

## Determination of Integrated Sequences of Non-T-Dna in Genome of Transgenic *Nicotiana Benthamiana* Plants

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### ABSTRACT

Genetic transformation of plants by useful genes is widely made by *Agrobacterium tumefaciens* harboring manipulated T-DNA sequences of the binary plasmid. Determining of the integrated T-DNA in the final step of the transformation is critical for understanding the mechanism of this process. The integration of non-T-DNA sequences in transgenic *Nicotiana benthamiana* plants obtained through *Agrobacterium* inoculation was investigated. Genomic DNAs from ten plants were subjected to PCR amplification on the sequences located outside the left or right T-DNA borders. Three plants out of ten were found to have 30 base pair of non-T-DNA sequences including the entire left T-DNA border. The same plants were also contained 107 base pair of the non-T-DNA sequences with the entire right border sequence. The percentage of integrated non-T-DNA sequences represented 30 % whether it belongs to sequences followed the right or left T-DNA side. Integrated T-DNA sequences were confirmed by southern blot analysis.

**Keywords:** T-DNA integration, PCR analysis, southern hybridization, vector backbone sequences, *Agrobacterium tumefaciens*.

### INTRODUCTION

Investigation of the functional genomics is the most important point in transgenic plants for discovering the biological function of particular genes and to uncover how genes work together leading to improvement of the quality and quantity of agronomic traits. Integration of one copy of the transgene reduces the potential for unintended insertional inactivation events and for transgene silencing associated with complex integration events (Chawla, *et al.*, 2006). It has been known for a long time that the pathogenic *Agrobacterium tumefaciens* is capable to transfer DNA to plant cells (Zupan *et al.* 2000). Therefore, it has been used for transformation of several plant species for the gaining of new characters. The T-DNA region located on the Ti (tumor-inducing) plasmid of the *Agrobacterium* is limited by left and right repeats called border sequences, facilitate the transfer of DNA (Gelvin 2003). Transformation of T-DNA from *Agrobacterium* cell to plant cell is facilitated by the *virA* and *virG* gene products. These genes are acting as transducer outer signals. Therefore, activating the rest of *vir* genes leading to the formation of a pilus for DNA transfer and the T-DNA is released by activated *virD1* and *virD2* endonucleases genes. These genes produce either one or double strand nick at the 25 bp border sequences of the T-DNA. Formation of a single T-strand or a double stranded T-DNA by the *virD1* and *virD2* might rely on the functions of virulence relating to the *Agrobacterium* strain (Steck 1997).

Both the right or left border sequence could initiate transferring the T-DNA, while it is more natural for starting the transferring of T-DNA at the right border because of incidence of a nearby overdrive sequence that is identified by the *VirC1* and *VirC2* proteins. These proteins are acting as transformation enhancers (Shaw *et al.* 1984).

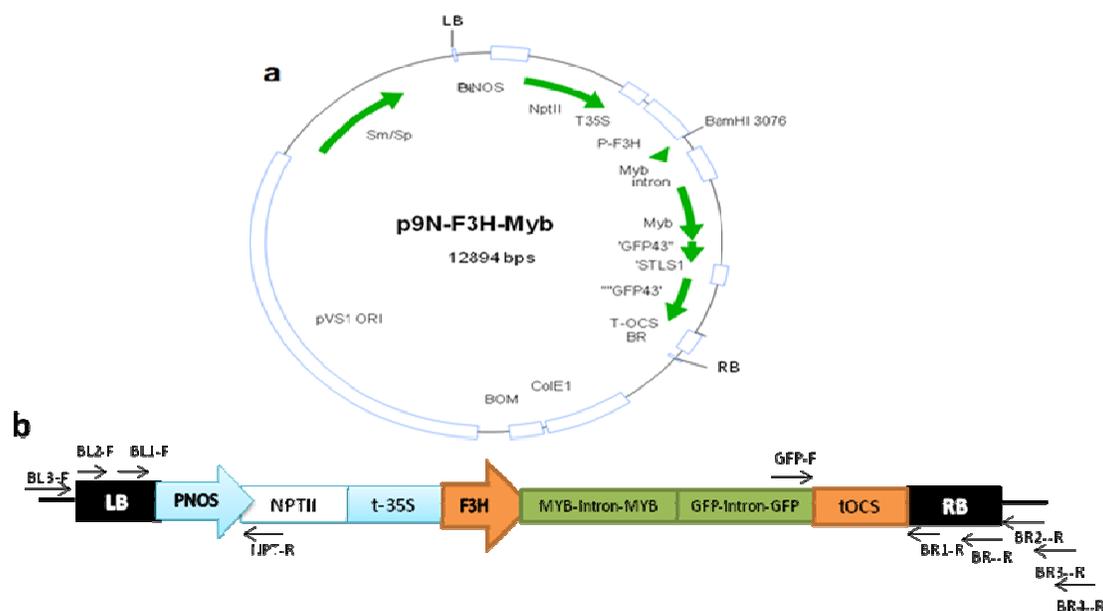
At the beginning of the technology of plant transformation researchers believed that the only

