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Application of Terminal Restriction Fragment Length Polymorphism (T-RFLP) Analysis to Monitor Effect of Biocontrol Agents on Rhizosphere Microbial Community of Hot Pepper (*Capsicum annuum* L.)

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Microbial communities in hot pepper (*Capsicum annuum* L.) cultivation fields under different cultivation methods were investigated by terminal restriction fragment length polymorphism (T-RFLP) analysis. Rhizosphere soil and leaf samples were collected from control, conventional and nature-friendly cultivation fields between May and July, 2009. Two *Bacillus subtilis* strains were applied to nature-friendly cultivation fields as biocontrol agents during the sampling period. Relative abundances of bacteria and plant pathogenic fungi related T-RFs were also measured to monitor the effect of biocontrol agents on potential plant pathogenic fungi. In the principal component analysis (PCA) based on T-RFLP profiles, the microbial communities from rhizosphere soil samples in July, including bacteria and fungi, showed distinct difference between nature-friendly cultivation fields and other cultivation fields. However, there was no correlation between cultivation methods and leaf microbial communities at any sampling period. Changes in the abundance of bacteria related T-RF in the rhizosphere of nature-friendly cultivation fields were observed clearly two months after application of biocontrol agent, while the abundance of plant pathogenic fungi related T-RFs significantly decreased.

***Keywords*:** terminal restriction fragment length polymorphism, nature-friendly cultivation, *B. subtilis*, biocontrol

Terminal restriction fragment length polymorphism (T-RFLP) analysis is one of culture-independent approaches that have been developed to examine microbial diversity of various habitats (Liu *et al*., 1997). Since differences in T-RFs represent the sequence polymorphism of 16S rDNA, the pattern of T-RFs reflects the composition of communities as molecular fingerprints of microbial populations (Edel-Hermaan *et al*., 2004; Wang *et al*., 2004; Culman *et al*., 2008). T-RFs also have been applied as quantitative indicators of the relative abundance of microorganisms in microbial communities based on its peak height and peak area (Blackwood *et al*., 2003; Castro *et al*., 2005; Jernberg *et al*., 2005; Sánchez *et al*., 2006).

Biocontrol agents have been widely used in agricultural fields to reduce plant disease, and commercialized for practical applications (Paulitz and Belanger, 2001; Fravel, 2005; Haas and Defago, 2005). While the identification of microbial populations associated with plant pathogen suppression is important for the development of sustainable biocontrol strategies (Yin *et al*., 2003; Mazzola, 2004; Borneman and Becker, 2007), monitoring the changes of microbial communities is necessary in understanding the ecology of disease management (McSpadden Gardener and Weller, 2001; Whipps, 2001; Bankhead *et al*., 2004; Berg *et al*., 2005). Since antagonistic functions of microbial stains interact with biological and environmental factors, especially microbial populations of crop fields, monitoring biocontrol agent strains after field application is also necessary in order to evaluate its persistence in fields, as well as its effectiveness in disease management, all of which will be important for the development of suitable application techniques. Molecular methods based on PCR amplification, including RAPD and real-time PCR, have been used for the detection and quantification of antagonistic microbial strains used as biocontrol agents (Schena *et al*., 2002; Joshi and McSpadden Gardener, 2006; Pujol *et al*., 2006; Larena and Melgarejo, 2009). T-RFLP analysis was also useful to study microbial communities and the diversity of biocontrol strains in agricultural samples (Bankhead *et al*., 2004; Benítez *et al*., 2007; Benítez and McSpadden Gardener, 2009).

In addition, T-RFLP analysis provides a cost-effective high-throughput approach to obtain information about the microbial community and for the quantification of the relative abundance of microbial species. In this study, changes of microbial community in the nature-friendly cultivation fields of hot pepper were monitored by T-RFLP analysis after application of two strains of *Bacillus subtilis* as biocontrol agents. The T-RFLP analysis was performed using 16S rDNA for bacteria and internal transcribed spacer (ITS) for fungi.

