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Site-independently integrated transgenes in the elite restorer rice line Minghui 63 allow removal of a selectable marker from the gene of interest by self-segregation

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Summary

In this study, we have demonstrated that two independent loci are involved in the integration of the insecticidal protein gene *cryIAb/cryIAc* and selectable marker gene *hph* in the recipient genome of the elite Chinese CMS restorer line Minghui 63. We have also documented the structural organization of these transgenes in each locus by restriction enzyme digestion and Southern blot analysis. The independent locus integration of different transgenes allowed us to remove the selectable marker gene *hph* from the gene of interest simply by self-segregation. Not having the selectable marker gene will enhance the commercial value of our transgenic line TT51-1, which showed a consistently high level of resistance against repeated infestations of yellow stem borers and natural outbreaks of leaf-folders, without a reduction in yield potential.

Introduction

The integration and function of the genes of interest play an important role in crop improvement. The production of a marker-free transgenic plant is highly desirable for its commercial applications. This is because the absence of marker genes removes the possibility of a transfer of antibiotic resistance genes to soil or gut bacteria or herbicide resistance genes to weedy relatives (Dale, 1992; Gresel, 1992) and reduces the likelihood for homologous sequence-dependent gene silencing when re-transforming transgenic plants to repeatedly stack transgenes using the same marker (Matzke and Matzke, 1991). A number of marker genes have been successfully used to introduce trait genes into rice, including *hph* (Datta *et al*., 1990), *nptII* (Peterhans *et al*., 1990), *ppt* (Datta *et al*., 1992), and *pmi* (Datta *et al*., 2003).

Recently, several systems have been developed to ensure the removal of selectable marker genes. These systems included intrachromosome recombination between attP regions from bacteriophage λ (Zubko *et al*., 2000), the site-specific recombination system R/RS from *Zygosaccharomyces rouxii* (Sugita *et al*., 1999), excision of selectable marker sequences via *cre/lox* recombination (Dale and Ow, 1991), altered metabolism (Negrotto *et al*., 2000; Wang *et al*., 2000), co-transformation with one *Agrobacterium tumefaciens* strain containing two binary plasmids for the nucleus genome transformation (Daley *et al*., 1998), and a plastid DNA recombination and cytoplasmic sorting system mediated by two or three short direct repeats for plastid transformation (Iamtham and Day, 2000). The development of new potentially less hazardous marker genes provides an alternative approach for excluding antibiotic and herbicide resistant markers from plants (Negrotto *et al*., 2000; Wang *et al*., 2000). However, none of these systems has been applied to a commercial crop cultivar, and there is an urgent need to demonstrate the utility of those technologies in developing the next generation of transgenic crops lacking selectable marker genes. On the other hand, the transgenic integration structure and molecular inheritance analysis are an essential step for determining whether the selectable marker gene is independently

integrated from the gene of interest. When a marker gene and a gene of interest integrate at distant genetic loci, meiosis ensures the segregation of these genes away from each other amongst the progeny. Moreover, this detailed molecular analysis is helpful for understanding the stable expression and functional features of the transgenes in the recipient genome. These stable expression and functional features of transgenes might be either transgenic integration structure-dependent or integration site-preferred (Iglesias *et al*., 1997; Fladung, 1999).

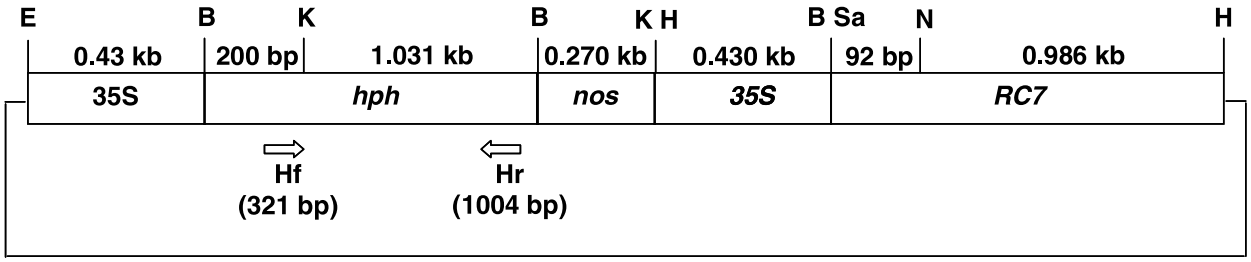
Based on these considerations, we designed a co-transformation procedure for introducing a fused *Bt* insecticidal protein gene (Tu *et al*., 1998a) and a separate plasmid that contains the selectable marker gene *hph* and a chitinase gene *RC7* (Datta *et al*., 1990, 2001) cloned from rice into the elite Chinese CMS restorer line Minghui 63 using a biolistic method (Datta *et al*., 1998; Tu *et al*., 2000). The transgenic line (TT51-1) developed afterwards, and its derived hybrid plants have been field-tested and showed excellent protection against stem borers without a reduction in yield (Tu *et al*., 2000). Here, we describe the integration patterns of transgenes at two unlinked genetic loci that allow the removal of the selectable marker gene from the gene of interest.

Results

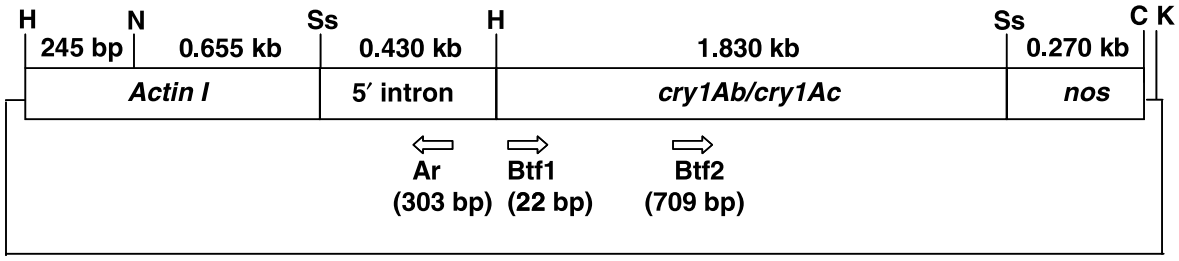
Rice transformation and molecular analysis of transgenic plants

For the co-transformation, the plasmid pFHBT1 that carries the fused *Bt* insecticidal protein gene made from *cry1Ab* and *cry1Ac* (Figure 1a) was mixed in a 4 : 1 ratio with the plasmid pGL2RC7 that carries the chitinase gene *RC7* cloned from rice and the selectable marker gene *hph* (Figure 1b). The DNAs were delivered into 980 freshly isolated immature embryos of the elite Chinese CMS restorer line Minghui 63 by the biolistic method, involving 11 bombardments. After transformation, 35 green plantlets were obtained, and 32 of them were fertile.

