Genome Editing in Alfalfa (*Medicago sativa*) To Hyper-Accumulate Phosphate

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INTRODUCTION

- Inorganic phosphate (P_i) is an essential nutrient for plant growth.
- Excess P_i from animal manure and fertilizer runoff can lead to water pollution.
- The goal of this project is to create a *Medicago sativa* (alfalfa) plant that hyper-accumulates Pi.
- Such a plant could be used to protect waterways from excessive P_i and reclaim P_i for future fertilizer use.
- *Arabidopsis* and *M. truncatula* mutants of the ubiquitin E2 conjugating enzyme PHO2 hyper-accumulate Pi (1,2).
- In *M. sativa* there are 4-6 *PHO2* related genes.
- We used CRISPRR/Cas9 to generate site-directed frame-shift mutations.
- Some of the indels observed in the T_0 generation were germline and transmitted following either self fertilization or outcrossing.
- In addition, we further characterized *PHO2* by analysis of its expression under different phosphate regimes.

RESULTS

Identification of *Medicago sativa* PHO2 orthologs

Three genes encoding PHO2 in *Medicago truncatula* were used to identify orthologs in alfalfa using the CADL V1 (www.alfalfatoolbox.org) genome assembly and the alfalfa gene index (plantgrn.noble.org/AGED/). Figure 1 shows the two orthologs identified in the CADL genome (MSAD_261291, MSAD_295423). Four distinct transcripts were found, three with 99%+ identity to MSAD_261291 (Mscontig_29822, MScontig_29823, Mscontig_81529) and one with 99%+ identity to MSAD_295423 (Mscontig_24721). Mscontig_29822 and Mscontig_81529 contained an additional 30 bp that is lacking in Mscontig_29823 due to the use of an alternate splice site (Fig. 1). Both alfalfa *PHO2* genes encode proteins of 912 amino acids or 902 amino acids when the alternate splice site is used in MSAD_261291 (Fig. 1). The exonic structure is fully supported by the full length cDNA sequences synthesized from RNA isolated from both leaf and root tissue.

Figure 1

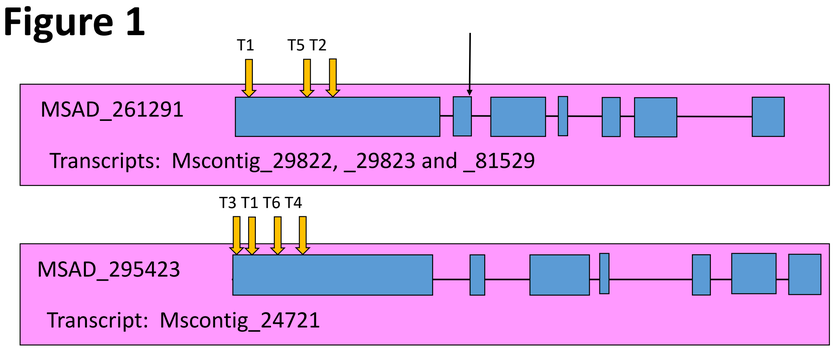

Figure 1. Structure of two *Medicago sativa* PHO2 genes. Blue boxes indicate exons, the black lines introns, the black arrow identifies the location of an alternate splice site, and the yellow arrows mark the CRISPR target sites. The transcripts of homologues found in tetraploid alfalfa are noted.

Phosphate Fertilization Experiment

Arabidopsis pho2 plants mount a constitutive P_i response with shoots accumulating high P_i levels under normal fertilization conditions (2). To gauge the response of alfalfa to changing P_i conditions, RegenSY27x plants were fertilized with low P_i (LP, 10 ppm), optimal P_i (OP, 40 ppm), and high P_i (HP, 60 ppm) 3x/week for 6 weeks.

Total plant P_i accumulation was measured by ICP analysis and the expression of the *PHO2* transcripts was measured by qRT-PCR (Fig. 2). Data was calculated by the ΔΔCt method using the OP mean as the reference sample. P_i accumulation correlated with P_i addition (Fig. 2A). *PHO2* transcript accumulation was reduced in the LP treatment compared to the OP treatment, consistent with previous reports showing that *PHO2* was repressed post-transcriptionally by P starvation (1, 3). The expression of both transcripts increased slightly under the HP conditions (Fig. 2B). The HP treatment brought about the greatest increase in *PHO2* transcript accumulation.

