CHARACTERIZATION OF PHOSPHATE PERMEASE IN *FUSARIUM OXYSPORUM* F. SP. *VASINFECTUM*: GROWTH, VIRULENCE AND SECONDARY METABOLISM

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Abstract

Microbial development depends on the effective sensing of environmental signals and appropriate acquisition of nutrients and other molecules critical for the organism's growth. Levels of nutrients and other molecules within the cell, and those outside of the cell, are usually relayed to the nucleus via signal transduction pathways. Inorganic phosphate (Pi) is one of the most important macronutrients in fungal cells and its intracellular levels are tightly regulated via the phosphate signaling transduction pathway (PHO). A Tfo1 transposon insertion has been found in a phosphate permease gene (pho84), a constituent of the PHO pathway, of highly virulent California race 4 (VCG0114) isolates of the cotton pathogen *Fusarium oxysporum* f. sp. *vasinfectum*. Strains where the insertion is present are more pathogenic, even in the absence of nematodes, than those lacking the insertion. Highly virulent VCG0114 isolates generally have high copy number of Tfo1 and mutator-like transposons, but isolate with high copy number can also lose pathogenicity, indicating that these transposons modulate the pathogenicity by targeting specific genes in the genome. We generated *Agrobacterium*-mediated knock-out mutants of the gene in four VCG0114 isolates displaying varying levels of virulence in soil drenching pathogenicity assays: CA9 (highly virulent), MD308 (highly virulent), MD312 (moderately virulent) and NRRL25433 (non-virulent). Three knock-out mutants per isolates were confirmed for the targeted gene replacement with a hygromycin resistance gene. Pathogenicity assays with the wild type progenitor and mutant isolates show no difference in plant weight or percent leaves wilted, except a reduced pathogenicity for the PHO84 deletion mutants of moderately virulent isolate MD312.

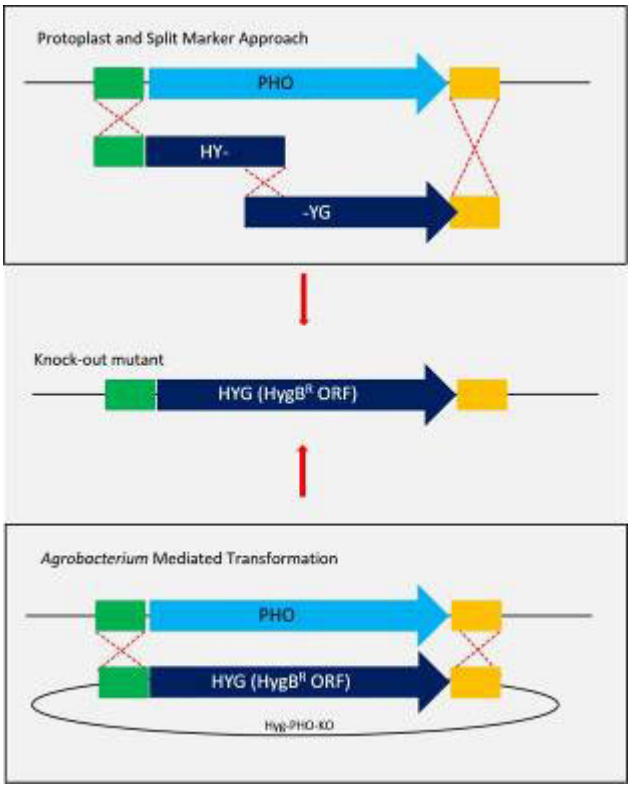
Introduction

Phosphorous is an essential component of biomolecules including nucleic acids and phospholipids. Phosphate also serves as a transmitter of biological signals that function in various cellular signaling pathways. A dual transport system, low- and high-affinity phosphate transporters, is utilized by fungi to acquire extracellular phosphate. When phosphate level became low, low-affinity transporters are replaced by high-affinity ones (Levy et al. 2011). Deletion of PHO84 (high-affinity) triggers constitutive expression of PHO pathway, lowering of low-affinity transporters, resulting in negligible phosphate uptake and poor growth in low phosphate medium. PHO pathway has been implicated in the virulence of a number of pathogenic fungi. A Tfo1 transposon insertion has been found in the high affinity phosphate permease gene (pho84) of highly virulent California race 4 isolates of the cotton wilt pathogen *Fusarium oxysporum* f. sp. *vasinfectum* (Fov) (Ortiz et al. 2017). Preliminary studies indicated that strains with presence of the insertion are more pathogenic, even in the absence of nematodes, than those lacking the insertion. The objectives of this study are: 1) to determine the correlation between the Tfo1 transposon abundance in the genome and virulence in various genotypes of Fov; 2) to determine the role of PHO84 in virulence, growth and secondary metabolism of Fov by gene-knock out approach.

