Genotypic variation in phosphorus acquisition from sparingly soluble P sources is related to root morphology and root exudates in *Brassica napus*

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Genotypic variations in the adaptive response to low-phosphorus (P) stress and P-uptake efficiency have been widely reported in many crops. We conducted a pot experiment to evaluate the P-acquisition ability of two rapeseed (*Brassica napus*) genotypes supplied with two sparingly soluble sources of P, Al-P and Fe-P. Then, the root morphology, proton concentrations, and carboxylate content were investigated in a solution experiment to examine the genotypic difference in P-acquisition efficiency. Both genotypes produced greater biomass and accumulated more P when supplied with Al-P than when supplied with Fe-P. The P-efficient genotype 102 showed a significantly greater ability to deplete sparingly soluble P from the rhizosphere soil because of its greater biomass and higher P uptake compared with those of the P-inefficient genotype 105. In the solution experiment, the P-efficient genotype under low-P conditions developed dominant root morphological traits, and it showed more intensive rhizosphere acidification because of greater H⁺ efflux, higher H⁺-ATPase activity, and greater exudation of carboxylates than the P-inefficient genotype. Thus, a combination of morphological and physiological mechanisms contributed to the genotypic variation in the utilization of different sparingly soluble P sources in *B. napus*.

*Brassica napus*, sparingly soluble P, genotypic variation, root morphology, H⁺ and carboxylate exudation

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Phosphorus (P) is one of the essential mineral nutrients required for plant growth, but it is one of the most immobile and inaccessible nutrients present in soils [1]. Low availability of P limits plant growth, metabolism, and production all over the world. This is particularly true of soils that are characterized by strong adsorption of PO₄³⁻ onto Ca, Al, and Fe oxides. Root-induced physiological and biochemical modifications of the rhizosphere may be involved in the mobilization and exploitation of sparingly soluble P sources in P-deficient soils. Such modifications include rhizosphere acidification, root exudation of organic anions, modification of root morphology, upregulation of ion transporters, and symbiotic associations with mycorrhizal fungi [2–4]. Genotypic differences in the adaptability to low-P stress and in P-uptake efficiency have been widely reported in many crops [5–7].

Root morphology is a key factor related to P acquisition in plants. The contribution of root morphology to P uptake has been reported for many plant species [8,9]. Rhizosphere acidification may play an important role in mobilizing phosphate from calcium phosphates [10]. Changes in pH in the rhizosphere can arise from protons or bicarbonate ions that are excreted by roots to counterbalance a net excess of cations or anions entering the roots [11,12]. Carboxylate
exudation, which sometimes results in rhizosphere acidification, is also an effective strategy to increase P uptake from sparingly soluble phosphate sources [3,13]. In addition, in some species, the carboxylate composition and concentration in root exudates varies depending on the forms of P sources in the soil [14]. Under low-P conditions, roots of rapeseed can actively extrude protons. This proton extrusion leads to soil acidification in the rhizosphere and the dissolution of sparingly soluble phosphate [3,12,15], probably resulting from increased H^+-ATPase activity in the root plasma membrane [16].

Brassica napus, also known as rapeseed, plays an important role in human nutrition all over the world, especially in China. It is one of the main oil crops with a high nutritional and economic value. Studies on the P-utilization efficiency of rapeseed are critical for the rational application of phosphate fertilizers for agricultural production in low-P soils. Previous studies have shown that, compared to onion phosphate fertilizers for agricultural production in low-P concentrations of sparingly soluble phosphate [3,12,15], probably resulting from increased H^+-ATPase activity in the root plasma membrane [16].

1 Materials and methods

1.1 Plant materials

We used two rapeseed genotypes, 102 (P-efficient) and 105 (P-inefficient), which were screened from 149 recombinant inbred lines (F9) derived from a cross between the P-efficient cultivar 97081 and the P-inefficient cultivar 97009 in low-P soil.

