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The AMT1 Arginine Methyltransferase Gene Is Important for Plant Infection and Normal Hyphal Growth in Fusarium graminearum

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Abstract

Arginine methylation of non-histone proteins by protein arginine methyltransferase (PRMT) has been shown to be important for various biological processes from yeast to human. Although PRMT genes are well conserved in fungi, none of them have been functionally characterized in plant pathogenic ascomycetes. In this study, we identified and characterized all of the four predicted PRMT genes in Fusarium graminearum, the causal agent of Fusarium head blight of wheat and barley. Whereas deletion of the other three PRMT genes had no obvious phenotypes, the Δamt1 mutant had pleiotropic defects. AMT1 is a predicted type I PRMT gene that is orthologous to HMT1 in Saccharomyces cerevisiae. The Δamt1 mutant was slightly reduced in vegetative growth but normal in asexual and sexual reproduction. It has increased sensitivities to oxidative and membrane stresses. DON mycotoxin production and virulence on flowering wheat heads were also reduced in the Δamt1 mutant. The introduction of the wild-type AMT1 allele fully complemented the defects of the Δamt1 mutant and AMT1-GFP fusion proteins mainly localized to the nucleus. Hrp1 and Nab2 are two hnRNPs in yeast that are methylated by Hmt1 for nuclear export. In F. graminearum, AMT1 is required for the nuclear export of FgHrp1 but not FgNab2, indicating that yeast and F. graminearum differ in the methylation and nucleo-cytoplasmic transport of hnRNPs. Because AMT2 also is a predicted type I PRMT with limited homology to yeast HMT1, we generated the Δamt1 Δamt2 double mutants. The Δamt1 single and Δamt1 Δamt2 double mutants had similar defects in all the phenotypes assayed, including reduced vegetative growth and virulence. Overall, data from this systematic analysis of PRMT genes suggest that AMT1, like its ortholog in yeast, is the predominant PRMT gene in F. graminearum and plays a role in hyphal growth, stress responses, and plant infection.

Introduction

In eukaryotic organisms, reversible phosphorylation of proteins by protein kinase and phosphatase is well known to regulate various growth and development processes. Protein methylation is another form of post-translational modifications that also play regulatory roles in various processes, including nucleo-cytoplasmic transport of proteins, transcriptional activation and elongation, mRNA precursors splicing, and signal transduction [1,2,3,4]. The majority of protein methylation occurred at the arginine residues are catalyzed by protein arginine methyltransferases (PRMTs), which are divided into four major classes. Type I and type II PRMTs catalyze asymmetric and symmetric ω N^G, N^O-dimethylation of arginine residues, respectively [2]. Whereas type III PRMTs catalyze ω N^G monomethylation of arginines, type IV PRMTs catalyze the formation of δ N^G-monodemethylarginine. In human, type I PRMTs include PRMT1, PRMT3, PRMT4, PRMT6, and PRMT8; PRMT5, PRMT7, and PRMT9 are type II PRMTs [5]. Whereas PRMT1, PRMT3, and PRMT5 are well conserved in eukaryotic organisms, PRMT2, PRMT8, and PRMT9 lack distinct orthologs in unicellular eukaryotes and may be required for tissue-specific functions in multicellular organisms [6,7].

The budding yeast Saccharomyces cerevisiae has only three PRMT genes, HMT1, RMT2, and HSL7 [8]. HMT1 (type I) is the major arginine methyltransferase and possesses similar functions of mammalian PRMT1. HMT1 is not essential for cell growth in yeast. However, deletion of HMT1 is synthetically lethal with mutations in the NPL3 or CBP80 genes [9]. RMT2 is a type IV PRMT gene that is found in fungi and plants but not in protozoa and human [2]. The HSL7 gene (type II) is orthologous to human PRMT5 [8]. In Arabidopsis, many RNA binding or processing proteins are methylated by AtPRMT5. Mutations in the AtPRMT5 gene affected RNA splicing in hundreds of genes involved in...
different biological processes and causes pleiotropic developmental defects, such as late flowering [10].

In S. cerevisiae, Hmt1 is a non-essential member of the heterogeneous nuclear ribonucleoproteins (hnRNPs) that are involved in mRNA biogenesis [9]. It confers SAM-dependent methylation to components of hnRNPs, which often contain C-terminal RGG-rich repeats as the sites of arginine methylation [11]. Hrp1, Nab2, and Npl3 are among the most studied hnRNPs that are methylated by Hmt1. Methylation by Hmt1 is important for their export from the nucleus [9,11,12]. Hrp1 is involved in the processing of 3′ ends of pre-mRNA, mRNA polyadenylation, and the nonsense mediated decay pathway [13,14,15]. The Nab2 protein is required for the export of pol(A) RNA and pol(A) tail length control [12,16]. Npl3 has been implicated in transcription elongation and termination [17]. Methylation by Hmt1 in the nucleus and phosphorylation by the SR protein kinase Sky1 in the cytoplasm regulate the nucleo-cytoplasmic transport of Npl3 [18].