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Materials and Methods

Cultivation practices

The cultivation of pepper (*Capsicum annuum* L.) was carried out in structured fields located in Chonnam Province from May to July, 2009. Three different practices were applied - cultivation without application of chemicals or biological agents (control), cultivation using chemical agents (conventional), and cultivation using nature-friendly agents. For the conventional cultivation, several chemicals including Ridomil, Deomani (Dongbu HiTek, Korea), Ongdalsaem (Syngenta, Korea), and Hint (Hankook Samgong, Korea) were used as fungicides, while A-pam, Boomerang, Mospiran, Stonet (Syngenta), Setis, Olgami, and Conido (Dongbu HiTek) were also used as insecticides, respectively. For nature-friendly cultivation, *B. subtilis* strains EML-BS1 and EML-CA1 were applied as soil and foliar treatments once a week. A volume of 100 ml solution containing microbial biocontrol agents were sprayed to the plant at the concentration of approximately 1×10^{11} cells/ml.

Isolation of DNAs from rhizosphere soil and leaf samples

Rhizosphere soil and leaf samples were collected from control, conventional, and nature-friendly cultivation fields every month from May to July, 2009. Collected rhizosphere soil samples were sieved using autoclaved cheese cloth. Soil DNA was then isolated from 0.5 g of sieved soil sample using the FastDNA Spin kit for Soil (MP Biomedicals, France). Fresh leaf samples were lyophilized and then ground to powder with autoclaved mortar and pestle. A portion of the 0.5 g ground leaf sample was mixed with 0.8 ml of CTAB solution (100 mM Tris-HCl; pH 8.0, 1.5 M NaCl, 100 mM EDTA; pH 8.0, 100 mM NaH_2PO_4; pH 8.0, 1% cetyltrimethyl ammonium bromide), and then frozen at -70°C and thawed at 60°C 5 times. To this, 50 μl of both achromopeptidase (10,000 units/ml) and lysozyme (50 mg/ml) were added and incubated at 37°C for 2 h. The suspension was then mixed with 0.1 ml of 20% SDS solution and incubated at 65°C for 1 h. The mixture was extracted twice with phenol/chloroform/isoamyl alcohol, and DNA was precipitated with 1/10 volume of 3 M sodium acetate and 2 volume of absolute ethanol. The pellet was washed with 70% ethanol and resuspended in TE buffer (10 mM Tris-HCl; pH 8.0, 1 mM EDTA; pH 8.0). The genomic DNA from the bacterial and fungal strains used in this study was also isolated using the CTAB method described above.

Terminal restriction fragment length polymorphism (T-RFLP) analysis

PCR primers to amplify bacterial 16S rDNA and fungal ITS region were selected based on previous studies (Chelius and Triplett, 2001; Manter and Vivanco, 2007). The bacterial 16S rDNA was amplified with primers 5' FAM-labeled 799f (5′-AAC MGG ATT AGA TAC CCK G-3′) and unlabeled 1492r (5′-GGY TAC CTT GTT ACG ACT T-3′). The fungal ITS region was amplified with primers 5' HEX-labeled ITS1f (5′-CTT GGT CAT TTA GAG GAA GTA A-3′) and unlabeled ITS4r (5′-TCC TCC GCT TAT TGA TAT GC-3′). PCR was performed in 50 μl reaction mixture containing 10 pmol of each primer by using AccuPower PCR PreMix (Bioneer, Korea). Thermal cycling conditions consisted of an initial denaturation at 95°C for 5 min followed by 32 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR products were purified using Gel & PCR purification system (Bioneer) and then 500 ng of purified PCR products were digested with 4 U of *Hha*I (Enzynomics, Korea) for 4 h. The electrophoretic runs of the digested products were performed using ABI 3730XL capillary DNA Sequencer (Applied Biosystems, USA), and the resultant T-RFLP profiles were analyzed using the Peak Scanner Software (version 1.0, Applied Biosystems).

