Journal of Plant Biology, March 2004, 47(1) : 1-7 **RESEARCH ARTICLE**

Studies on the Mobilization of a Maize Transposable Family, *Ac/Ds*, in Pepper Using In Vivo Transient Assay System

Chul Min Kim, Byoung Il Je, Ja Choon Koo, Hai Long Piao, Soon Ju Park, Joo Mi Jeon, Min Kyoung Kim, Sung Han Park, Jin Young Park, Eun Jin Lee, Woo Sik Chung[1,2], Kon Ho Lee[1,2], Kyu Young Kang[1], Sung-Ho Lee[2], and Chang-deok Han[1]

Division of Applied Life Science (BK21 program)
[1]Plant Molecular Biology & Biotechnology Research Center (PMBBRC)
[2]Environmental Biotechnology Research Center, Gyeongsang National University, Jinju 660-701, Korea

A maize transposable family, Ac/Ds, has been successfully utilized as a mutagenizing agent not only in monocot but also in dicot. In order to develop insertional mutagenesis system in pepper, the mobility of Ac/Ds has been examined. In this study, the excision of the elements was monitored via transient assay system with protoplasts. Two different systems were developed and compared; one- and two-elements systems. In a one-element system, Ac alone was introduced into cells. As a two-element system, Ac and Ds were cloned into a single vector and were expressed in protoplasts. Our data showed that both Ac and Ds elements were highly mobile in pepper cells. This is the first report suggesting that Ac/Ds mediated gene tagging system could be successfully operated in pepper.

Keywords; *Ac/Ds*, pepper, transient assay

Pepper is one of the most important vegetables in Korea. Understanding the pepper genome at the molecular genetic level is essential to improve the agronomic qualities of pepper plants. The pepper genome is around 2,702 Mb that is six times larger than rice genome (Arumanagathan and Earle, 1991). It is reasonable to speculate that chromosomal duplication and high repetition of various sizes of DNA blocks have taken place during evolution into such a large genome. Such high genomic complexity might lead to difficulty in conducting functional study on genomes and genes. To obtain molecular information and knowledge on pepper genes at the genomic scale, many strategies can be applied to explore gene function. Naturally, one of the most powerful methods is to conduct large scale insertional mutagenesis throughout the pepper genome. However, the efficiency of production of transgenic plants is known to be very poor in pepper. Therefore, in order to generate insertional pepper lines, transposon-mediated gene tagging systems that are operated by genetic manipulation (e.g., crossing or selfing) is the choice in developing insertionally mutagenized population. As heterologous elements, *Ac/Ds* and *Spm/dSpm* have been propagated and utilized as gene-tagging tool in Arabidopsis, tobacco, and rice (Wisman et al., 1998; Dooner et al., 1991; Chin et al., 1999). Especially, *Ac* (Activator) and *Ds* (Dissociation) in maize (McClintock, 1946) are the transposable element family that has been most extensively analyzed in heterologous plants. A variety of modified forms of *Ac* or *Ds* have been examined to dissect molecular mechanisms of transposition. The minimal sequence has been determined that is required for the mobility of *Ds* (Coupland et al., 1989). Extensive studies have demonstrated that *Ac/Ds* are frequently translocated to linked sites both in maize and in dicot such as tobacco and *Arabidopsis* (Jones et al., 1991; Long et al., 1993).

In this study, we tested the potential of the *Ac/Ds* transposable family as a gene tagging vehicle in pepper. So far, there has been no report whether heterologous transposable elements can be mobilized in pepper genomes. In this study, via transient assay system using protoplasts, we showed that *Ac* and *Ds* were highly mobile in pepper nuclei. Two different types of *Ac/Ds* mediated gene tagging systems were developed and compared; one- and two-elements systems. To test one-element system, *Ac* alone is introduced into cells. For two-element system, *Ac* and *Ds* were cloned into a single vector and were expressed in protoplasts. Our data suggested that both systems could be effectively operated in pepper plants.

