Fast-Tracking Determination of Homozygous Transgenic Lines and Transgene Stacking Using a Reliable Quantitative Real-Time PCR Assay

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Abstract The selection of homozygous lines is a crucial step in the characterization of newly generated transgenic plants. This is particularly time- and labor-consuming when transgenic stacking is required. Here, we report a fast and accurate method based on quantitative real-time PCR with a rice gene *RBE4* as a reference gene for selection of homozygous lines when using multiple transgenic stacking in rice. Use of this method allowed can be used to determine the stacking of up to three transgenes within four generations. Selection accuracy reached 100 % for a single locus and 92.3 % for two loci. This method confers distinct advantages over current transgenic research methodologies, as it is more accurate, rapid, and reliable. Therefore, this protocol could be used to efficiently select homozygous plants and to expedite time- and labor-consuming processes normally required for multiple transgene stacking. This protocol was standardized for determination of multiple gene stacking in molecular breeding via marker-assisted selection.

Keywords Transgenic stacking · Quantitative real-time PCR · Transgenic rice · Molecular breeding · Rice *RBE4*

Introduction

Genetic transformation is widely used in basic and applied research to generate genetically modified plants. It has led to the development of commercial crops with improved agronomic characteristics [1]. Rapidly obtaining homozygous lines shortens the breeding duration for generating transgenes, particularly when multiple genes or transgenes are stacked once the viability of primary transformants has been obtained. Screening of homozygotes and estimation of transgene copy number are vital for the selection and cultivation of transgenic plants, making them indispensable research techniques. Fast and accurate identification of transgenic

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homozygotes in subsequent generations is both highly desirable and beneficial, especially in crops requiring large amounts of planting space to produce their next generation. Therefore, a robust and reliable method of identifying homozygotes, especially for transgenic pyramiding or stacking, and for ascertaining transgene copy number in early generations is important for molecular marker-assisted selection (MAS) in transgenic breeding programs. Traditionally, target gene PCR and Southern blot are used to analyze transgenic homozygotes and copy number. Unfortunately, these methods are both laborious and time-consuming, requiring considerable amounts of DNA from fresh or frozen samples, and may involve hazardous radioisotopes [2].

To overcome these limitations, quantitative real-time PCR (qPCR) is often applied to analyze transgene integration. qPCR collects data throughout the PCR process, thus combining amplification and detection in a single step. Detection has been achieved using a variety of fluorescent molecules, in which PCR product concentration is correlated with fluorescence intensity [3]. The quantitative endpoint for qPCR is the threshold cycle (Ct) [4], and this is defined as the PCR cycle during which the fluorescent signal of the reporter dye crosses an arbitrarily determined threshold. Presenting data as a Ct ensures that quantification occurs during the exponential phase of amplification. The value of the Ct is inversely related to the amount of amplicons in the reaction [5]. The greater the quantity of target DNA in the starting material, the faster a significant increase in fluorescent signal will appear, thus yielding a lower Ct [6].

Although qPCR has been used to screen homozygous transgenic plants [7–9], accuracy and reliability for transgenic pyramiding or stacking have not been well resolved and practiced in breeding programs. In this study, we optimized the rice endogenous gene *RBE4* as a reference gene for determination of transgenically stacked homozygous lines. Accuracy reached 100 % for a single locus and 92.3 % for two loci. The accuracy of insertion locus/loci and copy number determination reached 100 %. The reliability of the homozygous lines was verified by PCR, and the accuracy of the insertion locus/loci was confirmed by Southern blotting and genetic analysis in the next generation. This methodology was successfully applied to three transgenic stackings and had the distinct advantages of higher accuracy, speed, and reliability and less time- and labor-consuming over Southern blotting process and other current transgenic research methodologies. This protocol was standardized for multiple gene stacking in molecular breeding programs via MAS.

