Integration and Characterization of T-DNA Insertion in Upland Cotton

Xiaojie YANG[1,2], Fuguang LI[1], Xueyan ZHANG[1], Kun LIU[1], Qianhua WANG[1], Chaojun ZHANG[1], Chuanliang LIU[1], Wei ZHU[3], Guofang SHAN[1], Chee-Kok CHIN[4] and Weiping FANG[2]

[1]State Key Laboratory of Cotton Biology, Institute of Cotton Research, Chinese Academy of Agriculture Sciences (CAAS), Anyang, Henan, P.R. China; [2]Economic Crop Research Institute, Henan Academy of Agricultural Sciences, Zhengzhou, P.R. China; [3]Agronomy College of Henan Agricultural University, Zhengzhou, P.R. China; [4]Department of Plant Science, Rutgers University, New Jersey, USA

Abstract

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Copy numbers were evaluated by real-time quantitative PCR, and 149 junctions of T-DNA were isolated by thermal asymmetric interlaced PCR from 92 independent transgenic cotton lines transformed by *Agrobacterium tumefaciens* strain LBA4404. Real-time quantitative PCR results showed that 46% had integration of one or two T-DNA copies, 54% had three or more copies. Among 63 amplified products at LB junctions, 51% showed co-transformation of the vector backbone, 30% retained a portion of LB ranging from 3 to 23 bp, and 19% showed deletions ranging from 1 to 148 bp from the LB inner end. In contrast, all of the cleavage sites were located in the inner region of RB. The distribution of T-DNA insertions in upland cotton genome included coding sequences, transposons, plastid-derived sequences and microsatellites.

Keywords: cotton (*Gossypium hirsutum* L.); deletion of border; genetic transformation; transgene copy; vector integration

Agrobacterium tumefaciens-mediated T-DNA (Transfer DNA) transfer is the most widely used transformation system to introduce foreign genes into plant genome. Cotton, an economically important crop cultivated in more than 80 countries, is the most important natural source of fibre for textiles and is also a source of relatively high-quality protein and oil (Sunilkumar *et al*. 2006). Although cotton is one of the most successful genetically modified crops in China, detailed knowledge of cotton transgenic integration is still very limited (Zhang *et al*. 2008). Many factors contribute to variation in transgene expression, including the integration site, the number of transgene copies, transgene mutation and epigenetic gene silencing (Hobbs *et al*. 1990; Fladung 1999; Maqbool & Christou 1999). Therefore, not only for cotton molecular breeding but also for the study of the molecular mechanisms of T-DNA integration, it is essential to investigate the characteristics of

T-DNA integration into transgenic cotton, including integration site sequence, copy number, etc.

As for integration sites, distribution of T-DNA insertions in intergenic sequence versus gene sequence appeared randomly in *Arabidopsis* genome (Forsbach *et al.* 2003). In rice, about 45% of T-DNA integration occurred into genes which represented only 10–25% of the genome (Jeong *et al.* 2006). In cotton, about 13% of T-DNA integration was into repetitive sequence, less than the predicted composition of 30–36% of repetitive sequences in tetraploid cotton genome (Zhao *et al.* 1995).

In research reported here, a total of 108 T_0 plants and corresponding 92 inbred T_1 transgenic T-DNA-tagged lines, wherein each of them coming from an independent transformation event by *Agrobacterium tumefaciens*, were generated, and copy number and more than 140 T-DNA/cotton genome junctions and flanking sequences were evaluated and isolated.

MATERIAL AND METHODS

Plant transformation. *Gossypium hirsutum* cv. CCRI 24 was transformed according to previous methods (Sumilkumar & Rathore 2001; Rathore *et al.* 2006). *Agrobacterium tumefaciens* strain LBA4404 containing the vector pCAMBIA2300 (CAMBIA Australia) was used to transfer T-DNA. Aseptic seedling hypocotyls of cotton were used as the explants for generating transgenic cotton lines. The antibiotic chloramphenicol was added into *Agrobacterium* culture before co-cultivation. About 50 mg/l of kanamycin was included in the callus, embryogenic callus, and somatic embryo induction media for selection of positive transformants. 108 T_0 putative transgenic plants, wherein each of them coming from an independent transformation event, were maintained in a greenhouse and used for kanamycin, molecular identification, and copy number analysis. A total of 92 transgenic positive inbred T_1 lines were used for further analysis.

