Effects of Transgenic Bt+CpTI cotton on the abundance and diversity of rhizosphere ammonia oxidizing bacteria and archaea

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Abstract

Genetically modified crops (GMCs) hold great promise for improving agricultural output, but at the same time present challenges in terms of environmental safety assessment. Ammonia oxidizers, including ammonia oxidizing bacteria (AOB) and archaea (AOA), are very important functional microbial groups in nitrogen cycle. The abundance and diversity of AOA and AOB in the rhizosphere of genetically modified cotton (SGK321) and non-GM cotton (SY321) across growth stages were investigated using real time quantitative PCR (qPCR) and terminal restriction fragment length polymorphism (T-RFLP). Results showed that cotton genotype had a significant effect on the change in abundance of AOA and AOB, as indicated by amoA copy number. Variations in AOB abundance in rhizosphere of SY321 differed from those in SGK321. The number of AOB in the rhizosphere of SY321 fluctuated considerably: It dramatically decreased from 1.2×10^6 copies g^-1 dry soil to 3×10^4 copies g^-1 dry soil during the flowering stage and then increased to 1.1×10^5 copies g^-1 and 1.5×10^5 copies g^-1 at the belling and boll opening stages, respectively. However, abundance of AOB in the rhizosphere of SGK321 was relatively stable during all the stages of growth. The effect of SGK321 and SY321 on AOA number was quite similar to that of AOB: AOA abundance in SGK321 increased smoothly from 1.0×10^6 copies g^-1 dry soil to 1.4×10^6 copies g^-1 dry soil during growth, but that in SY321 fluctuated. Correspondence analysis (CA), canonical CA (CCA), and partial CCA (pCCA) of T-RFLP profiles of AOA and AOB showed that AOB community changed across growth stages in both cotton genotypes, and cotton genotype was the most important factor affecting the AOA community. In conclusion, the current findings indicated no adverse effect of GM cotton on functional microorganisms.

Key words

Ammonium oxidizing archaea, Ammonium oxidizing bacteria, Bt+CpTI cotton, Genetically modified crop

Introduction

The global planting area of genetically modified crops (GMCs) has increased more than 100-fold from 1.7 million hectares in 1996 to over 175 million hectares in 2013 (Clives, 2012). This fast adoption rate can be considered as evidence of resilience and benefits that these crops deliver to farmers and consumers. GMCs have facilitated the production of agronomically desirable crops that are resistant to pests, herbicides, pathogens and environmental stress. Along with these benefits, however, comes the possibility of adverse ecological effects from the engineered traits and persistence of transgenic plants and their residues in the environment. The risks of GMCs to the environment and especially to microbial biodiversity, have been extensively assessed before and during their commercial cultivation (Lee et al., 2011; Chun et al., 2012). The effect of GMCs on soil microorganisms are important for risk assessment.
Molecular biological techniques introduced in the past two decades have facilitated examination of soil-borne microbial communities and the effects of GMCs on their diversity (Lee et al., 2011; Liu et al., 2008). Although majority of research on this subject has been concerned on the effect of GMCs on diversity of the overall soil-borne microbial community (Chun et al., 2012), little information about the impact of GMCs on functional microorganism is available.

Nitrification is a microbial-driven essential process in the global nitrogen cycle. Ammonia oxidation, the first rate-limiting step of nitrification, is carried out by ammonia oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA) (Francis et al., 2005). For both AOA and AOB, the amoA gene encoding for a-subunit of ammonia monooxygenase is in widespread use as a functional marker for investigation of their distribution and abundance in natural environments due to its great advantages over 16S rRNA gene in resolving the genetic diversity of microbial ammonia oxidizers (Francis et al., 2005; Purkhold et al., 2000). For this reason, it is relevant to monitor the AOB and AOA structure in the rhizospheres of GMCs by analyzing the amoA gene marker.

