

## ORIGINAL ARTICLE

# The plasma membrane H<sup>+</sup>-ATPase FgPMA1 regulates the development, pathogenicity, and phenamacril sensitivity of *Fusarium graminearum* by interacting with FgMyo-5 and FgBmh2

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## Abstract

*Fusarium graminearum*, as the causal agent of Fusarium head blight (FHB), not only causes yield loss, but also contaminates the quality of wheat by producing mycotoxins, such as deoxynivalenol (DON). The plasma membrane H<sup>+</sup>-ATPases play important roles in many growth stages in plants and yeasts, but their functions and regulation in phytopathogenic fungi remain largely unknown. Here we characterized two plasma membrane H<sup>+</sup>-ATPases: FgPMA1 and FgPMA2 in *F. graminearum*. The FgPMA1 deletion mutant ( $\Delta$ FgPMA1), but not FgPMA2 deletion mutant ( $\Delta$ FgPMA2), was impaired in vegetative growth, pathogenicity, and sexual and asexual development. FgPMA1 was localized to the plasma membrane, and  $\Delta$ FgPMA1 displayed reduced integrity of plasma membrane.  $\Delta$ FgPMA1 not only impaired the formation of the toxisome, which is a compartment where DON is produced, but also suppressed the expression level of DON biosynthetic enzymes, decreased DON production, and decreased the amount of mycelial invasion, leading to impaired pathogenicity by exclusively developing disease on inoculation sites of wheat ears and coleoptiles.  $\Delta$ FgPMA1 exhibited decreased sensitivity to some osmotic stresses, a cell wall-damaging agent (Congo red), a cell membrane-damaging agent (sodium dodecyl sulphate), and heat shock stress. FgMyo-5 is the target of phenamacril used for controlling FHB. We found FgPMA1 interacted with FgMyo-5, and  $\Delta$ FgPMA1 showed an increased expression level of FgMyo-5, resulting in increased sensitivity to phenamacril, but not to other fungicides. Furthermore, co-immunoprecipitation confirmed that FgPMA1, FgMyo-5, and FgBmh2 (a 14-3-3 protein) form a complex to regulate the sensitivity to phenamacril and biological functions. Collectively, this study identified a novel regulating mechanism of FgPMA1 in pathogenicity and phenamacril sensitivity of *F. graminearum*.

## KEYWORDS

DON production, FgPMA1, *Fusarium graminearum*, pathogenicity, phenamacril

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## 1 | INTRODUCTION

Fungal and plant plasma membrane  $H^+$ -ATPases are abundant in the plasma membrane and play important roles in cell physiology (Ambesi et al., 2000). Plasma membrane  $H^+$ -ATPases belonging to the P-type cations-translocating ATPase family regulate cell growth, intracellular pH, and activity of serine/threonine protein kinases by producing and maintaining a transmembrane electrochemical proton gradient that drives the uptake of a number of ions and nutrients (de la Fuente & Portillo, 2000; Keith & Schreiber, 1995; Perlin et al., 1989; Sanders et al., 1981; Zhang, Chen, Yin, et al., 2015). In *Neurospora crassa*, plasma membrane  $H^+$ -ATPases provide energy for nutrient-proton pumps and regulate intracellular pH (Bowman & Bowman, 1986). In mycorrhizal fungi, plasma membrane  $H^+$ -ATPases increase proton pumping activity and energize nutrient uptake during the interaction of plants and fungi (Requena et al., 2003; Wang et al., 2014). Plasma membrane  $H^+$ -ATPases have been proved to be important for pathogenicity in *Leptosphaeria maculans* (Remy et al., 2008). However, the function and regulation of plasma membrane  $H^+$ -ATPases in *Fusarium graminearum* are poorly known.

*F. graminearum* (teleomorph *Gibberella zeae*) is an economically important plant pathogen that is the causal agent of Fusarium head blight (FHB) on many cereal crops, including wheat, barley, maize, rye, and oats (Goswami & Kistler, 2004; Starkey et al., 2007). Importantly, *F. graminearum* threatens the health of humans and animals by producing mycotoxins such as deoxynivalenol (DON) and zearalenone (McMullen et al., 1997; Pestka & Smolinski, 2005). Previous studies showed that plasma membrane  $H^+$ -ATPases regulate secondary metabolism biosynthesis. In *Salvia miltiorrhiza*, plasma membrane  $H^+$ -ATPase SmPHA4 negatively regulates the biosynthesis of tanshinones (Li et al., 2021). DON is a mycotoxin that is a secondary metabolite in *F. graminearum*, contaminating wheat and causing harmful consequences to humans and animals (Alexander et al., 2009). The biosynthetic pathway of DON is regulated by trichothecene (*TRI*) genes, and many *TRI* genes have been characterized (Desjardins et al., 1993; Kimura et al., 2001, 2007). A previous study reported that plasma membrane  $H^+$ -ATPase might be involved in the DON biosynthesis pathway by interacting with a *TRI* pathway protein (Boenisch et al., 2017). Therefore, further exploration of the function and uncovering the underlying mechanism of plasma membrane  $H^+$ -ATPase in the DON biosynthesis pathway is required.

At present, the principal method to control FHB relies on fungicides; phenamacril is a widely used and effective fungicide for controlling FHB (Chen et al., 2008; Li et al., 2008). Previous studies showed that myosin-5 is the target of phenamacril (Zhang, Chen, Yin, et al., 2015; Zheng, Hou, et al., 2015; Zhou, Zhou, et al., 2020). However, other proteins have also been reported to affect sensitivity to phenamacril in *F. graminearum*. For example, deletion of the Fimbrin gene (*FgFim*) encoding an actin-binding protein in *F. graminearum* reduces resistance to phenamacril (Zheng et al., 2014). *FaSym1*, a myosin passenger protein gene in *F. asiaticum*, positively

regulates resistance to phenamacril; deletion of *FaSym1* reduces resistance to phenamacril (Liu et al., 2017). In this study, for the first time, we characterized the functional roles of a plasma membrane  $H^+$ -ATPase gene (*FgPMA1*) in the development of mycelial growth, spore production, pathogenicity, DON production, and sensitivity to phenamacril. Deletion of *FgPMA1* increased the sensitivity of *F. graminearum* to phenamacril by interacting with *FgMyo-5* and *FgBmh2*. Our findings show that *FgPMA1* has potential as a novel target for controlling FHB.

## 2 | RESULTS

### 2.1 | Identification, deletion, and complementation of *FgPMA1* and *FgPMA2*

By searching the *Fusarium* genome database ([http://www.broadinstitute.org/annotation/genome/fusarium\\_group/MultiHome](http://www.broadinstitute.org/annotation/genome/fusarium_group/MultiHome)) with the BLAST algorithm using *Saccharomyces cerevisiae* plasma membrane  $H^+$ -ATPase (PMA1) protein as a query, two putative  $H^+$ -ATPase genes, *FgPMA1* (FGSG\_01425) and *FgPMA2* (FGSG\_08343), in *F. graminearum* were retrieved. *FgPMA1*, a 3181 bp gene with two introns, is predicted to encode a 922-amino acid protein that shares 76.4% identity with *S. cerevisiae* PMA1. *FgPMA2*, a 3365 bp gene with two introns, is predicted to encode a 1006-amino acid protein that shares 48.8% identity with *S. cerevisiae* PMA2. We also retrieved PMA1 and PMA2 in other important pathogens from National Center for Biotechnology Information (NCBI) and analysed these sequences by a phylogenetic tree (Figure S1). Both *FgPMA1* and *FgPMA2* contain a Cation\_ATPase\_N domain, six transmembrane domains, an E1-E2\_ATPase domain, and a hydrolase domain, which is similar to *S. cerevisiae* PMA1 (Figure S1). To further determine the relationship between *FgPMA1* and *FgPMA2*, we conducted a yeast two-hybrid (Y2H) experiment and the results showed that *FgPMA1* and *FgPMA2* did not directly interact (Figure S2).

To determine the function of *FgPMA1* and *FgPMA2*, we deleted *FgPMA1* and *FgPMA2* in *F. graminearum* using a homologous recombination strategy. For each gene, two independent deletion mutants were obtained, with similar phenotypes as described below. To confirm that the phenotypic changes observed in  $\Delta FgPMA1$  and  $\Delta FgPMA2$  were caused by gene deletion, the mutants were complemented with a full-length wild-type *FgPMA1* and *FgPMA2*, respectively. All mutants were confirmed by Southern blot (Figure S3). The construction of a  $\Delta FgPMA1\Delta FgPMA2$  double mutant failed after several tries, suggesting that the  $\Delta FgPMA1\Delta FgPMA2$  double mutant is lethal.

