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RESEARCH ARTICLE

Detection of transgenes in three genetically modified rice lines by fluorescence *in situ* hybridization

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Abstract
Fluorescence in situ hybridization (FISH) using T-DNA probes was applied to localize transgenes onto specific chromosomes and confirm the steady integration of transferred genes in three genetically modified (GM) rice lines, LS28 (event LS30-32-20-1), Cry1Ac1 (event C7-1-9-1) and LS28×Cry1Ac1 (event L/C1-1-3-1), which are a rice leaf blast-resistant single trait GM line, a leaf folder-resistant single trait GM line, and a rice leaf blast-resistant and leaf folder-resistant stacked GM hybrid line, respectively. The FISH signals were clearly detected on the arms of one homologous chromosome pair for LS28, and on the arms of another chromosome pair for Cry1Ac1 when using the transformation vector pSBM AtCK containing the rice leaf blast-resistant gene *(LS28)* and pMJ-RTB containing the leaf folder-resistant gene *(mCry1Ac1*) as a probe, respectively. As expected, we detected two pairs of FISH signals, each on the arms of different chromosome pairs in the stacked GM rice line LS28×Cry1Ac1 when using both pSBM AtCK and pMJ-RTB as probes. These results indicate that the transgenes are located at specific homologous loci and show position stability among generations in both single trait and stacked GM rice lines. The usefulness and the necessity of FISH to detect inserted genes in transformed plants will be discussed for the purpose of future studies to develop breeding programs and conduct risk assessment of GM plants.

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Introduction

The development of GM crops is progressing rapidly around the world with goals targeted toward food, medicine and horticulture production. At the onset of plant genetic engineering, single traits were introduced into GM crops such as insect-resistance or herbicide-resistance (James, 2006); however, the development of stacked GM crops containing multi-traits has been increasingly reported in recent years (Akiyama et al., 2005, 2008; Shin et al., 2009b). Recently, the Rural Development Administration in Korea developed two transgenic rice lines, LS28 and Cry1Ac1, which are rice leaf blast-resistant and leaf folder-resistant, respectively. Moreover, these lines were crossbred to produce the stacked GM rice hybrid lines, LS28×Cry1Ac1, which are rice leaf blast and leaf folder-resistant. Various studies are being conducted to evaluate the relationship between these transgenic rice plants (Shin et al., 2009a).

It is very important to locate the inserted chromosomal position of alien DNA segment in addition to molecular characterization for GM research and risk assessment. However, studies confirming that cytological identification of the inserted segments under stable conditions are still limited. Until recently, the inserted genes in transformed plants were commonly analyzed and detected through molecular studies using PCR, southern blot, flanking sequence analysis, etc. However, these analyses are only possible when sequence information regarding the plant genome and the T-DNA is well understood. Accordingly, the use of molecular analyses alone can lead to mistakes in the location of transferred genes on a specific chromosome when sequence similarities are present. Therefore,

complementary methods that reveal the physical location of the inserted gene on a chromosome such as fluorescence *in situ* hybridization (FISH) are needed to confirm the molecular identification of the transgene position.

FISH is a powerful tool that enables identification of the location of specific DNA sequences on somatic or meiotic chromosomes and can be very useful for the detection of locations of transgenes. FISH detection of transgenes in cereal crops has been reported in wheat, barley, triticale (Pedersen et al., 1997), oat (Leggett et al., 2000; Svitashev et al., 2000) and maize (Castro and Williams, 1997). FISH applications for rice genome research have been conducted in many studies (Jiang et al., 1995, 1996a, 1996b; Dong et al., 2001; Kharb et al. 2001; Jin et al., 2002). Indeed, a great deal of information has been obtained from the visualization of transgenes by FISH on metaphase or interphase spreads, and this has led to supplement molecular interpretation of the position of the inserted genes based on PCR, southern blot hybridization, sequencing, etc. (Santos et al., 2006). However, FISH based molecular cytogenetic studies on GM rice has not been conducted in Korea until now.