Genetically modified cotton (GM cotton) is commercially planted in China and its planting area is increasing every year. The transgenic Bt (Guo Kang 12, GK12) and Bt+CpTI cotton (Shuang Guo Kang 321, SGK321), which expresses proteins that make it more resistant to pests, is the most popular GMC planted in China. However, the transgenic Bt cotton was gradually replaced by transgenic Bt+CpTI cotton due to its strong resistance to both lepidoptera pests and homopteran pests. The increasing planting area of transgenic Bt+CpTI cotton has intensified public anxiety about its safety to the environment. It has been reported that two of the three transgenic Bt cotton lines have caused a significant transient increase in total bacterial and fungal population, indicating that the response was specific to plant lineage (Donegan et al., 1995). Yudina and Burteeva (1997) also reported a temporary increase in total bacteria and fungus population caused by four transgenic Bt cotton lines. Oger et al. (1997) found that the concentration of total bacteria in the rhizosphere of both genetically modified plants (producing opin minnopine) and normal plants did not differ significantly. However, the minnopine utilizing appeared to be 80 times more abundant in the rhizospheres of genetically modified plants than in those of normal plants. This indicates that the production and release in the rhizosphere of minnopine by GM plant could alter the size of the population of bacteria that utilize the minnopine. It is possible that different rhizosphere secretions of two types of plants could change the micro-environment (Trevors et al., 1994). This could change the size of specific populations further. It has been reported that the root exudates from transgenic cotton can simulate spore germination of Verticillium dahliae (Li et al., 2009). Recently, Dong et al. (2014a) reported a significant difference in the AOB abundance between transgenic Bt+CpTI cotton and control cotton plants. However, little information about the effect of transgenic Bt+CpTI cotton on AOB and AOA community is available. In view of the above, the study was carried out to investigate the effects of GM Bt+CpTI cotton on AOA and AOB community structure.

Materials and Methods

Soil characteristics and sampling: Soil was collected from the field in cotton (Shiyuan321 or SGK321)-spring maize (Triticum aestivum L.) rotation at the Chinese Academy of Agricultural Science, PingGu, Beijing, China, in May 2012 before the cotton planting. The transgenic cotton was SGK321, which was genetically modified from cotton SY321. Soils was coarse loam in texture with acidic pH (6.1), as determined by a 1:1 soil-water suspension. Total organic carbon concentration was 10.2 g kg\(^{-1}\), and total nitrogen concentration was 1.00 g kg\(^{-1}\), as determined by combustion (Leco CNS-1000). To measure ammonium and nitrate concentrations, 100 ml of 0.01 mol l\(^{-1}\) CaCl\(_2\) was added to 10 g of soil, shaken for 1 hr on a reciprocating shaker, and centrifuged at 4100 g for 5 min at 5°C. The supernatant was collected and stored at -20°C until further analysis. NH\(_4\)\(^+\) and NO\(_3\)\(^-\) concentration in the extracts were determined using a continuous flow analytical system (TRAACS 2000, Bran and Lubbe, Norderstedt, Germany). Nitrification potential (NP) was measured in accordance with the protocol reported by Wang and Zhang (2006). The result of NH\(_4\)\(^+\) and NO\(_3\)\(^-\) concentration and NP has been reported in previous study by Dong et al. (2014a). Soil was frozen at -20°C to limit biological activity. At the start of the experiment, soil was thawed at room temperature, homogenized and passed through a 2-mm sieve.

Rhizosphere soil was collected from the same fields at different stages of cotton growth. Cotton plants were sampled at three sites in field including the two ends and the middle. Two plants per site were collected for a total of six SGK321 and six SY321 plants per stage. Surrounding soil was gently shaken off the roots and the soil still adhering to the roots was considered rhizospheric. The rhizosphere soil was collected at budding, flowering, belling and boll opening stages, and stored at -20°C until further use.

DNA extraction and purification: Total soil genomic DNA was extracted and purified using a MOBIO DNA extraction kit (MOBIO Laboratories Inc., Carlsbad, CA, U.S.) according to the manufacturer's instructions. 0.25 g soil was used to extract DNA and the purified DNA was stored at -
20°C and kept ready for any downstream application.

**Quantification of amoA in AOA and AOB in the rhizosphere soil using quantitative PCR (qPCR):**

Quantification of amoA gene in AOA (A-amoA) and AOB (B-amoA) in the rhizosphere soil were performed on Roche 480 (Roche, Basel, Switzerland) with plasmid pNIM-005 as standard. Taqman probe assay was used to quantify the B-amoA gene and SYBR green assay was used to quantify the A-amoA gene (Francis et al., 2005). The primers and probe are listed in Table 1. For Taqman assay, PCR amplifications were performed in a 25-μl reaction volume consisting of 12.5 μl of 2× Taqman Environmental Master Mix 2.0 (Life Technology, CA, U.S.), 0.5 μl of 10 μM forward and reverse primers, 0.25 μl of 10 μM probe, and 5 μl of DNA template. The qPCR thermal cycling for B-amoA gene ran with the following procedures: 2 min at 50°C and 10 min at 95°C, 50 cycles of 45 sec at 94°C, 60 sec at 55°C and 45 sec at 72°C.