### 2.2 | *FgPMA1* regulates vegetative growth

To examine the role of *FgPMA1* in regulating vegetative growth of *F. graminearum*, we tested the growth rate of mutant strains in different conditions in comparison to the wild-type strain.  $\Delta FgPMA1$

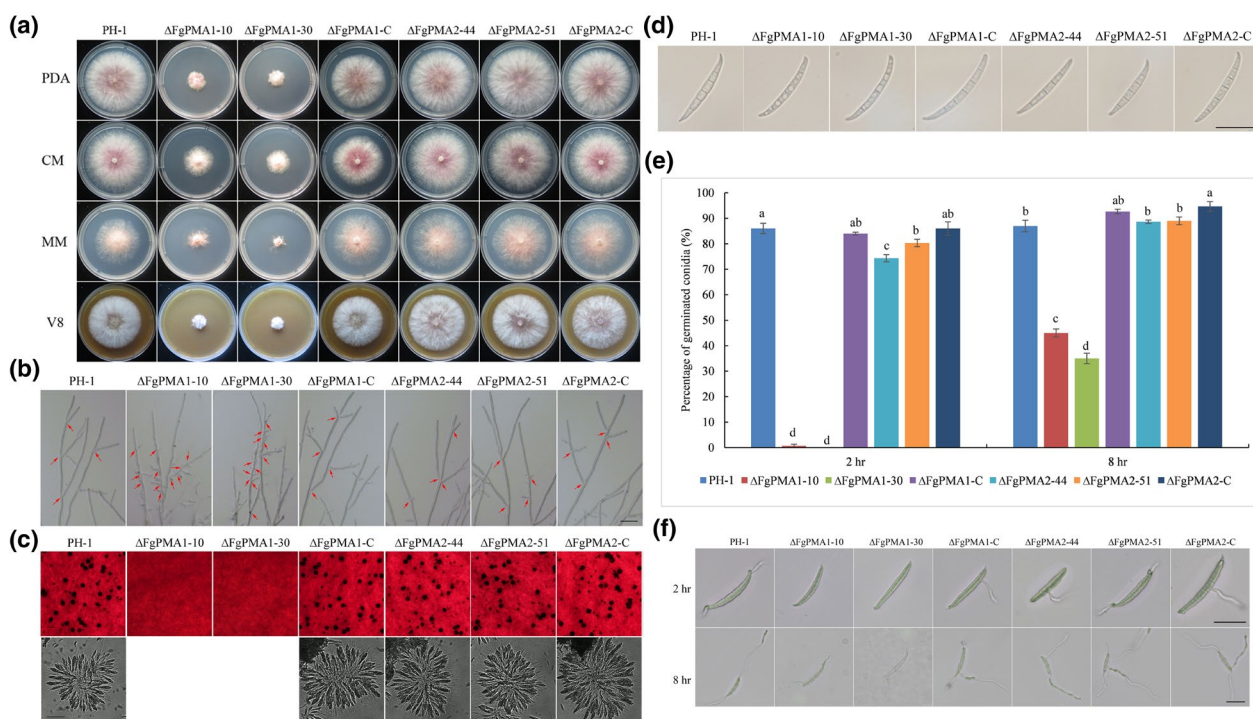
showed a reduced growth rate of hyphae on potato dextrose agar (PDA), complete medium (CM), minimum medium (MM), and V8 (Figure 1a and Table 1). Microscopic examination revealed that the number of hyphal branches of  $\Delta FgPMA1$  increased compared to the wild-type and complemented strains (Figure 1b and Table 1), suggesting that *FgPMA1* plays a role in vegetative development. However,  $\Delta FgPMA2$  did not show significant changes in vegetative growth.

### 2.3 | *FgPMA1* is involved in sexual and asexual development

To determine the role of *FgPMA1* in sexual development, fresh mycelial plugs of each strain were inoculated in carrot medium plates under black-light conditions to induce sexual reproduction, which is evident by perithecia formation. Surprisingly, no asci were observed in  $\Delta FgPMA1$ , unlike the wild-type strain (Figure 1c). However, the deletion of *FgPMA2* did not produce a similar phenotype. Therefore, our results demonstrate that *FgPMA1*, but not *FgPMA2*, is essential for sexual development.

To further investigate the roles of the plasma membrane  $H^+$ -ATPase genes in asexual development, we examined the conidia production of the mutants as compared to the wild-type and complemented strains. We found that only  $\Delta FgPMA1$  produced a significantly lower amount of conidia than the wild-type strain in carboxymethylcellulose (CMC) medium (Table 1). In addition, we visualized the morphology of the conidia obtained from the knockout strains and found that some of the conidia produced by  $\Delta FgPMA1$ , but not  $\Delta FgPMA2$ , had larger vacuoles than the wild-type and complemented strains (Figure 1d). Both  $\Delta FgPMA1$  and  $\Delta FgPMA2$  had fewer septa than the wild type (Table 1). These results suggest that *FgPMA1* affects conidial morphology and the number of septa, and *FgPMA2* only affects the number of septa.

The germination and germ tube growth of conidia from the knockout strains were assessed in water agar medium. The conidial germination of  $\Delta FgPMA1$  was delayed and the germ tube growth of conidia was slower compared to the wild type and complemented strains (Figure 1e,f), indicating that *FgPMA1* plays important roles not only in conidial production and conidial morphology, but also in subsequent conidial germination in *F. graminearum*, while *FgPMA2* is dispensable in asexual development.



**FIGURE 1** Impact of *FgPMA1* and *FgPMA2* deletion mutants on vegetative growth, and sexual and asexual development. (a) The wild-type PH-1, *FgPMA1* deletion mutant ( $\Delta FgPMA1$ ), *FgPMA2* deletion mutant ( $\Delta FgPMA2$ ), and complemented strains ( $\Delta FgPMA1-C$  and  $\Delta FgPMA2-C$ ) were grown on potato dextrose agar (PDA), complete medium (CM), minimal medium (MM), and V8 at 25°C for 3 days. (b) Hyphal branching pattern of each strain growing on water agar (WA). The branching of  $\Delta FgPMA1$  was increased in the extension zone of the colony. Bar = 72  $\mu$ m. (c) The mutant  $\Delta FgPMA1$  was defective in sexual development. Strains grown on carrot agar (CA) were self-fertilized. Bar = 200  $\mu$ m. Photographs of perithecia were taken after 10 days of incubation and photographs of ascospores were taken after 2 weeks of incubation. Bar = 250  $\mu$ m. (d) Conidia of the same set of strains were examined by differential interference contrast (DIC) microscopy. Bar = 20  $\mu$ m. (e) Conidia of each strain were covered on the WA surface. After 2 and 8 h of incubation, germination of 300 conidia was examined under a microscope. Error bars in each column denote SE of three experiments. Bars with the same letter indicate no significant difference at  $p = 0.05$ . (f) The length of germ tube of the strains after incubation for 2 and 8 h (bar = 20  $\mu$ m)

TABLE 1 Functional analyses of *Fusarium graminearum* wild type (PH-1), deletion mutants, and complemented (C) strains

Strain	Vegetative growth (%)				Lateral branch/ main branch	No. of septa in conidia (%)					Conidiation ( $\times 10^5$ /ml)
	PDA	CM	MM	V8		$\leq 2$	3	4	5	$\geq 6$	
PH-1	6.53 $\pm$ 0.16 a	6.61 $\pm$ 0.24 a	5.48 $\pm$ 0.36 b	5.60 $\pm$ 0.04 a	5.00 $\pm$ 0.58 b	9	29	19	36	7	16.86 $\pm$ 1.24 a
$\Delta$ FgPMA1-10	1.63 $\pm$ 0.11 c	4.04 $\pm$ 0.56 b	2.66 $\pm$ 0.22 c	1.53 $\pm$ 0.10 b	11.33 $\pm$ 0.33 a	33	45	16	5	1	10.53 $\pm$ 1.37 b
$\Delta$ FgPMA1-30	1.60 $\pm$ 0.05 c	3.87 $\pm$ 0.65 b	2.01 $\pm$ 0.40 c	1.43 $\pm$ 0.15 b	11.00 $\pm$ 1.00 a	52	35	9	4	0	8.53 $\pm$ 0.56 b
$\Delta$ FgPMA1-C	6.07 $\pm$ 0.13 b	6.07 $\pm$ 0.17 a	5.77 $\pm$ 0.09 ab	5.57 $\pm$ 0.11 a	5.67 $\pm$ 0.88 b	5	27	21	35	12	17.93 $\pm$ 1.35 a
$\Delta$ FgPMA2-44	6.68 $\pm$ 0.22 a	6.81 $\pm$ 0.28 a	6.03 $\pm$ 0.10 ab	5.63 $\pm$ 0.16 a	4.67 $\pm$ 0.88 b	10	62	20	7	1	18.17 $\pm$ 1.20 a
$\Delta$ FgPMA2-51	6.68 $\pm$ 0.15 a	6.63 $\pm$ 0.24 a	5.91 $\pm$ 0.08 ab	5.71 $\pm$ 0.10 a	4.46 $\pm$ 0.33 b	8	63	24	5	0	17.08 $\pm$ 2.62 a
$\Delta$ FgPMA2-C	6.62 $\pm$ 0.11 a	6.42 $\pm$ 0.30 a	6.12 $\pm$ 0.10 a	5.46 $\pm$ 0.06 a	5.00 $\pm$ 0.58 b	8	49	26	16	1	18.08 $\pm$ 3.60 a

Note: Data followed by same letters indicate no significant difference at  $p = 0.05$ .

