Bioengineering beans for phosphate-deficient soils in southern Africa

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Approximately eight species of southern African legumes are currently used as sustainable food crops. Biotechnology has the potential to improve the productivity of growing these plants by small-scale farmers who cannot afford sufficient phosphate fertilizer to optimize their nitrogen fixation and hence conversion to edible protein. The metabolic adaptations that enable legumes to fix atmospheric nitrogen are currently being investigated by our group for the purpose of genetic modification to enhance crop yields. Until now, attempts at modifying host plants or symbiotic bacteria have not significantly enhanced N_2 fixation. We propose, instead, to bioengineer the key enzymes that control the mechanisms involved in protein formation. This may lead to enhanced seed protein content, which would be of advantage to poor communities that rely on this source of food. We postulate that misregulation of phosphoenolpyruvate carboxylase (PEPc) could be exploited by biotechnology to improve N_2 fixation and protein content. We have found that, as distinct from their roots, legume nodules are under permanent phosphate stress, even during optimal phosphate supply to the host plant, implying that the development of phosphate stress may engage different forms of PEPc to ensure continued nodule functioning.

Legumes around the world

Legumes are grown on approximately 275 million hectares, or nearly 11% of arable land worldwide[1] and provide at least one third of human protein requirements. In the tropics and subtropics legumes can satisfy up to 80% of protein needs. Grain legumes are important as food and feed proteins and in many regions of the world they are the only source of protein in the diet,[2] because of the high price of animal protein. All legume seed proteins are relatively low in sulphur-containing amino acids and tryptophane, but the amount of lysine, another essential amino acid, is much greater than in cereal grains.[3] Most food in the poor countries of the tropics is grown on small, family-managed farms where, especially in Africa and South America, the use of fertilizer and other agrochemicals is minimal. Raising N_2-fixing grain legume crops in such circumstances provides excellent opportunities for producing high protein food sources without additional nitrogenous fertilizer (predominantly in the form of NH_4^+).[4] It takes 1.3 tons of fossil fuel to manufacture one ton of nitrogenous fertilizer. Since legumes do not require nitrogen as fertilizer, growing these plants as a source of protein would not consume non-renewable fuels. The reliance on legumes is therefore basic to sustainable and economic production of food and feed proteins.[5]

In southern Africa, indigenous leguminous crops are used as food and as a feed source for livestock. They may also be economically important, as they allow farmers on smallholdings to trade with any surplus. These crops include the following:[6]

- Wild coffee bean (*Bauhinia petersiana*) and jack bean (*Canavalia ensifolia*) — widely used as a coffee substitute; the seeds and pods may also serve as a food source.
- Pigeon pea (*Cajanus cajan*) — used for seeds and also as a green vegetable; it is grown almost exclusively for subsistence, with only small quantities reaching local and international markets.
- Copalwood (*Guibortia coleosperma*) — the seeds are a primary food source of the !Khu San (Bushmen) of northeastern Namibia, who use its oily arils for food during periods of famine, and various parts of the plant for traditional medicine.
- Karoo boer-bean (*Schotia afra* var. *afra*).
- Marama bean (*Tylosema esculenta*) — has a large woody, below-ground tuber with a moisture content of 81%, which makes it a valuable resource when water is scarce. The young pods are eaten as a vegetable and the large seeds are consumed roasted. It is an important part of the diet of rural people in the Kalahari, the Kaokoveld and Mozambique.
- Bambara groundnut (*Vigna subterranea*) — grown exclusively as a protein source, and included in many traditional recipes. The immature beans can be eaten raw or cooked, while ripe beans can be pounded into a flour or soaked and then cooked.[6] Bambara is considered a substitute for meat and the ripe beans are very nutritious.[7] One of the most under-rated and underdeveloped of crop plants, it produces reasonably well under extreme conditions such as drought and poor soil.[6]
- Cowpea (*Vigna unguiculata*) and mung bean (*Vigna radiata*) — these food plants are popular for their beans and also as green vegetables in southern Africa.
- Wild sweetpea (*Vigna vexillata*).[6]