For SYBR green qPCR reaction, a 25-μl reaction volume contained 12.5 μl of 2× Fast SYBR Green Master Mix (4385612, Life Technology, CA, U.S.), forward and reverse primers (Table 1) and 5 μl of DNA template. The working concentrations of the primers for each gene are listed in Table 1. Data were analyzed with LightCycler 480 software version 1.5.0 (Roche). PCR thermal cycling for A-amoA gene consisted of a 5 min activation period at 95°C followed by 6 cycles of touchdown PCR thermal profile of 15 sec at 95°C for denaturation, 30 sec at 63°C to 58°C for annealing, 45 sec at 72°C for extension and 15 sec at 80°C, then 39 cycles of 15 sec at 95°C for denaturation and 20 sec at 58°C for annealing and 30 sec at 72°C for extension. This was finally followed by 15 sec at 80°C with a melting curve program of 15 s at 95°C and 20 sec at 60°C.

The AOA and AOB population size reported in the present study were corrected for efficiency of extraction from soil by dividing the copies per ml from qPCR results by the DNA concentration (ng ml⁻¹) used in qPCR to obtain copies ng⁻¹ DNA. Then, this value was multiplied by the total amount of DNA (ng DNA per extraction) extracted from soil to get the copies per extraction. Lastly, dividing copies per extraction by dry weight of soil per extraction yields the normalized copies g⁻¹ dry soil.

**Terminal restriction fragment length polymorphism (T-RFLP):** Differences in AOB and AOA community composition were determined through T-RFLP analysis of amplified amoA gene for AOB and AOA. B-amoA was amplified using a nested PCR approach due to low abundance of AOB, with aliquots of the first-round PCR products used as templates in the second round of PCR. The first-round PCR primers used were amoA-2F/5R (Webster et al., 2002), and amoA-1F/2R in the second round (Rotthauwe et al., 1997). For AOA, the sequence of the primer pairs (A-amoA-F/A-amoA-R) used in T-RFLP analysis was same as that used in the real time PCR. The A-amoA-F and amoA-1F primers were labeled with carboxyfluorescein (6-FAM) for detection of terminal restriction fragments (TRF).

Amplification was carried out in 25 μl reactions containing 12.5 μl 2× PCR mastermix, 0.5 μl of each primer (10 μM), 5.5 μl sterile water and 1 μl DNA template. Amplification was performed with a PTC-200 Thermocycler (Biorad, Hercules, CA, U.S.). The cycling program for amoA amplification used touchdown PCR (Webster et al., 2002) for the first round, and the conditions for the second-round PCR were as follows: 95°C/5 min followed by 40 cycles of 95°C/30 s, 60°C/30 s, 72°C/40 s, and a 10 min final extension step at 72°C. Each PCR product (4 μl) was visualized after electrophoresis on 1% agarose TAE gels and subsequent staining with GelRed for 15 min. All reactions were performed in triplicate under the conditions described above. Triplicates were pooled (150 μl) and purified using QIA quick columns (Qiagen, Düsseldorf, Germany) to remove unincorporated nucleotides and labeled primers. DNA was eluted in a final volume of 50 μl.

Purified PCR products were digested for 3 hr in a water bath using TaqI (TCCG) for B-amoA at 65°C (Hoz et al., 2000) and Hha I (GCGC) for A-amoA. The restriction–digestion mixture contained 6 μl of purified PCR product, 2 μl of enzyme buffer, 11 μl sterile water and 1 μl of 10 U of restriction endonuclease. Aliquots (20 μl) of the digest were purified by ethanol precipitation. The precipitate was mixed with 0.3 ml of GeneScan ROX 500 size standard (Life Technology, CA, U.S.) and 10 ml of deionized formamide. The fluorescent labeled terminal fragments were separated by capillary electrophoresis using an ABI PRISM.

