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Rearrangements of large-insert T-DNAs in transgenic rice

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Abstract Introduction of large-DNA fragments into cereals by *Agrobacterium*-mediated transformation is a useful technique for map-based cloning and molecular breeding. However, little is known about the organization and stability of large fragments of foreign DNA introduced into plant genomes. In this study, we produced transgenic rice plants by *Agrobacterium*-mediated transformation with a large-insert T-DNA containing a 92-kb region of the wheat genome. The structures of the T-DNA in four independent transgenic lines were visualized by fluorescence in situ hybridization on extended DNA fibers (fiber FISH). By using this cytogenetic technique, we showed that rearrangements of the large-insert T-DNA, involving duplication, deletion and insertion, had occurred in all four lines. Deletion of long stretches of the large-insert DNA was also observed in *Agrobacterium*.

Keywords *Agrobacterium*-mediated transformation · Fluorescence in situ hybridization on extended DNA fibers (fiber FISH) · *Oryza sativa* · DNA rearrangement · Large T-DNA inserts

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Introduction

Agrobacterium-mediated plant transformation is the most popular and reliable method for introducing foreign genes into plants. Although monocots are not natural hosts for *Agrobacterium*, recent production of transgenic cereals has also been achieved by *Agrobacterium*-mediated transformation (Chan et al. 1992; Hiei et al. 1994; Ishida et al. 1996; Cheng et al. 1997). *Agrobacterium* transports a single stranded T-DNA carrying foreign genes into the plant genome: the T-DNA is delimited by 25-bp direct repeats, referred to as right border (RB) and left border (LB) sequences, which flank the DNA of interest. It has generally been postulated that T-DNA integration is initiated from the RB and terminated at the LB. The structure of the junctions between T-DNA and plant DNA has been extensively studied in transgenic dicotyledonous plants (Krizkova and Hrouda 1998; Buck et al. 1999; Windels et al. 2003) and in monocot plants (Kumar and Fladung 2002; Kim et al. 2003). These analyses showed that insertions of filler DNA and small deletions can occur at the junctions. Vector backbone sequences were also frequently observed in the regions flanking the T-DNA (Martineau et al. 1994; Kononov et al. 1997). Recent studies of transgene integration and organization have been reviewed by Kohli et al. (2003).

Binary vectors related to bacterial artificial chromosome (BAC) vectors, such as the binary BAC (BIBAC) and transformation-competent artificial chromosome (TAC), which can be used in *Agrobacterium*-mediated plant transformation, have been developed to transfer large DNA fragments into plant chromosomes (Hamilton et al. 1996; Liu et al. 1999). In addition, Tao and Zhang (1998) reported that over 100-kb segments of DNA could be inserted in conventional binary vectors. Transformation-ready large-insert libraries have been constructed to date for *Arabidopsis thaliana* (Liu et al. 1999), *Brassica napus* (Cui et al. 2000), tomato (Hamilton et al. 1999), wheat (Moullet et al. 1999; Liu et al.

2000), and petunia (McCubbin et al. 2000). These libraries are useful resources for introducing large and complex genomic regions bearing whole sets of genes into important crops, as well as for complementation analyses during map-based cloning. The progressive introduction of large foreign genomic fragments into plant genomes, which we refer to as 'genome fusion', would be a fascinating strategy for creating new crops by molecular breeding.

However, little information is available regarding the chromosomal integration and organization of large-insert T-DNA in transgenic plants, especially in transgenic cereals. In this study, we introduced a 92-kb fragment of the wheat genome into rice by *Agrobacterium*-mediated transformation. Rearrangements of the integrated T-DNA in transgenic rice plants were successfully visualized and analyzed by fluorescence in situ hybridization on extended DNA fibers (fiber FISH).

Materials and methods

Transformation

Binary cosmid DNA containing a 92-kb segment of the wheat *Ha*-locus region (Turnbull et al. 2003, Fig. 1) was purified using a Plasmid Midi Kit (Qiagen) and electroporated into the *Agrobacterium tumefaciens* strain EHA105 (Hood et al. 1993) using the Gene Pulser II (Bio-Rad) set to 2.5 kV, 25 μF, and 100 Ω. A cultivar of *japonica* rice, *Oryza sativa* L. Yamahoushi, was used to produce transgenic plants by *Agrobacterium*-mediated transformation as described by Yokoi and Toriyama (1999) with some modifications. Geneticin (G418, Sigma, 70 mg/l) was used as a selection agent, as the binary cosmid vector contains the neomycin phosphotransferase gene (*nptII*) in the T-DNA region (Fig. 1).

