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# Characterization of the Transgenic Rice Event TT51−1 and Construction of a Reference Plasmid

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**ABSTRACT:** Transgenic rice TT51−1 (BT63) is an insect resistant strain that was granted for safety certificate in China in 2009. This study characterizes the transgenic event TT51−1 using a GenomeWalker strategy. The organization of the transgenes indicated that the transgenes on two plasmids, pFHBT1 and pGL2RC7, had been integrated at the same locus. The sequence of the event TT51−1 spanned 8725 bp, including a truncated Cry1Ab/Ac cassette, an intact Cry1Ab/Ac cassette, two Amp gene segments, and an Hph gene segment. The 5′ and 3′ plant flanking sequences were isolated and used to locate the transgenes to chromosome 10 in TT51−1. The isolated TT51−1 fragment and a fragment of the rice PLD gene were integrated into a plasmid vector, to create plasmid pK-TT51 as a calibrator for detecting rice containing TT51−1. Analysis of unknown samples indicated that the reference plasmid was a reliable alternative to TT51−1 genomic DNA.

**KEYWORDS:** TT51−1, characterization, rearrangement, reference plasmid, calibrator

## INTRODUCTION

Between 1996 and 2008, the global planting area of genetically modified (GM) crops increased 74-fold, reaching 125 million hectares.[1] The commercial planting of GM crops contributes to global food, feed, fiber, and fuel security. Rice (*Oryza sativa*) is one of the most important food crops worldwide; most people from developing countries depend on rice as their staple food.[2] During the past 20 years, rice biotechnology has made tremendous progress since the first transgenic rice was produced.[3,4] Many transgenic rice varieties transformed with various agronomic traits, such as insect resistance, bacterial resistance, and herbicide tolerance, have been subjected to field-testing.[3] Bayer CropScience has developed three herbicide-tolerant rice events LLRice06, LLRice62, and LLRice601 containing the bialaphos resistance (Bar) gene by direct DNA transfer techniques (http://www.agbios.com/dbase.php). The three GM rice lines have been subjected to environmental and food safety assessment;[5,6] however, none have been commercially planted in any country. In China, the transgenic insect-resistant rice TT51−1 (synonym BT63) was granted a safety certificate for commercialization in August 2009, after food and environmental safety assessments.[7]

TT51−1 is an insect-resistant transgenic rice event created by microprojectile bombardment with two plasmids, pFHBT1 and pGL2RC7, into the elite Chinese cytoplasmic male sterile (CMS) restorer line, Minghui 63. The plasmid pFHBT1 harbors a hybrid Cry1Ab/Ac gene regulated by the rice actin 1 gene promoter and the nopaline synthase (NOS) terminator. Plasmid pGL2RC7 carries a Chitinase gene (RC7) and a selectable marker gene (Hph).[8,9] It has been documented that the selectable marker gene Hph had been removed from the gene of interest by simple self-segregation.[9] The TT51−1-derived hybrid "BT63" was generated by crossing with CMS line Zhenshan 97. Field-testing showed that TT51−1 could reduce pesticide application and increase rice production efficiency through resistance against yellow stem borers and leaf-folders, which cause high annual yield losses of rice in Asian countries.[10]

TT51−1 rice is the first transgenic food crop to be granted a safety certificate in China. Before BT rice was approved for planting, the adventitious presence of TT51−1-derived BT rice was found and reported many times in Chinese and European markets.[11] According to the regulations of China and many other countries, food or feed containing unauthorized GM organisms at any level is not permitted to be sold on the market.[12−17] Therefore, many governments and consumers are very concerned about the detection and labeling of TT51−1-derived products. The European Commission has adopted emergency measures to require compulsory certification for the imports of Chinese rice products that could contain the GMO BT63,[18] and has established construct-specific detection methods for TT51−1.[19,20] Meanwhile, China has established an event-specific detection method for TT51−1 to monitor the illegal planting and production of BT63 rice.[21]

Molecular characterization of the integration site of the TT51−1 event has not been available. Thus, the objective of this research was to characterize the structural organization of the transgenes at the integration site in the recipient genomic DNA, which can help to develop an event specific detection method and determine the genetic stability of the TT51−1 line. In practice, calibrators are essential for quantitative detection of GMO samples. At present, the approved TT51−1 derived rice varieties are only planted in central China Hubei province; it has been very difficult to obtain pure material to calibrate TT51−1 detection methods. The plasmid DNA harboring detection targets have been successfully used as alternative calibrators for GMO trace analysis in place of Certified Reference Materials (CRMs) since 2001.[22−27] Thus far, event-specific plasmid DNA calibrators have been described for Roundup Ready soybean,[22,28,29]

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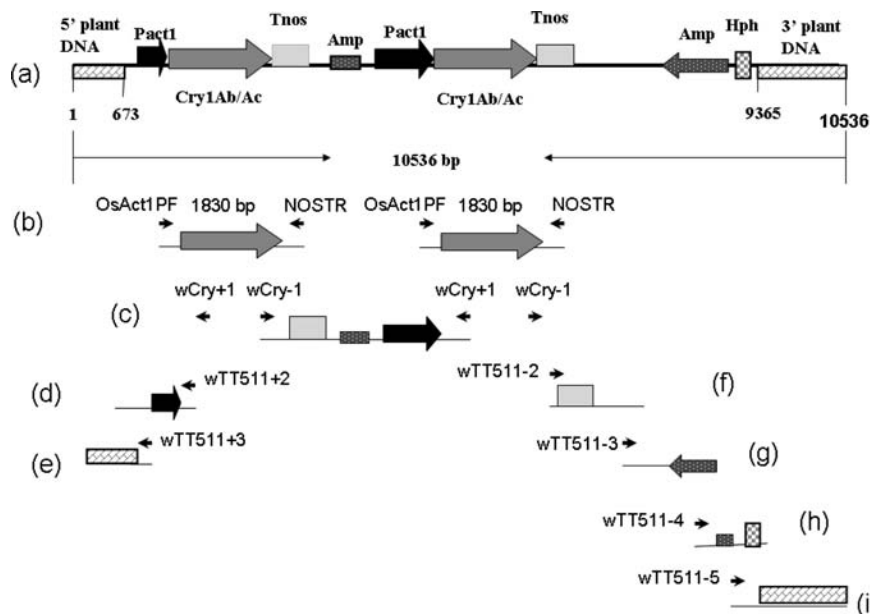