Co-transformation events in the T_0 generation were primarily confirmed by Southern blot analysis with a *Bt* probe. As a result, 3 of 32 fertile plants from which the field-tested homozygous line was developed showed the same band pattern and presence of the expected 1.8 kb *Hin*dIII-*Sst*1 fragment corresponding to the intact coding sequence of the *Bt* insecticidal protein gene (Figure 2a). These results demonstrate that these three positive plants were regenerated from the same transformation event. Apart from this, a smaller



(a) pGL2RC7 (5.966 kb)



(b) PFHBT1 (6.200 kb)

Figure 1 Diagram of plasmid constructs used in this study. (a). Structure of pGL2RC7, which contains a hygromycin resistance gene *hph* and a rice chitinase gene *RC7*. Both genes are under control of the CaMV35S promoter. The hygromycin resistance gene was used as a selectable marker during the transformation. (b). Structure of pFHBT1, which contains a fused *cry1Ab/cry1Ac* insecticidal protein gene under control of the rice *Actin*I promoter with its first intron and *nos* terminator. The coding sequences of both genes cut by corresponding restriction enzymes on the diagram of each construct were used as the specific probes for the Southern blot analysis. The white arrows under each construct indicate the locations of designated primers that specifically amplify the expected size of fragments from the *hph* coding sequence or junction part between the inserted transgenes. B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; N, *Nru*I; Sa, *Sac*I; Ss, *Sst*I.

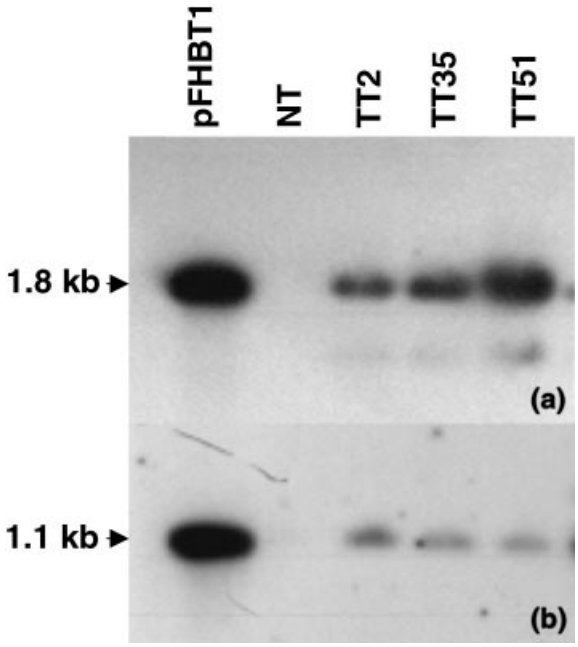


Figure 2 Southern blot analysis of T_0 transgenic plants. A total of 5 μg of plant genomic DNA and 30 pg of plasmid DNA were digested with the indicated coding sequence cutters for each gene and electrophoresed on 1% (w/v) agarose gels. The blots were then hybridized with (a) a *Bt*-specific probe and (b) an *hph*-specific probe. The arrows mark the expected 1.8 kb fragment-digested *Bt* plasmid DNA of pFHBT1 and 1.1 kb digested *hph* plasmid DNA of pGL2RC7. NT: non-transformed Minghui 63 control plant; TT2, TT35 and TT51: three T_0 transgenic plants.

than 1.8 kb band in all samples was also detected, indicating the presence of a deletion-type rearranged copy of the *Bt* transgene in the recipient genome. Similarly, the presence of the unique size of the 1.1 kb *Bam*HI fragment corresponding to the intact coding sequence of the *hph* gene in these three plants was also confirmed by Southern blot analysis with an *hph*-specific probe (Figure 2b). Additional data showing the same junction fragments digested with other restriction enzymes supported this clonal regeneration (data not shown). These results demonstrated that both the fused *Bt* gene and the selectable marker gene *hph* had been integrated into the genome of the restorer line Minghui 63. Further molecular analysis of the T_1 plants of TT51 showed a segregation band pattern of *Bt* transgenes. Fifteen out of 32 progeny plants screened by blot analysis are shown in Figure 3. Out of 32 plants detected, 17 possessed two loci with four bands, 6 possessed one locus with two upper bands, 7 possessed another locus with two bottom bands, and 2 did not carry any locus. The χ^2-test reveals that the segregation of these two loci fit a 9 : 3 : 3 : 1 ratio with a probability of more than 0.95. Therefore, further molecular analysis concentrated on this particular transformant, represented by TT51.

Integrative structure of *Bt* transgenes

The integration patterns of *Bt* transgenes in the recipient genome of the TT51 transformant were analysed by a restriction enzyme digestion of total DNA. The enzymes used in the experiments were the coding sequence cutters *Hin*dIII and *Sst*I, the plasmid unique cutters *Kpn*I and *Nru*I, the terminator end-associated cutter *Cla*I, and the plasmid non-cutter *Pac*I. Among them, *Pac*I is a cutting enzyme rare to the rice genome with a recognition site of 8 bp, whereas the rest is a common enzyme with a recognition site of 6 bp. The cleavage site of each enzyme is indicated on the plasmid vector diagram (Figure 1a,b). A putative model structural organization of transgenes is shown in Figure 4, which is well supported by patterns of transgenes integration, Southern, PCR, and restriction analysis (Figures 5–9). After digestion, both *Kpn*I and *Nru*I release four bands from their digested TT51 genome on the Southern blot, representing four copies of the *Bt* transgenes (Figure 5a). The two larger sizes of bands released by *Nru*I, however, are not distinguishable from each other because their sizes are more than 23 kb and the normal 1% agarose gel could not separate them properly. When the plasmid non-cutter *Pac*I was used to digest the genome samples of TT51, two bands of 48 and 50 kb were released and

