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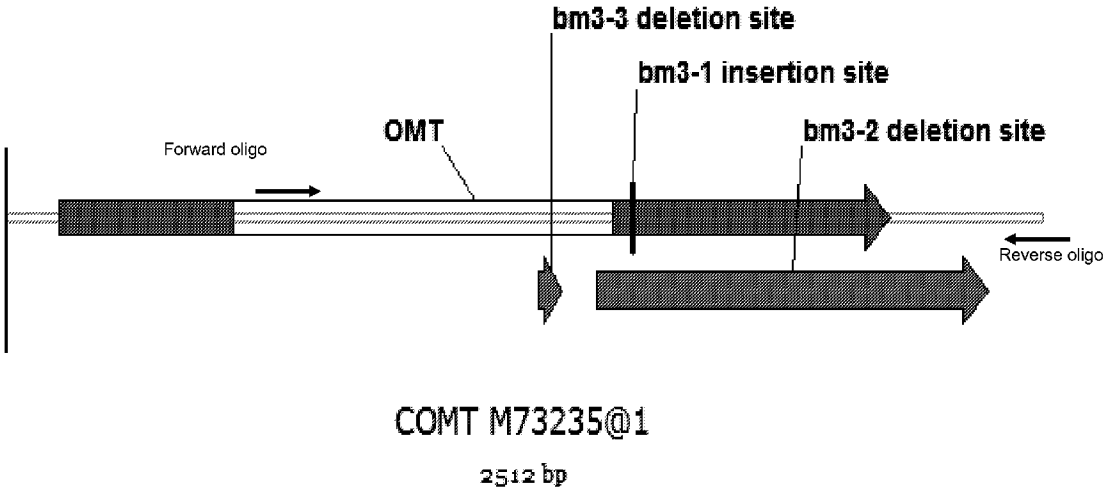
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(54) Titre : UTILISATION DE MARQUEURS SPECIFIQUES DU GENE BROWN MIDRIB-3 DANS LE MAIS A DES FINS D'INTROGRESSION DE CARACTERES

(54) Title: USE OF BROWN MIDRIB-3 GENE SPECIFIC MARKERS IN MAIZE FOR TRAIT INTROGRESSION



(57) Abrégé/Abstract:
This disclosure concerns compositions and methods for determining the zygosity of corn plants containing one or more brown midrib (BMR) mutations. The disclosure also concerns methods that are useful for enhancing the breeding process for BMR corn. In certain embodiments, compositions and methods for determining the zygosity of corn plants with respect to the bm3 allele.

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(54) **Title**: USE OF BROWN MIDRIB-3 GENE SPECIFIC MARKERS IN MAIZE FOR TRAIT INTROGRESSION

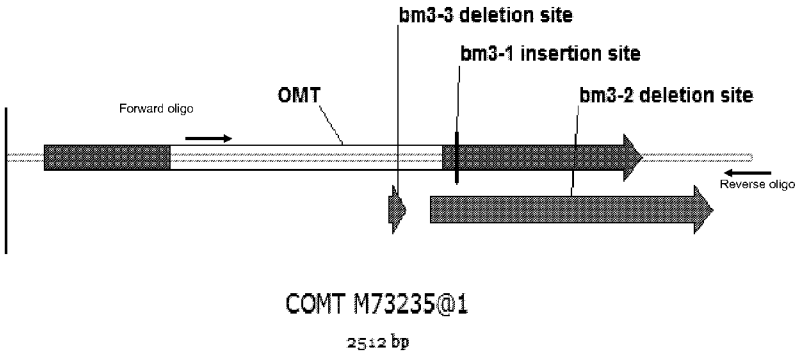


Fig. 1

(57) **Abstract**: This disclosure concerns compositions and methods for determining the zygosity of corn plants containing one or more *brown midrib* (BMR) mutations. The disclosure also concerns methods that are useful for enhancing the breeding process for BMR corn. In certain embodiments, compositions and methods for determining the zygosity of corn plants with respect to the *bm3* allele.

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USE OF BROWN MIDRIB-3 GENE SPECIFIC MARKERS IN MAIZE FOR TRAIT INTROGRESSION

PRIORITY CLAIM

This application claims the benefit of the filing date of United States Provisional Patent Application Serial Number 61/334,073, filed May 12, 2010, for "USE OF BROWN MIDRIB-3 GENE SPECIFIC MARKERS IN MAIZE FOR TRAIT INTROGRESSION."

TECHNICAL FIELD

The present disclosure relates generally to plant breeding. Methods are provided for determining the zygosity of plants containing *brown midrib* 3 (*bm3*) mutations. Methods of the disclosure are further useful for enhancing the breeding process for BMR-containing plant lines.

BACKGROUND

Lignins are universal components in plants that form cross-links with carbohydrates, such as hemicelluloses in the cell wall. Lignin polymers lower fiber digestion in ruminants, and the degree of lignifications may be inversely proportional to forage crop digestibility. Cherney *et al.* (1991) Adv. Agron. 46:157-98. Maize containing a *brown midrib* (BMR) mutation exhibit a reddish brown pigmentation of the leaf midrib that is associated with significantly reduced lignin content, altered lignin composition, and improved digestibility. At least four independent BMR mutations have been identified in maize. Kuc *et al.* (1968) Phytochemistry 7:1435-6. These mutations, termed "bm1, bm2, bm3, and bm4," all exhibit decreased lignin content when compared to control corn. *bm3* mutations include insertions (*bm3-1*), deletions (*bm3-2*), and insertion/deletions (*bm3-3*) within the *caffeic acid O-methyltransferase* (*COMT, e.g.*, GenBank Accession No. M73235) gene. Morrow *et al.* (1997) Mol. Breeding 3:351-7; Vignols *et al.* (1995) Plant Cell 7:407-16.

The *COMT* gene controls enzyme activities involved in lignin biosynthesis. *COMT* utilizes *S*-adenylsylmethionine to transmethylate caffeic acid, which results in the production of ferulic acid. Coniferyl alcohol and sinapyl alcohol are ultimately generated from the ferulic acid. The combination of coniferyl, ferulic, and sinapyl

alcohols in the presence of free radicals results in lignin production. *Bm3* mutations have been characterized, and are thought to inhibit the transmethylation of caffeic acid by gene inactivation. Guillet-Claude *et al.* (2004) Theor. Appl. Genet. 110: 126-35; Piquemal *et al.* (2002) Plant Physiology 130: 1-11; He *et al.* (2003) Crop Sci. 43:2240-51; Morrow *et al.* (1997) Mol. Breeding 3:351-7; Vignols *et al.* (1995) Plant Cell 7:407-16. However, a rapid method for specifically detecting and testing the zygosity of a particular plant at the *bm3* locus has not been developed.

DISCLOSURE OF THE INVENTION

Disclosed herein are methods for high-throughput PCR-based molecular characterization of BMR corn varieties (*e.g., bm3* mutants) that may greatly enhance the breeding process for BMR containing lines. Disclosed are methods for determining the zygosity of a plant tissue sample, and hence the plant from which the sample was prepared, by determining the presence or absence of a *bm3* mutant and the wild-type *COMT* alleles. Thus, an endpoint hydrolysis probe PCR-based zygosity assay (which is referenced as a TaqMan® assay herein) is provided that specifically detects and tests the zygosity status at the *bm3* locus. Disclosed are assays that utilize a biplex of oligonucleotides specific to a *bm3* mutant and to corresponding wild-type sequences in the same assay.

The present invention as claimed relates to:

- a method for determining the zygosity and/or presence/absence of a bm3 allele using corn plant tissue, the method comprising: obtaining a sample of isolated genomic DNA from the corn plant tissue; performing polymerase chain reaction (PCR) that comprises contacting the isolated genomic DNA with a set of four nucleic acid primer molecules in an amplification reaction to generate amplified genomic DNA, wherein each of the primers comprises (i) SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:7, and SEQ ID NO:8 or (ii) the complement of SEQ ID NO:4, complement of SEQ ID NO:5, complement of SEQ ID NO:7, and complement of SEQ ID NO:8; contacting the amplified genomic DNA with a first oligonucleotide probe comprising SEQ ID NO:6 or its complementary sequence and a second oligonucleotide probe comprising SEQ ID NO:9 or its complementary sequence, wherein the first oligonucleotide probe is labeled with a first reporter, and the second oligonucleotide probe is labeled with a second reporter, and the first reporter and the second reporter are fluorescent reporters with distinguishable excitation/emission; and detecting relative amount of fluorescent signals from the first reporter and second reporter, wherein the relative amount of

fluorescent signals indicate the zygosity, and/or presence or absence, respectively, of a bm3 allele in the genomic DNA; and

- a method for reliably and predictably introgressing a bm3 allele into plant germplasm, said method comprising: crossing a plant having a mutation in the COMT gene with another plant, to produce progeny; amplifying genomic DNA from each individual of the progeny; determining the zygosity and/or presence/absence of a bm3 allele in the genomic DNA from individual progeny according to the method of the invention; and selecting at least one of the individual progeny in which the relative amount of signal from the first reporter indicates that the bm3 allele has been introgressed into the germplasm of the plant.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 includes a schematic representation of *COMT* gene with *bm3* mutations and oligonucleotides designed for partial amplification of *COMT* gene (~1.8 kbp).

FIG. 2 includes a depiction of the amplification of partial *COMT* gene sequence from twelve *bm3* lines and three non-*bm3* lines (wild-type lines). PCR products were visualized on a 2% E-Gel® and then extracted through a 0.8% E-Gel® Clone Well for subsequent cloning into pCR4-TOPO® vector.

FIG. 3 includes an alignment of consensus sequences from *bm3* mutants and wild-type *COMT* genes. Three previously described deletion/insertion mutations are underlined.

FIG. 4 includes a schematic presentation of *COMT* gene with primers specific to *bm3* mutation and intact *COMT* gene. (A) *COMT* gene with dotted lines represents

bm3 deletion. *COMT* gene specific primers are within the deletion site. (B) *bm3* mutant gene. *bm3* specific oligonucleotides are in the junction where the forward and reverse oligonucleotides flanking the deletion site and the probe span the deleted region.

FIGs. 5a and 5b include real-time PCR amplification plots where relative fluorescence units (RFU) are shown for *bm3* (a) with FAM and (b) wild-type *COMT* with VIC (replaced with Hex because of the limitation of the real time PCR machine). Exponential amplification phase was observed from cycle 22 to 36 for both *bm3* and wild-type *COMT* genes.

FIG. 6 includes genotype determinations made using an allelic discrimination assay based on relative fluorescence units of FAM as allele 1 (homozygous *bm3*) and VIC (replaced with Hex because of the limitation of the machine) as allele 2 (homozygous wild-type *COMT*) at cycle 30.

FIG. 7 includes *bm3* zygosity determinations made with an end-point TaqMan® assay. On completion of PCR and fluorescence readings, a distribution graph was generated as described below: SOB1 = Signal over the background of FAM (sample signal over average of background signal at 535 nm), SOB2 = Signal over background of VIC (sample signal over average of background signal at 560 nm). Genotype determinations were made with SOB1/SOB2 < 1 for wild-type. 1< SOB1/SOB2< 5 for hemizygotes, and SOB1/SOB2 > 5 for homozygotes with a logarithmic scale.