Materials and Methods

Plasmid Construction and Generation of Transgenic Rice Plants

α-1,6-Fucosyltransferase gene (*FUT8*; GenBank accession No. D89289.1) and β-1,4-galactosyltransferase gene (*GalT*; GenBank accession No. M22921.1) were synthesized by GenScript Corporation (NJ, USA) using rice-preferred genetic codons. A mediated construct with endosperm-specific expression cassette with a globulin (*Glb*) promoter designated as *pOsPMP512* was used in this study. The synthesized genes were obtained by sequential digestion with *Sch*I and *Xho*I and then cloned into *pOsPMP512* that was digested by *Nae*I and *Xho*I, resulting in *pOsPMP513* (*FUT8*) and *pOsPMP514* (*GalT*). The plasmids of *pOsPMP513* and *pOsPMP514* were digested with *Hin*dIII and *Eco*RI and cloned into a binary vector *JH2600* that was digested by the same restriction enzymes. The resulting binary vectors were designated as *pOsPMP515* and *pOsPMP516* (Supplementary Fig. S1). The plasmids were transformed into *Agrobacterium tumefaciens* strain *EHA 105*. A *pOsPMP122* containing a hygromycin phosphotransferase (*HPT*) gene under control of a *CP* promoter was used as

selective marker (Supplementary Fig. S1). The target gene plasmid and the selective marker *pOsPMP122* were co-transformed into calli derived from a rice variety TP 309 through *Agrobacterium*-mediated transformation [10].

The hygromycin resistance-positive transformants were identified by PCR using the gene-specific primer pair *HPT-F/HPT-R* (Supplementary Table 1). A transgenic line (132-17) expressing a human *α1-antitrypsin* (*AAT*) was used in this study [11]. Co-transformants containing *HPT* gene and either *FUT8* or *GalT* gene were identified by PCR using target gene-specific primers *FUT8-F2/FUT8-R2* or *GalT-F2/GalT-R2* (Supplementary Table 1). A homozygous transgenic line 515 crossed with a homozygous line 516 first, and then, the F_1 of 515×516 crossed with line 132-17. An F_2 population derived from the cross (515×516) × 132-17 was used for identifying and confirming homozygous and heterozygous plants through application of the method developed.

DNA Extraction

Rice genomic DNA used for qPCR analysis was extracted from fresh leaves using the cetyltriemethylammonium bromide (CTAB) method [12]. The concentration of genomic DNA was measured with UV absorption at 260 nm, and DNA quality was evaluated using UV absorption ratio at 260/280 nm. DNA samples were analyzed using 1 % agarose gel electrophoresis.

PCR Amplification and Primers

Oligonucleotide primers were designed using Primer 5.0 software (Supplementary Table 1). For enhanced amplification efficiency, amplicons for the SYBR Green qPCR primers were designed to be smaller than 200 bp. An *RBE4-F/RBE4-R* primer pair for the endogenous reference gene *RBE4*, a *FUT8-F1/FUT8-R1* primer pair for the target gene *FUT8*, a *GalT-F1/GalT-R1* primer pair for the target gene *GalT*, and an *AAT-F1/AAT-R1* primer pair for the target gene *AAT* were used for the SYBR Green qPCR assay, yielding amplified fragments of 106, 118, 96, and 91 bp, respectively. A *FUT8-F2/FUT8-R2* primer pair for the target gene *FUT8*, a *GalT-F2/GalT-R2* primer pair for the target gene *GalT*, and an *AAT-F2/AAT-R2* primer pair for the target gene *AAT* were used for PCR, yielding amplified fragments of 965, 645, and 644 bp, respectively. The qPCR efficiency for each gene was determined using serial dilutions to obtain appropriate standard curves.

SYBR Green qPCR

Amplification reactions were performed on a qPCR platform (Applied Biosystems, Foster City, CA, USA). qPCR was carried out in 10-μl reaction mixtures containing 5-μl PCR buffer mix (Invitrogen, Grand Island, NY, USA), 1-μl DNA template (30 ng), and 500-nM gene-specific primers. The PCR program comprised 1 cycle of 10 min at 95 °C, followed by 40 cycles of 10 s at 95 °C and 20 s at 60 °C. The Ct value of the gene was determined systematically. Amplification data were analyzed using StepOne software (Applied Biosystems, Foster City, CA, USA). Each sample was quantified using three replicates and in triplicate for each replicate.

