Effects of chitinase-transgenic (*McChit1*) tobacco on the rhizospheric microflora and enzyme activities of the purple soil

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ABSTRACT

In order to evaluate the bio-security of genetically modified (GM) plants in the purple soil, we carried out a pot experiment about rhizospheric microflora at different development stages of a chitinase-transgenic (*McChit1*) tobacco (T-Chit), a plasmid-transgenic tobacco (T-Vi), and a non-transgenic tobacco (Nt-X) that were grown in the same purple soil, and surveyed the growth of three tobaccos and the properties of soil (i.e. the dynamic changes of the cultivable rhizospheric bacteria and fungi, soil enzyme activity and pH). The results showed that, compared with Nt-X plant as a control, T-Chit and T-Vi at the stages of flowering and mature significantly decreased the number of cultivable rhizospheric bacteria, but at their stubble stage the bacteria number returned to the same levels. Moreover, there were no significant differences about the number of cultivable rhizospheric fungi and the ratio of fungi to bacteria (F/B) among three treatments. It was of interest that soil catalase activities of T-Chit and T-Vi were lower than that of Nt-X during the same period, and urease activities of T-Vi and T-Chit were also lower than that of Nt-X at the stages of budding and stubble. Protease activity and the biomass of tobacco, however, showed no significant difference. This indicated that 1-year-old transgenic tobacco plants (T-Vi and T-Chit) inhibited the catalase and urease activities of the purple soil. In conclusion, the results revealed that 1-year-old T-Chit and T-Vi plants were non-toxic to the colony-forming units of cultivable bacteria and fungi in the studied purple soil during tobacco growth.

Keywords: GM tobacco; chitinase; soil ecosystem; soil microbe; enzymatic activity

Since 1980s, a large number of genetically modified (GM) plant products have been commercialized and released for cultivation (Cook 1999). The planting acreage of GM crops reached 160 million hectares in 2011, an 8% increase over 2010 (James 2011). With the continuous and worldwide release and use of GM plants and their replacement of traditional crops, the potential risks of GM plants to the environment and human health have aroused great concerns in recent years (Nap et al. 2003).

Soil is an important place for material cycle, energy conversion and information exchange in the ecosystem. As a part of it, the rhizosphere is that zone where living plant roots interact with surrounding minerals, organic, and microbial components of the soil. After growing in the same soil for a long period, GM plants will not only change the diversity or functions of microbial communities in the rhizospheric soil as well as the quality, structure, and functions of the rhizospheric soil,

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but also affect its enzyme synthesis and activity and such soil processes as decomposition and mineralization of the litter (Siciliano and Germida 1999, Dunfield and Germida 2004, Wu et al. 2004).

Pervious studies reported that cultivation of transgenic cotton had significant negative effects on the microbial properties and the enzyme activities in rhizospheric soil (Chen et al. 2012). *Bt* transgenic maize greatly affects the microbial community structure (Castaldini et al. 2005). In addition, although there were some transient differences, no strong evidence to demonstrate apparent effects of the GM plants on soil enzyme activities, microbial community composition and functional diversity when compared with the non-GM plants (Wu et al. 2004, Wei et al. 2008).

Tobacco is an important economic crop and a genetically modified model plant. Previous reports confirmed that the transgenic tobacco with overexpression of *Mcchit1*, a gene coding for balsam pear chitinase, led to enhanced disease resistance to fungus (Xiao et al. 2007). Although the transgenic tobaccos exhibited much higher resistance against the pathogens than the wild types (Luo et al. 2005, Xiao et al. 2007), there is still lack of experimental evidence in respect of the GM plants with their biological safety.

Purple soil is one of typical soils and the 'red beds' of the main types of sedimentary rocks, which is widely distributed in the Sichuan Basin that is located in subtropical regions of Southwest China (He 2003). Until now, there are few reports about the bio-safety of the environment in rhizosphere of the purple soil planted with GM plants. In this study, we used the purple soil from the Chongqing area belongs to the Sichuan Basin as a tested soil and examined the effects of transgenic tobacco on the rhizospheric microbial community and the activities of soil enzymes that are involved in major soil processes. The results would be important to the commercialization of the transgenic tobacco and could, hopefully, provide useful information for the assessment of the potential environmental risks of GM crops.

