Microbes Environ. Vol. 21, No. 4, 261–271, 2006
http://wwwsoc.nii.ac.jp/jsme2/

Microbial Community Analysis in the Rhizosphere of a Transgenic Tomato that Overexpresses 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase

SEISHI IKEDA[1,*,†], TOSHIKAZU OMURA[2], NOZOMI YTOW[1], HISAYUKI KOMAKI[3], KIWAMU MINAMISAWA[4], HIROSHI EZURA[1] and TATSUHITO FUJIMURA[1]

[1] *Gene Research Center, University of Tsukuba, 1–1–1 Tennoudai, Tsukuba, Ibaraki 305–8572, Japan*
[2] *Graduate School of Life and Environmental Sciences, University of Tsukuba, 1–1–1 Tennoudai, Tsukuba, Ibaraki 305–8572, Japan*
[3] *NITE Biological Resource Center (NBRC), National Institute of Technology and Evaluation (NITE), 2–5–8 Kazusakamatari, Kisarazu, Chiba 292–0818, Japan*
[4] *Graduate School of Life Sciences, Tohoku University, 2–1–1 Katahira, Aoba-ku, Sendai, Miyagi 980–8577, Japan*

(Received July 31, 2006—Accepted October 10, 2006)

The impact of overexpression of the 3-hydroxy-3-methylglutaryl coenzyme A reductase gene on microbial diversity in the rhizosphere of a transgenic tomato was examined using a ribosomal intergenic spacer analysis (RISA) and a terminal restriction fragment length polymorphism (T-RFLP) analysis of two functional bacterial genes (bacterial chitinase and *nifH* genes). While the overall profiles of both RISA and T-RFLP revealed high similarity between the transgenic and non-trangenic tomato, several polymorphic DNAs were detected as differential bands in both the qualitative and quantitative evaluations. Sequence analysis suggested that differential bands cloned in RISA showed low levels of similarity to known species but were most likely derived from uncultured microbes. The phylogenetic analyses of two differential bands cloned in the T-RFLP analysis of the chitinase and *nifH* genes indicated that these bands belonged to *Streptomyces* like chitinase group and cyanobacterial *nifH* group, respectively.

Key words: chitinase, *nifH*, ribosomal intergenic spacer analysis, terminal restriction fragment length polymorphism, tomato

The rhizosphere is defined as the soil environment that is directly under the influence of living roots[30]. The soil microorganisms colonizing the rhizosphere assist plants in the uptake of several vital nutrients from the soil, such as phosphorous, potassium and nitrogen[13,17,48,49]. These soil microbes can exert considerable influence upon the overall health of the plant[44] and plants themselves have a significant influence on soil microbial diversity in the rhizosphere[50]. In this regard, a series of reports have suggested that the microbial community structures in the rhizosphere are in fact dependent upon the types of plant species[18,19,52], including the genotypes within a species in the case of transgenic plants[42]. Culture-independent methodologies have now revealed that the majority of rhizosphere-associated microbes have not been cultured in the laboratory[20,30]. These studies have thus provided vital clues regarding the abundance and spatial distribution of specific microbial groups in the rhizosphere. However, the relationships between plants and rhizosphere microbes are still largely unknown as the rhizosphere contains a broad spectrum of microbes in terms of their degree of interaction with the plants themselves. These range from neutral microorganisms with no obvious effect on the host plant to patho-

* Corresponding author; E-mail: sikeda@nbrc.nite.go.jp, Tel: +81–438–20–5764, Fax: +81–438–52–2314
† Present address: NITE Biological Resource Center (NBRC), National Institute of Technology and Evaluation (NITE), 2–5–8 Kazusakamatari, Kisarazu, Chiba 292–0818, Japan

gens and mutualistic symbionts with deleterious and beneficial effects, respectively.

To date, the impact of transgenic plants on their rhizospheres has been examined in several reports, mainly because of a number of concerns regarding the detrimental effects of their cultivation on soil ecosystems[8,23,28]. While these studies have shown that the microbial community analysis of rhizospheres is a useful benchmark in the evaluation of the impact of transgenic plants on the soil microbial diversity, an important environmental safety issue, the findings from these reports also suggest that transgenic plants are a useful genetic resource for unraveling the close relationships between plants and microbial communities in the rhizosphere.

One of the main problems with analyzing microbial communities is the difficulty in interpreting changes of diversity indices in terms of biological evaluation due to the use of rRNA gene regions. This is because most of the microbes in nature are unculturable[5] and therefore, the functionality of these microbes can not be predicted accurately. In addition, a rRNA gene region-based phylogenetic assignment could imply certain biological roles for specific groups of microbes in an environment, but can not be used to predict all functionalities in diverse environments. More importantly, many microbiological functionalities in nature may be redundant among different phylogenetic groups.

In order to circumvent the problems described above, assessments of molecular diversity have been made for functional genes in diverse environments. In the rhizosphere, there are several microbial groups which are considered to be important for plant growth and disease protection[13,17,44,48,49]. Among these, diazotroph and chitinolytic bacterial communities are well characterized at the molecular level. The diazotroph is characterized by the possession of a *nifH* gene which is a subunit of the nitrogenase gene responsible for taking up nitrogen from the atmosphere, and several bacteria of this group are now recognized as plant growth promoters[29]. Similarly, many chitinolytic bacteria have been found to be important for disease control in the rhizosphere[55]. These findings imply that assessments of the molecular diversity of these functional genes may provide useful information regarding the functionality of the microbial community in the rhizosphere.

Kobayashi *et al.*[31] constructed a transgenic tomato that overexpresses the 3-hydroxy-3-methyglutaryl coenzyme A reductase (HMGR) gene from melon, and reported various unique phenotypes in this plant. HMGR catalyzes the irreversible conversion of 3-hydroxy-3-methylgluraryl coenzyme A into mevalonic acid, which is the rate-limiting step during isoprenoid biosynthesis[10,11,33,45,51]. The isoprenoid compounds have diverse functions in a number of physiological response pathways, including the synthesis of membrane sterols and plant growth regulators (cytokinin, abscisic acid, gibberellins, and brassinosteroids), electron transport, and the production of sesquiterpenoid phytoalexins, which confer resistance to pathogens[45]. With regard to the roles of the HMGR gene in plant-microbe interactions, elicitor-inducible HMGR genes have been characterized in a range of plants including tobacco, tomato, potato, and rice[12,16,35,39]. The results of these studies suggest the potential importance of HMGR gene expression in plant-microbe interactions, and that the disrupted expression of this gene can affect such interactions and thus may influence microbial community structures in the phytosphere. Moreover, over the past two decades, a number of reports have described genetic modifications of secondary metabolic pathways in a diverse range of plants, but none of the transgenic plants in these studies were examined in the context of plant-microbe interactions at the microbial community level.

