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**Technical Note**

# Visualization of multiple T-DNA loci by FISH on extended DNA fibers

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**Abstract** Introduction of T-DNAs with foreign genes by *Agrobacterium*-mediated transformation is widely used in plants. Multiple-introduced complex T-DNA loci, however, are difficult to clarify by conventional DNA gel blot analysis. We performed fluorescence *in situ* hybridization on extended DNA fibers (fiber FISH) of transgenic tobacco plants harboring multiple 37-kb T-DNA constructs. Five and seven types of integrations were successfully visualized in two transgenic lines. Most of the loci suffered duplication, deletion and/or translocation, indicating the complex integration events of the medium-size T-DNA. We concluded that fiber-FISH analysis is a powerful tool to analyze organization of multiple T-DNA loci in detail.

**Key words:** *Agrobacterium*-mediated transformation, Fiber FISH, Multiple integration, T-DNA, Transgenic tobacco.

Many transgenic plants have been produced by *Agrobacterium*-mediated plant transformation. *Agrobacterium* transports a single stranded T-DNA carrying foreign genes into the plant genome. This T-DNA integration is generally initiated from the right border (RB) and terminated at the left border (LB), both of which are 25-bp direct repeats delimiting the T-DNA region. The *Agrobacterium*-mediated transformation system is highly sophisticated and reliable, and a number of binary Ti vectors, marker genes, and *Agrobacterium* strains are available. In addition, large DNA fragments (~100 kb) can be introduced into plants by using *Agrobacterium*-mediated transformation with BIBAC (binary bacterial artificial chromosome)-related vectors (Hamilton et al. 1996), making it possible to introduce a complex genomic region with a set of genes for molecular breeding or complementation analysis.

Although transgene integration and organization have been extensively studied to better understand the mechanisms of the T-DNA integration (Kohli et al. 2003), most of the transgenic plants produced for other purposes were analyzed only by PCR or DNA gel blot analysis to identify the existence of transgenes and their copy number. The size of the T-DNA region including selection marker genes is usually less than 20 kb, and the genomic organization of the integrated T-DNAs can be easily characterized by DNA gel blot analysis. However, precise organization of multiple or large T-DNAs in the plant genome is difficult to determine only by DNA gel blot analysis.

To overcome such a disadvantage, fluorescence *in situ* hybridization on the extended DNA fibers (fiber FISH) is an effective tool for analysis of transgene organization (Wolters et al. 1998; Nakano et al. 2005). Wolters et al. (1998) visualized the three genomic regions of transgenic potato (*ca*. 40 to 90 kb in length) containing tandemly integrated multiple T-DNAs by using the two-color fiber FISH with the 5.1-kb T-DNA and 7.1-kb vector probes, and they showed that complex T-DNA loci consisting of tandem arrangement of multiple short T-DNAs can be analyzed by the fiber FISH. In the case of Nakano et al. (2005), large T-DNA loci (*ca*. 85 to 140 kb in length) in four lines of rice transformants were visualized by the two-color fiber FISH with the 119-kb whole construct and 6.3-kb T-DNA probes, demonstrating the rearrangements of the large-insert T-DNA in transgenic plants. In addition to these reports, further fiber-FISH experiments are necessary to analyze various types of complex integrations of T-DNA, and to reveal useful technology that can be applied in the field of plant genetics.

In the present study, we introduced a 37-kb binary cosmid clone, CENT14-S (Figure 1A), into tobacco, *Nicotiana tabacum* cv. Petit Havana SR1, by

Abbreviations: BIBAC, binary bacterial artificial chromosome; fiber FISH, fluorescence *in situ* hybridization on extended DNA fibers; LB, left border; RB, right border.
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*Agrobacterium*-mediated transformation to examine the structure of the multiple-integrated T-DNAs in transgenic plants. The CENT14-S construct contains a 30-kb wheat genomic fragment with retrotransposon-related repeats, which are localized at the wheat centromere (Fukui et al. 2001; Suzuki and Mukai 2004), and the existence of transgenes in the transformants was confirmed by PCR