CRISPR/Cas9 Gene Editing in *Medicago sativa*

A 6-plex CRISPR/Cas9 reagent targeting *PHO2* (Fig. 3) was assembled by Golden Gate Reaction (GGR) (4). Target amplicons of 67 T_0 plants were sequenced and mutations were found to be present in 10 plants. The PCR products from these plants were further analyzed by a heteroduplex assay also indicating the presence of mutated sequence (Fig. 4).

Figure 2

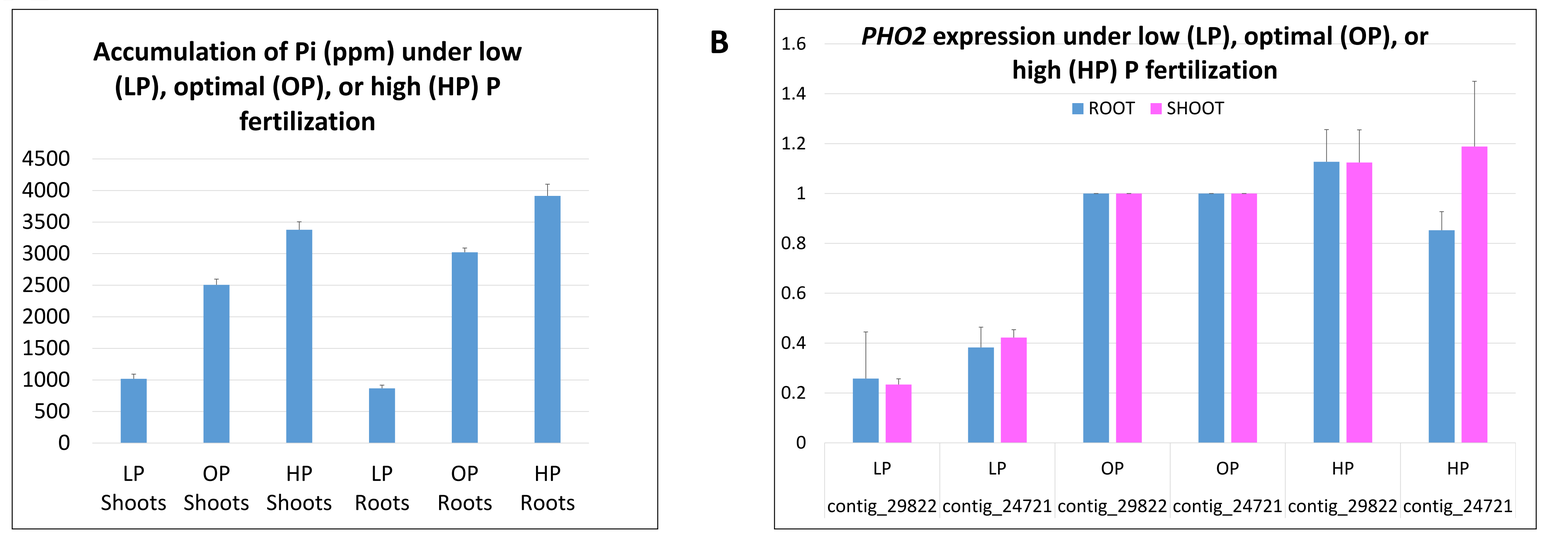

Figure 3

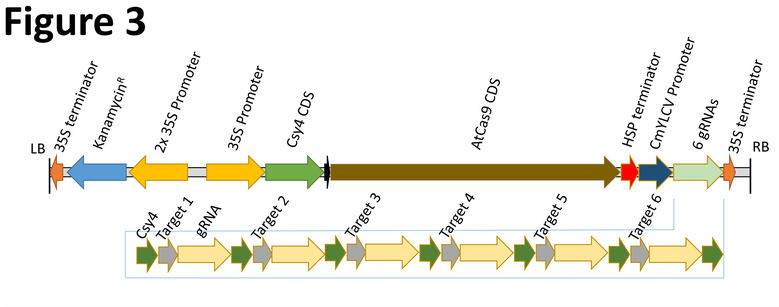

Figure 3. tDNA region of pDIRECT_22c vector containing 6-plex of gRNA targets for *PHO2*.

The target amplicons were cloned and sequenced with the results showing multiple mutant sequences in T_0 plants (Table 1). Combinations of somatic and potential germline mutations are commonly observed T_0 plants (2). Although M108 showed little discernable difference to the wildtype amplicon in this analysis, it has a 1 bp insertion in some *PHO2* allele(s) and is the only T_0 plant that has a deletion in the MSAD_261291-like gene (Fig. 5A). Mutated alleles were also confirmed in plants M20 and M23 (Fig. 5B). Target 1, found in both *PHO2* genes, appears to have the highest mutation rate. Only two of the six targets were found to be edited.