### Table 1. Primer and probe sequence and concentration for quantitative PCR

<table>
<thead>
<tr>
<th>Primers/Probe</th>
<th>Target</th>
<th>Sequence (5’ to 3’)</th>
<th>Concentration (nM)</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A189F amoA-2R’</td>
<td>amoA (AOB)</td>
<td>GGHGACTGGGAYTTCTGG CTCGASAAGGCTTCTC TAMRA</td>
<td>200</td>
<td>670</td>
<td>Okano et al., 2004</td>
</tr>
<tr>
<td>A337</td>
<td></td>
<td>FAM-TTCTACTGGTGTCRCA</td>
<td>200</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arch-amoAF Arch-amoAR</td>
<td>amoA (AOA)</td>
<td>STAATGGCTGCTTAGACG GCCGCCATCATCGTATGT</td>
<td>200</td>
<td>635</td>
<td>Francis et al., 2005</td>
</tr>
</tbody>
</table>
Fig. 1: The abundance of ammonia oxidizer and denitrifier in genetically modified cotton (SGK321) and non genetically modified cotton (SY321) rhizosphere soil by quantifying ammonia oxidizing bacteria amoA (a) and ammonia oxidizing archaea amoA (b). Pre-planting, sample before planting cotton; budding, flowering belling and boll opening are sampled at each corresponding stage of cotton growth. The data represents mean with standard deviation of five replicates.

A standard curve was generated by plotting serial dilutions of the plasmid standard (Dong et al., 2014b) ranging from 10⁰ to 10⁷ copies of plasmid containing the AOB amoA against their corresponding Ct values. The standard curve (Ct = -3.5×log 10 (amoA) + 47.77) had an R² = 0.999, indicating a strong linear relationship between Ct value and log 10 number of B-amoA copies over 6 orders of magnitude, and the reaction efficiency for B-amoA gene was calculated to be 93% (based on the formula E = 10 (-1/slope)-1). The AOB abundance (Fig. 1a) was about 2.5×10⁶ copies g⁻¹ dry soil in SGK321 and SY321 at pre-planting stage. At budding stage, AOB population size in rhizosphere of SGK321 and SY321 increased significantly, to 9×10⁶ copies g⁻¹ dry soil and 1.2×10⁷ copies g⁻¹ dry soil, respectively. During flowering
Fig. 2: AOB TRFLP data analysis by correspondence analysis (CA) (a), canonical CA (CCA) (b), and partial CCA (pCCA) (c). Pre, the stage of before planting cotton; Bud, stage of budding; Boll, stage of boll opening; Bell, stage of belling; Flower, stage of flowering; SGK321, the AOB TRFLP profile in rhizosphere of transgenic cotton; SY321, the AOB TRFLP profile in rhizosphere of normal cotton.

Fig. 3: AOA TRFLP data analysis by correspondence analysis (CA) (a), canonical CA (CCA) (b), and partial CCA (pCCA) (c). Circle, the samples taken from rhizosphere of SY321; square, the samples taken from rhizosphere of SGK321; triangle, the detected TRF of all samples; Pre, the stage of before planting cotton; Bud, stage of budding; Boll, stage of boll opening; Bell, stage of belling; Flower, stage of flowering; SGK321, the AOB TRFLP profile in rhizosphere of transgenic cotton; SY321, the AOB TRFLP profile in rhizosphere of normal cotton.
stage, the AOB number in rhizosphere of SY321 clearly decreased to $3 \times 10^5$ copies g$^{-1}$ dry soil, but that in SGK321 did not change significantly. During belling stage, the AOB number in rhizosphere of SY321 suddenly increased to $1.1 \times 10^6$ copies g$^{-1}$ dry soil and that in rhizosphere of SGK321 remained about $9 \times 10^5$ copies g$^{-1}$ dry soil. During boll opening stage, the AOB population size in rhizosphere of SY321 increased to be $1.5 \times 10^6$ copies g$^{-1}$ dry soil, and that in rhizosphere of SGK321 increased to be $1.0 \times 10^6$ copies g$^{-1}$ dry soil. Significant differences in the abundance of AOB were observed between the rhizosphere of SGK321 and SY321 at flowering, belling and boll opening stages ($p<0.05$). The AOB amoA gene abundance ranged from $3 \times 10^5$ copies g$^{-1}$ dry soil to $2 \times 10^6$ copies g$^{-1}$ dry soil, which was comparable with the results of a previous report quantified by droplet digital PCR (ddPCR) (Dong et al., 2014a). Variations in AOB abundance in rhizosphere of non-transgenic cotton SY321 differed from those in transgenic cotton SGK321. The number of AOB in the rhizosphere of SY321 fluctuated considerably: It decreased dramatically during flowering stage and then increased 4 times and 6 times at belling and boll opening stages, respectively. However, the abundance of AOB in the rhizosphere of SGK321 was relatively stable during all the stages of growth. This was closely consistent with previous study in which AOB abundance was quantified by ddPCR (Dong et al., 2014a).