sequences located between T-DNA right and left borders are transferred to the plant cell. However, it is now evident that more than a few reports have come into view explaining the integration of other sequences like vector backbone into the genome of transgenic plants. Wenck *et al.* (1997) reported that the entire binary vector containing sequences of the backbone in addition to the sequences of T-DNA might be integrated in the genome of *Nicotiana plumbaginifolia* and *Arabidopsis thaliana*. Kononov *et al.* (1997) deeply analyzed the sequences of binary vector backbone, tree fourth of transgenic tobacco plants having sequences of vector backbone. This was explained as such transfer might be the outcome of the initiation of transferring T-DNA from the left border to get vector backbone sequences into plant genome or from skipping the left T-DNA border when T-DNA was moved from the binary vector. This study aimed provide evidences of integration of non-T-DNA into *Nicotiana benthamiana* plants through characterizing the border sequences of T-DNA and vector backbone in the genome of *Nicotiana benthamiana* plants.

### MATERIALS AND METHODS

#### Plant material, Plasmids, bacterial strain

Seeds of *Nicotiana benthamiana* were surfaced sterilized according to Khidr *et al* (2012). Sterilized seeds were incubated on MS medium (Murashige and Skoog, 1962) in darkness at 25° C. *Agrobacterium tumefaciens*, LBA4404 strain, harboring the binary vector p9N-F3H-Myb was used for genetic transformation experiment. The T-DNA of the binary vector p9N-F3H-Myb contained a selectable marker *npII* controlled by promoter of *nos* gene and terminated by 35S terminator and the *MYB-GFP* fusion gene driven by *F3H* promoter and terminated by *ocs* terminator. The plasmid map and T-DNA construct are shown in Figure 1a and 1b, respectively.



**Figure 1. a, plasmid map; b, schematic representation of T-DNA construction. RB and LB represent the T-DNA right and left borders. The selectable marker *nptII* is driven by *nos* and terminated by 35S terminator, MYB10-GFP fusion driven by F3'H and terminated by OCS terminator; arrows indicate the primers binding positions.**

**Plant transformation and regeneration**

Leaves were detached from 10 days old of *in vitro* grown seedling and incubated with *Agrobacterium* as described by Khidr and Nasr (2012). Briefly, 50 leaf discs of *in vitro* grown of 10 *Nicotiana bentamiana* plants were cocultivated with *Agrobacterium* for two days at 25 °C on MS medium (Murashige and Skoog 1962) with 0.1 g/L NAA and 1mg/L BAP. The leaf discs were moved to the same media with 100 mg/L Kanamycin and 500 mg/L Carbenicillin. The explants were subcultured every 3 weeks on fresh medium until formation of callus and the shoots are starting to form in six to eight weeks. After development of the shoots, they were isolated separately and transferred onto MS media without plant growth regulators for two weeks. Regenerated plantlets with roots were transferred to watered soil in pots and covered with plastic bags for acclimatization.

**DNA isolation and PCR analysis**

DNA was extracted from 100 mg leaf tissues of 10 putative transgenic lines by the DNeasy Plant Mini Kit (Qiagen, Germany). Analysis of left and right border sequences of T-DNA construct including vector backbone was performed using appropriate PCR primer pairs listed in Table 1. The PCR reaction was carried out in 25 µl containing 20-50 ng of template DNA, 1X Dream Tag™ buffer, 0.2 mM deoxynucleoside triphosphates (dNPTs), 0.5 µM of each primer and 0.5 unit Dream Tag™ DNA polymerase (MBI Ferments, Germany). The program of PCR was carried out as following: 94 °C for 2 min in the initial denaturation, followed denaturation at 94 °C for 30s (30 cycles), annealing at 53-60 °C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 5 min.

