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Wenxue Zhai · Caiyan Chen · Xuefeng Zhu ·
Xuewei Chen · Dechun Zhang · Xiaobing Li ·
Lihuang Zhu

# Analysis of T-DNA-*Xa21* loci and bacterial blight resistance effects of the transgene *Xa21* in transgenic rice

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**Abstract** The genetic loci and phenotypic effects of the transgene *Xa21*, a bacterial blight (BB) resistance gene cloned from rice, were investigated in transgenic rice produced through an *Agrobacterium*-mediated transformation system. The flanking sequences of integrated T-DNAs were isolated from *Xa21* transgenic rice lines using thermal asymmetric interlaced PCR. Based on the analysis of 24 T-DNA- *Xa21* flanking sequences, T-DNA loci in rice could be classified into three types: the typical T-DNA integration with the definite left and right borders, the T-DNA integration linked with the adjacent vector backbone sequences and the T-DNA integration involved in a complicated recombination in the flanking sequences. The T-DNA integration in rice was similar to that in dicotyledonous genomes but was significantly different from the integration produced through direct DNA transformation approaches. All three types of integrated transgene *Xa21* could be stably inherited and expressed the BB resistance through derived generations in their respective transgenic lines. The flanking sequences of the typical T-DNA integration consisted of actual rice genomic DNA and could be used as probes to locate the transgene on the rice genetic map. A total of 15 different rice T-DNA flanking sequences were identified. They displayed restriction fragment length polymorphisms (RFLPs) between two rice varieties, ZYQ8 and JX17, and were mapped on rice chromosomes 1, 3, 4, 5, 7, 9, 10, 11 and 12, respectively, by using a double haploid population derived from a cross between ZYQ8 and JX17. The blast search and homology comparison of the rice T-DNA flanking sequences with the rice chromosome-anchored sequence database confirmed the RFLP mapping results. On the basis of genetic mapping of the T-DNA- *Xa21* loci, the BB resistance effects of the transgene *Xa21* at different chromosome locations were investigated using homozygous transgenic lines with only one copy of the transgene. Among the transgenic lines, no obvious position effects of the transgene *Xa21* were observed. In addition, the BB resistance levels of the *Xa21* transgenic plants with different transgene copy numbers and on different genetic backgrounds were also investigated. It was observed that genetic background (or genome) effects were more obvious than dosage effects and position effects on the BB resistance level of the transgenic plants.

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W. Zhai · C. Chen · X. Zhu · X. Chen · D. Zhang · X. Li ·
L. Zhu (✉)
National Key Laboratory of Plant Genomics, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences,
Beijing, 100101, China
e-mail: lhzhu@genetics.ac.cn
Tel.: +86-10-64870491
Fax: +86-10-64873428

## Introduction

*Agrobacterium tumefaciens*-mediated T-DNA transfer is the most widely used transformation system to introduce foreign genes into the plant genome. Intensive investigation of the mechanism of T-DNA transfer over the past several decades has resulted in a detailed understanding of this process (Zupan et al. 2000). It has been assumed that T-DNA transfer involves only DNA sequences between the two T-DNA borders. In the case of typical T-DNA transfer, the integrated T-DNA is flanked by plant genomic sequences. However, there is evidence in the literature suggesting that DNA sequences residing outside the T-DNA borders (non-T-DNA) may also occasionally be transferred into the plant during *Agrobacterium*-mediated plant transformation (Ooms et al. 1982; Kononov et al. 1997; De Buck et al. 2000; Yin and Wang 2000; Vain et al. 2003). The T-DNA flanking sequences can be easily analyzed by using PCR-based methods, and the positions of the T-DNA flanking sequences whose nature is plant genomic DNA can be determined on plant molecular maps (Liu et al. 1995). The molecular analysis of T-DNA loci in transgenic plants is a necessary step for the study of insertion mutants and transgenic breeding. Moreover, biosafety regulations are demanding that transgenic plants

to be commercialized are devoid of vector backbone sequences.

Both basic research and commercial plant breeding programs require that the transgenes are stably integrated and expressed through generations. However, unexpected variation in transgene expression is often observed among transgenic plants (Peach and Velton 1991). Many factors can contribute to variation in transgene expression, including integration site (position effects), transgene copy number (dosage effects), transgene mutation and epigenetic gene silencing (Hobbs et al. 1990; Fladung 1999; Maqbool and Christou 1999; Matzke et al. 2000; James et al. 2002). However, results from studies on transgene expression and associated phenotypes, particularly in cereal crops, are still limited and sometimes even contradictory. The actual relationship between transgene expression and the influencing factors need to be investigated in more instances.