Orthologs of yeast PRMT genes are well conserved in filamentous plant pathogenic ascomycetes. However, none of them have been experimentally characterized for their biological functions in plant pathogenic ascomycetes. F. graminearum is a major causal agent of wheat and barley head blight or scab worldwide [19,20]. Fusarium head blight (FHB) poses as a serious problem in wheat production by causing severe yield losses and contamination of infested kernels with harmful mycotoxins, including deoxynivalenol (DON) and zearalenone [19,21]. Because of the importance of PRMT genes in eukaryotes [2,5], in this study we identified and functionally characterized all of the four predicted PRMT genes in F. graminearum. Whereas deletion of the other three PRMT genes had no obvious phenotypes, the Δamt1 mutant was significantly reduced in virulence and DON production in infection assays with flowering wheat heads. Our results indicate that AMT1, like its ortholog HMT1 in yeast, is the predominant arginine methyltransferase in F. graminearum. Although dispensable for sexual and asexual reproduction, AMT1 is important for normal growth rate, stress responses, plant infection, and nucleo-cytoplasmic transport of FgHrp1.

Results

Identification of the HMT1 ortholog, AMT1, in F. graminearum

The genome of F. graminearum contains four PRMT genes, FGSG_01134 (XP_381310), FGSG_10718 (XP_390894), FGSG_00501 (XP_380677), and FGSG_10756 (XP_390932) that are named AMT1-AMT4 (for arginine methyltransferase genes) in this study. FGSG_01134 (AMT1) is orthologous to HMT1, which is the main arginine methyltransferase gene in S. cerevisiae. The 345-amino acid protein encoded by HMT1 has a typical arginine methyltransferase domain. FGSG_10718 (AMT2) encodes a PRMT3-like protein that also shares significant homology with yeast HMT1. FGSG_00501 (AMT3) and FGSG_10756 (AMT4) are orthologous to yeast RMT2 and HSL7, respectively (Figure S1). Unlike the budding yeast, filamentous ascomycetes such as Magnaporthe oryzae and Aspergillus nidulans (Figure S1) have four PRMT genes.

Generation of Δamt1 deletion mutants

The AMT1 gene replacement construct (Fig. 1A) was generated with the split-marker approach and transformed into the wild-type strain PH-1. Putative Δamt1 mutants were identified by PCR and confirmed by Southern blot analysis (Fig. 1B). In the wild type, a 7.0-kb BamHI band was detected with an AMT1 fragment amplified with primers AMT1/5F and AMT1/6R (Table S2) as the probe A (Fig. 1B). The same probe had no hybridization signal in transformants M1, M2, and M3 (Table 1). When probed with a fragment of the hph gene, PH-1 had no hybridization signals. Transformants M1 and M2 had a 6.4-kb band (Fig. 1B), which is similar to the expected size derived from the gene replacement event (Fig. 1A). Transformant M3 had a weak 6.4-kb band but a strong 10-kb band, suggesting that besides targeted homologous recombination, multiple copies of the AMT1 gene replacement construct were integrated ectopically during transformation. Therefore, only transformants M1 and M2 were the expected Δamt1 deletion mutants with no additional integration events. Mutants M1 and M2 had the same phenotype although only data with mutant M2 were described below.

When assayed for growth on CM medium, the Δamt1 mutant produced less aerial hyphae than the wild type (Fig. 1C) and had approximately 24% reduction in growth rate (Table 2). It was also reduced in aerial hyphal growth and growth rate on PDA, 5×YEG, and YEPD plates (Figure S2). When the wild-type AMT1 allele was transformed into the Δamt1 mutant, defects in hyphal growth and other phenotypes described below were rescued in the resulting Δamt1/AMT1 transformant C2 (Table 2). These results

Figure 1. The AMT1 gene replacement construct and deletion mutants. A. The AMT1 locus and gene replacement construct. The AMT1 and hph genes are marked with empty and black arrows, respectively. 1F, 2R, 3F, and 4R are primers used to amplify the flanking sequences. BamHI (B). B. Southern blot analysis with the wild type (PH-1) and Δamt1 transformants (M1, M2, and M3). All DNA samples were digested with BamHI. The blots were hybridized with probe A (left) amplified with primers AMT1/5F and AMT1/6R and probe B (right) amplified with H852 and H850. C. Colony morphology of the PH-1, Δamt1 mutant M2, and Δamt1/AMT1 transformant C2 cultures grown on CM. Photographs were taken after incubation for 3 days.