1.2 Pot experiments

We used low-P eluvial soil (20-cm depth) collected from Shizi Mountain on the campus of Huazhong Agricultural University, China. The soil properties were as follows: pH in water of 6.5 (1:2.5 w/w soil to water ratio), organic matter content of 0.6 g kg\(^{-1}\), total nitrogen content of 0.85 g kg\(^{-1}\), total phosphate content of 0.21 g kg\(^{-1}\), and available phosphate content of 1.78 mg kg\(^{-1}\). The pot experiment consisted of four P treatments with four replicates in a greenhouse maintained at 18–26°C and 70%–85% relative humidity (RH). Five kilograms of air-dried and sieved (2 mm) soil was mixed uniformly and divided into two portions: One contained no P (0) and the other was supplemented with 31 mg P kg\(^{-1}\) soil in the form of AlPO\(_4\) (Al-P), FePO\(_4\) (Fe-P), and KH\(_2\)PO\(_4\) (K-P). The soil was placed in black plastic pots lined with polyethylene bags. All other nutrients were dissolved in water and applied to each pot. The compositions of nutrients were as follows (all values in mg kg\(^{-1}\) soil): N, 200; K, 166; Mg, 50; Ca, 140; B, 0.2; and Mo, 0.1. All pots were watered and kept for 2 weeks in the greenhouse to reach a nutrient balance in the soil before sowing. Ten seeds of two genotypes were sown directly in the soil and covered with a small amount of fine soil on the surface of the pots to prevent evaporation. The seedlings were thinned to six plants per pot after 1 week. The pots were watered daily to 70% of field capacity and re-randomized weekly.

Plants were harvested after 45 d growth, and their shoot fresh weights were recorded. Soil samples were taken from the rhizosphere (close to the roots) with a special soil auger and were air-dried to determine pH, available P concentration, and inorganic P fraction. Intact plant roots adhering to the soil were taken from the pots and washed carefully to minimize root damage. The shoot and roots of each plant were dried at 65°C for 72 h, weighed, and then ground to a fine powder in a stainless steel ball mill. The powder was digested in concentrated H\(_2\)SO\(_4\) and HClO\(_4\) for total P determination by the Westernman vanadomolybdate method [19]. The available P in the soil was determined using the Bray and Kurtz [20] method, and Pi fractions (Al-P, Fe-P and Ca-P) were estimated by the Peterson and Corey method [21].

1.3 Solution culture experiment

The solution culture experiment was conducted at Huazhong Agricultural University, China. The seedlings were maintained in a growth chamber under the following controlled climatic conditions: 60%–75% RH, 25/18°C (16/8 h) day/night temperature, and average daytime photosynthetically active radiation of 400 μmol m\(^{-2}\) s\(^{-1}\). The germinated seeds were sown on gauze and grown for 5 d with distilled water only. Uniform seedlings were transferred to 5-L plastic pots containing nutrient solution with the following composition: Ca(NO\(_3\))\(_2\) (4 mmol L\(^{-1}\)), KNO\(_3\) (6 mmol L\(^{-1}\)), KH\(_2\)PO\(_4\) (1 mmol L\(^{-1}\)), MgSO\(_4\) (2 mmol L\(^{-1}\)), H\(_2\)BO\(_3\) (46 μmol L\(^{-1}\)), MnCl\(_2\) (9 μmol L\(^{-1}\)), ZnSO\(_4\) (0.7 μmol L\(^{-1}\)), CuSO\(_4\) (0.3 μmol L\(^{-1}\)), (NH\(_4\))\(_2\)MoO\(_4\)\(_2\) (0.02
μmol L⁻¹), and EDTA-Fe (50 μmol L⁻¹). Three P treatments were used in this experiment: (i) low P (5 μmol L⁻¹ P), (ii) high P (1 mmol L⁻¹ P), and (iii) no P. After 14 d growth in the high-P treatment, the seedlings were transferred to nutrient solution without P. The nutrient solution was refreshed every 3 d, and the pH was adjusted to 5.8 every day.

1.4 Determination and in situ visualization of fluxed H⁺ and H⁺-ATPase activity from roots

At 18 d after transplanting, five seedlings per treatment were carefully removed from the low-P and high-P solutions, and the root morphological parameters were estimated by a root scanner and the WINRhizo optical scanner-based image analysis system. At the same time, the capacity of the roots to release protons and their H⁺-ATPase activity were determined by the methods of M’Sehli et al. [22] and Shen et al. [23], respectively. An in situ visualization technology, as described by Li et al. [24] and Yan et al. [16], was used to estimate the difference in rhizosphere acidification of both genotypes.

