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Two-year field monitoring shows little evidence that transgenic potato containing *ABF3* significantly alters its rhizosphere microbial community structure

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Abstract

Background: Plants over-expressing *Arabidopsis ABF3* (abscisic acid-responsive element-binding factor 3) have enhanced tolerance to various environmental stresses, especially drought. Using terminal restriction fragment length polymorphism (T-RFLP) analysis, we compared the rhizosphere-associated structures of microbial communities for transgenic potato containing this gene and conventional "Jopoong" plants.

Results: During a 2-year field experiment, fungal richness, evenness, and diversity varied by year, increasing in 2010 when a moderate water deficit occurred. By contrast, the bacterial richness decreased in 2010 while evenness and diversity were similar in both years. No significant difference was observed in any indices for either sampling time or plant line. Although the composition of the microbial communities (defined as T-RF profiles) changed according to year and sampling time, differences were not significant between the transgenic and control plants.

Conclusions: The results in this study suggest that the insertion of *ABF3* into potato has no detectable (by current T-RFLP technique) effects on rhizosphere communities, and that any possible influences, if any, can be masked by seasonal or yearly variations.

Keywords: Biosafety, Community composition, Drought tolerance, T-RFLP, Water deficit

Background

Plant responses to various environmental stresses are complex and sophisticated processes that involve numerous genes and pathways. These processes are largely controlled by the phytohormone, abscisic acid (ABA). The ABA is an important regulator at several development stages, including germination, vegetative growth, and flowering (Domagalska et al. 2010). It also mediates plant physiological responses to various environmental stresses, e.g., drought, high temperature, or salinity (Tuteja 2007).

A large number of stress-responsive genes are induced by ABA. These genes contain cis-regulatory elements in their promoter regions, such as the abscisic acid-responsive element (ABRE), which is regulated by various upstream transcription factors, or TFs (Tuteja 2007). Among those TFs, a small family of ABRE-binding proteins has been identified, i.e., ABRE-binding factors (ABFs) or ABA-responsive element-binding proteins (AREBs) (Kim et al. 2004). Expression of these proteins is also induced by ABA (Choi et al. 2000). While attempting to generate plants with greater tolerance to various abiotic stresses, Kang et al. (2002) have shown that over-expression of *ABF3* in *Arabidopsis* alters the expression of ABA-responsive genes and produces ABA-related phenotypes. Moreover, expression of this gene in other plants, such as lettuce (*Lactuca sativa*) and rice (*Oryza sativa*), results in enhanced tolerance to abiotic stresses (Vanjildorj et al. 2005).

Arabidopsis ABF3 has been genetically inserted into potato (*Solanum tuberosum* L. subsp. *tuberosum*). Its over-expression has been linked with improved tolerance to abiotic stresses, especially drought (Kim 2007). However,

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biochemical and physiological alterations have also occurred in these transformed plants. Because *ABF3* in *Arabidopsis* acts as an upstream TF in ABA-dependent pathways, controlling the expression of several related genes, one can potentially modify the genetic networks associated with ABA-dependent pathways in potato because of these possible potential interactions. The functions of *ABF3* are not fully understood, and some ancient roles that developed over the evolutionary history of this gene could still remain (Abdeen et al. 2010). Therefore, we cannot exclude possible, unexpected pleiotrophic influences. Because this protein is constitutively expressed (as demonstrated in studies with the cauliflower mosaic virus 35S promoter), those effects can accumulate over time.

These alterations in transgenic potato plants could also affect their local soil microbial communities, either directly or indirectly. It has been shown that the community structure of rhizosphere bacteria associated with transgenic potato plants was significantly different from that of bacteria inhabiting on the rhizosphere of non-transgenic counterpart (Heuer et al. 2002). The direct detection of transgene proteins released into soil from transgenic potato plants has not been reported, presumably due to technical difficulties, but there is some evidence indicating that transgene proteins produced in transgenic plants can be released into the soil, where they can remain active long term. For example, researchers have detected the *Bacillus thuringiensis* (Bt) toxin in root exudates from three events of transgenic corn (*Zea mays* L.) (Saxena et al. 2002), and purified Bt toxin in the soil can remain active against the *Manduca sexta* insect after 234 days (Tapp and Stotzky 1998). Changes in plant physiology during genetic manipulations and ensuing modifications in root exudates can also have indirect consequences. Microbes in the rhizosphere can interact with the plants, with these close interactions thought to occur through rhizo-deposit substances, in particular root exudates (Raaijmakers et al. 2009). In alfalfa (*Medicago sativa* L.), Tesfaye et al. (2001) have found that over-expression of nodule-enhanced malate dehydrogenase, which is involved in malate synthesis, unexpectedly increases the amount and composition of other organic acids, such as citrate, oxalate, succinate, and acetate, in the roots and exudates.