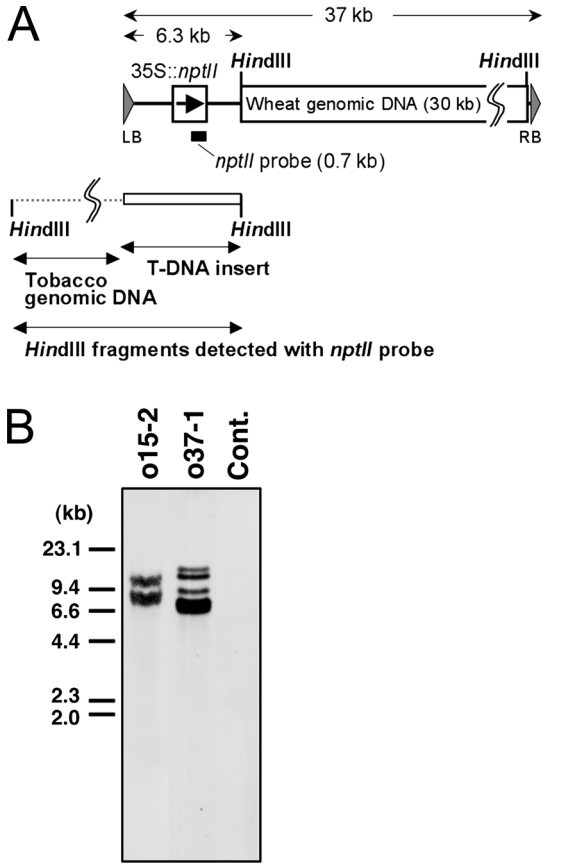


Figure 1. Copy number of *nptII* in the transgenic tobacco lines estimated by DNA gel blot analysis. (A) The CENT14-S construct and position of probes used in the DNA gel blot analysis are schematically represented. The digoxigenin-labeled *nptII* probe was prepared by PCR reaction using the PCR DIG labeling mix (Roche Diagnostics). As we illustrate below the construct, the *nptII* probe hybridizes to *Hin*dIII fragments, each of which contains the border of the T-DNA insert and tobacco genomic DNA, resulting in detection of different sizes of bands for different copies. (B) Genomic DNA (about 3 $\mu$g) of the two transgenic tobacco lines (o15-2 and o37-1) and an untransformant control plant (Cont.) were digested with *Hin*dIII and applied to DNA gel blot analysis with the *nptII* probe. Hybridization was carried out in 5×SSC, 0.5% blocking reagent (Roche Diagnostics), 0.1% sodium *N*-lauroyl sarcosinate and 0.02% SDS at 65°C, and washing was done twice in 0.1×SSC, 0.1% SDS at 65°C for 20 min each. The digoxigenin-labeled *nptII* probe was detected according to the instructions in the manual supplied with the dig luminescent detection kit (Roche Diagnostics). The copy number of *nptII* can be estimated from the number and intensity of detected bands. Each of two intense bands (*ca*. 8-, and 10-kb) detected in the o15-2 line might contain more than three copies of *nptII*, concluding that the o15-2 line possesses more than 6 *nptII* copies in total. In the case of the o37-1 line, four bands were detected, and the 7-kb intense band might correspond to at least 5 copies of *nptII*, indicating that the o37-1 line has more than 8 *nptII* copies in total.

(data not shown). In the present study, two lines (o15-2 and o37-1) were selected to dissect the genomic organization of the integrated T-DNAs, because DNA gel blot analysis of *Hin*dIII-digested genomic DNA with an *nptII* probe showed that these lines contained multiple T-DNAs (Figure 1B). The cloning site of the binary cosmid vector inserting the 30-kb wheat genomic fragments is a *Hin*dIII-recognition site, resulting in the appearance of different sizes of *Hin*dIII bands hybridized with the *nptII* probe, which corresponds to an outside region of the cloning site (Figure 1A). We estimated the copy number of the *nptII* gene for each line by counting the bands obtained from the DNA gel blot analysis; more than 6 and 8 copies of the *nptII* could be identified in the o15-2 and o37-1, respectively (Figure 1B). Several intense bands seen were due to more than one *nptII* fragment in the same length, and this suggested the tandem organization of the *nptII* regions of T-DNAs, including LB-RB junctions of the directly repeated T-DNAs. However, it is difficult to determine the detailed organization of multi-copies of the CENT14-S constructs in these lines only by this DNA gel blot.