*Corresponding author; fax +82-55-759-9363
e-mail cdhan@nongae.gsnu.ac.kr

MATERIALS AND METHOD

Construction of Vectors

An one-element vector, pCAMBIA::Ac, was constructed by inserting 4.7 kb genomic *Ac* DNA into the *Bam*HI site of 6.5 kb pCAMBIA T-DNA vector carrying HPT (hygromycin phosphotransferase), and GFP driven by 35S promoter (Fig. 1A). Whole *Ac* genomic DNA was donated by Jonathan Jones (John Innes Center, UK). A two-element vector, pMS9, was developed by inserting *Ac* and *Ds* into a single T-DNA vector, pCAMBIA1300. CaMV 35S promoter was used to express crippled *Ac* genomic DNA in which one end including the promoter region of the element was deleted by digestion of *Nae*I. Inside *Ds*, a 35S promoter carrying double enhancer was placed so that the promoter could transcribe the transcription of a coding region outside the element. *iaaH* (indole acetic acid hydrase) and NTPII were placed in the same transcription orientation and were used for a negative dominant marker and transformation-selection marker, respectively. *iaaH* gene was expressed by a p2 promoter from Ti plasmid and NOS terminator, which was of 2.1kb. NTPII was expressed by CaMV35S promoter and OCS terminator, which was of 1.4 kb. Both expression cassettes were originally derived from pWS1 (Sundaresan et al., 1995). As a *Ds* selection marker, a *bar* gene was installed inside the element. The *bar* gene was expressed by a p2 promoter from Ti plasmid and NOS terminator.

Protoplast Isolation and PEG-Mediated Transfection

The seeds were sterilized with 70% (v/v) ethanol for 1 min and with 3% sodium hypochlorite including 1 or 2 drops of Triton X-100 for 25 min and then they were rinsed several times in sterile distilled water. After sterilization, the seeds were sown in glass bottles containing hormone free MS basal medium and cultured for 3 weeks in light (16 hr per day at 5,000 lux from fluorescent lamps) at 25°C. Fully expanded leaves were used for protoplast isolation. Leaf tissues were cut into small (2-3 mm long) pieces and incubated overnight in an enzyme mixture containing 1 mgL^{-1} ascorbic acid, 1.2% cellulase R10 and 0.3% macerozyme R10 (Yakult Honsha Co. Ltd., Japan) in CPW 13 M solution (Power and Chapman, 1985) with agitation at 26°C in the dark. The digested tissue was passed through nylon sieves (100, 64 and 45 μm pore sizes) with gentle washing using CPW 13M solution. Protoplasts were pelleted by centrifugation and freed of cell debris by sucrose density gradient centrifugation with CPW 21S medium containing CPW salts with 21% (w/v) sucrose. Protoplasts were transferred to a mixture of KM8P and KM8 (2:1) media (Kao and Michayluk, 1975). A 300 μl

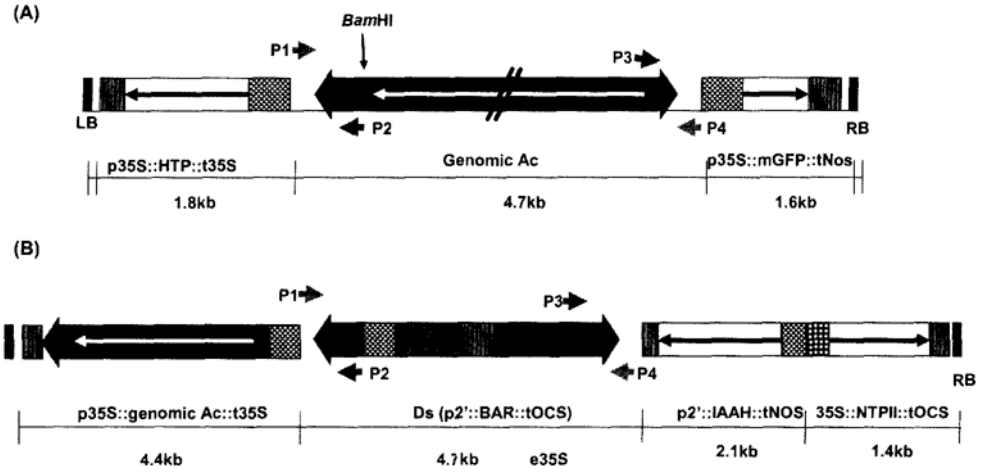

Figure 1. T-DNA vectors for one-element (A) and two-element systems (B), pCAMBIA::Ac and pMS9, respectively. **(A)** In a one-element system, the vector carried only *Ac* alone. Into the 6.5 kb CAMBIA backbone vector DNA, 4.7 kb genomic *Ac*, HPT (hygromycin phosphotransferase), and GFP driven by a 35S promoter were placed. HTP and GFP genes were expressed by 35S promoters. **(B)** In a two-element vector named pMS9, *Ac* and *Ds* were introduced into a single T-DNA vector, pCAMBIA1300. A CaMV 35S promoter was used to express *Ac* genomic DNA. Inside *Ds*, a *bar* gene was installed as a selection marker and double enhancer of a 35S promoter was placed at the near end of the element. As a counter selection marker, *iaaH* driven by a p2′ promoter from Ti plasmid was placed. Also, NTPII was expressed to be used for a transformation marker. The location o primers was indicated as arrow heads. Boxes containing arrows show coding regions and their transcription orientation. The boxes in the front of these arrow-boxes indicate promoters while ones behind do terminators.

