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 had increased sensitivities to oxidative and membrane stresses. DON mycotoxin production and virulence on flowering wheat heads also were 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 hnRNP components. 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 Nε2,Nε3-dimethylation of arginine residues, respectively [2]. Whereas type III PRMTs catalyze ω Nε3 monomethylation of arginines, type IV PRMTs catalyze the formation of δ Nε1-monomethylarginine. In human, type I PRMTs include PRMT1, PRMT3, PRMT4, PRMT6, and PRMT8, PRMT3, 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 poly(A) RNA and poly(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 343-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 amt1 deletion mutants**

The ΔAMT1 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 ΔAMT1 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...

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![Figure 1. The ΔAMT1 gene replacement construct and deletion mutants. A. The ΔAMT1 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. doi:10.1371/journal.pone.0038324.g001](http://example.com/figure1)
The wild-type and mutant strains of _Fusarium graminearum_ used in this study.

| Strains Brief descriptions | Reference |
|--------------------------|-----------|
| PH-1 Wild-type           | [55]      |
| M1 Δamt1 mutant of PH-1  | This study |
| M2 Δamt1 mutant of PH-1  | This study |
| M3 Δamt1 mutant of PH-1  | This study |
| C2 Δamt1/AMT1 transformant of M2 | This study |
| Y5 Δamt1/AMT1-GFP transformant of M2 | This study |
| HP10 Transformant of PH-1 expressing FgHRP1-GFP | This study |
| HA11 Transformant of M2 expressing FgHRP1-GFP | This study |
| NP12 Transformant of PH-1 expressing FgNAB2-GFP | This study |
| NA14 Transformant of M2 expressing FgNAB2-GFP | This study |
| DM7 Δamt1 Δam2 double mutant | This study |
| DM12 Δamt1 Δam2 double mutant | This study |
| KS2 Δam2 (FGSG_10718) deletion mutant of PH-1 | This study |
| KT3 Δam3 (FGSG_00501) deletion mutant of PH-1 | This study |
| KF4 Δam4 (FGSG_10756) deletion mutant of PH-1 | This study |

![Defects of the Δamt1 mutant in growth, conidiation, and plant infection.](image-url)

| Table 2. Defects of the Δamt1 mutant in growth, conidiation, and plant infection. |
|-------------------------|-------------------------|-------------------------|-------------------------|
|                        | Growth rate (mm/d)      | Conidiation (10⁶/ml)    | Disease Index           | DON (ppm)            |
| PH-1 (WT)              | 14.0±0.3a               | 1.39±0.16a              | 13.8±3.8a               | 15896±359.4a         |
| M2 (Δamt1)             | 10.6±0.3a               | 1.45±0.25a              | 4.3±3.7a                | 397.0±188.7a         |
| C2 (Δamt1/AMT1)        | 14.3±0.2a               | 1.42±0.21a              | 14.0±3.4a               | 1782.8±279.6a        |

*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 spikeletes 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).
The Δamt1 mutant has increased sensitivity to oxidative and membrane stresses

To determine whether the Δamt1 mutant was defective in stress responses, we assayed its growth on PDA plates with 0.05% H₂O₂, 0.01% SDS, and 0.7 M NaCl. It appears that AMT1 is dispensable for responses to hyperosmotic stress because the wild type and Δamt1 mutant strains 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 Δamt1 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 Δamt1 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.

Amt1 influences the nuclear transport of FgHrp1

In S. cerevisiae, Hmt1 is involved in the regulation of nucleo-cytoplasmic 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
Figure 6. Amt1 regulates the nucleo-cytoplasmic transport of FgHrp1. Fresh conidia were harvested from transformants of PH-1 (HP10) and Amt1 mutant M2 (HA11) expressing the FgHRP1-GFP fusion construct and incubated in liquid YEPD medium for 12 h. Germ tubes were then stained with DAPI and examined by fluorescence microscopy with the GFP- and DAPI-specific filters. FgHrp1-GFP fusion proteins mainly localized to the nucleus in the Δamt1 mutant but to the cytoplasm in the wild type. Bar = 20 μm. doi:10.1371/journal.pone.0038324.g006

(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 rate with the wild type (PH-1), M2 (Δamt1), KS2 (Δamt2), KT3 (Δamt3), KF4 (Δamt4), and DM7 (Δamt1 Δamt2). 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.