**Figure 1.** Schematic for the isolation of the insert DNA and flanking genomic DNA. The arrows indicate the location and orientation of primers. (a) The structural organization of the isolated DNA sequence. (b) Amplification of the Cry1Ab/Ac gene with primer pair OsAct1PF/NOSTR. (c) Amplification of the junction fragment between the two repeated Cry1Ab/Ac genes with primers wCry+1 and wCry−1. (d) The first round GenomeWalker PCR for isolating the sequence adjacent to the upstream incomplete Cry1Ab/Ac cassette with primer sets wCry+1/AP1 and wTT511+2/AP2. (e) The second round GenomeWalker PCR for amplifying the rice DNA with wTT511+2/AP1 and wTT511+3/AP2. (f) The first round GenomeWalker PCR for isolating the sequence adjacent to the downstream intact Cry1Ab/Ac cassette with primer sets wCry−1/AP1 and wTT511−2/AP2. (g) The second round GenomeWalker PCR with TT511−2/AP1 and wTT511−3/AP2. (h) The third round GenomeWalker PCR with TT511−3/AP1 and wTT511−4/AP2. (i) The fourth round GenomeWalker PCR with TT511−4/AP1 and wTT511−5/AP2.

Mon810 maize,[23] and NK603 maize.[24] In this study, we cointegrated the rice reference gene PLD and the isolated fragment including the TT51−1 insert DNA and flanking genome sequence into an appropriate plasmid to construct a reference plasmid. Furthermore, the utility of the reference plasmid was compared to that of the matrix material of TT51−1.

## MATERIALS AND METHODS

**Plant Materials.** Genuine seeds of a homozygous line of GM rice (*O. sativa*) containing the TT51−1 event were provided by Huazhong Agricultural University. The recipient material of TT51−1, nontransgenic *O. sativa* cv. Minghui 63, was purchased from a local market. Seeds of TT51−1 and Minghui 63 were sown and cultivated in a greenhouse. Fresh leaves were collected for DNA extraction.

**DNA Extraction.** Genomic DNA was extracted and purified from young leaves of TT51−1 and nontransgenic isoline Minghui63 following a cetyltrimethylammonium bromide (CTAB)-based protocol.[30] DNA samples from rice seeds were extracted using a DNA Extraction Kit for GMO Detection Ver. 2.0 (Takara, Shiga, Japan). Plasmid DNA was prepared using a QIAfilter Plasmid Giga kit (QIAGEN, Crawley, U.K.). DNA concentrations were estimated using the Picogreen dye method in a VersaFluor Fluorometer System (Bio-Rad, Hercules, CA, USA).

**Foreign DNA Amplification.** The PCR reaction was carried out with a KOD -Plus- kit (Toyobo, Osaka, Japan) in a volume of 50 $\mu$L containing 100 ng of genomic DNA, 1×KOD -Plus- buffer, 1 mM $MgSO_4$, 200 $\mu$M each of dNTPs, 100 nM each prime, and 1 unit KOD -Plus- DNA polymerase. The PCR reactions were performed on a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA). The reaction conditions comprised a 94 °C initial denaturation step for 2 min; 35 cycles of 15 s at 94 °C (denaturation), 3 min at 68 °C (annealing and extension), and terminated with an extension at 68 °C for 7 min.

The PCR products were checked by electrophoresis on 1% agarose gels (TAE buffer) and subcloned into the pZErO-2 vector (Invitrogen, Carlsbad, CA, USA) through an *Eco*RV restriction enzyme site. All the plasmids containing the PCR products were sequenced using the M13 forward primer and M13 reverse primer (Sunbiotech, Beijing, China).

**GenomeWalker PCR.** The BD GenomeWalker Universal Kit (Clontech, Mountain View, CA, USA) was used to isolate the exogenous DNA sequence and the flanking rice genomic sequence in TT51−1 according to the BD GenomeWalker Universal Kit User Manual. GenomeWalker primers (gene specific primer, GSP) were designed based on the isolated insert DNA. The positions of the GenomeWalker Primers are shown in Figure 1, and primer sequences are detailed in Table 1.

For GenomeWalker PCR, the primary and secondary PCR reactions were performed according to Wu et al.[31] PCR products with a single clear band were directly sequenced with the inner primer after purification (Sunbiotech, Beijing, China). Each sequence was used as a query in a BLASTN search of sequences in the GenBank database to confirm that the isolated fragments truly contain the expected sequence. If one round of GenomeWalker PCR was insufficient to isolate the plant DNA adjacent to the exogenous DNA, a second or third round of GenomeWalker PCR would subsequently be performed.

**Construction of a Reference Plasmid.** After characterizing the rice insertion event, its complete inserted DNA and flanking genomic DNA sequences were amplified by an overlapping PCR strategy and subcloned into plasmid vector pZErO-2. Positive clones were selected and sequenced. Meanwhile, an amplicon of the rice endogenous reference gene PLD was amplified,[32] and subsequently integrated into the plasmid containing the complete TT51−1 insert DNA and flanking genomic sequence, giving rise to the reference plasmid pK-TT51.