Abbreviations: CM, complete medium; MM, minimal medium; PDA, potato dextrose agar; V8, V8 juice agar.

## 2.4 | FgPMA1 is localized to the plasma membrane and is crucial for the integrity of the plasma membrane

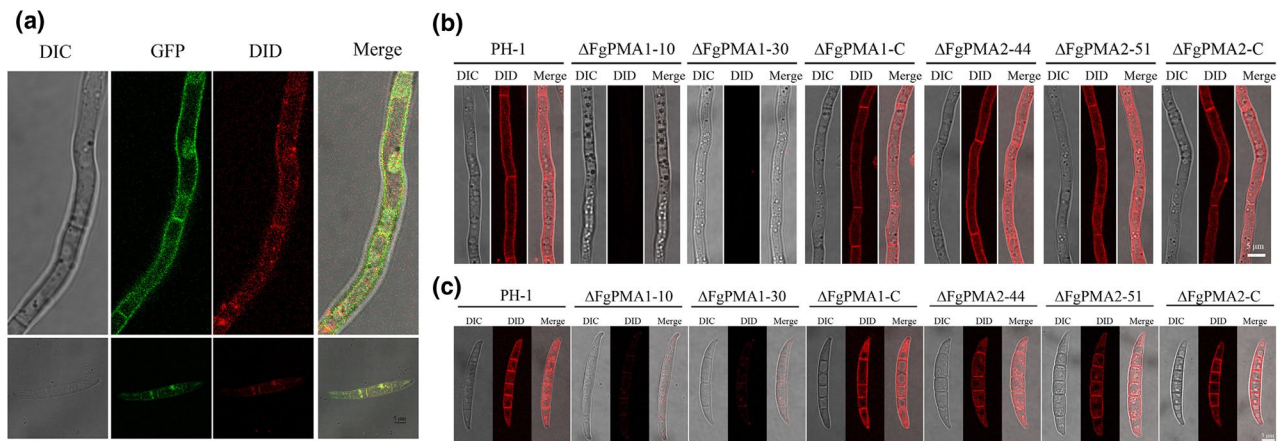
The plasma membrane has been proved to be essential in fungal pathogenicity (Flores-Huerta et al., 2020; Rella et al., 2016). To determine the role of FgPMA1 and FgPMA2 in the plasma membrane, we generated a strain carrying FgPMA1 tagged with green fluorescent protein (GFP); we also tried to construct the FgPMA2-GFP strain, but failed. The FgPMA1-GFP strain was used to observe the subcellular localization of FgPMA1. As we expected, a pattern of GFP signals was present in the plasma membrane (Figure 2a). Co-localization experiments were performed with FgPMA1-GFP and the plasma membrane indicator 3,3'-diiodo-4,4'-dimethoxydiphenylmethane perchlorate (DiD), and showed that the GFP and DiD signals clearly overlapped in mycelia and conidia (Figure 2a), which suggest that FgPMA1 is localized to the plasma membrane.

In *S. cerevisiae*, the ubiquitylation of the plasma membrane  $H^+$ -ATPase, Pma1-10, prevents cell-surface stability (Liu & Chang, 2006). Therefore, it is interesting to test whether the deletion of FgPMA genes affects the plasma membrane. As shown in Figure 2, the plasma membrane in the wild-type strain was clearly stained by DiD, while the DiD signal in  $\Delta$ FgPMA1 was weaker under the same conditions, suggesting that deletion of FgPMA1 disturbs the integrity of plasma membrane. Taken together, these results indicate that FgPMA1 is important for maintaining the integrity of plasma membrane in *F. graminearum*, but FgPMA2 is not required.

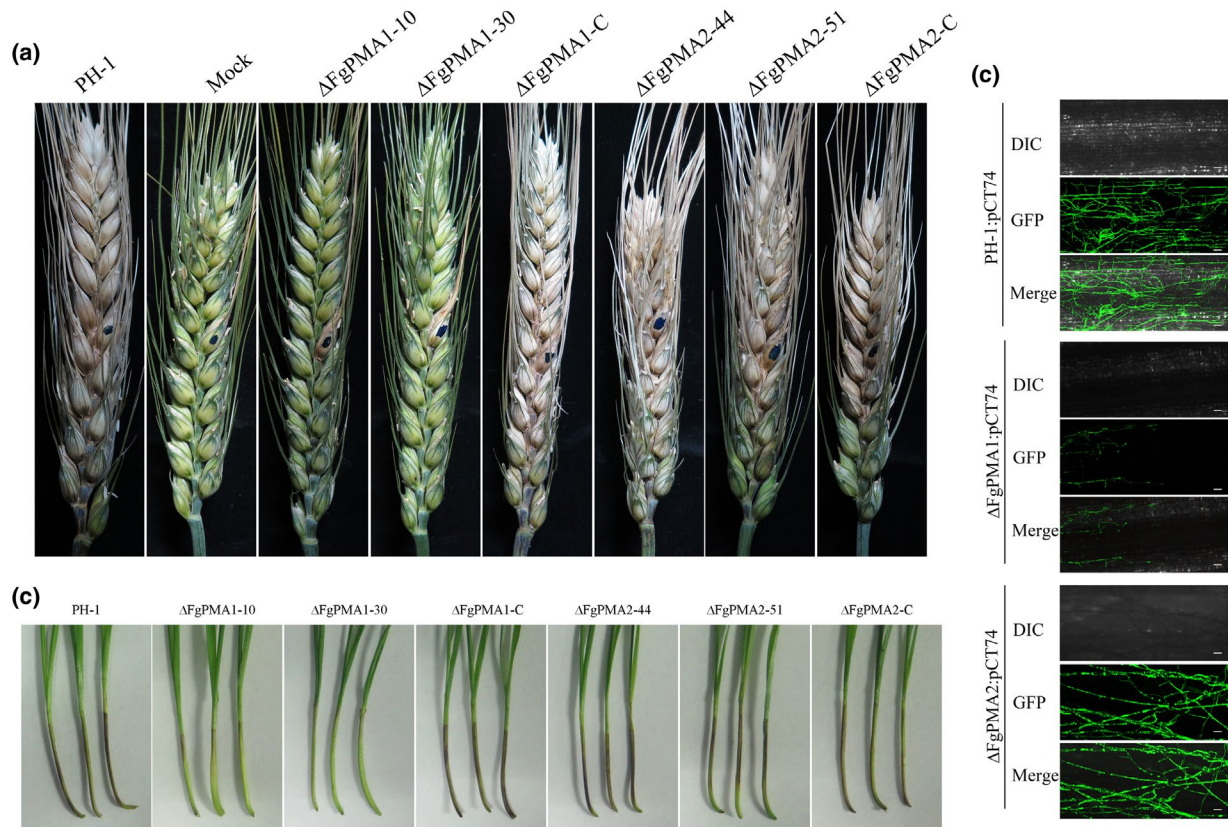
## 2.5 | FgPMA1 plays crucial roles in pathogenicity

To investigate the roles of FgPMA1 and FgPMA2 in *F. graminearum* pathogenicity, disease assays on wheat heads and coleoptiles were conducted. Two weeks after inoculation on wheat heads with conidia obtained from tested strains, the disease development was examined. Figure 3a shows that the  $\Delta$ FgPMA1 mutant displayed remarkably decreased virulence on wheat heads compared to the wild-type and complemented strains. To further confirm this observation, we also inoculated conidia on wheat coleoptiles. Consistent with the results of wheat heads,  $\Delta$ FgPMA1 developed shorter lesions compared with the wild-type and complementation strains (Figure 3b). However,  $\Delta$ FgPMA2 displayed a similar disease development to the wild-type strain. To determine whether the reduced pathogenicity is associated with the number of invading hyphae, a GFP plasmid (pCT74) was transformed into PH-1 (wild type),  $\Delta$ FgPMA1, and  $\Delta$ FgPMA2. Microscopic examination of infected wheat leaves by wild type PH-1:pCT74,  $\Delta$ FgPMA1:pCT74, and  $\Delta$ FgPMA2:pCT74 strains showed that  $\Delta$ FgPMA1:pCT74 exhibited limited growth in the intercellular space and on the surface of the wheat leaves (Figure 3c). Taken together, these findings indicate that FgPMA1 is essential for pathogenicity in *F. graminearum* during plant infection, but FgPMA2 is not required for the virulence of *F. graminearum*.