Figure 4

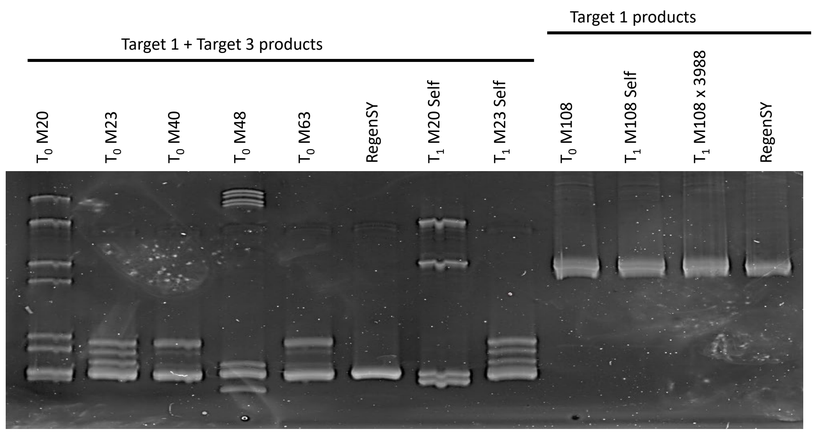

Figure 4. Heteroduplex analysis of PCR products of *PHO2* target regions from T_0 plants showing different banding patterns than wildtype RegenSY27x. PCR products from T_1 plants M20, M23, and M108 were found to retain the mutations as evidenced by retention of the same banding pattern.

Table 1. Identification of mutations in cloned targets.

	Mutated Sequences/Total Sequences		
T_0 Plant	Target 1	Target 3	Indels in T_0 Plant Subclones
M108	10/27	--	+1bp (9), S1bp (1)
M20	16/31	--	Δ2bp (10), Δ8bp (5)
M23	2/30	5/30	T1: +1bp (2) T3: Δ2bp (4)
M34	19/24	--	Δ8bp (9), S2bp (10)
M39	1/24	--	Δ1bp (1)
M40	2/24	2/24	T1: Δ1bp (1), S1bp (1) T3: Δ2bp (2)
M48	9/25	--	Δ25bp (5), Δ1bp (4)
M49	--	1/12	S1bp (1)
M63	1/25	2/25	T1: Δ1bp (1) T3: Δ2bp (1), S1bp (1)
M75	--	3/25	Δ2bp (3)
KEY:	Δ = deletion,	+ = insertion, S = substitution	

To confirm heritable transmission of targeted mutations, T_0 plants (M20, M23, M108) were self-fertilized and/or crossed to UMN 3988 (M108 only). Target amplicons from T_1 plants were sequenced revealing some of the identical mutations observed in T_0 plants (Fig. 5). While some M108 x UMN 3988 T_1 seedlings had the expected 1 bp insertion, many did not show evidence of targeted mutations, an expected result from segregation as only a fraction of the *PHO2* alleles in any T_0 plant were likely edited. No homozygotes in any *PHO2* gene were recovered out of any of the 109 T_1 plants screened.

Figure 5

Figure 5. CRISPR/Cas9 mutagenesis resulting in mutated alleles in two targets as compared to wildtype RegenSY27x sequence that were inherited in the T_1 generation. (A) Single mutation in MSAD_261291-like in T_0 plant M108. (B) Mutations in two T_0 plants (M20, M23) in MSAD_295423-like in Targets 1 and 3. Yellow boxes are PAM sites, target sequences are in bold face, base insertions are in red and substituted bases in blue.

Continuing Research and Conclusions

- Two additional CRISPR/Cas9 reagents targeting the *PHO2* genes have been assembled and tested.
- Preliminary results show these vectors have increased mutation frequency but not a significant overall increase in the number of edited plants generated. One T_0 plant has a homozygous mutation in the MSAD_261291-like *PHO2* gene and a second plant may have mutations in both MSAD-like *PHO2* genes.
- Experiments are currently in progress to measure P_i hyper-accumulation in T_1 progeny mutants to determine if the current mutation status is sufficient to alter *PHO2* expression levels.
- This work demonstrates the use of CRISPR/Cas9 to modify genes in a plant with complicated genetics.

References

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