Determination of Transgene Zygosity

Relative quantification by the comparative Ct ($2^{-\Delta\Delta Ct}$) method was used for identification of the homozygous plants. Because a homozygous plant contains twice as many transgenes as a

heterozygous one, the comparison of relative copy ratios of *FUT8* and *RBE4* between the transgenic plants is sufficient to quantitatively calculate the PCR products; the $2^{-\Delta\Delta Ct}$ value of a homozygote should be twice that of a heterozygote. The comparative Ct of *FUT8* and *RBE4* could be calculated using the following formula:

$$\Delta\Delta Ct = \left(C_{t,\,FUT8} - C_{t,RBE4}\right)_{Sample\,2} - \left(C_{t,FUT8} - C_{t,RBE4}\right)_{Sample\,1}$$

To calibrate qPCR efficiencies of the target and internal reference genes, the amplification efficiency of the internal reference gene was used for normalization of the amplification efficiency of the target gene. Each reaction had three technological replicates, repeated in triplicate for each replicate.

PCR for Homozygote Identification

Reactions were performed in 20-μl reaction mixtures (Fisher Scientific, Pittsburgh PA, USA) containing 1-μl DNA template (30 ng), 2 μl 10× PCR buffer, 0.8 μl 1 mmol l^{-1} dNTP, 1.2 μl 25 mmol l^{-1} Mg^{2+}, 1 unit *Taq* DNA polymerase, and 500-nM primers. The PCR program comprised 1 cycle of 10 min at 95 °C, followed by 32 cycles of 30 s at 95 °C, 30 s at 56 °C, and 30 s at 72 °C, and a final extension of 10 min at 72 °C. The sample was stored at 25 °C. Each reaction was repeated in triplicate.

For verification of homozygous plants identified in the previous generation, zygosity of the lines was monitored in the T_2 generation. T_1 plants were considered homozygous if no segregation was observed in the T_2 generation and were considered heterozygous if segregation was observed in the T_2 generation.

Determination of Transgene Copy Number

The absolute quantification calculated with the standard curves was used for determination of transgene copy number [4, 13]. UV absorption at 260 nm was used to calculate the copy number of the genes using the formula:

$$\text{Copies} = \text{Avogadro constant} \times \text{copies of positive plasmid concentration } \left(g\,ml^{-1}\right)$$
$$/\text{plasmid relative molecular weight } \left(g\,mol^{-1}\right)$$

Two standard curves for the two genes were obtained by plotting Ct values against log-transformed concentrations of serial tenfold dilutions (10^5, 10^4, 10^3, 10^2, and 10 copies μl^{-1}) from the genomic and plasmid DNA solutions. The absolute copy number of the target gene in each transgenic plant was calculated using Ct values based on their standard curves. *RBE4* is a single-copy gene, and the relative copy number of the target gene was calculated using the following formula:

$$\text{Copy number of target gene} = A/B \text{ or } \text{Copy number of target gene} = A/B \times 2$$

where A is the initial absolute copy number of the target gene and B is the initial copy number of the internal reference gene, *RBE4*.

RBE4 is unique in the rice genome. In the formulas, when a target gene is heterozygous, the copy number was multiplied by two. Each reaction had three replicates and was repeated three times.