Figure 7. Infection assays and stress response tests with mutants deleted of other PRMT genes. A. Corn silks inoculated with the wild type (PH-1), M2 (Δamt1), KS2 (Δamt2), KT3 (Δamt3), KF4 (Δamt4), and DM7 (Δamt1 Δamt2). B. Colonies of PH-1, DM7, M2, and KS2 grown on PDA plates with 0.05% H2O2 or 0.01% SDS. doi:10.1371/journal.pone.0038324.g007

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 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 PRMT1s [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 AMT genes, AMT1, AMT2, and AMT4, 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 in fungi and plants [31]. In yeast, Rmt2 specifically methylates ribosomal protein Rpl12 (L12) on Arg67 [32]. The rmt2 mutant is defective in Δ N^4- 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^4-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 Swi1 [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 Δamt1 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 ATRPMT5 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 (infectious growth) 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 CaHMT1 affects the expression and localization of NPL3 [31]. However, the function of CaHMT1 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 Δamt1 mutant, similar to the rmt1 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 infested 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 RRM-containing protein required for the cleavage and polyadenylation of pre-mRNA at the 3′-ends [13]. Nab2 is a nuclear polyA RNA-binding protein required for nuclear mRNA export and poly(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 nucleocytoplasmic 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 YEPE (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 AMT1/1F-2R and AMT1/3F-4R, respectively (Fig. 1A and Table S2). The resulting PCR products were connected to the hygromycin phosphotransferase (hph) fragment 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 AMT1/5-F6, AMT1/7-H853R, and H856F-AMT1R8 (Table S2). Potent 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 (NEO²) 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 Psl and BamHI, and cloned between the Psl and BamHI sites of the NEO² vector pHZ100 [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 \( \Delta \text{amt1} \) mutant M2. G418-resistant transformants harboring the transforming \( \text{AMT1-GFP} \), \( \text{HRP1-GFP} \), or \( \text{NAB2-GFP} \) 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 CM cultures and re-suspended in sterile distilled water to \( 2.0 \times 10^7 \) conidia/ml. The fifth spikelet from the base of the spike was inoculated with \( 10 \mu l \) of the conidial suspension as described [48]. 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 assayed for DON production as described [45]. For microscopic examinations, glumes and rachises were collected from inoculated spikelets and embedded in Spurr resins [49]. Thick sections (1 \( \mu m \)) were collected and placed on glass slides. After staining with aqueous 0.5\% (v/v) toluidine blue, sections were examined and photographed with an Olympus BX-51 microscope (Olympus Corporation, Japan). Infection assays with corn silks of cultivar Pioneer 2375 were conducted as described [50].

**qRT-PCR analysis**

RNA samples were isolated from 6 h germlings grown in liquid YEPD medium with the TRIzol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized with the Fermentas 1\(^{st} \) cDNA synthesis kit (Hanover, MD). All qRT-PCR reactions were performed with the Bio-Rad C1000 qRT-PCR machine. Primers used for qRT-PCR analysis were listed in Table S2. Relative expression levels of each gene were calculated by the 2\(^{-\Delta \Delta Ct} \) method [51] with the \( \text{F. graminearum} \) 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]. TEF-phosphorylation of Mgy1 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 \( \text{Fusarium graminearum} \), \( \text{Candida albicans} \), \( \text{Saccharomyces cerevisiae} \), \( \text{Schizosaccharomyces pombe} \), \( \text{Magnaporthe oryzae} \), \( \text{Neurospora crassa} \), \( \text{Aspergillus nidulans} \), and \( \text{Homo sapiens} \) were analyzed by the DNAmann5.0 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 \( \text{PRMT1} \), \( \text{PRMT2} \), and \( \text{PRMT5} \), 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 \( \Delta \text{amt1} \) mutant M2 grown on PDA, 5\% YEG, and YEPD plates.

**Figure S3** Perithecia and cirrhi produced by the wild-type strain (PH-1) and the \( \Delta \text{amt1} \) (M2), \( \Delta \text{amt2} \) (KS2), \( \Delta \text{amt3} \) (KT3), \( \Delta \text{amt4} \) (KF4), and \( \Delta \text{amt1} \Delta \text{amt2} \) (DM7) mutants. Photographs were taken 14 days after fertilization.

**Figure S4** Deletion of AMT1 had no effects on the nucleo-cytoplasmic transport of FgNab2. In both transformants of PH-1 (NP12) and \( \Delta \text{amt1} \) (NA14) mutant expressing the \( \text{FgNAB2-GFP} \) fusion construct, GFP signals mainly localized to the nucleus. Bar = 20 \( \mu m \).

**Table S1** Disease index of AMT1 mutants in the wheat head infection.

**Table S2** PCR primers used in this study.

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**Author Contributions**

Conceived and designed the experiments: JRX. Performed the experiments: GHW CFW. Analyzed the data: GHW CFW. Wrote the paper: GHW CFW JRX.
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