Materials and Methods

Copy numbers of transposons Tfo1, Hop5 and MuDR in the genomes of various genotypes of Fov were determined by qPCR. Pathogenicity assays were conducted by inoculating soil with 5 ml of $1x10^6$ conidia, transplanting pre-germinated Pima S7 seeds, and incubation at 23˚C day / 18˚C night in a growth chamber for 5 weeks. Generation of

PHO84 knock out mutants for isolates CA-9, Race 4, Race 7, MD308 and MD312 were accomplished by either protoplast or *Agrobacterium*-mediated transformation as illustrated in the scheme bellow. Fungal growth and colony morphology were determined on various media.



Results and Discussion

In addition to Tfo1 insertions in PHO84, we also observed presence of mutator-like elements MuDR and Hop5 inserted in the Tfo1 in the California race 4 and China race 7 isolates, both of which belong to VCG0114. General screening of various genotypes of Fov revealed proliferations of these transposons in the genomes of VCG0114 isolates and general trend of higher virulence corresponding to higher copy numbers of these transposons in the genome (Table 1). Isolates MD308 and Ch1972 have mutator transposon within Tfo1 in the PHO84 gene and were highly virulent. In the vascular competent genotypes of Fov (races 1, 2, 6, 8), the copy numbers were very low.

Table 1. Relative abundance of transposons Tfo1, MuDR, and Hop5 in the genomes, and the corresponding virulence of various genotypes of Fov[a].

Strain	Hop5	MuDR	Tfo1	Disease index
*CA-14	2.03	5.22	4.15	4
*CA-17	2.02	5.43	4.08	4
**CA-9	5.98	9.26	11.17	5
*CA-59	2.22	5.61	4.81	4
*MD305	2.23	4.91	3.13	5
*MD308	2.28	5.14	3.19	5
*MD312	2.06	4.95	3.01	2
*CH1889	1.3	5.92	3.58	2
*CH1913	1.6	6.09	4.12	4
*CH1972	2.85	5.81	7.59	5
*Aus1089	1.3	-8.86	1.56	4
*RACE 1	-10.36	-8.73	-1.8	0
*RACE 2	-7.58	-7.63	-1.13	0
*RACE 4	6.41	8.7	10.41	0
*RACE 6	-9.21	-7.09	-4.39	0
*RACE 7	6.62	9.73	10.88	0
*RACE 8	-2.87	-8.72	-1.91	0

[a]Values are ΔCT values normalized to actin gene. Disease index is on the scale of 0 to 5 with no disease at 0 and all leaves wilted or plant dead at 5 based on soil inoculation assay on Pima S7.

As the PHO84 was interrupted by Tfo1 which was itself interrupted by a mutator-like element in some of the most virulent VCG0114 isolates and PHO pathway was known to be involved in virulence in fungi, we characterized PHO84 in selected VCG0114 isolates with varying degree virulence, and Tfo1 and mutator insertion states by generating gene knock out mutants. Targeted knock out mutants were confirmed by Southern blot analyses (Fig. 1).