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The wild-type and mutant strains of *Fusarium graminearum* used in this study.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Brief descriptions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH-1</td>
<td>Wild-type</td>
<td>[55]</td>
</tr>
<tr>
<td>M1</td>
<td>Δamt1 mutant of PH-1</td>
<td>This study</td>
</tr>
<tr>
<td>M2</td>
<td>Δamt1 mutant of PH-1</td>
<td>This study</td>
</tr>
<tr>
<td>M3</td>
<td>Δamt1 mutant of PH-1</td>
<td>This study</td>
</tr>
<tr>
<td>C2</td>
<td>Δamt1/AMT1 transformant of M2</td>
<td>This study</td>
</tr>
<tr>
<td>Y5</td>
<td>Δamt1/AMT1-GFP transformant of M2</td>
<td>This study</td>
</tr>
<tr>
<td>HP10</td>
<td>Transformant of PH-1 expressing FgHRP1-GFP</td>
<td>This study</td>
</tr>
<tr>
<td>HA11</td>
<td>Transformant of M2 expressing FgHRP1-GFP</td>
<td>This study</td>
</tr>
<tr>
<td>NP12</td>
<td>Transformant of PH-1 expressing FgNAB2-GFP</td>
<td>This study</td>
</tr>
<tr>
<td>NA14</td>
<td>Transformant of M2 expressing FgNAB2-GFP</td>
<td>This study</td>
</tr>
<tr>
<td>DM7</td>
<td>Δamt1 Δamt2 double mutant</td>
<td>This study</td>
</tr>
<tr>
<td>DM12</td>
<td>Δamt1 Δamt2 double mutant</td>
<td>This study</td>
</tr>
<tr>
<td>KS2</td>
<td>Δamt2 (FGSG_10718) deletion mutant of PH-1</td>
<td>This study</td>
</tr>
<tr>
<td>KT3</td>
<td>Δamt3 (FGSG_00501) deletion mutant of PH-1</td>
<td>This study</td>
</tr>
<tr>
<td>KF4</td>
<td>Δamt4 (FGSG_10756) deletion mutant of PH-1</td>
<td>This study</td>
</tr>
</tbody>
</table>

To characterize the defects of the Δamt1 mutant in plant infection, inoculated flowering wheat heads were sampled, fixed, and examined for hyphal growth. At 48 h post-inoculation (hpi), fungal growth was observed on the surface and inside glume tissues inoculated with the wild type (Fig. 3). In wheat heads inoculated with the Δamt1 mutant, fungal growth was abundant on the surface and rarely in glume tissues (Fig. 3). At 120 hpi, the wild type had colonized the vascular and other tissues of the rachis and produced abundant intracellular hyphae (Fig. 3). In contrast, fungal growth was limited or much sparse in the rachis near the spikelet inoculated with the Δamt1 mutant (Fig. 3), indicating that AMT1 is important for invasion and spreading in plant tissues. These observations are consistent with reduced virulence of the Δamt1 mutant.

The Δamt1 mutant is significantly reduced in virulence

In infection assays with flowering wheat heads, the Δamt1 mutant caused typical scab symptoms in the inoculated kernels and was able to spread to nearby spikelets (Fig. 2A). However, it was significantly reduced in virulence compared to PH-1 (Fig. 2A). The average disease index, a measurement for virulence by counting diseased spikelets per wheat head, of the Δamt1 mutant and PH-1 was 4.3 and 13.8, respectively (Table 2), indicating that the Δamt1 mutant was defective in disease spreading.

Corn also is a host to *F. graminearum*. In infection assays with corn silks, the Δamt1 mutant caused only limited discoloration near the inoculation sites. Under the same conditions, extensive discoloration was observed in corn silks inoculated with PH-1 (Fig. 2B), confirming that AMT1 is important for virulence in *F. graminearum*.

Table 2. Defects of the Δamt1 mutant in growth, conidiation, and plant infection.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Growth rate (mm/d)*</th>
<th>Conidiation (10⁶/ml)</th>
<th>Disease Indexa</th>
<th>DON (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH-1 (WT)</td>
<td>14.0±0.07c</td>
<td>1.39±0.16a</td>
<td>13.8±3.8c</td>
<td>1589.6±359.4c</td>
</tr>
<tr>
<td>M2 (Δamt1)</td>
<td>10.6±0.3a</td>
<td>1.45±0.25a</td>
<td>4.3±3.7a</td>
<td>397.0±188.7a</td>
</tr>
<tr>
<td>C2 (Δamt1/AMT1)</td>
<td>14.3±0.2a</td>
<td>1.42±0.21a</td>
<td>14.0±3.4a</td>
<td>1782.8±279.6a</td>
</tr>
</tbody>
</table>

*Average growth rate/conidiation and standard error (mean ± standard error) were calculated from at least three independent measurements.