The rice gene *Xa21* confers resistance against the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) race 6. Due to its wide spectrum of bacterial blight (BB) resistance, *Xa21* is of great value when breeding for rice varieties with BB resistance (Huang et al. 1999; Li et al. 2001). The gene has been cloned through a map-based strategy, which provides an significant opportunity for improving BB resistance of rice by genetic transformation (Song et al. 1995). In addition, this rice-originated *Xa21* is also a valuable model gene to study the relationship between transgene expression and factors influencing its expression in rice because of the simplicity of measuring the resistance level. Through particle bombardment, the cloned *Xa21* gene has been transferred into several rice varieties and even pyramided with other resistance genes (Wang et al. 1996; Tu et al. 1998, 2000; Zhang et al. 1998; Datta et al. 2002). However, as variations in BB resistance provided by the transgene have been observed in some transgenic progenies, it would appear to be worthwhile to investigate the BB resistance of the transgene *Xa21* under different factors that may influence its expression.

An effective *Agrobacterium*-mediated transformation system has been established successfully in cultivated rice (Hiei et al. 1994). We used this system to transfer the *Xa21* gene into eight Chinese rice varieties in a series of experiments (Zhai et al. 2001, unpublished data). In the investigation reported here, the T-DNA- *Xa21* loci and BB resistance effects of the transgene *Xa21* were analyzed in transgenic lines with different genetic backgrounds, different transgene copy numbers and different transgene positions through several generations.

## Materials and methods

### Rice varieties and transgenic lines

Primary transformants ($T_0$) were obtained from eight Chinese rice (*Oryza sativa* L.) varieties, including *Minghui63*, *Yanhui559*, *Zhenxian97B*, *Peiai64*, C418, *Taihujing6*, 8706, and *Zhonghua11*, through an *Agrobacterium*-mediated transformation system using the vector pCXK1301 (Zhai et al. 2001), which was constructed by inserting the complete 9.9-kb *Xa21* gene (Song et al. 1995) into the *Kpn*I site of pCAMBIA1301 (R.A. Jefferson, CAMBIA, Australia). $T_1$, $T_2$ and $T_3$ progenies were obtained by self-pollination of $T_0$, $T_1$ and $T_2$ transgenic plants, respectively. The copy or locus numbers of the transgene *Xa21* in transgenic rice plants had been revealed through Southern hybridization following the protocol of Zhai et al. (2001). For PCR amplification and Southern hybridization, genomic DNAs of transgenic plants were extracted according to the method of McCouch et al. (1988).

### Isolation of T-DNA flanking sequences from *Xa21* transgenic plants using thermal asymmetric interlaced-PCR (TAIL-PCR) amplification

TAIL-PCR has been described in detail by Liu and Whittier (1995). To amplify the T-DNA flanking sequences of transgenic plants, we designed the specific primers according to the end sequences of T-DNA on the vector pCXK1301 as shown in Fig. 1. The primer sequences are as follows:

| | |
|---|---|
| $L_1$ | 5′-GTTTCGCTCATGTGTTGAGCA-3′ |
| $L_2$ | 5′-TCAGTACATTAAAAACGTCCGCA-3′ |
| $L_3$ | 5′-ACGTCCGCAATGTGTTATTAAGTT-3′ |
| $R_1$ | 5′-GTTTTTATGATTAGAGTCCCGC-3′ |
| $R_2$ | 5′-AATATAGCGCGCAAACTAGGA-3′ |
| $R_3$ | 5′-GATCGGGAATTAAACTATCAGTG-3′ |
| $AD_1$ | 5′-TGAGNAGTANCAGAGA-3′ |
| $AD_2$ | 5′-AGTGNAGAANCAAAGG-3′ |
| $AD_3$ | 5′-CATCGNCNGANACGAA-3′ |
| $AD_4$ | 5′-TCGTNCGNACNTAGGA-3′ |
| $AD_5$ | 5′-NTCGA(G/C)T(A/T)T(G/C)G(A/T)GTT-3′ |
| $AD_6$ | 5′-NGTCGA(G/C)(A/T)GANA(A/T)GAA-3′ |
| $AD_7$ | 5′-TG(A/T)GNAG(A/T)ANCA(G/C)AGA-3′ |
| $AD_8$ | 5′-AG(A/T)GNAG(A/T)ANCA(A/T)AGG-3′ |
| $AD_9$ | 5′-CA(A/T)CGICNGAIA(G/C)GGA-3′ |
| $AD_{10}$ | 5′-(A/T)GTGNAG(A/T)ANCANAGA-3′ |

PCR reactions were performed in a PE-9600 thermocycler (Perkin-Elmer, Foster City, Calif.) using the procedures listed in Table 1. PCR products were separated in a 1.4% agarose gel in 1× TAE buffer and the fragments recovered using the Agarose Gel DNA Extraction kit (Boehringer Mannheim, Indianapolis, Ind.) and cloned into pGEM-T Easy Vector (Promega, Madison, Wis.) for sequencing. The sequences of the PCR fragments were compared with each other or with the vector sequences, and with the rice genome sequence data using the DNASTAR program and the BLAST program.