PCR amplification from leaf samples with primers 799f and 1492r gave plant mitochondrial products that were approximately 1,090 bp in length, and bacterial products approximately 735 bp in length as described by Chelius and Triplett (2001). The digestion of mitochondrial products with *Hha*I yielded a T-RF length of 188±1 bp, was therefore subtracted from the T-RFLP profiles of the leaf samples.

In each sample, T-RFs of less than 50 bp and higher than 500 bp were excluded from the analysis to avoid primer peaks and uncertainties in size determination, and the T-RF peak heights were normalized by dividing the peak heights of each T-RF by the sum of the total peak heights of all T-RFs.

Statistical analysis

Principal component analysis (PCA) based on the normalized peak heights of T-RFs was conducted to examine the microbial community structure of each field sample. The analysis of variance (ANOVA) and multiple range test of Duncan (1955) were performed to compare the abundances of bacteria or fungi using the relative peak areas of its T-RFs. All statistical analyses were performed by using the SPSS program (version 10.0.7, SPSS, USA).

Results

Terminal restriction fragments (T-RF) of *B. subtilis* and plant pathogenic fungi

Two different *B. subtilis* strains used as biocontrol agents and five species of plant pathogenic fungi were analyzed to identify their T-RF sizes prior to the investigation of field samples. The two *B. subtilis* strains EML-BS1 and EML-CA1 showed the same T-RF length of 327±1 bp, and each plant pathogenic fungal species showed characteristic T-RF sizes ranging from 171±1 to 390±1 bp. These characteristic T-RFs were also found in the T-RFLP profiles from field samples and designated B1, F1, F2, F3, F4, and F5 (Fig. 1 and Table 1).

Microbial community analysis from rhizosphere soil and leaf samples

Differences among the T-RFLP profiles of amplified bacterial 16S rDNA and fungal ITS from rhizosphere samples taken in July were observed based on the presence or absence of T-RFs. Though some major T-RFs (B1, F1, F2, F3, F4, and F5) were found in all field samples, most of the other T-RFs (indicated

Table 1. Expected sizes of terminal restriction fragment (T-RF) of each strain used in the present study

Strain No.	Species	T-RF (bp)	Related T-RF group from field samples
EML-BS1	*Bacillus subtilis*	327±1	B1
EML-CA1	*Bacillus subtilis*	327±1	B1
KACC 40003	*Colletotrichum gloeosporioides*	171±1	F1
KACC 41083	*Fusarium oxysporum*	329±1	F2
KACC 41065	*Sclerotinia sclerotiorum*	332±1	F3
KACC 42131	*Alternaria alternata*	346±1	F4
KACC 40113	*Rhizoctonia solani*	390±1	F5

