African Journal of Biotechnology Vol. 10(59), pp. 12614-12625, 3 October, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.728
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

T-DNA integration patterns in transgenic maize lines mediated by *Agrobacterium tumefaciens*

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Accepted 1 September, 2011

To explore transfer deoxyribonucleic acid (T-DNA) integration patterns in the maize genome, we improved the protocol of thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR), and amplified the flanking sequences around T-DNA integration sites from 70 independent transgenic maize lines mediated by *Agrobacterium tumefaciens*. Out of 64 specific amplified fragments, 32 and 9 are homologous to the sequences of the maize genome and the expression plasmid, respectively. For 26 of them, a filler sequence was found flanking the cleavage sites. These results demonstrate that cleavage occurs not only during the T-DNA borders but also inside or outside the borders. The border sequences and some inside sequences can be deleted, and filler sequences can be inserted. Illegitimate recombination is a major pattern of T-DNA integration, while some hot spots and preference are present on maize chromosomes.

Key words: *Agrobacterium tumefaciens,* maize, thermal asymmetric interlaced PCR, transfer DNA, transgenics.

INTRODUCTION

Agrobacterium tumefaciens-mediated transformation is the most widely utilized technique to generate transgenic events of plants (Kole et al., 2010). The transfer deoxyribonucleic acid (T-DNA), carrying the engineered expression construct, is transported from the bacterial tumour-inducing plasmid and integrated into the plant genome. This property of T-DNA is also used for the inactivation of plant genes by insertion mutagenesis (Zhu et al., 2010). The integration and structure of a transgene locus can have profound effects on the level and stability of transgene expression (Kole et al., 2010; Zeng et al., 2010). A lot of effort has been paid to elucidation of the integration mechanism of T-DNA in the host genome. Illegitimate integration by non-homologous recombination was suggested for T-DNA integration in plant chromo-somes (Kim et al., 2007), whereas site-specific integra-tion and homologous recombination were identi-fied in many other transformed events (Thomas and Jones, 2007; Zhang et al., 2007). Sometimes, the integration of T-DNA can induce chromosomal rearrange-ment including translocation, inversion, deletion and insertion (Zeng et al., 2010; Zhu et al., 2010). Furthermore, various lengths of the bacterial plasmid backbone DNA sequence were found contained in the host genome of *Agrobacterium*-mediated transformats (Shou et al., 2004; Windels et al., 2003; Zeng et al., 2010). Based on the sequence analysis of 236 T-DNA transgenic rice lines (Zhu et al., 2006), believed that mul-tiple mechanisms are involved in T-DNA integration in plants.

In *Arabidopsis* and tobacco, the T-DNA integration

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Abbreviations: T-DNA, Transfer deoxyribonucleic; **CTAB**, cetyl trimethylammonium bromide; **TAIL-PCR**, thermal asymmetric interlaced polymerase chain reaction; **AD**, arbitrary degenerate primers.