Identification of kanamycin resistance in seedlings. The kanamycin resistance of the transgenic T_0 seedlings was evaluated by spreading 1500 mg/l kanamycin on the seedling true leaves. Plants were categorized as resistant (green leaves) or sensitive (withering).

Genomic DNA isolation and PCR identification. Genomic DNAs were isolated from the leaf material of transgenic plants according to a modified cetyl trimethyl ammonium bromide method (Paterson 1993). Primers (F: 5'-GCCATGTGGGCATTCAG T-3'; R: 5'-TACGCCATCAGCACGTTATCG-3') (Figure 1) were designed to amplify a coding region fragment of the *nptII* (kanamycin resistance) gene. Each PCR reaction mixture (20 µl) consisted of 10–20 ng of cotton genomic DNA, 0.2mM of dNTPs, 0.6µM of each primer, 1× PCR buffer and 0.15 U r-Taq DNA polymerase (TAKARA, Dalian, P.R. China). Thermal cycling was done at 94°C for 10 min followed by 32 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 30 s. After the final cycle, the reactions were maintained at 72°C for 10 min before completion. Reactions were conducted using a PTC-100 thermal cycler (MJ Research, Foster, USA).

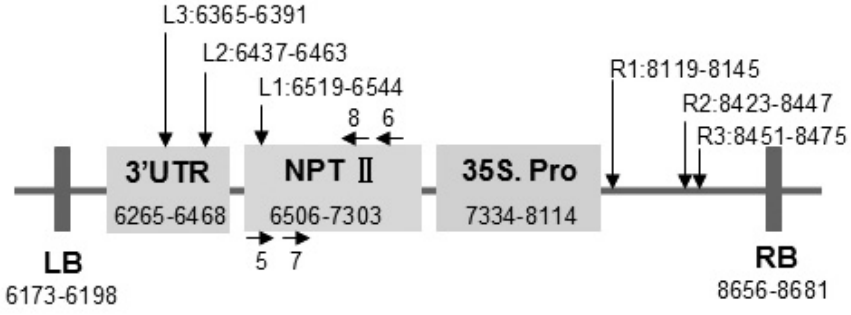

Figure 1. Map of the T-DNA region with binary vector pCAMBIA2300 for cotton callus transformation (see GenBank accession AF234315)

RB and LB represent the left border and the right border, respectively; L1-3 and R1-3 represent the Tail-PCR primers adjacent to LB and RB; numbers 5/6 are used to identify the positive transformants and used as hybridization probe, and 7/8 was used as real-time quantitative PCR primers for the analysis of transgenic copies; the arrows indicate the direction of primers from the 5' to the 3' end

Transgene copy number evaluated by real-time quantitative PCR. Q-PCR (Quantitative PCR) was used to evaluate the T-DNA insertion copy number with the endogenous upland cotton *SadI* gene (GenBank No. AJ132636) primer pair (F: 5'-ACGGCCTGTGGGCATTCAGT-3'; R: 5'-TGCACCATCAGCACGTTATCG-3') and the *nptII* gene primer pair (F: 5'-TGCGCCCAAGCTGCATCAT-3'; R: TGAACTCTCACCGCGACGTCTGT-3') (Figure 1). Q-PCR assays were carried out in a 7500 Real-Time PCR System (Applied Biosystems, Foster, USA). Fluorescence was monitored during each PCR cycle at the extension step. About 80–100 ng of DNA was used as the template for PCR amplification with 0.2μM of each primer, 2 μl ROX Reference Dye II and 25 μl of SYBR Premix Ex Taq (2×) (Takara, Dalian, P.R. China) in 50 μl of reaction solution. The PCR reactions were initiated by heating the samples at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 58°C for 29 s, and 72°C for 35 s and additional 10 min of extension at 72°C to complete PCRs. Modified CT method (Yang *et al*. 2005a) was used to quantify the transgene copies.