FIG. 8 includes *bm3* zygosity determinations made with an end-point TaqMan® assay. The raw fluorescence intensity data directly from the plate reader was analyzed in KLIMS (KBioscience laboratory information management system). A graph with RFU (relative fluorescence units) of FAM plotted on the x-axis and VIC plotted on the y-axis was generated. Zygosity determinations were made based on the cluster separation in a cluster view.

SEQUENCES

The nucleic acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, as defined in 37 C.F.R. § 1.822. Only one strand of each nucleic acid sequence is shown, but the

complementary strand is understood as included by any reference to the displayed strand. In the accompanying sequence listing:

SEQ ID NO:1 shows a forward primer sequence (BM35_F) used to amplify a corn *COMT* partial gene: TACTCTACTGGCTGCGGCTAGC.

SEQ ID NO:2 shows a reverse primer sequence (BM35_R) used to amplify a corn *COMT* partial gene: TAACCTTGATTGTTATTACTCGCACATGG.

SEQ ID NO:3 shows an oligonucleotide sequence (BM1234R) from the *COMT* gene used to sequence a corn *COMT* partial gene: ATCAGCATCAGCCAGGCAGG.

SEQ ID NO:4 shows a forward primer sequence (BM3_F) used to identify the mutant *bm3* allele: AAAAAGAACGAGGTTGCAAAAGATA.

SEQ ID NO:5 shows a reverse primer sequence (BM3_R) used to identify the mutant *bm3* allele: TTAGAATCCACGACATGCAAGAG.

SEQ ID NO:6 shows a probe sequence (BM3_Probe) used to identify the mutant *bm3* allele, with FAM at the 5' end, and MGBNFQ at the 3' end: FAM-ACAAACCAAAGGATGTCG-MGBNFQ.

SEQ ID NO:7 shows a forward primer sequence (COMT_F) used to identify the wild-type *COMT* allele: CGCACTCGACGACGATGAC.

SEQ ID NO:8 shows a reverse primer sequence (COMT_R) used to identify the wild-type *COMT* allele: CACGCTGCTCAAGAACTGCTA.

SEQ ID NO:9 shows a probe sequence (COMT _Probe) used to identify the wild-type *COMT* allele, with VIC at the 5' end, and MGBNFQ at the 3' end: VIC-CATTTTCCGGCAGCGC-MGBNFQ.

SEQ ID NO:10 shows a *bm3* nucleotide sequence.

SEQ ID NO:11 shows a partial *COMT* nucleotide sequence.

MODE(S) FOR CARRYING OUT THE INVENTION

I. Overview of several embodiments

Disclosed herein are methods for high-throughput PCR-based molecular characterization of *bm3* corn varieties that may greatly enhance the breeding process for BMR containing plant lines. In particular embodiments, the method may comprise obtaining a sample of isolated genomic DNA from a corn plant, contacting the isolated genomic DNA with at least one nucleic acid molecule comprising a nucleotide

sequence capable of hybridizing to SEQ ID NO:10 under high stringency conditions and at least one nucleic acid molecule comprising a nucleotide sequence capable of hybridizing to SEQ ID NO:11 under high stringency conditions, and determining the zygosity of a *bm3* mutation in the isolated genomic DNA from the corn plant. In particular embodiments, at least one nucleic acid molecule comprising a nucleotide sequence capable of hybridizing to SEQ ID NO:10 under high stringency conditions and at least one nucleic acid molecule comprising a nucleotide sequence capable of hybridizing to SEQ ID NO:11 under high stringency conditions are between 10 and 35 nucleotides in length. In some embodiments, at least one nucleic acid molecule comprising a nucleotide sequence capable of hybridizing to SEQ ID NO:10 under high stringency conditions is at least 95% identical to between 10 and 35 contiguous nucleotides of SEQ ID NO:10. In some embodiments, at least one nucleic acid molecule comprising a nucleotide sequence capable of hybridizing to SEQ ID NO:11 under high stringency conditions is at least 95% identical to between 10 and 35 contiguous nucleotides of SEQ ID NO:11. In some embodiments, a nucleotide sequence capable of hybridizing to SEQ ID NO:11 under high stringency conditions is not capable of hybridizing to SEQ ID NO:10 under high stringency conditions. In certain embodiments, at least one nucleic acid molecule comprising a nucleotide sequence capable of hybridizing to SEQ ID NO:10 or SEQ ID NO:11 is selected from the group consisting of SEQ ID NOs:1-9.

Also disclosed are methods for identifying a BMR mutation in corn plants. In some embodiments, the method may comprise exposing genomic DNA from a corn plant to a nucleic acid molecule capable of hybridizing to SEQ ID NO:10 under high stringency conditions. In particular embodiments, a nucleic acid molecule capable of hybridizing to SEQ ID NO:10 under high stringency conditions may be selected from the group consisting of SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6.

Further disclosed are methods for reliably and predictably introgressing a trait for low lignin content (*e.g.*, through introgression of a *bm3* allele) into plant germplasm. In some embodiments, the method may comprise backcrossing a plant having a mutation in the *COMT* gene with another plant, obtaining a sample of isolated genomic DNA from a progeny plant produced by the cross, contacting the sample of isolated genomic DNA with at least one nucleic acid molecule comprising a nucleotide

sequence capable of hybridizing to SEQ ID NO:11 under high stringency conditions, and selecting progeny from the cross that comprise a mutation in the *COMT* gene by reproducing a plant from which a sample of isolated genomic DNA was obtained that binds with high stringency to at least one nucleic acid molecule comprising a nucleotide sequence capable of hybridizing to SEQ ID NO:11 under high stringency conditions, thereby producing a genetically engineered plant wherein a trait for low lignin content has been introgressed into the germplasm of the genetically engineered plant.

Also disclosed are corn plants with genotype and/or zygosity determined according to high-throughput PCR-based molecular methods of the disclosure, as well as genetically engineered corn plants exhibiting a trait for low lignin content that are produced according to methods of the disclosure for reliably and predictably introgressing a trait for low lignin content into corn plant germplasm.

II. Abbreviations

BMR	*brown midrib*
MGBNFQ	Minor Grove Binding Non Flourescence Quencher™ I
FAM	6-carboxy-fluorescein
PCR	polymerase chain reaction
RFU	relative fluorescence units
VIC	6-carboxy-rhodamine

III. Terms

In the description and tables, which follow, a number of terms are used. In order to provide a clear and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided:

Backcrossing. Backcrossing is a process in which a breeder repeatedly crosses hybrid progeny back to one of the parents, for example, a first generation hybrid F_1 with one of the parental genotypes of the F_1 hybrid.

BMR corn: As used herein, the term "BMR corn" refers to corn varieties that contain a *brown midrib* mutation, such as the mutations characterized as *bm1*, *bm2*, *bm3*, and *bm4*. BMR corn varieties typically exhibit a reddish brown pigmentation of

the leaf midrib. BMR corn is also typically characterized by lower lignin content, higher fiber digestibility, and higher dry matter intake. Non-limiting examples of BMR corn varieties include F2F297, F2F383, F2F488, F2F449, F2F566, F2F610, F2F622, F2F665, F2F633, F2F682, F2F721, F2F700, and F2F797.

Hybridization: Oligonucleotides and their analogs hybridize by hydrogen bonding, which includes Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary bases. Generally, nucleic acid molecules consist of nitrogenous bases that are either pyrimidines (cytosine (C), uracil (U), and thymine (T)) or purines (adenine (A) and guanine (G)). These nitrogenous bases form hydrogen bonds between a pyrimidine and a purine, and the bonding of the pyrimidine to the purine is referred to as "base pairing." More specifically, A will hydrogen bond to T or U, and G will bond to C. "Complementary" refers to the base pairing that occurs between two distinct nucleic acid sequences or two distinct regions of the same nucleic acid sequence.

"Specifically hybridizable" and "specifically complementary" are terms that indicate a sufficient degree of complementarity such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. The oligonucleotide need not be 100% complementary to its target sequence to be specifically hybridizable. An oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA, and there is sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide to non-target sequences under conditions where specific binding is desired, for example under physiological conditions in the case of *in vivo* assays or systems. Such binding is referred to as specific hybridization.

Hybridization conditions resulting in particular degrees of stringency will vary depending upon the nature of the chosen hybridization method and the composition and length of the hybridizing nucleic acid sequences. Generally, the temperature of hybridization and the ionic strength (especially the Na^+ and/or Mg^{2+} concentration) of the hybridization buffer will contribute to the stringency of hybridization, though wash times also influence stringency. Calculations regarding hybridization conditions required for attaining particular degrees of stringency are discussed in Sambrook *et al.*

(ed.), Molecular Cloning: A Laboratory Manual, 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, chs. 9 and 11.

For purposes of the present disclosure, "stringent conditions" encompass conditions under which hybridization will occur if there is less than 25% mismatch between the hybridization molecule and the target sequence. "Stringent conditions" can be further defined into particular levels of stringency. Thus, as used herein, "moderate stringency" conditions are those under which molecules with more than 25% mismatch will not hybridize. Conditions of "medium stringency" are those under which molecules with more than 15% mismatch will not hybridize, and conditions of "high stringency" are those under which sequence with more than 10% mismatch will not hybridize. Conditions of "very high stringency" are those under which sequences with more than 6% mismatch will not hybridize.

In particular embodiments, stringent conditions can include hybridization at 60°C in TaqMan® genotyping master mix (Applied Biosystems, Foster City, CA, Catalog #4371355), diluted according to the manufacturer's instructions.

Isolated: An "isolated" biological component (such as a nucleic acid or protein) has been substantially separated, produced apart from, or purified away from other biological components in the cell of the organism in which the component naturally occurs, *i.e.*, other chromosomal and extra-chromosomal DNA and RNA, and proteins. Nucleic acid molecules and proteins that have been "isolated" include nucleic acid molecules and proteins purified by standard purification methods. The term also embraces nucleic acids and proteins prepared by recombinant expression in a host cell, as well as chemically-synthesized nucleic acid molecules, proteins, and peptides.

Oligonucleotide: An oligonucleotide is a short nucleic acid polymer. Oligonucleotides may be formed by cleavage of longer nucleic acid segments, or by polymerizing individual nucleotide precursors. Automated synthesizers allow the synthesis of oligonucleotides up to several hundred base pairs in length. Because oligonucleotides may bind to a complementary nucleotide sequence, they may be used as probes for detecting DNA or RNA. Oligonucleotides composed of DNA (oligodeoxyribonucleotides) may be used in PCR, a technique for the amplification of small DNA sequences. In PCR, the oligonucleotide is typically referred to as a

"primer," which allows a DNA polymerase to extend the oligonucleotide and replicate the complementary strand.

Zygosity: As used herein, the term "zygosity" refers to the similarity, or lack thereof, of alleles of a gene for an inherited trait in an organism. If both alleles are the same, the organism is "homozygous" for the trait. If both alleles are different, the organism is "heterozygous" for that trait. If one allele is missing, it is "hemizygous." If both alleles are missing, it is "nullizygous."