Disease was rated by the number of symptomatic spikelets 14 days after inoculation. Mean and standard error were calculated with results from three independent experiments. At least 10 wheat heads were examined in each repeat.

Data from three replicates were analyzed with the protected Fisher’s Least Significant Difference (LSD) test. The same letter indicated that there was no significant difference. Different letters were used to mark statistically significant difference (P=0.05).

*Correlation coefficients for the disease index and the growth rate were 0.56 (P<0.01) and 0.52 (P<0.01), respectively.

*Correlation coefficients for the disease index and the conidiation were 0.65 (P<0.01) and 0.62 (P<0.01), respectively.
In the presence of 0.05% H2O2 or 0.01% SDS, the AMT1 gene is likely involved in responses to oxidative and membrane stresses. Growth on PDA plates with 0.7 M NaCl (Fig. 4). However, AMT1 activity was more significantly reduced in growth rate than the wild type and AMT1 mutant stains had no obvious difference in vegetative growth on PDA plates with 0.7 M NaCl (Fig. 4). However, AMT1 is likely involved in responses to oxidative and membrane stresses. In the presence of 0.05% H2O2 or 0.01% SDS, the AMT1 mutant was more significantly reduced in growth rate than the wild type (Fig. 4).

Subcellular localization of AMT1-GFP fusion

To determine the expression and localization of AMT1, we generated an AMT1-GFP fusion construct and transformed it into the AMT1 mutant. After screened by PCR and confirmed by Southern blot analysis, transformant Y5 (Table 1) was identified as one of the transformants expressing the AMT1-GFP construct under the control of its native promoter. Similar to the complemented strain C2, defects of the Δamt1 mutant were rescued in the Δamt1/AMT1-GFP transformant. GFP signals were present in both cytoplasm and nuclei in conidia and 7 h germlings of transformant Y5 (Fig. 5). However, nuclei had stronger fluorescence signals than the cytoplasm, indicating that majority of Amt1-GFP proteins localized to the nucleus.

Aamt1 influences the nuclear transport of FgHrp1

In S. cerevisiae, Hmt1 is involved in the regulation of nucleocytoplasmic transport of hnRNP components, including Hrp1 and Nab2 [9]. Orthologs of HRP1 and NAB2 in F. graminearum are FGSG_13728.3 and FGSG_01282.3, respectively. We constructed the FgHRP1-GFP and FgNAB2-GFP fusion constructs and transformed them into the wild-type and Δamt1 mutant strains. In the resulting transformants expressing the FgHRP1-GFP construct, the subcellular localization of FgHrp1-GFP fusion proteins differed significantly between the wild-type and Δamt1 mutant (Fig. 6). In the Δamt1 mutant, GFP signals were detected mainly in the nucleus. Each nucleus had one or more dots of bright GFP signals that may correspond to hnRNP particles associated with FgHrp1. In the wild type, GFP signals were primarily observed in the cytoplasm, indicating that deletion of AMT1 affected the nuclear export of FgHrp1 proteins. Fluorescent particles in the cytoplasm may represent protein complexes that are associated with FgHrp1 after its exit from the nucleus.

In contrast, FgNab2-GFP proteins were distributed mainly in the nucleus in both wild-type and Δamt1 mutant strains (Figure S4), indicating that AMT1 is not important for the subcellular localization of FgNab2 in F. graminearum. Therefore, arginine methylation may play different roles in the nucleo-cytoplasmic transport of different hnRNP components in F. graminearum and S. cerevisiae.

Functional characterization of the other three PRMT genes in F. graminearum

To determine the functions of other three putative PRMT genes, the split-marker approach was used to generate the AMT2 (FGSG_10718), AMT3 (FGSG_00501), and AMT4 genes in the Wheat Scab Fungus.
(FGSG_10756) gene replacement constructs. The resulting PCR products were transformed into protoplasts of the wild-type strain PH-1. The Δamt2, Δamt3, and Δamt4 knockout mutants (Table 1) were identified by PCR and confirmed by Southern blot analyses. In comparison with the wild type, the Δamt2, Δamt3, and Δamt4 mutants had no obvious defects in vegetative growth, conidiation, and production of perithecia and ascospores (Figure S3; Figure S5). They also had similar growth rates with the wild type on PDA plates with 0.05% H2O2, 0.01% SDS, or 0.7 M NaCl, or 300 μg/ml Congo red (Figure S6). In infection assay with corn silks, the Δamt2 and Δamt3 mutants were as virulent as the wild type but the Δamt4 was slightly reduced in virulence (Fig. 7A; Table S1). These results indicate that AMT2, AMT3, and AMT4 genes are dispensable for vegetative growth, asexual and sexual reproduction, and stress responses. While AMT2 and AMT3 were dispensable for plant infection, AMT4 was required for full virulence.

