Microbial community structure in the rhizosphere of tobacco plants engineered to release phytase from their roots

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ABSTRACT

Transgenic plants that release microbial phytase from their roots have been developed and offer potential for improving the utilisation of inositol phosphates, which are the major form of organic P found in most soils. However, the efficacy of interaction between enzyme and substrate in the rhizosphere is dependent on various microbial, biochemical and physical interactions. In this study, the effect of the presence of soil microorganisms on P-accumulation by transgenic tobacco plants that express a fungal phytase (along with control plants) was investigated in a P-deficient soil following various treatments designed to perturb the soil microbial population. The structure of microbial communities within the rhizosphere was assessed using a TRFLP-based approach on amplified 16S rDNA genes. Whilst an interaction between the presence of soil microorganisms and plant P nutrition was evident, expression of the fungal phytase in the transgenic lines had little or no impact on the structure of the microbial community. Although soil microorganisms have an implicit role in the availability of P to plants, the community around roots appears to be resilient to the impact of single-gene changes that are designed to specifically modify the biochemistry of the rhizosphere in relation to mechanisms associated with nutrient cycling.
KEYWORDS: bacteria, fungi, inositol phosphate, phytase, transgenic plants

INTRODUCTION

In order to give plants access to P present in soil as phytate, transgenic plants (*Arabidopsis thaliana, Nicotiana tabacum* L., *Trifolium subterraneum* L.) that express phytase genes from soil microorganisms (eg *Aspergillus* sp) have been developed and characterized (Richardson et al., 2001; George et al., 2004; 2005). These plants exude heterologous phytase into the rhizosphere and, when grown in controlled environments (e.g., sterile and non-sorbing media such as agar), can accumulate significantly more P than controls when supplied solely with phytate (Richardson et al., 2001; George et al., 2004; 2005a). However, the benefit of exuding phytase from roots is compromised in soil environments, where transgenic plants achieved only small (up to 20%) and inconsistent increases in P accumulation (George et al. 2004). A possible reason for the relatively poor capacity of transgenic plants to acquire P from phytate in soil is the presence of microorganisms (phytase exuding or otherwise) which may compensate for the lack of phytase exuded by wild-type plants or out compete the plant for released P. Soil microorganisms are involved in many soil functions (Dunfield and Germida, 2001) including the cycling of nutrients. More specifically, soil microorganisms are considered to be critical to the mobilisation and cycling of P within the rhizosphere. For this and other reasons the impact of transgenic plants on the presence of individual species of microorganisms, or the biodiversity and functionality of the soil microbial biomass itself, has been of some interest (Bruisnma et al. 2003). Most studies looking at interactions between microorganisms and transgenic plants have focussed on the impact of plants modified to produce herbicide tolerance or resistance to insect pests or plant pathogens (Kowalchuk et al. 2003) and not those which directly alter rhizosphere biochemistry, such as transgenic plants which exude phytase. Here we investigate the impact of rhizosphere microorganisms on the P nutrition of plants and whether direct manipulation of rhizosphere biochemistry through genetic engineering has any impact on microbial community structure.

METHODS

Transformation of *Nicotiana tabacum*

N. tabacum plants were independently transformed with phytase genes (*phyA*) from *Aspergillus niger* (An) and *Peniophora lycii* (Pl) using Agrobacterium-mediated transformation. Primary transformant calli were selected on kanamycin (100 μg mL^{-1}) and verified by Southern blot. Vector control plants, which were transformed with the empty transformation cassette were also generated. A large number (~75) of independent lines of both transgenic and vector controls were initially selected. Transformed lines and vector control plants for subsequent experimentation were further selected from segregating T1 seedlings

Growth and P uptake by plants in amended soil

Soil was collected from Tentsmuir Point, Fife, UK and was identified as a Spodosol (USDA Soil Taxonomy). Soils were air-dried, mixed and passed through a 2-mm sieve and either left unamended (live soil) or amended by the addition of inorganic P to levels sufficient for unlimited plant growth, sterilization by γ-irradiation and the sterilized soil re-inoculated either with a soil-wash or with 5% (w/w) live soil. Five replicate pots containing 450g (weight at 80% field capacity) of each soil treatment were sown with three tobacco seedlings of each plant line separately and maintained at ~80% field capacity during growth. All nutrients, except P, were supplied weekly by addition of 5 mL of nutrient solution. Plants were grown in a randomized design in a glasshouse, between 14 and 22°C with an approximate daylight length of 16h. Shoots were harvested after 28 days growth and biomass determined after oven drying at 65°C. Shoot materials were milled and analyzed for total P content after digestion.