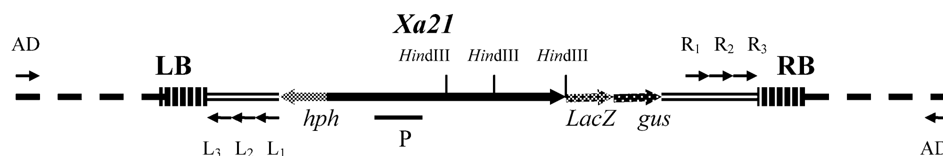


**Fig. 1** Schematic map of integrated T-DNA-*Xa21* and the locations of primers used in TAIL-PCR. *AD* An arbitrary degenerate primer, *LB* and *RB* left and right T-DNA border repeat, *L₁*, *L₂*, *L₃* nested specific primers at left T-DNA border, *R₁*, *R₂*, *R₃* nested specific primers at right T-DNA border, *hph* hygromycin phosphotransferase, *gus* β-glucuronidase, *LacZ* β-galactosidase alpha, *Xa21* the rice *Xa21* gene, *P* the position of the *Xa21* probe used in Southern hybridization

**Table 1** Reaction mixtures and cycle settings used for TAIL-PCR

| Reaction | Mixture contents (in 20 μl) | File no. | Cycle no. | Thermal settings |
|---|---|---|---|---|
| Primary | 1× PCR buffer (10 m*M* Tris-HCl PH 9.0, 50 m*M* KCl, 1.8 m*M* $MgCl_2$, 0.1% TritonX-100), 200 μ*M* each of dNTPs, about 20 ng genomic DNA, 0.8 U *Taq* polymerase, 0.2 μ*M* primer L1 (R1), 4 μ*M* one AD primer | 1 | 1 | 93°C, 1 min; 95°C, 1 min |
| | | 2 | 8 | 94°C, 30 s; 60°C, 1 min; 72°C, 2.5 min |
| | | 3 | 2 | 94°C, 30 s; 25°C, 3 min; ramping to 72°C over 3 min; 72°C, 2.5 min |
| | | 4 | 15 | 94°C, 15 s; 65°C, 1 min; 72°C, 2.5 min<br>94°C, 15 s; 65°C, 1 min; 72°C, 2.5 min<br>94°C, 15 s; 43°C, 1 min; 72°C, 2.5 min |
| | | 5 | 1 | 72°C, 5 min |
| Secondary | 1× PCR buffer, 200 μ*M* each of dNTPs, 1 μl 50-fold dilution of the primary PCR product, 0.6 U *Taq* polymerase, 0.2 μ*M* the primer L2 (R2), 2 μ*M* the same AD primer | 6 | 13 | 94°C, 15 s; 63°C, 1 min; 72°C, 2.5 min<br>94°C, 15 s; 63°C, 1 min; 72°C, 2.5 min<br>94°C, 15 s; 43°C, 1 min; 72°C, 2.5 min |
| | | 5 | 1 | 72°C, 5 min |
| Tertiary | 1× PCR buffer, 200 μ*M* each of dNTPs, 1 μl 50-fold dilution of the secondary PCR product, 0.6 U *Taq* polymerase, 0.2 μ*M* primer L3 (R3), 2 μ*M* the same AD primer | 7 | 25 | 94°C, 25 s; 43°C, 1 min; 72°C, 2.5 min |
| | | 5 | 1 | 72°C, 5 min |

### Genetic mapping of T-DNA flanking rice genomic sequences

The double haploid (DH) population containing 127 lines derived from a cross between ZYQ8 and JX17 (http://www.gramene.org/cmap/viewer/) was used to map the T-DNA flanking genomic sequences. Genomic DNA extraction and population analysis were carried out as described by McCouch et al. (1988). DNA was digested with *Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Sca*I and *Xba*I (Amersham, UK), respectively. The T-DNA flanking genomic sequences were used as probes for restriction fragment length polymorphism (RFLP) analysis. Southern hybridization was carried out as described by the manufacturer (Amersham). Genetic mapping was carried out by using MAPMAKER/EXP (Lincoln et al. 1992). The blast search and homology comparison of the flanking rice sequences was carried out at the TIGR Rice Database (http://www.tigr.org/tdb/e2 k1/osa1/) to find the anchored-bacterical artificial chromosome (BAC) clones.

### BB resistance analysis of transgenic lines with the transgene *Xa21*

Transgenic plants were investigated for their resistance to the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) Philippine race 6, PXO99, using the leaf-clipping method as described by Zhai et al. (2001). The growing and inoculation conditions for transgenic plants and wild controls in a designed experiment or generation were adjusted to be as similar as possible, although they may have varied slightly among different experiments and generations. The lesion length was measured on each of the inoculated leaves 16–18 days after inoculation when the susceptible check's lesion became obvious and stable.