Table 1. Primers Used in This Study

| purpose | name | sequence (5′−3′) | location |
|---|---|---|---|
| GenomeWalker Kit Adapter Primers | AP1 | GTAATACGACTCACTATAGGGC | GenomeWalker Kit Adapter |
| | AP2 | ACTATAGGGCACGCGTGGT | |
| isolation of the hybrid CryAb/Ac gene | OsAct1PF | AATCCCTCAGCATTGTTCATCG | actin1 promoter |
| | NOSTR | TGCGGGACTCTAATCATAAAAACC | NOS terminator |
| GenomeWalker of TT51−1 | wCry+1 | CCTGGCACGAACTCGCTGAGCAGAAAC | 5′ region of Cry1Ab/Ac gene |
| | wTT511+2 | CGGTTTCAATGCGTTCTCCACCAAGTA | junction between Pactin1 and Cry1Ab/Ac gene |
| | wTT511+3 | GACGAAGCAGCAGCTGCGCTCGGT | junction between Pactin1 and plasmid backbone |
| | wCry−1 | TGCCAATGCTTTTACATCTTCACTCGG | 3′ region of CryAb/Ac gene |
| | wTT511−2 | ATGGGTTTTTATGATTAGAGTCCCGCA | junction between Cry1Ab/Ac and Nos terminator |
| | wTT511−3 | AGAGGTGGCGAAACCCGACAGGACTAT | plasmid backbone |
| | wTT511−4 | ACTTTATCCGCCTCCATCCAGTCTATT | Amp gene |
| | wTT511−5 | GCCACATAGCAGAACTTTAACCCCCGA | junction between Amp gene and Hph gene |
| PLD real-time PCR | KVM159 | TGGTGAGCGTTTTGCAGTCT | rice PLD gene |
| | KVM160 | CTGATCCACTAGCAGGAGGTCC | |
| | TM013 | FAM-TGTTGTGCTGCCAATGTGGCCTG-BHQ1 | |
| TT51−1 real-time PCR | TT511C | GCGTCCAGAAGGAAAAGGAATA | 3′ flanking genome DNA |
| | TT511G | AGAGACTGGTGATTTCAGCGGG | insert DNA |
| | TT511P | FAM-ATCTGCCCCAGCACTCGTCCG-BHQ1 | spanning the 3′ junction |

**Real-Time PCR.** The event-specific detection method for TT51−1 established by Wu et al. was used to assess plasmid pK-TT51. The quantitative PCR conditions for TT51−1 and rice reference gene PLD were the same as in the published paper.[21] PCR primers and probes are shown in Table 1. The probe contained a reporter dye (FAM) at the 5′-end and a quencher dye (BHQ) at the 3′-end. The real-time PCR reactions were performed on a fluorescence thermal cycler (CFX96 Real-Time PCR Detection System, Bio-Rad, USA). Fluorescence signals were monitored and analyzed by the CFX Manager software.

**Determination of Conversion Factors (Cf) for the Reference Plasmid.** In practice, identical copies of genomic and plasmid DNA show a difference in their threshold cycle (Ct) values with the same primer/probe set. In this study, the Cf value was defined as the difference of Ct value between plasmid and genomic DNA under the same copy number. Reactions were performed on a plasmid DNA series (80, 400, 2000, 10000, and 50000 copies per reaction) as well as a genomic DNA series (0.04, 0.19, 0.94, 4.70, and 20.0 ng per reaction, corresponding to 80, 400, 2000, 10000, and 42500 copies) to determine the Cf value. Standard curves were constructed by plotting the Ct values against the logarithm of the DNA copy number. The Cf value was calculated by eq 1:

$$Cf = Ct_p - Ct_g \quad (1)$$

$Ct_p$ represented the Ct value of the plasmid molecule, estimated by standard curves based on the reference plasmid molecule; $Ct_g$ represented the Ct value of the genomic DNA, estimated by standard curves based on the genomic DNA. The final Cf value was the average of the two calculated Cfs of 42500 and 80 copies of the plasmid and genomic DNA.

**Estimation of GM Content of Unknown Samples.** A solution containing plasmid pK-TT51 was serially diluted with salmon sperm DNA (10 ng/$\mu$L, Sigma D-9156). A plasmid DNA series with copy numbers of 80, 400, 2000, 10000, and 50000 (absolute amount in 4 $\mu$L) were used as calibrators to construct the standard curves. After the real-time PCR reactions were finished, the obtained Ct values were plotted against the logarithm of the absolute number of plasmid DNA copies in each PCR tube to construct the standard curve. According to the standard curves, the sum of Ct values of samples obtained from real-time PCR and Cf values were used to determine the copy number of TT51−1 and total rice genomic DNA of the unknown samples. The relative content of TT51−1 to total rice DNA (%) was computed as (mean copy number of TT51−1 of four parallels)/(mean copy number of total rice DNA of four parallels) × 100.[21]

## RESULTS

**Organization of the Hybrid Cry1Ab/Ac Gene in TT51−1.** The hybrid Cry1Ab/Ac gene is driven by the rice Actin1 promoter with its first intron and NOS terminator.[9] To obtain the sequence information of the Cry1Ab/Ac gene, the primers OsAct1PF and NOSTR were designed according to the Actin1 promoter sequence (Accession No. Os03g50890) and the NOS terminator sequence (Accession No. AF485783), respectively (Figure 1b). The primer pair OsAct1PF/NOSTR yielded a 2138 bp amplicon from the genomic DNA of TT51−1. Sequence analysis indicated that this 2138 bp amplicon contained 1830 bp of opening reading frame (ORF), showing 95% identity with two members of Cry gene family (Y09787 and AY126450). This ORF was speculated to be the trait gene Cry1Ab/Ac in event TT51−1.

To determine the organization of the inserted Cry1Ab/Ac gene in TT51−1 rice, two primers, wCry+1 and wCry−1, were designed to anneal to the 5′ and 3′ terminal ends of the hybrid Cry1Ab/Ac gene. As these two primers face away from each other in the Cry1Ab/Ac gene, as shown in Figure 1c, an amplified fragment would most likely result from the head-to-tail multimerization of two copies of the Cry1Ab/Ac gene inserted close to each other. A product of about 2800 bp was amplified from the genome DNA of TT51−1 using the primers wCry+1 and wCry−1. With the primer sets wCry+1/wCry+1 and wCry−1/wCry−1, no amplicon was observed. The results indicated that two copies of the Cry1Ab/Ac gene may be integrated at the same site as a head-to-tail concatemer, which agrees with previous data published by Tu et al.[9] Sequencing of the amplicon showed that it comprised an Actin1 promoter, an incomplete ampicillin (Amp) resistance gene, and a NOS terminator (Figure 1c).