of protoplast mixture (1.5×10^6 protoplasts) was put in a 15 ml glass tube and 15 µg of plasmid DNA was added to protoplasts, immediately followed by dropwise addition of the 300 µl $Ca(NO_3)_2$-PEG solution containing 40% PEG6000 (MW), 0.4 M $Ca(NO_3)_2$, and 0.45 M mannitol to the protoplast-DNA suspension. After 25 min incubation at room temperature, 5 ml washing solution (CPW13M) was added slowly. Cells were collected by centrifugation.

Transient Assay with PCR (Polymerase Chain Reaction)

Genomic DNA was prepared from protoplasts through urea extraction procedures. PCR was performed with 1 µg genomic DNA. To analyze the excision of *Ds* from each vector, four primers (primer 1, 2, 3 and 4) were used in this study. The amplification program included an initial step at 94°C for 3 min, 30 cycles (1 min at 94°C, 30 sec at 65°C, 30 sec at 72 °C) and a final step at 72°C for 10 min. For pCAMBIA::Ac, primer 1 and primer 2 recognized vector sequences adjacent to the 5′ and 3′ ends of *Ac*, respectively. Primer 3′ and 4′ recognized the 5′ and 3′ end of genomic *Ac*, respectively. The sequences of primer 1, 2, 3 and 4 were 5′-atgttggcaagctgctctag-3′, 5′-gatcagattgtcgtttcccg-3′, 5′-atacgataacggtcgg-3′, and 5′-tatgaaaatgaaaacggtagagg-3′, respectively. The sequences of four primers for the analysis of pMS9, primer 1, 2, 3 and 4, are 5′-atgttggcaagctgctctag-3′, 5′-gatcagattgtcgtttcccg-3′, 5′-tggaaagcgggcagtgagc-3′, and 5′-agcttgcatgcctgcaggtc-3′, respectively. Amplified DNA was size-separated by electrophoresis on a 1.2% agarose gel.

RESULTS

Generation of One-Element and Two-Element Vectors

T-DNA vectors were constructed such that the transfection of vector DNA and the excision of *Ac* or *Ds* was able to be detected in a visual way or by PCR. Two different *Ac/Ds* systems were constructed and compared for excision activity of *Ac* or *Ds*. Schematic diagrams of these two vectors used in this study are shown in Fig. 1. The first vector, pCAMBIA::Ac, carried only *Ac* alone. In the 6.5 kb CAMBIA backbone vector, 4.7 kb genomic *Ac*, HPT (hygromycin phosphotransferase), and GFP driven by a 35S promoter were placed (Fig. 1A). In the second vector named pMS9 (Fig. 1B), a two-element system was constructed. *Ac* and *Ds* were introduced into a single T-DNA vector, pCAMBIA1300. A CaMV 35S promoter was used to express crippled *Ac* genomic DNA in which one end including the promoter region of *Ac* was deleted up to a NaeI site. Immobile *Ac* supplies transposases to mobilize a non-autonomous *Ds* element. Inside *Ds*, double enhancer of the 35S promoter was placed so that the promoter could transcribe a coding region outside the element. As a counter selection marker, *iaaH* driven by a p2 promoter from Ti plasmid was placed. Also, a *bar* gene was installed inside *Ds*, which would serve as a *Ds* selection marker.

In Vivo Transient Assay for the Activity of Ac/Ds

To introduce each vector DNA into cells, protoplasts were infected with about 15 µg of DNA in PEG solution. To verify that vector DNA was transferred into nuclei, and then genes in the vectors were expressed, pCAMBIA::Ac-infected protoplasts were examined for the expression of GFP. Fig. 2 shows the expression of GFP under the fluorescent light microscope. Around 30% of the protoplasts expressed GFP gene that was introduced via pCAMBIA::Ac vector. After 24 hr, infected protoplasts were collected and incubated in urea-basis DNA extraction buffer. Total genomic DNA was extracted from protoplasts.