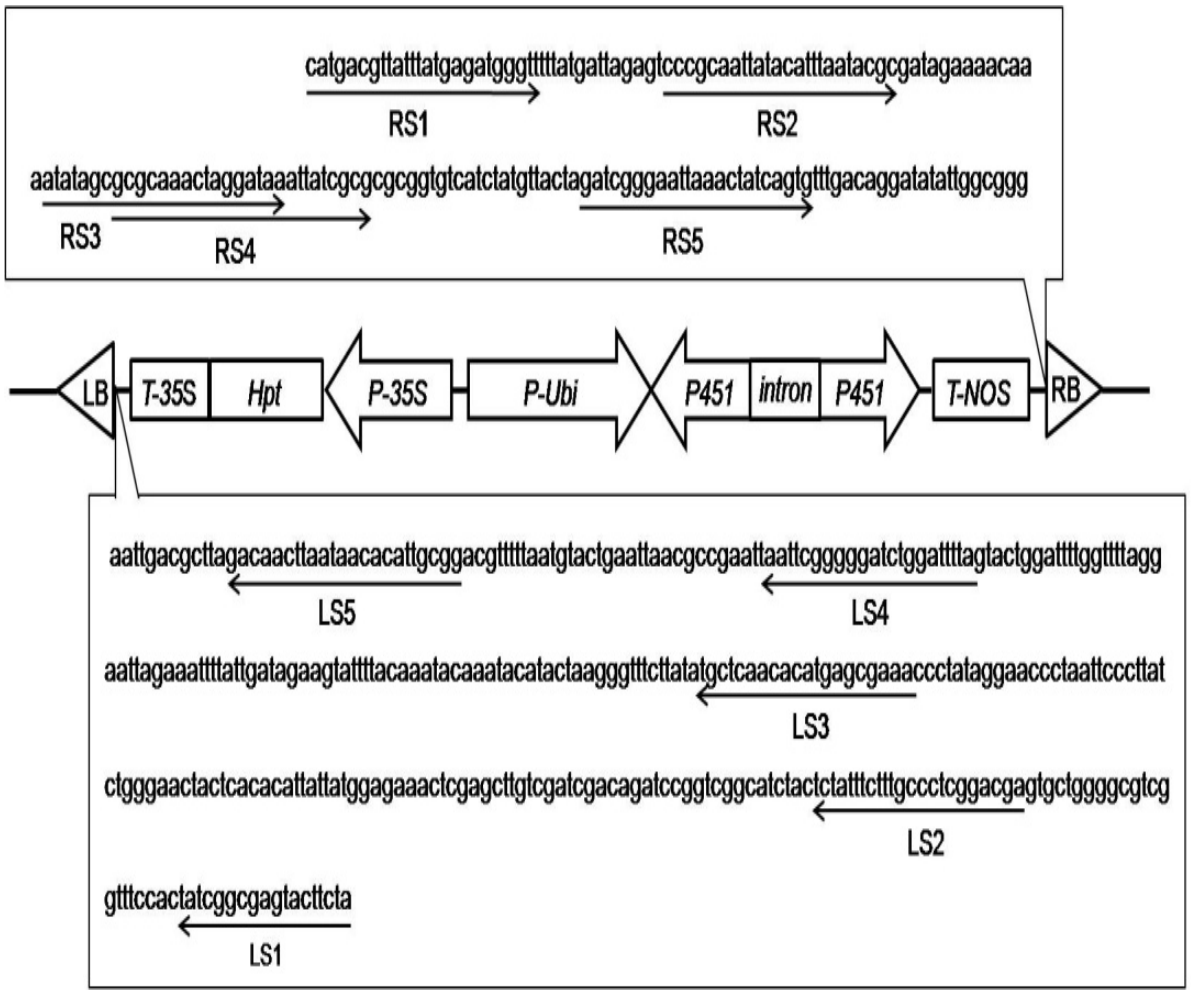

Figure 1. Nested specific primers complementary to inside flanking sequences of T-DNA left border and right border in plasmid pCAMBIA1390. **LS1, LS2, LS3, LS4 and LS5**, nested specific primers complementary to the inside flanking sequence of the T-DNA left border; **RS1, RS2, RS3, RS4 and RS5,** nested specific primers complementary to the inside flanking sequence of the T-DNA right border in plasmid pCAMBIA1390; **LB**, the left border; **RB**, the right border; ***P-Ubi***, maize ubiquitin promoter; ***P451***, 451 bp fragment homologous to P1 protein (protease) gene of maize dwarf mosaic virus; *intron*, intron of maize actin gene; ***T-nos***, terminator of nopaline synthase; ***P-35S***, cauliflower mosaic virus *35S* promoter; ***Hpt***, hygromycin phosphotransferase gene; ***T-35S***, cauliflower mosaic virus *35S* terminator.

pattern was found to be highly determined by the transformed target cell (De Buck et al., 2009; Shimizu et al., 2001). Maize was domesticated from the grass teosine in central America over the last ~ 10,000 years (Doebley et al., 2006). The maize genome has undergone several rounds of genome duplication. Its 10 chromosomes are structurally diverse and have endured dynamic changes in chromatin composition. Over the last 3,000,000 years, the size of the maize genome has expanded dramatically to 2.3 gigabases via proliferation of long terminal repeat retrotransposons (SanMiguel et al., 1998). The complex repetition and diversity of the maize genome make it a bigger challenge to explore T-DNA integration mechanism than other plants (Schnable et al., 2009; Zhou et al., 2009). Up to now, few documents have been available on T-DNA integration patterns in maize (Shou et al., 2004; Zhao et al., 2003). In the present study, we improved the protocol of thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) (TAIL-PCR, Liu et al., 1995; Sessions et al., 2002), amplified the flanking sequences around T-DNA integration sites from 70 independent transgenic maize lines mediated by *A. tumefaciens*, and explore the T-DNA integration patterns in the maize genome.

MATERIALS AND METHODS

Transformed maize lines and template DNA extraction

Template DNA samples were extracted by cetyl trimethylammonium bromide (CTAB) method from 70 transgenic maize lines of homologous T_2 generation. All of these lines were independently