Environmental constraints

The impoverishment of the soil is a growing constraint on sustainable development in the Third World, particularly in sub-Saharan Africa. The use of fertilizer in this part of the continent averages only 5–10 kg ha^{-1}, with many soils progressively being denuded of their nutrients.[8] The concentration of available phosphorus for plants is normally very low in soils, because most of the element combines with iron, aluminium and calcium to form relatively insoluble compounds.[9] Phosphorus is present in the soil water in the ionic forms $H_2PO_4^-$ and HPO_4^{2-} (ref. 10). Smallholders in southern Africa apply less inorganic phosphorus fertilizer (P*i*, as inorganic mineral salts) than is removed during harvesting, thereby depleting soil reserves.[11] P*i* deficiency is thought to be one of the factors limiting nitrogen fixation[9] owing to the high energy requirement of plants engaged in nitrogen fixation for nitrogenase function.[12] P*i* deficiency has important implications for the metabolic P*i* and adenylate pools of plants, which influence respiration and nitrogen fixation.[13] An alternative route of pyruvate supply during P*i* stress has been proposed by Theodorou and Plaxton.[13] This involves the combined activities of phosphoenolpyruvate carboxylase (PEPc), malate dehydrogenase and NAD-malic enzyme supplying pyruvate to the mitochondrion in response to P*i* stress.

Nodules impose an energy cost on host resources. The *Rhizobium* bacteria inside the nodules reduce N_2 to NH_4^+ in exchange for reduced carbon compounds from the plant (Fig. 1). Sucrose from the shoot is the principal source of reduced carbon for the nodule.[14] This sucrose is metabolized via the action of sucrose synthase and glycolytic enzymes; it is generally accepted that organic acids are the main products of sucrose degradation supplied to bacteroids to support nitrogen fixation in most legumes.[15] The carbon costs of N_2 fixation vary with host

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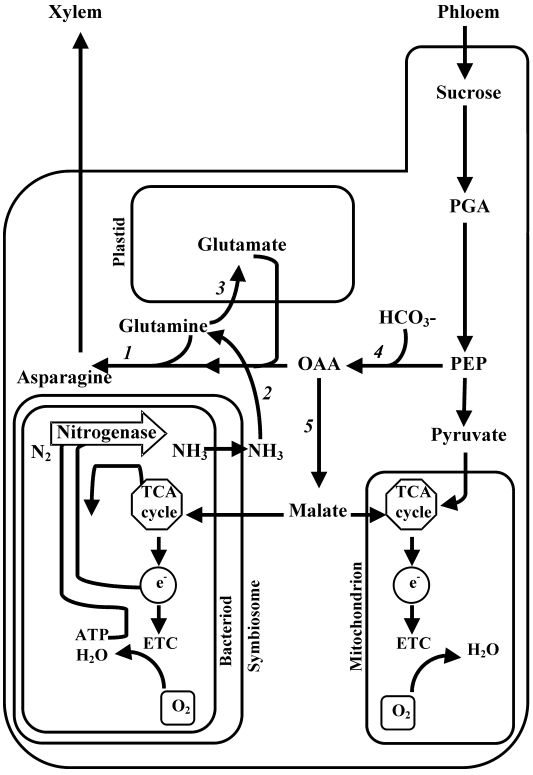

Fig. 1. A summary of key pathways of carbon and nitrogen metabolism and their location in the infected and uninfected cells of a legume nodule. The key enzymes of metabolic pathway regulation are: 1, asparagine synthetase; 2, glutamine synthetase; 3, glutamate synthase; 4, phosphoenolpyruvate carboxylase; 5, malate dehydrogenase. Modified from Layzell and Atkins.[19]

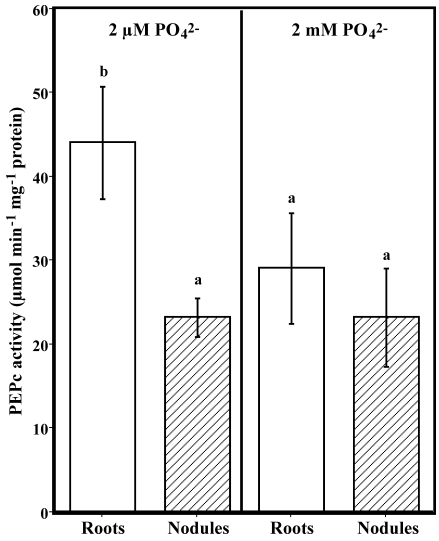

Fig. 2. Phosphoenolpyruvate carboxylase activities for 7-week-old *Lupinus angustifolius* plants grown hydroponically under either P-deficient (2 µM PO_4^{2}) or P-sufficient (2 mM PO_4^{2}) conditions. P-deficiency in roots caused an increase in phosphoenolpyruvate carboxylase activity, but had no significant effect in nodules. The different letters above the error bars indicate significant differences between treatments determined using analysis of variance (ANOVA) with post-hoc LSD tests. The values represent the means of four replicates and the standard error is less than 10%.