The Δamt1 Δamt2 double mutant has similar defects in plant infection with the Δamt1 mutant

The AMT1 and AMT2 genes are the only two predicted type I arginine methyltransferase genes in F. graminearum. They both share sequence similarity with yeast Hmt1 (Figure S1). To determine their functional relationship, we generated the Δamt1 Δamt2 double mutant by deletion of AMT2 in the Δamt1 mutant M2. The resulting double mutant (Table 1), similar to the Δamt1 mutant, was normal in conidiation and sexual reproduction (Figure S3) but slightly reduced in vegetative growth (Figure S5). In plant infection assays, the Δamt1 Δamt2 had similar defects in virulence with the Δamt1 mutant (Fig. 7A). On PDA plates with 0.05% H2O2 or 0.01% SDS, the Δamt1 and Δamt1 Δamt2 mutants also had similar growth defects (Fig. 7B). Therefore, the Δamt1 and Δamt1 Δamt2 mutants had no significant differences in growth, stress responses, and virulence. These results suggest that AMT1 and AMT2 have no overlapping functions.

Deletion of AMT1 results in less than 2-fold changes in the expression of AMT2, AMT3, and AMT4

RNA samples were isolated from vegetative hyphae of PH-1 and the Δamt1 mutant grown in liquid CM for 6 h. In comparison with the wild type, the expression levels of AMT2, AMT3, and AMT4 were reduced approximately 21%, 10%, and 44%, respectively, in the Δamt1 mutant (Fig. 8A). However, none of them had over 2-fold changes in the expression level between the wild type and Δamt1 mutant strains.

Deletion of AMT1 affects the expression of genes adjacent to the telomere

Because deletion of HMT1 is known to affect the formation of silent chromatin [22], we assayed the expression of three genes, FGSG_14027, FGSG_11614, and FGSG_11613 that are within 10 kb from the telomeric repeat sequences (TTAGGG) on supercontig 14 (Chromosome 4, Fig. 8B). The current version of F. graminearum assembly contains no other telomeric repeat sequences. FGSG_14027 (795–1625) encodes a putative histone deacetylase gene orthologous to yeast HOS4. It is less than 1 kb away from the telomeric repeats. FGSG_11614 (3250–5086) and FGSG_11613 (5679–7261) encode hypothetical proteins that are conserved in filamentous ascomycetes but not in yeast. Whereas the expression level of FGSG_14027 and FGSG_11614 was increased over approximately 4- and 5-fold, respectively, expression of FGSG_11613 was slightly increased but not significantly in the Δamt1 mutant compared to the wild type (Fig. 8B). Since FGSG_11613 is more distal to the telomeric repeats than the other
two genes, it is likely that silencing of genes adjacent to the telomere is affected by deletion of AMT1.

The expression and activation of Mgv1, Gpmk1, and FgHog1 MAP kinases in the amt1 mutant

Because PRMTs are known to affect signal transduction in mammalian cells [3], we assayed the phosphorylation of all three MAP kinases that have been characterized in F. graminearum and known to be important for plant infection [23,24,25,26,27]. In comparison with the wild type strain, the amt1 mutant was normal in the expression and phosphorylation levels of Mgv1 and FgHog1 (Fig. 9). For Gpmk1, the expression level was normal but the phosphorylation level was slightly but not significantly reduced (Fig. 9). These data indicate that Amt1 does not significantly affect the expression and activation of these MAP kinases in F. graminearum.

Discussion

Methylation of the arginine residues by arginine methyltransferases plays important roles in various cellular processes in eukaryotic organisms such as nucleo-cytoplasmic transport and mRNA biogenesis [1,3,4]. The genome of F. graminearum contains four predicted arginine methyltransferase genes that belong to the type I, type II, and type VI of PRMTs [28,29]. Phenotype analyses with targeted deletion mutants of these PRMT genes indicated that only the Δamt1 mutant had obvious defects in growth and plant infection. Mutants deleted of the other three PRMT genes had no significant phenotypes. Therefore, similar to its ortholog in yeast, AMT1 must be the predominant arginine methyltransferase in F. graminearum. In A. nidulans, three AMT1 genes, rmtA, rmtB, and rmtC, that are orthologous to AMT1, AMT2, and AMT4, respectively, have been characterized. None of the rmtA, rmtB, and rmtC deletion mutants had obvious defects in vegetative growth, sexual, and asexual reproduction on normal growth conditions [30], suggesting that A. nidulans may lack a predominant PRMT gene.