Unless otherwise specifically explained, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which this disclosure belongs. Definitions of common terms in molecular biology can be found in, for example, Lewin B., Genes V, Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew *et al.* (eds.), The Encyclopedia of Molecular Biology, Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Meyers R.A. (ed.), Molecular Biology and Biotechnology: A Comprehensive Desk Reference, VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

IV. High-throughput method for genotype and zygosity determinations of bmr corn

A. Overview

Described herein is a gene specific end-point TaqMan® PCR assay, generally useful for zygosity analysis of BMR corn or putative BMR corn. Particular examples utilize a biplex of oligonucleotides specific to a *bm3* deletion, and to the corresponding wild-type sequences in the same assay. In these examples, zygosity may be determined by the presence/absence of the *bm3* mutant and the wild-type *COMT* alleles. The assay has been submitted to high-throughput genomic analysis group for further implementation and may greatly enhance the breeding process for BMR introgression projects.

B. *Brown midrib* corn

Brown midrib (BMR) corn plants are characterized by brown pigmentation in the leaf midrib at the V4 to V6 stage and a light brown coloration of the pith after tasselling. *Brown midrib* hybrid corn contains a gene mutation that causes lower lignin content in corn plant tissue, for example, a *bm2* mutation, or a *bm3* mutation. The

brown midrib3 gene is located on the short arm of chromosome 4, and the *bm3* allele is recessive. The *brown midrib2* gene is located on the long arm of chromosome 1, and the *bm2* allele is also recessive. Lignin polymers limit the digestibility of the fiber in the corn plant. The reduced lignin in *brown midrib* corn results in silage with fiber that is more digestible than normal corn. Animal feeding trials have shown about 10 percent greater intake and increased milk production with BMR corn silage, as compared to normal silage.

Methods are provided that identify *bm3* mutations. Methods of the disclosure may be useful, for example, in the identification of a particular corn plant, or particular corn variety, as BMR corn. Disclosed methods may also be useful in the reliable and predictable introgression of a BMR trait into corn germplasm by crossing BMR corn with other corn varieties, or by crossing BMR corn containing a first BMR mutation with BMR corn containing a second BMR mutation (for example, crossing a *bm1* corn variety with a *bm3* corn variety). Many BMR mutations are known to those of skill in the art, and some BMR mutations have been characterized (*e.g.*, mapped and sequenced). Methods of the disclosure may be used to identify a BMR mutation, determine the genotype and/or zygosity of a BMR corn plant or variety, and for the introgression of any BMR mutation into corn germplasm.

C. End-point PCR detection assay

Described herein is a gene specific end-point TaqMan® PCR assay, generally useful for zygosity analysis of BMR corn or putative BMR corn. In particular embodiments, a gene specific end-point TaqMan® PCR assay may be used to analyze the zygosity of corn for a *bm3* mutation.

Primers and probes for use in a gene specific end-point TaqMan® PCR assay may be designed based on a known mutation in the gene of interest. For example, primers and probes for a *bm3*-specific assay may be designed based on a 978 bp deletion at the 3' end of the caffeic acid *O*-methyltransferase (*COMT*) gene. In biplex reactions, wherein oligonucleotides specific to the mutation (*e.g.*, *bm3*) and the undisrupted wild-type gene (*e.g.*, *COMT*) are used in the same assay, the specific oligonucleotides will selectively amplify sequence from either or both of the mutated gene and/or the wild-type gene that is present in a genomic DNA sample.

In some embodiments, a *bm3*-specific assay amplifies a fragment that is 71 bp in length, which is unique to the junction site where 978 bp of nucleotide sequence was deleted from the wild-type *COMT* gene. In certain embodiments, a target-specific oligonucleotide probe (*e.g.*, BM3_Probe (SEQ ID NO:6)) hybridizes under high stringency conditions to a target sequence in a genomic DNA sample between two PCR primers (*e.g.*, BM3_F (SEQ ID NO:4), and BM3_R(SEQ ID NO:5)).

In some embodiments, a wild-type *COMT* gene-specific assay amplifies a fragment of the *COMT* gene that is 65 bp in length and which is located within exon 2 (deleted in *bm3* mutants), such that non-*bm3* sequences at the *COMT* locus may not be amplified. In certain embodiments, a target-specific oligonucleotide probe (*e.g.*, COMT_Probe (SEQ ID NO:9)) hybridizes under high stringency conditions to a target sequence in a genomic DNA sample between two PCR primers (*e.g.*, COMT_F (SEQ ID NO:7), and COMT_R(SEQ ID NO:8)).

Target-specific oligonucleotides may be labeled, for example, with fluorescent dyes (*e.g.*, FAM, VIC, and MGBNFQ), which may allow rapid quantification of a target-specific fluorescent signal. PCR products may be measured after a pre-determined number of cycles, for example, when the reaction is in the early exponential phase. Negative control samples may comprise genomic DNA from any corn variety, for example, without a *bm3* mutation. Positive control samples may comprise genomic DNA from a corn variety with a BMR mutation, such as a *bm3* deletion mutation in the *COMT* gene. Control hemizygous samples may comprise either genomic DNA from a corn variety predetermined to be hemizygous for a *bm3* mutation; or a hemizygous sample may comprise equal proportions of negative control DNA to DNA from a corn variety predetermined to be homozygous for a *bm3*.

DNA may be isolated (for example, extracted, and purified) from corn plant tissue by methods known to those of skill in the art. Commercial kits for DNA isolation are available, for example, from Qiagen, Inc. In some embodiments, leaf discs from a particular plant are punched and transferred into collection tubes. The puncher may be cleaned after each sampling with 70% alcohol, rinsing in water, and drying. DNA extraction buffers may be prepared according to the manufacturer's recommendations. DNA may then be isolated using the kit according to the manufacturer's instructions. Finally, the concentration of the isolated DNA may be

determined using, for example, a Quant-iT™ PicoGreen® Quantfication Kit (Invitrogen, Carlsbad, CA) and a spectrophotometer, or by any other suitable technique.

Once primers, probes, and genomic DNA sample(s) have been prepared or otherwise made available, a PCR reaction may be conducted to identify nucleic acid sequences of interest (*e.g.*, sequences particular to a BMR mutation) in the genomic DNA sample(s). In particular embodiments, individual PCR reaction mixtures are prepared that contain all the reaction components, except the genomic DNA sample(s). For a biplex reaction comprising primers and gene-specific probes for *bm3* mutant and wild-type *COMT* corn, the reaction mixture may comprise enzyme, reaction buffer, forward and reverse primers for the bm3 mutation, forward and reverse primers for the wild-type *COMT* gene, a gene-specific probe for the bm3 mutation, a gene-specific probe for the *COMT* gene, and water. In some PCR assay systems (*e.g.*, TaqMan® PCR assays), enzyme and buffer may be present in a single kit component (*e.g.*, TaqMan® genotyping master mix; Applied Biosystems, Foster City, CA, Catalog # 4371355).

Once the reaction mixture is otherwise prepared, genomic DNA sample(s) may be added, and the reaction commenced. It is not necessary to normalize the amounts of genomic DNA in a sample. However, the skilled artisan may attain best results by using genomic DNA samples with relatively equal concentrations.

In some embodiments, a PCR assay (*e.g.*, a TaqMan® PCR assay) can be set up with appropriate controls. For example, a reaction in a multi-well plate may be performed with control wells comprising: (1) negative control(s) with reagents but no DNA sample; (2) homozygous positive control(s) comprising *bm3* corn genomic DNA; (3) and hemizygous positive control(s), as described above. DNA is then amplified by PCR under suitable cycle conditions. For example, in some embodiments using a GenAmp® PCR System 9700, there may be a single initial denaturation cycle at 95°C for 15 minutes, followed by 30 cycles of denaturation (92°C for 15 seconds) and annealing/extension (60°C for 60 seconds). Those of skill in the art understand that PCR cycle conditions may be varied according to the practitioner's discretion, and comparable results obtained.

D. Determination of genotype and/or zygosity

A PCR assay (*e.g.*, an end-point TaqMan® PCR assay) may be used for genotype and/or zygosity analysis of BMR corn or putative BMR corn. In some embodiments, a gene-specific oligonucleotide probe may be labeled with a reporter (*e.g.*, a fluorescent moiety). For assays using fluorometric detection, PCR reaction products may be analyzed in a spectrofluorometer (*e.g.*, Tecan GENios™; Männedorf, Switzerland) using excitation and emission wavelength settings appropriate for the detection of the probe(s). For example, the fluorescent dye, FAM, may be measured with an excitation wavelength of 485 nm, and an emission wavelength of 535 nm. Alternatively, the fluorescent dye, VIC, may be measured with an excitation wavelength of 525 nm, and an emission wavelength of 560 nm.

Following the completion of the PCR reaction and probe detection, a table and distribution graph may be generated using, for example, any suitable computer graphics software. Results obtained with wild-type, hemizygous, and homozygous DNA of similar genotypic background may serve as negative and positive controls. In a segregating population, three clusters of data points may be obtained allowing the visual determination of a sample result as likely belonging to one of the segregated clusters. Alternatively, data analysis computer software may be used to calculate the probability that a sample result belongs to each segregated cluster, with the most probable cluster serving as the sample designation. When a visual determination is made, the boundary of each cluster may be arbitrary, for example, when three clusters of data points are clearly visible.

Raw fluorescence intensity data may also be analyzed directly from a plate reader using a suitable analysis package, such as KLIMS (KBioscience laboratory information management system). A graph with the relative fluorescence units (RFU) of a fluorescence signal generated by a specific probe for a mutant allele plotted on one axis, and the RFU of a fluorescence signal generated by a specific probe for the wild-type allele plotted on the other axis may be generated. Zygosity determinations may then be made based on the cluster separation in a graphical display of the data.

Samples that do not contain mutant genomic DNA (*e.g.*, a BMR mutation) may only result in fluorescence readings of the wild-type PCR product. Samples containing hemizygous or homozygous mutant genomic DNA may result in RFU readings for the

mutant-specific probe higher than that of a negative background control. If a sample yields no adequate results, the genomic DNA in the sample may not be of adequate quality and/or quantity, and a new DNA preparation and/or new PCR reaction should be performed. Preferably, a negative control sample containing no DNA sample shows very low detection of gene-specific probe(s). It is also preferable that known homozygous controls show only high detection of the mutant or wild-type DNA in the control, and that known hemizygous controls show both high detection of the mutant and wild-type DNA.

A "test run" of the PCR method and genotype and/or zygosity determination may be performed with all appropriate controls prior to screening of samples. Further optimization of the methods may be desirable for components that may differ among uses (*e.g.*, method of genomic DNA preparation, Taq DNA polymerase, oligonucleotides, laboratory equipment, etc.). PCR and thermal cycling conditions may be established that amplify both mutant and/or wild-type sequences in a known genomic DNA template with acceptable levels of probe detection (*e.g.*, acceptable RFU for fluorescently labeled oligonucleotide probes).

E. Introgression of a trait for low lignin content into plant germplasm

Described herein are methods for producing a corn plant with low lignin content (*e.g.*, BMR corn), through conventional plant breeding involving sexual reproduction. Methods may comprise crossing a first parent corn plant that comprises in its genome at least one copy of a BMR mutation (*e.g.*, *bm3*, *bm3-1*, *bm3-2*) to a second parent corn plant, so as to produce F_1 progeny. The first plant can be any BMR corn plant including, for example, BMR corn varieties F2F297, F2F383, F2F488, F2F449, F2F566, F2F610, F2F622, F2F665, F2F633, F2F682, F2F721, F2F700, and F2F797. The second parent corn plant can be any corn plant that is capable of producing viable progeny corn plants (*i.e.*, seeds) when crossed with the first corn plant. The first and second parent corn plants may be of the same corn species (*e.g.*, *Zea mays* (maize)). The methods may further involve selfing the F_1 progeny to produce F_2 progeny. Methods may further involve one or more generations of backcrossing the F_1 or F_2 progeny plants to a plant of the same line or genotype as either the first or second parent corn plant. Alternatively, the F_1 progeny of the first

cross, or any subsequent cross, can be crossed to a third corn plant that is of a different line or genotype than either the first or second plant.