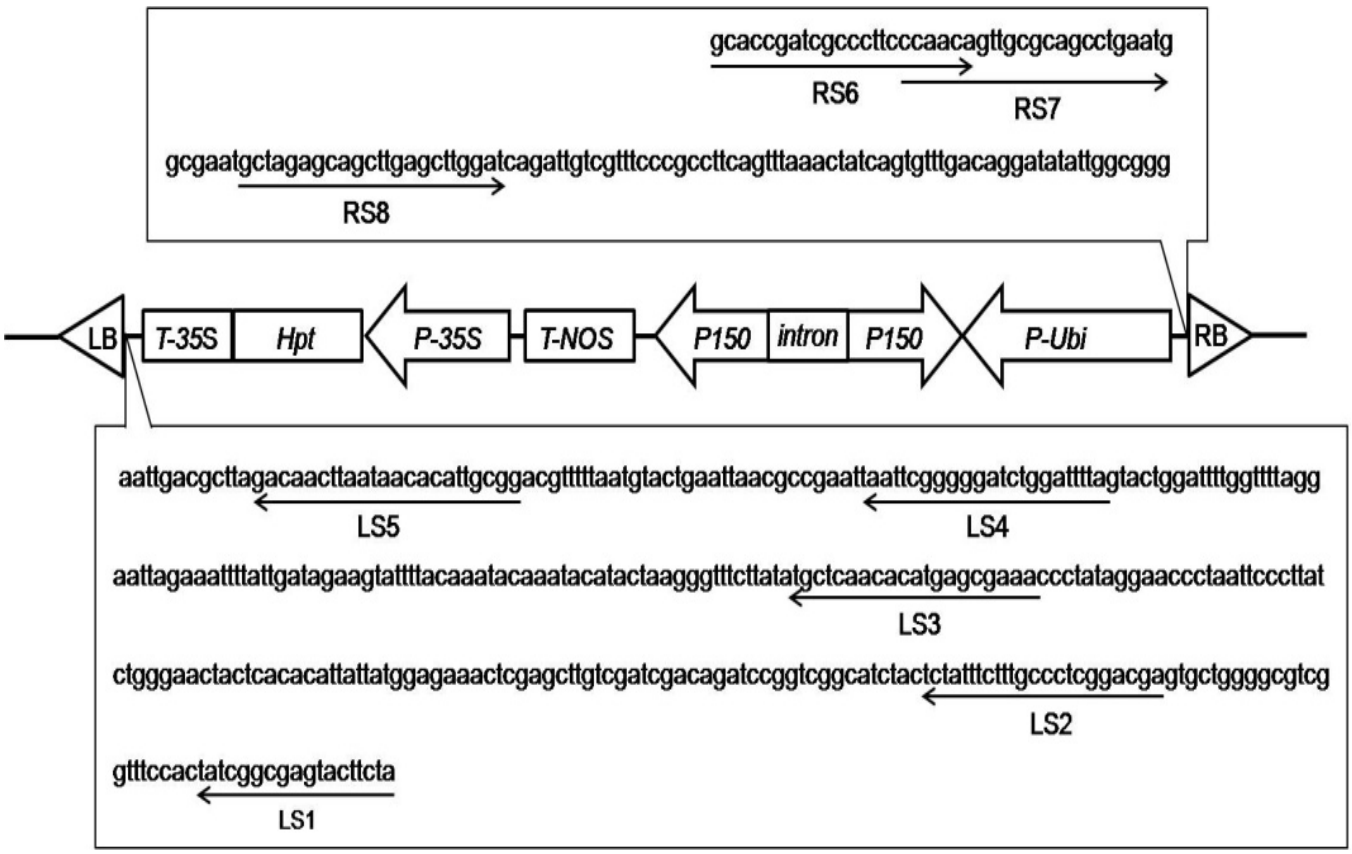

Figure 2. Nested specific primers complementary to inside flanking sequences of T-DNA left border and right border in plasmid pCAMBIA1300. **LS1, LS2, LS3, LS4 and LS5**, nested specific primers complementary to the inside flanking sequence of the T-DNA left border; **RS6, RS7 and RS8**, nested specific primers complementary to the inside flanking sequence of the T-DNA right border in plasmid pCAMBIA1300; **LB**, the left border; **RB**, the right border; ***P-Ubi***, maize ubiquitin promoter; ***P150***, 150 bp fragment homologous to P1 protein (protease) gene of maize dwarf mosaic virus; ***intron***, intron of maize actin gene; T-nos, terminator of nopaline synthase; ***P-35S***, cauliflower mosaic virus 35S promoter; ***Hpt***, hygromycin phosphotransferase gene; ***T-35S***, cauliflower mosaic virus 35S terminator.

derived from the positive calli, which were isolated from immature embryos of maize inbred line "18-599", and transformed by *A. tumefaciens* EHA105. This microbe strain harboured the engineered plasmids pCAMBIA1390 and pCAMBIA1300, that contained a hairpin expression construct of 451 and 150 bp fragments homologous to P1 protein (protease) gene of maize dwarf mosaic virus, respectively (Figures 1 and 2). The T-DNA integration into the maize genome had been identified to be single-copy by southern blotting (Zhang et al., 2010, the data of pCAMBIA1390 unpublished).

Amplification of flanking sequences by TAIL-PCR

For TAIL-PCR amplification of the flanking sequences around the T-DNA integration sites, 6 arbitrary degenerate primers (AD) of 15-17 bp length were designed according to the conserved amino acid sequences of the universal proteins in eukaryotes (Table 1, Liu et al., 1995). Five nested specific primers (LS1, LS2, LS3, LS4 and LS5) complementary to the inside flanking sequence of the T-DNA left border, 5 nested specific primers (RS1, RS2, RS3, RS4 and RS5) complementary to the inside flanking sequence of the T-DNA right border in plasmid pCAMBIA1390, and 3 nested specific primers (RS6, RS7 and RS8) complementary to the inside flanking sequence of the T-DNA right border in pCAMBIA1300 (Figures 1 and 2), were designed and synthesized at Invitrogen. The reaction system of 25 µl contained 12.5 µl of 2×Taq PCR MasterMix (Biomed-Tech), 20 ng of the template DNA, 4 pmol of one of the nested specific primers, and 40 pmol of one of the six arbitrary degenerate primers. For the secondary and tertiary rounds of the amplification, the products of the former round amplification was diluted 10-fold and used as templates. The products of the secondary and tertiary round amplification were separated by 2% argarose gel electrophoresis. The specific bands were recovered using Agarose Gel DNA Purification Kit Ver 2.0 (TaKaRa).