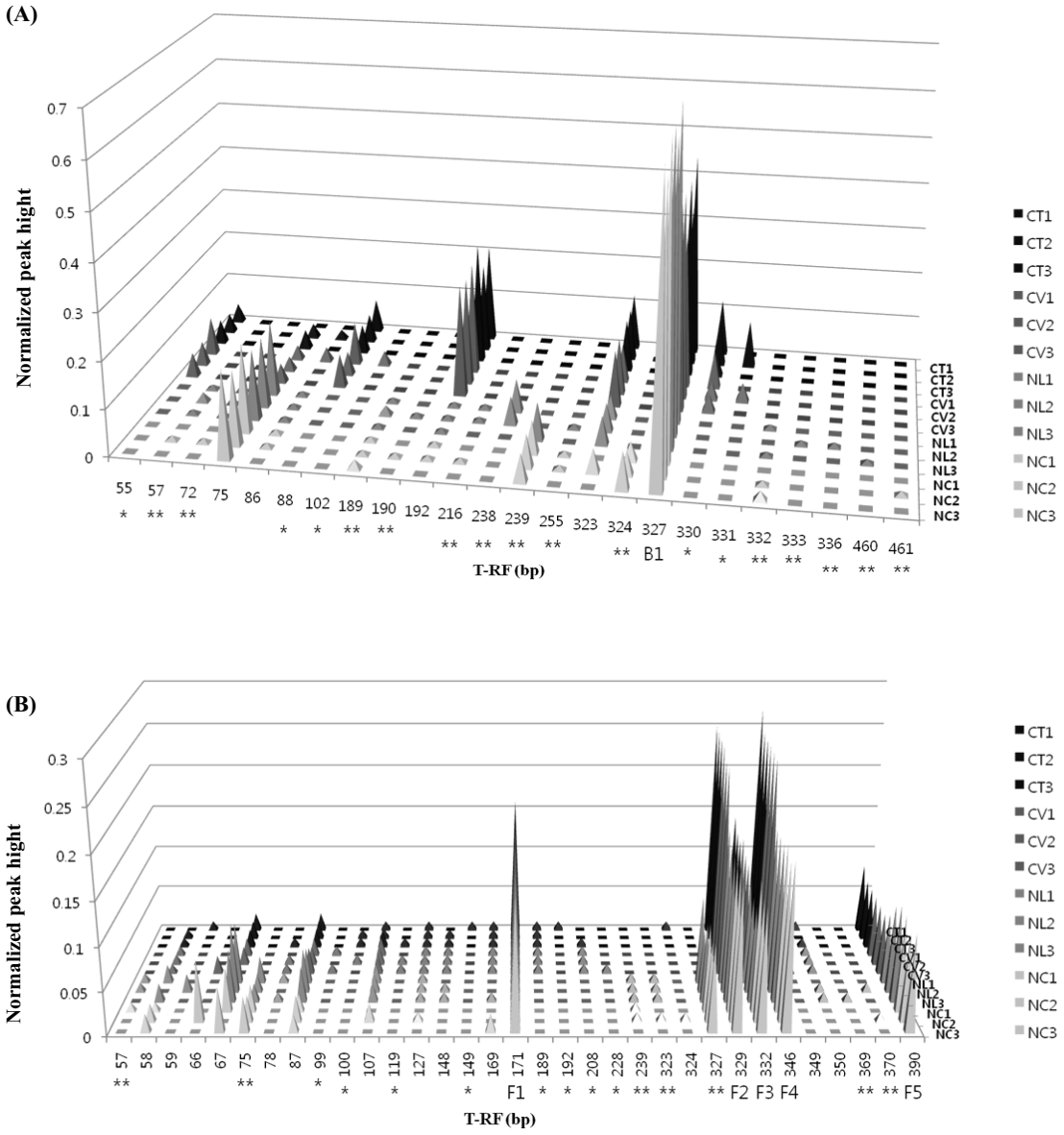

Fig. 1. T-RFLP profiles of amplified PCR amplicons of bacterial 16S rDNA (A) and fungal ITS (B) from rhizosphere soil samples collected in July. Each sample represents three replicates from control (CT1, CT2, CT3), conventional cultivation (CV1, CV2, CV3) and nature-friendly cultivation field with biocontrol stain EML-BS1 (NL1, NL2, NL3) and strain EML-CA1 (NC1, NC2, NC3). Asterisk symbols indicate T-RFs which represent differences between nature-friendly cultivation fields and other cultivation fields by presence or absence of T-RF peaks. B1, F1, F2, F3, F4, and F5 represent major T-RFs found from all samples.

with asterisk symbols in Fig. 1) were variably present between nature-friendly cultivation fields and the other field samples. T-RFs indicated by double asterisk symbols were found only from nature-friendly cultivation field samples (Fig. 1).