In some embodiments, progeny plants are subjected to a genotype and/or zygosity determination of the disclosure. Once progeny plants have been genotyped, and/or their zygosity has been determined, the skilled artisan may select those progeny plants that have a desired genetic composition. Such selected progeny plants may be used in further crosses, selfing, or cultivation. Methods of introgression of a BMR mutation that are directed according to methods of the disclosure reduce or eliminate the cultivation and/or reproduction of plants that do not have a desired genetic composition, and thereby provide desirable reliability and predictability (through expected Mendelian patterns of inheritance).

The following Examples are provided to illustrate certain particular features and/or embodiments. The following Examples should not be construed to limit the disclosure to the particular features or embodiments described.

EXAMPLES

Example 1: Materials and Methods

Plant and genetic material. Corn leaf samples containing homozygous *bm3* alleles and homozygous wild type *COMT* alleles were provided.

Isolation of total genomic DNA and quantification. Eight leaf discs were punched per sample, and were ground to a fine powder using a Genogrinder® 2000. DNA was extracted with Qiagen DNeasy™ 96-well kit (Valencia, CA). Prior to PCR, DNA samples were quantified with Quant-iT™ PicoGreen® Quantification Kit (Invitrogen, Carlsbad, CA) using the manufacturer's instructions.

Cloning and sequencing of partial *COMT* gene. Corn genomic DNA fragments of a partial *COMT* gene were amplified from twelve *bm3* lines and three non-*bm3* samples (see Table 1) with oligos BM35_F (5'-TACTCTACTGGCTGCGGCTAGC -3'; SEQ ID NO:1) and BM34_R (5'-TAACCTTGATTGTTATTACTCGCACATGG -3'; SEQ ID NO:2) using ABI GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA) in reactions containing 2.5 units of TaKaRa LA Taq™ (Takara Bio Inc., Shiga, Japan), 400 nM dNTP, 200 nM forward (BM35_F) and reverse primer (BM34_R) and 30 ng of genomic DNA. The PCR program began with two minutes of

denaturing at 94° C, followed by 30 cycles of 94° C for 45 seconds, 55° C for 45 seconds, and 72° C for two minutes. PCR products were visualized on a 2% E-Gel® (Invitrogen, Carlsbad, CA), and then extracted on 0.8% E-Gel® CloneWells™. Purified PCR products were then cloned into pCR4-TOPO® vector (Invitrogen, Carlsbad, CA), according to the manufacturer's instruction. Selected colonies were grown overnight in 1X freezer media containing 2.5% LB (10g tryptone, 10g NaCl, and 5g yeast extract for 1L LB media), 36 mM K_2HPO_4, 13 mM KH_2PO_4, 1.7 mM sodium citrate, 6.8 mM $(NH_4)_2SO_4$, 4.4% glycerol, 0.4 mM $MgSO_4 \cdot 7H_2O$, 12.5 µg/mL chloramphenicol (added immediately before use), and 50 µg/ml Kanamycin. Selected colonies were then sent to Cogenics® (Houston, TX) for sequencing with T7 and T3 promoter, as well as BM1234R (5'- ATCAGCATCAGCCAGGCAGG- 3'; SEQ ID NO:3), which is located within the *COMT* gene. Sequences were analyzed using Sequencher® 4.8 software. *Bm3* mutations were identified by comparing consensus sequences from *bm3* lines to wild type *COMT* samples.

Table 1: List of twelve *bm3* and three non-*bm3* inbred lines used for cloning and sequencing of *COMT* genes.

Genotype	Inbred lines
bm3 lines	SMM06BM
	SLD32BM
	SRD02BM
	11084BM
	BE1146BMR
	IAA18BM
	3633BMR
	PIA05BM
	PED04BM
	LLD44BM
	PEA55BM
	SMA96BM
COMT wild type	SLM07
	6XN442
	515Dbm1

TaqMan® PCR assay design and validation. Based on the DNA consensus sequences from *bm3* lines, primers (BM3_F; SEQ ID NO:4 and BM3_R; SEQ ID NO:5), and probe (BM3_Probe; SEQ ID NO:6) specific to the junction where partial sequences from *COMT* gene at the 3' end was deleted for identifying the mutant allele;

primers (COMT_F and COMT_R) and probe (COMT_probe) within the deleted region for identifying the wild type alleles were designed. Primer Express 3.0 was used for TaqMan® assay design. All primers, and dual labeled probes with FAM or VIC and Minor Grove Binding Non Flourescence Quencher™ I (MGBNFQ) dyes, were synthesized by Applied Biosystems (Foster City, CA). All primers and probes were dissolved in 1X Tris-EDTA to 100 µM. TaqMan® genotyping master mix (Applied Biosystems, Foster City, CA, Catalog #4371355) were used for all PCR reactions.

Real time PCR reactions in 20 µL volume were set up according to Table 2 using 96-well plate on an iCycler® optical system (BioRad, Hercules, CA), starting with 15 minutes of denaturing at 95° C, followed by 50 cycles of 92° C for 15 seconds, and 60° C for 1 minute. Fluorescence signals were recorded at the end of each cycle.

Table 2: PCR mixtures for each biplex reaction with 20 µL final volume.

Component	Volume (µL)
Taqman Genotyping Master Mix (2X, cat# 4371355)	10
BM3_F (100 µM)	0.18
BM3_R (100 µM)	0.18
COMT_F (100 µM)	0.18
COMT_R (100 µM)	0.18
BM3_Probe (100 µM)	0.04
COMT_Probe (100 µM)	0.04
DNA (20 to 50ng/µl)	1
Water	8.2
Total volume	20

End-point TaqMan® PCR assays in 10 µl volume were set up according to Table 3 using 384-well plates. ABI GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA) was used for amplification, starting with 15 minutes of denaturing at 95° C, followed by 30 cycles of 92° C for 15 seconds, and 60° C for 1 minute. PCR products were measured by a spectrofluorometer (Tecan GENios™, Männedorf, Switzerland) after an optimal number of cycles, when the reactions were in the early exponential phase with recommended instrument settings (FAM (*bm3* mutant): Excitation- 485 nm, Emission- 535 nm; VIC (wild-type *COMT*): Excitation- 525 nm, Emission- 560 nm).

Table 3: PCR mixtures for each biplex reaction with 10 µl final volume.

Component	Volume (µL)
Taqman Genotyping Master Mix (2X, cat# 4371355)	5
BM3_F (100 µM)	0.09
BM3_R (100 µM)	0.09
COMT_F (100 µM)	0.09
COMT_R (100 µM)	0.09
BM3_Probe (100 µM)	0.02
COMT_Probe (100 µM)	0.02
DNA (10 to 20 ng/µl)	1
water	3.6
Total volume	10

Data analysis. For real-time PCR, the fluorescence threshold was calculated automatically with its value slightly above the background by iCycler® software. The threshold cycle (Ct value) was determined by the number of cycles needed to generate fluorescence above the established threshold. An allelic discrimination assay was then used to determine genotypes.

Following completion of the endpoint TaqMan® PCR and fluorescence reading, signal over the background of report dye 1 (SOB1) and signal over background of report dye 2 (SOB2) were calculated. A distribution graph between sample numbers and absolute ratios of SOB1/SOB2 in a logarithmic scale were generated. Samples of wild-type, hemizygous, and homozygous plants of similar genotypic background were used as controls. Genotype determinations were made based on the cluster separations.

The raw fluorescence intensity data directly from the plate reader were also analyzed using KLIMS (KBioscience laboratory information management system). Graphs with RFU (relative fluorescence units) of FAM plotted on the x-axis and VIC plotted on the y-axis were generated. Zygosity determinations were made based on the cluster separation in a cluster view.

Example 2: Sequence Analysis of partial *bm3* mutations of the *COMT* gene

To design a gene specific TaqMan® assay for *bm3* mutants, precise sequence information was needed. Therefore, two oligonucleotides were designed to amplify partial *COMT* gene (FIG. 1) from genomic DNA from twelve *bm3* and three non-*bm3*

lines. FIG. 2. These fragments were cloned into pCR4-TOPO® vector, and sent to Cogenics, Inc. for sequencing with T7 and T3 promoter, as well as BM1234R (SEQ ID NO:3). BM1234R is located in the middle of the *COMT* gene and was used to achieve the full-length sequence information. High quality sequences from nine *bm3* lines were analyzed and aligned using Sequencher® 4.8. Consensus sequences from *bm3* lines were compared with wild-type samples. Multiple deletion/insertion mutations at the 3' end of the *COMT* gene were identified. For example, three mutations were confirmed and underlined. FIG. 3.

Gene specific assay design and validation. All the *bm3* lines we have tested contain a large deletion mutation at the 3' exon of the *COMT* gene. This mutation was then used for gene specific TaqMan® assay design. FIG. 4 includes a schematic representation showing the locations of oligonucleotides. Wild-type *COMT* gene specific primers and probes are located within the 978 bp deletion site. *Bm3* specific primers and probes are located in the junction where the forward and reverse oligonucleotides flanking the deletion site and the probe span the deleted region.

The twelve known *bm3* lines and the three non-*bm3* lines were used to test the gene specific TaqMan® assay. Six hemizygous samples were made by combining equal amounts of DNA from *bm3* lines with non-*bm3* lines. Real time PCR was used to test the efficiency of the assay. Oligonucleotides specific to the *bm3* mutants and to the corresponding wild-type sequences were combined in the same assay. FAM was used to monitor the amplicon of *bm3*, and VIC was used to monitor the amplicon for wild-type *COMT*. Exponential amplification phase was observed from cycle 22 to 36 for both *bm3* and wild-type *COMT* genes. FIGs. 5a and 5b. Correct genotype determinations were generated using an allelic discrimination assay based on FAM as allele 1 and VIC as allele 2 at cycle 30. FIG. 6.

Validation of end-point TaqMan® zygosity analysis. One 96-well plate of DNA samples containing *bm3* populations segregating as homozygous, hemizygous, and null (wild -type) was used to test and validate an end-point TaqMan® PCR assay. One advantage of end-point TaqMan® PCR is the ease of use. End-point TaqMan® PCR does not require a more expensive real-time PCR machine. Any regular PCR machine fit for 96- or 384-well plates, and a plate reader capable of reading the relevant fluorescent signal are sufficient to carry out the assay.

Based on the results from real-time PCR, 30 cycles of PCR reaction can efficiently separate the *bm3* genes from the wild-type *COMT* genes. The PCR reaction was terminated at cycle 30. Following completion of the TaqMan® PCR and fluorescence reading, fluorescence signals of FAM (*bm3*) over background (H_2O), as signal over background 1 (SOB1); and VIC (wild-type *COMT*) over background 2 (SOB2) were calculated. A distribution graph was generated using SOB1/SOB2 in a logarithmic scale. FIG. 7. Wild-type, hemizygous, and homozygous controls of similar genotypic background served as negative and positive controls. In a segregating population, three clusters of data points were obtained, allowing the cut-off points to be visually determined. For the example in FIG. 7, three clusters of data points were clearly visible. The data points for wild-type are less than 1, those for hemizygous samples range from 1 to 5, and those for homozygous are above 5.