**Determination of the Flanking Sequence of the Repeated Cry1Ab/Ac Genes.** To determine the flanking sequence upstream of the repeated Cry1Ab/Ac, a first round of GenomeWalker PCR was performed with primer sets wCry+1/AP1 and wTT511 + 2/AP2, generating a fragment containing an incomplete actin1 promoter (Figure 1d). A subsequent round of GenomeWalker PCR was carried out to further isolate the unknown DNA sequence adjacent to the incomplete actin1 promoter, using primer sets wTT511+2/AP1 and wTT511+3/AP2. The obtained amplicon contained a 673 bp fragment of plant DNA, showing 94−96% identity with the rice genome DNA (Accession No. AC113336, AC137073, CR855079 and so on). The results showed that an incomplete actin1 promoter was located in the 5′-upstream region of the repeated Cry1Ab/Ac genes, and that 673 bp of rice DNA adjacent to the foreign construct DNA had been isolated from TT51−1 (Figure 1a).

We were not able to reach the rice genomic DNA flanking the 3′-end of the repeated CryIAb/Ac gene by performing one round of GenomeWalking PCR. Three rounds of sequential GenomeWalking PCR reactions were carried out. The first round of GenomeWalker PCR only produced a fragment homologous to the backbone of the transgenic construct (Figure 1f) with primer set wCry−1/AP1 and wTT511−2/AP2. Using primer set wTT511−2/AP1 and wTT511−3/AP2, the second round of GenomeWalker PCR amplified a product harboring an incomplete ampicillin resistance gene (653 bp) (Figure 1g). The third GenomeWalker PCR amplified an amplicon carrying an incomplete hph gene (50 bp), using primer sets wTT511−3/AP1 and wTT511−4/AP2 (Figure 1h). Finally, after the fourth GenomeWalking PCR, 1172 bp of plant DNA was amplified from the region 3′ to the insert DNA, using primer sets wTT511−4/AP1 and wTT511−5/AP2 (Figure 1i). This plant sequence showed 99% identity with the rice genome sequence from chromosome 10 (Accession No. AC122146 and AC091238). The results indicated that the 3′-end-point of the repeated Cry1Ab/Ac genes was followed by an intact Nos terminator, an incomplete Amp resistance gene, and an incomplete Hph resistance gene. Finally, adjacent to the incomplete Hph gene, 1172 bp of rice DNA was isolated, as shown in Figure 1a.

**Characterization of the Insert DNA in TT51−1.** A 10536 bp fragment was retrieved by overlapping the obtained sequences presented in this paper. This 10536 bp fragment is made up of 8725 bp of insert DNA, 639 bp of rice DNA flanking the 5′-end of the insert DNA, and 1172 bp of rice DNA flanking the 3′-end of the insert DNA (EU880444) (Figure 1a). Analysis of the 8725 bp sequence allowed the structure of the transgenic locus in TT51−1 rice to be clearly elucidated (Figure 1a). The segment harboring the Cry1Ab/Ac cassette was from transformation construct pFHBT1, and the segment carrying the Hph gene was from the selective construct pGL2RC7.[9] The joint presence of the transgenes from pFHBT1 and pGL2RC7 at the same integration locus indicated that a rearrangement and ligation reaction had occurred between the two different transgenic constructs during transformation.

The structure of the transgenes suggested two tandem repeats of the Cry1Ab/Ac cassette in the insert DNA. A truncated Cry1Ab/Ac cassette with the actin1 promoter partially deleted is present in a direct repeat orientation at the 5′-end of the intact Cry1Ab/Ac cassette (Actin1 promoter-Cry1Ab/Ac-Nos terminator). The incomplete actin promoter was 149 bp in length, and showed high homology with intron 1 of the rice Actin1 gene, thus indicating that, during transformation, the truncated

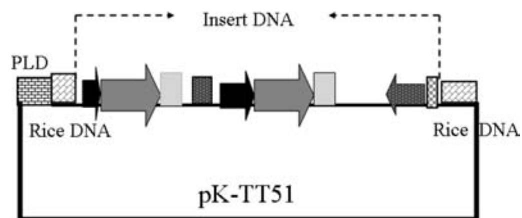


**Figure 2.** Schematic diagram of the integrated fragments in the constructed plasmid pK-TT51. PLD, partial fragment of rice endogenous PLD gene. Rice DNA, isolated flanking genomic DNA fragment in TT51−1. Insert DNA, rearranged transgenes in TT51−1.

actin1 promoter might have lost the necessary elements for its activity. The previous study showed that the region 834 bp upstream of the actin1 transcription initiation site contained all the regulatory elements necessary for maximal gene expression in transformed rice protoplasts.[33] Therefore, we concluded that only one Cry1Ab/Ac gene cassette is complete and can encode a functional protein in TT51−1. The other Cry1Ab/Ac cassette cannot be expressed because the truncated Actin1 promoter (149 bp) was not sufficient to activate the target gene expression. In addition to the target gene cassette, 83 bp of Amp gene sequence was located between the two tandem repeats of the Cry1Ab/Ac cassette (3388−3470), and 653 bp of Amp resistance gene sequence, together with 50 bp of the hygromycin resistance gene, were observed in the 3′-downstream region of the complete Cry1Ab/Ac cassette (Figure 1).

The 5′-end of the insert terminated within the fragmented transgenic vector backbone and was flanked by 673 bp from the rice genome. The 3′-end of the insert DNA terminated within a segment of pGL2RC7 and was followed by 1172 bp of rice genomic DNA. Whereas the 3′ flanking region displayed 99% identity with rice genomic DNA in chromosome 10, the 673 bp of the 5′ flanking sequence produced more than 20 targets showing 94−96% identity and 100% query coverage on a BLASTN search. These matches were scattered over different rice chromosomes, including chromosome 1, 3, 4, 5, 8, 9, 10, and 12. Furthermore, several copies of homologous targets were scattered in chromosome 10 (GenBank Accession Number AP008216, AC113336) as direct or inverted repeats. Thus, we concluded that the exogenous DNA was integrated in a long interspersed repeated segment in TT51−1. The 3′ flanking region showed sequence homology to chromosome 10, and the 5′ flanking region was inserted in a repeated DNA sequence motif; therefore, it was difficult to determine the exact integration site of exogenous DNA in TT51−1. On chromosome 10, the nearest homologous repeated DNA segment is at a distance of more than 300 kilobase pairs (kbp) from the position of the 3′ flanking region of TT51−1. We concluded that the target site was not simply disrupted by the transgenes, but that a large deletion at the target site or a translocation may have occurred when the foreign DNA integrated to form event TT51−1.