**Table 1. Primers used for examining the presence of integrated T-DNA borders and vector backbone in the plant genome.**

Primer		Sequence 5'>3'	Annealing [°C]	
BL (left border flanking region)	1F <sup>1</sup>	agcggcggcaggatatattca	60	
	2F <sup>1</sup>	gctcgattgtacctcggtca		
	3F <sup>1</sup>	caaggtagtcggcaaataatgtct		
nptII-Tail	R	agaacctgcgtgcaatccatc	60	
	F	cttcaaggacgacggaatta		
GFP	1R <sup>2</sup>	aaggcgggaaacgacaatct	53	
	2R <sup>2</sup>	tcttaggttaccgccaat		
BR (right border flanking region)	3R <sup>2</sup>	gcacatacaaatggacgaecgg	55	
	4R <sup>2</sup>	atcaagccacagcagcccact		
	5R <sup>2</sup>	tatttctgtggagtcccgcc		
				54

<sup>(1)</sup> primers were used in combination with primer nptII-Tail-R; <sup>(2)</sup> primers were used in combination with primer GFP\_F; F: forward primer, R: reverse primer.

**Southern hybridization**

Detection of integrated T-DNA copies in the plant genome was carried out by southern blot analysis; the DNA was extracted from 100-200 mg plant leaf

tissues of the lines number 7, 9 and 10 using modified cetyl trimethyl ammonium bromide (CTAB) extraction protocol as described by Roger and Bendich (1988). 10 µg DNA were digested with 100 units of Bam HI (MBI

Ferments) at 37 °C overnight. The digested DNA was separated in agarose gel (1%) and transferred on nylon membrane (Roche Diagnostics). Digoxigenin-labeled probes were amplified with forward and reverse primers on the T-DNA border and were used for hybridization. Detection and hybridization were achieved with the ECF-Random-Prime-Labeling and Detection Kit (Amersham Biosciences) according to the manual of manufacturer.

## RESULTS AND DISCUSSION

### Molecular evaluation of T-DNA borders and vector backbone sequences

#### ▪ PCR analysis of the right and left T-DNA borders and vector backbone sequences

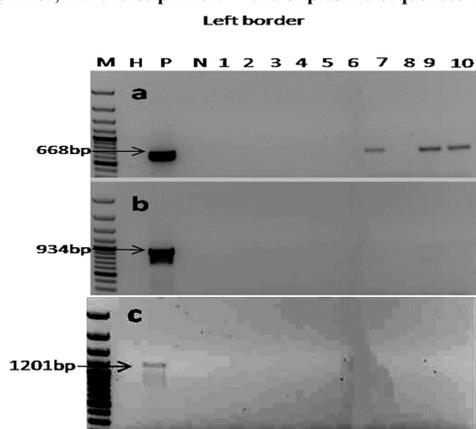
Testing the presence or absent of right and left T-DNA borders, and vector backbone sequences linked to the transferred T-DNA in the genome of transgenic *Nicotiana benthamiana* plants was evaluated by PCR using eight primer pairs. Tail-PCR with three primer pairs were used to amplify 668, 934, and 1201 bps on the flanking region of the left border and vector backbone sequences. Results in Figure (2) showed that only one primer pair was able to amplify a fragment of 668 bp in three DNA samples (number 7, 9 and 10) of

the transgenic lines, 638 bp of the amplified product located inside the T-DNA border and only 30 bp located on the vector backbone, outside the T-DNA (Table 2). While, no amplification was occurred with the other two primer pairs in all of the examined transgenic lines. On the other hand, PCR was carried out using five primer pairs flanking the right border region of the transferred T-DNA and vector backbone sequences to amplify 691, 742, 819, 985 and 1192 bps. The results in Figure (3) revealed a fragment of 691 bps with the first primer pair in all transgenic lines except line number 2. The amplified product is located inside the border sequences, No part of its sequences is belong to the vector backbone. The second primer pairs revealed a fragment of 742 bps with DNA samples number 7, 9 and 10 of transgenic lines, 712 bp of this product belong to the right border and 30 bp of them presented in the vector backbone sequences, outside the T-DNA (Table 2). Three transgenic lines number 7, 9 and 10 were positive with the third primer pair and produced a fragment of 819 bp in length, 712 bp of this amplicon belong to the right border and the remaining 107 bp located on the vector backbone (Table 2). Whereas, no amplification was observed on the DNA samples of all transgenic lines with the other tested three primer pairs on the right border and vector backbone.