## Results

### Analysis of integrated T-DNA-*Xa21* flanking sequences in transgenic rice plants

T-DNA flanking sequences in transgenic lines with the transgene *Xa21* were amplified through TAIL-PCR as described in Fig. 1 and Table 1. Twenty-four flanking sequences were obtained from 16 independent transformants, and these are listed in Table 2. We were able to classify these 24 sequences into three types on the basis of the nature of their sequence. Fifteen of these were actually rice DNA (Type A); six were the vector backbone sequence, which is referred to the bacterial replicon region just adjacent to T-DNA on the vector (Kononov et al. 1997) (Type B); the remaining three sequences may have resulted from complex recombination during T-DNA integration (Type C).

Of the 15 Type-A flanking sequences, 12 were at the left border (LB) of T-DNA and three at the right border (RB). Some sequences of the T-DNA end-joining region in Type-A sequences are compiled in a linear form (Fig. 2). No homology was found between these sequences and the sequence of the transfer vector pCXK1301. Most of them were found to have homologs at the TIGR Rice Database (http://www.tigr.org/tdb/e2 k1/osa1/) when a BLAST search was performed, although the sequences adjacent to the T-DNA ends in the flanking fragments did not match well

**Table 2** Flanking sequences and genetic mapping of T-DNA-*Xa21* loci

| Transformants (receptor variety) | Copies or loci[a,c] | Flanking sequences[b] | Sequence nature[c] | Located chromosome number[c] | Linked markers beside the locus | Anchored BAC clones |
|---|---|---|---|---|---|---|
| W3 (*Minghui63*) or W6[d], W71[d], W72[d] | 2 | W3aL | Rice DNA | 9 | C10403, C313 | P0501E09 |
| | | W3aR | Rice DNA | 9 | | |
| | | W3bL | Rice DNA | 5 | | |
| | | W3bR | Rice DNA | 5 | R566, R569 | OSJNBb0067H15 |
| W75 (*Minghui63*) | 2 | W75aL | Rice DNA | 11 | G320, RZ638 | |
| | | W75R | Vector DNA | | | |
| W76 (*Minghui63*) or $M_2$[e] | 1 | W76L | Rice DNA | 4 | C513, G271 CT206 | H0811E11 |
| | | W76R | – | – | | |
| W8 (*Minghui63*) or $M_1$[e] | 1 | W8L | Vector DNA | – | | |
| | | W8R | Vector DNA | – | | |
| W10 (*Yanhui559*) | 2 | W10L | Rice DNA | 3 | G249, G164 | OSJNBb0038E06 |
| | | W10R | Rice DNA | 3 | | |
| W68 (Yanhui559) | 1 | W68L | Rice DNA | 1 | R210, G270 | B1102E12 |
| | | W68R | – | – | | |
| W50 (*Zhenxian97B*) | 4 | W50L | Rice DNA | 1 | R210, G270 | OSJNBa0042P21 |
| | | W50R | – | – | | |
| W51 (*Zhenxian97B*) | 1 | W51L | Vector DNA | – | | |
| | | W51R | Vector DNA | – | | |
| W52 (*Zhenxian97B*) | 4 | W52L | Vector DNA (*X*a21) | – | | |
| | | W52R | Vector DNA | – | | |
| W53 (*Zhenxian97B*) | 3 | W53L | Rice DNA | 7 | G20, C285 | P0683C09 |
| | | W53R | – | – | | |
| W57 (*Zhenxian97B*) | 2 | W57L | – | – | | |
| | | W57R | Vector DNA | | | |
| W58 (*Zhenxian97B*) | 2 | W58L | Rice DNA | 10 | CT387, L169 | OSJNBa0079B05 |
| | | W58R | – | – | | |
| W60 (*Zhenxian97B*) | 1 | W60L | Rice DNA | 12 | G1391, RZ397 | OSJNBa0021D06 |
| | | W60R | – | – | | |
| W45 (*Taihujing6*) or W47[d], W49[d] | 2 | W45L | Rice DNA | 5 | G81, C246 | OJ1087C03 |
| | | W45R | – | – | | |
| W106 (8706) or W110[d] | – | W106L | Rice DNA | 5 | G81, C246 | OJ1362G11 |
| | | W106R | – | | | |
| W108 (8706) | – | W108L | Vector DNA | – | | |
| | | W108R | – | – | | |

[a]These were revealed through Southern hybridization according to Zhai et al. (2001)
[b]For an analyzed transformant, “a” or “b” following the transformant number indicates a clearly revealed T-DNA- *Xa21* locus; “L” or “R” following the transformant number indicates its T-DNA left or right flanking sequence, respectively
[c]–, Not obtained or could not be localized
[d]Transformants derived in a same transformation event
[e]The same transformant code as in the reference (Zhai et al. 2001)

with the homologs, which might be attributed to minor recombination during T-DNA integration. Moreover, strong hybridization signals could be detected when they were hybridized with genomic DNA of an indica variety, ZYQ8, and a japonica variety, JX17. The copy numbers of these sequences in the rice genome were determined following hybridization with rice genome DNA, and most of were determined to be single copy. All of these flanking sequence fragments had an AT-content below 70%, of which W68L, W58L and W53L had an AT-content lower than 61.5%, which is the average AT-content of the rice genome DNA (data not shown). In addition, the sequences neighboring the rice homologous sites of these flanking sequences were also searched and analyzed at the TIGR Rice Database, but no common sequence characteristic was found.

