Detection of Alien Genes and Analysis of their Integration Position in Transgenic Rice by Fluorescence *in situ* Hybridization

Wei-Wei Jin1, Shun-Bin Ning1, Rui Qin1, Si-Luo Huang1, Ding-Hou Ling2 and Yun-Chun Song*1

1) Key Lab of MOE for Plant Developmental Biology, Wuhan University, Wuhan 430072 P. R. China
2) South China Institute of Botany, the Chinese Academy of Sciences, Guangzhou 510650 P. R. China

Using fluorescence *in situ* hybridization (FISH), we localized transferred barnase-psl and pHctinG DNA sequences onto chromosomes in a group of rice transgenic plants cotransformed by microprojectile bombardment. In all of the tested rice transgenic lines, the detected cells showed 1-3 signal spots and they were located in the central and terminal regions of chromosome arms. The signals of both barnase-psl and pHctinG genes were mostly detected in close contact on chromosomes, which could not be resolved without using two-color *in situ* hybridization. In a few cells, the signals were separated from each other for barnase-psl and pHctinG.

Key Words: transgenic rice, fluorescence *in situ* hybridization, integration pattern.

Introduction

The lack of ideal male sterile lines in rice in China has limited the production of hybrid rice. One strategy consisting of the transformation of the barnase gene, which is one kind of RNase cloned from *Bacillus amyloliquefaciens*, could be used to generate new male sterile lines in rice. Using a modified method of particle bombardment for the barnase-psl and pHctinG genes, we produced a number of transgenic male sterile plants in rice (Ling et al. 1998).

Although gene transfer has been developed rapidly in plants (Christou et al. 1989, Cheng et al. 1998), transgene integration patterns, stability and expression within the recipients have not been fully elucidated. One of the main constraints in the application of transformation is that the alien genes, which were transferred into plants, show a variation in expression from generation to generation or among different transgenic plants, and that display a low level of expression or become silent. The silencing of the barnase-psl gene and the variability of its expression have also been observed in different transformed lines (Ling et al. 1998).

Transformation, expression and stability of a gene depend on various factors. In examining the factors that influence transgene expression, an important consideration is the influence of chromosome location where the alien genes are integrated, referred to as the "position effect" (Jorgensen and Andersen 1994, Frello et al. 1995, Pedersen et al. 1997). The position effect has been studied extensively in plants including rice (Itoh et al. 1997, Kumpatla et al. 1997, Koli et al. 1999). *In situ* hybridization (ISH) is one of the most powerful tools to study the position effect of transgenes, "cold or hot spots" and patterns of their integration (Legeott et al. 2000, Jiang et al. 1994). However, in rice, to our knowledge, reports on the determination of the physical location of transformed genes are not available.

In this study, the barnase-psl (about 4.8 kb) and pHctinG (about 8.0 kb) genes have been physically located for the first time on the chromosomes of 8 lines of the tested transgenic rice lines by fluorescence *in situ* hybridization (FISH). Meanwhile, based on the results of FISH, the integration patterns of the transgenes have been analyzed.

Materials and Methods

Plant materials

Eight lines of transgenic rice, which were produced by particle bombardment, were used in the present investigation. All the eight transgenic lines at the T0 generation were obtained by co-transferring the barnase-psl and pHctinG genes to the recipient, *Oryza sativa* japonica cv Qiu Guang. Southern hybridization confirmed that 3, 6, 4, 7, 2, 4, 5 and 2 copies of barnase and 3, 5, 5, 6, 2, 5, 8 and 4 copies of hph were integrated in the transgenic lines (Ling et al. 1998). The sterility data of the transformed lines are indicated in Table 1.

Histochemical assay of *Gus*

Detection of the *Gus* activity was performed as described by Jefferson (1987).

DNA probes and chromosome preparation for FISH

The barnase-psl clone is about 6.5 kb in size and it contains the ps1 promoter (1.7 kb), barnase gene and the other part of the clone. To prevent homologous sequence from influencing the results of hybridization, the ps1 promoter was cut off before labeling, because the ps1 was cloned from rice. The pHctinG clone about 8.0 kb in size, carries hph, *Gus* genes and 35S promoter. The barnase and pHctinG genes were labeled with digoxigenin and biotin, respectively, according to the nick translation protocol sup-
plied by the kit (Sino-American Biotechnology Company, China).

Root tips were cut from actively growing transgenic rice plants and fixed immediately in ethanol-acetic acid (3:1) at 4°C overnight. After being fully washed with distilled water, the root tips were treated with a mixture of 2% pectinase (SERVA) and 2% cellulase (SERVA) at 28°C for approximately 3 hr. Finally, the treated root tips were squashed on slides and dried over a flame (Song et al. 1995). The slides were kept in a −20°C freezer for FISH.