The primary objective of this study was to test if transgenic modification (insertion of *ABF3* gene into potato) makes any influences on associated rhizosphere microbial (bacterial or fungal) communities under field conditions. The terminal restriction fragment length polymorphism (T-RFLP) method allows one to discriminate among the structures of different microbial communities and, in particular, has been successfully applied in comparative studies on microbes inhabiting the rhizosphere of genetically modified plants or their controls (Chun et al. 2012). In the present study, T-RFLP method was utilized to investigate the bacterial and fungal communities in the rhizosphere of transgenic potato plants that constitutively express *Arabidopsis ABF3* and their parental counterparts, control.

Methods

Plants

A transgenic line of potato (Line 207, T_4 generation) was developed by inserting *ABF3* into plants of the non-transgenic cultivar "Jopoong" (Kim et al. 2010). Expression of this group of genes is controlled by the cauliflower mosaic virus 35S promoter which is one of the most widely used, general-purpose constitutive promoters (Odell et al. 1985; Sanders et al. 1987), and the nos terminator. The neomycin phosphotransferase II gene (*nptII*) is also introduced for kanamycin selection.

Climatic data

Data for daily air temperature, rainfall, and water contents at 10- and 30-cm soil depths were obtained for years 2009 and 2010 from the Cheongju Weather Station (Korea Meteorological Administration 2010), located 8 km from our field site. Daily and monthly mean air temperatures and mean precipitation averaged over 30 years (1981–2010) were also obtained.

Using these data, we created two drought indices, monthly percent of normal precipitation (%Normal Precip) and monthly standardized precipitation index (SPI). These indices are widely utilized as indicators of meteorological drought when monitoring precipitation over a specified time period (Mkhabela et al. 2010). The monthly %Normal Precip was calculated as actual monthly rainfall (the data from Cheongju Weather station utilized) divided by the monthly long-term normal (1981–2010) (Mkhabela et al. 2010). The SPI was computed based on the concept of standardized precipitation, which is the difference between total precipitation for a certain period of time and the long-term (30 years) mean total precipitation for that same period, divided by the standard deviation (Tabari et al. 2012).

Site description, experimental design, and soil sampling

Experiments were conducted in a field at the Korea Research Institute of Bioscience and Biotechnology (KRIBB), Cheongju, Republic of Korea (36° 43′ 04″ N, 127° 26′ 07″ E; elevation, 37 m). Soil characteristics at the site are described in Table 1.

The potato lines were arranged in a randomized complete block design (three replicated plots for each line). In 2009, each plot had four planting rows mulched with black plastic film. Tubers from transgenic plants or the Jopoong control (16 tubers each per plot) were planted on 16 April at a depth of 10 to 20 cm. In 2010,

Table 1 Soil characteristics at the experiment site ($n = 3$ for all parameters). Figures in parentheses represent standard errors; different letters indicate significant differences between groups at $p < 0.05$

Soil characteristics	2009		2010	
	Non-transgenic	Transgenic	Non-transgenic	Transgenic
pH	7.00 (0.05)a	7.06 (0.05)a	6.69 (0.03)b	6.80 (0.03)b
Organic matter content (%)	4.0 (0.2)a	4.0 (0.2)a	5.3 (0.2)b	5.2 (0.4)b
N (g/kg)	1.03 (0.18)a	1.15 (0.21)a	1.33 (0.23)a	1.92 (0.33)a
Available P (mg/kg)	271.0 (20.7)a	273.4 (62.0)a	120.8 (16.0)b	148.9(22.4)b
Exchangeable K (mg/kg)	56.3 (12.6)a	52.1 (6.3)a	87.4 (22.0)a	65.9 (10.6)a
Sand (%)	77.8 (1.2)a	77.1 (1.5)a	69.8 (1.3)b	69.1 (1.8)b
Clay (%)	6.7 (0.9)a	6.4 (0.9)a	12.2 (0.02)b	13.2 (1.5)b
Silt (%)	15.5 (0.3)a	16.5 (0.7)a	18.0 (1.3)a	17.6 (0.9)a

tubers from each line (20 tubers per plot) were planted at the same depth on 12 April.