PCR

To confirm the presence of the *puroindoline-a* and *GSP-1* genes in *Agrobacterium* and transgenic plants, PCR was performed with ExTaq polymerase (Takara), for 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min, followed by a final extension for 5 min, by using the Personal DNA thermal cycler (Takara). The primers used to amplify the *puroindoline-a* sequence were PA-F (5′-ATGAAGGCCCTCTTCCTCATAGG-3′) and PA-R (5′-TCACCAGTAATAGGCAATAGTGCC-3′), and GSP-F (5′-GCGATCTAAGTGGCTTCAAG-3′) and GSP-R (5′-GCTAGTGATGGGGATGTTGC-3′) were used to amplify the *GSP-1* sequence.

DNA gel-blot analysis

Total DNA was extracted from young leaf tissue of transgenic and non-transgenic rice plants by the procedure described by Murray and Thompson (1980). Total DNA (about 1 µg) and binary cosmid DNA (about 5 ng) isolated from *Escherichia coli* or *A. tumefaciens* were digested with *Hin*dIII, and fractionated on a 0.8% agarose gel. After electrophoresis, DNA was transferred to nylon membranes (Roche Diagnostics). Hybridization was carried out in 5×SSC, 0.5% blocking reagent (Roche), 0.1% sodium *N*-lauroyl sarcosinate and 0.02% SDS at 65°C. The membrane was washed twice in 0.1SSC, 0.1% SDS at 65°C for 20 min each. The digoxigenin (dig)-labeled *nptII* and BAC 8 probes (Fig. 1) were prepared by PCR using PCR DIG Labeling Mix (Roche) and random priming using the DIG DNA Labeling Kit (Roche), respectively. Detection of the hybridized probe was carried out according to the instructions in the manual supplied with the DIG Luminescent Detection Kit (Roche) using CSPD as the substrate.

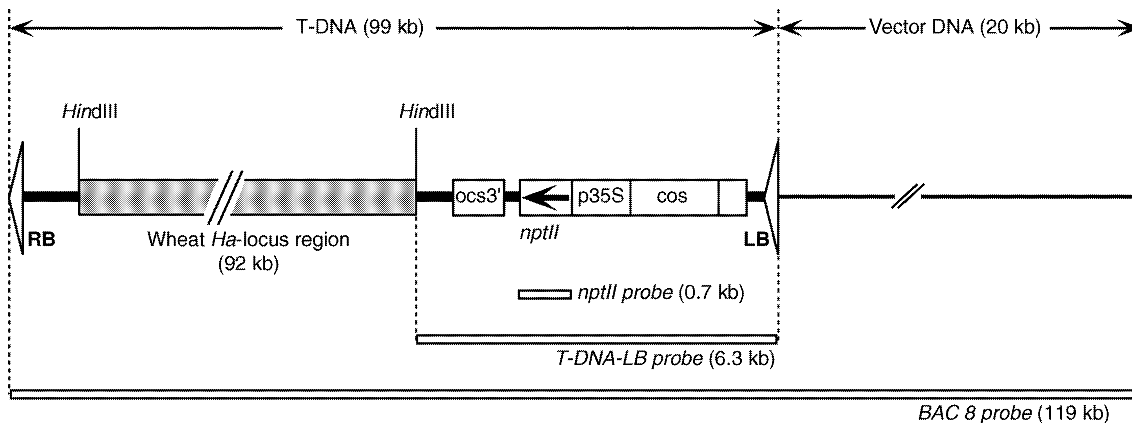

Fig. 1 Structure of the original BAC 8 construct used in this study. The relative positions of the probes employed are indicated by the *open bars below* the construct. A 92-kb genomic fragment from wheat containing the genes *puroindoline-a* and *GSP-1* was cloned in the binary cosmid vector pCLD04541 (Turnbull et al. 2003)