In the present study, using a HMGR gene over-expressing tomato plant as our experimental system, we evaluated the usefulness of utilizing a transgenic plant, which was constructed primarily to undertake studies of plant secondary metabolism, to analyze the nature of microbial diversity in the rhizosphere. These microbial community analyses in the rhizosphere were conducted using a ribosomal intergenic spacer analysis (RISA) of both bacteria and fungi.

In addition, the impact of plant genotype on the diversity of two functional bacterial genes (bacterial chitinase and *nifH* genes) in the rhizosphere was also examined in conjunction with the assessment of terminal restriction fragment length polymorphisms (T-RFLP).

Materials and methods

Plant material

The seeds of a non-transgenic parental tomato line (*Lycopersicon esculentum* Mill. cultivar "Moneymaker") and its transgenic counterpart harboring a melon HMGR gene under the control of the 35S CaMV promoter[31], were sequentially surface-sterilized in 70% ethanol for 2 min and 1% (v/v) sodium hypochloride with 2 drops of Tween-20 for 20 min. The seeds were then rinsed with sterilized water three times for 5 min each and germinated in a plant box with 40 ml of MS medium[34] supplemented with 15 g/l sucrose and 3 g/l Gelrite. The pH was adjusted to 5.8 prior to autoclaving. All cultures were maintained at 25°C under

Table 1. Characteristics of the soil used in the present study

Sample name	pH	EC[a]	PAC[b]	%C[c]	%N[d]	%OC[e]	%HC[f]
UT3	5.9	72	2270	3.5	0.3	13.7	1.2

[a] Electrical conductivity. [b] Phosphate adsorption coefficient.
[c] Total carbon level. [d] Total nitrogen level.
[e] Organic content. [f] Humic acid content.

a 16 h light/8 h dark cycle with fluorescent light (irradiance of 60 μmol/m^{2}s) for three weeks, then transferred to a cell tray (3×3 cm) using a commercial soil preparation (Kureha engei baido, Kureha Chemical Industry, Chuo-ku, Tokyo, Japan) for nursery cultivation under the same growth conditions for an additional 4 days. The seedlings were then transferred to pots (10 cm in diameter and 15 cm in height) using soil mixture that was prepared from equal amounts of commercially obtained a soil (Kureha engei baido, Kureha Chemical Industry) and arable soil from an experimental field at the University of Tsukuba on May 15 2005. The characteristics of the soil sample were analyzed by Kankyo Engineering Co., Ltd. (Chiyoda-ku, Tokyo, Japan), as previously described[27] (Table 1). The plants were maintained at a minimum temperature of 15°C under a 16 h light/8 h dark cycle using natural daylight and fluorescent light (irradiance of 60 μmol/m^{2}s) for four weeks. The sampling of the rhizospheres was conducted for three plants of each genotype.

Extraction of DNA from rhizosphere soil

The root systems were carefully removed of the surrounding soil and gently washed with distilled water. After excess water had been removed, the rhizosphere soil remaining on the roots was subjected to DNA extraction. A few root tips from each plant (approximately 5 cm in length and 0.25 g wet weight) were collected into 2-ml screw-capped tubes as sub samples, and three such samples were prepared from each plant. DNA extraction was subsequently performed using a FastDNA SPIN Kit for soil (Qbiogene, Carlsbad, CA, USA) according to the manufacturer's protocol, except that skim milk (8 mg/g soil weight) was added to the extraction buffer prior to bead beating to obtain a stable yield of soil DNA[27] and the washing step was repeated three times. Finally, one composite DNA sample was prepared by mixing an equal volume of three sub sample DNA extracts prepared from the same plant for subsequent PCR analysis.

Ribosomal intergenic spacer analysis (RISA)

RISA was carried out as previously described by Ikeda *et al.*[26]. Briefly, the primer sets used for bacteria and fungi were ITSF/ITSReub[9] and 1406f/3126T[22], respectively. Each PCR mixture (final volume of 50 μl) contained 5 μl of 10×buffer, 10 μg of BSA, 0.5 μM of each primer, 200 μM dNTPs, 4U of Ex *Taq* HS DNA polymerase (TaKaRa, Kyoto, Japan) and 8 ng of DNA extract. For fluorescence detection, the 5' end of each forward primer was labeled with 6-carboxyrhodamine (Sigma Genosys Japan, Ishikari, Hokkaido, Japan). The PCR amplification program consisted of 2 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 60°C and 2 min at 72°C, and a final extension step of 7 min at 72°C. The resulting PCR products (3 μl) were then mixed with 2.5 μl of loading dye (95% formamide, 10 mM EDTA, pH 8.0, and 0.1% bromophenol blue). After incubation at 80°C for 2.5 min, the samples were chilled on ice for 5 min, and directly loaded onto 5% polyacrylamide gels (19:1, acrylamide: bisacrylamide, 0.4 mm thick, 40 cm long) containing 7.7 M urea and 0.5×TBE. Electrophoresis was conducted at a constant voltage of 2000 V in 1×TBE for 2.5 h. Triplicate samples were electrophoresed for both non-transgenic and transgenic plant samples. Following gel electrophoresis, digital fingerprinting images were obtained using a fluorescent scanner (Molecular Imager FX, BIO-RAD Laboratories, Inc., Hercules, CA, USA).

Terminal restriction fragment length polymorphism (T-RFLP) analysis of bacterial chitinase and nifH genes

Terminal restriction fragment length polymorphism (T-RFLP) analyses of bacterial functional genes were carried out with slight modifications to methods previously reported by Ikeda *et al.*[26]. Briefly, the PCR primers used to amplify the bacterial chitinase gene and the *nifH* gene were GA1F/GA1R[53] and nifH-F/nifH-R, respectively[40]. For fluorescence detection, the 5' ends of the GA1R and nifH-F primers were labeled with 6-carboxyrhodamine (Sigma Genosys Japan). The PCR mixtures were prepared as described for RISA. The PCR amplification programs for both the bacterial chitinase and *nifH* genes were: 2 min at 94°C, 35 cycles of 30 sec at 94°C and 30 sec at 68°C, and a final extension time of 7 min at 72°C.

The size of the PCR products (approximately 400 bp for both the bacterial chitinase and *nifH* genes) was confirmed by 1% agarose gel electrophoresis in 0.5×TBE running buffer. The PCR products (50 μl) were then purified with

NucleoSpin Extract II (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) and eluted in 25 µl of elution buffer. Aliquots (2.5 µl) were digested with 1 U of *Afa*I, *Hha*I or *Mbo*I (TaKaRa) according to the manufacturer's instructions in 5 µl at 37°C for 1 hour. The digested PCR products (2.5 µl) were mixed with 2.5 µl of loading dye (95% formamide, 10 mM EDTA, pH 8.0, and 0.1% bromophenol blue).

