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Review

A transgenic approach to enhance phosphorus use efficiency in crops as part of a comprehensive strategy for sustainable agriculture

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ABSTRACT

Concerns about phosphorus (P) sustainability in agriculture arise not only from the potential of P scarcity but also from the known effects of agricultural P use beyond the field, i.e., eutrophication leading to dead zones in lakes, rivers and coastal oceans due to runoffs from fertilized fields. Plants possess a large number of adaptive responses to P_i (orthophosphate) limitation that provide potential raw materials to enhance P_i scavenging abilities of crop plants. Understanding and engineering these adaptive responses to increase the efficiency of crop capture of natural and fertilizer P_i in soils is one way to optimize P_i use efficiency (PUE) and, together with other approaches, help to meet the P sustainability challenge in agriculture. Research on the molecular and physiological basis of P_i uptake is facilitating the generation of plants with enhanced P_i use efficiency by genetic engineering. Here we describe work done in this direction with emphasis on the up-regulation of plant proton-translocating pyrophosphatases (H^+-PPases).

Contents

1. Introduction

Farmers know that insufficient P fertilizer reduces crop yields, so they continually add P to their fields. P is necessary to stimulate early growth, strong local root formation and fuels sustained growth and development. Thus, modern agricultural systems are dependent on continual addition of phosphate fertilizers derived from phosphate rock. Indeed, the US Geological Survey estimates that global P mining extracts about 22 million tons (MT) a year. US farmers use around 4.3 MT a year (Jasinski, 2008). Unfortunately, the P in applied inorganic fertilizers bonds quickly with other compounds in the soil, reducing its bioavailability. Thus, bioavailable P may constitute less than 0.1% of the total P present in the soil (Khan et al., 2010). Consequently, farmers apply P_i fertilizers to keep a steady concentration of P_i in the soil solution available for plant absorption. It is worth mentioning that P_i fertilization regimes depend on the type of soil; acidic or high-iron and/or high-aluminum soils require frequent applications (Kochian et al., 2004).

Given that global consumption of P is increasing about 3% annually and about 40% of the world's P comes from one country (Morocco), concerns have begun to arise about the long-term prospects of the global P supply and its geopolitical implications (Jasinski, 2008; Cordell et al., 2009). Once P mines are depleted, the only likely sources will be recycled waste streams or ocean water. Nei-

Abbreviations: AVP1, arabidopsis vacuolar pyrophosphatase 1; P, phosphorus; P_i, orthophosphate; H^+-PPases, proton-translocating pyrophosphatases.

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ther option is practical because those sources are too salty for direct use and would require far too much energy (Stewart et al., 2005). These forecasts raise important questions about the dependence of current agricultural approaches on mine-derived fertilizer supplies.

Concerns about P sustainability in agriculture arise not only from the potential of P scarcity but also from the known effects of agricultural P use beyond the field. Runoff of excess P from agricultural fields contributes to eutrophication, the increases in algal biomass in lakes, rivers, and coastal oceans that often lead to "dead zones" in which oxygen has been depleted by microbial decomposition of decaying blooms. The P content of freshwater systems worldwide is at least 75% greater than pre-industrial levels and P fluxes to oceans have increased from 8 MT yr^{-1} to 22 MT yr^{-1} during the same time period (Bennett et al., 2001). More than 400 coastal dead zones at the mouth of rivers discharging P and nitrogen (N) have been identified worldwide and are expanding at 10% a decade (Diaz and Rosenberg, 2008). Economic damages due to eutrophication of freshwaters in the US have been estimated at $2.2 billion annually (Dodds et al., 2009).

Together, the long-standing issue of eutrophication and the emerging issue of scarcity call for new ways of thinking about how P cycles in the human socio-environmental system, especially in agriculture as fertilizer use and food consumption now dominate the global P cycle (Falkowski et al., 2000). Meeting these dual issues is especially challenging given the essential role of P in sustaining biological production.