Restriction enzyme digestion of genomic DNA and Southern blotting. The following components were included in the genomic DNA *Eco*RI restriction enzyme digestion system (400 μl): 30 μg of genomic DNA, 40 μl of 10× H buffer (10× T buffer), 40 μl of *Eco*RI (8-20 U/μl), and sterile ddH_2O to a final volume of 400 μl. The mixture was vortexed and centrifuged briefly to ensure complete mixing. Digestion was performed at 37°C overnight, and then it was concentrated to 40μl by a QUATTRO sample concentrator (Genevac, Ipswich, UK). The PCR (F: 5'-TGCGCCCAAGCTGCATCAT-3'; R: TGAACTCTCACCGCGACGTCTGT-3') fragment of the *nptII* gene was used as a probe for Southern blotting. Probe labelling and hybridization were performed according to the instructions of the DIG High Prime DNA Labelling and Detection Starter I Kit (Roche, Mannheim, Germany).

Isolation of T-DNA border junctions and cotton flanking sequences by Tail-PCR (Thermal asymmetric interlaced PCR). Tail-PCR was performed in accordance with the literature (Liu & Whittier 1995). Specific primers were designed according to the inside adjacent sequences of the T-DNA border in pCAMBIA2300 (Figure 1). PCR reactions were performed in the PTC-100 Thermal Cycler (Applied Biosystems, Foster City, USA). The special and arbitrary degenerated primer sequences are as follows:

L1 5'-GCAAGCTGCTCTAGCCAATACGCAAAC-3'
L2 5'-AAACCCTGGCGTTACCCAACTTAAT-3'
L3 5'-GCAAGCTGCTCTAGCCAATACGCAAAC-3'
R1 5'- GCAAGCTGCTCTAGCCAATACGCAAAC -3'
R2 5'- CCGTCGTTTTACAACGTCGT10.5

RESULTS

Identification of transgenic cotton T_0 plants

Transformants were selected by kanamycin resistance during transformation and plantlet regeneration. Therefore, plants with a silenced *nptII* gene were not included in this study. Kanamycin resistance and PCR amplification were carried out to investigate the T-DNA integration into transgenic cotton plants. Most of the plants (81%, 92/108) were kanamycin-resistant, and all of the resistant plants showed the specific amplified band of the *nptII* gene (Figure 2).

Transgene copy number analysis

Q-PCR was conducted to check the copy number of T-DNA insertions in 92 T_0 independent transgenic cotton plants. The *SadI* gene (Yang *et al*. 2005b) was used as the endogenous reference

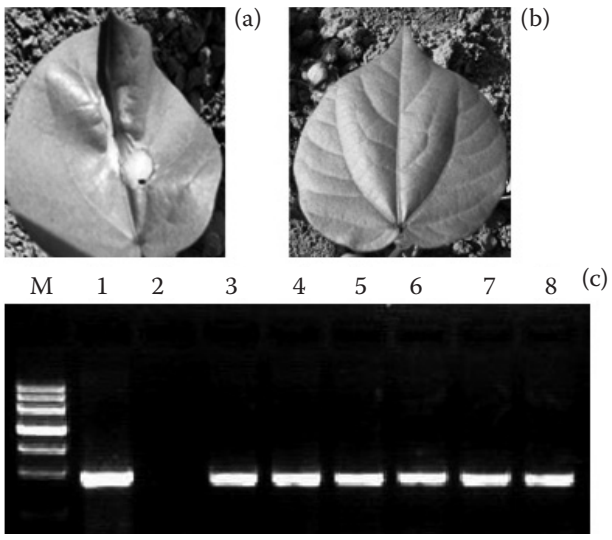

Figure 2. Identification of positive transgenic cotton plants: (a) kanamycin-sensitive, (b) kanamycin-resistant plants, (c) shows a conventional PCR of *nptII* M: marker; 1: positive plasmid control, 2: non-transgenic plant, 3–8: transgenic plants