After incubation at 80°C for 2.5 min, the samples were chilled on ice for 5 min, and electrophoresis was carried out with 6% polyacrylamide gel at a constant 2000 V in 1×TBE for 1.5 h. Triplicate samples were electrophoresed for both non-transgenic and transgenic plant samples. Following the gel electrophoresis, digital fingerprinting images were obtained using a fluorescence scanner (Molecular Imager FX, BIO-RAD Laboratories).

Digital image analysis

The banding patterns from the RISA and T-RFLP experiments were analyzed with Bio-Rad Quantity One™ software (BIO-RAD Laboratories, Inc.) to assess the relative abundance of the bands differing between the transgenic and non-transgenic plants. Lane background subtraction was carried out with a rolling disk size of 50 and band detection was performed using the default parameters. The DNA size ranges for the data collections were 1722 to 176 nt and 536 to 94 nt for RISA and T-RFLP, respectively. The relative abundance of each DNA band of interest from either RISA or T-RFLP was then calculated relative to the normalized intensity in a given sample.

Molecular cloning and sequencing analysis

For comparisons between the transgenic and non-transgenic lines, differential RISA bands which were consistent within triplicate samples were selected, and were later excised from the gel for cloning and sequencing analysis. The cloning and sequencing of amplicons from the RISA profiles were carried out as previously described in a previous report from our laboratory[25]. Five clones were sequenced for each polymorphic DNA band obtained by RISA. The clones corresponding to differential bands in the T-RFLP profiles were screened as follows: PCR amplification (50 µl) was performed essentially as described for the initial T-RFLP analysis, except that unlabelled primers were used. The resulting PCR products were then purified with NucleoSpin Extract II (MACHEREY-NAGEL GmbH & Co. KG) and resuspended in 25 µl of elution buffer. These purified PCR products were subsequently ligated to the pGEM-T Easy plasmid vector and introduced into *E. coli* using pGEM-T Easy Vector System I (Promega., Madison, WI, USA). After the culture of transformants at 37°C on LB agar plates containing 50 µg/ml ampicillin sodium overnight, a total of 192 colonies were selected at random for each functional gene. Colony PCR was then conducted in the same manner as the T-RFLP analysis, except that each colony was used as the template DNA. Plasmid clones were classified according to the T-RFLP fingerprinting patterns following digestion with *Afa*I or *Hha*I of the bacterial chitinase or *nifH* gene, respectively. The clones corresponding to the bands in the T-RFLP profiles differing between the parental and transgenic lines were then selected for sequencing based on the size of the terminal fragments.

The selected clones were grown at 37°C in 4.5-ml cultures of LB medium containing 50 µg/ml ampicillin sodium overnight, and plasmid DNA preparations and sequencing reactions were performed as described by Ikeda *et al.*[25]. Sequences were manually edited to remove the vector backbone, primer regions, and ambiguous sequences. All sequence data in the present study have been deposited in the DDBJ/EMBL/GenBank data library, and were aligned to public database entries using the BLASTN subroutine[3]. The predicted amino acid sequence data for functional genes were compared to public database entries using BLASTX[4] and sequence matches were considered to be significant when the score was >50[36].

Phylogenetic analysis of functional bacterial genes

For the phylogenetic analysis, alignments of deduced amino acid sequences were performed using the CLUSTAL W program[46]. The neighbor-joining method was used for building the trees[41]. The Phylip format tree output was applied using the bootstrapping procedure[15]; the number of bootstrap trials used was 1000. The trees were constructed with Tree View software[38].

Accession numbers of nucleotide sequences

Nucleotide sequences for the PCR clones isolated by RISA have been deposited in the DDBJ database under the accession numbers AB266364-AB266373. Nucleotide sequences for the PCR clones isolated in the T-RFLP analyses of chitinase and *nifH* genes have been deposited in the DDBJ database under the accession numbers AB266340-AB266352 and AB266353-AB266363, respectively.

Results

Microbial community analyses of the tomato rhizosphere by RISA

The complexity of the profiles obtained from the bacterial RISA was greater than that of the patterns generated by the fungal RISA (Fig. 1), and the overall patterns within each assay were highly similar among the samples examined. Five bacterial RISA bands differed in intensity between the parental and transgenic tomato lines (bands a, b, c, d, and e in Fig. 1-A). The intensities of two Bacterial RISA bands in the parental line were also found to be decreased in the transgenic line (bands a and b in Fig. 1-A), and the relative abundance of these two bands was statistically high compared with the transgenic line (Table 2). In contrast, increased intensities were observed for two bacterial RISA bands in the transgenic line (bands c and d in Fig. 1-A) and one weak band was detectable only in the transgenic line (band e in Fig. 1-A).

The fungal RISA showed a high degree of variability among the profiles in terms of both the overall patterns and the band intensities, compared with the bacterial RISA results. However, one differential fungal band was detected in the transgenic line (band f in Fig. 1-B).

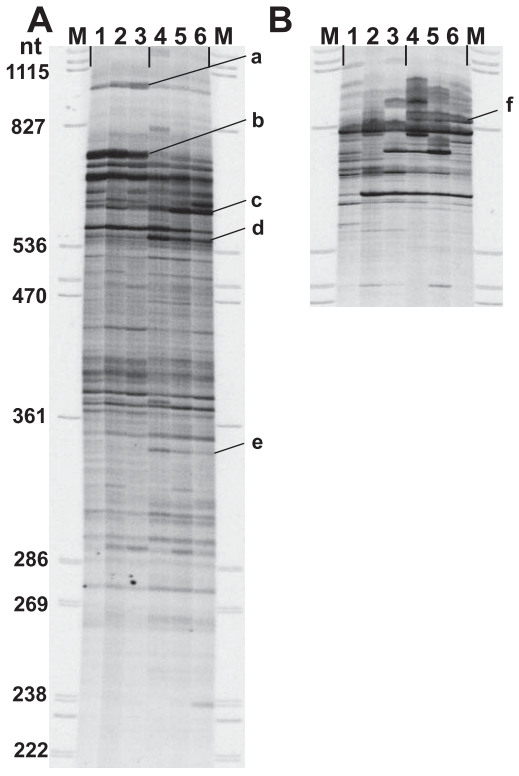

Fig. 1. RISA profiles of the microbial community in rhizospheres of the parental non-transgenic and the transgenic tomato plant line. (A) Bacterial RISA (B) Fungal RISA: M, GeneScan-2500 ROX molecular size markers (Applied Biosystems); lane 1 to 3 and 4 to 6 indicate different plants of parental and transgenic lines, respectively. Triplicate results for each line are shown. Numbers indicate marker fragment lengths.