The raw fluorescence intensity data directly from the plate reader was also analyzed in KLIMS (KBioscience laboratory information management system). A graph with RFU (relative fluorescence unit) of FAM plotted on the x-axis, and VIC plotted on the y-axis was generated. Zygosity determinations were made based on the cluster separation in a cluster view. FIG. 8. Since FAM was used to monitor the amplification of *bm3*, and VIC was used to monitor the amplification of wild-type *COMT*, samples with strong signals of FAM, and little or no VIC signal, are homozygous *bm3*; samples with strong signals of VIC, and little or no FAM, are wild-type *COMT* (nulls); and samples with strong signals of both FAM and VIC are hemizygous.

Genotype and zygosity determinations from this *bm3* gene specific end-point TaqMan® PCR assay matched 100% with phenotypic data collected in the field. This assay provides an easy and accurate way to characterize the *bm3* zygosity status in a high-throughput manner.

Example 3: Introgression of *bm3* mutation

The zygosity of a corn plant with respect to the *bm3* mutation is determined as described, *supra*. It is determined that the corn plant is homozygous for the *bm3* mutation. The plant is crossed through conventional plant breeding with a corn plant known to be homozygous for the wild-type *COMT* gene, and a corn plant homozygous

for the *bm3* mutation. F_1 progeny of the cross are produced. F_1 progeny of the cross are then selfed to produce F_2 progeny. Samples of genomic DNA of the F_2 progeny are prepared and the zygosity of the F_2 progeny plants are determined as described, *supra*.

F_2 progeny plants are selected that are homozygous for the *bm3* mutation. The selected progeny are then assayed for low lignin content, and those progeny that exhibit desirably low lignin levels are further reproduced by crossing and selfing, and the resulting progeny are cultivated.

While the present invention has been described herein with respect to certain preferred embodiments, those of ordinary skill in the art will recognize and appreciate that it is not so limited. Rather, many additions, deletions, and modifications to the preferred embodiments may be made without departing from the scope of the invention as hereinafter claimed. In addition, features from one embodiment may be combined with features of another embodiment while still being encompassed within the scope of the invention as contemplated by the inventors.

SEQUENCE LISTING IN ELECTRONIC FORM

In accordance with Section 111(1) of the Patent Rules, this description contains a sequence listing in electronic form in ASCII text format (file: 68483-80 Seq 11-SEP-12 v1.txt).

A copy of the sequence listing in electronic form is available from the Canadian Intellectual Property Office.

CLAIMS:

1. A method for determining the zygosity and/or presence/absence of a *bm3* allele using corn plant tissue, the method comprising:

obtaining a sample of isolated genomic DNA from the corn plant tissue;

performing polymerase chain reaction (PCR) that comprises contacting the isolated genomic DNA with a set of four nucleic acid primer molecules in an amplification reaction to generate amplified genomic DNA, wherein each of the primers comprises (i) SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:7, and SEQ ID NO:8 or (ii) the complement of SEQ ID NO:4, complement of SEQ ID NO:5, complement of SEQ ID NO:7, and complement of SEQ ID NO:8;

contacting the amplified genomic DNA with a first oligonucleotide probe comprising SEQ ID NO:6 or its complementary sequence and a second oligonucleotide probe comprising SEQ ID NO:9 or its complementary sequence, wherein the first oligonucleotide probe is labeled with a first reporter, and the second oligonucleotide probe is labeled with a second reporter, and the first reporter and the second reporter are fluorescent reporters with distinguishable excitation/emission; and

detecting relative amount of fluorescent signals from the first reporter and second reporter, wherein the relative amount of fluorescent signals indicate the zygosity, and/or presence or absence, respectively, of a *bm3* allele in the genomic DNA.

2. The method of claim 1, wherein the first or second reporter is 6-carboxy-fluorescein (FAM), and wherein the other reporter is 6-carboxy-rhodamine (VIC).

3. A method for reliably and predictably introgressing a *bm3* allele into plant germplasm, said method comprising:

crossing a plant having a mutation in the *COMT* gene with another plant, to produce progeny;

amplifying genomic DNA from each individual of the progeny;

determining the zygosity and/or presence/absence of a *bm3* allele in the genomic DNA from individual progeny according to the method of claim 1; and

selecting at least one of the individual progeny in which the relative amount of signal from the first reporter indicates that the *bm3* allele has been introgressed into the germplasm of the plant.

4. The method of claim 3, wherein the step of amplifying genomic DNA comprises performing polymerase chain reaction (PCR).

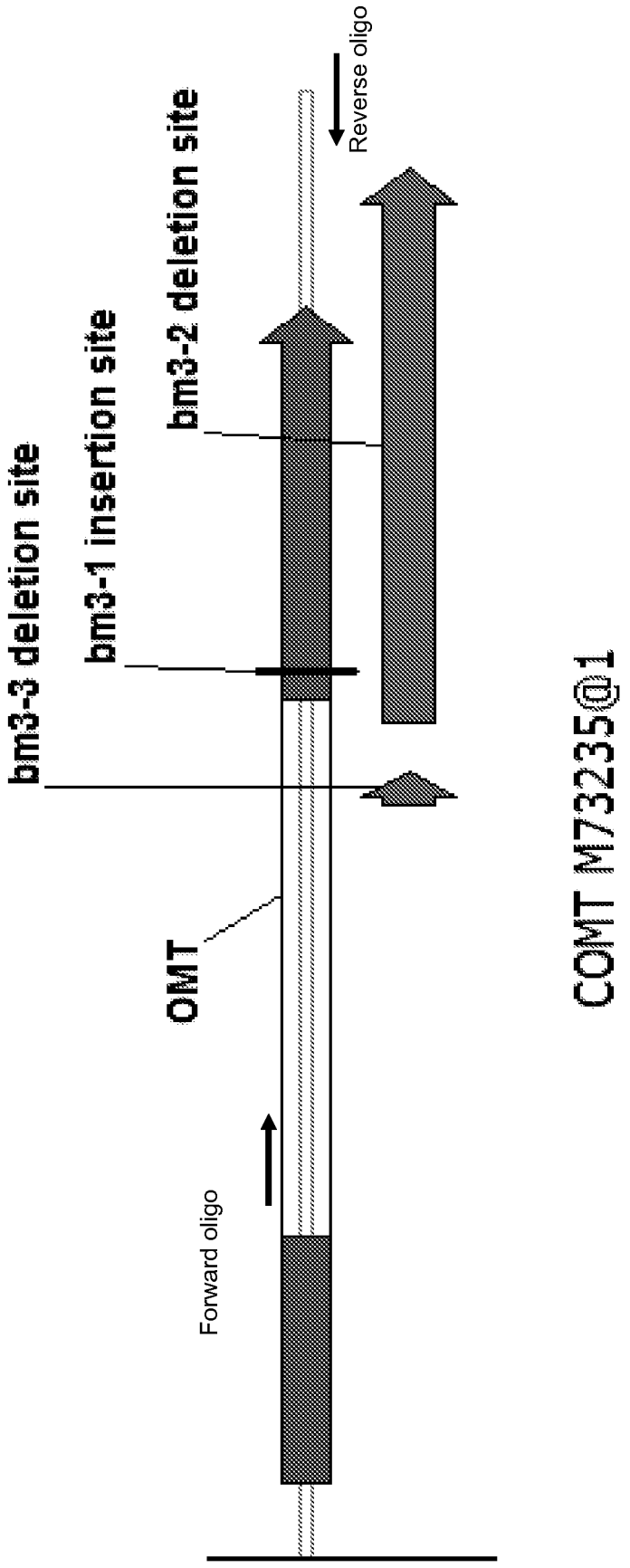

Reverse oligo
bm3-2 deletion site
bm3-1 insertion site
bm3-3 deletion site
OMT
Forward oligo
COMT M73235@1
2512 bp