Here, we report the application of FISH to elucidate the positions of transgenes within the chromosomes of two single trait GM rice lines and their crossbred stacked GM rice hybrid line. The results will complement PCR-based molecular studies and will be useful for breeding and risk assessment of GM rice.

Materials and methods

Plant Materials

In this study, we used two event lines, LS28 (event LS30-32-20-1) and Cry1Ac1 (event C7-1-9-1), and one stacked line (event L/C1-1-3-1) derived from crossing two event lines. LS28 is a rice leaf blast-resistant single trait GM rice line into which transgene *LS28* was introduced. Cry1Ac1 is a leaf folder-resistant single trait GM rice line into which transgene *mCry1Ac1* was introduced. The crossbred stacked GM rice hybrid line of LS28×Cry1Ac1 is leaf blast and leaf folder-resistant line into which both transgenes *LS28* and *mCry1Ac1* were introduced. The seeds of single trait and stacked GM rice lines were kindly provided by Bio-safety Division, National Academy of Agricultural Science in Korea, which developed the transgenic rice lines from the rice *Oryza sativa* L. *japonica* by agrobacterium-mediated transformation (Shin et al., 2009a). The introduced traits include the *OsCKI* (cholin kinase) gene in LS28 and the *mCry1Ac1* gene in Cry1Ac1. The fresh root tips germinated from the seeds on wet filter-paper in a petri-dish at 25℃ in the growth chamber were used as the experimental materials.

Chromosome Preparation

The fresh root tips were cut and pretreated with 1-bromonaphthalene for 3 hrs at room temperature, after which they were fixed in an acetic acid: ethanol (1:3 v/v) solution for 2~24 hrs in the refrigerator. After washing thoroughly in distilled water, the growing points were excised from the root tips and treated with an enzyme mixture solution (2% cellulose - Yakult, 2% pectolyase - Sigma, USA, in 0.01M CA-SC buffer, pH 4.5) at 37℃ for 4.5 hrs. The root tips were then transferred onto a glass slide and squashed in a drop of 60% acetic acid solution. The preparations were made by alcohol flame-drying technique (Kurata and Omura, 1978) after a drop of fixation solution was added to the slides. The prepared slide was used for FISH after air drying.

Probe Preparation

The transformation vectors of pSBM AtCK and pMJ-RTB

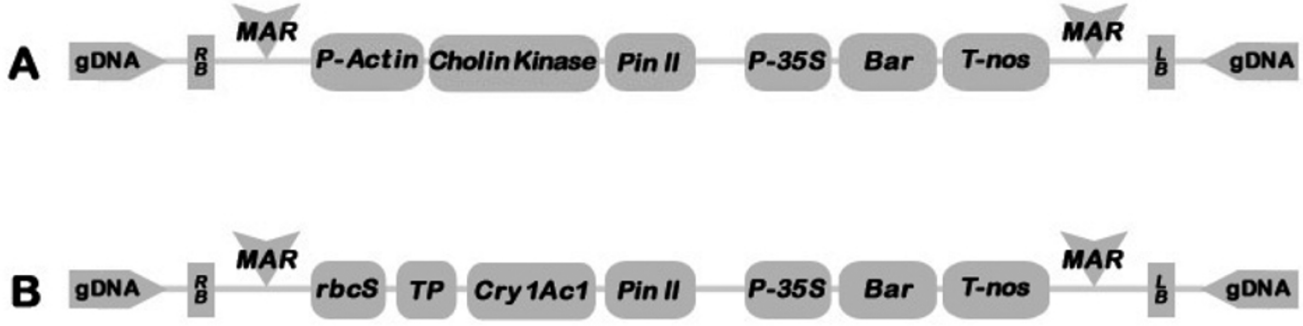

Figure 1. Schematic representation of the T-DNA structures of LS28 (event LS30-32-20-1) (A) and Cry1Ac1 (event C7-1-9-1) (B) (Shin et al., 2009).