During each growing season, soil samples were collected twice: (1) vegetative growth stage/tuber initiation and (2) tuber maturation. On each date, five plants were chosen per plot. Their entire root systems were carefully excavated and then shaken to remove bulk soil. Any substrates still adhering afterward was considered rhizosphere soil. For processing, 50 g of root samples (10 g per plant) were washed with 200 mL of distilled water. The suspension was centrifuged (4 °C, 11,590 × *g*) for 30 min, lyophilized in a freeze dryer, and passed through a 1-mm mesh sieve prior to T-RFLP analysis.

DNA extraction, PCR, and enzyme digestion

Genomic DNA was extracted from 0.8 g of each soil sample, using FastDNA® Spin Kit for Soil (Qbiogene, USA) according to manufacturer's instructions. The bacterial 16S rRNA genes were amplified by PCR with FAM-labeled forward primers 8F (5′-AGAGTTTGATCTGGCTCAG-3′) and unlabeled reverse primer 1492R (5′-[6FAM]TACGGTTACCTTGTTACGACTT-3′) (Gürtler and Stanisich 1996). Each PCR was performed in 50 μL of a reaction mixture containing 50 ng of template DNA, 0.2 μM of each primer, 2 U of *Taq* DNA polymerase, 1× reaction buffer, and 50 μM of each dNTP. Amplification conditions included an initial denaturation at 94 °C for 3 min; followed by 25 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s, and extension at 70 °C for 2 min; then a final extension at 72 °C for 7 min.

The fungal internal transcribed spacer (ITS) DNA was amplified with the FAM-labeled forward primer EF3RCNL (5′-[6FAM]CAAACTTGGTCATTTAGAGGA-3′) (Lord et al. 2002) and the unlabeled reverse primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990). The reaction mixture contained 100 ng of template DNA, 1 μM of each primer, 2 U of *Taq* DNA polymerase, 1× reaction buffer, and 350 μM of each dNTP. Conditions included an initial denaturation at 94 °C for 2 min; then 30 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min, and extension at 72 °C for 1 min; followed by a final extension at 72 °C for 10 min. The PCR products were purified with a QIAquick PCR Purification Kit (Qiagen, Germany).

The PCR products were digested separately, using one of three restriction endonucleases: *Hae*III (GG'CC), *Hha*I (GCG'C), or *Hinf*I (G'ANTC). Reaction mixtures consisted of 1 μg/μL of purified PCR products, 2 μL of 10× buffer, 2 μL of 10× bovine serum albumin, and 10 U of restriction enzyme. The reactions were conducted at 37 °C for 2 h, followed by inactivation at −20 °C. The resulting digestion products were checked on a 2% agarose gel.

T-RFLP analysis

To determine the length of the digestion products (restriction fragments), we mixed 1.5 μL of each digestion product with 0.6 μL of a size standard and 9 μL of formamide. After the mixture was denatured at 95 °C for 3 min, it was placed on ice for 5 min. The fragments were separated on an automated ABI 3130 DNA sequencer (Applied Biosystems, USA). Sizes of the fluorescently labeled terminal restriction fragments (T-RFs) were determined according to a size standard. GeneMapper® version 3.7 (Applied Biosystems) was used for analyzing sizes and heights of the T-RFs. Only fragments longer than 50 bp and with relative fluorescence units (rfu) >50 were used. For data normalization, individual peak height values were divided by the sum of all peak height values in the corresponding profile. Those results were used to calculate relative peak heights, such that peaks occupying less than 1% of the total height in each sample were excluded. All peaks were aligned via Ribo-Sort (Scallan et al. 2008).