**FIGURE 2** FgPMA1 affects the integrity of the plasma membrane. (a) FgPMA1 was localized to the plasma membrane. (b) FgPMA1 regulated the integrity of the plasma membrane in mycelium. All strains were incubated in yeast exact peptone dextrose (YEPD) for 12 h. Wild type, PH-1; complemented strains, -C. Mycelium was stained with the plasma membrane tracker 3,3'-diiododiphenylcarbazone (DID). (c) FgPMA1 regulated the integrity of the plasma membrane in conidia. All mutants were incubated in carboxymethyl-cellulose (CMC) for 3 days. All the photographs were taken under a confocal microscope (Leica)

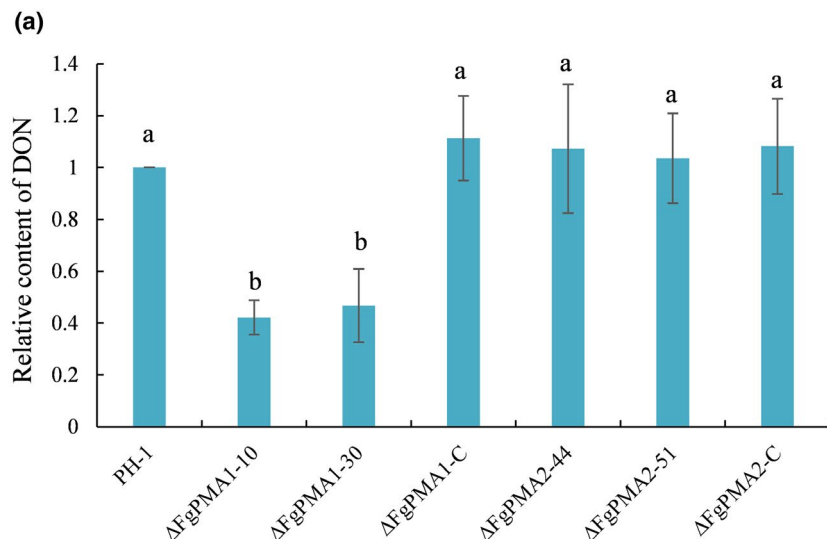


**FIGURE 3** FgPMA1 is important for pathogenicity of *Fusarium graminearum*. (a) Wheat heads infected by various strains were examined at 14 days after inoculation with a conidial suspension. The inoculation sites are indicated by black dots. Wild type, PH-1; complemented strains, -C. (b) The lesion length on wheat coleoptiles infected by various strains. The photographs were taken at 14 days postinoculation (dpi). (c) Confocal images of various strains growing inside wheat leaves at 5 dpi

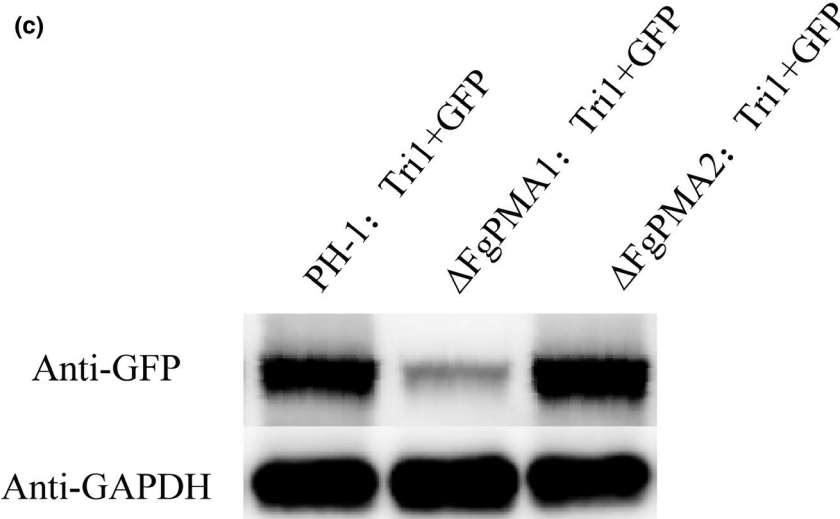
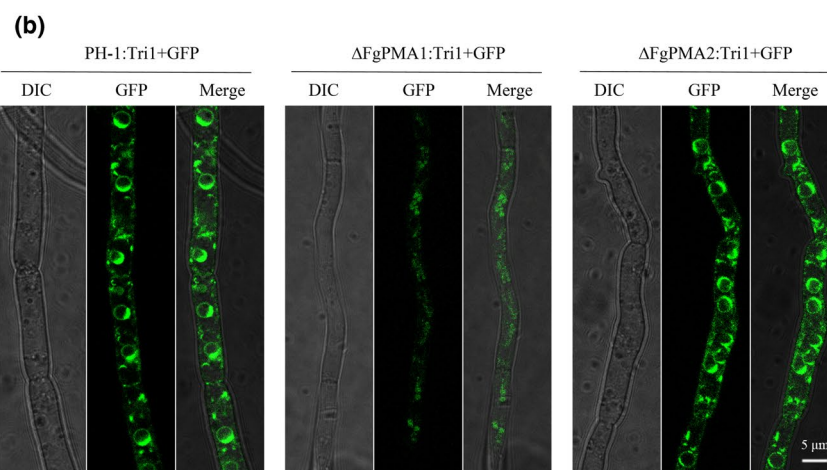
## 2.6 | FgPMA1 negatively regulates DON biosynthesis

Previous studies suggested that DON is a virulence factor in *F. graminearum* (Proctor et al., 1995). Therefore, to determine the

roles of FgPMA1 and FgPMA2 in DON biosynthesis, DON production was induced in the wild type, mutants, and complemented strains and quantitatively measured using a competitive ELISA-based DON detection plate kit.  $\Delta FgPMA1$  exhibited a significant decrease in DON production (Figure 4a) compared to that in wild-type,



**FIGURE 4** FgPMA1 affects deoxynivalenol (DON) synthesis. (a)  $\Delta FgPMA1$  displayed decreased DON content. Wild type, PH-1; complemented strains, -C. All strains were incubated in trichothecene biosynthesis induction (TBI) medium for 7 days. Bars with the same letter indicate no significant difference at  $p = 0.05$ . (b) FgPMA1 impaired the formation of DON toxismes, visualized by labelling the toxosome with Tri-GFP. Strains were incubated in TBI for 36 h. (c)  $\Delta FgPMA1$  showed a decreased expression level of Tri-GFP. Strains were incubated in TBI for 36 h and mycelia were harvested for western blot analysis



