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Induction of toluene degradation and growth promotion in corn and wheat by horizontal gene transfer within endophytic bacteria

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ABSTRACT

Some experiments involving important crop plants, corn and wheat, were carried out to characterize the agronomic and environmental application of *Burkholderia cepacia* strain FX2 able to degrade toluene and containing plasmids with the gene encoding for catechol 2, 3-dioxygenase (C23O), a key enzyme in the degradation pathway of monocyclic aromatic compounds. The inoculation of corn and wheat with FX2 led to the promotion of plant growth and reduction in evapotranspiration of toluene into the air. Endophytic bacteria able to grow on toluene as the only source of carbon and containing a C23O gene were found in the plants inoculated with FX2 but not in their non-inoculated controls. Compared to control plants, a greater number of toluene-degrading, phosphate-solubilizing and siderophore-producing endophytes were found in inoculated plants. Furthermore, a direct correlation occurred between plant biomass responses and the magnitude of C23O-containing endophytes. Phylogenetic tree comparison, plasmid analysis and filter mating assays showed that the C23O gene was transferred horizontally from FX2 to the natural endophytic bacteria of corn and wheat. Horizontal gene transfer among endophytic bacteria might contribute to pollutant degradation, growth promotion and potential for disease suppression in corn and wheat.

1. Introduction

Certain endophytic bacteria are able to promote phytoremediation of highly volatile organic pollutants, such as phenol and toluene which cannot be completely degraded by the plant thus resulting in phytotoxicity or volatilization of chemicals through the leaves (van der Lelie et al., 2001; Doty, 2008; Ryan et al., 2008; Weyens et al., 2009a). Some endophytic bacteria are capable of using certain pollutants as a nutrient source and are therefore able to degrade these target pollutants (Siciliano et al., 2001; Barac et al., 2004; Kuiper et al., 2004; Wang et al., 2007). The genetic information required for the efficient degradation of a pollutant is a predetermined feature of pollutant-degrading endophytic bacteria and is located on mobile genetic elements such as plasmids or bacterial chromosomes (Shields et al., 1995; Barac et al., 2004; Kuiper et al., 2004; Jussila et al., 2007). Horizontal transfer of this genetic information within the microbial population is a major mechanism by which microorganisms acquire new metabolic traits and rapidly adapt to new environmental stresses (Eltis and Bolin, 1996; Dong et al., 1998; Mars et al., 1999; van der Lelie et al., 2005). Recent research reports have shown that endophytic bacteria equipped with a toluene-degradation pathway are able to reduce pollutant phytotoxicity and improve phytoremediation of these pollutants (Barac et al., 2004; Newman and Reynolds, 2005; Taghavi et al., 2005; van der Lelie et al., 2005).

Other attractive properties of endophytic bacteria which make them suitable for agronomic applications include the induction of systemic resistance in plants against soil-borne pathogenic microorganisms, suppression of plant-pathogenic fungi, and promotion of plant growth (Weyens et al., 2009b). The endophytic bacteria, *Bacillus* strain EPB22, is able to enhance the growth of banana plants, and reduce the damage caused by banana bunchy top virus (Kavino et al., 2007). Individual bacterial isolates or mixtures of endophytic bacteria can induce growth promotion and suppression of wilt disease in oilseed rape and tomato (Nejad and Johnson, 2000). It has been reported that many of the cultivated endophytic bacteria, belong to the genera of *Burkholderia*, *Pantoea*, *Pseudomonas*, and *Microbacterium*, isolated from inside the roots and stems of sugarcane plants, were shown to produce the plant growth hormone indoleacetic acid. Many of the *Burkholderia* isolates produced the antifungal metabolite pyrrolnitrin (Mendes et al., 2007). Some potato-associated endophytes have been found to antagonize fungal as well as bacterial pathogens and showed a high production of active compounds (Sessitsch et al., 2004). Endophytic bacterial

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Enterobacter sp. strain 638 exhibits a beneficial effect on growth and development of poplar trees (Taghavi et al., 2009).

In our previous work, several strains of endophytic bacteria (FX1-FX5), classified as *Pseudomonas fluorescens* and *Burkholderia cepacia*, were isolated from a corn plant (*Zea mays*), and found to degrade phenol. The strains were found to possess the plasmids loaded with a gene encoding the catechol 2, 3-dioxygenase (C23O), a key enzyme in the degradation pathway of monocyclic aromatic compounds. Horizontal transfer of this gene has been found to occur between the bacteria living within a plant and its rhizosphere (Wang et al., 2007). In addition, strain FX2 was found to degrade toluene. However, characterization of the agronomic and environmental applications of these endophytic bacteria, especially to important crop plants, has not been carried out to date. In this paper we describe experiments that have been carried out on the potential applications of *B. cepacia* strain FX2 to important crop plants, corn and wheat. It was observed that this endophytic strain induced environmental remediation and the promotion plant growth, and even the potential for disease suppression, which might be a possible result of horizontal transfer of the C23O gene among those endophytic bacteria in corn and wheat.