Southern Blot Analysis

Genomic DNA (10 μg) was obtained from fresh young leaves using the CTAB method [12]. Genomic DNA was digested with *Hin*dIII, *Eco*RI, or a *Hin*dIII/*Eco*RI and then separated using 0.8 % agarose gel electrophoresis alongside relevant DNA size markers (λ DNA digested with *Hin*dIII). Separated DNA was transferred to a nitrocellulose membrane according to the manufacturer's instructions (Gene Company, Hong Kong, China). A 965 bp fragment derived from *FUT8* coding region was used as a probe, which was prepared by PCR using primers *FUT8-F2/FUT8-R2*. The DNA probe was labeled using a random primer labeling kit according to the manufacturer's instructions (Roche, Basel, Switzerland). Membrane baking, pre-hybridization, and hybridization were performed according to the manufacturer's instructions (DIG High Prime DNA Labeling and Detection Starter Kit I, Roche, Basel, Switzerland).

Results

Optimization of the Internal Reference for the Identification of Homozygotes

To reliably identify homozygous plants using qPCR, it is critical that an appropriate endogenous reference gene is used to normalize data. This eliminates sample-to-sample variations and allows calibration of the Ct value. It is important that the amplification of the internal reference does not change, even under conditions when target gene amplification may alter dramatically [14]. Ideally, an internal reference gene should be species-specific, having single or low copy number per haploid genome, and exhibiting low heterogeneity across genotypes within a species [1, 15]. To screen genes appropriate for use as an internal reference, the following eight genes, present as one or two copies in the rice genome, were chosen: starch branching enzyme (*RBE4*) [16], sucrose phosphate synthase (*SPS*), rice root-specific gene (*gos9*), eukaryotic elongation factor 1-*alpha* (*eEF1α*), rice actin gene (*RAc1*), 1-deoxy-D-xylulose-5-phosphate reductoisomerase (*dxr*), and trs-like genes (*Os3bet3* and *Os4trs20*). To achieve optimal amplification efficiency, 21 primer pairs were tested. Serial dilutions of the DNA template were used in the qPCR assay. The amplification plot, standard curve (including the slope of the line, average correlation coefficients (R^2), and PCR amplification efficiency), and melting curve of the eight genes were compared. The standard amplicons curve of *RBE4* had a high correlation coefficient ($R^2=0.994$; Fig. 1b). The PCR amplification efficiency was
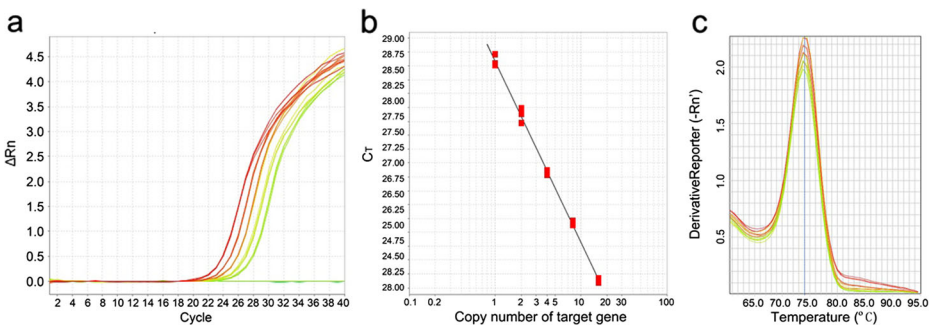

Fig. 1 Amplification plot, standard curve, and melting curve of *RBE4*. **a** Amplification plot. **b** Standard curve. **c** Melting curve. Ct represents threshold cycle, and ΔRn represents normalized reporter− baseline

nearly 100 %. The other genes tested did not completely satisfy all criteria as an internal reference gene or were not as efficient as *RBE4* (Table 1). Therefore, the single-copy gene, *RBE4*, was considered the best for use as an internal reference gene and chosen for further analysis (Fig. 1).