Based on T-RFLP profiles of bacterial 16S rDNA and fungal ITS region amplified from each field sample, principal component analysis (PCA) was performed to compare bacterial and fungal community among samples. Although the bacterial or fungal communities from rhizosphere in May and June were divided into a few clusters by PCA, the T-RFLP profiles from different cultivation fields were mixed together, and thus showed no obvious distinction among the cultivation fields. In July, however, there were two clustered groups in the bacterial and fungal communities respectively, one associated with the nature-friendly cultivation field samples NL and NC, the other with the control and conventional cultivation field samples. This indicates that there was a distinct difference in the microbial community structure between nature-friendly cultivation fields and other cultivation fields in July (Fig. 2).

As for the leaf samples collected in May and July, bacterial T-RFLP profiles of most samples were grouped into one cluster, thus showing few variations in the bacterial communities

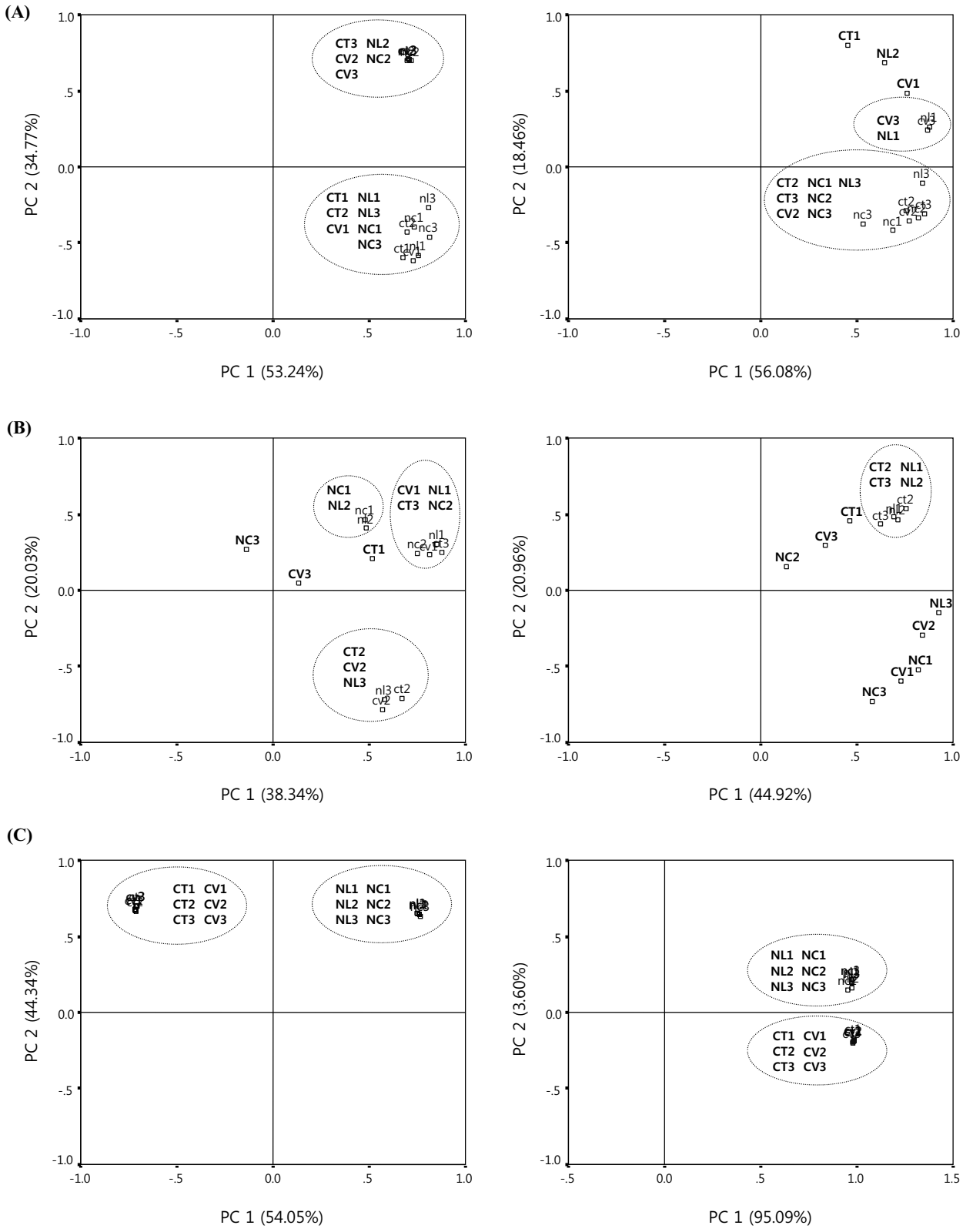

Fig. 2. Principal component analysis of T-RFLP profiles from PCR amplicons of bacterial 16S rDNA (Left) and fungal ITS (Right) which were obtained from rhizosphere soil samples collected in May (A), June (B), and July (C). Each sample represents three replicates from control (CT1, CT2, CT3), conventional cultivation (CV1, CV2, CV3) and nature-friendly cultivation field with biocontrol stain EML-BS1 (NL1, NL2, NL3) and strain EML-CA1 (NC1, NC2, NC3).

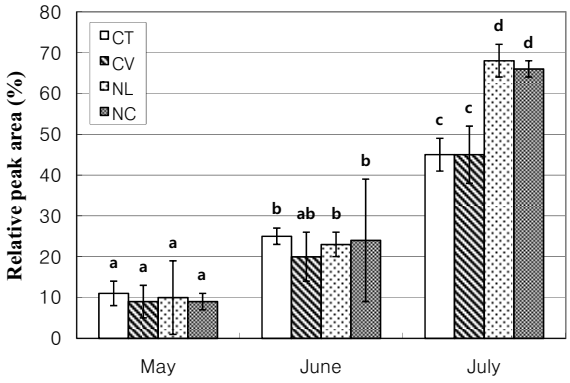