DNA extraction, PCR amplification, T-RFLP and sequencing

For each of the five replicate pots, rhizosphere soil was shaken from roots, collected and frozen in liquid N_2. Total DNA was extracted from and PCR amplification and community structure analysis was performed using T-RFLP. Template was amplified in a 25 μL volume reaction containing Platinum Taq buffer with 15 mM $MgCl_2$, 100 nM of each of the dNTPs, 200nM of each of the primers 16f27 (AGAGTTTGATCCTGGCTCAG, Amann et al. 1995) and 1392R (ACGGGCGRTGTGTACA, Blackwood et al. 2003) 20 mg mL-1 BSA and 0.725 units of Platinum Taq DNA polymerase (Promega UK). The 16f27 primer was 5' labelled with the fluorophore FAM and 1392R with VIC. Prior to amplification the PCR mastermix was digested with HhaI (40min at 37ºC) to remove contaminating template. PCR product was subjected to digestion using *Alu*I as follows: 5 μL of PCR product was digested with 0.5 U of enzyme at 37ºC for two hours followed by 10 minutes enzyme denaturation at 65ºC. The terminal restriction fragments marked with fluorophore were analysed by electrophoresis with an automated DNA sequencer (ABI PRISM™ 3730, USA).

RESULTS AND DISCUSSION

Plant growth and P accumulation

There was no significant effect due to plant line on P accumulation, although there was a significant effect ($p<0.001$) caused by the soil treatments, so that plants in all treatments accumulated more P compared to the live soil, and with all treatments being significantly different to each another (Fig. 1).

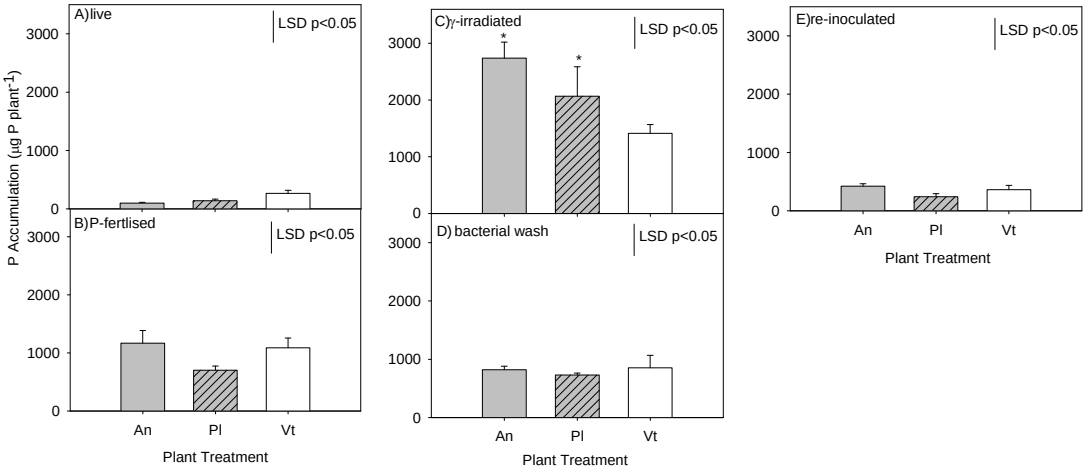

Figure 1: P accumulation (µg P plant^{-1}) for two independently transformed *N. tabacum* lines that express *phyA* from either *Aspergillus niger* (An) or *Peniophora lycii* (Pl) compared to a transformed vector control line (Vt). Plants were grown in soil that was either A) live; B) fertilised with KH_2PO_4; C) sterilized by γ-irradiation and sterilized soil re-inoculated with D) a bacterial wash or E) live soil.