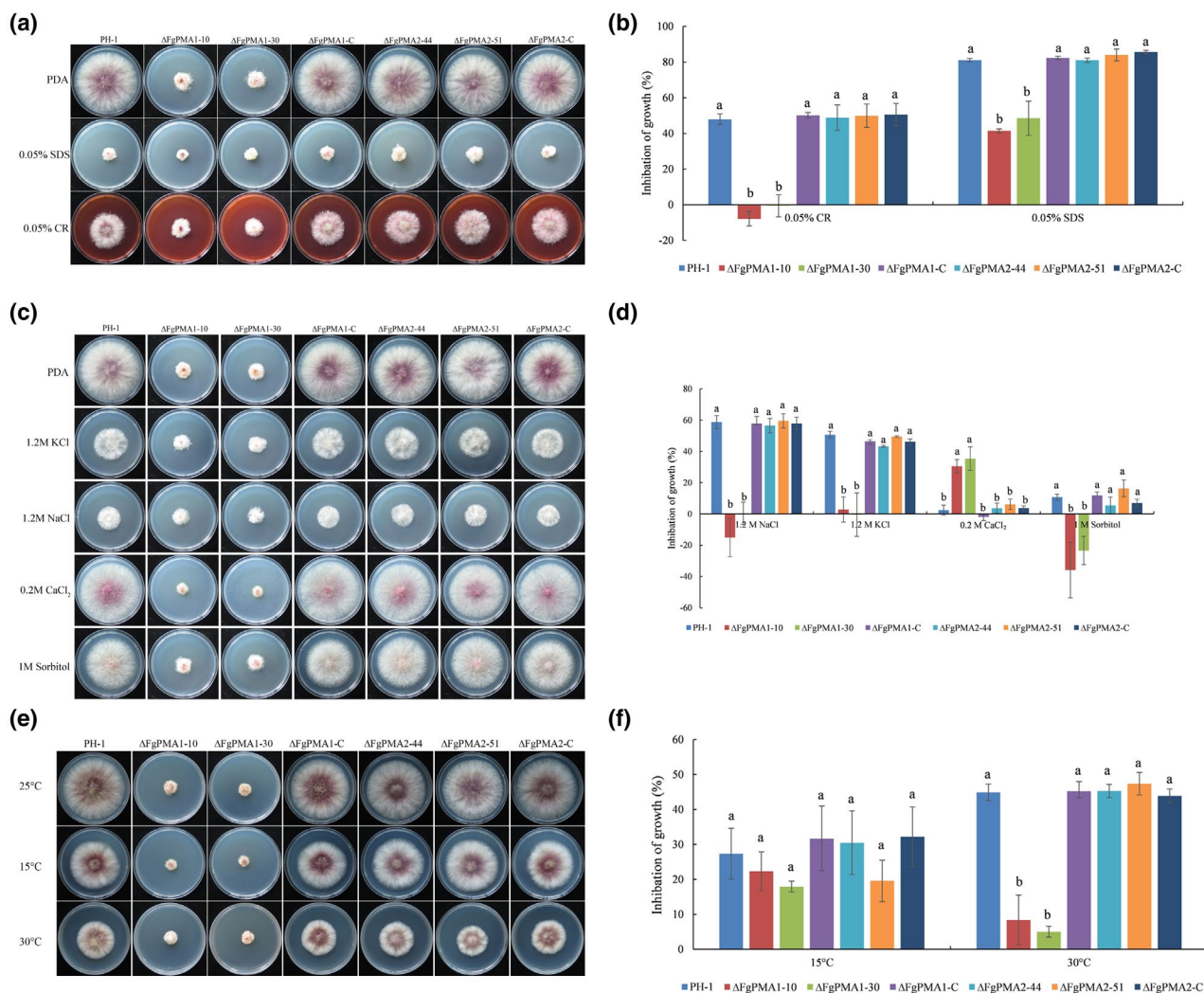
complemented, and  $\Delta FgPMA2$  strains. These findings indicate that FgPMA1 negatively regulates DON production, while FgPMA2 is not a regulator, which is consistent with the decreased pathogenicity in  $\Delta FgPMA1$ .

Previous reports demonstrated that the toxosome is a compartment where DON biosynthesis occurs and Tri1 protein (a cytochrome P450 oxygenases) is localized (Boenisch et al., 2017; Chen et al., 2019). FgMyo-5 interacts with Tri1 (Tang et al., 2018).

Therefore, we decided to check the formation of toxismes in  $\Delta FgPMA1$ ,  $\Delta FgPMA2$ , and the wild-type strains. These strains were incubated in trichothecene biosynthesis induction (TBI) medium for 36 h, and toxismes were observed in each strain. In the wild-type and  $\Delta FgPMA2$  strains, typical spherical toxismes were clearly observed (Figure 4b). However, toxismes were not visible in the  $\Delta FgPMA1$  mutant, suggesting that the formation of toxismes might be impaired in this mutant (Figure 4b). Western blot analysis of whole-cell extracts with the anti-GFP antibody confirmed that the protein level of Tri1-GFP was reduced in  $\Delta FgPMA1$  (Figure 4c). These results suggest that FgPMA1 is important for DON biosynthesis in *F. graminearum*, and FgPMA2 is not important for DON biosynthesis.

## 2.7 | FgPMA1 regulates the sensitivity to various stresses

During plant infection, the sensitivity to environmental stresses is important in *F. graminearum*. The roles of FgPMA1 and FgPMA2 were characterized in response to diverse environmental stresses, including osmotic stress, a cell wall-damaging agent (Congo red, CR), a cell membrane-damaging agent (sodium dodecyl sulphate, SDS), and heat shock stresses. As shown in Figure 5a,b,  $\Delta FgPMA1$  exhibited decreased sensitivity to 0.05% CR and 0.05% SDS. As shown in Figure 5c,d,  $\Delta FgPMA1$  displayed significantly decreased sensitivity to osmotic stresses generated by 1.2 M KCl, 1.2 M NaCl, and 1 M sorbitol, but significantly increased sensitivity to 0.2 M  $CaCl_2$ .



**FIGURE 5** Phenotypes of strains in response to various stresses. (a)  $\Delta FgPMA1$  decreased the sensitivity towards a cell membrane-damaging agent (sodium dodecyl sulphate, SDS) and a cell wall-damaging agent (Congo red, CR). (b) Statistical analysis of the growth inhibition rate of all strains under CR and SDS stresses. (c) Colony morphology is shown after 3 days of incubation on potato dextrose agar (PDA). Growth phenotype of wild type (PH-1), mutant, and complementation strains (-C) on PDA with or without supplementation of KCl, NaCl,  $CaCl_2$ , and sorbitol after 3 days of incubation at 25°C. (d) Statistical analysis of the growth inhibition rate of all strains under osmotic stress. (e)  $\Delta FgPMA1$  was more tolerant to heat at 30°C and did not show any difference compared with the wild type and complementation strains at 15°C. (f) Statistical analysis of the growth inhibition rate of all strains under heat shock. Bars with the same letter indicate no significant difference at  $p = 0.05$



The susceptibility of the mutants towards heat shock was also examined under 15, 25, and 30°C conditions, and the results showed that all mutants displayed similar growth as the wild type at 15°C, but  $\Delta FgPMA1$  showed decreased sensitivity at 30°C (Figure 5e,f). All these stress responses observed in the  $\Delta FgPMA1$  mutants were restored to the wild-type level in the complemented strains. In addition, changed sensitivities to various stresses were not observed in the  $\Delta FgPMA2$  deletion mutant. These findings indicate that *FgPMA1* is involved in the regulation of sensitivity to various stresses.

## 2.8 | Knocking out *FgPMA1* particularly increases the sensitivity to phenamacril by enhancing the expression of *FgMyo-5*