2. P as an essential nutrient for agriculture

P is an essential macronutrient for all living organisms. It performs basic biological functions as a structural element in nucleic acids and phospholipids, in energy metabolism, in the activation of metabolic intermediates, as a component in signal transduction cascades, and in the regulation of enzymes (Sterner and Elser, 2002). In plants, P plays a pivotal role in photosynthetic regulation, energy conservation, and in carbon metabolism (Abel et al., 2002). After nitrogen, P is considered to be the second most limiting nutrient in agricultural production (Halvin et al., 2005), and in natural ecosystems is now seen as co-equal in limiting production in terrestrial and freshwater ecosystems (Elser et al., 2007). Importantly, P availability in the soils of many developing countries (i.e., India, Spain, Australia, etc.) is at a critical low point (Gahoonia and Nielsen, 2004).

Plants absorb orthophosphate (P_i) either as $H_2PO_4^-$ or HPO_4^{2-} depending on soil pH. At pH 7.2 there are similar amounts of $H_2PO_4^-$ and HPO_4^{2-}. Uptake of $H_2PO_4^-$ is much faster than uptake of HPO_4^{2-}. The average agricultural soil solution P_i concentration is about 0.05 part per million (ppm) but this varies widely among soils (Halvin et al., 2005). Sub-optimal levels of P_i result in yield losses, although the severity and economical relevance of the losses varies from crop to crop.

P_i deficiency is critical in highly weathered soils of tropics and subtropics, as well as calcareous/alkaline soils of Mediterranean basin (Hinsinger, 2001). P_i can be adsorbed or precipitated by calcium salts or iron and aluminum oxide complexes and thus becomes trapped in minerals that greatly reduce availability (Holford, 1997). Thus, when phosphatic fertilizers are applied to soils, a complex set of chemical reactions occur among the phosphate, soil constituents, and the non-phosphatic fertilizer compounds that remove P_i from the soil solution. In soil science, this phenomenon is classically known as "P_i fixation" or "P_i retention". Soil pH is the most important factor in P_i fixation. In acid soils, P_i precipitates as iron (Fe)–P or aluminum (Al)–P secondary minerals and/or is adsorbed to surfaces of Fe/Al oxide and clay minerals. In neutral and alkaline calcareous soils, P_i forms secondary minerals of calcium (Ca)–P and magnesium (Mg)–P and/or is bound to surfaces of clay minerals and $CaCO_3$ (Bar-Yosef, 1991). Therefore, although the total amount of phosphorus in the soil may be high, much of it is unavailable for plant uptake. In a classic study, Al-Abbas and Barber (1964) showed that many soils have large reserves of total P_i, often 100-times higher than the P_i available to the crops. Thus, a key challenge to raising the P_i efficiency of agriculture is to raise the availability of these soil P_i reserves to crop plants.

3. Plant responses to limiting P_i

P_i deficiency symptoms in plants commonly include overall stunting and a dark green coloration of leaves. With increasing P_i deficiency, the dark green changes to a grayish-green to bluish-green metallic luster but in some plants dark green leaves change to brown with conspicuous venation (Marschner, 2002). "Purple leaf" is commonly observed in corn and other grasses due to the accumulation of anthocyanins under P_i limitation. Lower leaves become necrotic under severely P_i-deficient conditions as P_i is translocated from old leaves to newly developing tissues. In the reproductive stage, P_i is translocated to fruits and seeds. Thus, seed development and fruit maturity will be affected if P_i deficiency happens late in the growing season. As expected under P_i-deficient conditions, crop yields are severely reduced (Halvin et al., 2005).

In response to limiting P_i availability, plants undergo dramatic morphological and architectural changes in their root system in order to increase the absorptive surface area. These changes include increased extension rates of root growth, an enhanced frequency of lateral root formation and a higher recruitment of atrichoblasts (epidermal root cells that undergo tip growth) to form root hairs (Abel et al., 2002; Poirier and Bucher, 2002). Studies with the model plant *Arabidopsis thaliana* have shown that plants grown in low P_i develop root systems with higher numbers of lateral roots and larger root hairs (Lopez-Bucio et al., 2002, 2003). Of note, these P_i-starved *Arabidopsis* plants are more sensitive to the plant growth hormone auxin. Interestingly, this enhanced auxin sensitivity has been associated with the modifications in root architecture (Lopez-Bucio et al., 2002, 2003).