The complexity of our bacterial and fungal communities (T-RFs in the samples) was described according to three indices: species richness, Shannon diversity index, and species evenness. Species richness represents *S*, the number of total T-RFs. The Shannon diversity index was

estimated using the formula $H' = -\Sigma p_i \ln(p_i)$, where p_i is N_i/N (N is the sum of heights of T-RFs). Species evenness was determined as $J' = H'/\ln S$. All indices were calculated in PRIMER 6 (Clarke and Gorley 2006) and compared using factorial ANOVAs in R software (R_Core_Team 2013). It is to note that the T-RFs are not exactly comparable to biological "species," so the word "species" in species indices in the present study does not mean biological species but rather individual T-RFs.

The composition of our bacterial and fungal communities (T-RF profiles) was visualized and compared using a non-metric multidimensional scaling (NMDS) and permutational multivariate ANOVA (PerMANOVA). Data obtained from separate digestive restrictions by the three enzymes were normalized, aligned, and combined into one matrix. Afterward, the T-RFs in each matrix were converted to distance matrices using the Sørenson similarity index so that NMDS could be performed. When conducting PerMANOVA, we considered "genetic line" and "year" as fixed factors, whereas "sampling time" was nested in year and considered as a random factor. In all, 9999 permutations were applied. Both NMDS and PerMANOVA were performed in PRIMER with PerMANOVA add-ons.

Results and discussion

Based on two meteorological drought indices—%Normal Precip and SPI—the range for water supply was normal during the 2009 growing season but showed moderate (and sometimes severe) deficits for a considerable length of time in 2010 (Fig. 1b, c). During those deficit periods in the second year, mean daily air temperatures were generally above the long-term averages (Fig. 1a). Although soil moisture (%volume) fluctuated at 10 cm depth, its pattern was similar to that of %Normal Precip during the growing season in both years (Fig. 1d).

Bacterial richness varied across the 2 years but did not differ between sampling times or genetic lines (Fig. 2a). Both evenness and diversity were similar among sampling years, times, and genetic lines. In the case of fungi, all three indices varied across years, but differences were not significant for either sampling times or genetic lines (Fig. 2b).