AMT2 encodes a predicted type I PRMT protein that shares significant sequence similarity to PRMT3 in human. Its orthologs are well conserved in filamentous fungi, including M. oryzae, A. nidulans, and Neurospora crassa and the fission yeast Schizosaccharomyces pombe. However, S. cerevisiae and Candida albicans lack a distinct ortholog of AMT2, suggesting that this gene may have been lost in some Saccharomycetales species during evolution. As the only other predicted type I PRMT gene in F. graminearum, AMT2 shares limited homology with AMT1 and yeast RMT1. AlthoughAmt2 has a C2H2 zinc finger domain that is absent in Amt1, deletion of the AMT2 gene had no obvious phenotypic changes. In A. nidulans, deletion of the rmtB gene also lacked any detectable phenotype [30]. To determine the relationship between AMT1 and AMT2, we deleted the AMT2 gene in the Δamt1 mutant. The Δamt1 mutant and the Δamt1 Δamt2 double mutant had no significant differences in the phenotypes assayed, including growth rate, sensitivities to oxidative stress, and virulence. Deletion of AMT1 also had no significant impact on the expression level of AMT2 (Fig. 8A). Therefore, it is unlikely for AMT2 to have overlapping functions with AMT1 in F. graminearum.

For the other two PRMT genes in F. graminearum, AMT3 and AMT4 are orthologous to the RMT2 and HLS7 genes in yeast, respectively. Rmt2 and its related PRMT genes are specific to fungi and plants [31]. In yeast, Rmt2 specifically methylates ribosomal protein Rpl12 (L12) on Arg67 [32]. The rmt2 mutant is defective in δ N\textsuperscript{6}-methylarginine modifications but normal in growth and reproduction [33]. In C. albicans, the rmt2/rmt2 mutant grew as robustly as the reconstituted or heterozygous strains in rich media but the level of δ N\textsuperscript{6}-monomethylarginine is reduced [31]. However, no data on virulence of the mutant were presented. The genome of A. nidulans contains the ortholog of AMT3 (Figure S1).
but it has not been experimentally characterized for its biological function [30].

In *S. cerevisiae*, Hsl7 is required along with Hsl1 kinase for bud neck recruitment, phosphorylation, and degradation of Swe1 [34]. The Δhsl7 mutant produces elongated, anucleate buds and has increased sensitivity to Calcofluor and CaCl₂ [34]. In *F. graminearum*, the AMT1 deletion mutant had no obvious defects but the rmtC mutant of *A. nidulans* had increased sensitivity to oxidative stress and elevated temperatures [30]. In *U. maydis*, the Hsl7 ortholog was identified as a Smu1 PAK kinase interacting protein. It regulates cell length and the filamentous response to solid SLAD (synthetic low ammonia plus 2% dextrose) but is dispensable for plant infection. Although the *amt3* and *amt4* mutants of *F. graminearum* had no obvious defects in phenotypes assayed in this study, *AMT3* and *AMT4* genes are well conserved in filamentous fungi [35]. It is likely that they are functional in some biological processes that remain to be characterized in *F. graminearum*.

In *S. cerevisiae*, Hmt1 is a non-essential component of the hnRNP complex [9]. Hmt1 affects the nucleo-cytoplasmic transport of other hnRNP components that are important for mRNA biogenesis. In *F. graminearum*, the Δhmt1 mutant was reduced approximately 24% in vegetative growth but normal in conidiation and ascospore production. If it also is a component of hnRNP in *F. graminearum*, Amt1 may be dispensable for mRNA processing of genes that are important for sexual reproduction and conidiation. In Arabidopsis, the *AtPRMT5* gene only affects RNA splicing in a subset of genes [10]. It is likely that only subsets of genes important for vegetative growth and plant infection (fungal infection) are affected in the Δamt1 mutant in *F. graminearum*. AMT1 appears to play no or only minor roles in genes involved in sexual and asexual reproduction.