Beyond morphological shifts in the roots, the physiological and cellular traits of the roots also change under P deficiency. For example, white lupine (*Lupinus albus* L.), a plant with a very efficient P_i scavenging capacity, up-regulates the abundance and activity of the P-ATPase enzyme as a response to limiting P_i (Yan et al., 2002). It also copes with sparingly available P_i by producing specialized structures called cluster roots that release substantial amounts of carboxylates (organic acids, such as citrates) and concomitantly acidify the rhizosphere. Another study showed that upon onset of P_i limitation citrate exudation increases transiently and reached a maximum after 5 h (Tomasi et al., 2009). This effect was accompanied by strong acidification of the external medium and a transient alkalinization of the cytosol. The increase in proton secretion was due to both an increased transcription level of a H^+-ATPase gene as well as post-translational modifications of H^+-ATPase protein involving binding of activating 14-3-3 protein (Tomasi et al., 2009). These studies reveal that plants possess a large repertoire of adaptive responses to P_i limitation that provide potential raw materials to enhance P_i scavenging abilities of crop plants.

Indeed, biochemical and molecular studies have revealed a conserved multigenic P_i starvation-inducible rescue system in plants (Abel et al., 2002) with major effects on plant P_i economy. For example, in *Arabidopsis*, the coordinate induction of more than 600 genes under conditions of P_i deprivation has been reported (Misson et al., 2005). Among the members of this inducible rescue system we find high-affinity P_i transporters (Raghothama, 2000), phosphatases, ribonucleases, and glycolytic enzymes such as phos-

phoenolpyruvate carboxylase and pyrophosphate-dependent phosphofructokinase (Abel et al., 2002). Increased expression of these genes enhances both the P_i uptake capacity and P_i cycling rate in plants. The induction of both the phosphoenolpyruvate phosphatase and the pyrophosphate-dependent phosphofructokinase has been proposed as a P_i recycling system that bypasses adenylate-requiring steps in glycolysis to allow carbon metabolism in P_i-starved cells (Plaxton, 2004). The vacuolar H^+-PPase also bypasses an ATP-dependent reaction, thereby conserving limited cellular ATP while simultaneously promoting intracellular P_i recycling from PP_i (Plaxton, 2004). Up-regulation of the vacuolar H^+-PPase has also been documented in *Brassica napus* (rapeseed) suspension cultures grown under limiting P_i conditions. The authors proposed that H^+-PPase facilitates the conservation of limited ATP pools and P_i recycling during P_i scarcity stress (Palma et al., 2000). A rice transcription factor (TF) (OsPTF1) involved in the phosphate starvation response has been reported (Yi et al., 2005). OsPTF1 is expressed in phloem cells of the primary root, leaves and lateral roots. Overexpression of OsPTF enhances rice tolerance to P_i starvation. Interestingly, microarray data on this OsPTF transgenic rice plants showed a concomitantly enhanced expression of rice H^+-PPases (Yi et al., 2005). Of note, in *A. thaliana* P_i starvation triggers increases in transcript and protein abundance of both AVP1 and the plasma membrane H^+-ATPase (Yang et al., 2007). In sum, emerging studies indicate the existence of deep reservoirs of capacity for increased P_i use efficiency in crop plants. Tapping this reservoir to increase the efficiency of crop capture of natural and fertilizer P_i in soils is likely one of the key ways to meet the P sustainability challenge in agriculture.

4. Genetic engineering strategies to increase phosphorus use efficiency in crops

The optimization of P_i fertilization is one aspect that could help meet agricultural P sustainability challenge in the near future and at the same time have a positive impact in reducing the ecological problems triggered by runoff of the added P_i fertilizers. Attempts to improve soil phosphate availability using rhizospheric bacteria have not been particularly successful for various reasons, such as poor ecological fitness, low metabolite production, variable inoculant-delivery systems, and inconsistent performance in field applications (reviewed in (Shenoy and Kalagudi, 2005). However, there are other agricultural practices that have been shown to effectively improve soil phosphate availability, such as liming, optimized fertilization timing and placement (www.epa.gov/owow_keep/msbasin/pdf/symposia_ia_session7.pdf). An alternative, but not mutually exclusive, strategy is to enhance plant phosphorus use efficiency by improving acquisition, translocation and internal utilization of P_i. This can be achieved via the selection of cultivars with an enhanced P_i uptake or use efficiency by means of traditional plant breeding programs or genetic engineering (Zhu et al., 2005; Cao et al., 2009). Research on the molecular and physiological basis of P_i uptake, translocation, and internal utilization is facilitating the generation of plants with enhanced P_i use efficiency by genetic engineering.