Probes for FISH analysis

Binary cosmid DNA was purified with the Plasmid Midi Kit (Qiagen). For use as a BAC 8 probe (Fig. 1) for FISH analysis, the binary cosmid DNA was labeled with dig-11-dUTP or biotin-16-dUTP using a Nick Translation Kit (Roche). A PCR product amplified from the binary cosmid DNA was labeled with dig- or biotin-dUTP (Roche) and used as the T-DNA-LB probe (Fig. 1). The primers employed for amplification of the T-DNA-LB region were pCLD-F (5′-CCGGTACGTACCAGCTTTTG-3′) and pCLD-R (5′-AAATGTAGATGTCCGCAGCG-3′).

FISH on extended DNA fibers

Extended DNA fibers were prepared from young leaves, according to the protocol of Fransz et al. (1996a) with minor modifications. Multicolor FISH on the extended DNA fibers was performed by using the BAC 8 and T-DNA-LB probes. The dig-labeled probe was detected using an anti-Dig mouse antibody (Roche) and Cy3 conjugated anti-mouse IgG (Sigma). For detection of the biotin-labeled probe, two layers of Fluorescein Avidin DCS (Vector Laboratories) and one layer of biotinylated anti-avidin D (Vector Laboratories) were applied. Slides were counterstained with DAPI and mounted in an anti-fade solution (DABCO in 90% glycerol).

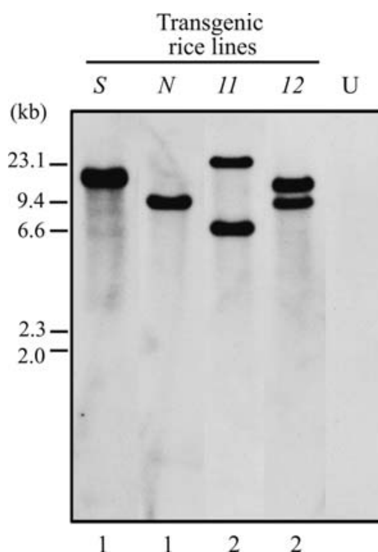

Fig. 2 Determination of the copy number of the *nptII* gene in the four transgenic lines *S*, *N*, *11*, and *12*. Genomic DNA (1 μg) from the transgenic rice lines (*S*, *N*, *11*, and *12*) and an untransformed host (U) was digested with *Hin*dIII and subjected to DNA gel-blot analysis with the *nptII* probe. The estimated copy number of the *nptII* sequence is indicated *below* each lane

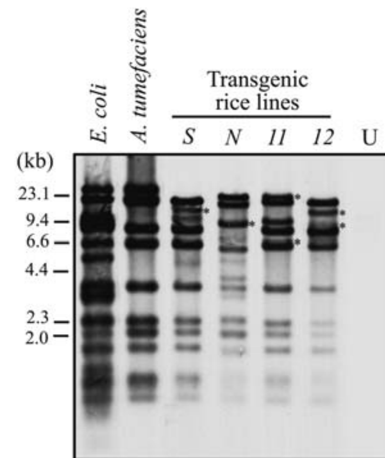

Fig. 3 Rearrangements of the BAC 8 construct occur both in *Agrobacterium* and in transgenic rice plants. Aliquots of the binary cosmid DNA (5 ng) isolated from *E. coli* or *A. tumefaciens*, and of genomic DNA (1 μg) from representative T_1 plants of the four transgenic lines (*S*, *N*, *11*, and *12*) and an untransformed control (U) were digested with *Hin*dIII and subjected to DNA gel-blot analysis with the BAC 8 probe, which consists of the complete BAC 8 construct including vector DNA. The *asterisks* indicate fragments that also hybridized with the *nptII* probe (Fig. 2)