species, bacterial strain and plant development. Nodules may consume up to 50% of photosynthates produced by N_2-fixing plants. For example, average values of carbon costs for different N_2-fixing legumes range as follows: 36–39% of carbon is required for nodulation, nodule growth and maintenance respiration, 42–45% for nitrogenase activity, and 16–22% for amino acid synthesis from NH_4^+ assimilation and subsequent export.[16] About 50% of photosynthates consumed by nodules are respired as CO_2. Nodules are able to reassimilate between 25% and 30% of this respired CO_2 via PEPc. This additional activity of PEPc can provide up to 25% of the carbon required for amino acid synthesis.[16] Oxaloacetate, the product of PEPc refixation, can be used in bacterial metabolism or exported to the host in the form of amino acids.[17] Legumes are categorized as amide or ureide exporters, depending on the organic form of nitrogen despatched from the nodules. This is important for carbon costs because amide exporters rely more heavily on the activity of PEPc than ureide exporters.

Progress in biotechnological modification of legumes

Herbicide-resistant soybeans, which are ureide exporters, were the first genetically modified legumes, in 1996, made commercially available. Today, more than 1000 of these glyphosate-resistant varieties are available.[18] Direct molecular modification of host plants or bacteria has not yet resulted in improved N_2 fixation,[8] but we propose that modifying the host component of the nodule could lead to enhanced seed protein content, which would be to the advantage to those who depend on these seeds as a primary source of protein. The interaction between carbon and nitrogen metabolism in the symbiotic system is regulated at three levels[19] by key enzymes, which offer possibilities for genetic modification (Fig. 1).

In our studies, *Lupinus angustifolius* serves as a model system for studying the physiology and metabolism of these levels of regulation, with a view to applying such knowledge to legume crops in Africa. The first level of regulation involves glutamine synthetase and glutamate synthase, which control the assimilation of NH_4^+ into glutamine and glutamate.[19] The second level of regulation concerns the combined activities of aspartate aminotransferase and asparagine synthetase, which regulate the flow between organic and amino acids.[19] The third level of regulation comprises the PEPc and malate dehydrogenase enzymes, which control the replenishment of the organic acid pool with anaplerotic carbon.[19] The fate of the anaplerotic carbon in the organic acid pool in P*i*-stressed legumes is the research focus of our group.

Marczewski[20] purified three iso-enzymes of PEPc from lupin nodules and roots, with two forms being nodule specific. The same study also reported that these two forms, one of which appeared to be closely associated with nitrogen fixation,[20] differed substantially in their kinetic properties and were also different from a third form of PEPc. The two nodular isoforms of PEPc could be the products of separate genes, or could be from the same gene, but may undergo different post-translational modification.[21] The different PEPc molecules could possibly contribute to different metabolic mechanisms in the nodule.[17]

We aim to help poor farmers to improve the protein quantity of the legumes they grow. Our focus on carbon metabolism via PEPc may facilitate the control of the carbon used during amino acid synthesis. To this end, we are investigating phosphorus stress in legume root systems and how it influences the nodular PEPc isoenzymes and pyruvate synthesis.

The key focus areas are:

1) The mechanism of pyruvate synthesis during P*i* stress in roots and nodules. The P*i*-stress-induced reactions have not hitherto been investigated in roots

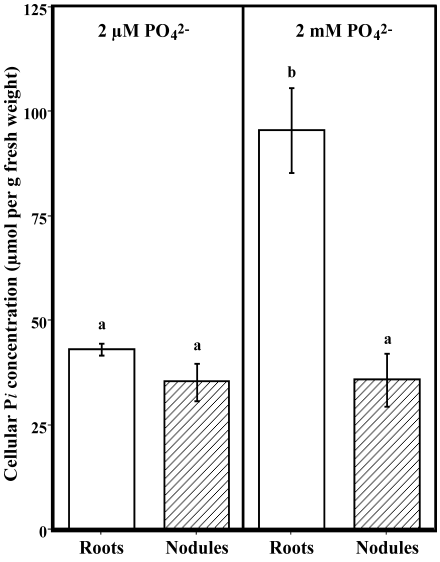

Fig. 3. Cellular P*i* concentrations for 7-week-old *Lupinus angustifolius* plants grown hydroponically under either P-deficient (2 µM PO_4^{2}) or P-sufficient (2 mM PO_4^{2}) conditions. Cellular P*i* concentration is higher in P-sufficient than in P-deficient roots, but remained constant in nodules. The different letters above the error bars indicate significant differences between treatments determined using analysis of variance (ANOVA) with post-hoc LSD tests. The values represent the means of four replicates and the standard error is less than 5%.