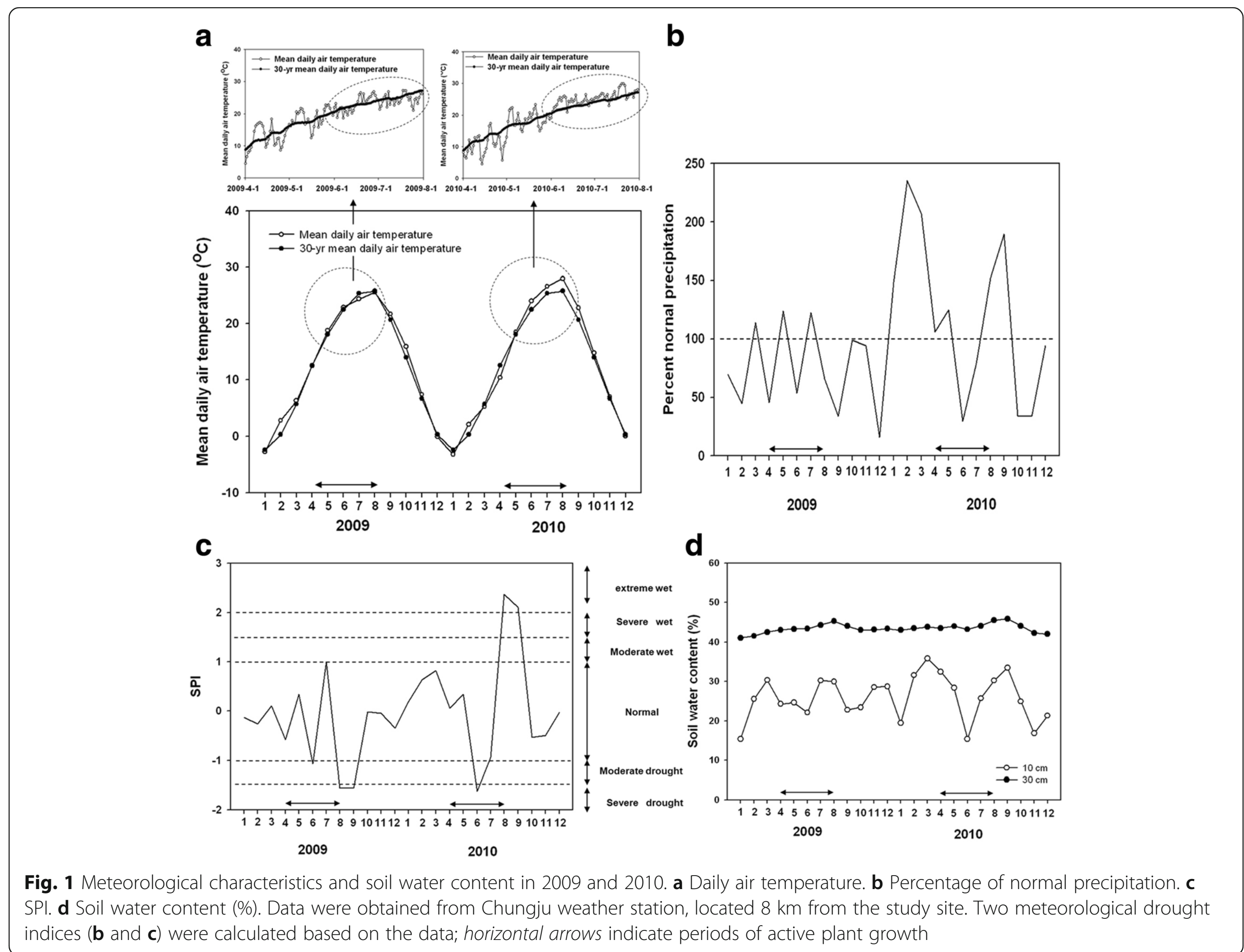

Fig. 1 Meteorological characteristics and soil water content in 2009 and 2010. **a** Daily air temperature. **b** Percentage of normal precipitation. **c** SPI. **d** Soil water content (%). Data were obtained from Chungju weather station, located 8 km from the study site. Two meteorological drought indices (**b** and **c**) were calculated based on the data; *horizontal arrows* indicate periods of active plant growth

