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Improvement of Phosphate Solubilization and Medicago Plant Yield by an Indole-3-Acetic Acid-Overproducing Strain of Sinorhizobium meliloti†‡

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Nitrogen (N) and phosphorus (P) are the most limiting factors for plant growth. Some microorganisms improve the uptake and availability of N and P, minimizing chemical fertilizer dependence. It has been published that the RD64 strain, a Sinorhizobium meliloti 1021 strain engineered to overproduce indole-3-acetic acid (IAA), showed improved nitrogen fixation ability compared to the wild-type 1021 strain. Here, we present data showing that RD64 is also highly effective in mobilizing P from insoluble sources, such as phosphate rock (PR). Under P-limiting conditions, the higher level of P-mobilizing activity of RD64 than of the 1021 wild-type strain is connected with the upregulation of genes coding for the high-affinity P transport system, the induction of acid phosphatase activity, and the increased secretion into the growth medium of malic, succinic, and fumaric acids. Medicago truncatula plants nodulated by RD64 (Mt-RD64), when grown under P-deficient conditions, released larger amounts of another P-solubilizing organic acid, 2-hydroxyglutaric acid, than plants nodulated by the wild-type strain (Mt-1021). It has already been shown that Mt-RD64 plants exhibited higher levels of dry-weight production than Mt-1021 plants. Here, we also report that P-starved Mt-RD64 plants show significant increases in both shoot and root fresh weights when compared to P-starved Mt-1021 plants. We discuss how, in a Rhizobium-legume model system, a balanced interplay of different factors linked to bacterial IAA overproduction rather than IAA production per se stimulates plant growth under stressful environmental conditions and, in particular, under P starvation.

Compared with the other major nutrients, such as nitrogen, phosphorus (P) is by far the least mobile and available to plants under most soil conditions. Although P is abundant in soils in both organic and inorganic forms, it is frequently a major or even the prime limiting factor for plant growth. Many soils throughout the world are P deficient, because the free concentration (the form available to the plant), even in fertile soils, is generally low due to high reactivity of soluble P with calcium, iron, or aluminum that leads to P precipitation (36, 41). In addition, in developing countries, chemical fertilizers, which provide the three major plant nutrients (N, P, and potassium), are not widely used, due to cost limitations. In these areas, the direct application of ground phosphate rock (PR) is increasingly used, even if the level of P released from PR is often too low for crop growth (9, 38). It is known that many microorganisms, in particular those of the genera Pseudomonas, Bacillus, and Rhizobium, have the ability to change their metabolism in response to the phosphorus available for cellular growth. The switch in metabolism is mediated through the repression and induction of various genes whose products are involved in processes ranging from uptake and acquisition of P sources to de novo synthesis of new cellular components (18, 36). Furthermore, in vitro studies showed that for some of these bacteria, the P-solubilizing activity and the production of the auxin indole-3-acetic acid (IAA) were coexpressed (17, 39), although a direct correlation linking IAA production to P solubilization was not demonstrated.

P uptake in various microorganisms has been investigated. Many bacterial species, including Sinorhizobium meliloti, have at least two P transport systems, consistent with the high- and low-affinity transport systems. The high-affinity system is encoded by the phoDET operon, and the low-affinity system is encoded by pit (in the orfA-pit operon). In S. meliloti, the expression of genes encoding both P transport systems is controlled by the PhoB activator. Under P excess conditions, PhoB is inactive, and the phoDET genes are not expressed. Under P-limiting conditions, the low-affinity Pit permease system is repressed by activated PhoB, while the high-affinity PhoCDET system is induced and becomes the primary mechanism of P transport (10). Many bacterial strains contain PstSCAB homologs that function as high-affinity phosphate transporters. For S. meliloti 1021, a 1-bp deletion in the pscC open reading frame (ORF) is probably responsible (via PhoB) for the moderate constitutive activation of 12 phosphate starvation-inducible genes, observed in the absence of phosphate stress (24, 43).

In both plants and microorganisms, the primary mechanisms of PR solubilization are H⁺ excretion, organic acid production, and acid phosphatase biosynthesis (2, 3). Organic acids, including acetate, lactate, malate, oxalate, succinate, citrate, gluconate, ketogluconate, etc., can form complexes with the iron or aluminum in ferric and aluminum phosphates, thus releasing plant-available phosphate into the soil (18, 22). Organic acids may also increase P availability by blocking P absorption sites.
on soil particles or by forming complexes with cations on the soil mineral surface (36).