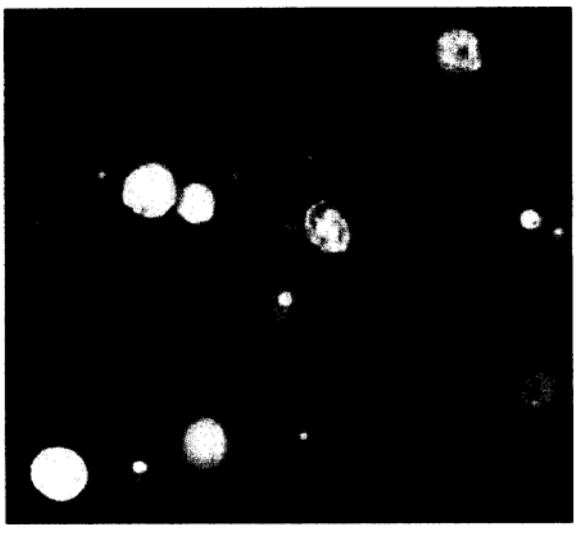

Figure 2. Expression of GFP of pCAMBIA::Ac in pepper protoplasts. Protoplasts were prepared from pepper leaves. DNA of pCAMBIA::Ac was transfected into protoplasts using PEG method. To examine the expression of the vector genes, pCAMBIA::Ac-transfected protoplasts were observed for the expression of GFP under a fluorescent light microscope. After 24 hr, around 30% of protoplasts expressed GFP gene that was introduced via pCAMBIA::Ac vector.

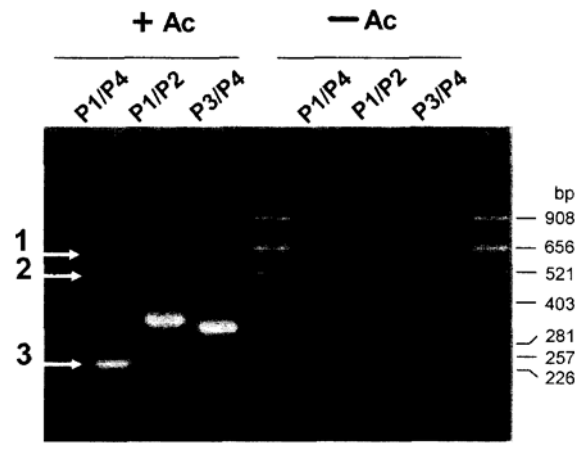

Figure 3. Detection of excision of *Ac* by PCR. Genomic DNA was prepared from protoplasts 24 hr after transfection (+Ac). For DNA source of a negative control (-Ac), protoplasts from normal pepper leaves were used. To detect the excision of *Ac* from pCAMBIA::Ac, the genomic DNA cut with BamHI was subjected to amplification with primers P1 and P4 (Fig. 1A). Three different sizes of DNA, 520, 500, and 250 bp, were detected (arrows in the figure). To test the integrity of vector DNA in pepper cells, the 5′ and 3′ ends of *Ac* were amplified by two primer sets, P1/P2 and P3/P4, respectively. The expected sizes from the amplification of the 5′ and 3′ ends were around 350 and 330 bp, respectively.