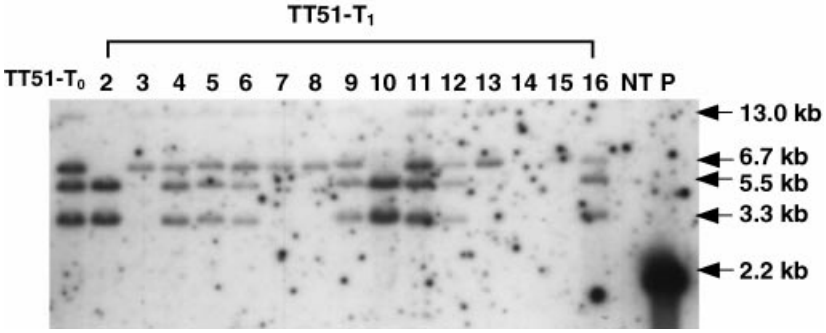


Figure 3 Southern blot analysis of the segregation of *Bt* transgenic loci in the T_1 plant of TT51. The genomic DNAs from 32 plants were digested with nucleus restriction enzyme *Hin*dIII and probed with the coding region of *cry1Ab/cry1Ac*. Data is only shown for 16 plants. Lane 1: TT51 T_0 plant; lanes 2–16: TT51 T_1 plants; NT: non-transformed control plant; P: plasmid DNA. Segregants 4, 5, 6, 9, 11, 12 and 16 contain transgenes at two loci whilst segregants 2 and 10 contain transgenes at locus 1 and segregants 3, 7, 8, 13 and 15 belong to different group with the transgene at the 2nd locus, segregant 14 lacks transgenes at both loci.

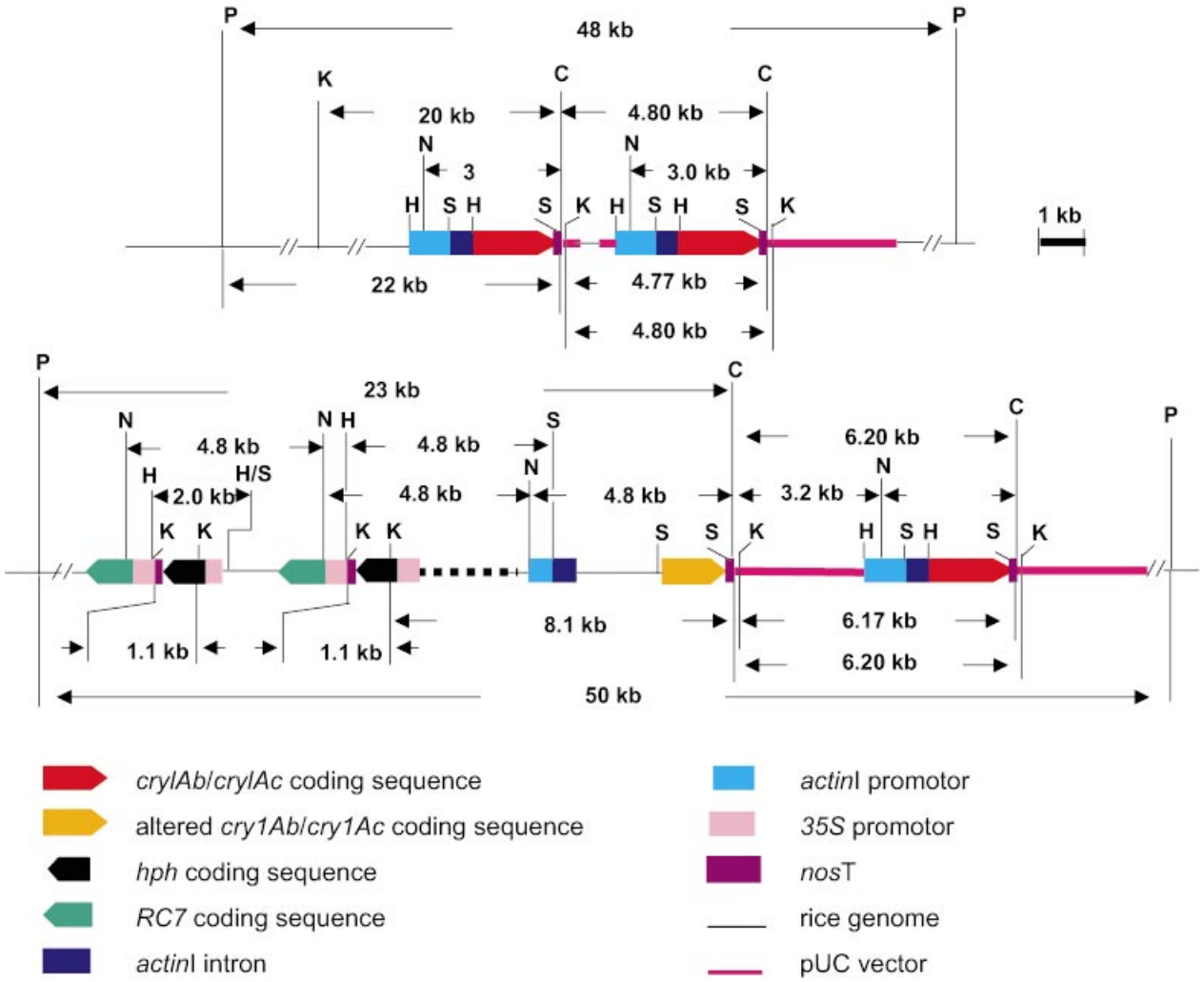


Figure 4 A model putative structural organization of the transgenes in the recipient genome.

confirmed by pulse-field electrophoresis (Figure 5b). From this configuration of the structural organization of both genes, a *Kpn*I cleavage site in the adjacent *hph*-containing insert DNA sequence (Figure 4) can explain the resulting length of the 8.1-kb fragment that contains the rearranged copy of the *Bt* transgene and is present in the TT51 *Kpn*I digestion lanes (Figure 5). The *Sst*I site at 0.65 kb downstream from the *Nru*I site in the rearranged copy at the *Bt* transgenic locus (Figure 4) can explain the resulting length of the 4.8 kb fragment that contains the *hph* gene and is present in the TT51 *Hin*dIII/*Sst*I double digestion lane (Figure 6b). On the other hand, the presence of a 4.8 kb fragment in the TT51 *Hin*dIII/*Sst*I double digestion lane (Figure 6b) indicated that a *Hin*dIII site at 0.245 kb upstream from the *Nru*I site (Figure 1b) according to the *actin*I coding sequence was lost during the rearrangement of a copy of the *Bt* transgene. From the configuration, it is also clear that, after removal of the *hph* transgenes together with their linked *Bt* transgenic loci, only two intact copies of the *Bt* transgenes remained in the recipient genome of TT51-1 (Figure 4) and can adequately protect the plants against attack from insect pests (Figure 10).