Mineralization of most organic phosphorus compounds is carried out by means of phosphatase enzymes. The major source of these enzymes in soil is considered to be of microbial origin. In particular, phosphatase activity is substantially increased in the rhizosphere. The pHs of most soils range from acid to neutral values. Thus, acid phosphatases should play the major role in this process (36).

In the present study, the P-solubilizing ability of an *S. meliloti* 1021 strain, RD64, and its effect on the growth of a *Medicago* host plant were analyzed. We used the *S. meliloti-Medicago truncatula* system since the microarrays were available for the bacterium and *Medicago* is a well-recognized model system for indeterminate nodule development. The RD64 strain has previously been engineered to overproduce IAA (11, 35), showing that this strain is able to release into liquid growth medium up to 78-fold more IAA than wild-type 1021 (12, 21). It was also previously reported that, as found for IAA-treated *Escherichia coli* cells (7), RD64 is more resistant to salinity and other abiotic stresses than 1021 (5). Medicago plants nodulated by this strain have a higher degree of protection against oxidative damage induced by salt stress than 1021-nodulated plants (5).

It was previously shown that IAA triggers induction of tri-carboxylic acid (TCA) cycle enzymes in quite-distant systems, such as transformed human cells (15), *E. coli* (8) and *S. meliloti* (21), with a mechanism not yet understood. To evaluate the global effects triggered by IAA overproduction in *S. meliloti* RD64, the gene expression pattern of wild-type 1021 was compared with those of RD64 and 1021 treated with IAA and four other chemically or functionally related molecules by microarray analysis.

Among the genes differentially expressed in RD64 and IAA-treated 1021 cells, we found two genes of the *pho* operon: *phoT*, coding for the phosphate uptake ABC transporter permease protein, and *phoC*, coding for the phosphate uptake ABC transporter ATP binding protein. This unexpected finding led us to examine the mechanisms for mineral P solubilization in RD64 and the potential ability of this strain to improve *Medicago* growth under P-starved conditions. Increases in acid phosphatase activity and organic acid excretion were observed for the RD64 strain under free-living conditions. Furthermore, the amount of organic acids exuded from the roots of *Medicago* plants nodulated by this strain was larger than that measured for plants nodulated by the 1021 wild-type strain. This effect was connected to the enhanced P solubilization and plant dry weight production observed for these plants.

**MATERIALS AND METHODS**

**Bacterial growth conditions.** The *S. meliloti* wild-type 1021 strain and the IAA-overproducing RD64 strain, differing from wild-type 1021 only in the presence of the p-saaMms2 construct, were as previously described (12, 21). Standard mannitol minimal medium for *Rhizobium* (RMM) (19) was modified to contain 1% (wt/vol) mannitol as a carbon source, 1% (wt/vol) ammonium chloride, 10 mM morpholine propanesulfonic acid (MOPS, pH 7.0) to buffer and P (KH₂PO₄) added to give a final concentration of 1 mM (P starvation) or 13 mM (P sufficiency). Antibiotics were included as required (5).

For starvation experiments, cells of 1021 wild-type and RD64 strains were grown at 30°C to mid-exponential phase (optical density at 600 nm [OD₆₀₀] = 0.6) in RMM broth containing 1% (wt/vol) mannitol as a carbon source and 13 mM P, washed with RMM containing no P, resuspended in the same medium, and then divided into three cultures. No P (P₀ cells), 1.0 mM P (P-starved cells), or 13 mM P (P⁺ cells) was added into the three cultures. The P-starved and P⁺ 1021 wild-type cells were treated for 3 h with 0.5 mM IAA. To test the specificity of IAA effects, four other selected compounds, indole (Ind), tryptophan (Trp), indole-3-carboxylic acid (ICA), and 2,4-dichlorophenoxyacetic acid (2,4-D), whose acidities cover a range from the weak acid (pH 5.9) to the weak acid (pH 6.1), were dissolved in 50% (vol/vol) ethanol and added to P-starved and P⁺ 1021 wild-type cells to give a final concentration of 0.5 mM. The introduced IAA biosynthetic genes in RD64 use Trp to produce IAA, thus possibly incurring tryptophan limitation. To avoid Trp limitation, RD64 cells were also treated with 0.5 mM Trp and used for microarrays and reverse transcription-PCR (RT-PCR) analyses. We found that the expression of *pho* genes was unaffected or slightly reduced compared to that observed for untreated RD64 cells (data not shown). Finally, to avoid solvent interference, control cells were treated with a similar amount of ethanol solution. After 3 h of each treatment, cell batches were collected, frozen, and stored at −80°C for use in experiments.