used to develop the transgenic rice lines LS28 and Cry1Ac1, respectively, were kindly provided by the Bio-safety Division, National Institute of Agricultural Biotechnology in Korea and used as probes for FISH. The schematic representations of the T-DNA structure of transgenic rice lines LS28 and Cry1Ac1 are shown in Figure 1. The T-DNA size of LS28 and Cry1Ac1 were assumed to be in a range of 7~8 kb (Lee et al., 2009). The T-DNA of LS28 contains the *Oryza sativa Act1* promoter, *OsCKI* (cholin kinase) gene, 3' *PinII*, *35S* promoter, *bar* coding region, 3' *nos* and *MAR*, while that of Cry1Ac1 carries the *rbcS* promoter, *Tp* (transit peptide), *mCry1Ac*, 3' *PinII*, *35S* promoter, *bar* coding region, 3' *nos* and *MAR*. Occasionally, we also used a 45S rDNA probe to detect satellite chromosomes. The probes were labelled with digoxigenin-11-dUTP or biotin-16-dUTP individually according to the nick translation protocol supplied in the kit (Roche, Germany).

Fluorescence *in situ* Hybridization

Chromosomal DNA on the slide was treated with an enzyme mixture (2% cellulase -Yakult, 2% pectolyase - Sigma, USA) at 30℃ for 30 min. The slide was then washed with distilled water for 30 min followed by 2× SSC for 5 min. Next, the slide was treated with 100 μg/mℓ RNase A (Sigma, USA) in 2× SSC at 37℃ for 1 hr and then washed in 2× SSC three times each for 5 min, after which it was incubated in 0.01M HCl for 2 min. The chromosomal samples were treated with 100 μg/mℓ pepsin (Sigma, USA) in 0.01M HCl at 37℃ for 10 min, after which they were washed with distilled water for 2 min and 2× SSC two times each for 5 min. The hybridization mixture contained 50% formamide, 10% dextran sulfate, 2× SSC, 5 μg/μℓ salmon sperm DNA, and 500 ng /μℓ of probe DNA. The mixture was denatured at 90℃ for 10 min and then kept on ice for 5 min. This mixture was applied to the chromosomal DNA (20 μl for each preparation) and covered with a glass coverslip. The slides were then denatured at 80℃ for 4.5 hrs, after which they were placed in a humid chamber at 37℃ overnight. After hybridization, they were washed once in 2× SSC for 5 min and then washed with 0.1× SSC for 30 min at 42℃. For detection of the biotinylated probe, the slide was incubated with a series of antibodies Cy™ 3-streptavidin conjugate (Invitrogen, USA), biotinylated anti-streptavidin (Vector, USA), Cy™3-streptavidin conjugate (Invitrogen, USA) at 37℃ for 1 hr each. The Dig-labeled probe was then detected using a series of antibodies, monoclonal anti-digoxin FITC conjugate (Sigma, USA), anti-mouse IgG FITC conjugate (Sigma, USA), and monoclonal anti-rabbit IgG FITC conjugate (Sigma, USA) at 37℃ for 1 hr each. The slide was washed with detection buffer in each detection step. The chromosomes were counterstained with 2 μl/ml of 4'-6-diamidino-2-phenylindole (DAPI) in Vectashield (Vector, USA) and examined with an Olympus BX51 fluorescence microscope and CCD camera (CoolSNAP™ Cf). Image analysis was performed using Genus version 3.1 and the final image was edited using Adobe Photoshop version 7.0.

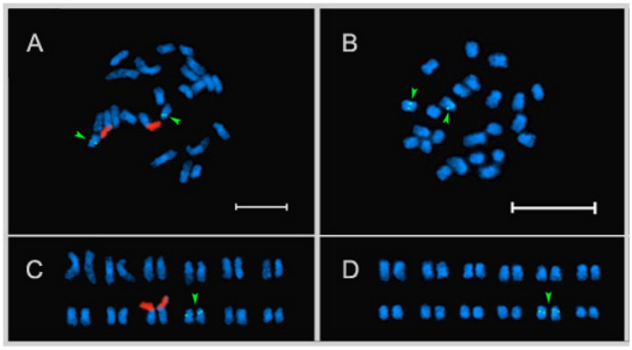

Figure 2. FISH pro-metaphases and their idiograms of single trait GM rice lines of LS28 (event LS30-32-20-1) (A, C) and Cry1Ac1 (event C7-1-9-1) (B, D). The arrowheads indicate the FISH signals (green) of T-DNAs which were localized on different chromosome pairs between LS28 and Cry1Ac1. The 45S rDNA signals (red) are also shown on another chromosome pair. Bars = 10μm.