Mitsukawa and collaborators reported that the over-expression of the P_i transporter gene Pht1:1 in tobacco cell cultures resulted in an increased rate of P_i uptake when cells were grown under P_i limited conditions (Mitsukawa et al., 1997). Unfortunately this enhanced uptake capacity has not been confirmed at the whole plant level. In another attempt to generate plants with an enhanced P_i uptake capacity Lopez-Bucio and collaborators metabolically engineered tobacco plants to produce more organic acids (specifically citrate). In a promising result, these citrate-overproducing plants yielded more leaf and fruit biomass than controls when grown under P_i-limiting conditions (Lopez-Bucio et al., 2000). These results point towards manipulation of biochemical pathways that increase the ability of the plant to release organic acids and thus acidify the soil microenvironment as a powerful means to improve P_i extraction from soils. Another interesting approach was to engineer *A. thaliana* plants with an alfalfa (*Medicago truncatula*) phytase gene. These transgenic plants displayed an improved acquisition capacity of organic phosphorus when grown on phytate as the only source of phosphorus (Xiao et al., 2005). Similarly, *Nicotiana tabacum* plants engineered to express a fungal phytase gene resulted in improved phosphorus nutrition when grown in amended soils (George et al., 2005). Here we describe in more details some recent work by one of us (Yang et al., 2007) along these lines.

5. Improving PUE by up-regulation of a type I H^+-pyrophosphatase

Prototypical plant proton-translocating pyrophosphatases (H^+-PPases) are highly conserved with amino-acid sequence identities of 85% or greater (Drozdowicz and Rea, 2001). Plants have two phylogenetically distinct H^+-PPases: type I and type II. Type I H^+-PPases depend on cytosolic K^+ for their activity and are moderately sensitive to inhibition by Ca^{2+} and type II H^+-PPases are K^+-insensitive but extremely Ca^{2+}-sensitive.

Over-expression of the type I H^+-PPase *AVP1* in *Arabidopsis* (*AtAVP1*OX) resulted in enhanced salt tolerance and drought resistance (Gaxiola et al., 2001). The salt tolerant phenotype was explained by an increased capacity for Na^+ uptake into vacuoles and drought resistance was attributed to an enhanced vacuolar osmoregulatory capacity (Gaxiola et al., 2001, 2002). Other groups have subsequently demonstrated that over-expression of this and other plant type I H^+-PPase genes can increase both salt- and drought-tolerance in diverse systems (Table 1). Interestingly, this genetic manipulation also triggered increased root and shoot proliferation in *Arabidopsis* and other plants (Table 1). To examine whether the robust root systems of *AtAVP1*OX plants are capable of responding to P_i scarcity, control and *AtAVP1*OX plants were grown under P_i-deficient (10 μM) conditions and the development of their root systems analyzed. Interestingly, *AtAVP1*OX seedlings developed significantly more robust root systems than wild type controls under P_i limitation (Yang et al., 2007). Furthermore, *AtAVP1*OX root hairs were also 2.5-fold larger and 1.5-fold denser than controls under P_i-deficient conditions increasing the absorptive area of the roots (Fig. 1). The more robust root systems developed by *AtAVP1*OX plants were shown to increase the acidification of P_i-deficient media, resulting in more efficient scavenging of P_i (Yang et al., 2007).