Relative to the control (live soil) plants accumulated 5.3-fold more P on γ-irradiated soils, 4.1-fold more on P-fertilised soils, 3.2-fold and 1.4-fold more on the bacterial wash and live soil inoculum treatments, respectively. Of particular interest was that in γ-irradiated soil both transgenic plant lines (35SAn and 35SPl) accumulated more P than the control plants (Fig. 1), whereas there was no significant difference between plant lines in all other soil treatments. This indicates that the heterologous expression of the fungal phytases in plants was effective in increasing the acquisition of P, but only in the soil where the microbial community had been severely disrupted and not re-introduced by inoculation

Impact on rhizosphere and rhizoplane microorganisms

Despite the apparent interaction of rhizosphere microorganisms and ability of the heterologous phytase to improve the P nutrition of plants, there was little impact of the different plant lines on the structure of the bacterial communities (Figure 2).

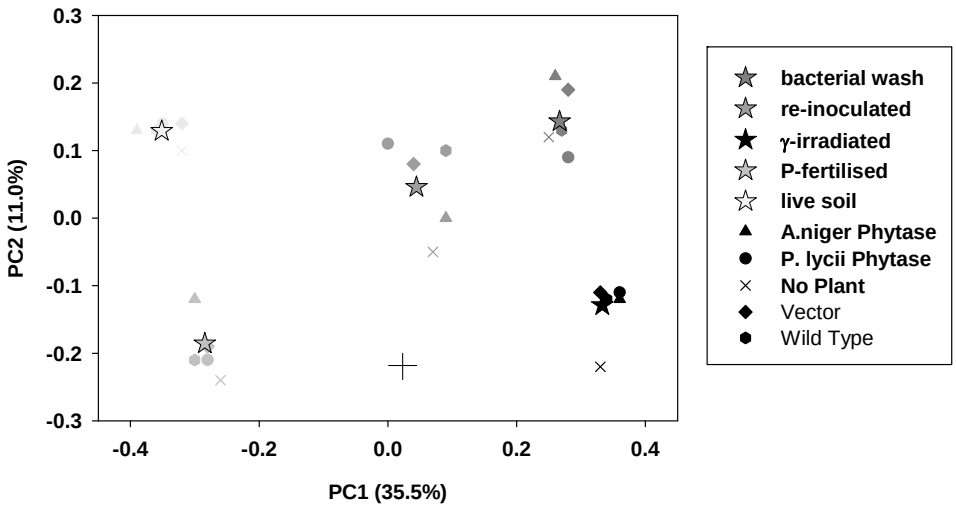

Figure 2: Mean loadings of principal components (PC) derived from rhizosphere bacterial community structure. The first two PC's to be significantly affected by the plant and soil treatments are plotted against each other. Bars show the LSD (p=0.05) for the interaction between plant and soil treatments in both dimensions.

The rhizosphere populations showed significantly different and distinct community structure between soil treatments, with the two live soils (live and P-fertilised) being more similar to each other than the various γ-irradiated treatments in PC1 (Figure 2). There were no significant differences between plant lines or any interaction between plant line and soil treatment. However, there were significant ($p<0.05$) differences between plant treatments in PC2 driven by the unplanted control. There were no significant difference in the structure of the rhizosphere community between the transgenic and control plant lines.

This study has for the first time investigated the impact of a heterologous protein designed specifically to modify the biochemistry of the rhizosphere and its impact on the structure of the microbial community. Nevertheless, the results are similar to other studies which have demonstrated that transgenic expression of heterologous genes does not impact the rhizosphere microbial community to any major extent. Our data suggests that while soil microorganisms appear to be intimately involved in the availability of P to plants, the microbial community in the rhizosphere appears to be relatively resistant to the impact of single-gene changes in plants designed to alter root biochemistry and nutrient cycling in the rhizosphere. Although it does remains possible that more subtle changes to specific microorganisms, or alteration to their function rather than to their presence, may have occurred. Nevertheless the experiments suggest that that transgenic technology aimed at improving the sustainability of agriculture by altering nutrient availability in the rhizosphere appears to have little impact with regard the ecology of the microbial community and thus the wider ecology of the agricultural system.

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