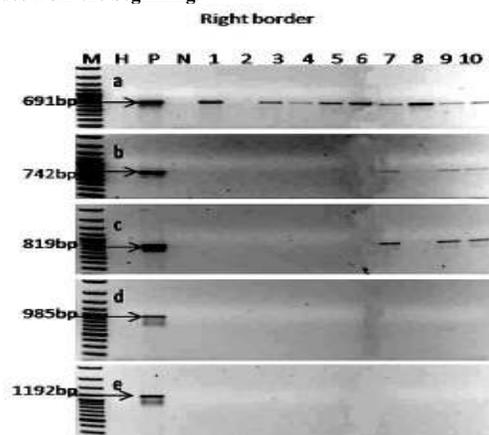
**Table 2. Represents the expected amplified size, size of fragment inside T-DNA border in bp and the size of sequences in the vector backbone in bp**

Primer name	Start-End base pair of amplified fragment	Expected amplified size (bp)	Size of sequence inside T-DNA border (bp)	Size of sequence in the vector backbone (bp)	Presence /absence
BL 1F <sup>1</sup> / nptII-Tail R	12587- (12894+360) <sup>3</sup> 13254	668	638	30	Yes
BL 2F <sup>1</sup> / nptII-Tail R	12321- (12894+360) <sup>3</sup> 13254	934	638	296	No
BL 3F <sup>1</sup> / nptII-Tail R	12054- (12894+360) <sup>3</sup> 13254	1201	638	563	No
GFP- F/ BR1-R <sup>2</sup>	4657 - 5347	691	691	0	Yes
GFP- F/ BR 2-R <sup>2</sup>	4657 - 5398	742	712	30	Yes
GFP- F/ BR 3-R <sup>2</sup>	4657 - 5475	819	712	107	Yes
GFP- F/ BR 4-R <sup>2</sup>	4657 - 5641	985	712	273	No
GFP- F/ BR 5-R <sup>2</sup>	4657 - 5848	1192	712	480	No

<sup>(1)</sup> primers were used in combination with primer nptII-Tail-R; <sup>(2)</sup> primers were used in combination with primer GFP\_F; F: forward primer, R: reverse primer. <sup>(3)</sup> end of plasmid sequences + 360 nucleotides from the beginning.



**Figure 2.** Detection of transferred left border and vector backbone sequences in genomic DNA of transgenic *Nicotiana benthamiana* plants by PCR. a) 668 bps, b) 924 bps and c) 1201 bps. M, DNA ladder Gene Ruler (100 bps), Fermentas; H, H<sub>2</sub>O as negative PCR; P, plasmid DNA as positive control; N, nontransgenic plant; 1-10, transgenic lines.



**Figure 3.** Detection of transferred right border and vector backbone sequences in genomic DNA of transgenic *Nicotiana benthamiana* plants by PCR. a) 691 bps, b) 742 bps, c) 819 bps, d) 985bps and e) 1192 bps. M, DNA ladder Gene Ruler (100 bps), Fermentas; H, H<sub>2</sub>O as negative PCR; P, plasmid DNA as positive control; N, nontransgenic plant; 1-10, transgenic lines.

#### ▪ Southern hybridization

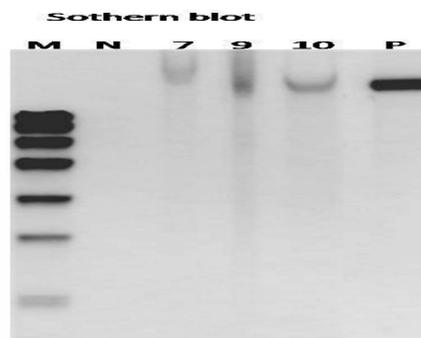
Integration of T-DNA border and vector backbone into the plant genome was analyzed by southern hybridization. Right border of the T-DNA signals was detected in all genomic DNA samples of the tested transgenic lines. All genomic DNA samples revealed one copy number (transgenic event) in all transgenic lines (Figure 4).