MATERIAL AND METHODS

Experimental soil. Purple soil was collected from the surface layer (20 cm) in a vegetable field at Beibei, Chongqing, China, where no GM plants had ever been planted. The soil was air-dried and sieved (< 2 mm). The properties of the experimental soil are shown in Table 1.

Experimental plant. Seeds of the T_3 generation of chitinase-transgenic (T-Chit) and plasmid-transgenic (T-Vi) tobacco were collected in 2010. The non-transgenic tobacco (Nt-X) *Nicotiana tabacum* cv. Xanthi, a parental plant of T-Chit and T-Vi, was regarded as control.

Experimental design. Ten seeds were separately germinated in plastic pots (27 cm in diameter and 22 cm in height). Each pot was filled with 10 kg of purple soil and the level of nitrogen application was 300 mg/kg, with an N:P:K fertilizer ratio of 1:1:2. The plants were grown in the chamber house in April 2011. Each type of surveyed tobaccos had three replicates (total 9 pots) and only two seedlings were kept in each pot. In spring time, all plants were watered every 2 days until three days before sampling and then, in summer time, watered every day until two days before sampling. The total duration of the experiment was 6 months.

Soil sampling and preparation. Soil samples, using a soil corer (1.5 cm diameter and 15 cm long), were taken from five sites of 3–18 cm depth in one pot by random and were mixed to one sample. 20 g soil sample was stored at 4°C for microbial enumeration, and the others were sieved (1 mm) after being air-dried at room temperature for 7 days and stored at 4°C for pH measurement and enzyme activity analysis. It was performed at five stages during the tobacco growth period: rosette, budding, flowering, mature and stubble stage.

Soil pH and biomass of plant. Soil pH was determined at 25°C in a 1:1 ratio of water/soil using a pH meter (pHS-3C$^+$). Plant height was measured and leaf number was recorded at the mature stage of the tobacco. Plant roots and shoot materials (stems and leaves) were separated by cutting the

Table 1. Properties of the experimental soil

Soil	Organic C	Total N	Total P	Total K	Alkalytic N	Readily available P	Readily available K	pH
	(g/kg)				(mg/kg)			
Purple soil	18.23	0.60	0.96	15.44	42.65	68.09	94.08	5.72

plant at the soil level at the stubble stage. Roots were washed to remove any adhering soil particles, and both roots and shoot materials were oven dried at 65°C for 48 h before dry biomass determination.

Microbial enumeration. Total soil aerobic bacteria and fungi were enumerated by a 10-fold dilution plate technique. Colony forming units (CFUs) of aerobic bacteria were determined by spreading 100 µL of diluted sample on LB agar medium. The number of CFUs of fungi was estimated on Martin's agar medium with 1.25 g streptomycin/L and 33 mg Rose Bengal/L (Ye et al. 1983).

Enzyme activity assays. Catalase activity in soil was assessed based on the rates of recovery of hydrogen peroxide, with the residual was determined by titration with 0.1 mol potassium permanganate in the presence of sulfuric acid (Johnson and Temple 1964). Urease activity was determined by the buffer method (Tabatabai 1994), and protease activity by the method of Ladd and Butler (1972), using gelatin.

Data analysis. All data of microbial counts (CFUs) and enzyme activities were compared statistically by ANOVA and the least significant difference (*LSD*) test at the 5% level with EXCEL (2007, Washington, USA) and SPSS (V 18.0, New York, USA).

RESULTS

Plant biomass. We first checked the biomass of T-Chit, T-Vi, and Nt-X plants and found that there were no significant differences in plant height, leaf number, shoot biomass, and root biomass from these three plants. This indicated that the growth and development of the plants were not affected by the genetic modification (Table 2).

Populations of bacteria and fungi. The total CFUs of bacteria in soil planted with Nt-X, T-Vi, and T-Chit were (15.33–30.89) × 10^6 cfu/g, (15.67–23.00) × 10^6 cfu/g and (14.89–23.17) × 10^6 cfu/g, respectively (Table 3). The maximum population of bacteria was detected at the flowering and mature stages of the plants in the fields grown with Nt-X (30.89 × 10^7 cfu/g), T-Vi (23.00 × 10^7 cfu/g), and T-Chit (23.17 × 10^7 cfu/g), respectively. The maximum of bacteria in T-Vi and T-Chit were 0.74 and 0.75 times of Nt-X. Comparing the treatments with different plants in the same growing stage, we found that the number of rhizospheric bacteria in the soil planted with T-Vi or T-Chit was generally lower than that planted with Nt-X, but the differences were observed only at the mature stage between T-Vi and Nt-X, and at the flowering stage between T-Chit and Nt-X. Furthermore, the number of rhizospheric bacteria of all three treatments was returned to the same levels at the stubble stage.