To detect the excision of *Ac* from pCAMBIA::Ac, the genomic DNA cut with BamHI was subjected to amplification with primers P1 and P4 (Fig. 1A). The *Ac* element of pCAMBIA::Ac carried only one BamHI site. These two primers recognized vector sequences next to the ends of *Ac*. Therefore, no DNA should be amplified from the pCAMBIA::Ac plasmid DNA that was digested with BamHI. Even if DNA could escape from enzymatic digestion, 4.7 kb of a genomic *Ac* element would be hardly amplified under the PCR condition of this experiment. In contrast, the pCAMBIA::Ac from which *Ac* is excised should produce around 520 bp DNA. Fig. 3 is an ethidium bromide stained gel of PCR products amplified by three different sets of two primers, P1/P4, P1/P2, and P3/P4. The P1/P4 set of primers was to detect the excision of *Ac*. P1/P2 was to amplify DNA between the 5′ end of *Ac* and the adjacent plasmid sequence. P3/P4 was for the amplification of DNA spanning from the 3′ end of *Ac* to the adjacent plasmid sequence. As a control, genomic DNA from un-infected protoplasts were used as a PCR template. As shown in Fig. 3, control PCR ("-Ac") did not produce any product, which indicated that PCR products of "+Ac" were derived from pCAMBIA::Ac vector. From excision sites, one major and two minor DNA species of different sizes were detected. Arrows in the lane "P1/P4" of "+Ac" in Fig. 3 show these three DNA bands of 520, 500 and 250 bp. Among them, the 250 bp DNA band (band 3) was the most abundant PCR product. Such heterogeneous sizes of *Ac* donor sites could be explained by previous reports that excision of *Ac/Ds* frequently accompanied DNA rearrangements such as deletions and additions (for details, see Gorbunova and Levy, 1997). However, it was still possible that the generation of multiple DNA could be related to instability of pCAMBIA vector DNA located inside pepper cells, rather than to *Ds* excisions. To test this possibility, DNA from both ends of *Ac* to the flanking DNA was amplified using P1/P2 and P3/P4, respectively. If the vector DNA *per se* underwent physical rearrangement inside cells, heterogeneous sizes were also expected from PCR products spanning either end of *Ac*. However, if the excision of *Ac* was responsible for heterogeneity of PCR product sizes, the same PCR reaction should generate only a single discrete PCR product from either end of *Ac* that still stayed in introduced vectors. PCR using either P1/P2 or P3/P4, whose location is indicated in Fig. 1, produced expected sizes of discrete DNA products. Expected size of DNA was 350 bp that spanned from the 5′ end of *Ac* to the flanking DNA. Also, the discreet band of 330 bp was detected when the 3′ end of *Ac* was amplified by primers 3 and 4. This experiment demonstrated that pCAMBIA::Ac was stably maintained in the cells and *Ac* was readily transposed.

To explore the nature of polymorphism, three major DNA products were cloned and sequenced. Based on sequence analysis of these three bands, it was found that "band 1" resulted from deletion of both sides of *Ac* insertion site and from replacement of new vector sequence. The sequence of "band 1" is presented in Table 1. Twenty one and sixty six bases of vector sequence were missing from the 5′ and 3′ ends of the element, respectively. Seventy five bases were filled into the deletion site. In "band 2", vector sequences adjacent to both sides of *Ac* were deleted as indicated in Fig. 4. The third band appeared to be generated by substantial deletion of vector sequence adjacent to the 3′ end of *Ac*. Most of the sequence from vector flanking to the 5′ end of *Ac* was missing. The sequence of "band 3" was presented in Tabe 1. Analysis of the excision products revealed that vector DNA adjacent to an *Ac* excision site was deleted in different extents. Fig. 4 shows the vector sequences and the breaking points are indicated by arrows. Previous studies on the foot-prints in other plants, such as

Table 1. Sequence of Bands 1 and 3 carrying new sequences at the excision site.

	Band 1	Band 3
Vector sequence flanking the 5′ end of *Ac*	CGGGAAACGACAATCTGATC.../// ..CTGGAAAGCGGGCAGTGAGCGCAACGCAATT AATGTGAGTTAGCTCACTCATTAGGCACCCCAG GCTTTACACTTTATGCTTCCGGCTCGTAT GTTGTGTGGAATTGTGAGCGGATAACAATTTCA CACAGGAAACAGCTATGACCATGATTACG(**AGC TCGGTACGGTACCC deleted)**	CGGGAAACGACAATCTGATC **(The rest of the sequence was** deleted)
New sequences at the excision sites	CCAAGCGCGCAATTAACCCTCACTAAAGGGAA CAAAAGCTGGAGCTCCACCGCGGTGGCGGCC GCTCTAGAACTAGTGGATCCCCCGGGCTGCAG GAATTCGATTATGTTGGCAAGCTGCTCTAGA	AGTCGACCTGCAGGCATGCAAGCTTGGCACTG GCCGT
Vector sequence flanking the 3′ end of *Ac*	GTCGACCTGCAGGCATGCAAGCTTGGCACTGG CCGTCGTTTTACAACGTCGTGACTGGGAAAAC CCTGGCGTTACCCAACTTAATCGCCTTGCAGCC ATCCCCCTTTCGCCAGCTGGCGTAATAGCGAA GAGGCCCGCACCGATCGCCCTTCCCAACAGTT G....///	(GTCGACCTGCAGGCATGCAAGCTTGGCACTG GCCGT deleted) CGTTTTACAACGTCGTGACTGGGAAAACCCTG GCGTTACCCAACTTAATCGCCTTGCAGCACATC CCCCTTTCGCCAGCTGGCGTAATAGCGAAGAG GCCCGCACCGATCGCCCTTCCCAACAGTTG.//