The combinations of the enzymes used above with terminator end-associated cutter *Cla*I were applied to the genome digestion of the same samples to further detect the integration patterns of the *Bt* transgenes in the recipient genome. Plasmid sequence data revealed that both the *Cla*I and *Kpn*I cutting sites are located at the 3′ end of the *cry1Ab*/*cry1Ac* coding sequence with 30 bp between them. After a double digestion of TT51 genome DNA with this enzyme pair, the expected reduction of 30 bp length for each *Kpn*I-released band corresponding to each copy of the transgenes was observed on the Southern blot (Figure 5a).

The *Nru*I site is 3.0 kb upstream from the *Cla*I site. This interval covers the downstream part (655 bp) of the *actin*I promoter plus its 5′ intron, plus the coding sequence of

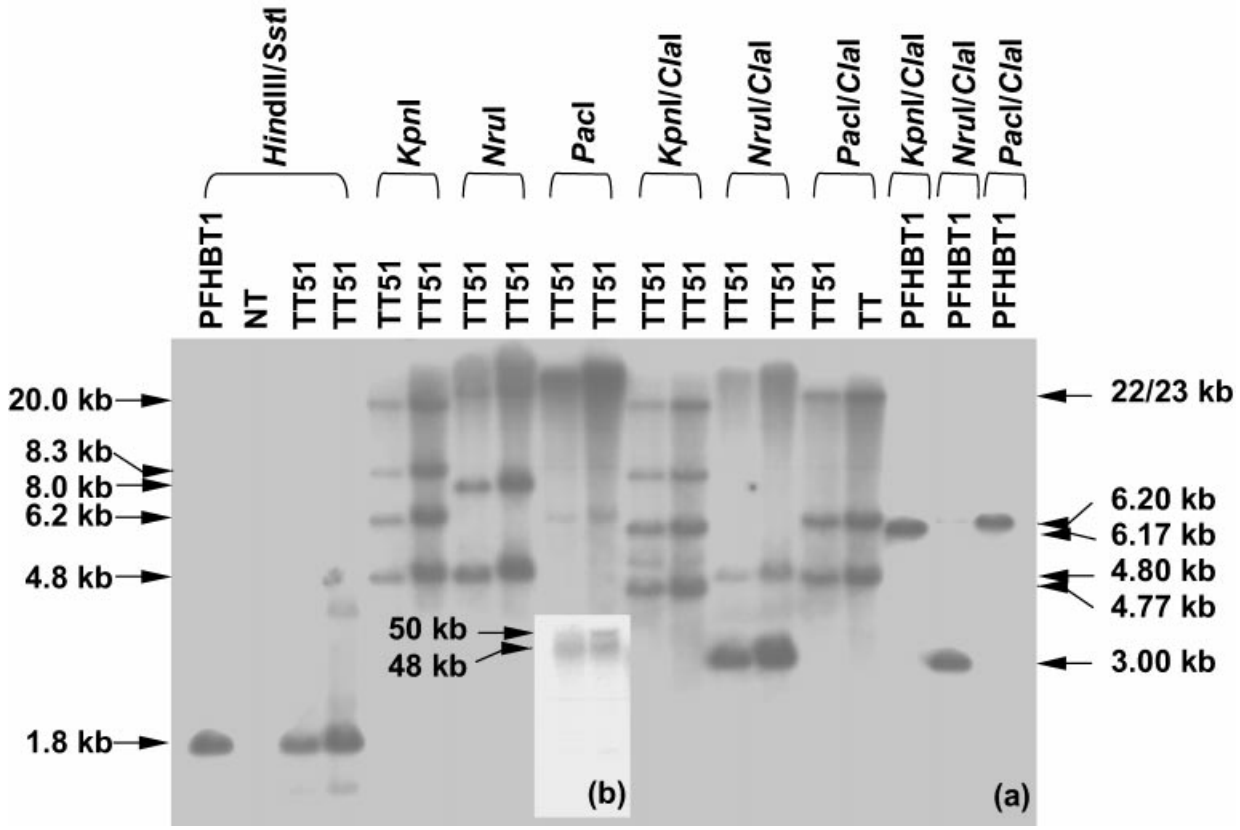


Figure 5 Southern blot analysis of the integration pattern of *Bt* transgenes in the genome of the primary transgenic plant TT51. (a). The genomic DNAs (5 μg) were digested with *Bt* coding sequence cutter *Hin*dIII/*Sst*I, the plasmid unique cutters *Kpn*I and *Nru*I, the plasmid non-cutter *Pac*I, and *Kpn*I, *Nru*I, and *Pac*I combined with *Cla*I and probed with the coding region of *cry1Ab*/*cry1Ac*. The plasmid DNAs (30 pg) digested with the above three enzyme pairs were used for positive and size control. NT: non-transformed control plant. (b). The *Pac*I digestion released two large fragments of 48 and 50 kb that were separated by pulsed field gradient gel electrophoresis (PFGE). Arrows mark each digested fragment.

cry1Ab/*cry1Ac* plus the *nos* terminator. After a double digestion of the TT51 genome DNA with this enzyme pair, an expected 3.0 kb darker band, reflecting the intact integration, and a weak 4.8 kb band, representing the insertion, resulting in a rearranged copy, were observed (Figure 5a). Further analysis revealed that the 4.8 and 6.2 kb sizes of two of these four *Pac*I/*Cla*I-digested fragments were exactly the same as those of two of the *Kpn*I-digested fragments (Figure 5a). These results imply that the integration of these four fragments of *Bt* transgenes in the recipient genome was two and two joined together in a head-to-tail concatemer manner first, and then these two transgenic concatemers interspersed into two separate loci. Otherwise, different enzymes could not release the exact same sizes of digested transgenic fragments. Moreover, the two *Kpn*I-digested fragments of 4.8 and 6.2 kb could not be equally shortened by 30 bp in length after further digestion with *Kpn*I/*Cla*I (Figure 5a). This provides further evidence which excludes three or more copies of the gene of interest in the 4.8 kb *Pac*I band.