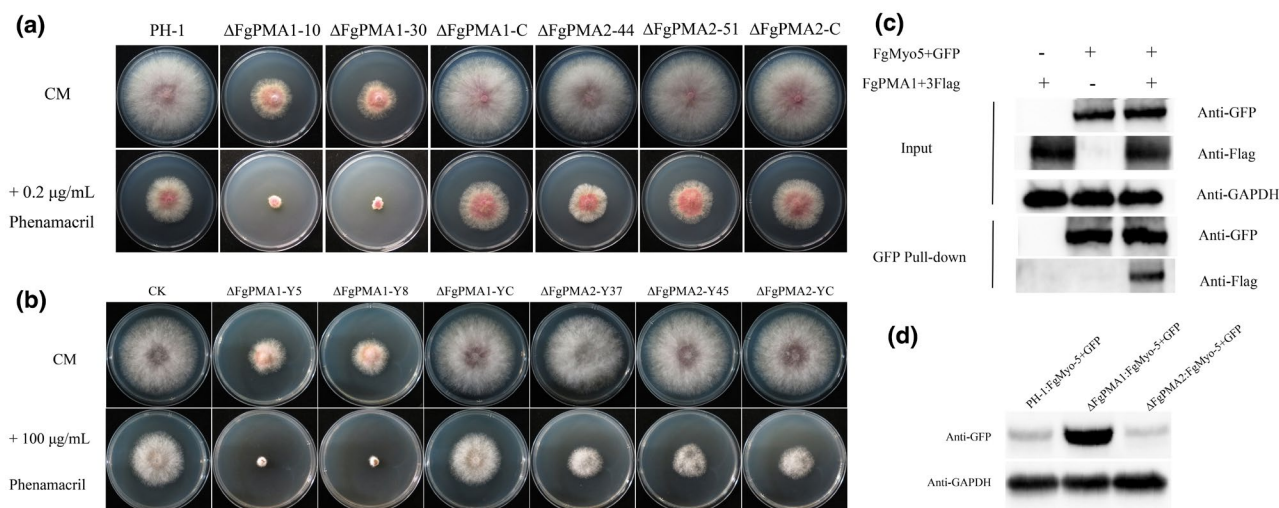
At present, the application of fungicides is considered to be the most effective method for controlling FHB. Therefore, we determined the sensitivity of  $\Delta FgPMA1$  and  $\Delta FgPMA2$  to widely used fungicides, including phenamacril, carbendazim, tebuconazole, pydiflumetofen, fluopyram, fluxapyroxad, pyraclostrobin, picoxystrobin, and azoxystrobin. As shown in Figure 6a,  $\Delta FgPMA1$  showed increased sensitivity to phenamacril compared to the wild-type,  $\Delta FgPMA2$ , and complemented strains. When treated with 0.2 µg/ml phenamacril, the growth of the wild-type strain decreased about 33.4%–38.3% and  $\Delta FgPMA2$  decreased 36.8%–46.3%, but  $\Delta FgPMA1$  decreased 68.0%–70.0%. However, both  $\Delta FgPMA1$  and  $\Delta FgPMA2$  did not show changed sensitivity to carbendazim, tebuconazole, pydiflumetofen, fluopyram, fluxapyroxad, pyraclostrobin, picoxystrobin, or azoxystrobin (Figure S4). To further determine the influence of  $\Delta FgPMA1$  to phenamacril, we knocked out *FgPMA1* in the phenamacril-resistant strain YP-1 ( $\Delta FgPMA1$ -Y). Knockout of *FgPMA2* ( $\Delta FgPMA2$ -Y) was

used as a control. Interestingly,  $\Delta FgPMA1$ -Y displayed decreased resistance to phenamacril (Figure 6b). When treated with 100 µg/ml phenamacril, the growth of the wild-type strain decreased 32.8%–35.1% and  $\Delta FgPMA2$ -Y decreased 47.1%–48.2%, but  $\Delta FgPMA1$ -Y decreased 66.0%–66.5%. Taken together, we can conclude that *FgPMA1* particularly regulates sensitivity to phenamacril.

A previous study showed that *FgMyo-5* (FGSG\_01410) is the target of phenamacril. As *FgPMA1* also participates in the tolerance to phenamacril, we decided to test whether *FgPMA1* can interact with *FgMyo-5*. Using *FgMyo-5* tagged with GFP as a bait, we conducted a pull-down assay followed by mass spectrometry. Surprisingly, *FgPMA1* was identified from the pull-down candidate proteins. To further confirm the interaction between *FgPMA1* and *FgMyo-5*, we generated a strain carrying *FgPMA1*-3×Flag and *FgMyo-5*-GFP. A co-immunoprecipitation (Co-IP) assay showed that *FgPMA1* interacted with *FgMyo-5* (Figure 6c). Furthermore, we analysed the expression level of *FgMyo-5* in  $\Delta FgPMA1$ . The results showed that the expression level of *FgMyo-5* increased in  $\Delta FgPMA1$  mutants (Figure 6d). Therefore,  $\Delta FgPMA1$  increased the sensitivity to phenamacril by enhancing the expression of *FgMyo-5*.

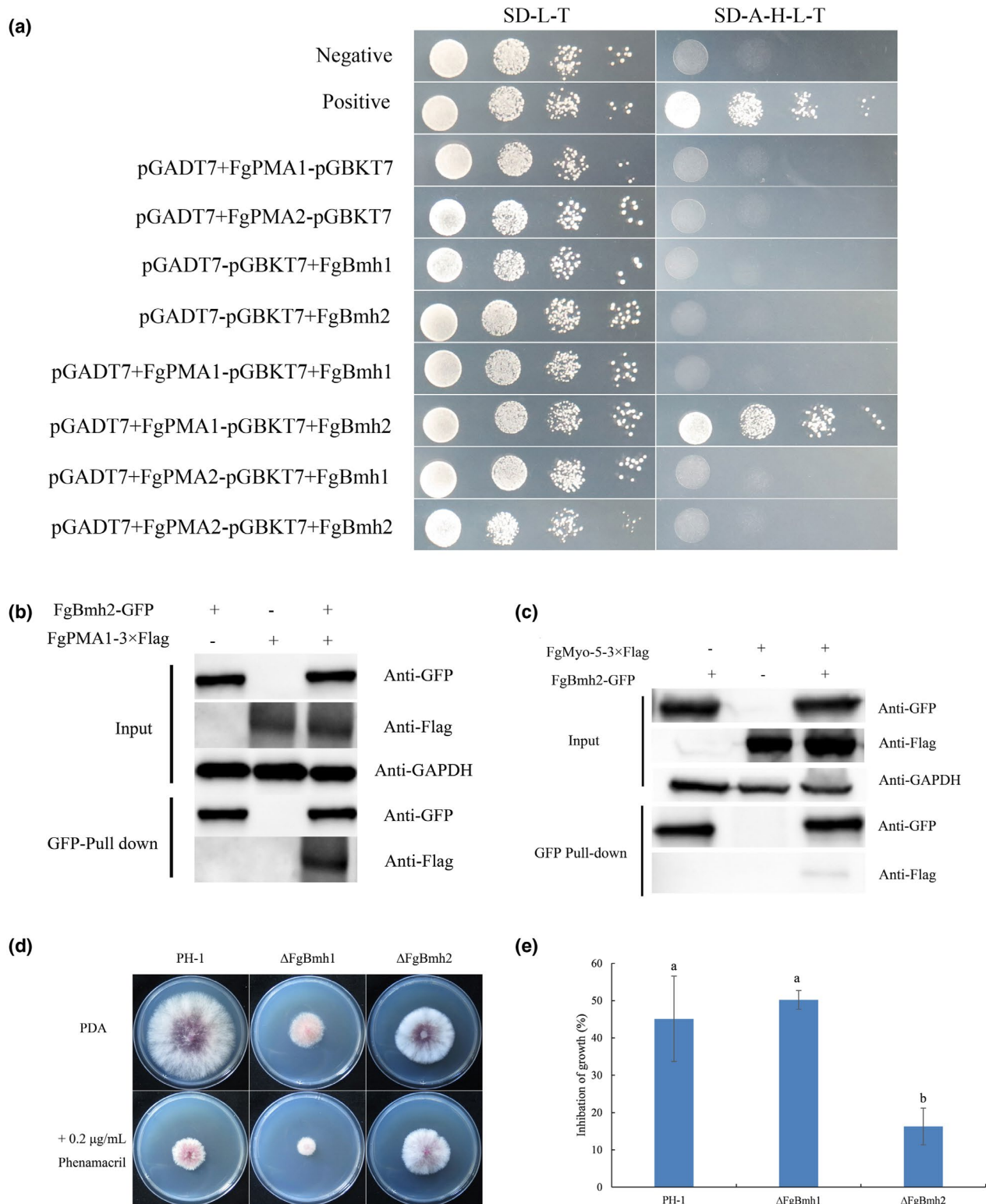
## 2.9 | *FgPMA1*, *FgMyo-5*, and *FgBmh2* form a complex and regulate sensitivity to phenamacril

Previous studies showed that 14-3-3 protein interacts directly with the plant plasma membrane  $H^+$ -ATPase, so we examined whether 14-3-3 protein interacts with the plasma membrane ATPase in *F. graminearum*. In *F. graminearum*, there are two 14-3-3 proteins, *FgBmh1* encoded by FGSG\_06847 and *FgBmh2* encoded by FGSG\_01241 (Brauer et al., 2020). By using Y2H, we found *FgBmh2*