In situ hybridization followed the procedures described by Song et al. (1995). For fluorescence detection, the slide was covered with goat anti-biotin FITC conjugate (Sigma) and/or mouse anti-dig (Boehringer Mannheim), and subsequently treated with rabbit anti-goat biotin conjugate (Sigma) and/or dig anti-mouse (Boehringer Mannheim). Then goat anti-biotin-FITC and/or rodamine anti-dig (Boehringer Mannheim) was added. The signals were amplified by immunological reaction, at 37°C for 30 min each and washing with PBS was conducted regular at intervals. And finally the slides were counterstained with a solution containing 8 μg/ml PI or 1 μg/ml DAPI. Chromosomes were observed with an Olympus BX60 fluorescence microscope equipped with a Sensys 1401E CCD camera. Red, green, and blue images were captured in black and white with different filters. The images were combined and pseudocolored in the computer using software V++.

Results

Gus activities

All the eight transgenic lines showed Gus activities except for the control (Table 1).

FISH of transgenes

All the eight transgenic lines with the barnase-psl probe (psl promoter was cut off) showed hybridization signals on their chromosomes (Fig. 1). These eight transgenic lines displayed different detection rates ranging from 55% to 75%. It was easy to locate the signals on the chromosome arms but it was difficult to assign them to the chromosome pairs because of the similarity in size and morphology of all the chromosomes in the karyotype of rice. Data for detection rates, arm ratios of the chromosomes on which signals were detected and fraction-length (FL) value, which is the percentage of the distance from the site of the signal to the centromere divided by the length of the chromosome arm carrying the signal, are presented in Table 2. The signals of the barnase probe were observed at 10 sites on 9 chromosome arms (Table 2, Fig. 1). Alien genes were introgressed into both long and short arms of the chromosomes. In the transgenic lines, Q1, Q2 and Q12, the hybridization signals of the barnase probe were observed only on long arms of the chromosomes and in Q4, Q5, Q9 and Q16, the signals were observed on short arms of the chromosomes, whereas, in the Q13 plant, the signals were observed on both long and short arms of the chromosomes. Furthermore it was observed that all the 10 integration sites were located in the interstitial regions instead of the centromeric regions. Fraction-length of the detection sites ranged from 24.4 to 92.4, indicating that the integration sites were distributed over a wide region of the chromosome arms (Table 2).

Double-color FISH was used to detect the hybridization signals of the two probes. Three hybridization sites were detected with a single color, one with red and two with green. Red signals revealed the hybridization sites of barnase and green ones showed those of Ph5inG. The yellow signals, nine in number, consisted of a mixture of red and green signals of barnase and Ph5inG. The present hybridization results revealed 10 integrated sites of barnase and 11 of Ph5inG in the 8 transgenic lines of rice.

Discussion

The signals on both sister chromatids could be observed simultaneously on partially detected cells (Fig. 1), but only one member of the homologues for each detection site showed the signal. These data indicated that all the tested plants were heterozygous instead of homozygous for the transformed genes. Our results showed that transformation by micro-projectile bombardment integrated alien genes over a wide region of different chromosome arms. Simulta-
neous detection of the signals at the same site in two members of each chromosome pair was nearly impossible.

Since the regions near the centromeres in which FL was less than 24.4 covered almost one-fourth of the whole arm length, it was considered that the integration of the transgenes in these regions would have obviously occurred. However, no signal was observed in these regions. This was not occasional and it could not be explained by random events. We consider that this finding may be attributed to two possible mechanisms. First, the regions close to centromeres are heterochromatin-rich and more condensed, and in a compact organization, presumably, it is more difficult to insert alien genes. Secondly, even if alien genes were integrated in these regions, since their expression would probably be affected by heterochromatin and decrease to some extent, they might have been easily discarded during selection. The decrease of the gene activity induced by heterochromatin could be detected in many cases (Ansari et al. 1999, Jakowitsch et al. 1999, Meyer and Sadeler 1996).

We did not observe any preferential integration sites because the signals were distributed over a wide region of the chromosome arms (Table 2). Our results were consistent with those obtained with micro-projectile bombardment transformation by other authors (Potrykus 1985, Saul and Potrykus 1990, Peng et al. 1992, Gharti-Chhetri et al. 1990). However, in Agrobacterium-mediated gene transfer, seven out of eleven T-DNA inserts were detected in the distal region in a group of transformants in petunia (Wang et al. 1995), three of five Crepis capillaris transformants were telomeric (Ambros et al. 1986), in tomato transformants most of the T-DNAs were observed in the distal region (Hoopen et al. 1996), and the integration sites of nine out of thirteen barley transformants were located in the distal 40% region of chromosome arms (Pedersen 1997). Comparison of the results between the two transformation methods indicated that whether integration sites were preferential or not probably depended on the transformation technique applied.

