Engineering Alfalfa Plants to Hyper-Accumulate & Recycle Phosphate

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Phosphate (P_i) is an essential nutrient for plant growth and plays a central role in photosynthesis, sugar metabolism, and other plant development processes. However, P_i is a finite resource with some estimates citing current rates of use will exhaust the world's supply in 20-40 years. Interestingly, the International Plant Nutrition Institute reported P_i surpluses for many U.S. croplands. Efforts to improve P_i management and conservation will require multiple strategies, including: 1) improved efforts to recover P_i from sewage, manure, and abattoir waste; 2) improved soil testing and fertilizer management; 3) development of plants with improved root architecture that can more effectively capture P_i; 4) biotechnological innovations to engineer plants that can bioremediate and recycle P_i.

P_i is applied to farmlands by either pelleted commercial fertilizer or manure and is one of the least available nutrients in the soil with only 20% or less taken up per season. Oftentimes, farmers may add extra P_i to the land, a consequence of the limitations of current soil testing. Animal farms, large and small, wrestle with manure management issues, in particular P_i over-saturation of soils which increases run-off and pollution of water in the drainage area.

Bioremediation is a strategy using plants to hyper-accumulate a target substance. A previously reported alfalfa plant that bioremediates and reduces nitrates in the soil is a good example of this strategy. Can alfalfa be re-tooled to bioremediate P_i and reduce levels in over-saturated land? Unfortunately, no current crop variety is capable of this task, since breeding for enhanced P_i uptake is not a focus of plant breeding. Understanding plant P_i use, acquisition, and remobilization will be necessary to engineer such a plant.

Gene editing reagents are a new technology generating specific genetic modifications in a wide range of crops amenable to genetic transformation. The effectiveness of these reagents has revolutionized crop plant breeding programs and they are especially useful for manipulating crops with complex genomes like alfalfa. The technology introduces specific breaks in the DNA which are then repaired by cellular processes in the plant. This repair is mostly seamless, however, in some cases errors are introduced leading to small insertions or deletions. These minor modifications disrupt the function of a gene and are used to create new traits in crops. In addition to precise edits, these plants are transgene-free and are not considered a regulated article in the U.S.

The mutagenesis of genes involved in the alfalfa phosphorus regulation pathway could potentially increase amounts of P_i taken up from the soil. These genes are called *Pho2* and mutants in *Arabidopsis* (a small mustard) have been shown to hyper-accumulate P_i. These plants allow the unregulated uptake of P_i even under high P_i conditions where a functional mechanism would normally reduce uptake. Many plants have between 0.1-0.9% P_i in stems and leaves. Concentrations higher than 1% are often toxic and can also cause deficiencies of other nutrients. As in nitrate bioremediation, the proposed alfalfa plant would accumulate excess P_i in the foliage that would be removed by harvesting.

Transformation experiments targeting alfalfa *Pho2* were performed and several mutated plants were recovered. Mutagenesis was confirmed by DNA sequencing and small base-pair deletions at target sites were observed. Moreover, heritable transmission of these mutations by self-fertilization and/or outcrossing was demonstrated in several plants. Alfalfa is an autotetraploid plant that has four highly conserved copies of each gene. It is expected that combinations of one to four copies of the target gene will be mutated in any given plant. For example, mutations of two and four copies of *Pho2* in two separate plants respectively have been confirmed. Currently, more advanced sequencing technologies are being deployed to thoroughly screen the collection of transformed plants and to identify single, double, triple, and quadruple mutant combinations. These mutants will be used in a series of phenotype analyses to measure both P_i uptake and accumulated P_i levels in the plant and help determine the optimal mutant combination.

These preliminary transformation experiments are the first steps in an ambitious project to engineer an alfalfa plant for bioremediation of P_i from over-saturated land. This current work will provide an indication of the efficiency of our gene editing platform and highlight areas of improvements. In addition, identifying an optimal mutant combination for P_i uptake and plant performance will be an important milestone for the project and will pave the way for additional research to improve the bioremediation of P_i.