When the junction sequences from the integrated T-DNA-*Xa21* insertions to their respective flanking rice DNAs were compared with each other and with the vector sequences, we observed that the T-DNA border and nearby

**Fig. 2a, b** Junction sequences of T-DNA-*Xa21* to flanking rice DNA. The *first line* in both **a** and **b** is the sequence of vector pCXK1301. The *vertical line* is the joint site of T-DNA (on the *left*) and rice DNA (on the *right*). The *shaded capital letters* are the border sequences of T-DNA. The positions and directions of nested specific primers are indicated by *arrows*

A. T-DNA Left Border/Rice DNA

$L_1$ → $L_2$ → $L_3$ →

| | T-DNA | Rice DNA |
|---|---|---|
| pCXK1301 | --acgtccgcaatgtgttattaagttgtctaagcgtcaatttGTTTACACCACAATATATCCTGCCAccagccagccaacagctccccga-- | |
| **W3aL** | --acgtc | gcatgctcccggccgccatggcggccg -- |
| **W10L** | --acgtccgcaatgtgttattaagttgtctaagcgtcaatttGTTTACACCACAATA | gaaagatacagtctcagaagaccaaagg-- |
| **W68L** | --acgtccgcaatgtgttattaagttgtctaagcgt | tggctattcatggcggctagcagatctgg-- |
| **W50L** | --acgtccgcaatgtgttattaagttgtctaagcgt | ggtggaatgctgacaccggtccgtcatc-- |
| **W53L** | --acgtccgcaatgtgttattaagttgt | ttggccatgtcgccgtgcgagacagcat-- |
| **W45L** | --acgtccgcaatgtgttattaagttgtctaagcgtcaatttGTTTACACCA | tgtttattatagtgggtcaccatggtatgat-- |
| **W60L** | --acgtccgcaatgtgttattaagttgtctaagcgtcaatttGTTTACAC | gattttgtagacgacaagttgagggacct-- |
| **W106L** | --acgtccgcaatgtgtt | gagcatataatggggcatgcaaccttatg-- |
| **W76L** | --acgtc | ggcggagagggagaggtgaccagtga-- |

B. T-DNA Right Border /Rice DNA

$R_1$ → $R_2$ → $R_3$ →

| | T-DNA | Rice DNA |
|---|---|---|
| pCXK1301 | --gatcgggaattaaactatcagtgttTGACAGGATATATTGGCGGGTAAACcgggtaaacctaagagaaaagagcgtttatt-- | |
| **W3bR** | --gatcgggaattaaactatcagtgttT | tgtgaagaacaaaatatttcaatctttgatctaactttgtttcatcattttaatatattgaatt-- |
| **W10R** | --gatcgggaattaaactatcagtgttTGA | tagccctttatttttgaaggacgtaattcatatatgatgatgagactggaattatcagct-- |

sequences were deleted to different degrees in different transgenic plants (Fig. 2). At the left end of the integrated T-DNA, the whole LB sequence and a number of nucleotides (nt) adjacent to the LB were deleted in flanking sequence fragments W3aL, W50L, W53L, W68L, W76L and W106L, whereas in some other flanking fragments the border remnants were retained—for example, 15 nt in W10L and 8 nt in W60L, although they were not the typical 22-nt border remnant. At the right end of the integrated T-DNA, however, the deletion of the whole right border (RB) was not found, and at least 1 nt (in W3bR) or 3 nt (in W10R) of the RB sequence was retained, which correlates with the identified positions of major and minor nick sites in T-DNA processing (Wang et al. 1987).

Type B flanking sequences consisted of vector backbone sequences directly linked to the T-DNA across either the LB or RB. In the three amplified fragments at LB (W8L, W51L and W108L) and another three amplified segments at RB (W8R, W51R and W57R), sequences linked to either border were found to read through the LB or RB. In addition, Southern hybridization showed that transgenic plants W8 and W51 contained only one copy of the transgene *Xa21* (Table 2). Therefore, it was possible that, in both cases, the whole backbone vector was integrated into the rice genome.