The high degree of identity at the amino acid level among the type I H^+-PPases across the plant kingdom (Drozdowicz and Rea, 2001), suggests that AVP1 from one species would be functional in another species. As expected transgenic tomatoes overexpressing the E229D gain-of-function mutant (*AVP1D*) of the *Arabidopsis* H^+-PPase (*LeAVP1D*OX) develop more robust root systems and are resistant to imposed soil water deficits (Park et al., 2005). Furthermore, as was seen with *AtAVP1OX*, both *LeAVP1D-1* and *-2* overexpression lines developed larger shoots, root systems and fruits than controls when grown under P_i-deficient conditions (Yang et al., 2007). Root and shoot dry weights of plants grown in the presence of 100 ppm NaH_2PO_4 were, on average, 13% and 16% higher ($P < 0.01$) in *LeAVP1D*OX than in controls, respectively. Of note, under the same low P_i conditions, fruit dry weight data and P_i content per plant were 82% and ~30% higher ($P < 0.01$) than in controls, respectively. Of note, these enhanced shoot and fruit yields suggest that, under the conditions tested, the physiological costs incurred by the AVP1 transgenic plants in developing larger root systems do not jeopardize their capacity to allocate sufficient

Table 1
Phenotypes triggered by the up-regulation of type I H^+-PPases in plants.

Transgenic plant	Origin of H^+-PPase	Phenotypes	Reference
Arabidopsis thaliana	*Arabidopsis thaliana*	Salt and drought tolerance	Gaxiola et al. (2001)
Arabidopsis thaliana	*Arabidopsis thaliana*	Enhanced biomass root and shoot Enhanced auxin transport Enhanced cell division	Li et al. (2005)
Arabidopsis thaliana, tomato and rice	*Arabidopsis thaliana*	Enhanced growth under P_i limitation Enhanced rhizosphere acidification capacity Enhanced size and density of root hairs	Yang et al. (2007)
Arabidopsis thaliana	*Arabidopsis thaliana*	50% Enhanced auxin and salicylic acid	Gonzalez et al. (2010)
Cotton (*Gossypium hirsutum*)	*Arabidopsis thaliana*	Salt and drought tolerance 20% Higher fiber yield	Pasapula et al. (2010)
Creeping bentgrass (*Agrostis stolonifera*)	*Arabidopsis thaliana*	Salt tolerance Enhanced root phosphorus content	Li et al. (2010)
Alfalfa (*Medicago sativa*)	*Arabidopsis thaliana*	Salt and drought tolerance Enhanced photosynthetic capacity Enhanced K^+ uptake	Bao et al. (2008)
Cotton (*Gossypium hirsutum*)	*Thelungiella halophila*	Enhanced shoot and root growth Drought resistance Enhanced chlorophyll content	Lv et al. (2009)
Cotton (*Gossypium hirsutum*)	*Thelungiella halophila*	Salt tolerance Enhanced photosynthetic performance	Lv et al. (2008)

photosynthate for shoot and fruit development (Lynch, 1995; Yang et al., 2007).

To determine whether increased H^+-PPase activity improves plant performance under P_i-deficient conditions in monocots, rice (*Oryza sativa* var. *japonica* 'Taipei 309') was engineered with a 35S: *AVP1D* cassette. These *AVP1D*OX rice lines (*OsAVP1D*OX) exhibited sustained shoot growth under P_i-deficient (10 μM) conditions while the controls grew poorly. Moreover, these lines developed more robust root systems than controls in both P_i-sufficient and P-deficient conditions (Fig. 2). Therefore, *AVP1* overexpression in both monocots and dicots results in enhanced root systems and increased soil acidification capacity under low P_i conditions. It also results in marked increases in crop growth and yield. Importantly, it also enhances the extraction capacity of P by crops under both P-deficient and P-sufficient conditions (Table 2).

6. Conclusions

These results discussed above suggest that engineering plants to overexpress AVP1 or orthologs appears to be a broadly applicable technology to a range of agriculturally important crops. It will be interesting to explore if this technology is applicable to crops with extremely high P_i fertilization requirements, such as corn. The generation of AVP1-transgenic corn with enhanced P_i uptake capacity could help to alleviate the severe environmental impacts currently associated with this crop (Diaz and Rosenberg, 2008; Donner and Kucharik, 2008). This technology could help alleviate agricultural losses due to P_i limitation in acid and calcareous soils

