Short communication

Effects of transgenic poplars expressing increased levels of cellular cytokinin on rhizosphere microbial communities

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ABSTRACT

Considerable effort has been made in biotechnology to increase plant biomass. Altering cellular levels of plant hormones, including cytokinin, by genetic modification, has been one way to achieve the goal as it is involved in a variety of processes related to plant growth and development. However, the alteration inevitably may change physiological and biochemical characteristics of plants, and thus could affect the relationship between plants and other organisms interacting with the plants such as microorganisms inhabiting in the rhizosphere. To determine if these indirect effects on rhizosphere microorganisms, mediated by hormonal changes in plants, do occur, we investigated the microbial biomass and community structure associated with transgenic *Populus* trees with altered cellular cytokinin levels, using phospholipid fatty acid (PLFA) analysis. Three transgenic lines expressing increased levels of cellular cytokinin (T1403, T1410, and T1413) and their non-transgenic isoline (BH) were planted at three locations (Suwon, Cheongwon, and Jinju) in 2011. Soil samples were collected near the base of each tree monthly, from May to September. Indicator PLFAs were utilized to calculate the microbial (bacterial and fungal) biomass, and PLFA profiles were developed to characterize the structure of those communities. Over the growing season, soils from Cheongwon and Jinju had similar microbial biomasses (PLFAs indicating functional groups) whereas, at Suwon, the biomass associated with the rhizosphere of Line T1413 was significantly different from that of the other transgenics and the control. At Cheongwon and Jinju, the structure of the rhizosphere microbial communities differed significantly between Lines T1403 or T1410 and BH, but only in May and June. By contrast, those structures were similar in all sampling months for each line at Suwon. Our results indicate that the influence resulting from genetic modification of the poplar trees on the rhizosphere microbial community is only temporary and inconsistent depending upon location and genetic line.

1. Introduction

Cytokinin is a phyto-hormone involved in a variety of processes related to plant growth and development [1]. Great attempts have been made in biotechnology to modify cellular cytokinin levels in plants to improve biomass production [2]. In line with those efforts, *Populus alba* × *Populus tremula* var. *glandulosa*, has been genetically engineered to express greater amounts of cellular cytokinin by inserting the prokaryotic *tzs* (trans-zeatin secretion locus) gene [3]. The resulting transgenics exhibit greater above-ground biomass. However, they also show more shoots, smaller leaves, and delayed height growth. In addition, their concentrations of leaf sugars, such as glucose, fructose, and sucrose, are increased, and apical bud formation in autumn is delayed [3], indicating significant physiological modifications due to genetic transformation.

Such alterations in plant physiology, mediated by changes in cytokinin levels, can have an impact on soil microbial communities in the rhizosphere. It is well known that soil microorganisms inhabiting the rhizosphere are affected by rhizodeposits, a wide range of substances originating from sloughed-off root cells or tissues, and soluble carbon-containing compounds secreted from the roots, referred as to root exudates [4]. Rhizodeposits, especially root exudates are likely to be affected by altered cellular cytokinin

levels. Empirical evidence has shown that changes in cellular cytokinin levels can influence photosynthate assimilation and allocation within plants, which in turn can alter the amount and composition of root exudates. For instance, cytokinins affect photosynthetic activities by regulating chlorophyll biosynthesis [5] and chloroplast development [6]. They are also involved in photosynthate transport and the distribution of assimilates [7].

Even though all these substantial indications, experimental evidence on the effects of changes in cytokinin levels in plants, especially trees, on the rhizosphere microbial community is scarce. Therefore, we investigated how physiological alterations in transgenic poplar, as mediated by increased levels of cellular cytokinin, affect rhizosphere microbial communities.

2. Materials and methods

2.1. Plants

We evaluated three transgenic poplar lines (T1403, T1410, and T1413) and their parental non-transgenic line (BH; a hybrid clone from *P. alba* × *P. tremula* var. *glandulosa*). Levels of cellular cytokinin (trans-zeatin) in the transgenics were increased from 0.01 to 0.20 ng per g fresh weight following transformation with an *Agrobacterium tumefaciens* vector that carried a bacterial *tzs* gene, pAUX-*tzs* [3].

2.2. Site description, experimental design, and soil sampling

Isolated field experiments were conducted at three locations (northern, middle, and southern regions in South Korea) with different soil characteristics (Table 1). The sites at Suwon (37°15′N, 126°57′E) and Jinju (35°12′N, 128°09′E) were established in 2002 and 2010, respectively, and had been cropped with poplars since then. Both fields were within experimental forests belonging to the National Forest Research Institute. The third field, at Cheongwon (36°43′N, 127°26′E), is a former vineyard that was converted to grow chili pepper and potato from 2005 to 2009, and then poplar since 2010.

All of the trees were initially grown in a nursery field at Suwon in 2010. In March 2011, cuttings were taken and transplanted into the experimental fields that had been fertilized with animal manure beforehand. At Suwon and Cheongwon, 20 cuttings per line were installed at a 60- × 60-cm spacing in two rows (10 cuttings per row). Due to limited availability at Jinju, 10 cuttings per line were planted at a 60- × 60-cm spacing in only one row.