Type C flanking sequences were more complicated. As in W75R and W52R, the flanking fragments outside the RB were the backbone sequence linked to LB in the vector. In W52L an unusual flanking sequence outside LB was found to be a segment of the exogenous gene *Xa21*. A more complicated sequence existed in W10L, which was 273 nt long and contained a 15-nt remnant of the LB. The sequence between its 57th nt and 162nd nt was a backbone fragment, but its two end segments (i.e. the segment between the 1st nt and 56th nt and the segment between the 163th nt and 273th nt) were not homologous to vector pCXK1301. Moreover, the whole fragment showed strong hybridization signals in Southern analysis probed with rice genomic DNA, indicating that both of its end segments originated from rice DNA. It seems that the Type C flanking sequences were long recombinant filler DNAs generated during the T-DNA integration at the junction of T-DNA and host genomic DNA or between tandem T-DNAs.

To study the inheritance of T-DNA-*Xa21* integration sites in some of the transformants, we analyzed the T-DNA flanking sequences from the different generations of $T_0$–$T_3$. No variations in the flanking sequences were observed through the generations, indicating that the T-DNA-*Xa21* integration sites were inherited with no changes. In addition, the same flanking sequence was obtained from transformants derived in the same transformation event, such as W3/W6/W71/W72, W45/W47/W49 and W106/W110, indicating that the flanking sequence analysis could also distinguish independent and non-independent transformants correctly.

## Mapping of T-DNA-*Xa21* loci on rice chromosomes

To determine the chromosomal locations of the T-DNA-*Xa21* loci, we carried out genetic mapping of T-DNA flanking rice sequences in a DH population derived from a cross between ZYQ8 and JX17. Fifteen T-DNA flanking rice sequences from independent *Xa21* transgenic lines were used as probes to survey their respective RFLPs between the two parents and then mapped onto rice chromosomes. For the T-DNA-*Xa21* loci from which both of the left and right flanking genome sequences could be obtained—such as W3aL and W3aR for the W3a locus and W10L and W10R for the W10 locus—both sequences

were used as probes to be located independently and were mapped onto the same chromosome site. In total, 12 T-DNA-*Xa21* loci were mapped on rice chromosomes 1, 3, 4, 5, 7, 9, 10, 11 and 12, separately, as shown in Table 2, where the two linked markers beside each integration site are also listed. This result showed that T-DNA- *Xa21* was integrated at different chromosome positions in rice.

In addition to RFLP mapping, T-DNA flanking rice sequences were also mapped by homology comparison with the rice chromosome-anchored sequences in the database. For example, W58L was anchored on the BAC clone OSJNBa0079B05 on rice chromosome 10. W10L and W10R matched with the sequences found on the same BAC clone—OSJNBb0038E06—on chromosome 3. In total, 11 T-DNA-*Xa21* loci were anchored on BAC sequences in the database (Table 2), which further confirmed the results of RFLP mapping.

## BB resistance effects of the transgene *Xa21*

On the basis of analysis and genetic mapping of T-DNA-*Xa21* loci, we investigated the influence of integration positions on the BB resistance of the transgene *Xa21*. Independent primary transformants ($T_0$) derived from two different indica varieties, *Minghui63* and *Zhenxian97B*, were measured for their resistance to Philippine *Xoo* race 6 (Table 3). We observed that different transformants with the same genetic background and transgene copy number displayed no obvious difference in resistance level despite the fact that the transgene had not been homozygous on the loci. For example, no difference in disease resistance could be found between *Minghui63* transformants W76 and W8, both with one copy of the transgene, and between W3 and W75, both with two copies of the transgene. This was also the case in *Zhenxian97B* transformants W60 and W51, both with one copy of the transgene, and W50 and W52, both with four copies of the transgene. These results indicate that the positions of the integration did not affect expression of the transgene *Xa21* in both varieties.

Subsequent investigation of the transgene position effect was carried out using homozygous transgenic lines with one copy of the transgene *Xa21* in their progenies. *Minghui63* transgenic lines with a single T-DNA-*Xa21* locus were selected from the $T_0$ to $T_3$ generations. We obtained six homozygous transgenic lines, W3a, W3b, W75a, W75b, W76 and W8, with the same genetic background as *Minghui63* from the $T_3$ generation. Southern hybridization as described by Zhai et al. (2001) (data not shown) verified that these lines possessed only one copy of the transgene located at different chromosome positions . These six transgenic lines were almost identical except for their different transgene loci and unknown small deletions or rearrangements. Therefore, they could be regarded as being near iso-transgenic (NIT) lines of *Minghui63* with respect to the transgene *Xa21*. BB resistance in these NIT lines would actually reflect the position effect of the transgene *Xa21*. The BB resistance level of the six NIT lines was investigated, and all had a lesion length of 2.0–2.3 cm (Table 4). This strongly indicated that there were no obvious differences in the resistance of the transgene *Xa21* among the six NIT lines.