Segregation patterns of *Bt* transgenes

To determine how the *Bt* transgenes in the different integration sites in the recipient genome of the field-tested homozygous line TT51-1 developed from the TT51 transformant and its derived hybrid, the DNA samples isolated from them were added for further digestion analysis. The original TT51 genome DNA samples were used as a control. After digestion and hybridization with a *Bt*-specific probe, two bands from either *Kpn*I or *Kpn*I/*Cla*I digestion lanes and one band from the *Pac*I/*Cla*I digestion lane were missing from this homozygous line and its derived hybrid DNA samples when compared with the digested TT51 DNA samples (Figure 6a). These results demonstrated that the two remaining fragments of 20 and 4.8 kb in the *Kpn*I digestion lane, or 20 and 4.77 kb in the *Kpn*I/*Cla*I digestion lane of the TT51 genome samples, joined together and integrated in a locus, whereas the two missing fragments of 8.3 and 6.17 kb in the *Kpn*I digestion lane or 8.1 and 6.0 kb in the *Kpn*I/*Cla*I digestion lane of the TT51 genome samples joined together and

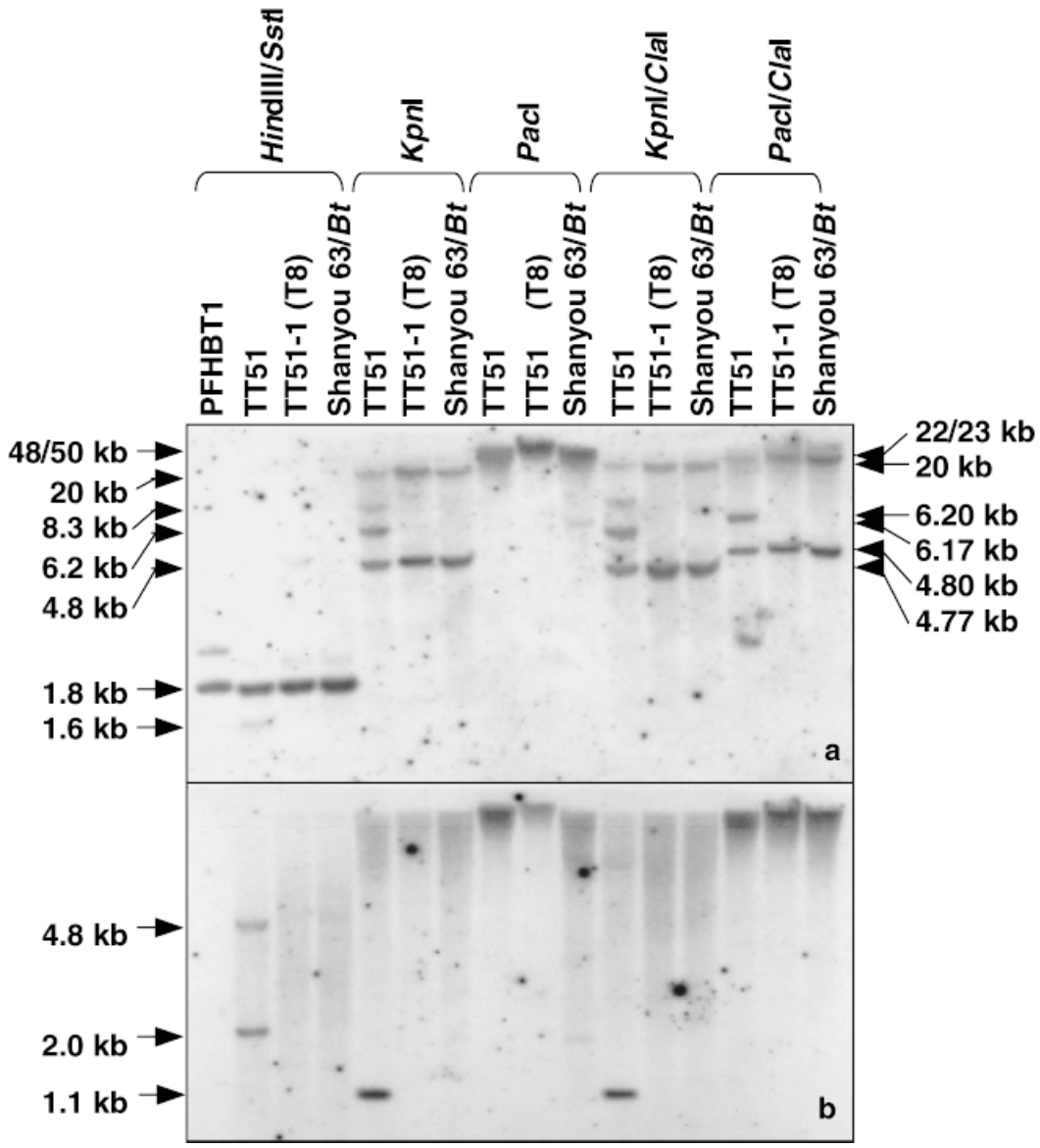


Figure 6 Southern blot comparison of the integration pattern of *Bt* transgenes in the primary transgenic plant TT51, the derived line TT51-1 (T8), and its hybrid Shanyou 63, confirming removal of the selectable marker gene *hph* from the gene of interest. (a) hybridized with *Bt*-specific probe; (b) hybridized with *hph* probe.

integrated in a separate locus. This result was consistent with a previous molecular genetic analysis (Figure 3) that showed an independent integration of the two loci.

In addition, the rearranged fragment of less than 1.8 kb and the weak hybridization signal appeared in the TT51 genome samples but disappeared from the homozygous line TT51-1 and its derived hybrid after digestion with the *Bt* coding sequence cutter *Hin*dIII/*Sst*I and detection by a *Bt*-specific probe. These results provide further evidence that the rearranged copy of the *Bt* transgene is joined to the intact-copy-containing fragment of 6.2 kb released by either *Kpn*I or *Pac*I/*Cla*I.

The head-to-tail joined concatemer of two intact copies of *Bt* transgenes was also confirmed by PCR analysis using two forward primers from the *cry1Ab*/*cry1Ac* coding sequence pairing with the same reverse primer from the *actin*I coding sequence (Figure 8). The amplified junction fragments in the locus that contains the intact copies of *Bt* genes have the expected size of 3.8 and 4.5 kb deduced from the digestion analysis.