Results

Characterization of transgenic lines by PCR and Southern hybridization

We introduced a 92-kb binary cosmid clone, BAC 8 (Fig. 1), into rice by *Agrobacterium*-mediated transformation in order to examine structure of the integrated large T-DNA in transgenic plants. BAC 8 contains a genomic fragment from wheat that includes the *puroindoline-a* and *GSP-1* genes, which are located at the grain hardness locus, *Ha* (Turnbull et al. 2003). Of the nine transgenic rice lines obtained, four (lines *S*, *N*, *11*, and *12*) were used to dissect the genomic organization of the integrated T-DNA. The presence of the *puroindoline-a* and *GSP-1* genes in the transformants was confirmed by PCR (data not shown), and the copy number of the *nptII* gene was estimated by DNA gel-blot analysis as 1, 1, 2 and 2 copies for the lines *S*, *N*, *11*, and *12*, respectively (Fig. 2). DNA gel blot analysis of the segregated T_1 plants showed that the two copies of *nptII* in each of the lines *11* and *12* were linked and unlinked, respectively (data not shown). A representative homozygous T_1 plant of the lines *S*, *N* and *12* was used for subsequent DNA gel blot and FISH analyses, but a T_0 plant was used for the FISH analysis of line *11*.

To analyze rearrangements of the T-DNA in the *Agrobacterium* and transgenic plants, DNA gel blot analysis was performed with a BAC 8 probe (Fig. 3). This probe can specifically hybridize with the whole BAC 8 construct including vector DNA (Fig. 1). Several *Hin*dIII bands detected in the original BAC 8 clone in *E. coli* were lost from the BAC 8 clone in *Agrobacterium*