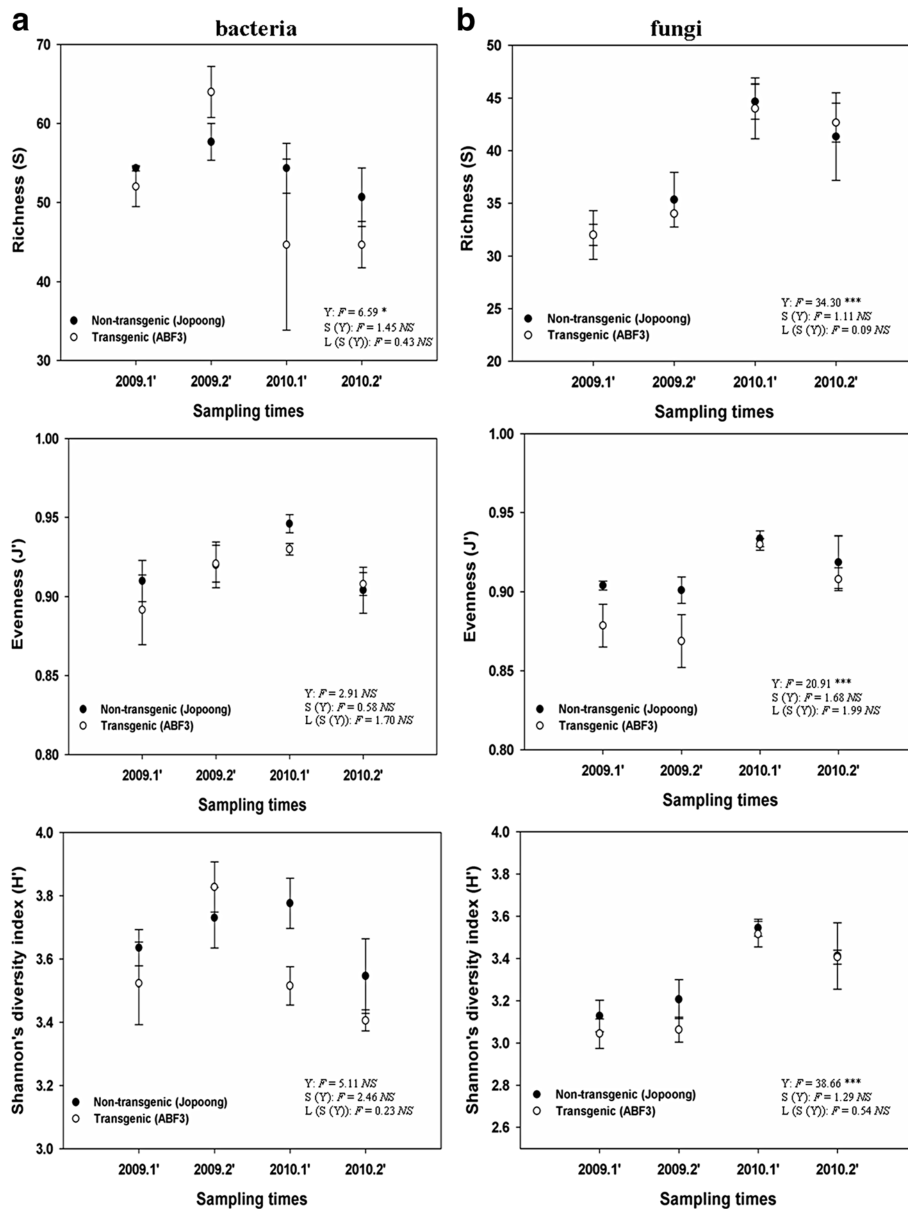

Fig. 2 Richness, evenness, and diversity of **a** bacterial and **b** fungal communities associated with rhizospheres of "Jopoong" control plants and transgenic potatoes containing *ABF3*. Individual T-RF was considered as species; richness was defined as number of T-RFs in sample. On *x*-axis, *2009.1* indicates first sampling in 2009. *Y* year, *S* sampling time, *L plant line*, *NS* non-significant difference. *F* values were obtained from two-way ANOVAs; * represents significant differences between groups at $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$

The structure of the bacterial and fungal community (T-RF profiles) associated with the potato plants varied according to year and sampling time but did not differ between the ABF3 transgenics and the Jopoong control plants (Fig. 3, Table 2).

Conflicting results have arisen from investigations of the response by such soil community to drought. For example, a decrease in water availability can reduce the richness and diversity of bacteria but have no effect on fungi (Barnard et al. 2013). In contrast, fungi can be more sensitive than bacteria to stress conditions (Bapiri et al. 2010). Although the reasons for this non-consensus are unknown, the resistance by bacteria or fungi that arises from water deficits is often attributed to physiological adaptations, e.g., sporulation (Landesman and Dighton 2010). Here, our soil bacteria appeared to be susceptible to a water deficit while the fungi were relatively resistant. The richness of bacterial species was greater in 2009 (under normal precipitation) than in 2010 (during a moderate water deficit) (Fig. 1). However, the indices for