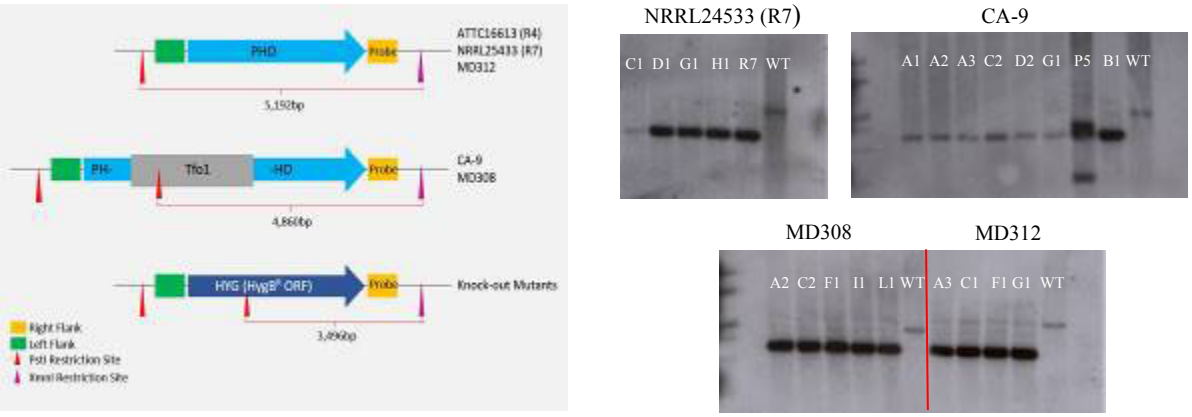


Figure 1. Digestion of wild-type and knock-out mutant DNA with PstI and XmnI restriction endonucleases (left) and Southern Blot confirmations of knock out mutants (right).

No significant differences were detected in vegetative growth on PDA or Czapek media (non-phosphate starving) upon PHO84 knock out, though small numeric decreases were observed for MD312 which had intact PHO84 gene (Figure 2). Secondary metabolite production were altered on several mutants, but not consistently among all three mutants derived from the same progenitors (Figure 3). Two of the mutant of MD312 produced purple compounds in heptaketide suppressing media not observed in the wild type or the other mutant. Though zinc has been reported to be also transported by PHO, the PHO knock out did not consistently alter fungal growth and secondary metabolite production (Figure 3).

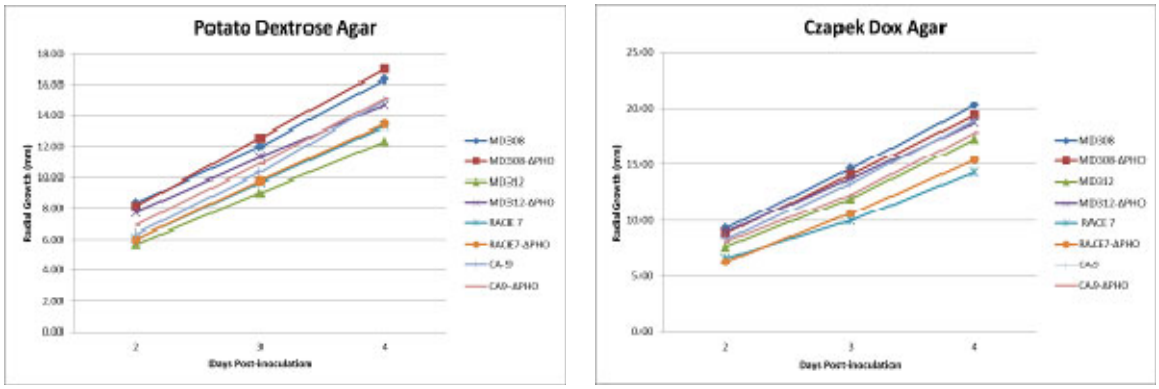


Figure 2. Effect of PHO knock out on the vegetative growth on potato dextrose agar (left) and Czapek Dox agar (right).

The effects of knocking out PHO84 in various VCG0114 isolates on their virulence were assessed by soil inoculation assays on Pima S7 (Table 2). Race 7 reference isolate is a non-pathogenic isolate with intact PHO84, and PHO84 deletion did not restore its virulence. Both Ca-9 and MD308 have a Tfo1 insertion but the latter has an additional short truncated mutator element in the Tfo1 at the PHO84 locus. Both isolates remained highly

pathogenic after the PHO84 with the insertions were deleted. PHO84 knock out in the mildly pathogenic isolate MD312 with intact PHO84, however, resulted in significate decreases in its virulence in the soil inoculation assay.