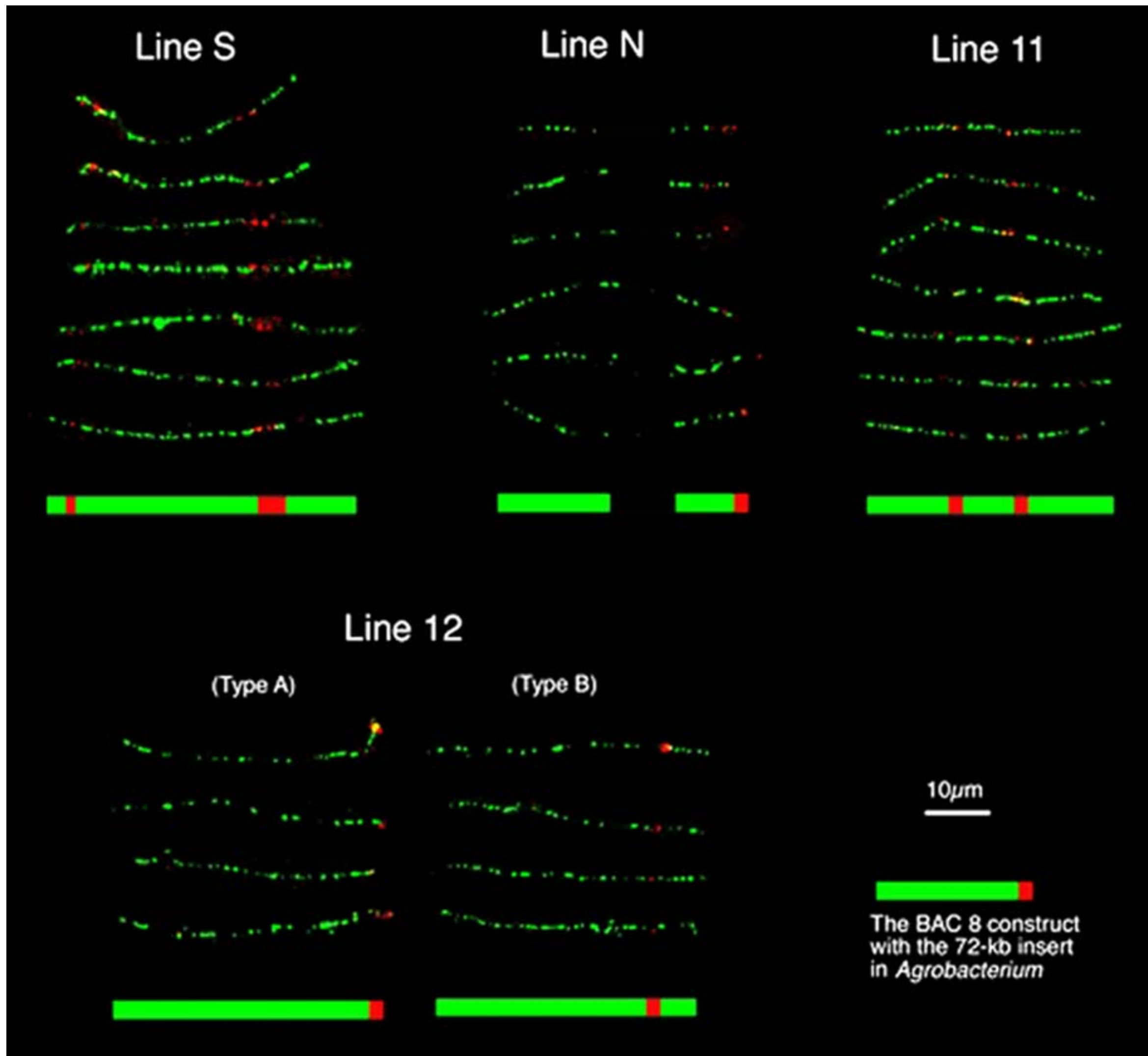

Fig. 4 Visualization of the large-insert T-DNA on extended DNA fibers from the four transgenic lines *S*, *N*, *11*, and *12*. Extended DNA fibers were prepared from the lines *S*, *N*, *11*, and *12*, and hybridized with BAC 8 (*green*) and T-DNA-LB (*red*) probes. The scale bar represents 10 μm. A schematic representation of the FISH signal for each line is shown *below* the hybridized preparation. The DNA fibers obtained from the lines *S*, *N*, and *12* were isolated from the same T_1 plants as those used for the DNA gel blot analysis shown in Fig. 3. The DNA fibers for line *11* were isolated from a T_0 plant. However, the patterns of bands detected in T_0 and T_1 plants by DNA gel blot analysis with the *nptII* and BAC 8 probes (data not shown) were found to be identical

(Fig. 3); in all, some 20 kb of the DNA was deleted in *Agrobacterium*. This rearrangement of the BAC 8 construct in *Agrobacterium* implies that actual size of the construct used for *Agrobacterium*-mediated gene transfer in the present experiment was approximately 72 kb. Most of the *Hin*dIII bands found in the transgenic lines *S*, *11* and *12*, were the same as those seen in *Agrobacterium*, whereas changes in band lengths were observed in line *N* (Fig. 3). The *Hin*dIII fragments including the *nptII* gene (marked by asterisks in Fig. 3) might contain T-DNA/plant DNA junctions at the LB, resulting in appearance of the bands different from those seen in *Agrobacterium*. The presence of several other bands that differed from those in *Agrobacterium* suggested that rearrangements of T-DNA had also occurred in the transgenic plants.

Analysis of the T-DNA inserts by fiber FISH

It is difficult to determine the complex structure of the large T-DNA loci by DNA gel blot analysis. Therefore, for further investigation of the genomic organization of the integrated T-DNA, we visualized the integrated regions by FISH on extended DNA fibers (fiber FISH) obtained from the transgenic rice plants. Representative results of the fiber FISH with the biotin-labeled BAC 8 probe (detected as green signals) and the Dig-labeled T-DNA-LB probe (detected as red signals) are depicted in Fig. 4. The BAC 8 probe can hybridize to all segments of the whole BAC 8 construct as in the case of DNA gel blot analysis, and the T-DNA-LB probe can detect the 6.5-kb LB region including the *nptII* gene (Fig. 1). The average lengths of the different signals were converted to