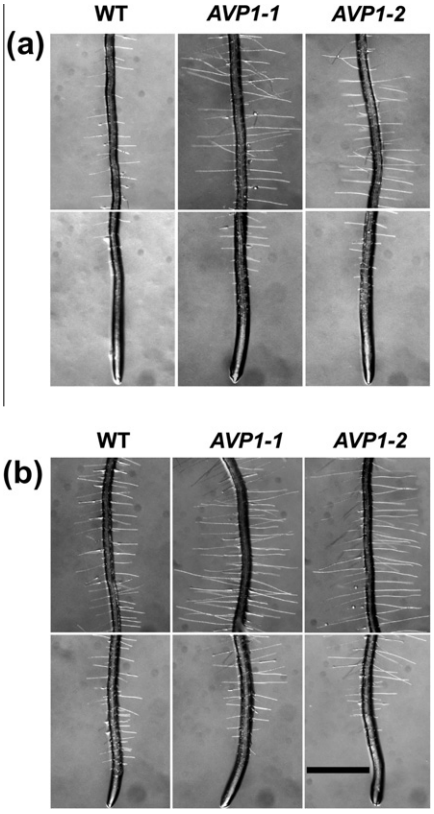

Fig. 1. Root hair development in control and *Arabidopsis AVP1*OX plants grown under normal or limiting P_i. Root hair development of seedlings (as indicated) grown for 7 d on (a) 1 mM P_i and (b) 10 μM P_i. Bar = 0.5 mm.

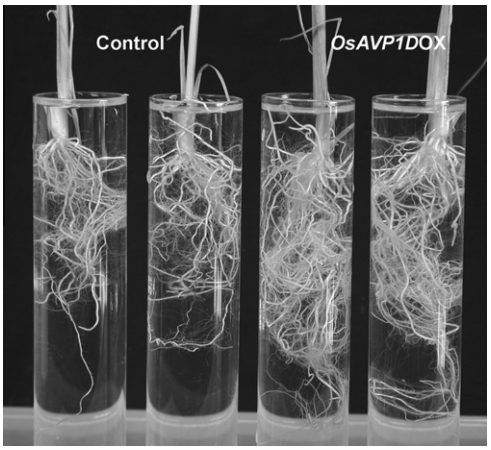

Fig. 2. Root phenotypes under P_i limitation. Control and *OsAVP1D*OX (rice) seedlings (as indicated) grown for 35 d under limiting P_i conditions (10 μM Pi).

(Raghothama, 1999), reducing the need for external fertilizer while allowing farmers to increase and maintain yields and thus maintain and improve their standards of living. Meeting the P sustainability challenge will require a wide suite of both low-tech and high-tech approaches (Cordell et al., 2009; Childers et al., 2011). We propose that, among these approaches, we should consider further development and implementation of transgenic technologies, such as AVP1 modified crops with increased PUE. These efforts would be part of a comprehensive strategy for sustainable global agriculture that can meet the forecasted needs for food production (Nature Editorial, 2010).

Table 2
Effect of P_i availability on P_i content of *AVP1*OX transgenic and control plants (modified from Yang et al. (2007)).

Genotype and conditions	Total P content (μg plant^{-1})	Genotype and conditions	Total P content (mg plant^{-1})	Genotype and conditions	Total P content (mg plant^{-1})
1 mM P		400 ppm		1 mM P	
Col-0	3.75 ± 0.41	LeWT	11.33 ± 1.08	Control	0.72 ± 0.09
AVP1–1	4.60 ± 0.12**	*LeAVP1D-1*	11.60 ± 1.11	*OsAVP1D-2*	1.03 ± 0.11**
AVP1–2	5.26 ± 0.10**	*LeAVP1D-2*	13.12 ± 1.16*		
10 μM P		100 ppm		10 μM P	
Col-0	0.30 ± 0.04	LeWT	7.76 ± 0.92	Control	0.051 ± 0.009
AVP1–1	0.37 ± 0.05**	*LeAVP1D-1*	9.63 ± 1.15**	*OsAVP1D-2*	0.089 ± 0.019**
AVP1–2	0.44 ± 0.04**	*LeAVP1D-2*	10.54 ± 1.21**		
		44 ppm			
		LeWT	0.76 ± 0.08		
		LeAVP1D-1	0.97 ± 0.06**		
		LeAVP1D-2	1.46 ± 0.12**		

* $P < 0.05$.
** $P < 0.01$.

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