The CFUs of rhizospheric fungi were relatively stable under tobacco cultivation. The population of fungi in T-Vi was significantly different between the budding stage and the flowering stage ($P < 0.05$). In the soils planted with T-Chit and Nt-X, the population of fungi was also maintained at the same levels. No significant differences were observed in the population of cultivable fungi in the rhizosphere among the tobacco genotypes studied in all growth stages except for the budding stage ($P < 0.05$) (Table 3).

A previous study reported that the ratio of fungi to bacteria (F/B) is more reliable to describe the microbial community structure and, to a certain extent, it also can characterize the degree of soil health (Ibekwe et al. 2002). Different types of microbes interact with each other in soil and affect the health of the soil. In the present study, however, the differences of the ratios of the F/B from different tobacco genotypes at the same growth stage were not significant ($P < 0.05$).

Effects of transgenic tobacco on enzyme activities in the rhizospheric soil. Catalase activity in the soil planted with three tobacco genotypes increased shapely at the budding stage, reached to the maximum at the budding or the flowering stage,

Table 2. Plant biomass

Treatment	Plant height (cm)	Leaf number (piece)	Shoot biomass (g)	Root biomass (g)
Nt-X	104.65 ± 4.99^A	30.50 ± 1.80^A	48.59 ± 3.03^A	8.94 ± 0.46^A
T-Vi	94.16 ± 11.07^A	30.33 ± 0.29^A	47.11 ± 2.17^A	8.83 ± 1.93^A
T-Chit	96.58 ± 3.88^A	29.83 ± 1.44^A	48.26 ± 1.19^A	8.45 ± 0.68^A

Values are means ± standard deviation ($n = 3$). Different letters (A, B, C) in the same column indicate a significant difference at $P < 0.05$. Nt-X – non-transgenic tobacco; T-Vi – plasmid-transgenic tobacco; T-Chit – chitinase-transgenic tobacco

Table 3. Colony forming units (CFUs) of soil microorganism and F/B at different growing stages of tobacco

Organism	Treatment	Tobacco growth stages				
		rosette	budding	flowering	mature	stubble
Bacteria (10^6 cfu/g)	Nt-X	15.33 ± 0.39^{cA}	19.60 ± 1.20^{bcA}	27.67 ± 5.51^{abA}	30.89 ± 7.07^{aA}	15.33 ± 5.81^{cA}
	T-Vi	16.00 ± 2.00^{bA}	15.67 ± 1.52^{bA}	23.00 ± 3.33^{aAB}	20.67 ± 1.84^{aB}	14.00 ± 3.00^{bA}
	T-Chit	14.89 ± 2.83^{bA}	18.44 ± 5.83^{abA}	19.67 ± 0.58^{abB}	23.17 ± 2.95^{aAB}	15.44 ± 2.14^{bA}
Fungi (10^4 cfu/g)	Nt-X	22.78 ± 9.43^{aA}	21.33 ± 1.15^{aA}	33.00 ± 1.90^{aA}	27.00 ± 0.39^{aA}	31.06 ± 4.42^{aA}
	T-Vi	21.89 ± 4.67^{abA}	16.89 ± 2.17^{bB}	34.33 ± 7.51^{aA}	32.11 ± 6.41^{abA}	21.56 ± 3.60^{abA}
	T-Chit	22.33 ± 8.09^{aA}	23.89 ± 2.71^{aA}	32.56 ± 2.37^{aA}	34.22 ± 2.83^{aA}	19.67 ± 8.41^{aA}
F/B ($1/10^2$)	Nt-X	1.50^{abA}	1.09^{bA}	0.99^{bA}	0.93^{bA}	2.23^{aA}
	T-Vi	1.18^{aA}	1.08^{bA}	1.51^{aA}	1.53^{aA}	1.58^{aA}
	T-Chit	1.48^{aA}	1.38^{aA}	1.65^{aA}	1.51^{aA}	1.24^{aA}

Values are means ± standard deviation (n = 3). Different letters in the same line (a, b, c) indicate a significant difference at $P < 0.05$. Different letters in the column (A, B) indicate a significant difference at $P < 0.05$. Similarly hereinafter. Nt-X – non-transgenic tobacco; T-Vi – plasmid-transgenic tobacco; T-Chit – chitinase-transgenic tobacco

and decreased thereafter (Figure 1A). Catalase activity of T-Vi and Nt-X showed a significant difference at the stubble stage.