In this study, it was found that only 30 bp of the left border and 107 bp of the vector backbone sequences were integrated into the genome of *Nicotiana benthamiana* plants. Similarly, different studies have been found that integrations of vector backbone sequences along with T-DNA into plant genomes are occurring frequently (Martineau *et al.*, 1994; Kononov, *et al.*, 1997; Wenck, *et al.*, 1997; De Buck, *et al.*, 2000). Graaff *et al.*, 1996 who inserted twenty nine T-DNAs in *Arabidopsis thaliana* plants and examined transgenic plants for the flanking plant DNA and the junction of the right border sequences. They found a whole sequence of right border repeat with the binary vector in 4 of the transgenic lines was present. In another study, cloning of *gus* gene between the vector backbone sequences outside the T-DNA resulted in about 75% of the analyzed plants expressed the *gus* gene (Kononov, *et al.*, 1997). Moreover, detection of 40 % to 90% positive *trfA* gene located outside the T-DNA in transgenic strawberry plants was found by Abdal-Aziz, *et al.* (2006). Zeng, *et al.* (2008) Detected a percentage of 76.7% in transgenic birch plants contained some DNA of the vector backbone. Yang, *et al.*, (2013) reported a percentage of co-transformation of the vector backbone with 51% among 63 amplified products at left border junctions, 30% preserved a fraction of Left border varied from three to 23 base pairs.

Integration of left or right T-DNA borders and vector backbone sequences have been explained by two hypotheses: first, the non identification of the left border via the endonucleases proteins *virD1* and *virD2* might facilitate starting of T-strand at right border to go on at the left border which known as read-through at the left border. In other meaning, it was thought that the sequences of vector backbone are transferred due to read through of the left border throughout the releasing of T-DNA. On the other hand, the *virD2* protein possibly continued covalently attached to this left border 5' end, leading to precise integration of this portion of the left border (Durenberger, *et al.*, 1989). Second, formation of T-strand might start at left border and continue towards right border, ending in the integration of the whole plasmid vector (Graaff, *et al.*, 1996).

Existence of the sequences of vector backbone in the plant genome may has consequential effects where, these vector sequences could elevate transgene expression (Iglesias, *et al.*, 1997). Conversely, vector backbone sequence might have a negative impact on transgene expression and stability. This most likely due to that backbone sequences complicate the production and development of transgenic traits. Furthermore, regulatory agency and consumers are insisting that

commercial transgenic plants be clean of needless genes, such as vector backbone sequences or selectable marker genes. Therefore, additional studies will be required to satisfy product safety standards (Que, *et al.*, 2014). In this study, we successfully identified precisely the integrated T-DNA borders and vector backbone sequences in the *Nicotiana benthamiana* plants using PCR and southern blot analysis.



**Figure 4.** Southern blot analysis of integrated DNA samples of transgenic *Nicotiana benthamiana* lines. DNA was digested with *Bam*HI and the right border probe was used for detection. M, labeled DNA marker; N, non-transgenic plant; 7, 9 and 10, transgenic lines; p, plasmid as positive control.

#### REFERENCES

- Abdal-Aziz, S.A., Pliego-Alfaro, F., Quesada, M.A., and J. A. Mercado (2006). Evidence of Frequent Integration of Non-T-DNA Vector Backbone Sequences in Transgenic Strawberry Plant. *Journal of Bioscience and Bioengineering*. 101(6):508–510.
- Chawla, R., Ariza-Nieto, M., Wilson, A.J., Moore, S.K. and V. Srivastava (2006). Transgene expression produced by biolistic-mediated, site-specific gene integration is consistently inherited by the subsequent generations. *Plant Biotechnol J* 4: 209–218.
- De Buck, S., De Wilde, C., Van Montagu, M., and A. Depicker (2000). T-DNA vector backbone sequences are frequently integrated into the genome of transgenic plants obtained by *Agrobacterium*-mediated transformation. *Mol. Breed.*, 6: 459–468.
- Durenberger, F., Crameri, A., Hohn, B. and Z. Koukolikova- Nicola (1989). Covalently bound *VirD2* protein of *Agrobacterium tumefaciens* protects the T-DNA from exonucleolytic degradation. *Proc Natl Acad Sci USA* 86:9154-9158.
- Gelvin, S.B. (2003). *Agrobacterium*-mediated plant transformation: the biology behind the “gene-jockeying” tool. *Microbiol. Mol. Biol. Rev.* 67, 16-37.
- Graaff, E., Dulk-Ras, A. and P. J.J. Hooykaas (1996). Deviating T-DNA transfer from *Agrobacterium tumefaciens* to plants. *Plant Molecular Biology* 31: 677-681, 1996. *Plant Molecular Biology* 31: 677-681, 1996.