Sequence analyses of polymorphic DNA bands detected by RISA

All of our bacterial clones either showed some similarity to uncultured strains or harbored partial sequences of known species, but none of the clones revealed a high level of similarity to a known bacterial strain within their overall sequences (Table 3). Clones, isolated from band a, revealed the highest degree of similarity to an uncultured bacterium deposited in the database as a rhizosphere microbe within their overall sequences.

Clones, isolated from band b, showed similarity to three different known sequences in the database. Two of these clones showed partial similarity to almost half of the intergenic spacer region of the rRNA gene in *Burkholderia thailandensis*. Another two clones, identified from band b, showed limited similarity to the tRNA-Ile coding region for *Desulfotalea psychrophila*. Clone AB266367 showed limited similarity to the tRNA coding region of an uncultured bacterium. No significant similarity was observed among the three groups of clones isolated from band d. Similar sequence variability was observed in clones isolated from

Table 2. Summary of the relative abundance of DNA bands differing between the parental and transgenic tomato lines based on RISA and T-RFLP analysis

Band name[a]	Relative abundance (%)	
	Parental line[b]	Transgenic line[b]
Band a	4.0±0.9	1.4±0.2**
Band b	10.1±2.1	2.2±1.1**
Band c	3.6±0.2	6.9±1.7
Band d	2.0±0.2	4.8±1.8
Band e	ND[c]	1.7±1.0
Band f	ND	7.4±1.1
Band g	2.2±0.5	ND
Band h	3.6±1.8	15.0±5.9*

[a] Corresponds to the bands highlighted in Figures 1 and 2.
[b] Mean of the relative abundance (intensity of a band/total intensity×100)±S.D. of three independent plant samples.
[c] Not determined because no DNA band could be detected.
*, ** Statistically significant differences between parental and transgenic lines at the 0.05 and 0.01 level measured using the t-test.

Table 3. Sequence similarities between PCR products amplified from the tomato rhizosphere using RISA and known microbial sequences

Clone ID[a]	Band type[b]	Length (bp)	Score[c]	Expect[d]	DB No.[e]	Organism
AB266364	a	946	745	0	AY484735	Rhizosphere bacterium
AB266365	b	687	396	3.00E-107	CP000085	*Burkholderia thailandensis*
AB266366	b	678	115	2.00E-22	CR522870	*Desulfotalea psychrophila*
AB266367	b	680	141	3.00E-30	AY534293	Uncultured bacterium
AB266368	c	558	250	4.00E-63	AY593480	*Collimonas fungivorans*
AB266369	c	562	119	1.00E-23	AY484727	Uncultured bacterium
AB266370	c	568	137	4.00E-29	DQ192547	*Moorella thermoacetica*
AB266371	d	514	246	6.00E-62	AY713457	*Burkholderia andropogonis*
AB266372	e	307	77.7	2.00E-11	AY934406	Uncultured gamma proteobacterium
AB266373	f	802	268	3.00E-68	AJ544651	*Sellaphora pupula*

[a] DDBJ accession number for representative PCR clones reported in the present study.
[b] The corresponding band types in Figure 1. [c] BLAST search score. [d] BLAST expect score.
[e] The accession number of sequences of the closest relative found in Genbank.

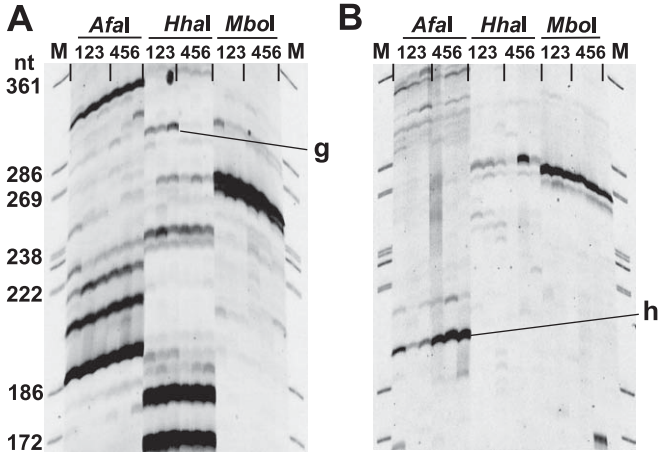

Fig. 2. T-RFLP analyses of bacterial chitinase (A) and *nifH* (B) genes in the microbial community from the rhizosphere of the parental and transgenic tomato plant lines. M, GeneScan-2500 ROX molecular size markers (Applied Biosystems); lanes 1 to 3 and 4 to 6 indicate different plants of parental and transgenic lines, respectively. Triplicate results for each line using three different restriction enzymes are shown. Numbers indicate marker fragment lengths.

band c. Three clones showed 74.4% similarity to *Collimonas fungivorans* within the overall sequence. The remaining two clones showed limited similarity to the tRNA-Ala coding region of both an uncultured bacterium and *Moorella thermoacetica*, respectively. No significant similarity was again observed among these three groups of clones isolated from band c. The clones isolated from band d showed the highest degree of similarity to marginally different sequences deposited in the database, which were mainly limited to tRNA coding regions. The clones isolated from band e showed limited similarity to a portion of the 16S rRNA gene from an uncultured gamma proteobacterium.

During the cloning and sequencing of the fungal RISA amplicons, there was some difficulty with band f, from which only 4 of 24 clones were successfully obtained based on selection for insert sizes in the plasmids. All four of these clones showed the highest level of similarity to a portion of the 18S rRNA and 5.8S rRNA gene regions of *Sellaphora pupula*.

T-RFLP analyses of bacterial chitinase and nifH genes

The T-RFLP analysis of both the bacterial chitinase gene and the *nifH* gene revealed a high degree of similarity for the overall patterns among the samples following treatment with all three restriction enzymes and regardless of genotype (Fig. 2). The numbers of DNA bands detected for the

chitinase and *nifH* genes were approximately 20 and 10, respectively. From the *Hha*I-digestion profile of the chitinase gene, one band was shown to be specific to the parental line as it was undetectable in the transgenic line at the corresponding location (band g in Fig. 2-A). From the T-RFLP analysis of the *nifH* gene, a lower number of bands and a greater degree of variability in both number and intensity was evident, compared with the T-RFLP profile of the chitinase gene. Increased intensity was observed in one band for the *Afa*I profile of the transgenic line (band h in Fig. 2-B), and its relative abundance was statistically higher than in the parental line (Table 2).