Urease activity was decreased at the rosette stage, increased at the mature stage, and reached the maximum level at the stubble stage (Figure 1B). It was worth mentioning that the urease of Nt-X was 4.84 mg at the stubble stage, being significantly higher than that of T-Vi (3.88 mg) and T-Chit (3.96 mg).

During the whole growing stages, protease activity was relatively stable and no significant difference was observed among different treatments (Figure 1C).

Dynamic change of pH. Compared with the pristine soil pH (5.72), the rhizopheric soil pH of Nt-X was decreased at first and then, with the growth of the tobacco, increased significantly due to the fertilizer application. The treatments of T-Vi and T-Chit exhibited the same trend as well.

Rhizopheric soil pH of T-Chit was much lower than that of the other two treatments at the rosette and budding stages (Table 4), but it gradually increased from the flowering stage and reached its

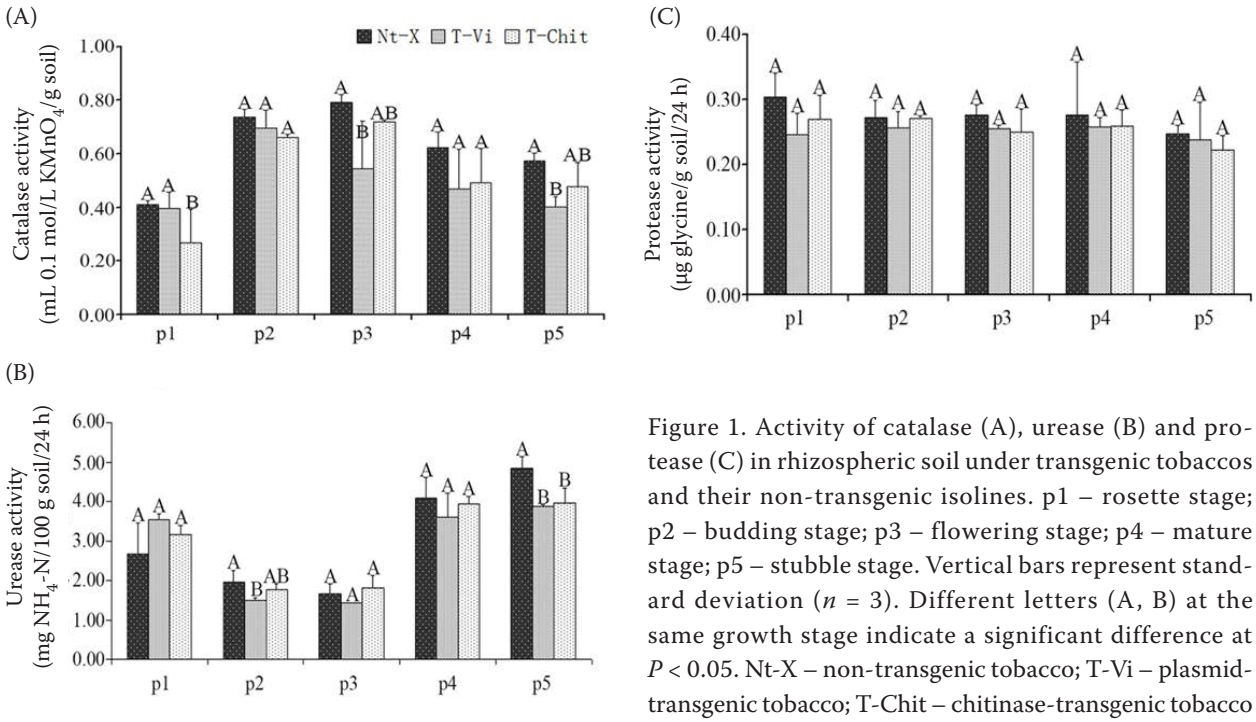

Figure 1. Activity of catalase (A), urease (B) and protease (C) in rhizospheric soil under transgenic tobaccos and their non-transgenic isolines. p1 – rosette stage; p2 – budding stage; p3 – flowering stage; p4 – mature stage; p5 – stubble stage. Vertical bars represent standard deviation (n = 3). Different letters (A, B) at the same growth stage indicate a significant difference at $P < 0.05$. Nt-X – non-transgenic tobacco; T-Vi – plasmid-transgenic tobacco; T-Chit – chitinase-transgenic tobacco