In order to determine organization of the multiple integrated T-DNAs, a $T_0$ plant for each line was used in the following fiber-FISH analysis. Extended DNA fibers were prepared from young leaves, according to the protocol of Fransz et al. (1996) with minor modifications. Multicolor FISH on the extended DNA fibers was performed by using digoxigenin- and biotin-labeled probes, which were detected with Cy3 and Fluorescein, respectively (Yamamoto and Mukai 2005). For FISH probes, the 37-kb whole construct (a CENT14 probe) and the 6.3-kb LB region including the *nptII* gene (a T-DNA-LB probe) were used (Figure 2A). Fiber-FISH results were highly reproducible, and the representative results are shown in Figure 2B; green signals corresponding to the biotin-labeled CENT14 probe, and red signals corresponding to the digoxigenin-labeled T-DNA-LB probe were detected. The same reproducible results were obtained by fiber-FISH with a reverse color combination of the probes (data not shown). In total, 181 and 192 fiber signals were obtained and characterized for the o15-2 and o37-1 lines, respectively. The stretching degree of 3.27 kb $\mu m^{-1}$ determined by Fransz et al. (1996) was applied to estimate physical length (kb) from the average length ($\mu$m) of the fiber-FISH signals (Table 1).

In the case of the o15-2 line, the 181 fiber-FISH signals were classified into five different types (*a–e* in Figure 2), which corresponded to the five different T-DNA loci integrated in the o15-2 transgenic plants. The average lengths of signals of 32, 30, 30, 60, and 29 samples were able to be analyzed in detail for the loci *a*, *b*, *c*, *d*, and *e*, respectively (Table 1). The selected signals of the *d*-locus occurred almost twice as often as those of

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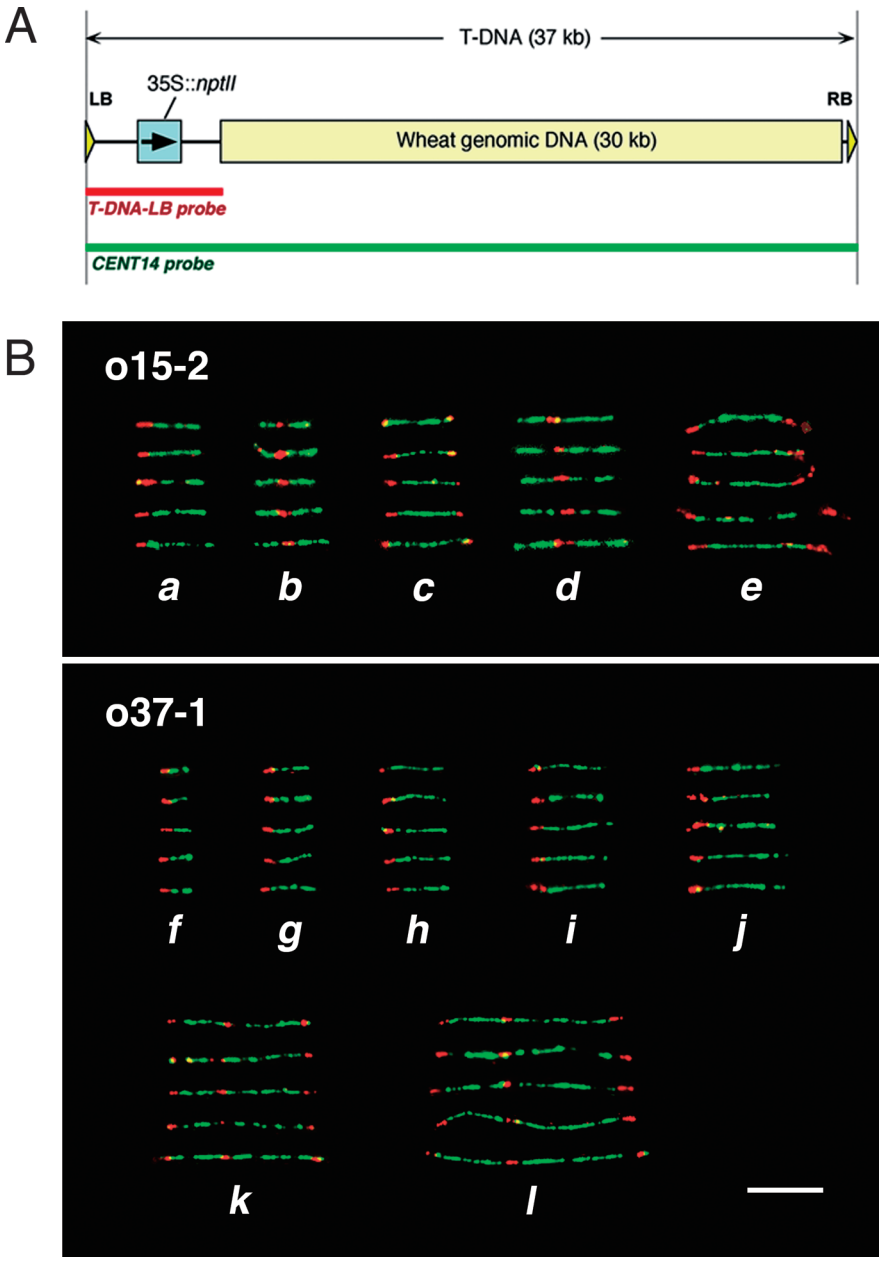