Removal of the selectable marker gene from the gene of interest

The results demonstrated that the four copies of *Bt* transgenes are two and two joined together and integrated in the two separate loci in the recipient genome of TT51. Assuming that the selectable marker gene is a single-site integration

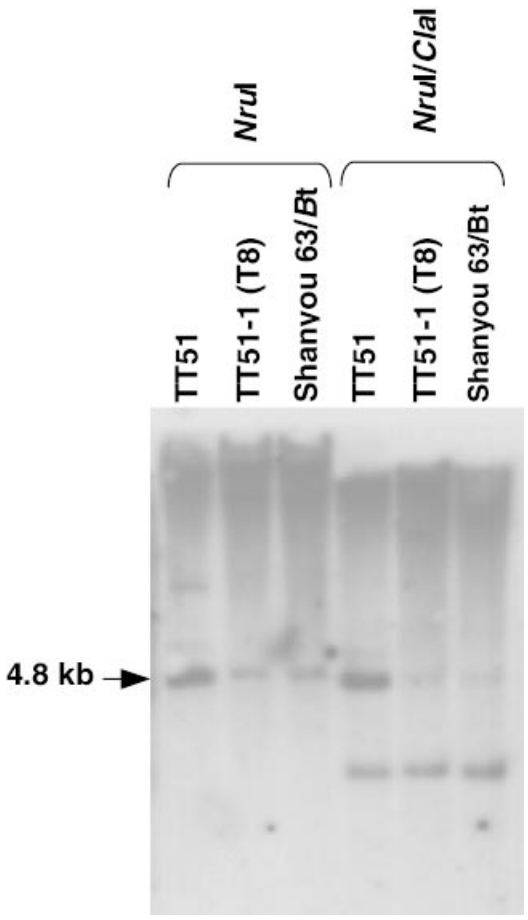


Figure 7 Southern blot confirmation of *Nru*I-digested *hph*-containing fragments of 4.8 kb. The plant genomic DNAs were digested with *Nru*I and *Nru*I/*Cla*I and first probed with the coding region of *cry1Ab/cry1Ac*. Without stripping off the *Bt* probe, the same blot was re-hybridized with the coding region of *hph*. After exposure to X-ray film, the expected strong signals appeared in the TT51 digestion lanes but not in the other digestion lanes.

and that this site is linked with one of the transgenic loci of the *Bt* gene mentioned above, it should be able to segregate-out together with the linked *Bt* locus from the segregating population. The segregants in which the integration site of the selectable marker gene has been segregated-out could then be identified by molecular analysis. Based on these deductions, the primer pairs Hf and Hr that specifically amplify a 0.68 kb fragment starting at 321 bp downstream from the start codon and ending at 1004 bp upstream from the stop codon of the *hph* coding sequence (Baisakh *et al*., 2000) (Figure 1a) were used to screen individual plants selected from different generations. The data shown in Figure 9 reveal that a DNA fragment with the expected size was amplified from the positive control of plasmid, from the primary transgenic plants, from two of the three T1 plants (TT51-4 and 5), and from the TT51-1 line homozygous for the *Bt* transgenic locus and its derived hybrid plant. The same *Bt* Southern blot used for integrative structure analysis (Figure 6a) re-hybridized with the *hph*-specific probe also confirmed these results. For instance, the positive band/s were observed in the TT51 digestion lanes but not in TT51-1 and its derived hybrid digestion lanes when *Kpn*I, *Kpn*I/*Cla*I and *Hin*dIII/*Sst*I were used (Figure 6b). These results thus proved the above assumptions that the *hph* transgenes are linked to a *Bt* transgenic locus that accommodates a rearranged copy and an intact copy of the *Bt* transgenes.

The integrative structure of *Bt* and *hph* transgenes

Hybridizing with the *hph*-specific probe, the TT51 genome DNA sample digested with the enzyme pair *Hin*dIII/*Sst*I releases two bands of 4.8 and 2.0 kb, respectively (Figure 6b). Considering that the cleave sites of both *Hin*dIII and *Sst*I are

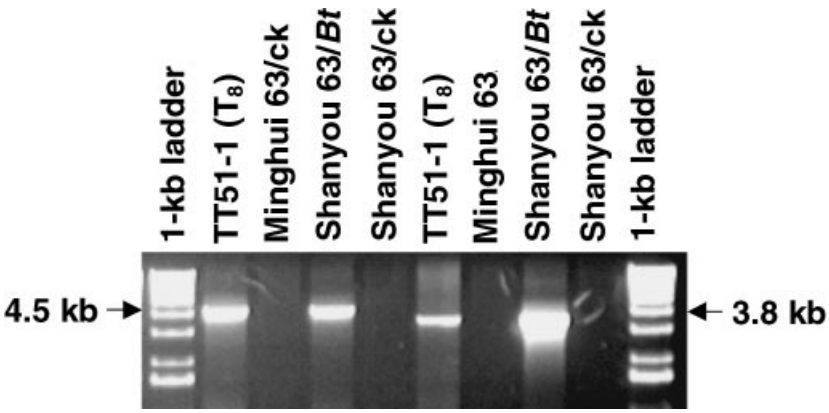


Figure 8 (Lanes according to the primers used.) PCR amplification of junction fragments between two intact copies of *cry1Ab/cry1Ac* at one integration site that contains only *Bt* transgenes. Two expected sizes of 4.5 and 3.8 kb fragments were observed. The 4.5 kb fragment was amplified by the pair of *Bt* forward primers starting at the 22nd bp and *actin*I reverse primer starting at the 1203rd bp, whereas the 3.8 kb fragment was amplified by the pair of *Bt* forward primers starting at the 709th bp and the same *actin*I reverse primer. TT51-1 (T8): the eighth generation of the transgenic line TT51-1; Minghui 63: the non-transgenic control; Shanyou 63/*Bt*: the *Bt* hybrid derived from the cross of TT51-1 with CMS line Zhenshang 97; Shanyou 63: the hybrid control. The arrows mark the expected sizes of two amplified fragments.

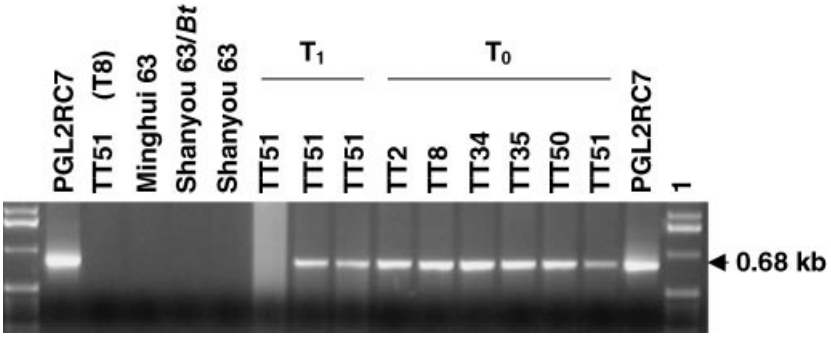


Figure 9 PCR analysis of *hph* transgenes in different generations. TT51-1 (T8): the eighth generation of the transgenic line TT51-1; Minghui 63: the non-transgenic control; Shanyou 63/*Bt*: the *Bt* hybrid derived from the cross of TT51-1 with CMS line Zhenshan 97; Shanyou 63: the non-transgenic hybrid control; TT51-1, 4, 5: three T1 plants of TT51; TT2, TT8, TT34, TT35, TT50, TT51: the primary transgenic plants; pGL2RC7: the plasmid that contains the *hph* and chitinase genes and that was used for the co-transformation with *Bt* plasmid in the experiment.