For phosphate solubilization experiments, 5% Moroccan phosphate rock (PR) (Sigma-Aldrich) was used as a P source. At least five independent experiments were always performed.

**Microarray analysis.** Previously described methods were used to compare the gene expression patterns of untreated 1021 cells (control) with those of RD64, 1021-IAA, 1021-Ind, 1021-ICA, and 1021-2,4-D cells grown under P-sufficient conditions as reported in Imperlini et al. (21). RT-PCR analysis. Total RNA from P⁺ and P-starved cells was isolated as previously described (5). cDNA was synthesized with the Stratascript reverse transcription reagents (Stratagene) and random hexamers as primers. Quantitative RT-PCR was performed with Power SYBR PCR master mix (Applied Biosystems). Reactions were run on an iCycler iQ (Bio-Rad). The thermocycling conditions were as follows: 15 min at 95°C, 40 cycles of denaturation at 95°C for 20 s and annealing for 20 s, and extension for 35 s at 72°C. The specific primer pairs, designed using the Primer3 software program, were as follows: for *phoB*, 5'-TTACGTCGTCAAGCCTCTT-3' and 5'-CCGTTGGAACGATAATATG-3'; for *phoC*, 5'-ACCTTTGAGGACGCTCACATG-3' and 5'-ACCTTTGAGGACGCTCACCTGC-3'; for *phoE*, 5'-GTCCTCATCCGTGTTCTTCCTC-3' and 5'-AGACCTTCTTGCCTTGCCTTAT-3'; for *phoT*, 5'-TGGCCCTGTTCTTCCTACGTA-3' and 5'-GTCTCTCTTTCGAGCTGACG-3'; for *smc00261*, 5'-CGAAGGTTGATGACGAAATGA-3' and 5'-ACCGACTTCTTCCGACAGAT-3'; and for *smc00128*, 5'-CTTCAAAGTGAGTAACAGAAGA-3' and 5'-AAAGAACCGCCCTAACCTTCTC-3'. smc00261 and smc00128 were used as housekeeping genes for data normalization using the comparative threshold cycle (*Ct*) method as previously described (8).

**Phosphatase activity and phosphate solubilization.** Alkaline and acid phosphatase enzymes under P-limiting conditions were assayed as previously reported (16). Units are reported as nanomoles per minute per milligram of protein. Protein concentrations were determined by the Bradford assay. 

The concentration of soluble phosphate was estimated using a modification of the Fiske-Subbarow method as described by Saheki et al. (37).

**Phosphate solubilization experiments.** Bacterium-free RMM broth containing 1 mM CaHPO₄ and 1.1 mM K₂HPO₄, respectively. These plants received, only during the first week, 0.02% PR. For collection of exudates, the roots of 4-week-old plants were washed, submerged in sterile water, and kept in a growth chamber for 48 h. Exudates were evaporated to dryness and analyzed by high-pressure liquid chromatography (HPLC). The identity of peaks was confirmed by gas chromatography-mass spectrometry (GC-MS).

**Plant growth conditions.** Seeds of *Medicago truncatula* cv. Jemalong 2HA were surface sterilized, germinated, and transferred into hydroponic units as previously reported (5). P-limiting conditions were achieved by providing a modified Jensen medium containing 1 mM CaCO₃ and 1.1 mM KCl instead of 7.3 mM CaHPO₄ and 1.1 mM K₂HPO₄, respectively. These plants received, only during the first week, 0.02% PR. For collection of exudates, the roots of 4-week-old plants were washed, submerged in sterile water, and kept in a growth chamber for 48 h. Exudates were evaporated to dryness and analyzed by high-pressure liquid chromatography (HPLC). The identity of peaks was confirmed by gas chromatography-mass spectrometry (GC-MS).