Fig. 1

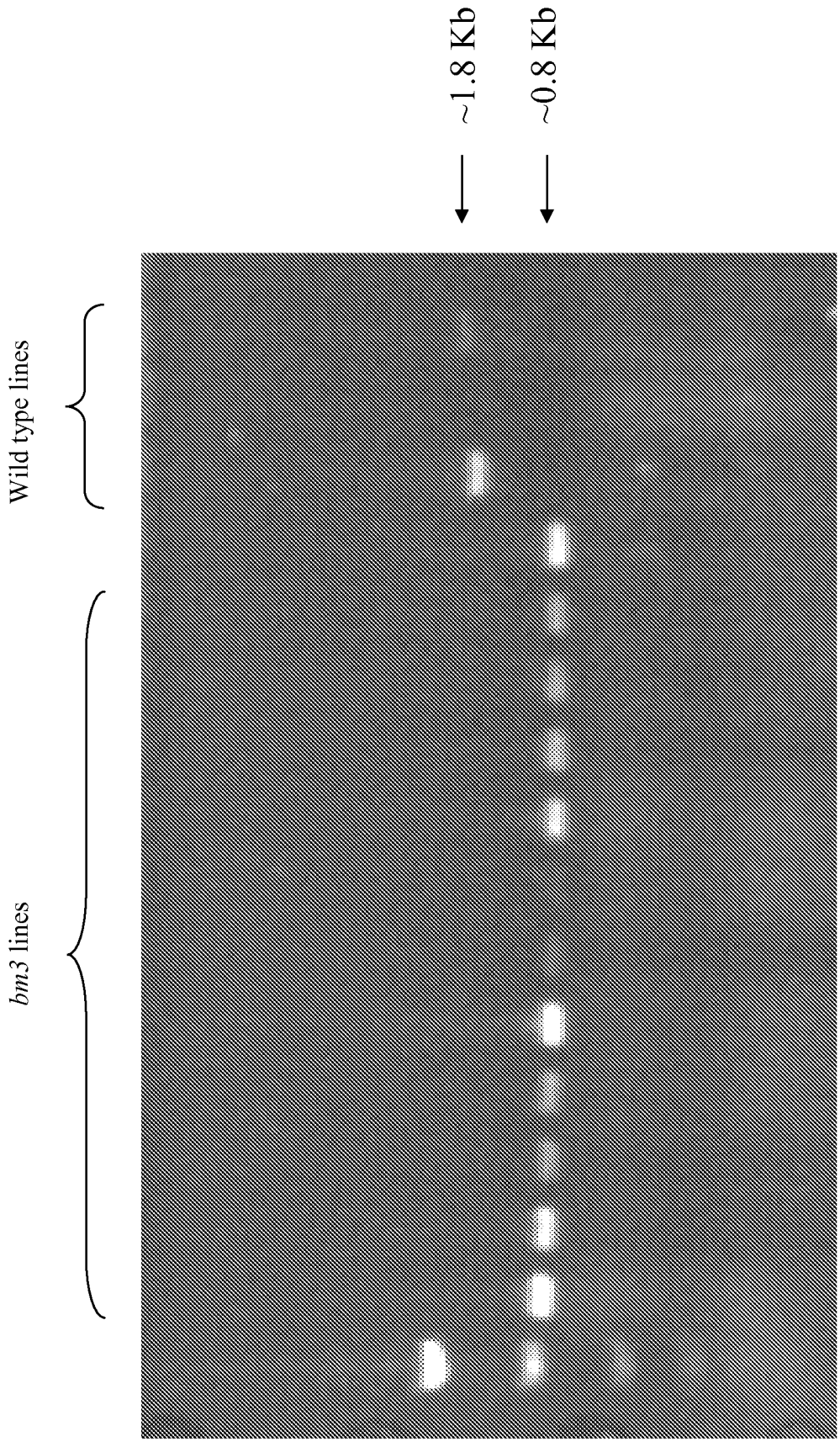


Fig. 2

```
bm3  SEQ ID NO 10       TACTGGCTGCGGCTAGCACAAGGCTGGAAATAGTTATTATCT-------ACGATATAATA 53
COMT  SEQ ID NO 11      TACTGGCTGCGGCTAGCACAAGGCTGGAAATAGTTGTTACTTGTTATACACGATATAATA 60
                        ********************************** ***  *       ***********

bm3  SEQ ID NO 10       TTTCCCTAGAACAAAAAAAGTTTTTTTTATAAAAAGCAAGAAAGAAAGAAGGAAAGAAAGT 113
COMT  SEQ ID NO 11      TTTCTCTAGAACAAAAAAGATTTTTTTTTTATAA-----AAAGCAAGCAAGAAAGAAAGT 115
                        **** *************  ******* * * **     **** *** * **********

bm3  SEQ ID NO 10       GAGTGACTTCAAGTTTTTCCTAAAAAAAAAGTTAGGAGTGGGATGGAAAAGTCAGCAAGG 173
COMT  SEQ ID NO 11      GAGTGACTTCATGTTTTTCCTAAAAAAAA-GTTAGGAGTGGGATGGAAAAGTCAGCAAGG 174
                        *********** ***************** ****************************

bm3  SEQ ID NO 10       ACCACTTGTTTGTTGTCCACTATCCATCCAGTGGGTGAGACTTTTTTGCGAGACGGAGCA 233
COMT  SEQ ID NO 11      ACCACTTGTTTGTTGTCCACTATCCATCCAGTGGGTGAGACTTTTTTGCGAGACGGAGCA 234
                        ************************************************************

bm3  SEQ ID NO 10       CTATATTATTGGCCGACTCCTTTTTCTGTATCCGCAAAACGGCAGCCGTCGATCGCCGGA 293
COMT  SEQ ID NO 11      CTATATTATTGGCCGAGTCCCTTTTCTGTATCCGCAAAACGGCAGCCGTCGATCGCCGGA 294
                        *************** *** ************************************

bm3  SEQ ID NO 10       CGGATCG-ACGGCTCACATGAGTGTCGAGTCCAATTCCAACCACGAGGGCTGGAAGGAAA 352
COMT  SEQ ID NO 11      CGGATCGCACGGCGACACTGAGTGTCGAGTCCAATTCCAACCACGAGGGCTGGAAGGAAA 354
                        ******* *****     ****************************************

bm3  SEQ ID NO 10       ACCATCCGTGCTGGTCTGGACTTTTTGCCAAACTCCATTCAGCCATTCGCCGACTGAAGG 412
COMT  SEQ ID NO 11      GCCATCCGTGCTGGTCTGGACTTTTTGCCAAACTCCATTCAGACGTTCGCCGACTGAAGG 414
                        ************************************ * ***************

bm3  SEQ ID NO 10       TGAATCTTCAGACA--GCCAGATTGGTTGGTGTCTAGTGTGTGCGAAGATGGCGTAGAAA 470
COMT  SEQ ID NO 11      TGAATCTTCAGACACAGCCAGATTGTTTGGTGTC-------TGCGAAGATGGCGTAGAAA 467
                        **************  ********* ********       ******************

bm3  SEQ ID NO 10       AGACTGAGAGACAGTTGGCTCACACAGACAATTGACAACTGACTATAGTATCTGCCTGCC 530
COMT  SEQ ID NO 11      AGACCAAGAGACAGTTGGCTCACACAGACAAGTGACAACTGACTATAGTATCTGCCTGCC 527
                        ****  ************************* ****************************

bm3  SEQ ID NO 10       TGGCTGATGCTGATAGAGATGGGGACTCTTGTCCTG-CTGTCTGTTCT-------TGTAT 582
COMT  SEQ ID NO 11      TGGCTGATGCTGATAGAGATAG--ACTCTTGCCCTGTCTGTTCCTTGTACAAACGTGGAC 585
                        ******************** *  ******* **** ****   ** *       ** *

bm3  SEQ ID NO 10       AAAT----------------------------------------CTCCGTTGTCAAATATTT 604