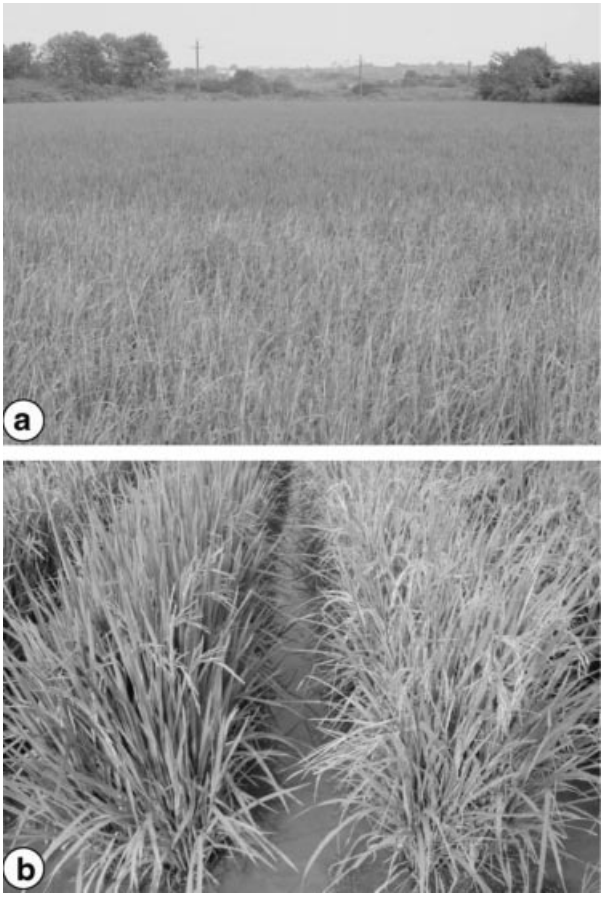


Figure 10 Field reactions of transgenic restorer line against stem borers. (a) Resistance reaction of restorer line against leaf-folders: Minghui 63/*Bt* plants (top) and Minghui 63 control plants (bottom); (b) Resistance reaction of restorer line against yellow stem borers: Minghui 63/*Bt* plants (left) and Minghui 63 control plants (right).

outside of the *hph* coding sequence, the two bands observed above represent the two intact copies of *hph* transgenes.

*Kpn*I cut a 1.1 kb fragment that contains the intact *hph* coding sequence from the plasmid pGL2RC7. After digestion with *Kpn*I and the enzyme pair *Kpn*I/*Cla*I, the band with the expected size of 1.1 kb cut from two copies of the integrated transgenes was observed in the TT51 digestion lanes (Figure 6b). These results further demonstrate the intact integration of the *hph* gene in the recipient genome.

The *Nru*I, however, is a unique enzyme to the plasmid pGL2RC7, and its cleavage site is located 92 bp from the start codon of the *RC7* coding sequence. *Nru*I in combination with *Cla*I was used to digest the genomic DNA of TT51, resulting in the release of the hybridized signals detected by the *hph*-specific probe appearing in the same position of 4.8 kb on the Southern blot (Figure 7). These results indicate that the integrated exogenous DNA fragments containing two copies of the intact *hph* transgenes were cut into the same size by three *Nru*I sites. Two of these three *Nru*I sites are already known from each of two integrated *hph*-containing exogenous DNA fragments (Figure 1a). Another *Nru*I site comes from the rearranged copy of the *Bt* transgenes at an adjacent integration locus. This adjacent integration of the *hph* transgenic locus to the *Bt* transgenic locus was supported by the observation that the selectable marker gene is removed together with its linked *Bt* transgenic locus, as previously mentioned (Figure 6a,b). In addition, the presence of the *hph* transgene in each resulting *Nru*I fragment (Figure 7) predicted that the two integrated *hph*-containing exogenous DNA fragments also join in a head-to-tail concatemer manner, and the head of this concatemer joins to the head of the rearranged copy at the adjacent *Bt* transgenic locus. On the other hand, because the two *Nru*I sites reside in each of two *hph*-containing exogenous DNA sequences that release a 4.8 kb band, this implies that a filler DNA of about 1 kb in length lies in between these two integrated exogenous DNAs. At the same time, this filler DNA brings a *Hin*dIII or *Sst*I site to pair with another *Hin*dIII site at the inserted exogenous DNA sequence to cut down the observed 2.0 kb band that contains an intact copy of the *hph* transgene from the TT51 genome DNA sample.

Performance of agronomic traits

The marker-free transgenic restorer line, TT51, and its-derived hybrid, *Bt*-Shanyou 63, have been repeatedly field-tested and

demonstrated an insect resistance (Figure 10) consistent with previous results (Tu *et al*., 2000), thus indicating that their stable phenotype resulted from their stable genotype. The average yields of this line and the hybrid plus *Bt*-Eryou 63 made from Er A/*Bt*-Minghui 63 (TT51), *Bt*-Xieyou 63 from Xieqingzao A/*Bt*-Minghui63 (TT51), and *Bt*-Mayou 63 from Maxie A/*Bt*-Minghui63 (TT51) in a field trial were 10.29–11.35 tons/ha, 21–65% more than that from the non-transgenic controls Minghui 63 and Shanyou 63 under treatment with chemical spray, and 9.30–12.8 tons/ha, 140–250% more than that from non-transgenic controls under treatment without chemical spray. The average yields in both cases are significantly different from those of controls at $P < 0.01$.

Discussion

In the present study we have demonstrated the integration of the insecticidal protein CryIAb/CryIAc and selectable marker gene *hph* at two unlinked genetic loci in the elite Chinese CMS restorer line Minghui 63. In addition, we have also determined the structural organization of these transgenes in each locus by restriction enzyme digestion and Southern hybridization analysis. The independent locus integration of both genes allowed us to remove the selectable marker gene *hph* from the gene of interest simply by self-segregation. A linearized minimal expression vector without a bacterial marker (ampicillin) gene is now being used and could be employed for future generations of product development. Not having the selectable marker gene will enhance the commercial value of our transgenic line TT51-1, which showed a consistent high level of resistance against a heavy repeated infestation of yellow stem borers and natural outbreaks of leaf-folders, without the reduction in yield potential which was reported previously (Tu *et al*., 2000), and with the data presented above.