Establishment of Ct ($2^{-\Delta\Delta Ct}$) for Identification of Homozygous and Heterozygous Plants

To test whether the internal reference gene *RBE4* could be applied in the identification of homozygous and heterozygous plants, we examined its accuracy in T_1 transgenic lines. Relative quantification was performed using the comparative Ct ($2^{-\Delta\Delta Ct}$) method. Firstly, standard curves were validated and qPCR amplification efficiencies confirmed for both internal reference and target genes. The R^2 of the standard curves for *RBE4* and for the *FUT8* target gene was 0.998 and 0.994, respectively. PCR amplification efficiencies for both genes reached 99 and 101 %, respectively, indicating that both the internal reference gene and the target gene were effective for homozygous plant identification. To determine the feasibility of the identification of homozygous plants, 100 individual plants each from five transgenic lines derived from the T_1 generation were used. It was assumed that the *FUT8*/*RBE4* gene ratio could be quantitatively determined by the ΔCt value [17]. Thus, the ratio of the absolute copy numbers of *FUT8*/*RBE4* between homozygotes and heterozygotes was reflected in their ΔCt. It was assumed that the value of $2^{-\Delta\Delta Ct}$ for a homozygote was twice that of a heterozygote. The $2^{-\Delta\Delta Ct}$ value for each sample was approximately 1 or 2 (Table 2), exactly reflecting the differences between the homozygotes and the heterozygotes.

Accuracy of Homozygous Plant Identification Validated in the T_2 Generation

To verify the reliability of the homozygous or the heterozygous plants identified by qPCR, we performed accuracy verification by monitoring genetic segregation in the T_2 generation that derived from the T_1 plants. Offspring from self-pollinated homozygous, heterozygous, or negative plant lines were used for verification by PCR using gene-specific primers (Table 3). The results indicated that 100 % accuracy for homozygous and negative line determination and 93.33 % for heterozygous line determination were reached when the transgenic lines carrying a single locus. The 92.31 % accuracy for homozygous lines and 86.67 % for heterozygous lines determination were confirmed for the transgenic line carrying two loci. These results demonstrated that this protocol was accurate for identifying homozygous lines in an early generation.

Table 1 Correlation coefficients (R^2) and PCR amplification efficiencies of all tested genes

Genes	Correlation coefficients (R^2)	PCR amplification efficiency (E %)
RBE4	0.994	101.00
SPS	0.983	116.36
gos9	0.986	95.14
eEF1α	0.953	123.33
RAc1	0.984	105.76
dxr	0.986	90.03
Os3bet3	0.991	95.43
Os4trs20	0.987	119.92

Table 2 $2^{-\Delta\Delta Ct}$ values from T_1 transgenic plants for determination of zygosity

Number	Ct value (FUT8)	Ct value (RBE4)	ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$	Zygosity
515-1-1	15.591	20.324	−4.733	0	1	Heterozygote
515-1-2	14.175	19.893	−5.718	−0.985	1.979	Homozygote
515-1-3	15.192	19.961	−4.77	−0.037	1.025	Heterozygote
515-1-4	14.477	20.016	−5.538	−0.805	1.747	Homozygote
515-1-5	15.209	19.794	−4.585	0.148	0.902	Heterozygote
515-1-6	15.26	19.994	−4.734	−0.001	1.001	Heterozygote

Determination of Homozygous Lines for Transgene Stacking

To determine the feasibility of this protocol for gene stacking, we tested its reliability for homozygous plant identification when stacking three genes of *FUT8*, *GalT*, and *AAT* (Fig. 2a). As shown in Table 4, a step-down approach was used for screening homozygous plants. Five hundred individual plants from an F_2 population derived from (515×516) × 132-17 were tested. Firstly, the plants containing *FUT8* from the F_2 population of the cross (515×516) × 132-17 were screened. A total of 133 *FUT8* homozygous plants were identified. These plants were used for a second round of screening to identify *GalT* homozygous plants. Thirty-six homozygous plants containing both *FUT8* and *GalT* were obtained. Finally, these plants were screened using *AAT* gene-specific primers, and eight homozygous plants stacked with *FUT8*, *GalT*, and *AAT* were obtained (Table 4).