**Construction of a Reference Plasmid.** We cointegrated the complete exogenous sequence, flanking genomic sequence, and rice reference gene PLD into a plasmid vector, to form reference plasmid pK-TT51 (Figure 2), which can be used for event-specific, construct-specific, or screening detection of the TT51−1 event.

**Construction of Calibration Curves and Determination of the Cf Value.** Five different amounts of matrix-TT51−1 DNA and plasmid-pK-TT51 molecules were analyzed to construct

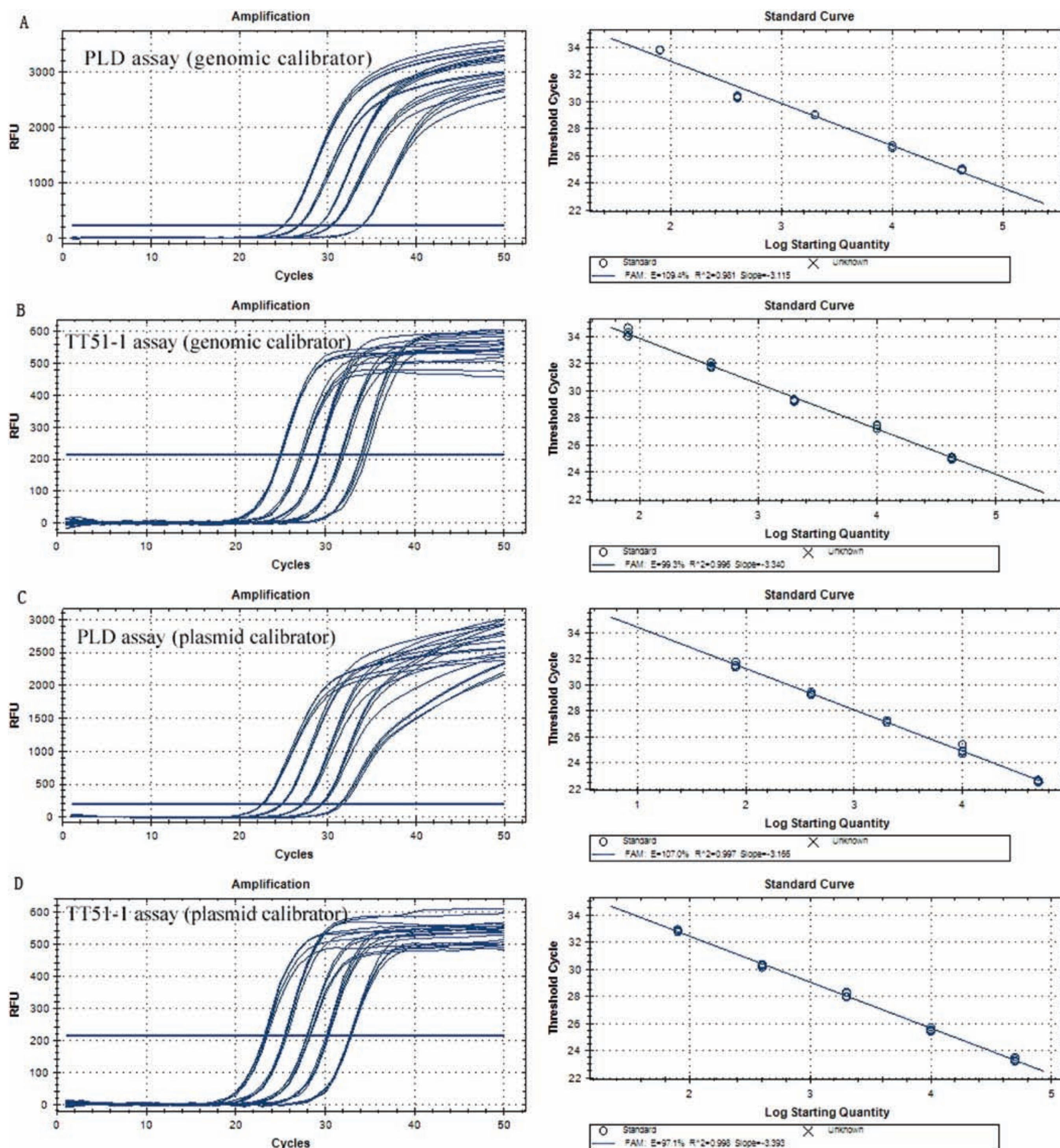


**Figure 3.** Amplification plot and standard curves based on the amplification data. A, amplification signal of the PLD assay and standard curve for PLD using genomic DNA as the calibrator; B, amplification signal of the TT51−1 assay and standard curve for TT51−1 using genomic DNA as the calibrator; C, amplification signal of the PLD assay and standard curve for PLD using plasmid DNA as the calibrator; D, amplification signal of the TT51−1 assay and standard curve of TT51−1 using plasmid DNA as the calibrator.

standard curves and to measure Cf values with four parallel reactions per template level. The amplification plots and standard curves are shown in Figure 3. The square regression coefficient ($R^2$) values for the TT51−1 assay were 0.996 and 0.998 using matrix-TT51−1 DNA and plasmid pK-TT51 as calibrators, respectively. The $R^2$ values for the PLD assay were 0.981 and 0.997 using matrix and plasmid DNA, respectively. All the $R^2$ values were higher than the minimum requirement of 0.98,[28] indicating good agreement between the Ct values and the amount of template for both types of calibrators.