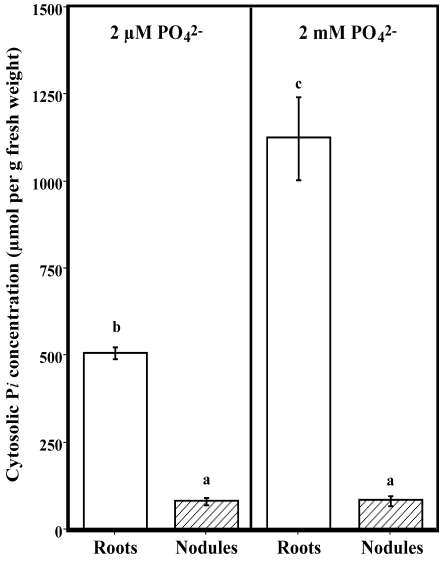

Fig. 4. Calculated cytosolic P*i* concentrations for 7-week-old *Lupinus angustifolius* plants grown hydroponically under either P-deficient (2 µM PO_4^{2}) or P-sufficient (2 mM PO_4^{2}) conditions. Cytosolic P*i* concentration is higher in P-sufficient than in P-deficient roots, but remained constant in nodules. The different letters above the error bars indicate significant differences between treatments determined using analysis of variance (ANOVA) with post-hoc LSD tests. The values represent the means of four replicates and the standard error is less than 5%.

and nodules of symbiotic legume root systems.

2) The regulation of PEPc in roots and nodules during P*i* stress, which also has not been studied in leguminous plants.
3) The role of the two nodular PEPc isoenzymes under P*i* stress. The two forms of the enzymes may have different functions and so it is essential for biotechnological manipulation to target the correct one.

Here we report for the first time the relative P*i* levels and PEPc presence in symbiotic nodules and roots of P-stressed legumes. We have found that PEPc activity is lower in phosphorus-sufficient roots than when phosphorus is deficient (Fig. 2); there were no significant differences between the PEPc activities of P-sufficient and P-deficient nodules (Fig. 2). We therefore postulate that the changes in PEPc activity may arise from nodules experiencing permanent P*i* stress, whereas roots experience P*i* stress only when phosphorus is in low supply in the nutrient solution. It should also be noted that in nodules, the two isoforms of PEPc may have different roles according to the levels of phosphorus availability. The total PEPc activities in nodules do not express the relative contributions of the two isoforms of the enzyme.

We also found that cellular P*i* concentration is higher in P-sufficient than in P-deficient roots (Fig. 3), but that cellular P*i* concentrations in the nodules remained constant even under phosphorus stress. The current study is the first to report levels of metabolically available phosphorus in P-deficient legume root systems. Since cellular P*i* is not an accurate reflection of the metabolic compartment where P*i* stress is experienced, the cytosolic P*i* pools were inferred (Fig. 4). The cytosolic pools of P*i* so determined indicate that the nodules do not experience fluctuating P*i* levels under phosphorus stress whereas the roots do. On the other hand, the cytosolic P*i* pools underwrite the results of the cellular P*i* concentrations, suggesting indirectly that the PEPc alternative route is engaged in roots.

The implication of these findings is that symbiotic nodules may not experience phosphorus stress even when the host roots do, or else that the nodules are able to maintain normal metabolic functions at very low P levels. This may also mean that under phosphorus-limiting conditions, the nodules may behave as aggressive scavengers for the element at the expense of the host root. This corresponds to the suggestion of Al Neimi *et al.*[12] that the bacterial symbiont may compete with the roots for phosphorus. Attempts to control the replenishment of nodular oxaloacetate and malate, under P stress, should therefore take into account the implied different metabolic roles of the PEPc isoforms.

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