In infection assays with flowering wheat heads and corn silks, the Δamt1 mutant was significantly reduced in virulence. Although AMT1 orthologs are well conserved, none of them have been characterized in plant pathogenic ascomycetes. In the human pathogen *Candida albicans*, deletion of CalHMT1 affects the expression and localization of NPL3 [31]. However, the function of CalHMT1 in virulence has not been reported. One common stress faced by hyphae of necrotrophic fungi in planta is reactive oxygen species (ROS) generated during oxidative burst [36,37]. The Δhmt1 mutant, similar to the rnt1 mutant of *A. nidulans* [30], had increased sensitivity to H₂O₂. It also had a slightly reduced growth rate and increased sensitivity to membrane stress. All of these defects may contribute to the defects of the Δamt1 mutant in plant infection. In addition, in diseased wheat kernels, the Δamt1 mutant was reduced in the production of DON, which is a well-characterized virulence factor in *F. graminearum* [38,39]. However, reduced DON production in infected plant tissues may be related to reduced fungal biomass of the Δamt1 mutant.

In *S. cerevisiae*, two of the well-characterized hnRNP components are Hrp1 and Nab2 [12,13]. HRP1 is an essential gene that encodes a KRM-containing protein required for the cleavage and polyadenylation of pre-mRNA at the 3′-ends [13]. Nab2 is a nuclear pol(A) RNA-binding protein required for nuclear mRNA export and pol(A) tail length control. Methylation by Hmt1 regulates the shuttle of Hrp1 and Nab2 between the nucleus and cytoplasm. Hrp1 and Nab2 fail to exit the nucleus in cells lacking Hmt1 [11,13]. In *F. graminearum*, FgHrp1-GFP fusion proteins were accumulated in the nucleus (Fig. 5), suggesting that Amt1 is required for the nucleocytoplasmic transport of FgHrp1. However, in transformants expressing the FgNAB2-GFP fusion construct, GFP signals mainly localized to the nucleus in both the wild type and Δamt1 mutant. The localization and nucleocytoplasmic transport of FgNab2 appears to be independent of Amt1. These results indicate that methylation by this PRMT and nucleo-cytoplasmic transport of hnRNP components may be different between *S. cerevisiae* and *F. graminearum*.

### Materials and Methods

#### Strains and culture conditions

The wild-type strain PH-1 and all the transformants of *F. graminearum* generated in this study were routinely cultured on PDA agar plates [24]. Growth rate and conidiation were assayed as described [40,41]. DNA and RNA were extracted from vegetative hyphae harvested from liquid YEED (1% yeast extract, 2% peptone, 2% glucose). Sexual reproduction, and protoplast preparation, and PEG-mediated transformation were performed as described [24]. Hygromycin B (Calbiochem, La Jolla, CA) and geneticin (Sigma, St. Louis, MO) were added to the final concentration of 300 and 350 μg/mL, respectively, to the TB3 medium for transformant selection. To test sensitivity against various stresses, vegetative growth was assayed on PDA plates with 0.05% H₂O₂ (v/v), 0.01% SDS (w/v), or 0.7 M NaCl as described [25,42].

#### Generation of Δamt1, Δamt2, Δamt3, Δamt4, and Δamt1 Δamt2 mutants

All the mutants were generated with the split-marker approach [43]. For AMT1, the 0.83-kb upstream and 0.65-kb downstream flanking sequences were amplified with primer pairs AMT1F1-2R and AMT1F3-4R, respectively (Fig. 1A and Table S2). The resulting PCR products were connected to hygromycin phosphotransferase (hph) fragments amplified with primers HY-R-YG/F and HYG/F-HYG/R by overlapping PCR and transformed into protoplasts of PH-1 as described [40,44]. Hygromycin-resistant transformants were screened for Δamt1 mutants by PCR with primer pairs AMT1F5-R6, AMT1F7-HB55R, and H856F-AMT1R8 (Table S2). Putative Δamt1 mutants were then analyzed by Southern blot hybridizations to confirm the gene replacement event. The same approach was used to generate the Δamt2, Δamt3, and Δamt4 mutants. To generate the Δamt1 Δamt2 double mutant, the AMT2 gene replacement construct generated with the neomycin resistance gene (*NEO*) was transformed into the Δamt1 mutant M2.

#### Complementation of the Δamt1 mutant

A fragment containing the entire AMT1 gene and its promoter and terminator sequences was amplified with primers AMT1-CM/F and AMT1-CM/R (Table S2), digested with *Pst* and BamHI, and cloned between the *Pst*I and *Bam*HI sites of the *NEO* vector pH100 [45]. The resulting construct, pAMT1, was transformed into protoplasts of the Δamt1 mutant M2. The Δamt1/AMT1 transformants were confirmed by PCR and Southern blot analyses.