1.5 Determination of carboxylates

Carboxylates were collected based on the method described by Ishikawa et al. [25]. At 19 d after transplanting, roots of nine seedlings were washed with deionized water and then immersed in 250 mL of aerated solution with 0.2 mmol L⁻¹ CaCl₂ (pH 5.3) for 6 h before collecting the root exudates of each plant for carboxylate measurement. The collected solution was passed through a cation exchange column containing 4 g cation-exchange resin and then through an anion exchange column containing 2 g anion-exchange anion resin. The carboxylates retained in the anion exchange resins were then eluted with 2 mol L⁻¹ hydrochloric acid. The eluent was concentrated to dryness under reduced pressure at 45°C using a rotary evaporator, and the residue was re-dissolved in 1 mL of ultra-pure water and filtered through a 0.45-μm filter. The carboxylates were detected by HPLC (Agilent 1200, America; equipped with a C18 250×4.6 mm (5 μm) ion-exclusion column, Alltima, America).

1.6 Statistical analyses

Data were analyzed using ANOVA tests with SPSS software. Significance was assigned at P<0.05 with an LSD test. All values represent the mean of three or four replicates±the standard error of the mean.

2 Results

2.1 Plant growth and P uptake in the pot experiment

The shoot and root dry weights of two rapeseed genotypes were greatest when plants were supplied with K-P, as compared with those of plants supplied with Fe-P or Al-P, which were in turn significantly greater than those of plants grown under the no-P treatment (Figure 1). For the two sparingly soluble P treatments, the shoot and root dry weights of the seedlings supplied with Al-P were greater than those of the seedlings supplied with Fe-P, regardless of the genotype. There were significant genotypic differences in shoot and root dry weights under the Al-P treatment, especially in root dry weight, which was 1.6 times greater in the P-efficient genotype 102 than in the P-inefficient genotype 105. There were no significant genotypic differences in shoot and root dry weights under the Fe-P treatment (Figure 1).

Tissue P accumulation was significantly affected by P sources. P accumulation in both the shoots and roots of both genotypes was greatest in plants supplied with K-P, followed by those supplied with Al-P, Fe-P, and no P (Figure 2). Consistent with the dry weight, there was significantly greater P accumulation in both genotypes in the Al-P treatment than in the Fe-P treatment (P<0.05). P uptake by the P-efficient genotype 102 was significantly greater than that by the P-inefficient genotype 105 under the Al-P treatment but not under the Fe-P treatment (P<0.05) (Figure 2).

2.2 Rhizosphere pH

After 45 d growth, soil pH in the rhizosphere of both genotypes supplied with sparingly soluble P sources (Al-P and Fe-P) decreased significantly compared with that of plants supplied with K-P. The shoot and root dry weights of two rapeseed genotypes were supplied with different P sources 45 d after sowing. Error bars represent standard errors of the means of four replicates. Different letters indicate statistical difference at P<0.05 between genotypes among all P treatments.

Figure 1 Shoot and root dry weight of two rapeseed genotypes supplied with different P sources 45 d after sowing. Error bars represent standard errors of the means of four replicates. Different letters indicate statistical difference at P<0.05 between genotypes among all P treatments.
grown under the K-P treatment \((P<0.05)\). The pH of the plant rhizosphere under the no-P treatment did not change during the experiment (Figure 3). There was no genotypic difference in rhizosphere soil pH when plants were supplied with two different sparingly soluble P sources (Figure 3).

### 2.3 Soil-available P and concentration of inorganic P in the rhizosphere

The concentration of available P in the rhizosphere of both genotypes increased progressively from the no-P treatment to the Al-P, Fe-P, and K-P treatments (Table 1). Moreover, the available P concentration in the rhizosphere soil of the P-efficient genotype 102 was lower than that of the P-inefficient genotype 105 under all P treatments, especially when plants were supplied with sparingly soluble P sources \((P<0.05)\) (Table 1).

The concentrations of both Al-P and Fe-P in the rhizosphere soil were higher for both rapeseed genotypes supplied with different P sources after 45 d growth, compared with those in the control treatment (no P) (Table 1). It appeared that Al-P was utilized more easily than Fe-P. There was a significant difference between the two genotypes in the concentration of Al-P, but not Fe-P, in the rhizosphere soil \((P<0.05)\). There was a significant decrease in the concentration of Ca-P in the rhizosphere soil of both genotypes. In addition, there were significant differences between the two genotypes in amount of available P in the rhizosphere of plants supplied with Al-P and Fe-P, but not K-P (Table 1).