Kohli *et al*. (1995) showed that multiple integration events occurring as a cluster gave rise to a single transgenic locus in all the rice lines they analysed. Our results show that two transgenes integrated into the two independent loci facilitated the removal of the selectable marker genes. In fact, several previous reports have suggested that the transgenes in the plants resulting from *Agrobacterium*-mediated transformation were integrated in single to multiple loci (Daley *et al*., 1998, 2000; De Block and Debrouwer, 1991; Komari *et al*., 1996; McKnight *et al*., 1987; Miller *et al*., 2002; Xing *et al*., 2000). Cooley *et al*. (1995) observed two unlinked transgenic loci in the recipient rice genome, although this event occurred at a very low frequency in their experiment. Besides the present report, we also observed several co-transformed transgenic lines, such as IR72 with *Bt*, Tulsi with *Chi11* bearing the separate locus integration of transgenes (unpublished data). From our studies it is clear that a biolistic method can introduce more copies of the transgenes and can integrate at distant loci allowing the isolation of marker-free plants. In addition, it is worthwhile noting that a mixture of the genes of interest with the selectable marker gene at a 4 : 1 molar ratio of gene of interest to selectable marker gene is important for enhancing the frequency of co-transformation and increasing the probability of excluding the marker gene from the gene of interest at secondary integration sites.

To our knowledge, this is the first report of a commercially valuable marker-free transgenic line in any cereal crop.

Experimental procedures

Plasmid vector

The two plasmid DNAs used for rice transformation are shown in Figure 1. Plasmid pFHBT1 contains 1830 bp of a fused *crylAb/crylAc* (Tu *et al*., 1998a) under control of the *actin*I promoter (McElroy *et al*., 1990), *actin*I 5′ intron, and *nos* terminator. Plasmid pGL2RC7 contains both 1031 bp of the selectable marker gene *hph* (Blochinger and Diggelmann, 1984; Datta *et al*., 1990) and 1078 bp of the rice chitinase gene *RC7* under control of the CaMV35S promoter and polyA terminator (Datta *et al*., 2001).

Plant transformation

The plant material used in the present study was Chinese cytoplasmic male sterile (CMS) restorer line Minghui 63. DNA preparation, isolation, preparation of immature embryos, particle bombardment and plant regeneration were performed as previously described (Datta *et al*., 1998; Tu *et al*., 1998a,b). Putative transgenic plants obtained then underwent to Southern blot analysis to confirm the stable integration of the transgenes in the T_0 generation followed by restriction digestion analysis to identify the integrative structure or organization of the transgenes in the recipient genome. The transgenic plants that showed segregation of the transgenic loci were further used to scan for the absence of the selectable marker gene. Then, the selected marker-free transgenic line (TT51-1) with improved fertility after confirming the resistance phenotype by stem-cutting assay was used to cross with different CMS lines including Zhenshan 97, Er A, Xieqingzao A and Maxie A to produce the hybrids. Then, this selected marker-free transgenic line and its derived hybrids were used for a field test. The plot design and insect

infestation method were the same as in an earlier report (Tu *et al*., 2000).

Southern blot analysis

Genomic DNA was extracted from leaf tissue harvested from primary transgenic plants and their progeny by an improved CTAB method based on the procedure described by Murray and Thompson (1980). Five micrograms of DNA of each sample, estimated by fluorometry after treatment with RNaseA, was digested with different restriction endonuclease (Gibco-BRL, Gaithersburg, MD) in a final volume of 50 mL. The digested DNA was electrophoresed on 0.8% or 1% (w/v) agarose gels. After electrophoresis, DNA fragments were denatured and transferred on to a hybond-N^+ nylon membrane (Amersham, Arlington Heights, IL) according to the manufacturer's instructions. The coding region of both the *hph* and *Bt* genes from plasmid digested with the corresponding enzyme was used as a hybridization probe after being labelled with (α-^{32}P) dCTP using a Rediprime Labeling Kit (Amersham, Arlington Heights, IL). The large DNA fragments resulting from *Pac*I digestion were separated by PFGE (Schwartz and Cantor, 1984) at 6 V/cm on a 1% agarose gel in 0.5 × TBE at 14 °C with a switching time of 5–15 s for 12 h.

PCR amplification

PCR analysis of *hph* transgenes (short fragments) was conducted using a PCR Core Kit (Roche Diagnostics GmbH, Indianapolis, IN, USA) based on the procedures described by Huang *et al*. (1997). The primers used for this analysis were 5′ primer (Hf), 5′-TACTTCACACAGCCATC-3′ and 3′ primer (Hr), 5′-TATGTCCTGCGGGTAAAT-3′ for the amplification of a 680 bp fragment from the *hph* gene. The PCR mixture contained 100 ng of template DNA, 50 ng of each primer, 0.16 mM dNTP, 2.1 mM $MgCl_2$, 1× PCR buffer (10 mM Tris, pH 8.4, 50 mM KCl), and 1 unit of Taq DNA polymerase in a volume of 25 μL. Template DNA was initially denatured at 94 °C for 5 min followed by 35 cycles of PCR amplification with the following parameters: 30 s denaturation at 94 °C, 30 s primer annealing at 60 °C, and 1 min primer extension at 72 °C, which allowed for a completion of primer extension. The PCR amplification of junction contigs (long fragments) was conducted using Elongase Enzyme Mix according to the procedures described by the manufacturer. The primers used for this analysis were 3′ primer (Ar) from the coding sequence of *actin*I, 5′-CGCACGAGGCTGCATTTGTCA-3′, 5′ primers (Btf1 and Btf2) from the coding sequence of *cry1Ab/cry1Ac*, 5′-ACAACTGCTTGAGTAACCCAGA, and 5′-ACATTGTGTCTCTCTTCCCGAA. The PCR mixture contained 100 ng of template DNA, 200 nM of each primer, 200 μM of each dNTP, 60 mM Tris-SO_4 (pH 9.1), 18 mM $(NH_4)_2SO_4$ with 1.8 mM $MgSO_4$, and 1 μL of Taq DNA polymerase in a volume of 50 μL. Template DNA was initially denatured at 94 °C for 30 s followed by 35 cycles of PCR amplification with the following parameters: 30 s of denaturation at 94 °C, 30 s of primer annealing at 60 °C, and 3 min of primer extension at 68 °C, which allowed for a completion of the primer extension. The amplified products were electrophoretically identified on a 0.8–1% agarose gel in 1× TAE buffer.

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