#### Generation of AMT1-GFP, HRP1-GFP, and NAB2-GFP fusion constructs

To generate the AMT1-GFP fusion, PCR products amplified with primers AMT1-YA/F and AMT1-YA/R (Table S2) were cloned into pFL2 by the yeast gap repair approach [46,47]. The same approach was used to generating the HRP1-GFP and NAB2-GFP fusion constructs. All GFP fusion constructs were verified by sequencing analysis and transformed into protoplasts of PH-1 or...
the Δamt1 mutant M2. G418-resistant transformants harboring the transforming AMT1-GFP, HRP1-GFP, or NAB2-GFP construct were identified by PCR and confirmed by the presence of GFP signals.

Infection and DON production assays

For infection assays with flowering wheat heads of cultivars XiaoYan 22 or Norm, conidia were harvested from 5-day-old CMC cultures and re-suspended in sterile distilled water to 2.0×10^7 conidia/ml. The fifth spikelet from the base of the spike was inoculated with 10 μl of the conidial suspension as described [46]. Inoculated wheat heads were capped with a plastic bag to keep humidity for 48 h. After removing the bags, wheat plants were cultured for another 12 days before examination for symptomatic spikelets. Infested kernels were harvested and used for qRT-PCR analysis were listed in Table S2. Relative expression levels of each gene were calculated by the 2-DDCt method [51] with the F. graminearum GAPDH gene [52] as the endogenous reference. Data from three biological replicates were used to calculate the mean and standard deviation.

Western blot analysis

Total proteins were isolated from 24 h germlings grown in CM, separated on a 12.5% SDS-PAGE, and transferred to nitrocellulose membranes for western blot analysis as described [47,53]. TEY-phosphorylation of Mvg1 and Gpmk1 and TGY-phosphorylation of FgHog1 were detected with the PhosphoPlus p44/42 and p38 MAP kinase antibody kits (Cell Signaling Technology, Danvers, MA) following the manufacturer’s instructions [54].

Supporting Information

Figure S1 Phylogenetic analysis of fungal PRMTs. The amino acid sequences encoded by PRMT genes from Fusarium graminearum, Candida albicans, Saccharomyces cerevisiae, Schizosaccharomyces pombe, Magnaporthe oryzae, Neurospora crassa, Aspergillus nidulans, and Homo sapiens were analyzed by the DNAman5.0 program to create the dendrogram. The branch length is proportional to the mean number of differences per residue along each branch. All of the filamentous ascomycetes analyzed have four PRMT genes. Whereas three of them are orthologous to human PRMT1, PRMT3, and PRMT5, the fourth one is specific to fungi and plants. Scale bar is equal to 5% sequence divergence.

Figure S2 Cultures of the wild type and Δamt1 mutant M2 grown on PDA, 5×YEG, and YEYP plates. (TIF)

Figure S3 Perithecia and cirri produced by the wild-type strain (PH-1) and the Δamt1 (M2), Δamt2 (KS2), Δamt3 (KT3), Δamt4 (KF4), and Δamt1 Δamt2 (DM7) mutants. Photographs were taken 14 days after fertilization. (TIF)

Figure S4 Deletion of AMT1 had no effects on the nucleo-cytoplasmic transport of FgNab2. In both transformants of PH-1 (NP12) and Δamt1 (NA14) mutant expressing the FgNAB2-GFP fusion construct, GFP signals mainly localized to the nucleus. Bar = 20 μm. (TIF)

Figure S5 Three-day-old PDA cultures of the wild-type strain (PH-1) and the Δamt2 (KS2), Δamt3 (KT3), and Δamt4 (KF4) mutants on PDA without or with 0.7 M NaCl, 300 μg/ml Congo red, 0.05% H2O2, or 0.01% SDS. Photographs were taken after incubation at 25°C for 3–5 days as labeled. (TIF)

Figure S6 Assays for defects in stress responses. Cultures of the wild type (PH-1) and the Δamt2 (KS2), Δamt3 (KT3), and Δamt4 (KF4) mutants on PDA without or with 0.7 M NaCl, 300 μg/ml Congo red, 0.05% H2O2, or 0.01% SDS. Photographs were taken after incubation at 25°C for 3–5 days as labeled. (TIF)

Table S1 Disease index of AMTs mutants in the wheat head infection. (DOC)

Table S2 PCR primers used in this study. (DOC)

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Author Contributions

Conceived and designed the experiments: JRX. Performed the experiments: GHW CFW. Contributed reagents/materials/analysis tools: GHW CFW. Wrote the paper: GHW CFW JRX. Contributed reagents/materials/analysis tools: GHW CFW RH XYZ GTL SJZ. Analyzed the data: GHW CFW. Conceived and designed the experiments: JRX. Performed the experiments: GHW CFW. Contributed reagents/materials/analysis tools: GHW CFW RH XYZ GTL SJZ. Analyzed the data: GHW CFW.

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