Table 2. FISH of barnase-psl and pHctinG on transgenic rice

<table>
<thead>
<tr>
<th>Code of Plant</th>
<th>No. of cells with signals</th>
<th>Detection rate (%)</th>
<th>Ratio of long to short arm</th>
<th>FL (%)</th>
<th>A arms with signals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1</td>
<td>63</td>
<td>55</td>
<td>1.43 ± 0.09</td>
<td>24.4 ± 3.5</td>
<td>L</td>
</tr>
<tr>
<td>Q2</td>
<td>49</td>
<td>65</td>
<td>1.25 ± 0.06</td>
<td>63.2 ± 4.0</td>
<td>L</td>
</tr>
<tr>
<td>Q4</td>
<td>59</td>
<td>70</td>
<td>1.38 ± 0.09</td>
<td>44.8 ± 2.9</td>
<td>S</td>
</tr>
<tr>
<td>Q5</td>
<td>52</td>
<td>75</td>
<td>1.12 ± 0.07</td>
<td>70.5 ± 3.5</td>
<td>S</td>
</tr>
<tr>
<td>Q9</td>
<td>44</td>
<td>70</td>
<td>1.28 ± 0.05</td>
<td>92.4 ± 3.6</td>
<td>S</td>
</tr>
<tr>
<td>Q12</td>
<td>72</td>
<td>65</td>
<td>1.76 ± 0.10</td>
<td>83.3 ± 4.9</td>
<td>L</td>
</tr>
<tr>
<td>Q13</td>
<td>71</td>
<td>65</td>
<td>1.76 ± 0.10</td>
<td>69.0 ± 3.4</td>
<td>L</td>
</tr>
<tr>
<td>Q14</td>
<td>60</td>
<td>65</td>
<td>1.30 ± 0.14</td>
<td>93.0 ± 5.0</td>
<td>L</td>
</tr>
<tr>
<td>CK</td>
<td>40</td>
<td>0</td>
<td>1.50 ± 0.08</td>
<td>91.7 ± 4.1</td>
<td>S</td>
</tr>
</tbody>
</table>

S: short arm, L: long arm. (1); standard deviation. (2); only detected for barnase-psl. (3); only detected for pHctinG. For the other sites: both barnase-psl and pHctinG.

A series of research data (Gheyser et al. 1991, Matsumoto et al. 1990, Kartzke et al. 1990, and Gharti-Chhetri et al. 1990) showed that the micro-projectile bombardment method was probably associated with different integration mechanisms as did the Agrobacterium-mediated gene transfer.

Usually, the micro-projectile bombardment method of transformation enables to integrate multiple copies of transgenes (Ritala et al. 1994). In this study, based on the results of Southern blot, in the tested lines, 2-7 copies of the barnase gene were integrated. However, our FISH results for barnase indicated that most of the tested lines showed only one detection site except for the lines Q12 and Q13 in which two detection sites were observed (Table 2). It is possible that each detection site may contain more than one copy of the transgenes. Our results were consistent with the observations of other authors (Christou et al. 1989, Spencer et al. 1992, Ritala et al. 1994), who reported that multiple copies of transgenes could be usually detected, and often co-segregated as a transgenic locus.

The cotransformation frequency of pHctinG and barnase-psl was very high up to about 94% (Ling et al. 1998). Wojciech (1996) indicated that the frequencies of cotransformation of transgenes derived from separate plasmids were similar to the cotransformation frequencies of two genes introduced into the same plasmid. Our results showed that the two plasmids mostly integrated into the adjacent position of the chromosome, which was consistent with the molecular biology results of Herve (1993) and Christou (1990). Koli (1998) suggested that in rice an initial site of integration may provide a “hot spot” for the subsequent integration of successive transgene sequences in the same region. Our results support this assumption. It would be important to determine whether the different transgenes are connected with each other to further elucidate the transgene integration mechanisms.
Fig. 1. A–I FISH with the barnase-ps1 and pHc11G probes on metaphase chromosomes of transgenic rice. Hybridization signals are denoted by arrows. A–C: hybridization with barnase-ps1 probe and counter-staining with PI. A Q13, B Q16, C Q9. D–I: hybridization of Q12 (D–F) and Q15 (G–I) and counter-staining with DAPI. D and G: merged images of separately detected images using a UV filter (detection of blue chromosomes when stained with DAPI) and Green filter (detection of red signals of barnase-ps1 labeled with digoxigenin). E and H: merged images of the separately detected images using UV filter and Blue filter (detection of green signals of pHc11G labeled with biotin). F and I: merged images of separately detected images using UV, Blue and Green filters.

Acknowledgements

We thank Dr. Alice Cheung (Yale University, USA) and Dr. J. Futterer (ETH, Switzerland) for kindly providing the barnase-ps1 and pHc11G plasmids. This work was supported by the National Natural Science Foundation of China (No. 30070376) and the Research Fund for the Doctoral Program of Higher Education (No. 207980112).

Literature Cited

Cheng, X.Y., R. Sardana, H. Kaplan and L. Altissara (1998) Agrobacterium-transformed rice plants expressing synthetic cry1(b) and cry1A(c) genes are highly toxic to striped stem borer and