A standard curve (Ct = $-3.44 \times \log_{10} (amoA) + 27.27$) with $R^2 = 0.998$ for quantification of A-amoA gene showed strong linear relationship between Ct value and log 10 number of A-amoA copies over 6 orders of magnitude. The numbers of AOA in both cotton fields at pre-planting were close to $1.0 \times 10^5$ copies g$^{-1}$ dry soil (Fig. 1b). AOA abundance in SGK321 displayed a continuous increase from $1.0 \times 10^5$ copies g$^{-1}$ dry soil to $1.4 \times 10^5$ copies g$^{-1}$ dry soil across all the stages of growth. In the rhizosphere of SY321, AOA was detected in a similar manner as that of AOB: AOA abundance first increased and then decreased during growth stage. A significant difference in AOA abundance was observed at budding, flowering and boll opening stage, between the rhizosphere of SGK321 and SY321.

The abundance of AOA amoA gene ranged from $9 \times 10^5$ copies g$^{-1}$ dry soil to $2 \times 10^6$ copies g$^{-1}$ dry soil, which was comparable to AOA abundance in other similar soil (Yao et al., 2011). The effect of SGK321 and SY321 on AOA number was quite similar to that on AOB: AOA abundance in SGK321 increased smoothly during growth, but it was fluctuated in SY321. This was closely consistent with the change in ammonium concentration in the rhizospheres of both kinds of cotton plants during growth stage. The ammonium and nitrate concentration at each growth stage of SGK321 and SY321 were determined in previous study (Dong et al., 2014a). Results showed that both ammonium and nitrate concentration in SGK321 changed more slowly than in SY321. It was speculated that the relatively stable increase in AOB and AOA numbers in SGK321 results in a slow decrease in ammonium concentration and slow accumulation in nitrate concentration in SGK321. This could in turn prevent competition between ammonia oxidizers and plants in ammonium absorbance, which favors plant growth.

AOB and AOA amoA gene copies were successfully quantified using a reference plasmid as a standard in qPCR (Dong et al., 2014b). qPCR is a popular method of determining the effects of GMCs on soil microbial abundance. However, it cannot be used to determine the number of gene copies by qPCR without DNA reference material. In most routine laboratories, in absence of DNA reference material, DNA standards are used to generate a standard curve for qPCR measurement. These standards are developed by cloning a plasmid or extracting genomic DNA from pure culture (Dong et al., 2009; Okano et al., 2004; Wallenstein and Vilgalys, 2005). These standards are then quantified by UV spectrophotometer. The accuracy of method in quantification of DNA reference material is important because quantitative results rely on the reference value determined by the method selected. The reference plasmid used for quantifying ammonia oxidizer in the study was characterized by well-validated methods of isotope dilution mass spectrometry and droplet digital PCR (Dong et al., 2014b; Dong et al., 2012). The comparability and reliability of the results of analysis performed at different laboratories can be achieved by using a universal and accurate DNA reference material. In this way, the results of the observed changes in abundance of ammonia oxidizer in the rhizosphere soil of SGK321 was reliable, because an accurate DNA reference standard was used.

Total bacteria abundance in the rhizospheres of both cotton strains was also quantified by ddPCR and it was about $2 \times 10^5$ copies g$^{-1}$ dry soil (Dong et al., 2014a). However, there was no significant difference in total bacteria number between the two types of cotton (Dong et al., 2014a). This is explainable because the ratio of AOB and total bacteria was only between 0.02% and 0.1% based on the number of AOB and total bacteria. In this way, such a small difference could not be considered indicative of the total bacteria abundance when only quantifying the total bacterial numbers. However, such a small amount of functional group would play an important role in nitrification. It is here proposed that the functional group could be used as an indicator to explore the real effect of GMCs on microbial diversity.

T-RFLP profiles of the AOB (Fig.2) and AOA (Fig.3) communities were explored using CA, CCA and pCCA. The first two CA ordination axes explained 47.7% of AOB
variation and 61.6% of AOA variation, respectively. The first CA axis separated the samples into two groups both for AOB (Fig. 2a, $p = 0.002$) and AOA (Fig. 3a, $p = 0.001$). For AOB community, one group included samples at the budding and boll opening stage and the other one included all other growth stages (pre-planting, flowering, and belling stage). This indicated that the diversity of AOB at the budding and boll opening stage was significantly different from that at other stages. Most TRFs congregated around the budding and boll opening stages and fewer TRFs gathered at the pre-planting, flowering, and belling stages. However, the first CA axis separated the AOA into two distinct groups along the lines of transgenic cotton and non-transgenic cotton, which indicated a significant difference in AOA diversity between transgenic and non-transgenic cotton.