Table 1. Copy numbers of the *nptII* gene in 92 transgenic plants

Copy No.	No. of transgenic plants	Percentage (%)
1	19	21.1
2	23	25.0
3	18	19.2
≥ 4	32	34.6

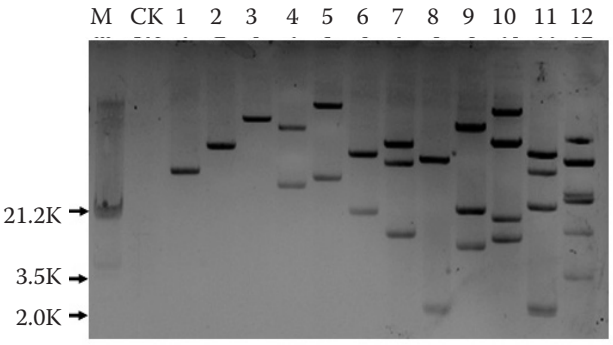

Figure 3. Transgene copy number identification by Southern blot analysis
M: molecular marker; CK: DNA from a non-transgenic cotton plant; lanes 1–3: one transgene copy evaluated by q-PCR, lanes 4–6: two copies, lane 7–9: three copies, and lanes 10–12: four, five and six copies, respectively; all of the genomes were digested by *Eco*R I

gene and transgenic upland cotton plant with one T-DNA insertion identified by Southern blot (data not shown) was selected as the calibrator (control) in relative analyses of q-PCR. Because the selected calibrator was homozygous at the T-DNA insertion site, according to the mathematical model, the copy number (CN) of the transgene of a sample is calculated based on E and Ct (calibrator-sample): $CN = 2\times (E_{nptII})^{Ct\ nptII}/(E_{SadI})^{Ct\ SadI}$. The analysis of transgene copies showed that among the 52 randomly selected T_0 transgenic plants, only 19 (21%) had a single copy of T-DNA, 41 (44%) had two or three copies, and 32 (35%) had four or more copies (Table 1).

Molecular characteristics of T-DNA insertions

The transgenic plant-specific DNA fragments (≥ 300 bp) that flanked the T-DNA were amplified by Tail-PCR. The successful amplification rate for amplification of specific products of the right border of T-DNA (RB) region was higher than that of the left border of T-DNA (LB) region (Figure 4). There was more than one junction amplified from some plants at the right border and/or left border, which indicated also the existence of multiple T-DNA integrations. A total of 63 fragments flanking LB and 86 fragments flanking RB out of the 92 transgenic plants were aligned to the LB and RB adjacent sequences to characterize the T-DNA integration sites in detail. Among the 59 examined amplified products flanking the LB region, 32 (51%) showed the co-transformation of T-DNA and vector backbone sequences integrated into the plant genome, 19 (30%) retained a portion of the LB ranging from 3 to 23 bp, and 12 (19%) showed deletions ranging from 1 to 148 bp from the LB inner end (Table 2 and Figure 5). All of the cleavage sites were located in the inner region of RB (Figure 6).

As mentioned above, compared with the LB, all of the cleavage sites were located in the inner region of RB, which was not typical and precise as the literature previously published. The cleavage sites were distributed at positions 8633, 8581, 8573, 8569, 8566, 8555, 8551, 8512 and 8391 bp, and the cleavage site 8391 bp was a fragment amplified