Molecular characterization of polymorphic DNA fragments by T-RFLP of functional bacterial genes

Eighteen out of the 192 clones corresponding to the polymorphic DNA band detected by the T-RFLP analysis of the bacterial chitinase gene were sequenced, and all of these species showed similarities ranging from 60 to 64% to a bacterial endochitinase gene, ChiC, of *Microbulbifer hydrolyticus* (AY646088). All of these clones were 361 bp in size. Based on the deduced amino acid sequences, the similarity among these clones ranged from 94.2 to 100% and 13 unique amino acid sequences were obtained. Phylogenetic analysis further revealed that these clones belong to the group of streptomyces-like chitinases and form a distinct cluster from other chitinases in known species (Fig. 3).

From the *nifH* gene profiles, eleven out of 192 clones corresponding to the polymorphic DNA band in the T-RFLP analysis were screened, and all showed a high degree of similarity (81 to 91%) to the *nifH* of *Nostoc punctiforme* (ZP 00112319). All of these clones were 406 bp in size and based on their deduced amino acid sequences, the similarity between them ranged from 94.8 to 100%. In total, seven unique amino acid sequences were obtained. Phylogenetic analysis revealed that these seven sequences belong to a group of cyanobacteria and form a distinct cluster from the other *nifH* genes in this group (Fig. 4).

Discussion

Several studies were initially conducted on microbial communities in the rhizospheres of transgenic plants due to concerns regarding possible, although unintentional, deleterious effects on soil microbial communities as a result of the introduction of antimicrobial genes[1,23,24,32]. More recently, it

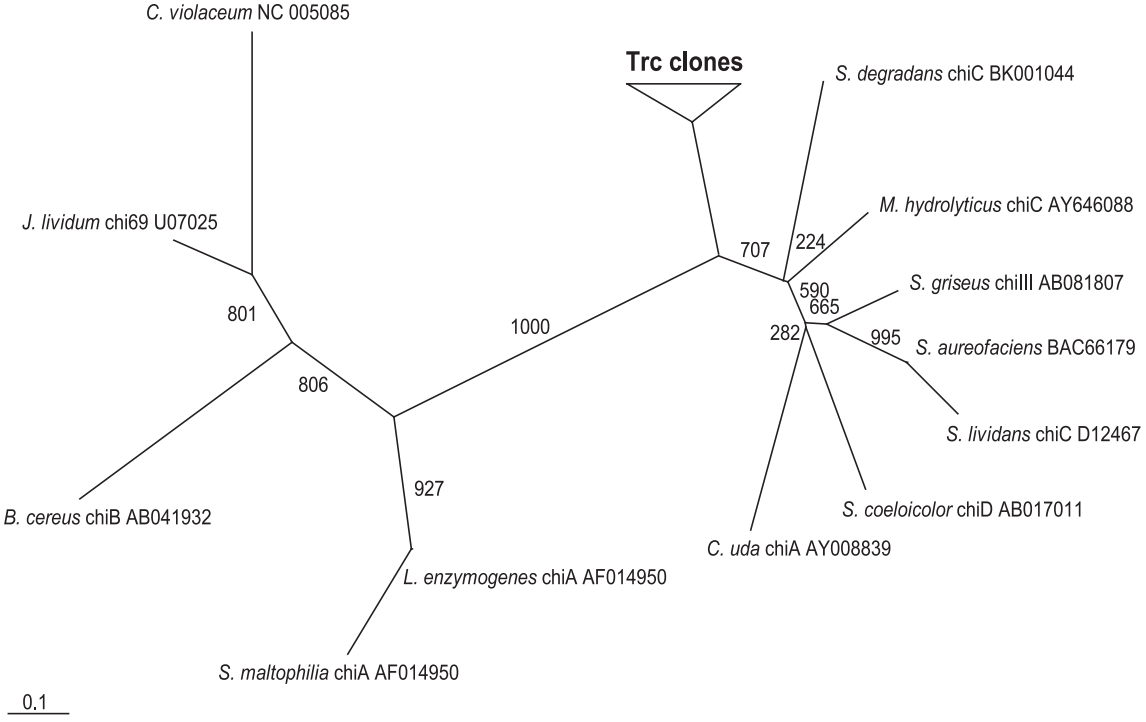

Fig. 3. Phylogenetic tree of bacterial chitinases constructed by the neighbor-joining method. The scale represents 0.1 substitutions per site. The numbers at the nodes are the proportions of 1000 bootstrap resamplings that support the topology shown. The bacterial chitinase gene clones (Trc clones) isolated in the present study are shown as a triangle indicating that they form a collapsed branch.