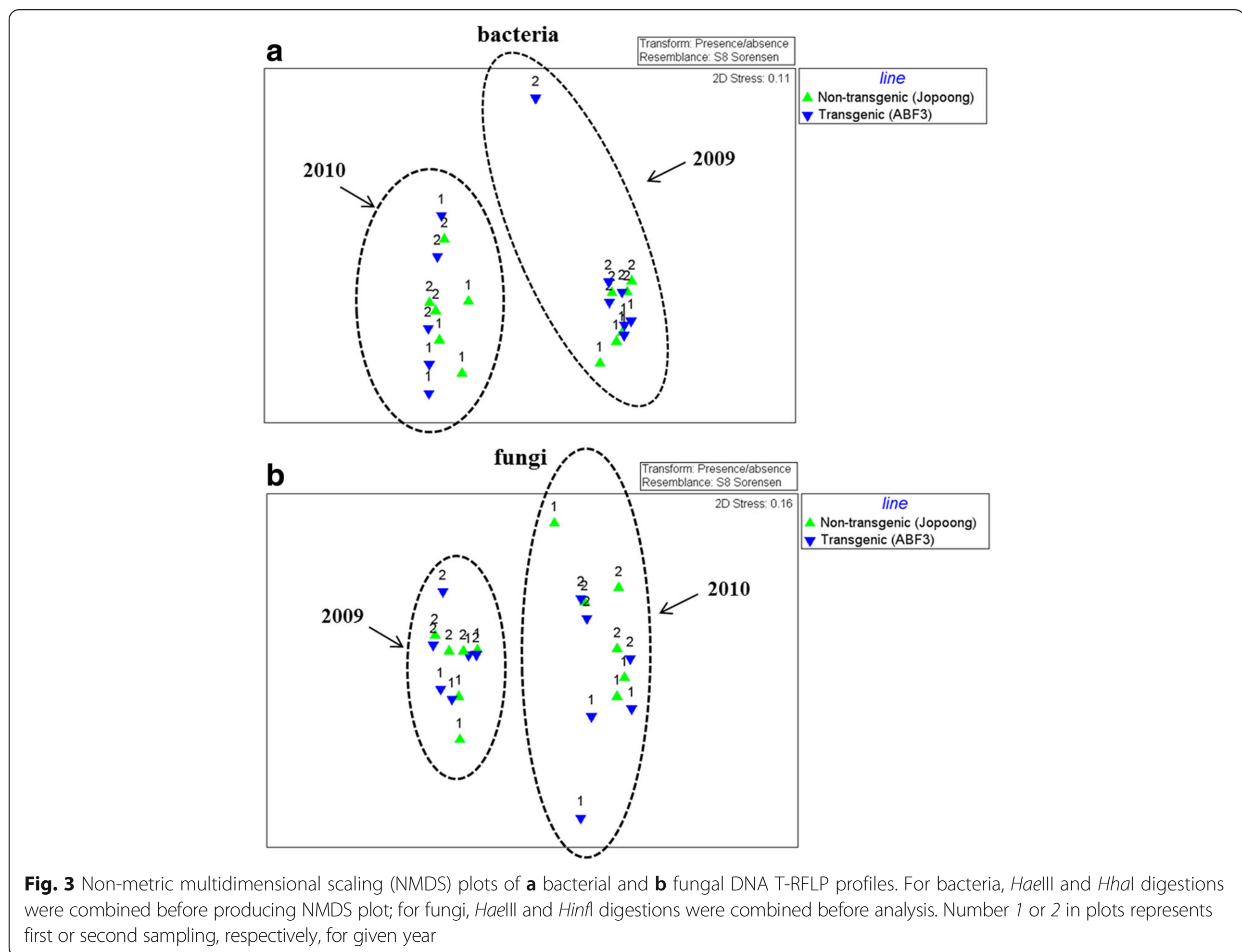

Fig. 3 Non-metric multidimensional scaling (NMDS) plots of **a** bacterial and **b** fungal DNA T-RFLP profiles. For bacteria, *Hae*III and *Hha*I digestions were combined before producing NMDS plot; for fungi, *Hae*III and *Hinf*I digestions were combined before analysis. Number *1* or *2* in plots represents first or second sampling, respectively, for given year

evenness and diversity did not differ between those two years. Because the community structures (T-RF profiles) varied between years (Fig. 3, Table 2), we might infer that a few bacteria, presumably the most abundant, may have been affected by drought conditions in 2010 while most of the others underwent only a small magnitude of change, possibly also favoring some minor species. For example, Barnard et al. (2013) have reported that populations of most bacteria do not vary much according to drying and rewetting cycles whereas considerable changes are found with Actinobacteria and Acidobacteria, the two most abundant bacterial groups. This leads to parallel stress responses by bacterial communities that have different compositions, soil types, and local microclimates.

In the case of fungi, most species here seemed to be resistant to stress conditions in 2010, with some even appearing to benefit, as manifested by the higher values for all diversity indices in 2010. Our results are comparable to those of Hawkes et al. (2011), whose experiments showed that the soil fungal community was more abundant and diverse under drought. Those authors speculated that environmental stresses, such as drought, may have moderated the fungal competition, leading to an increase in the diversity and abundance of soil fungi.