Results and discussion

We conducted FISH with digoxigenin-labeled or biotin-labeled T-DNA probes to localize transgenes on chromosomes and confirm the integration of the inserted genes in three GM rice lines, LS28, Cry1Ac1 and LS28×Cry1Ac1. The FISH pro-metaphases and their idiograms of LS28 (A, C) and Cry1Ac1 (B, D) are shown in Figure 2. In both images, the double spot signals of the inserted genes were clearly observed on the chromosomes. The FITC signals as shown in green double spots exhibit the inserted T-DNA location on homologous chromosomes. The FISH probes were hybridized onto one chromosome pair (j) in LS28 and onto another chromosome pair (k) in Cry1Ac1, as indicated by a pair of green dots and red dots when using pSBM AtCK and pMJ-RTB as probes, respectively. These findings suggest that the transgenes are located at specific homologous loci in both single trait GM rice lines of LS28 and Cry1Ac1. These results are in agreement with molecular analysis data (Lee et al., 2009; Shin et al., 2009a, 2009b) that showed a single gene insertion in the Cry1Ac1 lines. As one of the chromosome markers, a pair of FISH signals of 45S rDNA that appeared in red in Figure 2A is shown in the pro-metaphase chromosomes of LS28.

Lee et al. (2009) reported that the T-DNA containing *mCry1Ac1* was integrated into chromosome 1 from sequence

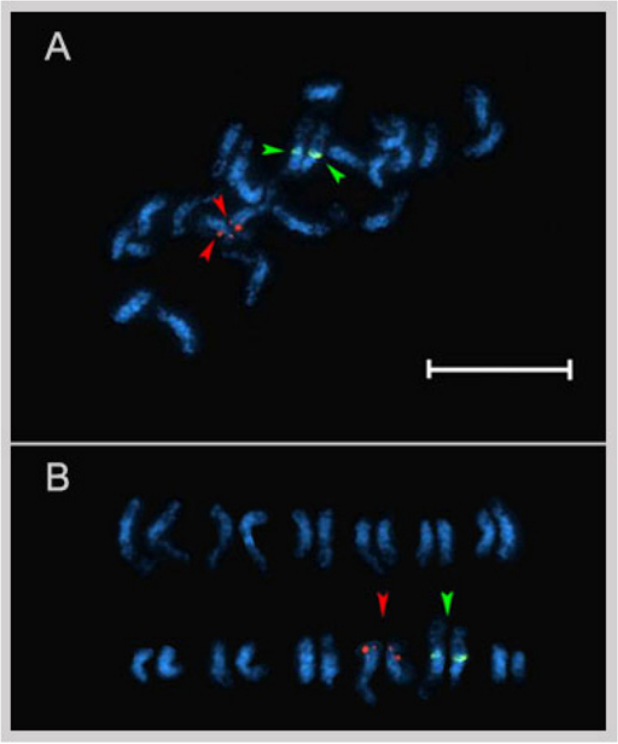

Figure 3. FISH pro-metaphase (A) and its idiogram (B) of the stacked GM rice hybrid line of LS28 × Cry1Ac1 (event L/C1-1-3-1). The arrowheads indicate FISH signals as shown as the red and green spots. T-DNAs hybridized to each chromosome pair j and k, respectively. Bars = 10μm.

analysis. It is known that the rice metaphase chromosomes are very small in a range of 1~2 μm (Kurata and Omura, 1978) and it is very difficult to identify each homologous chromosome exactly in metaphase. Even though by FISH we detected considerably small size of transgenes (7~8 kb) clearly on specific homologous chromosomes of each GM rice line, we still could not confirm the exact chromosome number into which transgene was integrated. To identify the T-DNA inserted chromosomes accurately by FISH, it needs to have additional bi-colour FISH experiment with both probes of T-DNA and chromosome specific BAC.