Figure 2. Visualization of multiple T-DNA loci of transgenic tobacco plants by using fiber FISH. (A) The CENT14-S construct and position of probes used in the fiber FISH are schematically represented. For a CENT14 probe, the binary cosmid DNA was labeled with digoxigenin-11-dUTP or biotin-16-dUTP using a nick translation kit (Roche Diagnostics). For a T-DNA-LB probe, a PCR product amplified from the binary cosmid DNA was labeled with digoxigenin- or biotin-dUTP (Roche Diagnostics). (B) Two $T_0$ lines (o15-2 and o37-1) of the CENT14-S-introduced transgenic tobacco were subjected to the fiber FISH analysis with the T-DNA-LB (red) and CENT14 (green) probes. Five types of integrations (*a–e*) and seven types of integrations (*f–l*) were observed in the o15-2 and o37-1 lines, respectively. Five representative signals for each type are indicated. The bar represents 10 $\mu$m.

the other loci, suggesting that the *d*-locus signals were derived from the two loci with similar structure. Based on the characteristics of the signals, we concluded that the *a*-locus contained one copy of the CENT14 transgene, and the *c*-locus contained one copy of the CENT14 with the additional truncated T-DNA-LB region (Figure 2, Table 1). Although the estimated physical length (kb) of the integrated copies of the *a*- and *c*- loci (e.g. 4.3-kb T-DNA-LB and 24.7-kb CENT14 in the *a*-locus) was almost 70% of the actual length of the construct (6.3-kb T-DNA-LB and 37-kb CENT14), each ratio of T-DNA-LB : CENT14 was *ca.* 1 : 6, which was the same as the actual ratio of the construct. Thus, the DNA fibers of these samples seemed to be less stretched than those of previous reports (Fransz et al. 1996; Suzuki et al. 2004). Alternatively, the one copy of the *a*- and *c*-locus might have been deleted in both the T-DNA-LB and CENT14 regions. As reported in Nakano et al.

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Table 1. Summary of the fiber FISH results.

| Lines | Loci | Probes | Signal length (μm) | | Physical length (kb) | |
|---|---|---|---|---|---|---|
| | | | average | S.D. | average | S.D. |
| o15-2 | *a* | T-DNA-LB | 1.31 | 0.56 | 4.30 | 1.84 |
| | (n=32) | CENT14 | 7.54 | 1.25 | 24.66 | 4.09 |
| | *b* | CENT14 | 3.65 | 0.80 | 11.93 | 2.61 |
| | (n=30) | T-DNA-LB | 1.24 | 0.39 | 4.04 | 1.29 |
| | | CENT14 | 4.66 | 0.84 | 15.25 | 2.76 |
| | *c* | T-DNA-LB | 1.28 | 0.50 | 4.19 | 1.64 |
| | (n=30) | CENT14 | 8.44 | 1.89 | 27.59 | 6.20 |
| | | T-DNA-LB | 0.73 | 0.27 | 2.38 | 0.89 |
| | *d* | CENT14 | 4.68 | 0.71 | 15.32 | 2.33 |
| | (n=60) | T-DNA-LB | 1.82 | 0.55 | 5.96 | 1.79 |
| | | CENT14 | 6.86 | 0.73 | 22.44 | 2.38 |
| | *e* | T-DNA-LB | 1.55 | 0.48 | 5.07 | 1.55 |
| | (n=29) | CENT14 | 13.12 | 0.97 | 42.89 | 3.17 |
| | | T-DNA-LB | 1.41 | 0.46 | 4.60 | 1.50 |
| o37-1 | *f* | T-DNA-LB | 1.09 | 0.39 | 3.57 | 1.28 |
| | (n=27) | CENT14 | 2.96 | 0.61 | 9.67 | 1.99 |
| | *g* | T-DNA-LB | 1.54 | 0.30 | 5.02 | 0.97 |
| | (n=42) | CENT14 | 5.28 | 0.63 | 17.26 | 2.05 |
| | *h* | T-DNA-LB | 1.91 | 0.45 | 6.26 | 1.48 |
| | (n=27) | CENT14 | 7.32 | 0.47 | 23.94 | 1.55 |
| | *i* | T-DNA-LB | 2.09 | 0.51 | 6.82 | 1.67 |
| | (n=27) | CENT14 | 8.79 | 0.80 | 28.76 | 2.60 |
| | *j* | T-DNA-LB | 2.22 | 0.62 | 7.26 | 2.02 |
| | (n=29) | CENT14 | 10.84 | 0.85 | 35.45 | 2.78 |
| | *k* | T-DNA-LB | 1.76 | 0.49 | 5.77 | 1.60 |
| | (n=21) | CENT14 | 6.29 | 0.54 | 20.58 | 1.76 |
| | | T-DNA-LB | 1.89 | 0.58 | 6.17 | 1.90 |
| | | CENT14 | 11.11 | 0.98 | 36.33 | 3.22 |
| | | T-DNA-LB | 1.90 | 0.54 | 6.21 | 1.78 |
| | *l* | T-DNA-LB | 2.06 | 0.35 | 6.73 | 1.13 |
| | (n=19) | CENT14 | 8.42 | 0.67 | 27.54 | 2.18 |
| | | T-DNA-LB | 2.17 | 0.52 | 7.11 | 1.70 |
| | | CENT14 | 13.25 | 0.96 | 43.34 | 3.14 |
| | | T-DNA-LB | 2.09 | 0.63 | 6.82 | 2.06 |