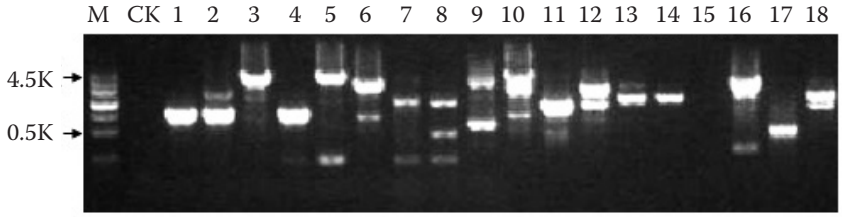

Figure 4. Products of T-DNA integration junctions by tertiary Tail-PCR
M: marker; CK: non-transgenic plant; 1–18: amplification fragments from transgenic lines, 1–9: the amplification of RB conjunctions, 10–18: the amplification of LB conjunctions; gel electrophoresis indicated the multi-band amplification of some transgenic plants

Table 2. Variation of T-DNA border adjacent regions in transgenic cotton plants

Border type	Number of LB region (%)	Number of RB region (%)
Border with backbone sequence	32 (51)	0 (0)
Intact border	0 (0)	0 (0)
Border of partial length	19 (30)	0 (0)
No border sequence	12 (19)	86 (100)
Total	63	86

by the primary Tail-PCR reaction. The cleavage frequency of the cleavage sites was not evenly distributed, three of which, including positions 8573, 8391 and 8633 bp, showed a higher cleavage frequency (38, 15 and 12, respectively), and cleavage nucleotide bases were mostly G/C, accounting for 73% (63/86) (see GenBank accession AF234315 and Figure 6). However, the cleavage nucleotide bases of LB adjacent region were almost all A/T, and their portion was up to 90.3% (28/31).

Distribution of T-DNA in the cotton genome

For 141 T-DNA flanking sequences (both RB and LB flanking sequences), except those T-DNA junctions (26 sequences were all the T-DNA plasmid backbone) with long vector sequence integration, a homology search against the NCBI nucleic acid non-redundant database was conducted, and the identities of these genomic border sequences are listed in Table 3. More than half (61.7%, 87/141) of T-DNA integrations were in microsatellites. Thirteen T-DNAs (5.7%) were inserted into protein-coding sequences, including those for an RNA recognition motif-containing protein, 2S albumin storage protein (Mat5-A), gland development-related protein, glycerol-3-phosphate dehydrogenase, gypsy-like retroelement hrs7 integrase, cotton Lea4-A, and one MADS box protein. We also noted that 7.8% of T-DNA integrations were in transposon/retrotransposon sequences and 1 was a chloroplast-derived sequence. Approximately 24.1% of the cotton genome flanking sequences returned no hits in the database (Table 3).

Figure 5. Conjunctions of the left border and cotton flanking sequences
The figure shows the results of T-DNA integration into the cotton genome; the sequences of T-DNA are shown in bold, underlined sequences represent the left border of T-DNA integration (TGGCAGGATATATTGTGGTGTAAAC, located from 6173 to 6198 bp; see GenBank accession AF234315); double slash (//) represents the recombination sites; the sequences before and after the double slash represent the T-DNA region and the cotton DNA, respectively; superscript Arabic numbers represent the nicked sites on CAMBIA 2300, and Arabic numbers represent the cleavage frequency

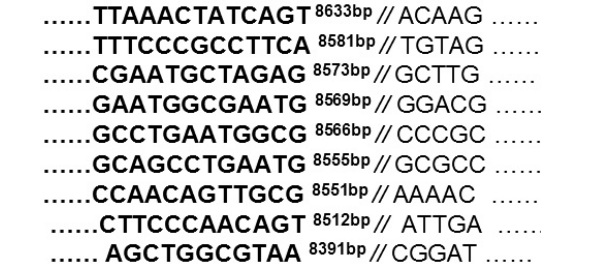

Figure 6. Conjunctions of the inner region of the right border and cotton flanking sequences
The figure shows the results of T-DNA integration into the cotton genome; the sequences of T-DNA are shown in bold, and double slash (//) represents the recombination sites; the sequences before and after the double slash represent the T-DNA region and the cotton DNA, respectively; Arabic numbers represent the nicked sites on CAMBIA 2300; right borders (TGACAGGATATATTGGCGGGTAAAC, located from 8637 to 8661 bp; see GenBank accession AE007871) in all the transgenic lines were nicked absolutely during transformation