Nevertheless, we might also attribute the greater fungal diversity in 2010 to the higher amount of organic matter that year. This is because fungi are influenced by organic substrate inputs (Chen et al. 2012). Here, although we conducted all of our experiments at the same location, some soil properties, such as pH, available P, and organic matter content, differed between 2009 and 2010. These variations could also have affected the rest of the soil microbial community. For example, bacteria are generally favored under a high soil pH (Pietri and Brookes 2009), and gram-negative bacteria are also competitive in a nutrient-rich environment (Leckie et al. 2004). Therefore, we cannot discount the additional possibility that the lower richness of bacteria in 2010 was associated with a slight but significant decline in soil pH coupled with a lower availability of soil P.

The T-RF profiles of the bacterial and fungal community noted here varied depending on year and sampling time (Fig. 3, Table 2). Our results are comparable to those obtained in earlier studies (Chun et al. 2012 and

Table 2 Results of permutational ANOVAs to investigate the effects of plant line (line), sampling time (time) and year (year) on the structure of rhizosphere microbial communities associated with untransformed and transgenic potatoes grown in an experimental field in 2009 and 2010. Analyses were based on Sørenson similarity matrices of square root arcsine-transformed data. All tests utilized 9999 permutations under the reduced model. Significant *p* values are italicized

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
(a) Bacteria							
Line	1	1623.2	1623.2	1.453	0.2987	799	0.2465
Year	1	23,139	23,139	3.9106	0.3356	3	*0.0271*
Time (year)	2	11,834	5916.9	3.9862	*0.0001*	9902	0.0001
Line × year	1	1908.3	1908.3	1.7082	0.2385	799	0.1775
Line × time (year)	2	2234.4	1117.2	0.75264	0.7867	9896	0.7033
Residuals	16	23,750	1484.4				
Total	23	64,488					
(b) Fungi							
Line	1	1623.2	1623.2	1.453	0.2987	799	0.2465
Year	1	23,139	23,139	3.9106	0.3356	3	*0.0271*
Time (year)	2	11,834	5916.9	3.9862	*0.0001*	9902	0.0001
Line × year	1	1908.3	1908.3	1.7082	0.2385	799	0.1775
Line × time (year)	2	2234.4	1117.2	0.75264	0.7867	9896	0.7033
Residuals	16	23,750	1484.4				
Total	23	64,488					

df degrees of freedom, *P*(*MC*) Monte Carlo *p* value used when the number of unique perms was too low

references therein). These variations are often attributed to fluctuations in environmental factors such as temperature and soil moisture. Plant-related factors, e.g., variations in root-released products associated with a particular developmental stage, are also major determinants. However, we found that the bacterial and fungal communities did not differ, whether associated with transgenic or control potato plants. Therefore, these results indicate that the genetic insertion of this gene into potato exerts little, if any, influence on the bacterial and fungal communities present in those rhizospheres. Furthermore, any possible minor affects may be masked by variations caused by seasonal or yearly factors.

Conclusions

Much effort and resulting significant development have been made to overcome various environmental stresses such as drought in order to improve productivity of crop plants, especially by utilizing genetic engineering. However, concerns associated with (intended or unintended) environmental implications due to artificial genetic modifications are also increasing. This study investigates possible impacts on soil rhizosphere microbial (bacterial and fungal) community due to physiological or biochemical changes in potato after genetic modifications. The results of this study suggest that the insertion of *ABF3*, a gene possibly involved in drought tolerance in *Arabidopsis*, into potato has no detectable (by current T-RFLP technique) effects on rhizosphere microbial communities, and that any possible influences, if any, can be masked by seasonal or yearly variations.

Abbreviations

%Normal Precip: Monthly percent of normal precipitation; ABA: Abscisic acid; *ABF3*: Abscisic acid-responsive element-binding factor 3; ABRE: Abscisic acid-responsive element; AREBs: ABA-responsive element-binding proteins; SPI: Monthly standardized precipitation index; TFs: Transcription factors; T-RFLP: Terminal restriction fragment length polymorphism

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Availability of data and materials

Please contact author for data requests.

Authors' contributions

CG, KH, and SY designed the research, and HJ and IS conducted the experiments. KJ analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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