Table 1
Soil characteristics of three study locations. Data are means ± standard errors ($n = 5$). Values within rows followed by the same letters are not significantly different (Tukey's HSD test, $P < 0.05$).

Soil characteristic	Location		
	Suwon	Cheongwon	Jinju
pH	5.46 ± 0.03c	6.30 ± 0.06a	5.72 ± 0.10b
Total N (%)	0.096 ± 0.003ab	0.105 ± 0.016a	0.066 ± 0.002b
Soil organic carbon (%)	2.64 ± 0.16a	2.91 ± 0.07a	2.56 ± 0.24a
C/N ratio	27.6 ± 2.4a	30.8 ± 5.2a	38.4 ± 2.9a
Available P (mg kg^{-1})	385.5 ± 81.9b	983.3 ± 147.3a	90.3 ± 4.1b
Exchangeable K (mg kg^{-1})	72.1 ± 3.4a	51.2 ± 3.8b	42.1 ± 2.5b
Sand (%)	80.9 ± 0.4ab	76.7 ± 0.9b	88.7 ± 4.6a
Clay (%)	8.5 ± 0.3a	6.9 ± 0.6a	2.6 ± 1.1b
Silt (%)	10.5 ± 0.4a	16.4 ± 0.4a	8.7 ± 3.6a
Soil texture	Loamy sand	Loamy sand	Sand
Soil type	Inceptisol	Inceptisol	Inceptisol

Soil samples were collected monthly throughout the growing season (from May to September). Three trees were randomly chosen per line, and one soil core (3 cm diam., 20 cm deep) was taken within 5 cm of the base of each tree. The samples were placed immediately in an ice chest, brought to the laboratory, then lyophilized and sieved through a 2-mm mesh. They were stored at −80 °C before processing. Soil characteristics were evaluated on the day of the first sampling in May 2011. Five samples were collected at a depth of 0–20 cm from each site to represent all of the soil types containing our four poplar lines.

2.3. Analysis of phospholipid fatty acids

Phospholipid fatty acids (PLFAs) were extracted as described by Bligh and Dyer [8], with slight modifications. Briefly, 8 g of each lyophilized soil sample was extracted twice in a mixture of chloroform, methanol, and phosphate buffer (23 mL total on a 1.0:2.0:0.8 volume basis). The extracts were fractionated into neutral lipids, glycolipids, and phospholipids in SPE-Pak R Silica Cartridges (Waters Corporation, USA). The phospholipid fraction was isolated and then trans-esterified with mild alkaline methanol. The resulting fatty acid methyl esters were identified and quantified on an Agilent 6890 Gas Chromatograph (GC) with flame ionization detector, using MIDI peak identification software (MIDI Inc., USA). The methyl ester of fatty acid 19:0 (2 μg mL^{-1}) was added as an internal standard for quantification before GC analysis. Conditions included an injection volume of 2 μL; flow rate, 0.4 mL min^{-1}; injection temperature, 250 °C; and detector temperature, 300 °C. The initial oven temperature of 170 °C was raised to 310 °C at 5 °C min^{-1}.

The PLFAs were designated according to the nomenclature described by Bååth et al. [9]. The total PLFA concentration (nmol per g DW of soil) was utilized as an index of the total viable microbial biomass. Bacterial biomass was estimated by summing PLFAs indicating Gram-positive bacteria (i15:0, a15:0, i16:0, 16:1ω7c, 16:1ω9c, i17:0, a17:0, ct17:0, and 18:0), Gram-negative bacteria (cy17:0, 18:1ω5c, 18:1ω7c, and cy19:0), and general bacteria (15:0 and 17:0) [10–13]. The fungal biomass was calculated by summing the 18:2ω6, 9c and 18:1ω9c PLFAs; the actinomycetes 10Me16:0, 10Me17:0, 10Me18:0, and 10Me19:0 PLFAs; and the arbuscular mycorrhizal (AM) fungi 16:1ω5 [10,14,15].

2.4. Statistical analysis

Since the major focus of this study was to detect differences associated with plant lines rather than site specificity, the data obtained from the three locations were analyzed separately, with each site considered as a block. Mauchly tests were performed to test the assumption of sphericity [16]; if not met, Greenhouse-Geisser's corrections for departure from sphericity were then made [17]. Pair-wise t-tests with a Bonferroni correction were utilized for all post hoc comparisons. To compare the microbial biomasses associated with each line through five sampling times, we used "car" package in R software [18] to conduct two-way repeated-measures ANOVAs (line × sampling time as factors).

Differences in microbial communities (defined as PLFA profiles in the soil samples) associated with each line were examined via permutational multivariate analysis of variance (PERMANOVA; [19]), based on a Bray–Curtis distance measure. Prior to analysis, individual concentrations were expressed as a percentage of the total PLFAs (mole %) in the samples, and were then arcsine square root-transformed. Only PLFAs constituting more than 1.0% of the total were included in the analysis. PERMANOVA models were employed with sampling time as the repeated factor and genetic line as the fixed factor [20]. Because PERMANOVA is sensitive to within-