**Organic acids and phosphate release.** On the basis of the results obtained in the analysis of organic acid production in culture supernatant, malic acid (MA), succinic acid (SU), fumaric acid (FU), and 2-hydroxyglutaric acid (2HG) were added in modified bacterium-free RMM, and soluble phosphate concentration was measured. For 1021 growth-simulating conditions, 1.4 mg/liter FU, 500 mg/liter MA, and 1 g/liter SU were added. For 1021-IAA growth-simulating conditions, 16 mg/liter FU, 860 mg/liter MA, and 860 mg/liter SU were added. For RD64 growth-simulating conditions, 5.6 mg/liter FU, 840 mg/liter MA, and 3.1 g/liter SU were added. For conditions simulating growth of *M. truncatula* plants nodulated by 1021 (MD-60), and MD-60 + IAA were added at final concentrations of 49.6 mg/liter and 115.2 mg/liter, respectively. Bacterium-free medium was also treated with 0.5 mM IAA solution.

**Analysis of organic acids using HPLC and GC-MS.** The organic acids were determined by HPLC with a reverse-phase Hypersil GOLD C₁₈ (100- by 4.6-mm)
RESULTS

Regulation of \( \text{pho} \) operon genes. We have evaluated, under P-sufficient conditions, the global effects triggered by IAA overproduction in \( S. \text{meliloti} \) cells by use of a transcriptional profiling approach. We compared the gene expression patterns of wild-type 1021 with those of RD64 and 1021 treated with IAA (1021-IAA). To verify the specificity of IAA effects, we conducted at a different time.

Microarray data accession number. Microarray data have been deposited in the Gene Expression Omnibus (GEO) database under accession number GSE21745.

Organic acid production and P solubilization. To evaluate the ability of RD64 to solubilize inorganic P when PR is used as the sole P source, the amount of P released into culture medium was measured. We show that the soluble P concentrations increased over time, reaching near-maximal levels within 6 days (Fig. 3). Interestingly, the measured P concentrations of RD64 and 1021-IAA in the growth medium were up to 80% higher than those found for untreated 1021 cells. In contrast, the concentration of soluble P was negligible in bacteria-free medium with or without the addition of IAA.

In order to study the relationship between phosphate solubilization and the production of organic acids, culture supernatants were filtered and analyzed by HPLC. Three of the major peaks (see Fig. 2A in the supplemental material) were identified as malic, succinic, and fumaric acids. The identification of these organic acids was confirmed by GC-MS. The concentrations of these acids were higher in both RD64 and 1021-IAA cells than in control cells, with the highest increment observed for succinic acid (Table 1).

We compared the amounts of soluble P released into bacteria-free medium upon external addition of malic, succinic, and fumaric acids. To simulate the growth of \( S. \text{meliloti} \) cells,
the organic acids were added into bacterium-free medium at the same relative ratios found in bacterial cultures. We found that P solubilization was more effective when the levels of added organic acids were comparable to those measured during the growth of RD64 and 1021-IAA cells than when the levels were comparable to those obtained with untreated 1021 cells (Fig. 4A). However, the level of P released under these conditions (purified acid addition to the liquid medium) was lower than that released from bacterial cultures.

TABLE 1. Organic acids exuded by *S. meliloti* 1021, 1021-IAA, and RD64 cells grown on minimal medium containing 5% PR as the sole phosphate source

<table>
<thead>
<tr>
<th>Sample</th>
<th>Organic acid content (mg/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Malate</td>
</tr>
<tr>
<td>1021</td>
<td>50 ± 5</td>
</tr>
<tr>
<td>1021-IAA</td>
<td>86 ± 10</td>
</tr>
<tr>
<td>RD64</td>
<td>84 ± 8</td>
</tr>
</tbody>
</table>

The IAA concentration added for 1021-IAA was 0.5 mM. All strains were grown in 1% mannitol RMM. The values reported are the means ± SD of results from at least five biological experiments, conducted at different times. All averages differ significantly according to Tukey’s test (*P* < 0.001).
Mt-1021 and Mt-RD64 plant growth. To evaluate the ability of *S. meliloti* to support plant growth under P-starved conditions, *Mt*-1021 and *Mt*-RD64 plants were grown under low-P (0.02% PR as P source) and high-P conditions, and fresh and dry weights were evaluated after 4 weeks of growth. When P-sufficient conditions were used, a significant improvement of *Mt*-RD64 plant growth was observed compared to the level for *Mt*-1021 plants. We show that for these plants, enhanced biomass productions of the aerial part (Tukey’s test; *P* < 0.001) and of the whole root apparatus (Tukey’s test; *P* < 0.001) were observed (Fig. 5).