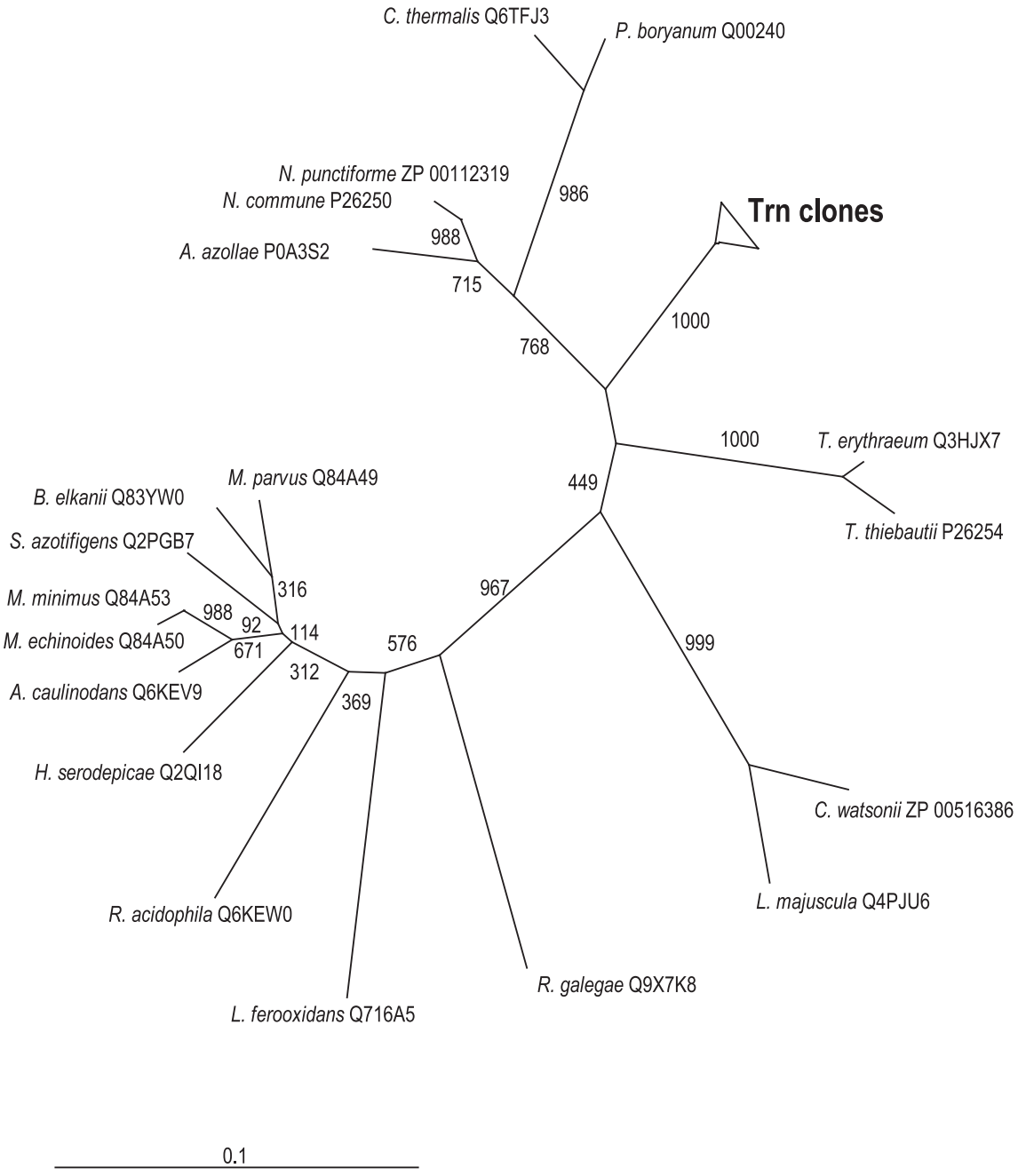

Fig. 4. Phylogenetic tree of *nifH* genes constructed by the neighbor-joining method. The scale represents 0.1 substitutions per site. The numbers at the nodes are the proportions of 1000 bootstrap resamplings that support the topology shown. The *nifH* gene clones (Trn clones) isolated in the present study are shown as a triangle indicating that they form a collapsed branch.

appears that unintended genotypic changes can be introduced into each transgenic plant, regardless of the *a priori* effects of transgenes caused by the transformation events and by ectopic expression, and could cause structural changes within microbial communities of their rhizospheres[14,42,21]. The results of these studies demonstrated that the genotypes of transgenic plants could have an impact on their associated microbial community structures. However, the degree of change is small compared with that caused by either seasonal changes, plant growth stages or other environmental factors[21,43]. In any event, analysis of subtle changes to microbial communities in transgenic

plants has the potential to provide an investigative tool for the study of plant-microbe interactions in the phytosphere, including the rhizosphere, particularly in relation to unculturable microbes.

In the present study, we examined the impact of a HMGR transgene introduced into a tomato plant on the microbial community structure in the rhizosphere, since it was expected that the genetic modification of secondary metabolic pathways would influence plant-microbe interactions[56]. As anticipated, both bacterial and fungal community analyses revealed structural differences in the microbial profiles between the parental and transgenic lines (Fig. 1). The subsequent sequence analysis revealed that differential bands cloned in RISA showed low levels of similarity to known species but were most likely derived from uncultured microbes. In addition, considerable sequence variability was observed in some clones derived from a differential band, such as band b in Figure 1, indicating that some of these polymorphic bands may be composed of different microorganisms.

Microbial community analyses using ribosomal RNA gene regions have become standard techniques due to the phylogenetic information that can be obtained and because of the considerable accumulation of the corresponding sequences from a wide variety of organisms in the current public databases. These techniques are therefore likely to be useful for analyzing overall microbial diversity, but may not prove to be as effective in assessing minor populations such as *Streptomyces* species, which have important ecological roles in nature. Moreover, these types of analyses will not be appropriate for the study of some functional microbial groups which can not be distinguished by the phylogeny of rRNA-related sequences. These are fundamental criticisms that have been directed at techniques that analyze microbial community profiles via rRNA gene regions, particularly when these methods have been applied to complex biological materials such as soil as in the present study.

Because of the limitations associated with microbial community analyses using rRNA gene regions, we also employed T-RFLP analyses in the current experiments for two functional bacterial genes currently available for microbial community analyses. We chose the bacterial chitinase gene and the *nifH* gene because of their potential importance in the rhizosphere. The T-RFLP analysis of both genes revealed bands differing between the parental and transgenic tomato lines (Fig. 2). Subsequent sequence analyses suggested that the differential bands in the T-RFLP profiles may reflect the presence or absence of specific bacterial groups in the rhizosphere. This indicated that the T-RFLP analysis of functional genes may be a feasible way to study the microbial community analysis in the rhizosphere.

The bacterial chitinase clones specifically identified in the parental tomato line in the present study belong to the group of Streptomyces like chitinases. The *Streptomyces* group is generally thought to be important in the rhizosphere, and several species have been shown to be antagonists for fungal pathogens and possess antifungal activities, including chitinase activity[54,55]. Although we detected only one differential band in our T-RFLP analysis, the genotypic changes in the transgenic line may have no obvious influence in terms of disease control, since the overall profiles of the parental and transgenic lines were almost identical and there are several known antagonists which could have redundant roles in the rhizosphere[2,37].

Recently, several non-symbiotic diazotrophs have been recognized as plant growth-promoting bacteria in the rhizosphere[29], and are predicted to be potential biofertilizers that could be utilized for commercial agriculture. A marker gene for N fixation in these diazotrophs is *nifH*, which codes for the small subunit of nitrogenase reductase[6]. In our current T-RFLP analysis of the *nifH* gene, we observed one differential band showing an increased intensity in the transgenic line. Sequence analysis revealed that the clones derived from this differential band belong to a member of the cyanobacteria. It has been reported that cyanobacteria can colonize plant roots, and that the growth of mangrove seedlings can be enhanced by the inoculation of a filamentous cyanobacterium, *Microcoleus chthonoplastes*[7,47]. Interestingly, and regardless of the slight suppression of total plant growth, the transgenic tomato examined in the present study showed an increase in fruit size and a higher chlorophyll content in leaves compared with the parental line, under greenhouse conditions[31]. Hence, the interpretation of phenotypic evaluations of transgenic plants may need to be undertaken with some caution under non-sterilized conditions, since the abundance or presence/absence of certain groups of microbes in the rhizosphere may also affect diverse plant phenotypes such as rate of growth, the uptake of various nutrients, and biotic/abiotic stress resistance.