Fig. 3. Relative abundance of T-RF B1 (327±1 bp) obtained from rhizosphere soil samples. Each bar represents the average with standard deviation from three replicated samples of control (CT), conventional cultivation (CV) and nature-friendly cultivation field with biocontrol stain EML-BS1 (NL) and strain EML-CA1 (NC). Bars with the same letter did not differ significantly at $P<0.05$ by ANOVA and the multiple range test of Duncan (1955).

regardless of cultivation fields (data not shown). The fungal community from the leaf samples also formed one cluster in May and two clusters in July, thus showing no significant difference among different cultivation fields (data not shown).

Quantification of bacteria and fungi related T-RFs

The relative peak area of bacteria related T-RF B1 from rhizosphere soil was always less than 20% of the total in samples collected in May, but increased gradually to higher than 40% in the samples collected in July. Notably, nature-friendly cultivation field samples NL and NC collected in July showed around 68% and 66% of relative peak area respectively, while those from the control and conventional cultivation fields represented around 45%. These differences were statistically significant, and indicated higher abundance of T-RF B1 in rhizosphere soil samples taken in July from nature-friendly cultivation fields than from other cultivation fields (Fig. 3). The relative peak areas of T-RF B1 from leaf samples collected in May and July were less than 10% and 30%, respectively, while there was no statistically significant difference in T-RF B1 among different cultivation fields during the whole period of investigation (data not shown). The relative peak areas of T-RF B1 from the leaf samples in May and July were also observed to be smaller than those from rhizosphere soil samples collected at the same periods.