We also observed that the BB resistance levels of transgenic plants with either one to two copies of transgene *Xa21* derived from *Minghui63* or with one to four copies of transgene *Xa21* derived from *Zhenxian97B* were almost the same within a given genetic background (Table 3). The resistance level of the transgenic rice had no tendency to increase with an increase in copy number. Obviously, dosage effect is not a main factor affecting expression of the transgene *Xa21*.

However, *Minghui63* transformants and *Zhenxian97B* transformants did show significant differences in resistance level (based on a *t*-test between the means of the two groups, $P<0.01$). The genetic backgrounds of the two varieties affected the resistance of the transgene *Xa21* more strongly than the transgene position and copy number. Differences in the resistance level of the transgene *Xa21* were also observed among the other six varieties, *Yanhui559*, *Peiai64*, C418, *Taihujing6*, 8706 and *Zhonghua11*. The genetic background effects of the transgene *Xa21* among the eight transgenic varieties were investigated through the $T_0$–$T_2$ generations, and all displayed

**Table 3** Resistance of independent *Xa21* transformants ($T_0$) in the *Minghui63* and *Zhenxian97B* genetic backgrounds to Philippine *Xoo* race 6

| Genetic background | Transformants | Hybridization fragment size of *Xa21* (kb)[a] | Copies or loci[a] | Lesion length (cm) |
|---|---|---|---|---|
| *Minghui63* | W76 | 9 | 1 | 2.4±0.2 |
| | W8 | 13 | 1 | 2.2±0.2 |
| | W3 | 6.8, 7.0 | 2 | 2.2±0.2 |
| | W75 | 8, 11 | 2 | 2.3±0.2 |
| | Non-transgenic control | | | 27.8±1.1 |
| *Zhenxian97B* | W60 | 8 | 1 | 1.0±0.2 |
| | W51 | 10 | 1 | 1.0±0.1 |
| | W57 | 9, 10 | 2 | 1.1±0.2 |
| | W53 | 7.5, 9.5, 15 | 3 | 1.0±0.1 |
| | W50 | 7.5, 9.5 (×2), 16 | 4 | 1.0±0.2 |
| | W52 | 7.5, 11, 15 (×2) | 4 | 1.0±0.1 |
| | Non-transgenic control | | | 15.6±1.1 |

[a]These were revealed through Southern analysis of *Hin*dIII-digested genomic DNA of the transformants using the 1.4-kb PCR fragment of the *Xa21* gene as a probe according to Zhai et al. (2001). The hybridization fragment size in W8 (or $M_1$) was estimated to be 11 kb by Zhai et al. (2001)

**Table 4** BB resistance of homozygous near-iso-transgenic lines (NIT) ($T_3$) of *Minghui63* to Philippine *Xoo* race 6

| NIT line | Hybridization fragment size of *Xa21*[a] (kb) | T-DNA-*Xa21* loci (linked markers on either side) | Lesion length[b] (cm) |
|---|---|---|---|
| W3a | 6.8 | Chromosome 9 (C10403, C313) | 2.1±0.2 |
| W3b | 7.0 | Chromosome 5 (R566, R569) | 2.0±0.2 |
| W75a | 8 | Chromosome 11 (G320, RZ638) | 2.2±0.2 |
| W75b | 11 | – | 2.2±0.2 |
| W76 | 9 | Chromosome 4 (C513, G271) | 2.1±0.1 |
| W8 | 13 | – | 2.3±0.2 |
| *Minghui63* (wild type) | | | 28.1±1.2 |
| IRBB21 (*Xa21* donor) | | Chromosome 11 (RG103) | 2.5±0.2 |

[a]These were revealed through Southern analysis of *Hin*dIII-digested genomic DNA of transformants using the 1.4-kb PCR fragment of the *Xa21* gene as a probe according to Zhai et al. (2001)
[b]Values are the average of at least ten $T_3$ plants, with five inoculated leaves on each plant for each NIT line

similar genetic background effects. The average lesion length of transgenic plants in the $T_1$ generation was 1.5–6.7 cm and obviously differed among the eight genetic backgrounds (Table 5). Moreover, the resistance value changed more significantly among the japonica varieties than among the indica varieties.

## Discussion

Until recently it has been assumed that during *Agrobacterium*-mediated plant transformation, only the sequence between the two T-DNA borders is transferred to the plant. However, evidence in the literature suggests that DNA sequences residing outside the T-DNA border may also occasionally be transferred to the plant (Ooms et al. 1982). Kononov et al. (1997) found backbone sequences in over 75% transgenic tobacco plants. De Buck et al. (2000) also detected vector sequences in 38–39% of the *Arabidopsis* and tobacco transformants. In rice, non-T-DNA sequences have been found in 33% and 45% of the transformants in two independent studies (Yin and Wang 2000; Vain et al. 2003). In the study reported here, 38% (9/24) of the sequenced flanking fragments or 6/16 independent rice transformants were found to have backbone sequences that were either linked to the T-DNA across the border sequence or independent of the T-DNA. Therefore, the frequent occurrence of vector backbone sequences in transgenic plants should be taken into consideration with respect to their commercial production.