COMT  SEQ ID NO 11      AAACATTAGTAGCAGTTTTCTTTGATCTACAAAAAGTACTACCTCAGTTTTTAAATATTT 645
                        ***                                        *** *** * ********

bm3  SEQ ID NO 10       ATCGTCCGATTATTTATTTYTAAACT-AAACAACGACAAATAAAAAAGAACGAGGTTGCA 663
COMT  SEQ ID NO 11      ATCGGCTGTTAGTTTATTTTTGAACTTAAACGACT------------------GGTTGCA 687
                        **** * * *  ******* * **** **** **                   *******

bm3  SEQ ID NO 10       AAAGATAGATACA----AACCAAAGGA---------------------------------- 686
COMT  SEQ ID NO 11      AAAGATAGATAGATACAAACGAAAGGATGTCGTCGCTGTGCGCTGATCTGATCACTGCCA 747
                        *********** *    *** ******

bm3  SEQ ID NO 10       ~ 978 bases deletion in bm3 mutant          ----------
COMT  SEQ ID NO 11                                                  GGTACTATCT 765

bm3  SEQ ID NO 10       ------------------------------------------------------------
COMT  SEQ ID NO 11      CAAGGACGCGGTGCTGGACGGCGGCATCCCGTTCAACAAGGCGTACGGGATGACGGCGTT 825


bm3  SEQ ID NO 10       ------------------------------------------------------------
COMT  SEQ ID NO 11      CGAGTACCACGGCACGGACGCGCGCTTCAACCGCGTGTTCAACGAGGGCATGAAGAACCA 885
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Fig. 3

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bm3  SEQ ID NO 10       ------------------------------------------------------------
COMT  SEQ ID NO 11      CTCGGTGATCATCACCAAGAAGCTGCTGGACTTCTACACGGGCTTCGAGGGCGTGTCGAC 945

bm3  SEQ ID NO 10       ------------------------------------------------------------
COMT  SEQ ID NO 11      GCTGGTGGACGTGGGCGGCGGCGTGGGCGCCACGCTGCACGCCATCACGTCCCGCCACCC 1005

bm3  SEQ ID NO 10       ------------------------------------------------------------
COMT  SEQ ID NO 11      GCACATCTCCGGGGTCAACTTCGACCTGCCGCACGTCATCTCCGAGGCGCCGCCGTTCCC 1065

bm3  SEQ ID NO 10       ------------------------------------------------------------
COMT  SEQ ID NO 11      CGGCGTGCGCCACGTGGGCGGGGACATGTTCGCGTCCGTGCCCGCCGGCGACGCCATCCT 1125

bm3  SEQ ID NO 10       ------------------------------------------------------------
COMT  SEQ ID NO 11      CATGAAGTGGATCCTCCACGACTGGAGCGACGCGCACTGCGCCACGCTGCTCAAGAACTG 1185

bm3  SEQ ID NO 10       ------------------------------------------------------------
COMT  SEQ ID NO 11      CTACGACGCGCTGCCGGAAAATGGCAAGGTCATCGTCGTCGAGTGCGTGCTGCCGGTCAA 1245

bm3  SEQ ID NO 10       ------------------------------------------------------------
COMT  SEQ ID NO 11      CACGGAGGCCACCCCCAAGGCGCAGGGCGTGTTCCACGTCGACATGATCATGCTCGCGCA 1305

bm3  SEQ ID NO 10       ------------------------------------------------------------
COMT  SEQ ID NO 11      CAACCCCGGCGGCAAGGAGCGGTACGAGCGCGAGTTCCGCGAGCTCGCCAAGGGCGCCGG 1365