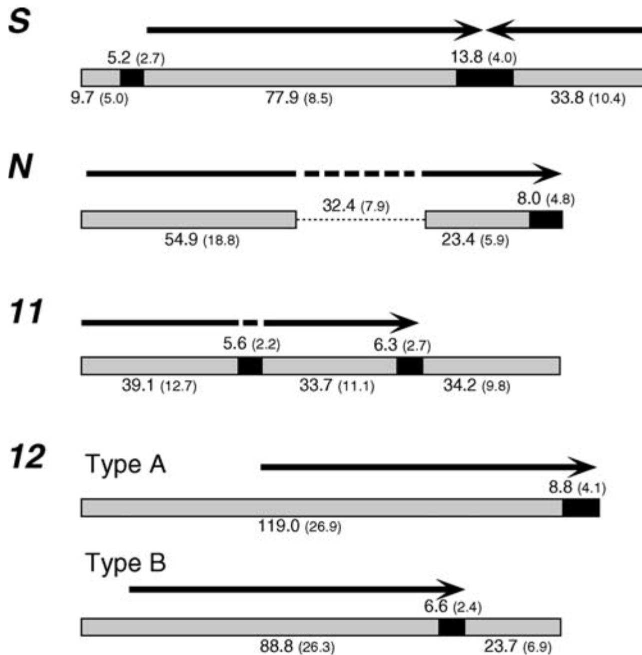

Fig. 5 Schematic representation of the deduced organization of the integrated T-DNAs in the four transgenic lines. The *gray* and *black bars* represent the integrated T-DNA regions that hybridized with BAC 8 and T-DNA-LB probes, respectively; a *broken line* between the *bars* denote rice genomic DNA. The average length (kb) of each region (±SD) is given in *parentheses*. The *arrows* indicate the estimated position and orientation (RB to LB) of the BAC 8 construct

kb lengths by using the stretching factor of 3.27 kb/µm reported by Fransz et al. (1996a). The predicted structure of the integrated region is schematically represented in Fig. 5.

In the case of the line *S*, a long and contiguous green signal (~50 µm) with two short red signals (approximately 2 and 4 µm) was observed on the DNA fiber (Fig. 4). The mean length of the integrated region in the line *S* was estimated to be 140.2 kb (SD = 12.0, *n* = 22). This total length suggested that two copies of the 72-kb construct had integrated in this line, although only one copy of the *nptII* gene was detected by DNA gel blot analysis (Fig. 2). This difference in the copy number estimate could be explained by assuming that the 4-µm red signal (13.8 kb, SD = 4.0) might correspond to two copies of the 6.5-kb T-DNA-LB region duplicated in opposite orientation (LB to LB orientation), because the single 13-kb *Hin*dIII band was scored as one copy in the DNA gel blot analysis with the *nptII* probe (Fig. 2; see the construct shown in Fig. 1). We assume that the 2-µm red signal (5.2 kb, SD = 2.7) does not include the *nptII* gene, and that this fragmented T-DNA has been copied from the neighboring construct with the short insert fragment (9.7 kb, SD = 5.0). The internal green signal (77.9 kb, SD = 8.5) with the two red signals on both sides might correspond to one copy of the intact BAC 8 insert, whereas the outer green signal (33.8 kb, SD = 10.4) on the same side as the 4-µm red signal might correspond to another copy of the deleted BAC 8 insert. To summarize the possible integration events in the line *S*, two copies of the BAC 8 construct have been integrated in inverted orientation, a deletion has occurred in one of the copies, and short segments of T-DNA and insert DNA have been duplicated and transferred to the terminal (Fig. 5).

In the case of the line *N*, a ~10-µm green signal with a red signal and a ~20-µm green signal, which were interrupted by a gap, were observed on the same DNA fiber (Fig. 4). The total length of the integrated region in the line *N* (without the gap) was estimated to be 86.3 kb (SD = 13.6, *n* = 23). This length and the presence of a signal from the T-DNA-LB region at one end indicated that it consisted of one copy of the BAC 8 construct. The fragment corresponding to the gap of 32.4 kb (SD = 7.9) must be derived from genomic DNA of rice inserted in one copy of the construct (Fig. 5). Additional small insertions may be present on the predicted RB-side of the construct (54.9 kb, SD = 18.8). However, the small gaps could not be distinguished from the artifacts of the fiber-FISH signals which are typically detected as a ‘beads-on-a-string’ pattern rather than continuous tracks (Fransz et al. 1996a).

The fiber-FISH signals in the line *11* were detected as an approximately 40-µm contiguous green signal with two red signals (Fig. 4). The length of the integrated region in the line *11* was calculated to be 119.0 kb (SD = 11.2, *n* = 19). The presence of the two red signals on the same fiber was consistent with the detection of two linked *nptII* copies by DNA gel blot analysis. One explanation for this arrangement in the line *11* is that one copy of the BAC 8 construct has been interrupted by the insertion of a T-DNA-LB region (Fig. 5). Alternatively, two deleted constructs might be arranged in tandem. In both cases, the existence of another integrated region (34.2 kb, SD = 9.8), detected as the green signal beyond the LB, might be due to co-integration of vector backbone sequences and/or internal duplication.