Repeat PCR amplification of TAIL-PCR products

To confirm that the separated bands were specific fragments amplified by a combination of an AD primer and a nested specific primer, the recovered product of the longest band in each electrophoretical lane of the secondary round was used as template to conduct repeat PCR amplification, with the same arbitrary degenerate primer and nested specific primers as used in the secondary and tertiary rounds of TAIL-PCR amplification. A consensus annealing temperature (52°C) was used to mediate the

Table 1. Arbitrary degenerate primes.

Primer	Sequence
AD1	NTC GAS TWT SGW GTT
AD2	NGT CGA SWG ANA WGA A
AD3	NGT ASA SWG TNA WCA A
AD4	STT GNT AST NCT NTG C
AD5	AGW GNA GWA NCA WAG G
AD6	TGW GNA GWA NCA SAG A

S, G/C; **W**, A/T; **N**, A/T/C/G.

difference of annealing temperatures between the AD primer and the nested specific primers. The elongation time was determined based on the speed of 1000 bp/min. The amplified product was separated by 2% argarose gel electrophoresis.

Sequence analysis

According to the specificity confirmation by the repeat PCR amplification, the recovered products of specific fragments amplified in the tertiary round TAIL-PCR were cloned into PMD18-T vector (TaKaRa), and sequenced with three replications at SinoGenoMax. After removing the sequences of PMD18-T vector and the expression constructs, local alignment was conducted between the sequences of the specific fragments and the maize genome (line B73), downloaded from maize Sequence (http://www.maizesequence.org), or the expression plasmid. The threshold identity and expect value were set to ≥90% and ≤e^{-100}, while the alignment coverage was more than 85% of the sequences of the specific fragments.

RESULTS

Specificity of TAIL-PCR products

From 60 of the total 70 transgenic maize lines, 42 and 33 fragments were amplified by the combinations of the AD and the nested specific primers complementary to the inside flanking sequence of the T-DNA left border and right border, respectively. By the repeat PCR amplification, 64 out of the 75 recovered products were confirmed to be specific fragments amplified from 57 transgenic maize lines. These fragments ranged in size from 400-1000 bp. The amplification efficiency of AD4 and AD6 was higher than the other AD primers (Figure 3). By the sequence analysis, 41 out of the 64 specific fragments were demonstrated to be the flanking sequences outside the left border (26 fragments) or right border (15 fragments) of the integrated T-DNA sequences. Thirty-two (78.0%) and nine (21.9%) of them are homologous to the sequences of the maize genome and the expression plasmid, respectively (Table 3). For the other 23 specific fragments, the identities and expect values of sequence alignment with the sequences of either the maize genome or the expression plasmid were out of the threshold criterions.

Flanking sequences around T-DNA integration sites

By the sequence analysis, 32 of the 41 flanking sequences were demonstrated to be homologous to the maize genome (Table 3). For 26 of them, a filler sequence of 3-63 bp long was found flanking the maize genomic sequence (Figure 4). These filler sequences were homologous neither among themselves nor to the sequences of the maize genome or the expression plasmids. For transgenic lines 1, 7, 8, 14, 15, 26, 32 and 46 (19.5%), the specific fragments were unable to be amplified by a nested specific primer the most adjacent to the left or right borders (LS5 or RS5), but by a nested specific primer farther from the left or right borders (LS3, RS3 or RS4, Figure 1). Nine of the specific fragments (21.9%) were found to be homologous to the backbone sequences of expression plasmids pCAMBIA 1300 or pCAMBIA1390. The length of the integrated plasmid sequences ranged from 360-1000 bp (Table 3).

T-DNA integration sites

Out of the 32 flanking sequences homologous to the maize genome, eleven were found homologous to the sequences at more than one physical site on three to ten chromosomes (Table 3), indicating that their integration sites are repetitive sequences. Because of the incompletion of the sequence data of the maize genome and the diversity of the genomic sequences between the acceptor maize line “18-599” and sequenced maize line “B73”, it was difficult to precisely identify the detail integration sites in the repetitive sequences which are highly homologous.