- Iglesias, V.A., Moscone, E.A., Papp, I., Neuhuber, F., Michalowski, S., Phelan, T., Spiker, S., Matzke M. and A. J. Matzke (1997). Molecular and cytogenetic analyses of stably and unstably expressed transgene loci in tobacco. *Plant Cell*, 91: 251-1264
- Khidr, Y.A., Amal M., El-Absawy, A.A., Hemeida, A. and M.E. Hasan (2012). Production of transgenic cucumber plantlets containing sequences from watermelon mosaic virus-II fused with gfp gene. *Egyptian J. of Genetics and Cytology* 41: 19-36.
- Khidr, Y.A., M.I. and Nasr (2012). Improvement of genetic transformation and plant regeneration via suspension cultures in Cucurbitaceae family. *Egyptian J. of Genetics and Cytology* 41: 1-18.
- Kononov, M. E., Bassuner, B., and S. B Gelvin (1997). Integration of T-DNA vector 'backbone' sequences into the tobacco genome: evidence for multiple complex patterns of integration. *Plant J.*, 11: 945-957.
- Martineau, B., T. A. Voelker, and R. A. Sanders. 1994. On defining T-DNA. *Plant Cell* 6:1032-1033.
- Murashige, T. and F. Skoog (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant*, 15: 473-497.
- Que, Q., Elumalai, S., Li, X., Zhong, H., Nalapalli, S., Schweiner, M., Fei, X., Nuccio, M., Kelliher, T., Gu, W., Chen, Z. and M. Chilton (2014) Maize transformation technology development for commercial event generation. *Frontiers in Plant Science* 5: 1-19.
- Shaw, C. H., M. D. Watson, G. H. Carter, and C. H. Shaw. (1984). The right hand copy of the nopaline Ti-plasmid 25 bp repeat is required for tumour
- Steck, T.R. (1997) Ti plasmid type affects T-DNA processing in *Agrobacterium tumefaciens*. *FEMS Microbiol Lett* 147:121-125
- Wenck, A., Czakó, M., Kanevski, I., and L. Márton (1997). Frequent collinear long transfer of DNA inclusive of the whole binary vector during *Agrobacterium*-mediated transformation. *Plant Mol. Biol.*, 34: 913-922.
- Yang, X., Li, F., Zhang, X., Liu, K., Wang, Q., Zhang, C., Liu, C., Zhu, W., Shan, G., Chin, C.K. and W. Fang (2013). Integration and characterization of T-DNA insertion in upland cotton. *Czech J. Genet. Plant Breed.*, 49: 51-57.
- Zeng, F-S., Zhan, Y-G., Su, T. and S. Dong (2008). Analysis of flanking sequences of T-DNAs in transgenic birch plants Based on Site Finding PCR. Conference Paper, 349-352 · June, DOI: 10.1109/ICBBE.2008.89 · Source: IEEE Xplore , 978-1-4244-1748-3/08/\$25.00 © 2008 IEEE
- Zupan, J., Muth, T.R., Draper, O. and P. Zambryski (2000). The transfer of DNA from *Agrobacterium tumefaciens* into plants: a feast of fundamental insights. *The Plant Journal* 23, 11-28.

### تحديد التتابعات المدمجة الغير T-DNA في جينوم نباتات الدخان المحول وراثياً

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يتم التحول الوراثي للنباتات بجينات مفيدة على نطاق واسع بواسطة أجروباكتريوم تؤوى التتابعات المعدلة المنقولة للبلازميد. تحديد تلك التتابعات للحمض النووي المنقول والمدمج في الخطوة النهائية من التحول الوراثي يكون أمر بالغ الأهمية لفهم آلية هذه العملية. بحثت وجود اندماج لتتابعات ليست تتابعات الحمض النووي المراد نقله في جينوم نباتات الدخان المعدلة وراثياً. تم تضخيم تتابعات الحمض النووي الواقعة خارج نطاق منطقة النقل T-DNA سواءاً من ناحية اليمين او اليسار لعدد 10 جينومات لتلك النباتات المعدلة وراثياً. أوضحت النتائج وجود ثلاث نباتات من أصل عشرة تحوى 30 نيوكليوتيدة من تتابعات ليست تتابعات منطقة النقل T-DNA من الناحية اليسارية لمنطقة النقل. وجد ان تلك الثلاث نباتات تحوى ايضا 107 نيوكليوتيدة بعد تتابعات الحدود اليمنى لمنطقة النقل ليست ضمن تتابعات منطقة النقل T-DNA. تمثل هذه التتابعات التي وجدت خارج منطقة التحول T-DNA نسبة 30% سواءاً كانت خلف الناحية اليمنى او اليسرى لحدود النقل. تم تأكيد وجود تلك التتابعات المدمجة الغير في جينوم نباتات الدخان المحول وراثياً بواسطة تحليل الساذرن.