The two independent copies of the BAC 8 construct in the line *12* were detected as two different types of signals (Fig. 4): a green signal with a terminal red signal (type A) and another with a subterminal red signal (type B). The integrated regions of types A and B were, on average, 128.0 kb (SD = 27.5, *n* = 20) and 119.1 kb (SD = 28.5, *n* = 19) long, respectively, indicating that the both copies were longer than the expected insert size (Fig. 5). Internal duplication might have occurred in the unlinked two T-DNA regions of the line *12*. In addition, type B showed the subterminal red signal because the integrated region possibly contained vector backbone sequences beyond the LB.

Thus, various types of rearrangements that occurred in the integrated large-insert DNA were successfully visualized in the four transgenic lines by two-color fiber FISH. The fiber FISH with a reverse combination of the probes was also performed to confirm the arrangement of the different signals (data not shown).

Discussion

FISH is an effective tool for the analysis of transgene organization. Single-copy T-DNA regions have been visualized by FISH on mitotic chromosomes (Dong et al. 2001; Khrustaleva and Kik 2001) and in interphase nuclei (ten Hoopen et al. 1999). Chromosome rearrangements in transgenic plants have also been characterized by FISH (Fransz et al. 1996b; Papp et al. 1996). Furthermore, recent improvements in the fiber FISH technique (Wolters et al. 1998) now permit detailed analysis of the rearrangement and tandem duplication of transgenes.

In this study, we successfully visualized large-insert T-DNA by fiber FISH in order to determine the length of the integrated region in transgenic rice plants. These visual data provide new and important information on the genomic organization of large transgenes in plants—an issue that has been quite difficult to analyze using traditional DNA gel-blot analysis. We were able to deduce the orientation, copy number, and extent of deletion of the large-insert T-DNA by using two-color fiber FISH with a combination of the probes for the 6.5-kb LB region (the T-DNA-LB probe) and a full-length 119-kb BAC construct (the BAC 8 probe).

The genomic organization of the integrated T-DNAs differed in the four transgenic lines analyzed in this study. All the integrated large transgenes had been rearranged. Inverted integration of two constructs and deletions in one copy were observed in the line *S*, an insertion of rice genomic DNA into the construct was detected in the line *N*, insertion of the T-DNA-LB region or the tandem duplication of deleted constructs was visualized in line *11*, and two longer constructs were present in line *12*. These duplication, deletion and insertion events might have occurred independently in the transgenic plants during or after the integration of the T-DNA. Our analysis suggested that the large T-DNAs integrated by *Agrobacterium*-mediated transformation tend to be rearranged in transgenic rice plants. Similar rearrangements were also observed in other transgenic rice plants harboring large genomic fragments from wheat (our unpublished results). The integration of intact T-DNA should therefore be routinely confirmed by cytogenetic analysis in the case of transgenic plants transformed with large transgenes using *Agrobacterium*.

To our surprise, the BAC 8 construct used in this study had suffered significant deletion in *Agrobacterium*; the 92-kb insert in the original BAC 8 construct was apparently converted into a 72-kb insert in *Agrobacterium* prior to infection of rice. Recently, Song et al. (2003) reported that potato BIBAC and TAC constructs containing genomic DNA fragments larger than 100 kb were unstable in *Agrobacterium*. Similarly, in the present investigation, deletions have been observed in the BAC 8 construct in *Agrobacterium*; these might be due to the presence of repetitive sequences in the 92-kb insert of wheat genomic DNA. In fact, FISH analysis with the BAC 8 probe on wheat mitotic chromosomes resulted in the strong signals over entire chromosomes, suggesting the existence of highly repetitive sequences in the BAC 8 insert (unpublished results). In transgenic experiments, it is therefore important that large-insert clones must be constructed to be as short as possible, and the constructs should be checked for rearrangements in *Agrobacterium* before using them for plant transformation. The initial identification of a stable clone is especially important, because clone instability in *Agrobacterium* may facilitate rearrangements in the transgene loci.

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