In conclusion, transgenic plants with modifications to secondary metabolic pathways such as isoprenoid biosynthesis are likely to be a valuable resource for analyzing the microbial community in the rhizosphere. Furthermore, in addition to microbial community analyses using rRNA gene regions, T-RFLP analyses of functional genes, such as the bacterial chitinase and *nifH* genes, could also be successfully utilized for the functional analysis of microbial communities in the rhizosphere. Hence, the combination of a

wide variety of plant genotypes, including transgenic plants, and the availability of diverse methodologies for analyzing microbial communities will facilitate future challenging investigations of plant-microbe interactions in rhizospheres.

References

1) Ahrenholtz, I., K. Harms, J. de Vries and W. Wackernagel. 2000. Increased killing of *Bacillus subtilis* on the hair roots of transgenic T4 lysozyme-producing potatoes. Appl. Environ. Microbiol. **66**: 1862–1865.
2) Akutsu, K., A. Hirata, M. Yamamoto, K. Hirayae, S. Okuyama and T. Hibi. 1993. Growth inhibition of *Botrytis* spp. by *Serratia marcescens* B2 isolated from tomato phylloplane. Ann. Phytopath. Soc. Jpn. **59**: 18–25.
3) Altschul, S.F., W. Gish, W. Miller, E.W. Myers and D. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. **215**: 1651–1656.
4) Altschul, S.F., T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller and D.J. Lipman. 1997. "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. **25**: 3389–3402.
5) Amann, R.I., W. Ludwig and K.H. Schleifer. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59**: 143–169.
6) Bairoch, A. 1993. The ENZYME data bank. Nucleic Acids Res. **21**: 3155–3156.
7) Bashan, Y., M.E. Puente, D.D. Myrold and G. Toledo. 1998. In vitro transfer of fixed nitrogen from diazothophic filamentous cyanobacteria to black mangrove seedlings. FEMS Microbiol. Ecol. **26**: 165–170.
8) Brusetti, L., P. Francia, C. Bertolini, A. Pagliuca, S. Borin, C. Sorlini, A. Abruzzese, G. Sacchi, C. Viti, L. Giovannetti, E. Giuntini, M. Bazzicalupo and D. Daffonchio. 2004. Bacterial communities associated with the rhizosphere of transgenic Bt 176 maize (*Zea mays*) and its non transgenic counterpart. Plant Soil **266**: 11–21.
9) Cardinale, M., L. Brusetti, P. Quatrini, S. Boron, A.M. Puglia, A. Rizzi, E. Zanardini, C. Sorlini, C. Corselli and D. Daffonchio. 2004. Comparison of different primer sets for use in automated ribosomal intergenic spacer analysis of complex bacterial communities. Appl. Envrion. Microbiol. **70**: 6147–6156.
10) Chappell, J. 1995. The biochemistry and molecular biology of isoprenoid metabolism. Plant Physiol. **107**: 1–6.
11) Chappell, J. 1995. Biochemistry and molecular biology of the isoprenoid biosynthetic pathway in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **46**: 521–547.
12) Choi, D., B.L. Ward and R.M. Bostock. 1992. Differential induction and suppression of potato 3-hydroxy-3-methylglutaryl coenzyme A reductase genes in response to *Phytophthora infestans* and to its elicitor arachidonic acid. Plant Cell **4**: 1333–1344.
13) Cocking, E.C. 2003. Endophytic colonisation of plant roots by nitrogen-fixing bacteria. Plant Soil **252**: 169–175.
14) Dunfield, K.E. and J.J. Germida. 2001. Diversity of bacterial communities in the rhizosphere and root interior of field-grown genetically modified *Brassica napus*. FEMS Microbiol. Ecol. **38**: 1–9.
15) Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution **39**: 783–791.
16) Genschik, P., M.C. Criqui, Y. Parmentier, J. Marbach, A. Durr, J. Fleck and E. Jamet. 1992. Isolation and characterization of a cDNA encoding a 3-hydroxy-3-methylglutaryl coenzyme A reductase from *Nicotiana sylvestris*. Plant Mol. Biol. **20**: 337–341.
17) George, E., H. Marschner and I. Jakobsen. 1995. Role of arbuscular mycorrhizal fungi in uptake of phosphorous and nitrogen from soil. Crit. Rev. Biotechnol. **15**: 257–270.
18) Germida, J.J., S.D. Siciliano, J.R. de Freitas and A.M. Seib. 1998. Diversity of root-associated bacteria associated with field grown canola (*Brassica napus* L.) and wheat (*Triticum aestivum* L.). FEMS Microbiol. Ecol. **26**: 43–50.
19) Grayston, S.J., S. Wang, C.D. Campbell and A.C. Edwards. 1998. Selective influence of plant species on microbial diversity in the rhizosphere. Soil. Biol. Biochem. **30**: 369–378.
20) Goodman, R.M., S.B. Bintrim, J. Handelsman, B.F. Quirino, J.C. Rosas, H.M. Simon and K.P. Smith. 1997. A dirty look: soil microflora and rhizosphere microbiology, pp. 219–234. *In* H.E. Flores, J.P. Lynch and D. Eissenstat (eds.), Radical biology: advances and perspectives on the function of plant roots. American Society of Plant Physiologist, Rockville, Md.
21) Gyamfi, S., U. Pfeifer, M. Stierschneider and A. Sessitsch. 2002. Effects of transgenic glufosinate-tolerant oilseed rape (*Brassica napus*) and the associated herbicide application on eubacterial and *Pseudomonas* communities in the rhizosphere. FEMS Microbiol. Ecol. **41**: 181–190.
22) Hansgate, A.M., P.D. Schloss, A.G. Hay and L.P. Walker. 2005. Molecular characterization of fungal community dynamics in the initial stages of composting. FEMS Microbiol. Ecol. **51**: 209–214.
23) Heuer, H., R.M. Kroppenstedt, J. Lottmann, G. Berg and K. Smalla. 2002. Effects of T4 lysozyme release from transgenic potato roots on bacterial rhizosphere communities are negligible relative to natural factors. Appl Environ. Microbiol. **68**: 1325–1335.
24) Heuer, H., M. Krsek, P. Baker, K. Smalla and E.M.H. Wellington. 1997. Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. Appl. Environ. Microbiol. **63**: 3233–3241.
25) Ikeda, S., S. Fuji, T. Sato, N. Ytow, H. Ezura, K. Minamisawa and T. Fujimura. 2006. Community analysis of seed-associated microbes in forage crops using culture-independent methods. Microbes Environ. **21**: 112–121.
26) Ikeda, S., D.M. Roberts, K.N. Watanabe and N. Ytow. 2004. Microbial community analyses using a simple, rapid detection method for DNA fingerprints with a fluorescence scanner. J. Biosci. Bioeng. **98**: 500–503.
27) Ikeda, S., K.N. Watanabe, K. Minamisawa and N. Ytow. 2004. Evaluation of soil DNA from arable land in Japan using a modified direct-extraction method. Microbes Environ. **19**: 301–309.
28) Ikeda, S., N. Ytow, H. Ezura, K. Minamisawa and T. Fujimura. 2006. Soil microbial community analysis in the environmental risk assessment of transgenic plants. Plant Biotech. **23**: 137–151.
29) Kennedy, I.R., A.T.M.A. Choudhury and M.L. Kecskés. 2004. Non-symbiotic diazotrophs in crop-farming systems: can their potential for plant growth promotion be better exploited? Soil Biol. Biochem. **36**: 1229–1244.
30) Kent, A.D. and E.W. Triplett. 2002. Microbial communities and their interactions in soil and rhizosphere ecosystems. Annu. Rev.