CCA was used to extract the correlation between environmental factors and AOB and AOA diversity. The first two axes of CCA explained 69.6% of AOB variation (Fig. 2b) and 76.5% of AOA variation (Fig. 3b), respectively. In AOB profiles (Fig. 2b), the projection of environmental variables revealed a strong correlation to the budding and boll opening stages. More TRFs were correlated with budding and boll opening stages. This indicated that the AOB community was richer during these two stages. In contrast, fewer TRFs presented at pre-planting, flowering and belling stages. This suggested that the AOB community was less rich at these times. The budding and boll opening stages were two factors most important to the AOB TRF patterns. They were followed by pH, transgenic cotton (SGK321) and control cotton (SY321), it means the significance of the other factors are pH>transgenic cotton>control cotton. Nitrification potential was found to be positively correlated with transgenic cotton and negatively correlated with the non-transgenic cotton. The two significant factors (budding and boll opening) served covariates in pCCA, and the first two axes explained 74.8% of the variation (Fig. 2c). The cotton genotype (transgenic cotton and non-transgenic cotton) was found to be the most significant factor. The other significant factors were pH, flowering stage and ammonium concentration, it means the significance of the other factors are pH>flowering stage>ammonium concentration.

The TRFs (283, 219, 48, 354, 441, and 491 bp) found in tested soil mainly included previously reported AOB lineages within b-Proteobacteria. The TRF of 283 bp, the most abundant TRF found in any of the samples, was found to be of *Nitrosospira* lineage. TRF 219 bp belonged to *Nitrosomonas europea*, and TRFs of 48, 354, 441, and 491 bp were of other *Nitrososomona* lineages specifically, *Nitrososomona oligotropha*, *Nitrososomona cryotolerans*, *Nitrososmonas marina* and *Nitrososmonas communis*, respectively (Park and Noguera, 2004; Siripong and Rittmann, 2007).

In addition to the above, AOB TRFs found in tested soil belonged to b-Proteobacteria, some other TRFs were also detected. These were 85, 205 and 334 bp in size. Some of these have been reported previously (Wang et al., 2010). These TRFs are not expected fragment sizes derived from TAP T-RFLP in silico analysis in Ribosomal Database Project version 8.10 (http://rdp.cme.msu.edu). They could be uncharacterized AOBs. It is speculated that there were more AOB species than are represented in the database. Further work identifying uncharacterized AOBs by metagenomics is necessary to improve the AOB database.

In AOA profile (Fig. 3b), after cotton genotype, ammonium concentration was the second most significant factor followed by pre-planting stage and pH. AOA population size was found to be positively correlated with nitrification potential and negatively correlated with ammonium concentration. This suggested that an increase in AOA numbers would increase nitrification potential and decrease ammonium concentration. Previous microcosm studies have proved the preference of AOA to ammonia limited in soil (Di et al., 2009, 2010; Pratscher et al., 2011; Verhamme et al., 2011). From the AOA T-RFLP pattern, the AOA diversity in transgenic cotton was higher than the non-transgenic cotton. TRF 160, 167, 170, 258, 354, 433, 434, and 450 bp were present in transgenic cotton SGK321 and TRF 260, 300, and 270 bp were more abundant in non-transgenic cotton SY321 than in transgenic cotton. Because CCA established the importance of transgenic cotton as an overarching controlling variable, cotton genotype was treated as covariable in pCCA to extract the effects of other variables on the AOA community T-RFLP profiles. The pCCA explained 59.0% and 21.1% of the variation on the first two axes. In the pCCA diagram (Fig. 3c), the most important variables were represented by longer arrows. Besides ammonium concentration, the main factors correlated with AOA T-RFLP pattern were pre-planting, pH, and flowering, it means the significance of the other factors are pre-planting>pH>flowering. Ammonium concentration was negatively correlated with nitrification potential, indicating that disappearance of ammonium led to accumulation of nitrate. In conclusion, the AOA and AOB population size was found to be positively correlated with nitrification potential and negatively correlated with ammonium concentration, indicating that disappearance of ammonium led to accumulation of nitrate. In conclusion, the AOA and AOB abundance were more stable in GM cotton and AOA diversity was more sensitive to cotton genotype and the current findings indicate the advantageous effect of GM cotton on functional microorganisms.

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