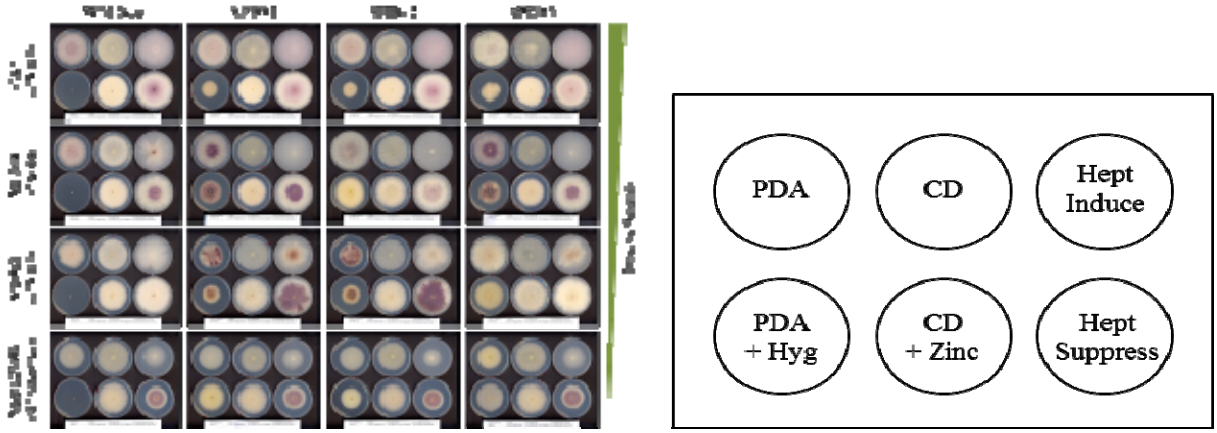


Figure 3. Colony morphology of wild type and PHO deletion mutants on various media grown for 7 days at 24°C (left). Plate arrangement of various media was illustrated at right. PDA: potato dextrose agar; PDA + Hyg: PDA amended with hygromycin B; CD: Czapex-Dox agar; CD + Zinc: CD amended with Zinc (10µg/ml); Hept Induce: heptaketide inducing agar; Hept Suppress: heptaketide suppressing agar. ΔPHO 1, 2, and 3 refer to each of the three knock-out mutants generated. Three plates per media type per isolate were used. Representative plates were selected and displayed in this figure. Disease severity graph is based on the strain's disease assay results obtained on Pima S7.

Table 1 PHO mutants pathogenicity assay on *Gossypium barbadense* cv. Pima S7[a]

Strain	Fresh Weight (gram)				Percent Leaf Wilted			
	M1[b]	M2	M3	WT	M1	M2	M3	WT
Race7	7.8 A a	7.1 A ab	6.7 B b	7.6 A ab	0 A a	0 A a	0 A a	0 A a
CA9	0.7 B a	0.6 B a	0.5 B a	1.3 B a	98 B a	100 B a	95 B a	95 B a
MD308	0.7 B a	0.9 B a	0.7 B a	0.7 B a	97 B a	95 B a	93 B a	100 B a
MD312	6.8 A a	4.8 B b	3.9 B c	2.8 B d	6 A a	33 B b	59 B c	68 B c
Control[c]	7.6 A				0 A			

[a]Values are the least squares means of five replications in each of the two trials except the controls which consisted of ten replications in each trial. Means were separated at P = 0.05 using least squares means with the Tukey-Kramer adjustment. Means with the same lower case letter within the row are not significantly different. Means with same capital letter compared to water control inoculation are not significantly different. Plants were evaluated 5 weeks after inoculating with $5x10^6$ conidial suspensions.
[b]M1, M2, M3, and WT refer to the three selected mutants and the wild type progenitor strain, respectively.
[c]Control: plants were treated with sterile water instead of fungal conidial suspension.

Conclusions

Highly virulent VCG0114 isolates generally have high copy number of Tfo1 and mutator-like transposons, but isolate with high copy number can also lose pathogenicity, indicating that these transposons modulate the pathogenicity by targeting specific genes in the genome. Further studies needed to identify the affected genes. Reduced pathogenicity for the PHO84 deletion mutants was only demonstrated in isolate MD312. The pathogenicity determinates in highly virulent VCG0114 isolates with Tfo1 insertion in their PHO84 need to be further elucidated.

Acknowledgements

We thank Cotton Incorporated for their financial support.

References

Levy, S., Kafri, M., Carmi, M., and Barkai, N. 2011. The competitive advantage of a dual-transporter system. Science 334:1408-1412.

Ortiz, C. S., Bell, A. A., Magill, C. W., and Liu, J. 2017. Specific PCR detection of *Fusarium oxysporum* f. sp. *vasinfectum* California race 4 based on a unique Tfo1 insertion event in the PHO gene. Plant Dis. 101:34-44