To verify the reliability of the homozygous plants identified by this protocol, a homozygous line stacked with the *FUT8*, *GalT*, and *AAT* genes was randomly chosen and used to produce a F_2/F_3 population. We checked genetic segregation by PCR using the target gene-specific primers. No segregation was found in the 100 plants tested in the F_2/F_3 population (Fig. 2b). The results demonstrated that the protocol was effective and reliable in the identification of homozygous lines for multiple gene stacking.

Estimation and Validation of Reliability for Insertion Locus/Loci Determination

Because the internal reference gene *RBE4* is single-copied in the rice genome, the copy number of a target gene can be determined by comparison with *RBE4*. To verify the feasibility of this method to determine copy number, we chose five independent *FUT8* transgenic lines with different loci, which were previously determined to be heterozygous

Table 3 Zygotic identification in the T_1 generation and verification by conventional PCR in the T_2 generation

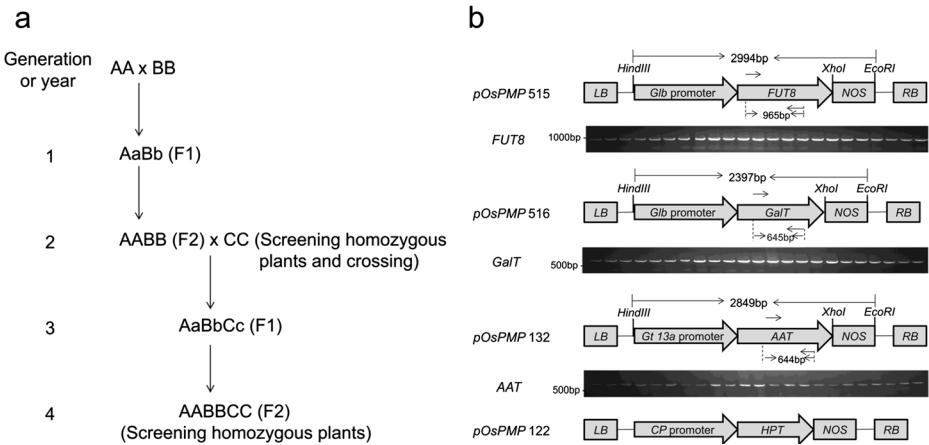| Type	Zygosity	Zygosity in T_1 qPCR	Zygosity in T_2 PCR	Accuracy (%)	Remark
Single-copy plant	Homozygous lines	13	13	100	
	Heterozygous lines	15	14	93.33	One plant is homozygous
	Negative lines	14	14	100	
Double-copy plant	Homozygous lines	13	12	92.31	One plant is heterozygous
	Heterozygous lines	15	13	86.67	Two plants are homozygous
	Negative lines	13	13	100	

Fig. 2 Transgene stacking and PCR amplification scheme. **a** Scheme of determining transgene stacking. **b** Diagrams of four plasmids and the examples of PCR using gene-specific primers for verification of the homozygous individuals that were determined by this protocol in F_2 generation. The size of PCR products and entire expression cassette between *HindIII* and *EcoRI* were shown. *A* represents FUT8 gene, *B* represents *GalT* gene, and *C* represents *AAT* gene. *Upper* and *lower letters* represent dominant locus and recessive locus, respectively

by this protocol and late confirmed by genetic analysis. Thirty T_2 individual plants each line derived from the heterozygous line out of the T_1 generation were analyzed. We detected the genetic segregation ratio of the positive and the negative plants for locus/loci determination of the target genes. The copy number of *FUT8* was determined by calculating the absolute initial quantity of each gene using its average Ct value from the standard curve with the internal reference gene *RBE4*. The results indicated that three lines possessed a single locus, and two lines presented two loci (Table 5). These results were consistent with the results from the genetic analysis.