The other 21 flanking sequences were found homologous to the sequences at a single physical site on one chromosome. These precisely indentified integration sites distributed on all the 10 chromosomes, while seven of them (33.3%) clustered on chromosome 1. Sixteen (76.2%) of the 21 integration sites had relative distances to the centromeres of the integrated chromosome arms more than 0.50, implying that T-DNA integration prefers to the distal ends of chromosomes. The integration sites of lines 14, 19 and 35 were close adjacent to those of lines 15, 26 and 47, and the integration sites of lines 12, 13, 21 and 46 were close adjacent each other. Especially, the integration sites of lines 12 and 13 are exactly overlapped. These two lines were speculated to be derived from the same transformed event. Of these 21 integration sites, the four were precisely located between adjacent base pairs A/T and T/A, 14 between A/T and G/C, and three between G/C and C/G. The T-DNA integration site in line 59 was found during the encoding sequence of nucleic acid binding protein

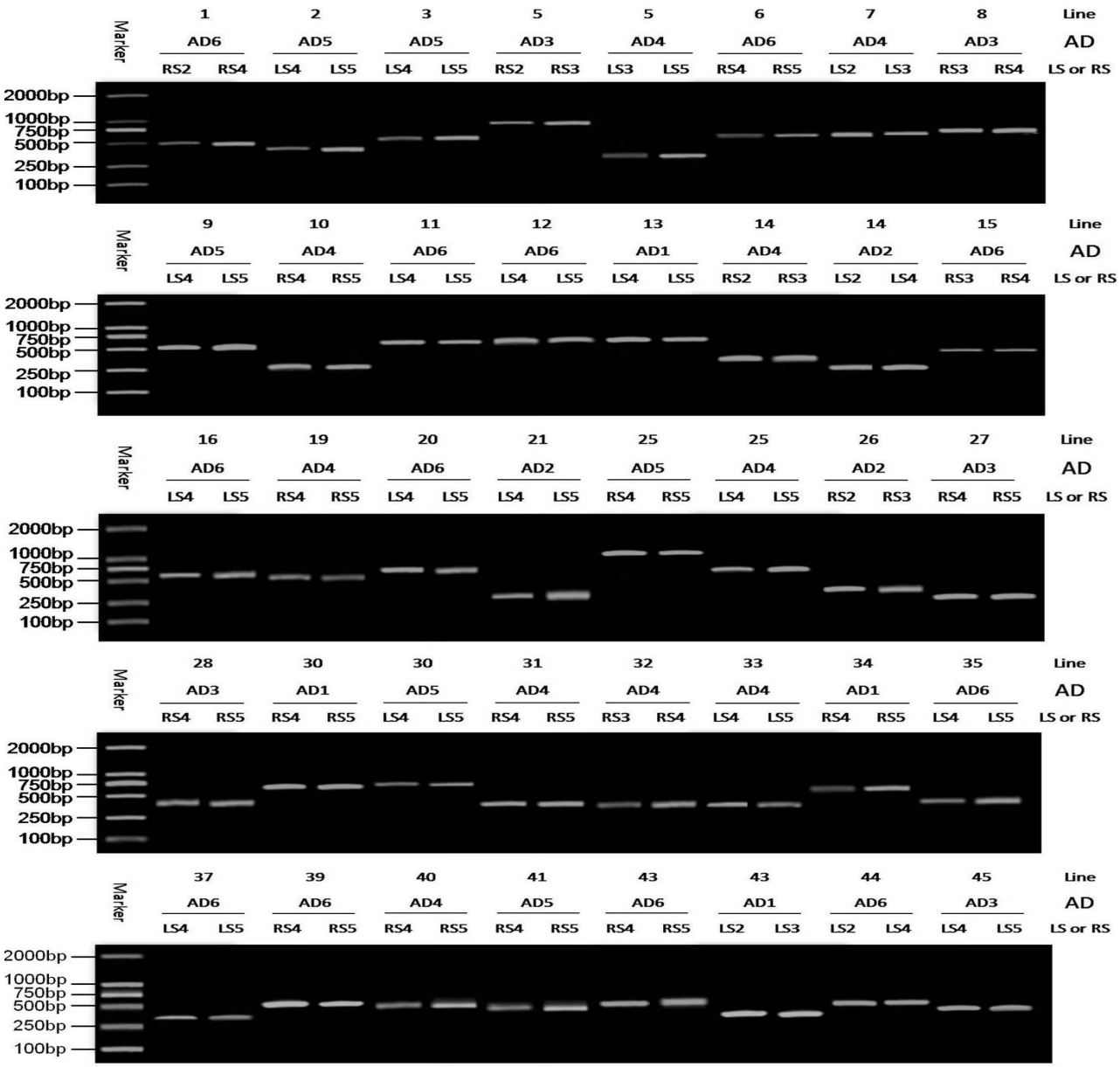

Figure 3. Specific bands amplified by the repeat PCR. **AD**, arbitrary degenerate primers; **LS**, nested specific primers complementary to the inside flanking sequence of the T-DNA left border; **RS**, nested specific primers complementary to the inside flanking sequence of the T-DNA right border. For each pair of the two electrophoresic lanes, the left lane was loaded with the repeat PCR product amplified using the secondary round product of TAIL-PCR as template, and the right lane was loaded with the repeat PCR product amplified using the tertiary round product of TAIL-PCR as template.

(LOC100280490, Table 3). Further study should be conducted to explore the influence of this integration to protein function and phenotype.