### 2.4 Root morphological traits in the solution experiment

There were significant differences in biomass weight and root morphology among the different P treatments and between the two genotypes (Table 2). Both genotypes showed greater shoot biomass under the high-P treatment than under the low-P treatment, while the opposite trend was observed for root biomass. P-stressed plants showed significantly increased total root length, root surface area, and root volume, and significantly more lateral roots compared with plants grown under the high-P treatment \((P<0.05)\). We observed wide genotypic variation in root morphological characteristics between the two genotypes under low-P conditions. The P-efficient genotype 102 developed more

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**Table 1** Soil-available P and inorganic P fraction concentrations in rhizospheres of two rapeseed genotypes supplied with different P sources 45 d after sowing. Different letters indicate statistical difference at \(P<0.05\) between genotypes in each P treatment \((n=4)\)

| Genotype | P source | Available P (mg P kg\(^{-1}\) soil) | Inorganic P fractions (mg P kg\(^{-1}\) soil) | Al-P | Fe-P | Ca-P |
|----------|----------|-----------------------------------|-----------------------------------------------|------|------|------|
|          |          |                                   |                                               |      |      |      |
| 102      | No-P     | 3.74 d                            |                                               | 2.42 d | 62.2 d  | 14.2 b |
|          | Fe-P     | 4.75 bc                           |                                               | 3.98 cd | 80.0 ab  | 8.2 f  |
|          | Al-P     | 4.18 cd                           |                                               | 4.36 c  | 76.8 bc  | 11.0 d  |
|          | K-P      | 7.36 a                            |                                               | 6.54 ab | 74.8 c   | 15.0 ab |
| 105      | No-P     | 4.23 cd                           |                                               | 2.48 d  | 64.2 d   | 15.3 a  |
|          | Fe-P     | 6.77 a                            |                                               | 6.12 b  | 83.5 a   | 9.9 e   |
|          | Al-P     | 5.51 b                            |                                               | 7.63 a  | 78.7 b   | 12.1 c  |
|          | K-P      | 7.59 a                            |                                               | 6.85 ab | 74.0 c   | 15.5 a  |
dominant root morphological traits than the P-inefficient genotype 105 ($P<0.05$) (Table 2).

### 2.5 Flux of $H^+$ from roots, $H^+$-ATPase activity in roots, and rhizosphere acidification

Under the high-P treatment, roots of both genotypes showed little proton flux, and there was no significant difference between the genotypes (Figure 4A). However, P deficiency resulted in increased proton flux from the roots, and the P-efficient genotype 102 showed greater proton flux than the P-inefficient genotype 105 (Figure 4A). In addition, under low-P conditions, $H^+$-ATPase activity was significantly increased in roots of genotype 102 but not genotype 105. Overall, the activity of $H^+$-ATPase in the roots was greater in the P-efficient genotype 102 than in the P-inefficient genotype 105 (Figure 4B).

For the two genotypes, the acidification of the rhizosphere under low-P or high-P conditions was visible as color changes in the agar gel on the 18th day after transfer (Figure 5). Under high-P conditions, the color of the agar gel in the rhizosphere was almost unchanged for both genotypes (Figure 5A and B). However, the agar gel became yellow under low-P conditions (Figure 5C and D), reflecting acidification of the rhizosphere under P-deficient conditions. The deeper yellow color of the agar gel indicated that the acidification of the rhizosphere was stronger for the P-efficient genotype 102 than the P-inefficient genotype 105.