To further validate the reliability of the method, Southern blotting was performed for a single-locus line (515-1) and a two-locus line (515-2). As shown in Fig. 3, there are two different size hybridization bands in line 515-2 when the genomic DNA was digested by *Hin*dIII and *Eco*RI, respectively. There is one hybridization bands with similar size in line 515-1 when the genomic DNA was digested by *Hin*dIII and *Eco*RI, respectively. In addition, both lines showed the single band when digested by *Hin*dIII/*Eco*RI, indicating that these loci have an entire expression cassette. The Southern analysis confirmed that 515-2 carried two loci and 515-1 carried one locus, which was consistent with the qPCR and genetic analysis (Table 5). These results again demonstrated that this qPCR protocol was an effective and accurate way to determine integration locus/loci of the transgene.

Table 4 Application of the qPCR protocol to gene pyramiding

	Genes	Total plants	Homozygotes	Hemizygotes	Negative plants	P value (χ^2) (3:1)
Round 1	*FUT8*	500	133	239	128	0.78
Round 2	*GalT*	133	36	62	35	0.89
Round 3	*AAT*	36	8	21	7	0.79

Table 5 Different insertion locus/loci as determined by genetic analysis and qPCR

Line number	No. of locus/loci (genetic analysis)	No. of plants detected in T_2	Theoretical no. of positive plants in T_2	Actual no. of positive plants in T_2	P value (χ^2)	Copy number (qPCR)
515-1	1	30	22.5	22	0.92 (3:1)	1
515-2	2	30	28.1	27	0.84 (15:1)	2
515-3	1	30	22.5	21	0.75 (3:1)	1
515-4	1	30	22.5	22	0.92 (3:1)	1
515-6	2	30	28.1	26	0.69 (15:1)	2

Discussion

A transgenic homozygote can be obtained from a T_1 generation; however, zygosity needs to be confirmed in the T_2 generation. Owing to difficulties in distinguishing plants with two identical copies from those with only one copy, neither PCR nor genomic Southern blot hybridization is typically used to identify transgenic homozygotes in a T_1 population [9]. Since there are no obvious visual differences between a homozygote and a heterozygote, there is a practical need to identify zygosity as early as possible. It usually takes an additional generation to confirm homozygosity through genetic analysis of transgene segregation in the T_2 population. In this study, we optimized a method that could effectively identify homozygous, heterozygous, and negative lines in the early segregation generation; this method could also determine transgene insertion locus/loci. Our results indicated that this protocol was accurate, effective, and reliable.
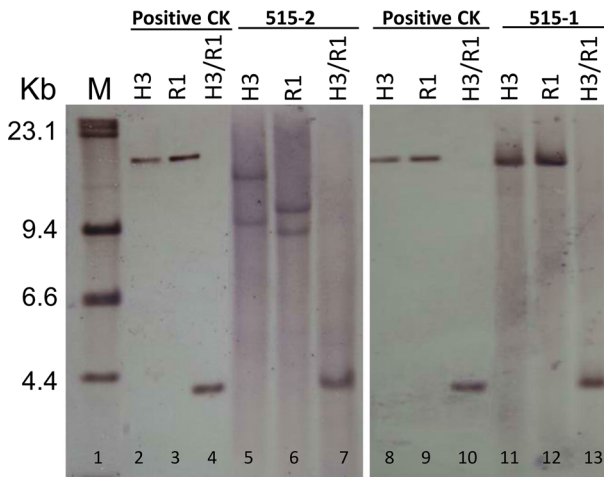

Fig. 3 Southern blot analyses of the transgenic lines 515-2 and 515-1. Genomic DNA was digested with *HindIII*, *EcoRI*, or *HindIII/EcoRI* and probed with the *FUT8* gene. A binary plasmid *pOsPMP515* digested by *HindIII*, *EcoRI*, and *HindIII/EcoRI*, respectively, was used as positive control. *M* represents λ DNA digested with *HindIII*, lanes *2–4* and *8–10* are the plasmid DNAs digested by *HindIII* (H3), *EcoRI* (R1), and *HindIII/EcoRI* (H3/R1), respectively, lanes *5–7* are the genomic DNAs of the transgenic line 515-2 digested by *HindIII*, *EcoRI*, and *HindIII/EcoRI*, respectively, and lanes *11–13* are the genomic DNAs of the transgenic line 515-1 digested by *HindIII*, *EcoRI*, and *HindIII/EcoRI*, respectively