We observed reductions of shoot and root fresh weights in *Mt*-1021 plants grown under P-starved conditions (with PR as the P source) compared to the level for *Mt*-1021 plants, while for *Mt*-RD64 plants the difference was statistically significant only for the shoot fresh weight (Tukey’s test; *P* < 0.001) and of the whole root apparatus (Tukey’s test; *P* < 0.001) were observed (Fig. 5).

DISCUSSION

It has previously been shown that, compared to 1021 cells, RD64 cells (i) release larger amounts of IAA, increase nitrogen fixation, and trigger the accumulation of storage compounds as poly-β-hydroxybutyrate (PHB) and starch (21), (ii) exhibit improved resistance against stress conditions (5), and (iii) show enhanced long-term cell survival (13). Moreover, *Mt*-RD64 plants show better root nodule development (21) and salt tolerance (5) than *Mt*-1021 plants and are able to release larger amounts of IAA, increase nitrogen fixation, and trigger the accumulation of storage compounds as poly-β-hydroxybutyrate (PHB) and starch (21), (ii) exhibit improved resistance against stress conditions (5), and (iii) show enhanced long-term cell survival (13). Moreover, *Mt*-RD64 plants show better root nodule development (21) and salt tolerance (5) than *Mt*-1021 plants and are able to
attenuate the local IAA imbalance by increasing the transcription of genes involved in the synthesis of cytokinins (6), considered important signals for nodule formation (31, 32, 34). We show here that under P-stressed conditions, RD64 improved mineral phosphate solubilization compared to the wild-type 1021 strain. The expression levels of the phoB activator gene and all genes in the phoCDDET regulon were induced in RD64 compared to the level for 1021, thus suggesting that the P transport rate increased via the high-affinity transport system. Similar results were obtained when 1021 cells were treated with IAA. This effect seems to be specific to the presence of IAA since the treatment with structurally or functionally similar molecules did not lead to significant alterations in gene expression. Considering that in S. meliloti 1021, a moderate level of activated PhoB is present even under phosphate sufficiency, we think that the degree of induction observed for these genes might be greater in S. meliloti strains lacking mutations in the pstC gene.

RD64 cells also showed higher levels of acid phosphatase enzymes, which facilitate the hydrolysis of organic P esters, and released larger amounts of organic acids, known to be highly effective in mobilizing P from insoluble sources (17, 23), than untreated 1021 cells. Similar results were obtained when 1021 cells were treated with IAA. Under P-starved conditions, a higher level of biomass accumulation was observed for Mt RD64 plants than for Mt-1021 plants. We think that this effect is linked to the release of a larger amount of 2-hydroxyglutaric acid exuded from the roots of Mt-RD64 plants and to the modifications of important root architecture traits, such as root branching, observed for these plants. Indeed, it is already reported that the ability of plants to use insoluble P compounds can be significantly enhanced by engineering plants to produce more organic acids (27) and that IAA plays an important role in root system architecture adjustment during P deprivation in Arabidopsis and other plant species (25, 26, 29, 33).

In the present work, we speculate that the upregulation of TCA cycle enzymes in RD64 cells (21) leads to the excretion of larger amounts of malic, succinic, and fumaric acids, three intermediates of the TCA cycle, resulting in positive effects in both P-sufficient and P-limiting conditions. The enhanced metabolic activity and the correlated production of more carboxylates in RD64 cells might also occur in bacteroids inside root nodules. Carboxylates accumulated inside nodules of Mt-RD64 plants might be exuded from the root into the rhizosphere (as we found for 2-hydroxyglutaric acid, a derivative of the TCA cycle intermediate 2-ketogluartaric acid), increasing the availability of P to plants. Further investigations are required to verify these hypotheses and therefore to determine the complex mechanism by which IAA promotes greater organic acid secretion.

Both free-living RD64 cells and Mt-RD64 plants are able to better overcome different stressful environmental conditions, including P starvation, than untreated 1021 cells and Mt-1021 plants (14). We thus speculate that rhizobia able to overproduce IAA might be selected in order to increase plant yield in extreme environments. Such an application would be particularly interesting in regions where high salinity is a substantial constraint to crop production, PR deposits are widespread but levels of soluble P are low, and the use of chemical N fertilizers, which strongly inhibited the symbiotic relationship between rhizobia and legumes, is reduced due to their cost. Finally, the combination of higher IAA release levels, P solubilization, and improved N fixation could make the Mt-RD64 system a good candidate for legume-cereal intercropping.

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