(2005), deletion events might easily occur during the *Agrobacterium*-mediated transformation because the CENT14-S construct is larger than typical constructs and contains repetitive sequences. Recombination events, such as deletion, duplication, and translocation, are necessary to account for the genomic organization of the CENT14 transgene in the *b*, *c*, *d*, and *e* loci (Figure 2, Table 1). For example, the *b*-locus seems to suffer translocation of T-DNA-LB from a normal position to the internal region of CENT14. The *d*-locus might consist of almost an intact copy and an additional duplicated part of the CENT14 region. The *e*-locus can be explained by two truncated copies of the transgene with reverse orientation having RB-RB junction. In total, the ~6 T-DNA loci corresponding to ~7 CENT14 copies with ~8 T-DNA-LB (including *nptII*) copies were observed in the o15-2 lines. This conclusion is consistent with the estimation of more than 6 *nptII* copies from the DNA gel blot analysis (Figure 1).

The one hundred ninety two fiber FISH signals of the o37-1 line were classified into seven different types of signals (*f*–*l* in Figure 2), which correspond to seven T-DNA loci. The average lengths of signals of 27, 42, 27, 27, 29, 21, and 19 samples were able to be analyzed in detail for the loci *f*, *g*, *h*, *i*, *j*, *k*, and *l*, respectively (Table 1). As in the case of the *d*-locus of the o15-1 line, the frequency of the *g*-locus was relatively high, indicating the possibility that the *g*-locus signals could be derived from the two different loci with similar structure. It was difficult to determine an intact copy in the *f*-, *g*-, *h*-, *i*-, and *j*-loci, which showed the typical characteristic of one-copy integration as a short red signal and a long green signal (Figure 2), because each ratio of T-DNA-LB : CENT14 varied and did not fit to 1 : 6. Duplication or deletion of short fragments might have occurred in these T-DNA loci. Each *k*- and *l*-locus was comprised of

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two or three copies of the construct with some deletions and/or duplications (Figure 2, Table 1). These tandem copies with direct or reverse orientation resulted in 75 to 90-kb long stretches of T-DNA regions. In total, the ~8 T-DNA loci corresponding to ~12 CENT14 copies with ~12 T-DNA-LB (including *nptII*) copies were observed in the o37-1 lines. This conclusion is also consistent with the estimation of more than 8 *nptII* copies from the DNA gel blot analysis (Figure 1).

The most attractive advantage of the present two-color fiber-FISH analysis is an easy understanding of complex multiple T-DNA loci by visual classification. We obtained various types of information from the visualized T-DNA loci as described above. In particular, both the o15-2 and o37-1 lines possess high copies of transgenes, so we could not determine the precise T-DNA organization without the fiber-FISH. This study suggested that combining fiber-FISH and DNA gel blot analyses is an effective procedure to determine an accurate copy number of integrated T-DNAs in the case of transgenic plants having multiple and/or larger T-DNA locus. Moreover, these molecular cytogenetic studies enabled us to analyze rearrangement and tandem duplication of transgenes in detail.

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