Although qPCR has previously been used to identify homozygous plants in early generations [7–9, 18], none of the previously reported qPCR methods can simultaneously achieve high accuracy, reliability, and simplicity for determining transgene homozygosis and transgene stacking (Supplementary Table 2). For example, only 15–46 % of qPCR for copy number determination has been confirmed by Southern blotting analysis in a study involving qPCR screening [18]. In another qPCR-based method, the accuracy only reached 83 % [8]. Although different internal reference genes, qPCR parameters, and the formula of analysis were used in those methods, the accuracy has not reached a satisfactory level. In the present study, the accuracy of homozygote and negative plant identification reached 100 % when the line had a single insertion locus, and this was confirmed by PCR. Accuracy of homozygous line identification reached 92.3 % when the line had two insertion loci. qPCR for copy number determination reached 100 %, consistent with the genetic segregation ratio and Southern blotting analysis. We successfully demonstrated that this protocol is a practical and reliable protocol for three-gene stacking.

In addition, none of the qPCR-based method has been utilized for gene stacking to show the accuracy highly consistent among qPCR, genetic segregation, and Southern Blotting analysis [7–9, 19]. Our results indicated that our protocol not only was more effective and accurate than previously published qPCR methods, but also offered the distinct advantages of relative simplicity, rapid screening, and comparable accuracy over traditional methods such as Southern Blotting and previously published qPCR methods. The higher accuracy obtained in the present method is largely attributed to the selection of the appropriate internal reference gene, optimizing the qPCR parameters and primer design. This protocol could be also used in other crops with diploid genomes via minor modifications. For crops with polyploid genomes, this method would be expected to work well if an appropriate internal reference gene is selected and qPCR parameters and primer design are optimized. Furthermore, this method could be used to determine the zygosity of the endogenous gene of interest, induced mutation by TILLING, insertional mutation, or site-directed mutation introduced through a genome editing.

Furthermore, cost is an important issue when using qPCR methods at a large-scale screening homozygous plants in breeding programs. Most current qPCR methods have used TaqMan probes [8, 9]. We choose SYBR Green rather than TaqMan probe in this protocol because it is much cheaper, easier to use than TaqMan probe. In addition, TaqMan probe is used only once, requiring new synthesis and luminophores each time, while SYBR Green is nonspecific as it binds to any double-stranded DNA.

It is essential to develop a fast, accurate, and precise method for identifying homozygous lines for transgenic and molecular breeding. This would accelerate breeding programs and shorten breeding time. Furthermore, these requirements are especially important for breeding programs that use gene stacking or pyramiding. The proposed protocol, using SYBR Green qPCR, provides a simple and feasible approach applicable to molecular breeding and transgenic research. When three-gene stacking is used, breeding programs could be possibly shortened by between 6 months and 2 years, along with savings in labor and costs associated with field trials. For example, in a conventional three-gene stacking program, the selection of a homozygous line requires an F_2 generation for the first crossing. And then, a homozygous line from the F_3 generation is crossed with a line containing the third gene. This follows the same time course as the first round of crossing and selection. Therefore, at least 7 years/generations are required. However, three-gene stacking or pyramiding, selection of homozygous plants, and crossing of the line with the third gene can simultaneously be accomplished in the F_2 generation using our procedure (Fig. 2a). Therefore, only 4 years/generations are required when using this protocol. Additionally, fewer F_2 and F_3 populations and field trials are required in comparison to conventional breeding programs. Conventionally, to obtain homozygous

lines from a segregation population containing three genes, a minimum of ten lines is required from the F_2 generation and at least 500 individual plants from each line are required in the F_3 population for each cross. This requires 5,000 PCR reactions for a single gene, and the workload is also similarly tremendous for second- and third-round identifications of homozygous plants. Therefore, this protocol has numerous advantages over conventional breeding programs and traditional molecular biology approaches.

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