Inorganic phosphate (Pi) is an essential nutrient for plant growth. Excess Pi, 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 prevent waterways from excessive Pi, and reclaim P, 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 CRISPR/Cas9 to generate site-directed frame-shift mutations. Some of the indels observed in the T0 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.alfalloatoolbox.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.

#### Phosphate Fertilization Experiment

Arabidopsis pho2 plants mount a constitutive Pi response with transcript accumulation of both PHO2 transcripts was measured by qRT-PCR (Fig. 2).

Total plant Pi accumulation was measured by ICP analysis and the expression of PHO2 transcripts was measured by qRTPCR (Fig. 2). Data was calculated by the ∆∆Ct method using the OP mean as the reference sample. Accumulation correlated with P 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 T0 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).

To confirm heritable transmission of targeted mutations, T0 plants (M20, M23, M108) were self-fertilized and/or crossed to UMN 3988 (M108 only). Target amplicons from T1 plants were sequenced revealing some of the identical mutations observed in T0 plants (Fig. 5). While some T0 x UMN 3988 T1 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 T0 plant were likely edited. No homologues in any PHO2 gene were recovered out of any of the 109 T1 plants screened.

### References