The objective of this study was to construct a reference plasmid molecule and to check whether the plasmid DNA can

**Table 2. Repeatability of TT51−1 and PLD Real-Time PCR Using Plasmid pK-TT51 as Calibrators**

| target | copy no. | repeat | Ct value 1 | 2 | 3 | 4 | mean of Ct values | SD | RSD (%) | mean of all Ct values | SDr | RSDr (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TT51−1 | 50000 | 1 | 23.51 | 23.38 | 23.26 | 23.19 | 23.33 | 0.14 | 0.61 | 23.30 | 0.15 | 0.62 |
| | | 2 | 23.70 | 23.54 | 23.34 | 23.37 | 23.49 | 0.17 | 0.72 | | | |
| | | 3 | 23.41 | 23.27 | 23.04 | 22.88 | 23.15 | 0.23 | 1.01 | | | |
| | | 4 | 23.49 | 23.31 | 23.10 | 23.05 | 23.24 | 0.20 | 0.87 | | | |
| | 10000 | 1 | 25.54 | 25.73 | 25.50 | 25.41 | 25.55 | 0.13 | 0.52 | 25.49 | 0.16 | 0.65 |
| | | 2 | 25.80 | 25.74 | 25.70 | 25.53 | 25.69 | 0.11 | 0.44 | | | |
| | | 3 | 25.30 | 25.45 | 25.25 | 25.22 | 25.31 | 0.10 | 0.41 | | | |
| | | 4 | 25.48 | 25.51 | 25.50 | 25.25 | 25.43 | 0.13 | 0.50 | | | |
| | 2000 | 1 | 28.31 | 28.25 | 28.01 | 27.95 | 28.13 | 0.18 | 0.64 | 28.19 | 0.15 | 0.52 |
| | | 2 | 28.63 | 28.49 | 28.26 | 28.15 | 28.38 | 0.22 | 0.77 | | | |
| | | 3 | 28.22 | 27.90 | 28.05 | 27.98 | 28.04 | 0.13 | 0.48 | | | |
| | | 4 | 28.44 | 28.21 | 28.13 | 28.09 | 28.22 | 0.15 | 0.55 | | | |
| | 400 | 1 | 30.25 | 30.31 | 30.12 | 30.38 | 30.27 | 0.11 | 0.37 | 28.24 | 0.13 | 0.47 |
| | | 2 | 30.45 | 30.57 | 30.38 | 30.68 | 30.52 | 0.13 | 0.43 | | | |
| | | 3 | 30.25 | 30.34 | 30.15 | 30.48 | 30.31 | 0.14 | 0.46 | | | |
| | | 4 | 30.08 | 30.26 | 30.18 | 30.36 | 30.22 | 0.12 | 0.39 | | | |
| | 80 | 1 | 32.92 | 32.88 | 32.78 | 32.74 | 32.83 | 0.08 | 0.26 | 32.80 | 0.14 | 0.42 |
| | | 2 | 32.91 | 32.73 | 33.05 | 33.13 | 32.95 | 0.17 | 0.52 | | | |
| | | 3 | 32.65 | 32.50 | 32.75 | 32.57 | 32.62 | 0.11 | 0.33 | | | |
| | | 4 | 33.12 | 32.71 | 32.78 | 32.63 | 32.81 | 0.22 | 0.66 | | | |
| PLD | 50000 | 1 | 22.72 | 22.57 | 22.54 | 22.67 | 22.62 | 0.08 | 0.37 | 22.68 | 0.07 | 0.31 |
| | | 2 | 22.79 | 22.71 | 22.68 | 22.76 | 22.74 | 0.05 | 0.21 | | | |
| | | 3 | 22.80 | 22.66 | 22.73 | 22.83 | 22.76 | 0.08 | 0.34 | | | |
| | | 4 | 22.72 | 22.57 | 22.54 | 22.67 | 22.62 | 0.08 | 0.37 | | | |
| | 10000 | 1 | 25.43 | 24.88 | 24.96 | 24.72 | 25.00 | 0.31 | 1.22 | 25.00 | 0.01 | 0.03 |
| | | 2 | 25.03 | 24.96 | 25.00 | 25.02 | 25.00 | 0.03 | 0.13 | | | |
| | | 3 | 24.97 | 25.02 | 24.86 | 25.08 | 24.98 | 0.09 | 0.37 | | | |
| | | 4 | 25.43 | 24.88 | 24.96 | 24.72 | 25.00 | 0.31 | 1.22 | | | |
| | 2000 | 1 | 27.11 | 27.16 | 27.14 | 27.28 | 27.17 | 0.07 | 0.28 | 27.25 | 0.09 | 0.32 |
| | | 2 | 27.27 | 27.39 | 27.21 | 27.39 | 27.32 | 0.09 | 0.33 | | | |
| | | 3 | 27.26 | 27.33 | 27.24 | 27.48 | 27.33 | 0.11 | 0.40 | | | |
| | | 4 | 27.11 | 27.16 | 27.14 | 27.28 | 27.17 | 0.07 | 0.28 | | | |
| | 400 | 1 | 29.26 | 29.23 | 29.34 | 29.44 | 29.32 | 0.09 | 0.32 | 29.39 | 0.08 | 0.28 |
| | | 2 | 29.46 | 29.53 | 29.46 | 29.50 | 29.49 | 0.03 | 0.12 | | | |
| | | 3 | 29.44 | 29.38 | 29.41 | 29.48 | 29.43 | 0.04 | 0.14 | | | |
| | | 4 | 29.26 | 29.23 | 29.34 | 29.44 | 29.32 | 0.09 | 0.32 | | | |
| | 80 | 1 | 31.48 | 31.77 | 31.34 | 31.50 | 31.52 | 0.18 | 0.56 | 31.57 | 0.12 | 0.37 |
| | | 2 | 31.71 | 31.91 | 31.37 | 31.99 | 31.74 | 0.28 | 0.87 | | | |
| | | 3 | 31.58 | 30.90 | 31.69 | 31.78 | 31.48 | 0.40 | 1.27 | | | |
| | | 4 | 31.48 | 31.77 | 31.34 | 31.50 | 31.52 | 0.18 | 0.56 | | | |