FISH signals of T-DNA were very rarely observed compared with those of 45S rDNA in the chromosomal preparations. This might have been due to the relatively small size of the T-DNA probe (7~8 kb) compared with the large size of the highly repetitive 45S rDNA. Nevertheless, FISH was sufficient to detect the T-DNA inserts in the GM rice lines.

The FISH pro-metaphase (Fig. 3A) in the stacked GM rice hybrid line of LS28 × Cry1Ac1 obtained using both pSBM AtCK and pMJ-RTB together as probes is shown in Figure 3. The T-DNA probes containing *LS28* and *mCry1Ac1*, respectively, are revealed as red and green spots on each homologous chromosome pair (j and k, respectively). As expected, we detected two pairs of FISH signals in the stacked GM rice hybrid line, LS28 × Cry1Ac1, each of which was derived from its parental single trait GM line of LS28 and Cry1Ac1, respectively.

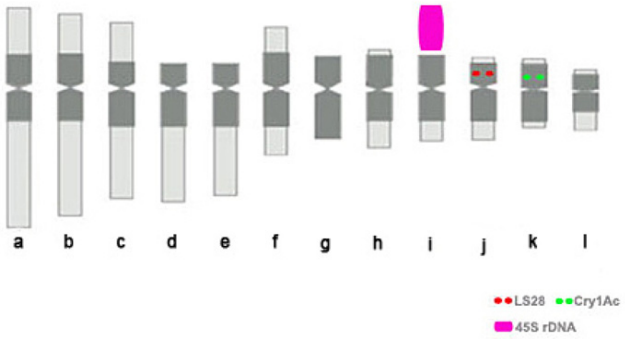

Figure 4. FISH pro-metaphase karyotypic diagram with combined FISH signals of 45S rDNA (pink), T-DNA containing LS28 (red), and T-DNA containing mCry1Ac1 (green) detected in three GM rice lines. The dark-gray areas indicate a well-condensed heterochromatic region, while light-gray areas indicate an insufficiently condensed euchromatic region in the pro-metaphase chromosome. Bars = 10μm.

The combined FISH pro-metaphase karyotypic diagram of three GM rice lines investigated in this study with FISH signals of *45S* rDNA, T-DNA containing *LS28* and T-DNA containing *mCry1Ac1* are presented in Figure 4. Each signal is shown in pink, red, and green, respectively. In the chromosomal diagram, the dark-gray areas indicate well-condensed heterochromatic regions, while light-gray areas indicate insufficiently condensed euchromatic regions in the pro-metaphase chromosome.

To commercialize transformed plants or GM crops, it is essential to investigate the chromosomal position and stability of the transgene in studies conducted for breeding programs and risk assessment of GM plants. To date, this has generally been accomplished using molecular approaches such as PCR, southern hybridization and DNA sequencing. However, such molecular studies frequently cannot represent conclusive evidence of the exact localization of inserted gene. If molecular cytogenetic data is available in addition to the molecular evidence, it would give more accurate information for the GM plant and that will be helpful to explain the safety problems such as uncertian or unstable gene functions. FISH has been used to complement molecular data, especially to visualize single copy transgenes on chromosomes (Abranches et al., 2000; Dong et al., 2001; Jackson et al., 2001; Salvo-Garrido et al., 2001, 2004; Choi et al., 2002; Svitashev et al., 2000; Romano et al. 2003; Harwood et al., 2005; Travella et al., 2005; Santos et al., 2006). This technique makes it possible to map trans-

genes on a specific chromosome accurately.

In this paper, we applied FISH techniques to recently developed GM rice lines to locate transgenes on chromosomes. FISH enabled identification of the transgenes at specific chromosomal loci and to demonstrate that they are inherited with steady integration among generations.

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