Table 4. The pH of the rhizospheric soil of transgenic tobacco and non-transgenic tobacco plants at different growth stages

Treatment	Rosette stage	Budding stage	Flowering Stage	Mature stage	Stubble stage
Nt-X	5.34 ± 0.007^{aA}	5.58 ± 0.007^{bA}	5.66 ± 0.007^{cA}	5.89 ± 0.007^{dB}	6.06 ± 0.014^{eB}
T-Vi	5.37 ± 0.007^{aA}	5.60 ± 0.007^{bA}	5.55 ± 0.007^{cB}	5.91 ± 0.007^{dB}	6.03 ± 0.007^{eC}
T-Chit	5.30 ± 0.014^{aB}	5.38 ± 0.007^{bB}	5.66 ± 0.014^{cA}	6.01 ± 0.007^{dA}	6.10 ± 0.007^{eA}

Values are means ± standard deviation (n = 3). Different letters in the same line (a, b, c) indicate a significant difference at $P < 0.05$. Different letters in the same column (A, B) indicate a significant difference at $P < 0.05$. Nt-X – non-transgenic tobacco; T-Vi – plasmid-transgenic tobacco; T-Chit – chitinase-transgenic tobacco

maximum at the stubble stage in all treatments, which might be associated with the root exudates of T-Chit tobacco. The population of bacteria and the activity of catalase in rhizospheric soil decreased likely due to the increase of the pH at the flowering stage.

DISCUSSION

Being at the center of the biosphere, soil ecosystem is of vital importance to the health of the overall ecosystem. To date, most studies concerning soil environmental safety of GM plants are focused on the diversity of soil microorganisms, soil enzyme activities, and chemical properties of the soil.

Several reports found that the population and diversity of soil microbial communities can be affected by plants, but there are no consistent results about whether transgenic plants can affect rhizospheric soil nutrient cycling, soil enzyme activities and indigenous microbial populations (Saxena and Stotzky 2001, Wu et al. 2004, Castaldini et al. 2005, Chen et al. 2012, Wei et al. 2012). In this study, by monitoring the growth of tobaccos in the three treatments, we found a consistent trend, namely the numbers of cultivable bacteria and fungi and three soil enzyme activities in the rhizospheric soil were mainly related to the metabolism of tobaccos. Rhizospheric microorganisms were affected in different ways and showed different responses to the changes of metabolic functions and root exudates at different growth periods of tobacco, especially in the transition period from vegetative growth to reproductive growth.

It was previously found that the expressed products of antibiotic resistance genes, such as *NPT II*, were released into soil through litter and root exudates, which could change the rhizospheric environment and soil microbial communities (Saxena and Stotzky 2001, Flores et al. 2005). In our study, compared with fungi, bacteria were more sensitive to environmental changes. The number of cultivable rhizospheric bacteria of T-Chit was significantly lower than the Nt-X at the flowering stage, and great difference was also existed between T-Vi and Nt-X. Interestingly, the activities of all the three soil enzymes (catalase, urease and protease) were lower in T-Chit and T-Vi than in Nt-X at the same growth stage, but the biomass of tobacco showed no difference in the three treatments. We hypothesize, therefore, that the metabolic functions and the root exudates of the tobacco plants might be changed due to the expression of the chitinase gene or antibiotic resistance genes (*NPT II*) in the vector. The number of cultivable bacteria, soil pH and enzyme activities in rhizosphere were affected by the gene expressed products. Based on our results, it is recommended that researches of non-antibiotic resistance genes should be strengthened so as to avoid the interference of the antibiotic resistance genes in the study of GM plants.

It is noted that only 0.1–1% of the microbes in soil can be cultivated (Losey et al. 1999). In this study, we only investigated the number of cultivable bacteria and fungi. To achieve a more accurate environmental assessment of GM plants, it is necessary to further study the diversity of microbial communities and other special floras in soil using biotechnologies, such as high-throughput sequencing (Taylor et al. 2008, Jun et al. 2012). Since the evaluation of the effects of GM plants on soil environment is a continuous process, longer-term field experiments and release experiments are essential for bio-security evaluation.

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