be used to quantify genomic DNA extracted from a matrix. Therefore, the plasmid DNA calibrator should be comparable to the genomic DNA calibrator, and the comparability between the plasmid and genomic DNA in the real-time PCR was reflected in the characteristics of the constructed standard curves (figure 3). Figure 3 allowed us to examine the slopes, intercepts, and $R^2$ values of genomic and plasmid DNA calibrators. For TT51−1 and PLD, the genomic and plasmid standard curves showed approximately equal slopes and different intercepts, indicating that the two types of DNA calibrators had the same amplification efficiencies in real-time PCR, but different Ct values under the same copy number. The Cf value should be used to correct the Ct values of genomic DNA samples, if the plasmid DNA calibrator would be applied for quantification of genomic DNA samples instead of the genome DNA calibrator. The Cf values for TT51−1 and PLD ($Cf_{TT51-1}$ and $Cf_{PLD}$) were calculated by the difference of Ct values between the plasmid and genomic DNA, based on the standard curves. The results showed that $Cf_{TT51-1}$ was −1.47, and the $Cf_{PLD}$ was −1.78.

Table 3. Repeatability of Ct Values in Four Repeats for Quantitative Detection of Samples S1 and S2

| sample | target | repeat | Ct value 1 | 2 | 3 | 4 | mean of Ct values | SD | RSD (%) | mean of all Ct values | SDr | RSDr (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S1 | TT51−1 | 1 | 29.34 | 29.25 | 29.28 | 29.28 | 29.29 | 0.04 | 0.12 | 29.34 | 0.20 | 0.66 |
| | | 2 | 29.53 | 29.37 | 29.52 | 29.38 | 29.45 | 0.09 | 0.29 | | | |
| | | 3 | 29.16 | 29.01 | 29.16 | 29.03 | 29.09 | 0.08 | 0.29 | | | |
| | | 4 | 29.69 | 29.54 | 29.57 | 29.41 | 29.55 | 0.12 | 0.40 | | | |
| | PLD | 1 | 25.43 | 24.39 | 24.47 | 24.60 | 24.72 | 0.48 | 1.94 | 24.67 | 0.35 | 1.43 |
| | | 2 | 25.03 | 24.39 | 24.47 | 24.60 | 24.62 | 0.29 | 1.16 | | | |
| | | 3 | 24.97 | 24.39 | 24.47 | 24.60 | 24.61 | 0.26 | 1.04 | | | |
| | | 4 | 25.43 | 24.39 | 24.47 | 24.60 | 24.72 | 0.48 | 1.94 | | | |
| S2 | TT51−1 | 1 | 32.98 | 32.87 | 33.33 | 32.77 | 32.99 | 0.24 | 0.73 | 32.92 | 0.19 | 0.57 |
| | | 2 | 33.26 | 33.03 | 32.96 | 33.15 | 33.10 | 0.13 | 0.40 | | | |
| | | 3 | 32.79 | 32.88 | 32.83 | 32.69 | 32.80 | 0.08 | 0.25 | | | |
| | | 4 | 32.71 | 32.83 | 32.75 | 32.87 | 32.79 | 0.07 | 0.22 | | | |
| | PLD | 1 | 24.73 | 24.78 | 24.69 | 24.96 | 24.79 | 0.12 | 0.47 | 24.74 | 0.12 | 0.49 |
| | | 2 | 24.61 | 24.62 | 24.62 | 24.79 | 24.66 | 0.09 | 0.35 | | | |
| | | 3 | 24.65 | 24.68 | 24.78 | 24.96 | 24.77 | 0.14 | 0.57 | | | |
| | | 4 | 24.93 | 24.64 | 24.63 | 24.72 | 24.73 | 0.14 | 0.57 | | | |

**Validation of Reference Plasmid Molecule pK-TT51 and Cf Values.** After the Cf values of TT51−1 and PLD were determined in the quantitative PCR assays, two rice samples containing different percentages of TT51−1 (5.0% and 0.5%) were used as unknown samples to evaluate the suitability of the constructed reference plasmid and the accuracy of the Cf values. The standard curve method was used in this study for quantitative assays. A solution series of plasmid pK-TT51 was used to build the standard curve. To determine the repeatability of the quantitative assay, the real-time PCR reactions were repeated four times, with four parallel reactions each time. The Ct values of the reference plasmid molecule and genomic DNA of samples are shown in Tables 2 and 3. The standard deviation (SD) and the relative standard deviation (RSD) of the four parallel reactions and the standard deviation ($SD^r$) and the relative standard deviation ($RSD^r$) of repeatability were in the acceptable range; the $RSD^r$ values of plasmid calibrators were in the range 0.03% to 0.65%, less than the acceptable value (≤25%).[34] The statistical analysis showed that both the TT51−1 and PLD assays had good repeatability for the reference plasmid DNA or genomic DNA of samples.

The characteristics (slope, intercept, and $R^2$ value) of the standard curves for PLD and TT51−1 are shown in Table 4. For samples S1 and S2, the Cf values of $Cf_{TT51-1}$ and $Cf_{PLD}$ were used to correct the Ct values of TT51−1 and PLD by adding the Cf value to Ct value from PCR of sample DNA. The copy numbers of the TT51−1 event and total rice genome DNA were calculated by subjecting the corrected Ct values to the standard curves from the plasmid calibrators. The content of the rice samples was estimated by the defined formula, as described in Material and Methods. For the sample S1 (theoretical content 5%), the estimated content of TT51−1 DNA ranged from 4.46% to 5.48%, with a bias between 0.71% and 10.72% in the four repeats. For the sample S2 (theoretical content 0.5%), the estimated proportion of TT51−1 DNA ranged from 0.43% to 0.51%, with a bias between 1.74% and 13.97 (Table 4). The calculated contents of these samples were very close to the expected theoretical values. Similar quantitative results were obtained by Wu et al. using matrix-TT51−1 DNA as calibrator.[21] The accuracy of the quantitative results was measured by the bias between the estimated GM content and the theoretical percentage. Compared to the accuracy of previously reported data from maize and soybean samples using plasmids as calibrators,[26,28,35] the quantitative results of these rice samples were acceptable. The quantitative assays demonstrated that plasmid pK-TT51 represents a valuable alternative to genomic DNA as a calibrator for the quantification of rice event TT51−1 in food and feed products, and the Cf values of $Cf_{PLD}$ (−1.78) and $Cf_{TT51}$ (−1.47) could be used as conversion factors for the pK-TT51 calibrator.