Microbiol. **56**: 211–236.
31) Kobayashi, T., S. Kato-Emori, K. Tomita and H. Ezura. 2003. Transformation of tomato with the melon 3-hydroxyl-3-methylglutaryl coenzyme A reductase leads to increase of fruit size. Plant Biotechnol. **20**: 297–303.
32) Lottman, J., H. Heuer, K. Smalla and G. Berg. 1999. Influence of transgenic T4-lysozyme-producing potato plants on potentially beneficial plant-associated bacteria. FEMS Microbiol. Ecol. **29**: 365–377.
33) McGarvey, D.J. and R. Croteau. 1995. Terpenoid metabolism. Plant Cell **7**: 1015–1026.
34) Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco cultures. Physiol. Plant **15**: 473–479.
35) Nelson, A.J., P.W. Doerner, Q. Zhu and C.J. Lamb. 1994. Isolation of a monocot 3-hydroxy-3-methylglutaryl coenzyme A reductase gene that is elicitor-inducible. Plant Mol. Biol. **25**: 401–412.
36) Newman, T., F.J. de Bruijin, P. Green, K. Keegstra, H. Kende, L. Mcintosh, J. Ohlrogge, N. Raikhel, S. Somerville, M. Thomashow, E. Retzel and C. Somerville. 1994. Gene Galore: a summary of methods for accessing results from large-scale sequencing of anonymous *Arabidopsis* cDNA clones. Plant Physiol. **103**: 359–370.
37) Ordentlich, A., Y. Elad and I. Chet. 1988. The role of chitinase of *Serratia marcescens* in biocontrol of *Sclerotium rolfsii*. Phytopathology **78**: 84–88.
38) Page, R.D. 1996. Tree View: an application to display phylogenic trees on personal computers. Comput. Appl. Biosci. **12**: 357–358.
39) Park, H.S., C.J. Denbow and C.L. Cramer. 1992. Structural and nucleotide sequence of tomato HMG2 encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase. Plant Mol. Biol. **20**: 327–331.
40) Rösch, C., A. Mergel and H. Bothe. 2002. Biodiversity of denitrifying and dinitrogen-fixing bacteria in an acid forest soil. Appl. Environ. Microbiol. **68**: 3818–3829.
41) Saitou, N. and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4**: 406–425.
42) Siciliano, S.D. and J.J. Germida. 1999. Taxonomic diversity of bacteria associated with the roots of field-grown transgenic *Brassica napus* cv. Quest, compared to the non-transgenic *B. napus* cv. Excel and *B. Rapa* cv. Parkland. FEMS Microbiol. Ecol. **29**: 263–272.
43) Smalla, K., G. Wieland, A. Buchner, A. Zock, J. Parzy, S. Kiser, R. Roskot, H. Heuer and G. Berg. 2001. Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal shifts revealed. Appl. Environ. Microbiol. **67**: 4742–4751.
44) Smith, K.P. and R.M. Goodman. 1999. Host variation for interactions with beneficial plant-associated microbes. Ann Rev Phytopathol **37**: 473–491.
45) Stermer, B.A., G.M. Bianchini and K.L. Korth. 1994. Regulation of HMG-CoA reductase activity in plants. J. Lipid Res. **35**: 1133–1140.
46) Thompson, J.D., D.G. Higgins and T.J. Gibson. 1994. CLASTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Comp Biol **22**: 4673–4680.
47) Toledo, G., Y. Bashan and A. Soeldner. 1995. In vitro colonization and increase in nitorogen fixation of seedling roots of black mangrove inoculated by a filamentous cyanobacteria. Can. J. Microbiol. **41**: 1012–1020.
48) Trolove, S.N., M.J. Hedley, G.J.D. Kirk, N.S. Bolan and P. Loganathan. 2003. Progress in selected areas of rhizosphere on P acquisition. Aust. J. Soil Res. **41**: 471–499.
49) Timonen, S., R.D. Finley RD, S. Olsson and B. Soderstrom. 1996. Dynamics of phosphorous translocation in intact ectomycorrhizal systems: non-destructive monitoring using a B-scanner. FEMS Mcrobiol. Ecol. **19**: 171–180.
50) Wall, D.H. and R.A. Virginia. 1999. Controls on soil biodiversity: insights from extreme environments. Appl. Soil Ecol. **13**: 137–150.
51) Weissenborn, D.L., C.J. Denbow, M. Kaine, S.S. Lang, Z. Yang, X. Yu and C.L. Cramer. 1995. HMG-CoA reductase and terpenoid phytoalexins: molecular specialization within a complex pathway. Plant Physiol. **93**: 393–400.
52) Westover, K.M., A.C. Kennedy and S.E. Kelleys. 1997. Patterns of rhizosphere microbial community structure associated with co-occuring plant species. J. Ecol. **85**: 863–873.
53) Williamson, N., P. Brian and E.M.H. Wellington. 2001. Molecular detection of bacterial and stremptomycete chitinases in the environment. Antonie Leeuwenhoek **78**: 315–321.
54) You, M.P., K. Sivasithamparam and D.I. Kurboke. 1996. Actinomycetes in organic mulch used in avocado plantations and their ability to suppress *Phytophthora cinnamomi*. Biol. Fertil. Soils, **22**: 237–242.
55) Yuan, W.M. and D.L. Crawford. 1995. Characterization of *Streptomyces lydicus* WYEC 108 as a potential biocontrol agent against fungal root and seed rots. Appl Environ. Microbiol. **61**: 3119–3128.
56) Zhao, J., L.C. Davis and R. Verpoorte. 2005. Elicitor signal transduction leading to production of plant secondary metabolites. Biotechnol. Adv. **23**: 283–333.