bm3  SEQ ID NO 10       ------------------------------------------------------------
COMT  SEQ ID NO 11      CTTCTCCGGGTTCAAGGCCACCTACATCTACGCCAACGCCTGGGCCATCGAGTTCATCAA 1425

bm3  SEQ ID NO 10       ------------------------------------------------------------
COMT  SEQ ID NO 11      GTGAATACGGCTACCACCGTCGCCGCGATGAGATGCATGGCTGCCACATGCATGCTTGCT 1485

bm3  SEQ ID NO 10       ------------------------------------------------------------
COMT  SEQ ID NO 11      TGCTTGGTCCTCGTATCGTACGTCGCCGTCGTCGTCTTCTTCTGGTTGCGCTGCTACCTT 1545

bm3  SEQ ID NO 10       ------------------------------------------------------------
COMT  SEQ ID NO 11      GCTGCTCTCGCCCTCGCGTATGCATGTACTTTTGCTTAATTTTCTTTCTTCATATCATGC 1605

bm3  SEQ ID NO 10       ------------------------------------------------------------TGTC 690
COMT  SEQ ID NO 11      ACTCTGGCTGGCCTAGACTGCCCCCGATCCATGGTGGCCATGTCTTCGGTACGTCTTGTC 1665
                                                                                    ****

bm3  SEQ ID NO 10       GAGCTCTTGCATGTCGTGGATTCTAAATTCTTCTTCTGCGTCGAATTGTCTCTGCCATGT 750
COMT  SEQ ID NO 11      GAGCTCTTGCATGTCGTGGATTCTAAATTCTTCTTCTGCGTCGAATTGTCTCTGCCATGT 1725
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bm3  SEQ ID NO 10       GCGAGTAATAACAATCAAGGTTATACTTACCATACAATTACATGGTGGTTTAATTGCTCT 810
COMT  SEQ ID NO 11      GCGAGTAATAACAATCAAGGTTATACTTACCATACAATTACATGGTGGTTTAATTGCTCT 1785
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bm3  SEQ ID NO 10       CTTTTAATTTGGTGA 825
COMT  SEQ ID NO 11      CTTTTAATTTGGTGA 1800
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Fig. 3 cont.

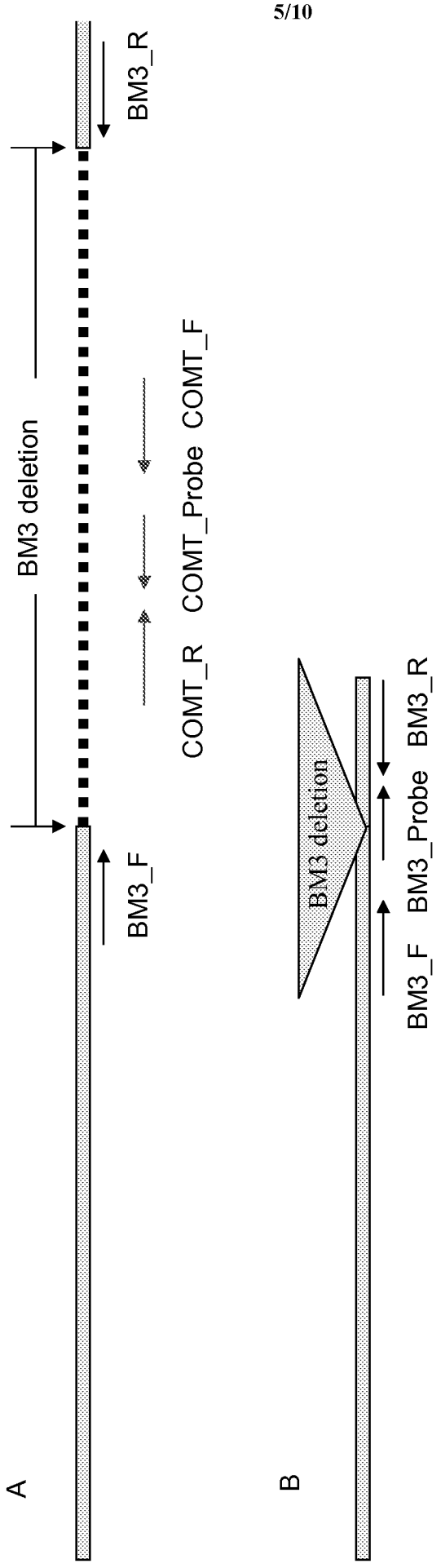

A
BM3_F
BM3 deletion
BM3_R
COMT_R
COMT_Probe
COMT_F
B
BM3 deletion
BM3_F
BM3_Probe
BM3_R

Fig. 4

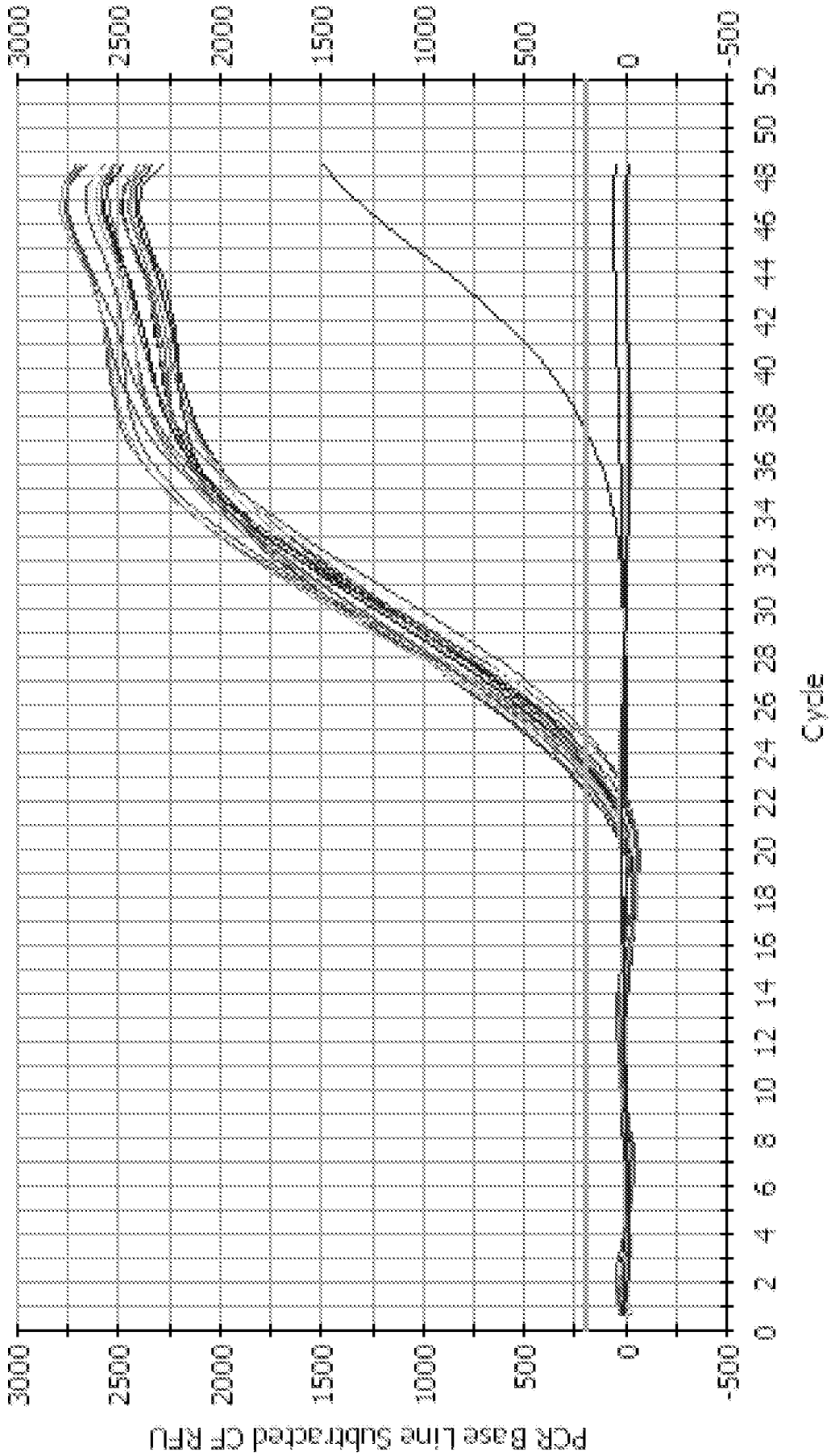

PCR Base Line Subtracted CF RFU
3000
2500
2000
1500
1000
500
0
-500
Cycle
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52

Fig. 5a

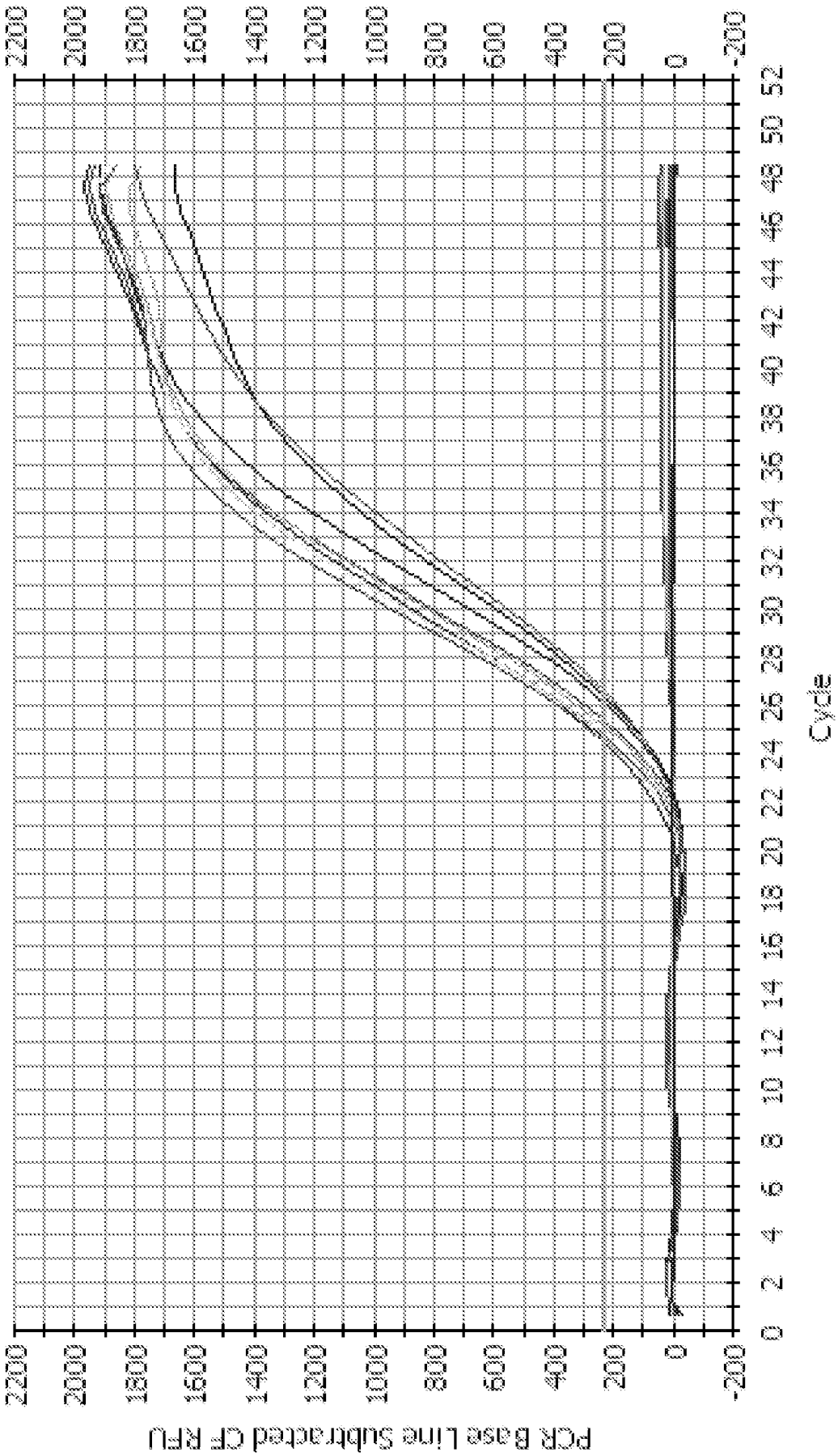

PCR Base Line Subtracted CF RFU
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
-200
Cycle
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52

Fig. 5b

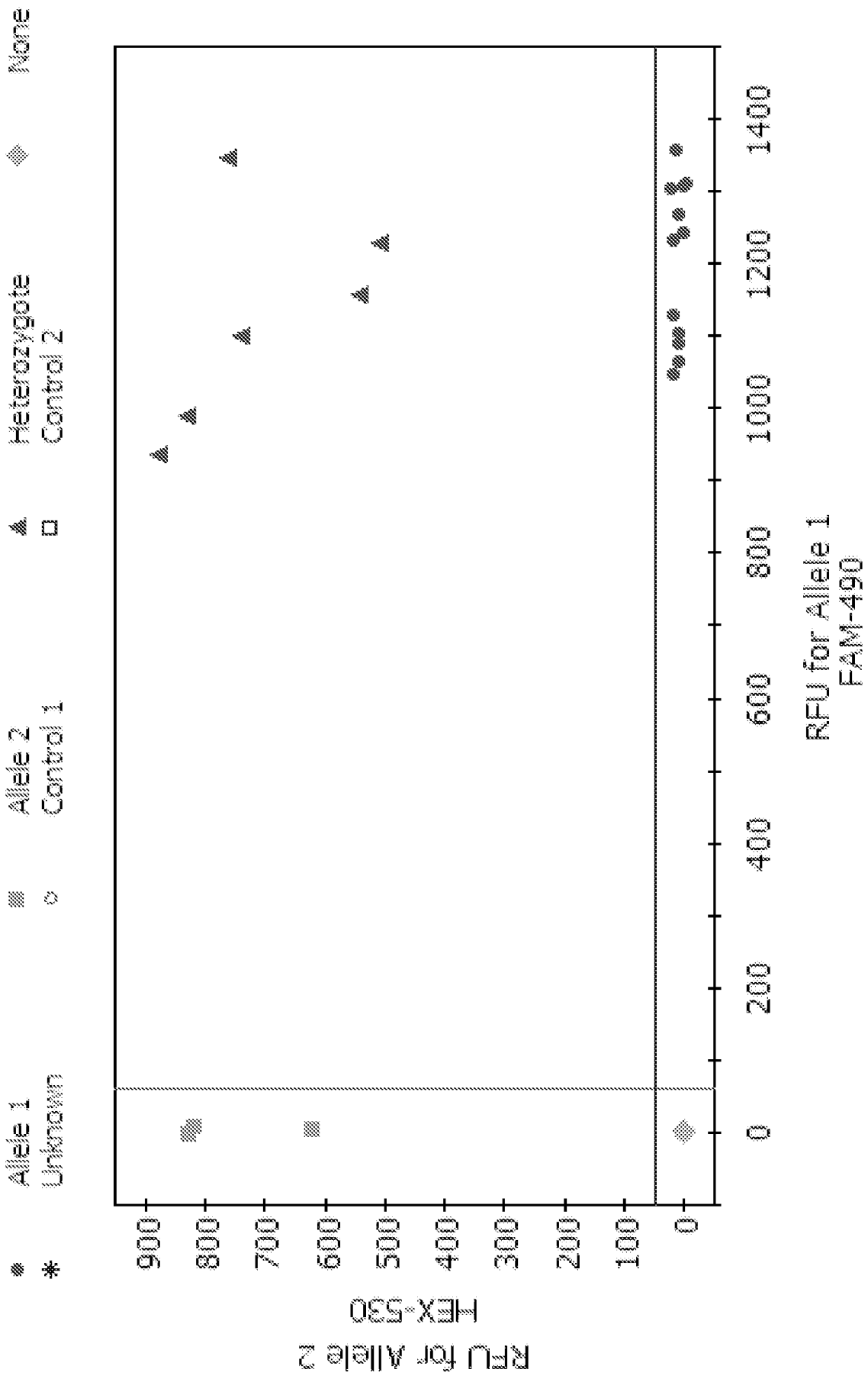

Allele 1
Unknown
Allele 2
Control 1
Heterozygote
Control 2
None
RFU for Allele 2
HEX-530
RFU for Allele 1
FAM-490
0
100
200
300
400
500
600
700
800
900
0
200
400
600
800
1000
1200
1400

Fig. 6

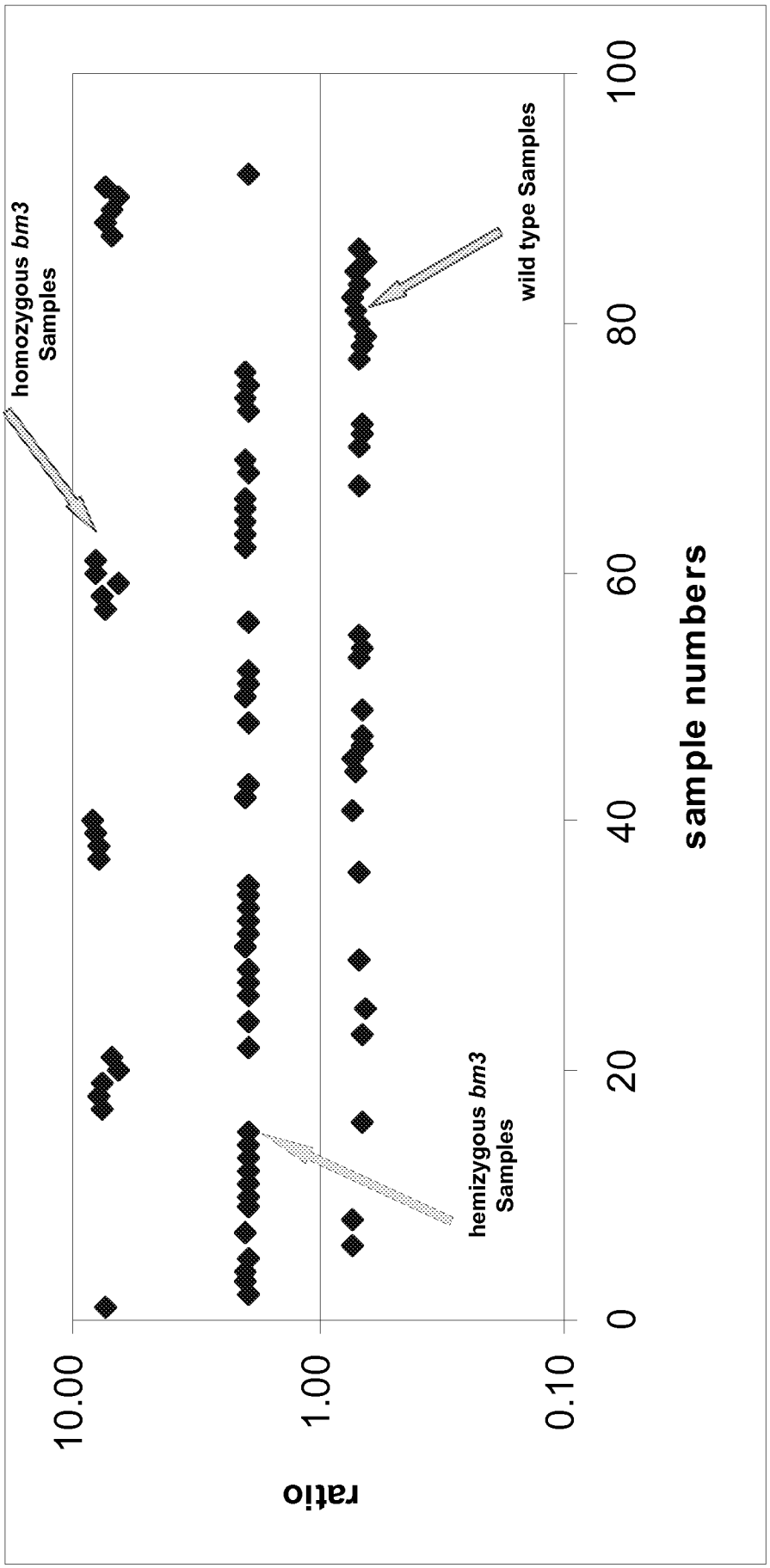

homozygous bm3 Samples
wild type Samples
hemizygous bm3 Samples
ratio
10.00
1.00
0.10
0
20
40
60
80
100
sample numbers

Fig. 7

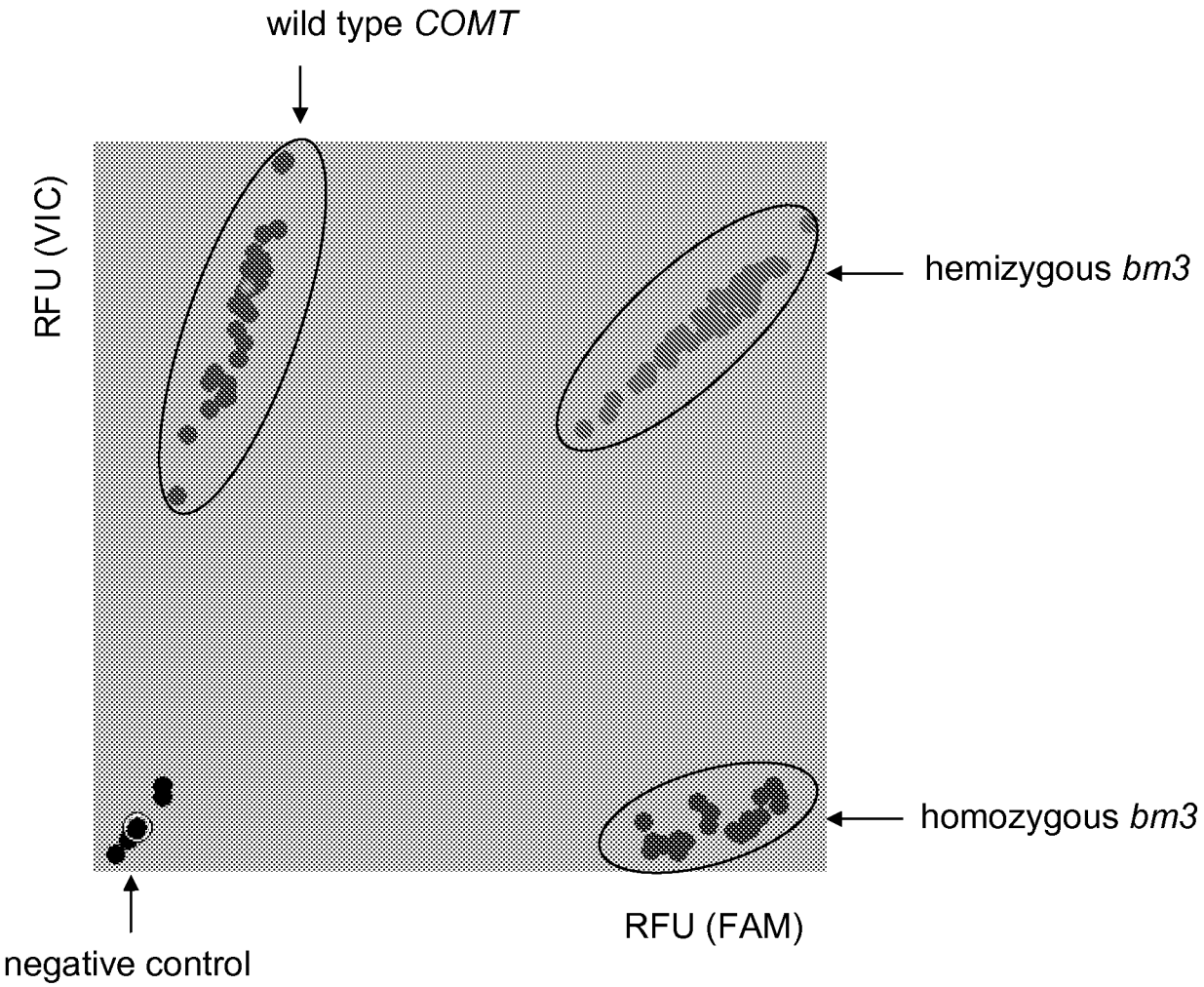
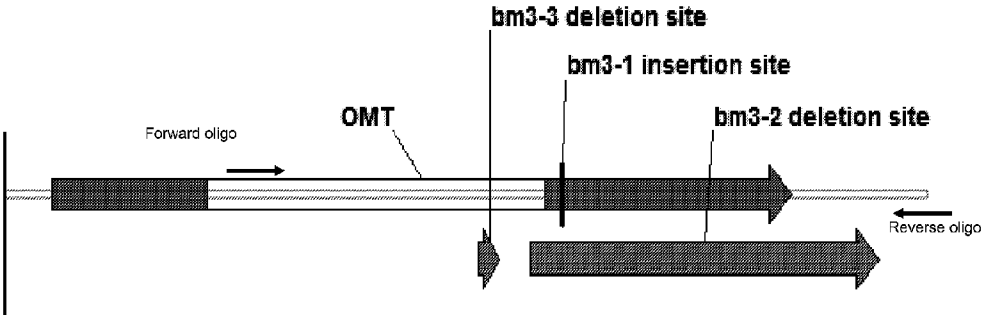

wild type *COMT*
RFU (VIC)
hemizygous *bm3*
homozygous *bm3*
RFU (FAM)
negative control

Fig. 8



COMT M73235@1

2512 bp