### Table 2 Dry weights and root morphological parameters of P-efficient genotype 102 and P-inefficient genotype 105 after 18 d growth in low-P (5 $\mu$mol L$^{-1}$ P) or high-P (1 mmol L$^{-1}$ P) nutrient solutions. Different letters indicate statistical significance at $P<0.05$ ($n=3$)

| Treatment | Genotype | Shoot dry weight (mg) | Root dry weight (mg) | Total length (cm) | Surface area (cm$^2$) | Root volume (cm$^3$) | Number of lateral root |
|-----------|----------|-----------------------|----------------------|-------------------|------------------------|----------------------|----------------------|
| High P    | 102      | 66.9 a                | 7.6 a                | 214 b             | 12.4 b                 | 0.058 c              | 1376 b               |
|           | 105      | 38.6 bc               | 3.5 b                | 140 d             | 6.3 c                  | 0.030 d              | 834 c                |
| Low P     | 102      | 48.0 b                | 8.3 a                | 291 a             | 18.4 a                 | 0.095 a              | 1725 a               |
|           | 105      | 23.1 c                | 4.9 b                | 168 c             | 12.4 b                 | 0.076 b              | 1270 b               |

**Figure 4** Flux of $H^+$ and $H^+$-ATPase activity in roots of two genotypes of rapeseed grown in low-P (5 $\mu$mol L$^{-1}$ P) and high-P (1 mmol L$^{-1}$ P) nutrition solutions during the treatment period (18 d). Values are the means of three replicates±SD at $P<0.05$. Different letters indicate statistical difference at $P<0.05$ between genotypes and P treatments.

**Figure 5** Visualization of rhizosphere acidification of two rapeseed genotypes 18 days after transfer into low-P (5 $\mu$mol L$^{-1}$ P) and high-P (1 mmol L$^{-1}$ P) treatments. High P-treatment of genotypes 102 (A) and 105 (B), and low-P treatment of genotypes 102 (C) and 105 (D). Roots were imbedded for 5 h in agar gel containing a pH indicator (bromocresol purple) and K$_2$SO$_4$ (2.5 mmol L$^{-1}$)/CaSO$_4$ (1 mmol L$^{-1}$) without a P supply. Yellow indicates acidification. Changes in pH are indicated by color changes.
2.6 Root exudation of carboxylates

We quantified tartrate, malate, acetate, citrate, and succinate in the root exudates of two genotypes (Figure 6). Large amounts of carboxylates were exuded from the roots of the two genotypes under low-P and no-P treatments. Under high P conditions, only small amounts of carboxylates were exuded by the plants, and there were no significant differences between the genotypes. In contrast, in the no-P solution, both genotypes released large amounts of carboxylates (Figure 6A). Malic and acetic acids were the predominant carboxylates exuded by the P-stressed genotypes (Figure 6B and C). There was no significant difference in the amount of total carboxylates released between the low-P and no-P treatments, despite the lower concentrations of malate and citrate released by roots under the low-P treatment compared with those under the no-P treatment. The P-efficient genotype 102 released a significantly larger amount of total carboxylates than the P-inefficient genotype 105 under the no-P treatment but not under the low-P treatment (Figure 6A).

3 Discussion

Low P availability has seriously limited plant productivity in acidic soils. However, it has been reported that there is wide genotypic variation in the utilization of sparingly soluble P among plant species and genotypes [26–28]. The results obtained in the present study show that genotype 102 was more P-efficient because it was able to produce up to 59% and 27% of its maximum dry weight when supplied with Al-P and Fe-P, respectively, whereas genotype 105 produced only 49% and 25%, respectively, of its maximum dry weight under the same conditions (Figure 1). This result supports our previous findings that genotype 102 had a higher rate of P uptake and growth in soils with low P availability [18]. These results suggest that there are different physiological or biological mechanisms between genotypes that contribute to the utilization of sparingly soluble P. These findings were further confirmed by the fact that genotype 102 could mobilize and uptake more P from the rhizosphere soil than genotype 105, as demonstrated by analysis of the biomass production, P uptake, and inorganic P fractions in the rhizosphere soil of both genotypes (Figure 2, Table 1).

Figure 6 Genotypic variation in organic acid exudation of plants grown in low-P (5 μmol L⁻¹ P), no-P (without P) and high-P (1 mmol L⁻¹ P) nutrient solutions during the 19-day treatment period. Values are means of three replicates±SD at $P<0.05$. Different letters indicate statistical difference at $P<0.05$ between genotypes and among P treatments.
It is noteworthy that both genotypes were better able to utilize P from Al-P than from Fe-P, producing greater dry matter and accumulating higher levels of P in the tissues (Figures 1 and 2). We believe that there may be two reasons for this: First, Al-P is more soluble than Fe-P. Similar results were reported in other studies, which suggested that because of its poor solubility, Fe-P was less available to a wide range of crops than either Ca-P or Al-P [29,30]. Second, the higher Al concentration resulting from soil acidification under the Al-P treatment might stimulate the release of organic acid anions and indirectly supplement the Al-P nutrition compared to the Fe-P treatment [31,32]. As has been reported previously [33–35], in the presence of external Al, Al-resistant species or genotypes exude a number of organic anions including citrate, malate, and oxalate, and the exudation of citrate might contribute to the detoxification of Al and to the increased phosphate availability in the rhizosphere in rapeseed [36].