Relative peak areas of the five fungi related T-RFs were also measured each month to monitor the change in fungal communities among the different cultivation fields and the influence of the biocontrol agent on the nature-friendly cultivation fields. From the rhizoshpere soil samples collected in May, only T-RF F2 was observed (Fig. 4A). T-RF F1, F2, and F4 increased gradually in the rhizosphere soil samples collected in June (Fig. 4B). T-RF F3 and F5 were found in rhizosphere soil samples taken in July (Fig. 4C). The relative peak areas of every fungi related T-RF also increased from June to July. Although there were no significant differences in the relative peak areas of fungi related T-RFs among rhizosphere soil samples collected in May and June from different cultivation fields, T-RF F1, F2, and F4 in rhizosphere soil samples taken in July showed smaller peak areas in both nature-friendly cultivation field samples NL and NC than in other cultivation field samples. The relative peak area of T-RF F3 in rhizosphere soil collected in July decreased only in the nature-friendly cultivation field sample NL, while there was no difference in the T-RF among other cultivation fields. In contrast, T-RF F5 showed relatively larger peak area in nature-friendly cultivation fields in July than in control and conventional cultivation fields. These differences among cultivation field samples were statistically supported by ANOVA and the multiple range test of Duncan (1955). As for leaf samples, four fungi related T-RFs (F1, F2, F4, and F5) except T-RF F3 were observed in May, and the numbers of fungi related T-RFs decreased in later period, that is, only T-RF F1 and F4 were found in July. The peak areas of T-RF from leaf samples were more variable than those from rhizosphere soil samples. Significant statistical differences were found in T-RF F4 in July, which showed larger peak area in nature-friendly cultivation field sample NC than other cultivation field samples (data not shown).

Discussion

The effect of biocontrol strains or microbial inoculants on indigenous microbial population has been analyzed using the T-RFLP technique in many other researches. T-RFLP profiles of 16S rDNA sequences showed that shifts in the structure of the rhizosphere bacterial community occurred in samples treated with the *Pseudomonas fluorescens* strain, when compared to untreated samples (Bankhead *et al*., 2004). The change of endophytic actinobacterial diversity was also detected by T-RFLP in wheat plants after treatment of commercial mixed microbial inoculants to soil environment (Conn and Franco, 2004). Similarly, in the present work, an increased abundance of bacteria related T-RF B1 was observed in rhizosphere soil samples that were collected in July from nature-friendly cultivation fields. Furthermore, bacterial and fungal communities of rhizosphere soil from nature-friendly cultivation fields were different from those of other cultivation fields, while some of fungi related T-RFs showed lower abundance in nature-friendly cultivation fields than in other cultivation fields. Increased abundance of specific bacterial T-RFs related with antagonistic function has also been reported from microbial