Studies on transgenic plants obtained through particle bombardment and calcium phosphate have revealed that transgenes introduced by these direct gene transfer techniques have a preference to integrate with the repeated sequences and AT-rich regions bearing S/MAR motifs in plant genomes (Takano et al. 1997; Sawasaki et al. 1998; Shimizu et al. 2001). The T-DNA-*Xa21* flanking sequences identified in this study were mainly single copy in the rice genome and did not contain AT-rich regions or S/MAR-like structures. Moreover, the T-DNA-*Xa21* was mapped onto different chromosome sites in rice, which is similar to what has been observed in *Petunia*, *Lycopersicon* and *Arabidopsis* (Wallroth et al. 1986; Chyi et al. 1986; Liu et al. 1995). It seems that T-DNA integration is rather autonomous and has no preferred sites in the plant genome. In addition, we found the T-DNA LB sequence to be partially or completely deleted while the RB was more perfect in *Xa21* transgenic rice, which is consistent with early observations in dicots (Bakkeren et al. 1989; Kononov et al. 1997; Fladung 1999) and

**Table 5** BB resistance of the transgene *Xa21* in the genetic backgrounds of eight rice varieties to Philippine *Xoo* race 6 in $T_1$ generation

| Genetic backgrounds | Variety type | Lesion length of wild type (cm) | Lesion length of transgenic plants[a] (cm) |
|---|---|---|---|
| *Minghui63* | Indica | 29.1±1.2 | 3.2±0.6 |
| *Yanhui559* | Indica | 32.1±1.2 | 2.5±0.3 |
| *Zhenxian97B* | Indica | 17.1±1.1 | 1.9±0.4 |
| *Peiai64S* | Indica | 22.8±1.5 | 2.2±0.6 |
| C418 | Japonica | 25.8±1.8 | 6.7±1.4 |
| *Taihujing6* | Japonica | 17.4±1.1 | 1.5±0.3 |
| 8706 | Japonica | 23.9±2.2 | 3.7±1.3 |
| *Zhonghua11* | Japonica | 15.5±1.4 | 1.7±0.4 |
| IRBB21[b] (*Xa21* donor) | Indica | | 3.8±0.8 |
| IR24[b] (control) | Indica | 39.3±1.7 | |

[a]The average lesion length was obtained from more than 50 random resistant plants ($T_1$) of at least five independent transformants irrespective of their transgene copy numbers, loci and homozygous or heterozygous states
[b]IRBB21 and IR24 are near isogenic lines (Huang et al. 1999; Li et al. 2001)

indicating that T-DNA integration in monocots possibly shares the same mechanism with dicots.

Homology comparison could be an alternative and convenient approach to fine mapping of the T-DNA loci, particularly with the completion of the whole chromosome-anchored sequences in rice. It can also provide more sequence information near the integrations. However, it is impossible through this approach to explore the integration sites within unsequenced regions. RFLP mapping has no such limitation. In the present study, the consistent mapping result of integration sites obtained through both approaches indicates that they are complimentary and the results confirmed each other.

Unexpected variations in transgene expression have been often observed in many transgenic plants, including rice (Hobbs et al. 1990; Peach and Velton 1991; Maqbool and Christou, 1999; James et al. 2002). Compared with the transgene expression in these earlier studies, the resistance phenotype of the transgene *Xa21* was rather different in this study. There were no obvious transgene position and dosage effects on the BB resistance of the transgene *Xa21* in the same genetic background, which is consistent with recent results in transgenic plants obtained using pGreen/pSoup vectors (Vain et al. 2003). This is possibly because the transgene *Xa21* originated from the rice genome in which the gene is properly expressed and regulated under the own promoter. Moreover, the whole 9.9-kb transgene fragment contained large non-coding sequences in addition to the *Xa21* coding region, which could protect the transgene from the influence of many factors. However, changes in resistance expression or silencing of the transgene *Xa21* have been reported in some transgenic plants obtained through particle bombardment, which might be attributed to damage in transgenes during transformation (Wang et al. 1996; Tu et al. 1998; Zhang et al. 1998). It would appear that *Agrobacterium*-mediated transformation has an advantage over particle bombardment with respect to the stable resistance of the transgene *Xa21*.

In this study, we observed distinct differences in resistance among the eight genetic backgrounds. This is possibly caused by the interactions of the transgene *Xa21* with other genes, particularly unknown BB resistance genes. An increase in resistance has been reported in pyramiding lines with multiple resistance genes obtained through transformation or marker-based selection (Tu et al. 1998, 2000; Huang et al. 1999; Li et al. 2001; Datta et al. 2002). Our study provides more evidence that the genetic background should be taken into consideration in the application of *Xa21* transgenic plants.

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