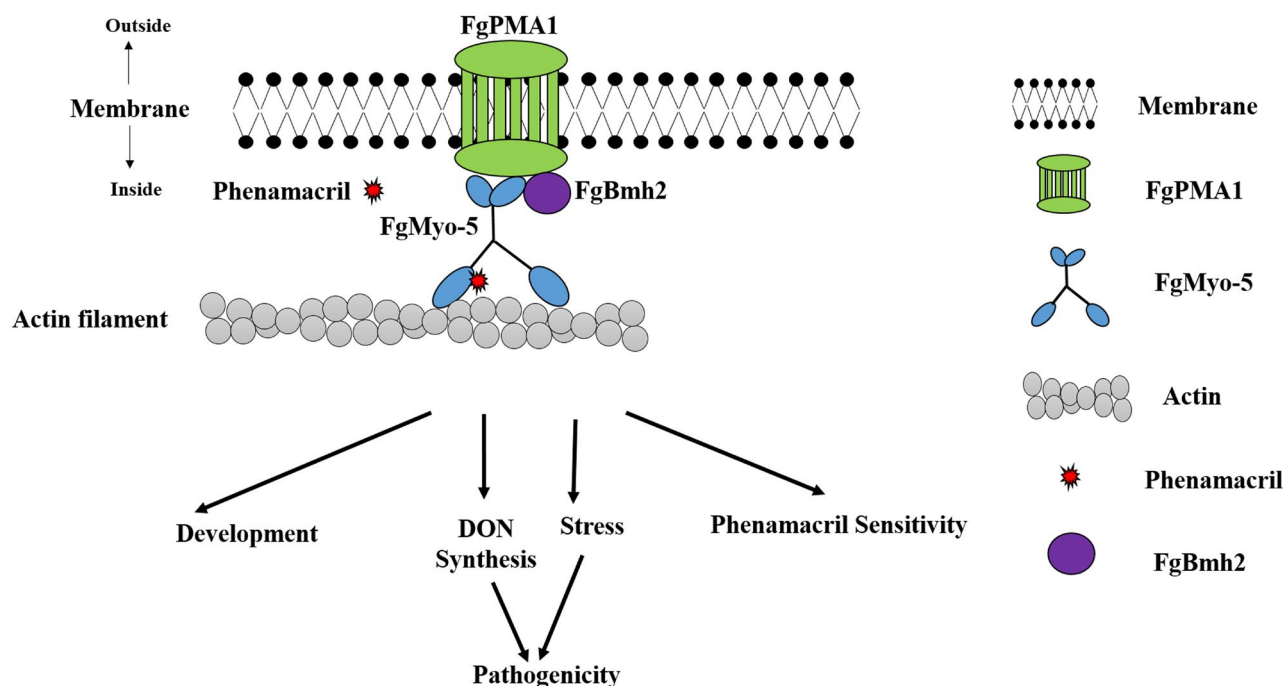


**FIGURE 6** *FgPMA1* regulates the sensitivity to phenamacril. (a) The  $\Delta FgPMA1$  mutation in sensitive strains increased the sensitivity to phenamacril. All strains were incubated on complete medium (CM) for 3 days. Wild-type phenamacril-sensitive strain, PH-1; wild-type phenamacril-resistant strain YP-1, -Y; complemented strains, -C; (b) The  $\Delta FgPMA1$  mutation in resistant strains decreased the resistance to phenamacril. All strains were incubated on CM for 3 days. (c) Co-immunoprecipitation confirmed the interaction between *FgPMA1* and *FgMyo-5*. Strains were cultured in yeast extract peptone dextrose (YEPD) medium for 36 h. (d) The *FgPMA1* deletion mutant increased the expression level of *FgMyo-5*. Strains were cultured in YEPD for 36 h





**FIGURE 7** FgPMA1, FgMyo-5, and FgBmh2 form a complex to regulate the sensitivity to phenamacril. (a) Yeast two-hybrid assay showed that FgPMA1 and FgBmh2 interacted directly. (b) Co-immunoprecipitation (Co-IP) confirmed the interaction between FgPMA1 and FgBmh2. (c) Co-IP confirmed the interaction between FgBmh2 and FgMyo-5. (d)  $\Delta$ FgBmh2 decreased the sensitivity to phenamacril. All the strains were incubated on potato dextrose agar (PDA) with or without phenamacril for 3 days. Wild type, PH-1. (e) Statistical analysis of the growth inhibition rate of all strains treated with phenamacril. Bars with the same letter indicate no significant difference at  $p = 0.05$



**FIGURE 8** A proposed model showing the role of FgPMA1-FgMyo-5-FgBmh2 complex. When FgPMA1 is deleted, this complex is disrupted, leading to the changed activity or stability of FgMyo-5 as evidenced by  $\Delta$ FgPMA1 affecting the sensitivity to phenamacril. This disrupted protein complex also affects the development and pathogenicity of *Fusarium graminearum*

interacted directly with FgPMA1 (Figure 7a), and we further confirmed the interaction of FgBmh2 and FgPMA1 by Co-IP (Figure 7b). Because FgPMA1 interacted with FgMyo-5, we hypothesized that FgPMA1, FgMyo-5, and FgBmh2 might form a protein complex. Interestingly, we also identified FgBmh2 from the pull-down candidate list using FgMyo-5 tagged with GFP as a bait. This interaction between FgBmh2 and FgMyo-5 was further confirmed by Co-IP (Figure 7c). Because FgPMA1 positively regulated the sensitivity to phenamacril, we examined whether FgBmh2 could regulate the sensitivity to phenamacril. We found that  $\Delta$ FgBmh2 exhibited decreased sensitivity to phenamacril (Figure 7d,e). Taken together, our results indicate that FgPMA1, FgMyo-5, and FgBmh2 form a complex to regulate the sensitivity to phenamacril (Figure 8).

### 3 | DISCUSSION

In *S. cerevisiae*, *Schizosaccharomyces pombe*, and *N. crassa*, the plasma membrane  $H^+$ -ATPase is encoded by the PMA1 gene and is essential to cell growth (Addison, 1986; Ghislain et al., 1987; Hager et al., 1986; Serrano et al., 1986; Ulaszewski et al., 1983), while the plasma membrane  $H^+$ -ATPase PMA2 gene is expressed at very low levels under normal growth conditions and is dispensable for growth (Ghislain & Goffeau, 1991; Schlessner et al., 1988). In this study, we found that FgPMA1 played an important role in vegetative growth and FgPMA2 was dispensable for growth (Figure 1a). Reverse transcription (RT)-PCR assays showed FgPMA1 was highly expressed and FgPMA2 had a lower expression (Figure S5). These

findings are consistent with the observations of PMA1 and PMA2 in other organisms, and demonstrate that FgPMA1 and FgPMA2 function differently due to their different expression levels. We generated a strain carrying FgPMA2-GFP, but no GFP signals were visualized and western blot did not detect this fusion protein. According to the RT-PCR assays, no GFP signal could be attributed to the low level of expression of FgPMA2.

*F. graminearum* can produce toxins, such as trichothecene (DON), in toxisomes, which contaminate wheat (Bian et al., 2021; Tang et al., 2018; Zhou, Duan, et al., 2020). Many TRI genes have been well characterized and are involved in DON biosynthesis (Meek et al., 2003; Nasmith et al., 2011), of which calonecristin oxygenase (Tri1) catalysing the late steps in the DON biosynthetic pathway is known to localize in toxisomes. FgMyo-5 has been reported as a linker between membranes and the actin cytoskeleton, and is essential for the formation of toxisomes. In addition, Tri1 interacts with FgMyo-5 in *F. graminearum* (Tang et al., 2018). In this study, we reported that FgPMA1 interacted with FgMyo-5 (Figure 6c), so we speculated that FgPMA1 might interact with Tri1. However, Figure S6 shows that no interaction was detected between FgPMA1 and Tri1. Our results showed that the deletion of FgPMA1 decreased the content of DON (Figure 4a). These observations indicate that FgPMA1 might interfere or interact with other DON biosynthesis components to influence DON production.

In plants, the plant plasma membrane  $H^+$ -ATPase has an autoinhibitory domain in the C-terminal (Palmgren et al., 1990, 1991). After deleting this autoinhibitory domain, the  $H^+$ -ATPase has a higher activity at physiological pH, and an increased coupling of  $H^+$  pumping to

ATP hydrolysis (Baunsgaard et al., 1996; Palmgren et al., 1990, 1991). Previous studies showed 14-3-3 proteins directly interact with the C-terminal region of the plant plasma membrane  $H^+$ -ATPase (Jahn et al., 1997). We identified two 14-3-3 protein homologs, FgBmh1 and FgBmh2, in *F. graminearum* but only FgBmh2 directly interacted with FgPMA1 (Figure 7a). Conversely, the last 98 amino acids in the C-terminal region of FgPMA1 were not required for the interaction with FgBmh2, suggesting that the C-terminal autoinhibitory domain shows divergent functions in plants and fungi.