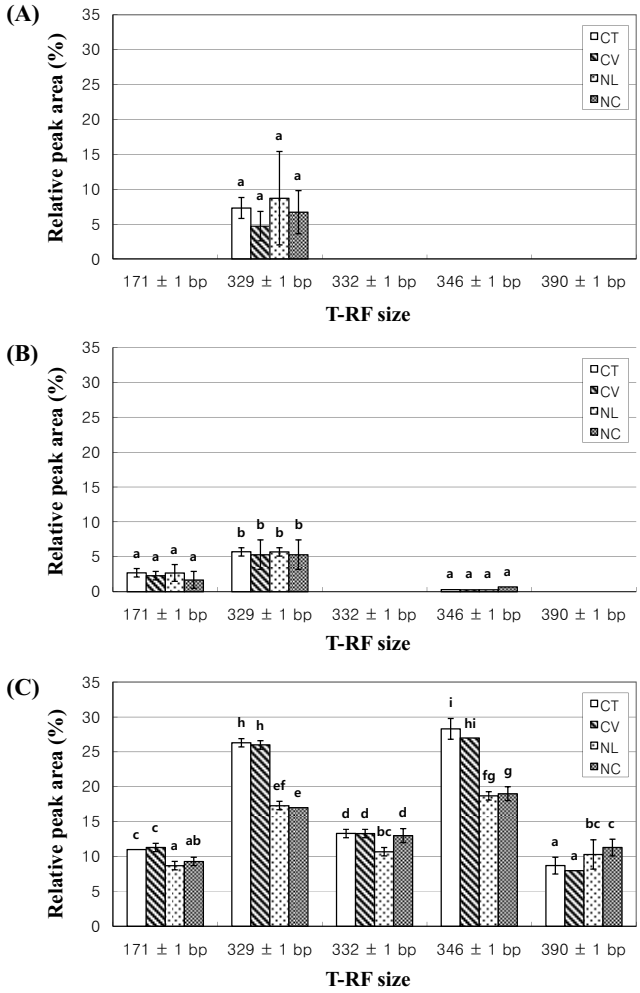

Fig. 4. Relative abundances of T-RF F1 (171±1 bp), F2 (329±1 bp), F3 (332±1 bp), F4 (346±1 bp), and F5 (390±1 bp) that were obtained from rhizosphere soil samples collected in May (A), June (B) and July (C). Each bar represents the average with standard deviation from three replicated samples of control (CT), conventional cultivation (CV) and nature-friendly cultivation field with biocontrol stain EML-BS1 (NL) and strain EML-CA1 (NC). Bars with the same letter did not differ significantly at $P<0.05$ by ANOVA and the multiple range test of Duncan (1955).

populations in plant pathogen suppressive soil (Hjort *et al*., 2007; Benítez and McSpadden Gardener, 2009). Two *B. subtilis* strains EML-BS1 and EML-CA1, used as biocontrol agents for nature-friendly cultivation in the present study, showed the same T-RF length of 327±1 bp corresponding to T-RF B1 (Table 1). Although this T-RF did not successfully distinguish applied strains from other indigenous *B. subtilis* or other bacterial species in fields, higher abundance of T-RF B1 was observed in rhizosphere soil samples in July from nature-friendly cultivation fields than from other cultivation field. Moreover, the abundances of three fungi related T-RFs (F1, F2, and F4) from the same samples decreased compared to those from other samples. These T-RF F1, F2, and F4 were corresponded to the T-RFs of plant pathogenic fungi *Colletotrichum gloeosporioides*, *Fusarium oxysporum*, and *Alternaria alternata*, respectively (Table 1). Even though there were ambiguities in accordance with predicted species based on T-RFs, it could be assumed that applied *B. subtilis* strains and other bacterial species in rhizosphere soil, related with T-RF B1, populated later than June although those biocontrol agent strains had been applied since May. In addition, the effects of treatment on potential plant pathogenic fungi became visible when the estimated relative peak area of T-RF B1 became higher than 60% of the total.

The abundance of T-RF B1 in rhizosphere soil from each cultivation field was observed to increase gradually from May to July. In leaf samples, however, the maximum relative peak area of T-RF B1 was lower than 30%, and no significant difference in the abundance of T-RF was observed from each cultivation field by sampling period or application of biocontrol agents. There was also no significant decrease in the abundance of fungi related T-RFs in leaf samples from nature-friendly cultivation fields, when compared to the T-RFs from other cultivation fields. Hence, bacterial populations represented by T-RF B1, including biocontrol agent strains EML-BS1 and EML-CA1, can be considered to survive better in the soil than in the foliar environment, and that control of potential plant pathogenic fungi was also more effective in soil than in foliar treatments.

T-RFLP analysis showed changes of microbial communities

and increased abundance of bacteria related T-RF B1 in rhizosphere soil samples from nature-friendly cultivation fields after two months from first application, while biocontrol agent *B. subtilis* strains were applied repeatedly at regular intervals. Thus, it may have taken at least a couple of months for applied antagonistic *B. subtilis* strains to adapt themselves to soil rhizosphere system and make an effect on indigenous or plant pathogenic fungal populations. This assumption can also be supported by the decreased abundance of some plant pathogenic fungi related T-RFs from rhizosphere soil samples that were collected in July.

The T-RFLP analysis was useful to investigate the effect of biocontrol agents on microbial communities and to examine the fate of biocontrol agent strains after field application. Furthermore, this approach provided a fast and cost-effective way to examine practical disease management strategies and to monitor potential plant pathogens in cultivation fields under biocontrol programs.

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