Table 3. Distribution of T-DNA loci in the cotton genome

Category	Frequency	Percentage
Coding sequence	8	5.7
Plastid derived sequence	1	0.7
Microsatellite	87	61.7
Transposon	11	7.8
Unknown	34	24.1
Total	141	100

DISCUSSION

Copy number of a transgene transformed by *Agrobacterium tumefaciens*

In our study, the transgene copies of 92 transformed plants were estimated by q-PCR. The results differ from previous results obtained by Southern blot analysis, for which as many as 37% of transformed plants had one T-DNA copy, 24% had two T-DNA copies and 39% had three or more copies (Zhang *et al*. 2008). But similar results in transgenic rice were also reported previously (Yang *et al*. 2005a). Southern blot hybridization was applied to validate the accuracy of the PCR method; the results showed that the other 10 copy numbers were consistent with those of q-PCR method except for two transgenic lines (lane 8 and 11) (Figure 3).

From the aspect of breeding transgenic plants, these results suggest that because of influences of transgene copies on the level of transgene expression and the ease of stabilizing expression in subsequent generations (Cervera *et al*. 2000), it is important that transgene copies in putative transformants (here T_0 plantlets) should be analysed as soon as possible so that only the most useful ones are taken through the steps of acclimation in soil, flowering, seed production, etc.

Recombination of border regions of T-DNA

Among the examined 52 LB junctions, 19 retained a portion of LB and all of them but two ended with an A or T nucleotide. As for RB junctions, RB ends were mostly G or C. This result corroborates a conclusion reported previously (Hobbs *et al*. 1990). However, we did not find that the RB was mostly nicked at the cleavage site for generating a single-stranded T-DNA fragment found in *Arabidopsis thaliana* (Hiei *et al*. 1994). This is a different situation from that in *A. thaliana*, for which T-DNA integration was precise for both LB and RB (Forsbach *et al*. 2003).

Deletions inside the T-DNA borders have also been reported in rice plants (Sha *et al*. 2004). In a report on aspen, 16 of 20 LB regions were deleted, ranging from 2 to 24 bp (Kumar & Fladung 2000). In a study of barley, 36 out of 39 LB regions were deleted, ranging from 1 to 95 bp from the cleavage site (Stahl *et al*. 2002). These results suggest that T-DNA integration mechanisms are similar between monocotyledonous and dicotyledonous species.

We also found that the LB junctions showed the co-transformation of T-DNA and vector backbone sequences into the cotton genome. In previous studies, only one of the two borders with vector sequences integrated into the plant genome was also found in transformed rice plants (Sha *et al*. 2004) and cotton plants (Zhang *et al*. 2008).

Sequence characteristics of frequent T-DNA integration sites

The distribution of T-DNA insertions in intergenic sequences versus gene sequences occurs randomly in the *Arabidopsis* genome (Forsbach *et al*. 2003). In rice, as many as 45% of T-DNA integrations occur into genes which represent only 10–25% of the genome (Jeong *et al*. 2006). In our study, 5.7% of T-DNA integrations occurred into coding genes. Of course, 25.1% of 141 flanking sequences had no sequence hits in the GenBank non-redundant nucleic acid database. This is possibly because of the very limited coverage of the cotton genome in the database.

Our results support the idea that the microhomologies at the RB integration site play a role in anchoring the T-DNA to the target DNA (Brunaud *et al*. 2002). The frequent presence of sequence homology between T-DNA and the genomic preinsertion site suggests that integration involves the annealing of at least one of the T-DNA border regions to a single-stranded region within the target DNA (Kim *et al*. 2003).

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Corresponding author:

Fuguang Li, Ph.D., Chinese Academy of Agriculture Sciences (CAAS), Institute of Cotton Research, State Key Laboratory of Cotton Biology, 455 000 Anyang, Henan, China
e-mail: lifug@cricaas.com.cn