DISCUSSION

From 10 of the total 70 transgenic maize lines, it was unsuccessful to amplify detectable products both in the secondary and tertiary rounds of TAIL-PCR. This might be due to the poor adaptability of the 6 AD primers to the flanking sequences of the T-DNA integrated sites of these 10 transgenic maize lines. 11 of the 75 recovered products of the secondary round amplification were not confirmed to be specific fragments by the repeat PCR amplification. In TAIL-PCR amplification, non-specific fragments were probably amplified by 2 AD primers because the concentration of AD primes was 10-fold of the nested specific primers, and the annealing temperatures were increased slowly from low

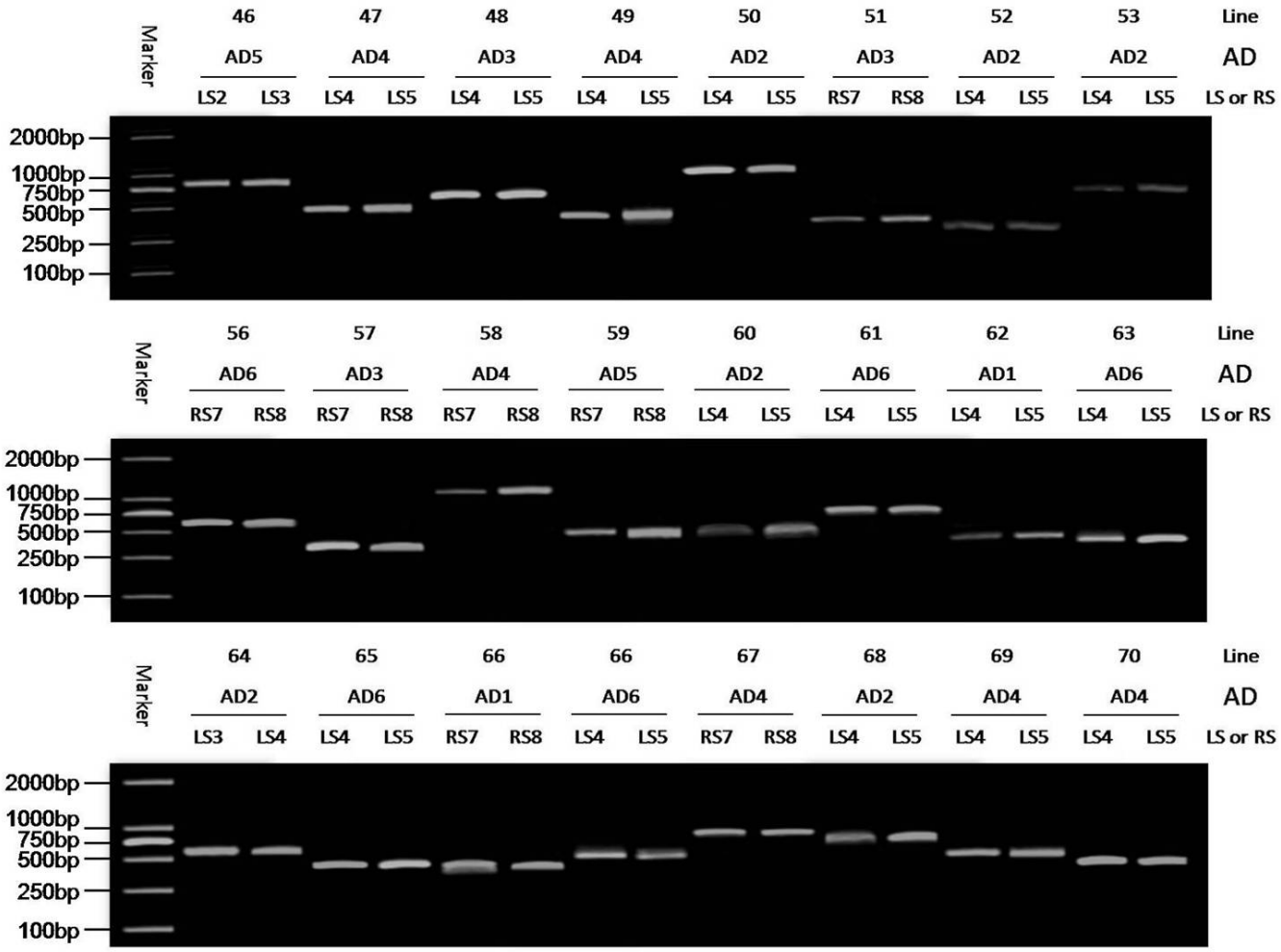

Figure 3. Contd

temperatures in several steps (Table 2).