2. Materials and methods

2.1. Biomass for plant under greenhouse conditions and in field trials

Seeds of corn (*Z. mays*) and wheat (*Triticum durum*) were surface sterilized with 0.1% $HgCl_2$ for 5 min and then washed thoroughly with distilled water. Seedlings were placed with their roots in the Hoagland's solution containing strain FX2 at a final concentration of 10^8 CFU/ml for 96 h (Hoagland and Arnon, 1950). The Hoagland's solution with strain FX2 was supplemented with 200 ml of LB medium per liter. The control plants were treated with the Hoagland's solution without strain FX2. The seedlings were then transferred into the pots containing sandy soil and either maintained in a greenhouse, or used in field trials. In the greenhouse studies, earthenware pots, 20 cm in height and 20 cm in diameter were used in the experiment, and 4 corn seedlings or 6 wheat seedlings were grown in each pot containing 5 kg of soil. Six pots per treatment were used. The treated seedlings and the control seedlings were watered for 5, 10 or 15 days with half-strength Hoagland's solution containing 0 or 400 mg toluene liter^{-1} (one time everyday). Field trials were conducted in a field with a history of toluene pollution (5.238 mg of toluene per kg of soil). The soil was finely pulverized clay–loam with a pH of 7.5. Plots of 2 × 1 m in size were prepared. Each plot contains 8 rows, and 8 plants were grown in per row, so, 64 plants were included in per plot. The seedlings treated with strain FX2 and their controls were planted in rows (10 cm apart) and watered for 10, 15 or 20 days with half-strength Hoagland's solution containing 0 or 400 mg toluene liter^{-1} (one time everyday). At the indicated time points, the seedlings were harvested, and the growth parameter, plant biomass (in gram), was determined (fresh weight) and designated Wt. The plant biomass was determined (fresh weight) before the addition of toluene and designated Wo. The value of (Wt−Wo)/Wo was then calculated. The data are the means of 5 replicates and the error bars indicate the standard deviation from the mean. Two-way analysis of variance (ANOVA) was used to determine statistical significance ($P < 0.05$).

2.2. Amount of toluene volatilized from the corn and wheat

Amount of toluene volatilized from the corn and wheat plants was determined essentially as previously described with the slight modifications (Barac et al., 2004). The corn and wheat seedlings inoculated with strain FX2 together with their controls were transferred into airtight glass jars with the half-strength sterile Hoagland's solution containing 100 mg l^{-1} toluene. In order to avoid gas exchange between the upper and lower areas of the jars, the latter were divided into upper and lower sections with glass plates which contained holes through which the roots of the plants were able to grow and make contact with the Hoagland's solution in the lower section of the jar. The glass jars were equipped with an injection port through which synthetic air was introduced. Activated charcoal tubes with a link to the headspace in the opening of the upper part of the glass jars were used for collecting the toluene that had volatilized from the plants. The glass jars containing the plants were placed in a growth chamber at a constant temperature of 22 °C and a cycle of 14 h of light (150 μmol m^{-2} s^{-1}) and 10 h of darkness. The toluene concentrations in the activated charcoal tubes were determined by GC–MS for a period of 120 h after the start of the experiment. The amount of toluene volatilized from those plants was calculated per unit of leaf surface area. The data are the means of 5 replicates and the error bars indicate the standard deviation from the mean. Two-way analysis of variance (ANOVA) was used to determine statistical significance ($P < 0.05$).

2.3. Isolation and characterization of endophytic bacteria

Isolation of endophytic bacteria was performed as described previously (Wang et al., 2007). In brief, soil was removed from the roots under running tap water. The tissue were rinsed with deionized water, washed with sterile distilled water, and drained. The tissue was sterilized using 0.2% $HgCl_2$ for 30 s, washed thoroughly with distilled water, cut into small pieces and homogenized in sterile distilled water. The cultures used to screen for toluene-degrading bacteria were grown in Stanier's minimal medium (MSB) (Stanier et al., 1966) supplemented with toluene in the vapor phase, which was introduced into the headspace through a foam plug in the opening of the flask. Mineral phosphate solubilization was assayed on TY medium supplemented with 5 g l^{-1} of $Ca_3(PO_4)_2$. Aliquots of fresh culture were spread onto plates, and incubated at 28 °C for 48 h. A clear zone around the colonies indicated solubilization. Siderophore production was detected by the formation of a bright zone with a yellowish fluorescence surrounding bacterial colonies on chrome azurol S (CAS) agar (Schwyn and Neilands, 1987) after 48 h of incubation at 28 °C.

2.4. Plasmid analysis, 16S rDNA amplification, clone of C23O-encoding sequence, southern hybridization

For each strain studied, a single colony was picked from a fresh culture, and resuspended in 50 μl sterile deionized water. The genomic DNA was isolated according to standard method (Sambrook et al., 1989). If needed, DNA solution was re-extracted with phenol, and chloroform, precipitated with isopropanol, and washed twice in ethanol. Plasmid extraction was conducted according to standard method (Sambrook et al., 1989). The plasmids were subject to HindIII digestion, and separated on a 0.7% agarose gel. PCR amplification of 16S rDNA based on the genomic DNA was performed with the universal primer pair: 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-TACCTTGTTACGACTT-3′). (Weissburg et al., 1991; Polz and Cavanaugh 1998; Martin-Laurent et al., 2001). PCR product was purified using the gel extraction kit (Pharmacia), sequenced through dideoxynucleotide sequencing. The PCR primers for clone of the C23O gene were designed based on the previous reports (Mars et al., 1999):

P1, 5′-GCTGCTCCATGGGTATTATGAGAATTGGC-3′;
P2, 5′-GACGTCGGATCCTCATCATGTGTACACGGTG-3′

A PCR product was ligated in the pGEM-T vector and was transformed into the cell of *E. coli* DH5a. Selection of transformants