Currently, the application of fungicides is a major method to control FHB. Phenamacril can effectively control FHB (Li et al., 2008). In this study, we found that FgPMA1 specifically regulated sensitivity to phenamacril (Figure 6a and Figure S4). In *N. crassa*, plasma membrane  $H^+$ -ATPases have been estimated to consume 38%–52% of the cell ATP (Gradmann et al., 1978). Therefore, we tried to determine whether FgPMA1 regulates sensitivity to phenamacril by affecting the content of ATP. However, the content of ATP did not show any significant change in the FgPMA1 deletion mutant (Figure S7), which indicates that FgPMA1 probably regulates sensitivity to phenamacril via other factors. To further dissect the relationship between FgPMA1 and FgMyo-5, we performed pull-down and Co-IP assays, and the results showed FgPMA1 interacted with FgMyo-5 (Figure 6c). Furthermore, we found both FgPMA1 and FgMyo-5 interact with FgBmh2. According to these results we propose a working model of a FgPMA1-FgMyo-5-FgBmh2 complex in regulating sensitivity to phenamacril in *F. graminearum* (Figure 8). When FgPMA1 is deleted, this complex is disrupted, leading to changed activity or stability of FgMyo-5 as evidenced by  $\Delta$ FgPMA1 affecting the sensitivity to phenamacril. This disrupted protein complex also affects the development and pathogenicity of *F. graminearum*.

## 4 | EXPERIMENTAL PROCEDURES

### 4.1 | Strains and sensitivity determination

*F. graminearum* wild-type strain PH-1 was used as a parental strain for transformation experiments in this study. Mycelial growth of the wild type and the mutants was assayed on PDA, CM, or MM as described previously (Gu et al., 2015; Zhang, Chen, Jiang, et al., 2015). To study the effect of phenamacril on the knockout mutant, the inhibition of mycelial growth at the same concentration of phenamacril was measured compared to that in the negative control without phenamacril, and the inhibition rate (%) was calculated by the following formula: (the averaged diameter of control – the averaged diameter in fungicide concentration)/(the averaged diameter of control – 5 mm of mycelial plug)  $\times$  100. To determine sensitivities to various stresses, 5-mm mycelial plugs of each strain taken from a 3-day-old colony edge were inoculated on PDA or CM supplemented without/with KCl, NaCl, SDS, CR, or phenamacril, and then incubated at 25°C for 3 days in the dark. Three biological replicates were used for each strain and each experiment was repeated three times independently.

### 4.2 | Generation of deletion mutants and construction of GFP and Flag fusion cassettes

Protoplast preparation and fungal transformations were performed following standard protocols as previously reported (Hou et al., 2002). A split-marker approach was used to generate gene replacement fragments for FgPMA1 and FgPMA2. The primers used to amplify the flanking sequences for each gene are listed in Table S1. Transformants were screened by PCR and further confirmed by Southern blot. For complementation of the  $\Delta$ FgPMA1 strain, a 5.3-kb segment of the FgPMA1 gene with 1191 bp upstream and 1103 bp downstream flanking sequences was amplified. The vector was transformed into the  $\Delta$ FgPMA1 strain. Using the same strategy, the complementation construct of the  $\Delta$ FgPMA2 and FgPMA1-3 $\times$ Flag fusion vector was constructed.

To construct the FgPMA1-GFP cassette, FgPMA1 containing the native promoter region and open reading frame (ORF) (excluding the stop codon) was amplified. Then the PCR product was cotransformed with XhoI-digested pYF11 into the yeast strain XK1-25 as described (Bruno et al., 2004). Subsequently, the FgPMA1-GFP fusion vector was recovered from the yeast transformant by using a Yeast Plasmid Kit (Solarbio) and then transferred into *Escherichia coli* DH5 $\alpha$  for amplification. With a similar strategy, other GFP fusion vectors were also constructed. Each plasmid was transformed into the wild-type strain PH-1. Using the same strategy, the FgBmh2-GFP fusion vector was constructed.

### 4.3 | Western blot assays

Each tested strain was incubated in yeast exact peptone dextrose (YEPD) at 25°C for 3 days, and then mycelia were harvested and washed with sterile water for protein extraction. About 100 mg of mycelia ground in liquid nitrogen was resuspended in 1 ml of extraction buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10  $\mu$ l of protease inhibitor cocktail [Yeast]). After homogenization with a vortex shaker, the lysate was centrifuged at 12,000  $\times$  g in a microcentrifuge for 20 min at 4°C. Fifteen microlitres of each sample was loaded onto 10% SDS-PAGE gels. Proteins separated on gels were transferred to Immobilon-P transfer membrane (Millipore). The monoclonal anti-GFP antibody 300943 (Zenbio) and monoclonal anti-Flag antibody 390002 (Zenbio) were used at a 1:1000 dilution for immunoblot analyses. Incubation with a secondary antibody and chemiluminescent detection were performed as described previously (Yang et al., 2012).

### 4.4 | Yeast two-hybrid assays

To construct plasmids for Y2H analyses, the coding sequence of each tested gene was amplified from the cDNA of PH-1 with the primer pairs indicated in Table 1. The cDNA fragments were inserted



into the yeast GAL4-binding domain vector pGBKT7 and GAL4 activation domain vector pGADT7 (Clontech). The pairs of Y2H plasmids were co-transformed into *S.cerevisiae* AH109 following the lithium acetate/single-stranded DNA/polyethylene glycol transformation protocol (Schiestl & Gietz, 1989). In addition, a pair of plasmids pGBKT7-53 and pGADT7 served as a positive control. A pair of plasmids pGBKT7-Lam and pGADT7 were used as negative controls. Transformants were grown at 30°C for 3 days on synthetic medium (SD) lacking Leu and Trp, and then transferred to SD lacking His, Leu, Trp, and Ade. Other vectors were generated using similar approaches. Three independent experiments were performed to confirm Y2H assay results.

#### 4.5 | Affinity capture-mass spectrometry analysis, immunoblot, and Co-IP assays

To analyse the interacting proteins of FgMyo-5, affinity capture was conducted using strain FgMyo-5-GFP. Strain FgMyo-5-GFP was incubated in YEPD at 25°C for 36 h, and total proteins were extracted using extraction buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 1% Triton X-100 and 10 µl of protease inhibitor cocktail 20124ES03 [Yeasen]). First, 35 µl of magnetize beads (Bio-Rad) were incubated with 3 µl anti-GFP antibody in 300 µl PBST (150 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4, 0.1% Tween 20) at 25°C for 20 min. Then, magnetized beads were washed three times with 1 ml PBST and were incubated with 1 ml total protein at 25°C for 1.5 h. Finally, total proteins were discarded, and magnetized beads were washed with 1 ml PBST three times, then the interacting proteins were identified by mass spectrometry analysis (Shanghai Applied Protein Technology Co., Ltd).

The FgMyo-5-GFP or FgPMA1-3×Flag-fusion constructs were verified by DNA sequencing and transformed into PH-1 or corresponding mutants. Transformants expressing FgMyo-5-GFP or FgPMA1-3×Flag were confirmed by western blot analysis. In addition, the transformants expressing a single tag protein were used as references. For Co-IP assays, magnetic beads (Bio-Rad) were first incubated with the anti-GFP antibody, following the manufacturer's protocol. Thereafter, the magnetic beads were incubated with total protein samples. Protein samples (10 µl) eluted from magnetic beads were assayed by western blot with anti-GFP antibody or anti-Flag antibody. Total protein samples were further assayed with monoclonal anti-GAPDH antibody 60004-1-Ig (ProteinTech) as a reference. All experiments were repeated twice.

#### 4.6 | Microscopic examinations

The localization of Tri1-GFP protein was observed with a TCS SP8 confocal microscope (Leica). For examination of toxisome assembly patterns in PH-1 and mutants, all strains labelled with Tri1-GFP were cultured in TBI medium for 2 days before observation. The following parameters for confocal microscopy were used:

Plan-Neofluar 100 × 1.30 oil differential interference contrast (DIC) objective, laser at 488 nm at 30% power for green fluorescence or at 561 nm at 40% power for red fluorescence (DiD, kgmp0025, Jiangsu KeyGEN BioTECH Co., Ltd), pinhole 100 µm, and digital gain 1.00.

#### 4.7 | Plant infection and DON production assays

Infection assays on flowering wheat heads and wheat coleoptiles were conducted as previously described (Zheng, Zheng, et al., 2015). For DON production assays, all the strains were grown in liquid TBI medium at 28°C for 7 days in the dark. DON was quantitatively measured using a competitive ELISA-based DON detection plate kit (Wise) and according to previous study (Li et al., 2019; Zheng et al., 2018).

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#### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## SUPPORTING INFORMATION

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