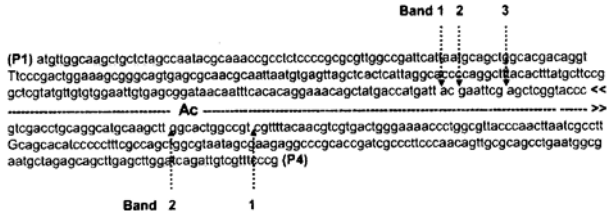

Figure 4. Location of deletion sites on vector sequences due to *Ac* excisions. From the excision sites, one major and two minor DNA species of different sizes were detected as shown in the lane 2 of Fig. 3. Based on the sequencing data on these three DNA of 520, 500 and 250 bp, deletions and replacement were detected in vector DNA adjacent to both ends of the *Ac* element. Arrows showed the break points of deletions. The underlined sequences were ones of P1 and P4 primers, as indicated in Fig. 1A.

maize and *Arabidopsis*, demonstrated that excision of transposable elements frequently accompanied DNA rearrangement such as deletion and replacement.

In the second strategy, pMS9 vector DNA of a two-element system was introduced into protoplasts under the same condition. As a control, pMS9-Ds was used in which *Ds* was absent. The only difference between pMS9 and pMS9-Ds was the presence or absence of a *Ds* element. Genomic DNA from protoplasts infected with either pMS9 or pMS9-Ds were subjected to PCR using three different sets of primers, P1/P4, P1/P2, and P3/P4 (Fig. 5B). The first set of primers (P1/P4) was to detect excision product. The second and third sets were used to examine integrity of the 5′ and 3′ ends of untransposed *Ds* elements, respectively, that still stayed in the vector DNA. PCR products were visualized on an ethidium bromide stained gel. As seen in lane 1 of Fig. 5A, a unique single DNA band was detected in excision product with P1/P4. This was the predicted size of a PCR product from an empty donor site, 200 bp, if DNA rearrangement did not take place while *Ds* was excised. As expected, the size was identical to one from the control vector, pMS9-Ds (lane 2 of Fig. 5A). It was in contrast to what was observed in protoplasts infected with pCAMBIA::Ac (see Fig. 3B). From amplification of the 5 end of pMS9-Ds with P1/P2 and of the 3′ end with P3/P4, PCR products were expected to be around 500 and 220 bp, respectively. In fact, PCR of the 3′ end produced a single DNA of 220 bp (lane 5 of Fig. 5A). However, the amplification of the 5′ end yielded several DNA bands (lane 3 of Fig. 5A). This was another difference from what was seen from pCAMBIA::Ac that produced a single DNA from PCR to amplify the 5′ end of *Ac*.

Considering the fact that pCAMBIA::Ac and pMS9 were infected into protoplasts isolated from the same variety, a major difference between two systems might be, at least in part, the expression level of *Ac* mRNA. The strength of the endogenous promoter of *Ac* has been known to be very weak. Meanwhile, 35S promoter is one of the strongest in dicot plants. Therefore, it is reasonable to argue that any observed difference might be due to the difference in the level of *Ac* protein. Previous reports suggested that the intracellular level of *Ac* protein was one of the determinants of the frequency of transposition (Scofield et al., 1993). Based on our data on *Ac* and *Ac/Ds* mobility, *Ac* excision from pCAMBIA::Ac led to the production of multiple