In the present study, the P-stressed genotypes released more H⁺ and acidified their rhizosphere more intensely than those under high-P conditions (Figures 4A and 5). This may be explained by the increased activity of H⁺-ATPase in the roots of both genotypes under the low-P treatment (Figure 4B). This observation is consistent with results from Yan et al. [16], who suggested that the enhanced activity of the plasma membrane H⁺-ATPase in cluster roots of white lupin under P-stressed conditions might be responsible for the increased H⁺ release. Rhizosphere acidification induced by P-stress can increase the availability of rock phosphate in neutral and alkaline calcareous soil [37,38]. Our results indicate that the Ca-P in the rhizosphere soil was depleted significantly when Al-P and Fe-P, but not K-P, were added. In addition, the soil pH for both genotypes decreased significantly when Al-P and Fe-P, but not K-P, were added. These changes allowed genotype 102 to increase P acquisition in rapeseed compared to that of the P-inefficient genotype 105 (Table 1).

In P-deficient conditions, plants develop more lateral roots, and show increases in root length, root surface area, and root volume [45,46]. These changes significantly alter the root architecture and morphology. In this study, P-stressed plants showed significantly increased total root length, root surface area, and root volume, and developed more lateral roots compared with those grown under the high-P treatment (Table 2). The results are similar to those observed in previous studies as mentioned above. A larger root system is critical for P uptake by plants because P is relatively unavailable and immobile in many soils [47,48]. Root length, surface area, and volume are particularly important morphological characteristics for P acquisition, which requires the expansion of the rhizosphere to enhance uptake [49–51]. The present results show that the P-efficient genotype 102 developed more dominant root morphological traits than did the P-inefficient genotype 105 (Table 2). These changes allowed genotype 102 to increase P acquisition from the rhizosphere because of greater root-soil contact.

In some studies, the root length, surface area, and root exudations per unit root biomass were compared to show differences in P-efficiency among genotypes. A more developed root system and/or greater exudation of carboxylates per unit root biomass may contribute greatly to the P-use efficiency of plants. In the present study, there was little difference between the two cultivars in terms of carboxylates exudation per unit root biomass (data not shown). However, from the view point of practical production, P efficiency of plants in low-P soil is primarily related to total root exudation and dominant root morphology, which are more significant for P uptake from soil, and consequently, for plant yield. Our results show that the total root length, surface area, and root secretions were much higher for the P-efficient genotype 102 than for the P-inefficient genotype 105. These root characteristics may activate more of the sparingly soluble P in soils and indirectly stimulate plant
growth.

The biomass and P uptake of plants grown in P-deficient soil are directly related to the ability of the plant to acquire P from sparingly soluble phosphates. In the present study, we found that there was significantly higher biomass dry weight and P accumulation for both genotypes under Al-P than under Fe-P treatment. Greater P uptake was also observed for the P-efficient genotype 102 than for the P-inefficient genotype 105 under Al-P treatment but not Fe-P treatment (P<0.05) (Figure 2). Therefore, we presume, as described by Pearse et al. [52], that greater ability of plants to access one form of sparingly soluble phosphate does not mean that they can efficiently utilize all forms of sparingly soluble P, and the ability to access different forms of P sources by plants may depend on different morphological and physiological mechanisms. In addition, as observed in the pot experiment, the sparingly soluble inorganic phosphate supplied to each treatment was not the sole P source. Organic phosphate in the soil can also be utilized after being mineralized by root-secreted phosphatases and microbes in the rhizosphere. Therefore, a combination of morphological and physiological mechanisms, including changes in root morphology and root exudates, can explain the genotypic differences in the utilization of different sparingly soluble P sources.

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