameresco. FLAG peptide (DYKDDDDK) was synthesized by Augt Co. (Beijing, China). Myosin-5a HMM, rabbit skeletal muscle, and actin were prepared as described previously.

### Expression and Purification of FgMyo1_{1Q2}, FgMyo1_{1Q2} (residues 1–757) wild-type was expressed in insect Sf9 cells and purified with Anti-FLAG M2 affinity chromatography as described previously, except that Sf9 cells were co-infected by two recombinant baculoviruses encoding FgMyo1_{1Q2} and FgCaM (see below), respectively. Purified proteins were aliquoted, quick-frozen in liquid nitrogen, and stored at ~80 °C. The protein concentration of FgMyo1_{1Q2} was determined by the absorbance at OD_{280nm} and using a molar extinction coefficient of 94,880 L mol^{-1} cm^{-1}. The amount of the FgMyo1_{1Q2}-bound FgCaM was determined by SDS-PAGE and Coomassie Brilliant Blue staining with the known concentration of FgCaM (see below) as a standard. The stoichiometry of FgCaM versus FgMyo1_{1Q2} was then calculated based on the molecular mass.
FgMyo1IQ2 mutants were created by QuikChange site-directed mutagenesis using Phusion Ultra DNA polymerase with FgMyo1IQ2/pFastBac as the template. The cDNAs generated by PCR were confirmed by DNA sequencing. The recombinant baculovirus encoding FgMyo1IQ2 was prepared as described previously.

**cDNA Cloning and Protein Expression of FgCaM.** The cDNA of FgCaM (gene locus: FGSG_01891) was obtained by RT-PCR using the total RNA of mycelia of *F. graminearum* strain PH-1 as the template. The FgCaM cDNA was cloned into the pET30a expression vector (Novagen-Merck Biosciences) using the NdeI and BamHI sites (the NdeI and BamHI sites are located before and after the His-tag coding region; thus, the obtained plasmid does not contain the His-tag sequence). FgCaM was expressed in BL21(DE3) and purified by phenyl-Sepharose chromatography as described for *Drosophila* CaM. The protein concentration of FgCaM was determined by the absorbance at OD280nm using a molar extinction coefficient of 1280 L mol⁻¹ cm⁻¹.

To prepare the recombinant baculovirus encoding FgCaM, the cDNA of FgCaM was subcloned into the baculovirus vector pFastBac (Invitrogen) using the EcoRI and the KpnI sites. The recombinant baculovirus was prepared using the Bac-to-Bac system (Invitrogen), as described previously.

**Identification of the Light Chain of FgMyo1.** To determine the light chain associated with FgMyo1, we took an approach similar to that for the identification of the light chain of mouse myosin-19. We expected that only a minority of proteins, including CaM and CaM-like light chain, will be recovered. We first prepared a crude extract of myosin light chains from the mycelia of *F. graminearum* strain PH-1. About 200 mg of the mycelia of *F. graminearum* strain PH-1 was homogenized in 5 mL of lysis buffer (30 mM Tris-HCl (pH 7.5), 8 M urea, 5 mM DTT, and 10 µg/mL leupeptin). The homogenization was clarified by centrifugation at 25000 rpm for 1 h at 4 °C. The proteins in the supernatant were denatured by 5% trichloroacetic acid and then subjected to centrifugation at 25000 rpm for 30 min at 4 °C. The precipitation was dissolved with 8 M urea (adjust the pH to 7.0 with 1 M Tris-base) and then dialyzed against 1 L of 30 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 1 mM DTT three times (6 h each). The dialyzed sample was clarified by centrifugation at 13000 rpm for 10 min at 4 °C, and the supernatant was used as the crude extract of myosin light chains.

The isolation and the identification of the light chain associated with FgMyo1 were performed as follows: FLAG-tagged FgMyo1IQ2 was expressed in Sf9 cells and absorbed onto Anti-FLAG M2 agarose. About 0.5 mL of Anti-FLAG M2 agarose containing ~0.2 mg of FgMyo1IQ2 was then incubated with 1 mL of the crude extract of myosin light chains in binding buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM MgCl₂, 1 mM EGTA, and 1 mM DTT) at 4 °C for 2 h. After washing the unbound proteins away with binding buffer, the bound protein was eluted with 0.2 mg/mL FLAG peptide in binding buffer. The eluted proteins were subjected to SDS-PAGE (~20%) and Coomassie Brilliant Blue staining. The ~20 kDa band in SDS-PAGE was excised and subjected to trypsin digestion and LC-MS/MS analysis.

**Actin Co-sedimentation Assay.** Purified FgMyo1IQ2 (1 µM) was incubated with rabbit skeletal muscle actin (10 µM) in a 50 µL solution consisting of 20 mM MOPS-KOH (pH 7.0), 50 mM KCl, 2 mM MgCl₂, 1 mM DTT, 1 mM EGTA, 0 or 1 mM ATP, and 0 or 1 mM phenamacril at 25 °C for 5 min and then centrifuged in a benchtop ultracentrifuge (Beckman Optima MAX-XP) at 80000 rpm for 10 min at 0 °C. The supernatant was recovered and added with 12 µL of 5XSDS-loading buffer. The pellet was resuspended in 60 µL of SDS-loading buffer. Equal portions of the supernatant and the pellet (20 µL each) were analyzed by SDS-PAGE and visualized by Coomassie Brilliant Blue staining.

**ATPase Assay.** The ATPase activity was measured at 25 °C using an ATP regeneration system, as described previously.

The basal ATPase activity of FgMyo1IQ2 was measured in a solution containing 20 mM MOPS-KOH (pH 7.0), 50 mM NaCl, 1 mM MgCl₂, 0.9 mM EGTA, 1 mM DTT, 0.25 mg/mL BSA, 4 µM FgCaM, 0.5 mM ATP, 2.5 mM phosphoenolpyruvate, 20 U/mL pyruvate kinase, and ~0.1 µM FgMyo1IQ2. The actin-activated ATPase activity of FgMyo1IQ2 was measured as described above, except using ~30 nM FgMyo1IQ2 and an indicated concentration of actin.

To analyze the phenamacril inhibition on the basal ATPase activity of FgMyo1IQ2, the ATPase assay was performed as the assay for the basal ATPase activity described above, except for the addition of an indicated concentration of phenamacril and 5% DMSO. To analyze the phenamacril inhibition on the actin-activated ATPase activity of FgMyo1IQ2, the ATPase assay was performed as the assay for the actin-activated ATPase activity, except using 40 µM actin, an indicated concentration of phenamacril, and 5% DMSO. The IC₅₀ was derived by a hyperbolic fit, V = (V_max – V_min)/(1 + [phenamacril]/IC₅₀) + V_min, where V_max is the activity in the absence of phenamacril, V_min is the activity in the presence of saturated phenamacril, and IC₅₀ is the concentration of phenamacril required to achieve a half-maximal degree of inhibition. Note that DMSO was used for dissolving phenamacril and we found that 5% DMSO slightly altered the actin-activated ATPase activity of FgMyo1IQ2.

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T.N. and M.Y. are the primary persons responsible for performing the experiments of this study. H.-H.J. contributed to the preparations of the cDNA constructs and recombinant proteins. G.T. analyzed the phenotype of F. graminearum. T.N., M.Y., Y.C., Z.M., and X.-d.L. designed research and analyzed the data. X.-d.L. wrote the manuscript. All authors reviewed the manuscript.

Notes

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Abbreviations

FgMyo1, the class I myosin of Fusarium graminearum; CaM, calmodulin; FgCaM, calmodulin of Fusarium graminearum

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