## DISCUSSION

The transgenic rice TT51−1 was generated by direct DNA transfer through cotransformation of two separate plasmids: pFHBT1 harboring the Cry1Ab/Ac gene and pGL2RC7 carrying Hph gene.[8] Particle bombardment favors integration of multiple copies of transgenes at a single locus and rearrangement of transgenes.[36−39] The joint presence of the Cry1Ab/Ac cassettes and the selectable gene Hph at the same locus indicates that, during the preintegration phase, the broken plasmid molecule pFHBT1 together with a fragment of the Hph gene from selective plasmid pGL2RC7 were ligated and integrated at the same locus, giving rise to the rearranged transgenes. The cotransformation of TT51−1 also supports a ligation model, as the transgenes on two plasmids are seen to integrate in the same locus.[40] Previous studies indicated that transferred DNA is preferentially inserted into plant retrotransposons and repeated sequences, as was the case with T25, MON810, and Bt11.[23,41,42] The 5′-end of the transgenes is integrated into a scattered repeated sequence motif in the TT51−1 event. As the 5′ region of the insert DNA is integrated into a scattered repeated motif, we

Table 4. Quantitative Analysis of Unknown Samples S1 and S2

| | | std curve | | | quantitative assay of S1 (5%) | | | | | quantitative assay of S2 (0.5%) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| repeat | target | slope | intercept | $R^2$ | mean of Ct values | mean + Cf[a] | copy no. | Estimated content | bias (%) | mean of Ct values | mean + Cf[a] | copy no. | est content | bias (%) |
| 1 | TT51−1 | −3.39 | 39.22 | 0.998 | 29.29 | 27.82 | 2301 | 5.48% | 9.53 | 32.99 | 31.51 | 187 | 0.47% | −6.62 |
| | PLD | −3.17 | 37.58 | 0.997 | 24.72 | 22.94 | 42011 | | | 24.79 | 23.01 | 40017 | | |
| 2 | TT51−1 | −3.40 | 39.43 | 0.997 | 29.45 | 27.98 | 2337 | 4.96% | −0.71 | 33.10 | 31.63 | 197 | 0.43% | −13.97 |
| | PLD | −3.22 | 37.88 | 0.998 | 24.62 | 22.84 | 47072 | | | 24.66 | 22.88 | 45861 | | |
| 3 | TT51−1 | −3.43 | 39.19 | 0.997 | 29.09 | 27.62 | 2385 | 4.81% | −3.80 | 32.80 | 31.33 | 197 | 0.45% | −10.4 |
| | PLD | −3.13 | 37.54 | 0.996 | 24.61 | 22.83 | 49593 | | | 24.77 | 22.99 | 43985 | | |
| 4 | TT51−1 | −3.42 | 39.29 | 0.996 | 29.55 | 28.08 | 1875 | 4.46% | −10.72 | 32.79 | 31.32 | 213 | 0.51% | 1.74 |
| | PLD | −3.17 | 37.58 | 0.997 | 24.72 | 22.94 | 42011 | | | 24.73 | 22.95 | 41787 | | |

[a] Cf value for TT51−1 (CfTT51−1) was −1.47, and Cf value for PLD ($Cf_{PLD}$) was −1.78.

cannot determine the exact integration locus of the exogenous genes in the recipient genome or the rearrangement of genomic DNA in TT51−1.

The characterization of the transgenes shows that the truncated Amp gene is integrated into the host genome, and the filler DNA (3241−3289) is present in the insert DNA, showing 100% identity with the coding sequence of the *O. sativa* Act2 gene (EU155408). This is in agreement with the results of a previous study, in which the plasmid−plasmid recombination junctions invariably associated with deletions at one or both ends of the recombining molecules, and contained filler DNA.[43] Kohli et al. (1998) also found that the integration locus of transgenes may have plant DNA separating the transgenic sequences.[39] Thus, we obtained the entire sequence of the inserted DNA and determined the rearrangement of the transforming plasmids in TT51−1. Although Tu et al. (2003) have documented the structural organization of Cry1Ab/Ac transgenes in the recipient genome of TT51−1 by restriction enzyme digestion and Southern blot analysis,[9] they did not find that complex recombination and ligation reactions occurred between the cotransformation plasmids pFHBT1and pGL2RC7 during transformation. Therefore, the data presented in this study provide further support for their conclusions.

The complete aligned sequence of event TT51−1 was submitted to GenBank under the Accession Number EU880444. Through integrating the aligned sequence into a plasmid vector, we developed a novel plasmid, pK-TT51, carrying the entire insert DNA sequence, the flanking genomic DNA fragment, and a rice endogenous PLD fragment. Quantitative assays demonstrated that plasmid pK-TT51 was applicable as an alternative calibrator for quantifying GM rice samples containing the TT51−1 event. Meanwhile, because pK-TT51 harbors the complete sequence of the insert DNA, it is also suitable for construct-specific detection and screening of GM rice.

Focusing on the difficulty of obtaining sufficient genuine TT51−1 material as calibrators for quantitative or qualitative assays, this study provides a reliable alternative to matrix material of the TT51−1 event. Owing to the advantages of plasmid standards in terms of easy preparation, cost-efficient production, high-speed propagation, and long-term stability, plasmid standards are thought to be "gold standards".[22,23,26,28] In contrast to the matrix TT51−1, plasmid pK-TT51 can be produced on a large scale without the need of agricultural products, and can be made available for a wide range of DNA target concentrations.

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## ABBREVIATIONS USED

NOS, nopaline synthase; Bar, bialaphos resistance; CMS, cytoplasm male sterile; Hph, hygromycin phosphotransferase; Amp, ampicillin; CRMs, Certified Reference Materials; PLD, phospholipase D; Cf, conversion factor; Ct, threshold cycle

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