For 23 of the 64 specific fragments, the identities and expect values of sequence alignment with the sequences of either the maize genome or the expression plasmid were out of the threshold criterions. This could be referred to the incompletion of the sequence data of the maize genome, and to the diversity of the genomic sequences between the acceptor maize line “18-599” and the sequenced maize line “B73”. Non-specific amplification is the major constraint of TAIL-PCR. On the basis of the standard TAIL-PCR (Liu et al., 1995) and its improved procedure (Sessions et al., 2002), we further improved the temperature cycles of the 3 successive rounds by gradient screening of optimal annealing temperature for the nested specific primers (Table 2), and verified the specificity of the amplified fragments by the repeat PCR. By this improved procedure, 64 out of the 75 recovered products were confirmed to be specific fragments (Figure 3). This amplification efficiency of specific fragments (85.3%) matches with the protocol of high-efficiency TAIL-PCR proposed by Liu et al. (2007). The simple improvements we made are useful for identification of flanking sequences around T-DNA integration sits.

In several other researches, the filler DNA sequences were found to be homologous to the sequences of the host genomes or the expression plasmids in some extent. It was explained by the molecular mechanism of microhomology-mediated end joining (De Buck et al., 1999; Windels et al., 2003; Zeng et al., 2010). In this study, the filler DNA sequences of 26 transgenic lines were homologous neither among themselves nor to the sequences of the maize genome or the expression plasmids. This result implies that the filler DNA sequences can be inserted by some other mechanisms such as modification and rearrangement of T-DNA sequence (Forsbach et al., 2002; Kole et al., 2010).

In available data, the left and right border sequences are considered as cleavage sites of T-DNA integration in the transformation mediated by *A. tumefaciens* (Kole et al., 2010). In this study, the backbone sequences of the expression plasmids were found around the T-DNA

Table 2. Temperature cycles of three TAIL-PCR rounds.

Reaction	Cycle	Thermal setting
Primary	1	93℃, 3 min; 95℃, 1 min
	5	94℃, 30 s; 68℃, 1 min; 72℃, 2.5 min
	1	94℃, 30 s; 25℃, 3 min, ramping to 72℃, over 3 min; 72℃, 2.5 min
	15	94℃, 15 s; 68℃, 1min; 72℃, 2.5 min;
		94℃, 15 s; 68℃, 1min; 72℃, 2.5 min
		94℃, 15 s. 44℃, 1min; 72℃, 2.5 min
	1	72℃, 5min
Secondary	12	94℃, 15 s; 68℃, 1 min; 72℃, 2.5 min
		94℃, 15 s; 68℃, 1 min; 72℃, 2.5 min
		94℃, 15 s. 44℃, 1 min; 72℃, 2.5 min
	1	72℃, 5 min
Tertiary	14	94℃, 40 s; 45℃, 1 min; 72℃, 2.5 min
	1	72℃ , 10 min

Table 3. T-DNA integration sites in the maize genome.

Transgenic maize lines	arbitrary degenerate primes	Nested specific primers	Integration site	Length (bp)	Similarity (%)	Nucleotides of inserted position	Relative distant to centromere
1	AD6	RS1, RS2, RS4	Chr5 111733188-111733633	445	99	T/G	0.06
2	AD5	LS2, LS4, LS5	Plasmid pCAMBIA 1390	368	99		
3	AD5	LS2, LS4, LS5	Plasmid pCAMBIA 1390	538	99		
6	AD6	RS1, RS4, RS5	Chr1, Chr2, Chr3, Chr4, Chr5, Chr6, Chr7, Chr8, Chr9, Chr10	500-550	>95		
7	AD4	LS1, LS2, LS3	Chr4 Chr6 Chr8	~500	>98		

Table 3. Contd.

8	AD3	RS2, RS3, RS4	Chr2 Chr5 Chr7	550-600	>99		
9	AD5	LS2, LS4, LS5	Chr1, Chr2, Chr3, Chr4, Chr5, Chr6, Chr7, Chr8, Chr9, Chr10	450-500	>97		
12	AD6	LS2, LS4, LS5	Chr1 288462705-288462064	641	97	C/G	0.93
13	AD1	LS2, LS4, LS5	Chr1 288462705-288462064	641	96	C/G	0.93
14	AD4	RS1, RS2, RS3	Chr3 72021965-72021615	350	98	G/A	0.24
15	AD6	RS2, RS3, RS4	Chr3 72021282-72021741	459	99	A/T	0.24
16	AD6	LS2, LS4, LS5	Chr9 112696401-112695807	594	99	T/G	0.53
19	AD4	RS1, RS4, RS5	Chr6 111104512-111103976	536	97	C/T	0.52
20	AD6	LS2, LS4, LS5	Plasmid pCAMBIA 1390	592	99		
21	AD2	LS2, LS4, LS5	Chr1 288462382-288462046	336	97	G/A	0.93
25	AD4	LS2, LS4, LS5	Plasmid pCAMBIA 1390	590	99		

Table 3. Contd.