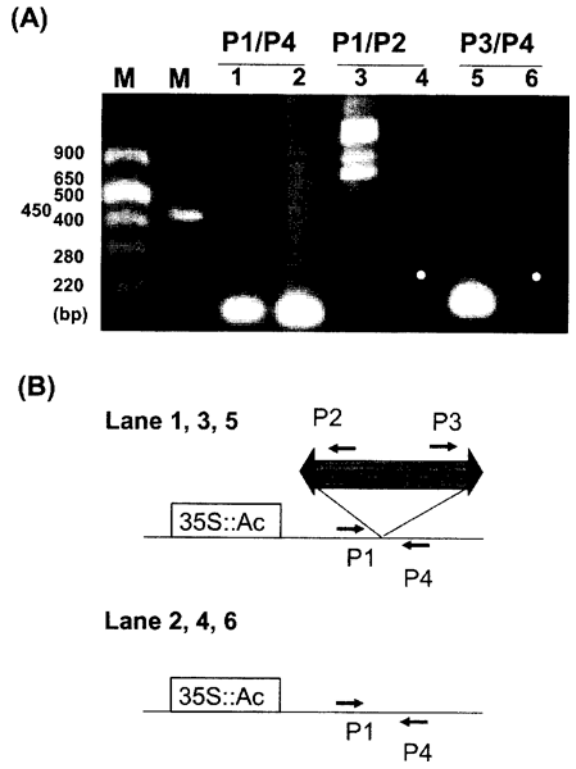

Figure 5. Detection of excision of *Ds* by PCR (A) and location of primers (B). (**A**) The excision activity of *Ds* from pMS9 vector DNA was examined after being introduced into protoplasts (lanes 1, 3 and 5). As a control, pMS9 was used in which *Ds* was absent (lanes 2, 4 and 6). Genomic DNA from protoplasts infected with either pMS9 or pMS9-Ds were subjected to PCR using three different sets of primers, P1/P4, P1/P2, and P3/P4. (**B**) The first set of primers (P1/P4) was to detect excision product. The second (P1/P2) and third (P3/P4) sets were used to examine integrity of the 5′ and 3′ ends of untransposed *Ds* elements, respectively, that still stayed in infected vector DNA. Very low amounts of PCR products of unknown source were detected in lanes 4 and 6 and indicated as "*" in the figure.

DNA bands while a single expected product was obtained from the *Ds* excision site of pMS9. Therefore, 35S promoter-driven expression of *Ac* tends to result in less DNA rearrangement at the donor site of the element. However, it appeared that the 5′ end of DNA was very unstable, by unknown reason, in the presence of the high level of *Ac*.

DISCUSSION

In understanding the biological function of pepper genes, a transposon-mediated gene tagging system could make significant contribution. Using transient assay system, this study demonstrated that *Ac/Ds* tagging system could be successfully operated in pepper plants. To effectively operate *Ac/Ds* mediated gene tagging system, it is required to generate a reasonable number of transgenic plants harboring *Ac* and *Ds*. Among these transgenics, ones that carry simple and single insertions of *Ac* and *Ds* T-DNA of a single copy should be selected and developed into 'starter lines'. Since a single pollination produces many seeds, a reasonable size of F_1 seed population could be generated even from limited pollination between *Ac* and *Ds* starters. This is an advantage in propagating the population of transposants in pepper. However, the large pepper genome (around 2,702 Mb) might contain complex chromosomal duplication and high repetition of various sizes of DNA blocks. Therefore, compared to plants of smaller sizes of genomes, phenotypic mutations from tagged genes could be less frequently detected in pepper. To effectively retrieve functional information from *Ds*-inserted locus, it might be necessary to establish advanced gene-tagging systems such as activation tagging or gene trapping. *Ds* has been successfully utilized in enhancer/gene trap vehicles in *Arabidopsis* and rice (Sundaresan et al, 1995; Chin et al., 1999) in which a reporter gene encoding either β-glucuronidase (*gusA*) or GFP is installed inside *Ds*. By visualizing the expression pattern or localization of genes via GUS or GFP, the function of tagged genes can be elucidated even though there is no noticeable phenotype of knockout plants. In plants, activation tagging systems have been operated to identify genes of duplicate function (Weigel et al., 2000). One of the most noticeable observations in this study is that *Ac/Ds* was actively mobilized without DNA replication. In monocots, such as maize and rice, it has been known that the transposition of *Ac* or *Ds* is coupled with replication (Greenblatt, 1984; Chin et al., 2000). However, in dicots such as petunia, *Ac* movement was detected using plasmid DNA that is not able to replicate inside cells (Houba-Hérin et al., 1990). The vectors used in this study contained no self-replication components in plant cells. Furthermore, excision was examined 24 hr after infection of DNA into protoplasts. Within 24 hr, there was no evidence of division of protoplasts. Therefore, in pepper, DNA replication is not required to the transposition of *Ac/Ds*.

Even though we demonstrated that *Ac/Ds* was active in pepper protoplasts, further experiments should be directed to obtain the information whether the activity or mobility of *Ds* can be maintained in subsequent cell divisions after vectors are integrated into the pepper genome.

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