26	AD2	RS1, RS2, RS3	Chr6 111103788-111104198	410	97	C/T	0.52
30	AD5	LS2, LS4, LS5	Plasmid pCAMBIA 1390	590	100		
31	AD4	RS1, RS4, RS5	Chr2 Chr5 Chr7	~400	>95		
32	AD4	RS2, RS3, RS4	Chr2 Chr5 Chr7	~400	>95		
33	AD4	LS2, LS4, LS5	Plasmid pCAMBIA 1390	386	96		
34	AD1	RS1, RS4, RS5	Chr1 102485634-102486191	557	99	A/C	0.23
35	AD6	LS2, LS4, LS5	Chr3 225058224-225057782	442	97	T/G	0.96
37	AD6	LS2, LS4, LS5	Chr1 275129001-275129338	337	97	T/T	0.85
39	AD6	RS1,RS 4,RS5	Chr1, Chr2, Chr3, Chr4, Chr5, Chr6, Chr7, Chr8, Chr9, Chr10	450-500	>99		
40	AD4	RS1,RS 4, RS5	Chr1 Chr3	~400	>97		
46	AD5	LS1, LS2, LS3	Chr1 288462876-288462064	812	96	A/C	0.93
47	AD4	LS2, LS4, LS5	Chr3 225058115-225057645	470	97	T/A	0.96
48	AD3	LS2, LS4, LS5	Plasmid pCAMBIA 1390	592	99		

Table 3. Contd.

49	AD4	LS2, LS4, LS5	Chr10 137382709-137383092	383	96	G/T	0.87
50	AD2	LS2, LS4, LS5	Plasmid pCAMBIA 1390	1086	99		
51	AD3	RS6, RS7, RS8	Chr 8 23230124-23230519	395	99	A/T	0.50
58	AD4	RS6, RS7, RS8	Chr1, Chr2, Chr3, Chr4, Chr5, Chr6, Chr7, Chr8, Chr9, Chr10	~1000	>95		
59	AD5	RS6, RS7, RS8	Chr4 240459215-240459685	470	91	A/G	0.96
60	AD2	LS2, LS4, LS5	Chr10 17568721-17568245	476	99	T/G	0.71
62	AD1	LS1, LS4, LS5	Chr2 13523648-13524073	425	100	A/G	0.85
65	AD6	LS1, LS4, LS5	Chr1 300174871-300175332	461	92	C/C	1.00
66	AD6	LS2, LS4, LS5	Chr1,Chr3,Chr6	~500	>95		
67	AD2	LS2, LS4, LS5	Plasmid pCAMBIA 1300	744	100		
68	AD4	LS2, LS4, LS5	Chr1,Chr3,Chr6, Chr9	~500	>98		
70	AD4	LS2, LS4, LS5	Chr4 141448908-141448482	426	93	T/C	0.30

Relative distance to centromere was calculated as the ratio of the distance of the integration site to centromere (Mb) divided by the full length (Mb) of the integrated chromosome arm. For transgenic lines 7, 8, 9, 10, 32, 33, 41, 42, 61, 70 and 74, the flanking sequences adjacent to the integration sites were found homologous to the maize genomic sequences at multiple physical sites on three to ten chromosomes.

integration sites (Table 3). Nine of the flanking sequences were amplified by the nested specific primers farther from the borders (LS3, RS3 or RS4, Figure 1). These results suggest that the cleavage occurs not only during the T-DNA borders but also inside or outside the borders. The border sequences of T-DNA are not cleavage sites but

Figure 4. Filler DNA sequences flanking the maize genomic sequences. The suspension points (…) represent sequences adjacent the left and right cleavage sites.

recognition sites of cleavage. If the cleavage site is inside the borders, the border sequences, as well as some sequences of different length inside the borders, can be deleted in the process of T-DNA integration. The nested specific primer cannot anneal at the most adjacent sequence to the left or right borders. If the cleavage site is outside the borders, some of the backbone sequences can be integrated into the maize genome. The length variation of the integrated plasmid sequences suggests that the cleavage sites are variable among the different transgenic lines. Similar results were obtained by Shou et al. (2004) and Zhu et al. (2006).

In previous studies (Brunaud et al., 2002), the adjacent base pairs of the integration sites were considered to have preference to A/T base pair, referring to its less stable pairing than G/C base pair. In this study, most of the adjacent base pairs of the integration sites were found to be other than A/T base pairs (Table 3). This result should also be explained by modification and rearrangement of T-DNA sequence (Forsbach et al., 2002; Kole et al., 2010).

According to the sequence analysis of integration sites, eleven out of thirty-two integration sites were found among repetitive sequences (Table 3). This ratio (34.4%) is much less than the proportion of repetitive sequences in the maize genome (SanMiguel et al., 1998; Zhou et al., 2009). The preference of T-DNA integration into non-repetitive sequences was also found in other plants (Szabados et al., 2002). Therefore, we conclude that T-DNA integration have some hot spots on maize chromosomes, and preference to the distal chromosomal ends and non-repetitive sequences, while illegitimate recombination is still a major pattern of T-DNA integration into the maize genome.

ACKNOWLEDGEMENTS

This work was supported by the Projects of Development Plan of the State Key Fundamental Research (973 Project) (2009CB118400), and the National Key Science and Technology Special Project (2008ZX08003-004 and

2009ZX08003-012B). We thank Professors Zhi-Zhong Chen and De Ye at China, Agricultural University for their kindly providing experiment facilities.

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