(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
29 January 2015 (29.01.2015)

WIPO | PCT

(10) International Publication Number
WO 2015/012783 A2

(51) International Patent Classification:
C12Q 1/68 (2006.01)

(21) International Application Number:
PCT/US2013/042997

(22) International Filing Date:
29 May 2013 (29.05.2013)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/653,287 30 May 2012 (30.05.2012) US

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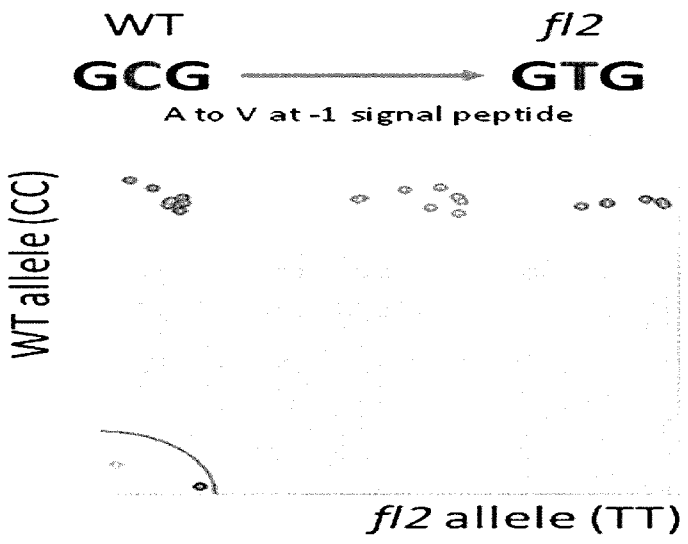
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,

[Continued on next page]

(54) Title: FLOURY 2 GENE-SPECIFIC ASSAY IN MAIZE FOR FLOURY (FL2) TRAIT INTROGRESSION

FIG. 3



(57) Abstract: The disclosure concerns compositions and methods for determining the zygosity of corn plants containing one or more floury2 (*fl2*) mutations. In embodiments, the disclosure concerns a gene specific PCR-based molecular (KASPar®) assay for identifying the floury2 trait in plants. In certain embodiments, compositions and methods are disclosed for determining the zygosity of corn plants with respect to the *fl2* allele. In particular embodiments, the assay may be used for *fl2* germplasm identification, accelerating introgression and molecular breeding programs in corn and other plants.

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TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

— *with sequence listing part of description (Rule 5.2(a))*

FLOURY 2 GENE-SPECIFIC ASSAY IN MAIZE FOR FLOURY (FL2) TRAIT INTROGRESSION

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority under 35 U.S.C. § 119(e) to U.S. Provisional Application Serial No. 61/653,287, filed May 30, 2012, the disclosure of which is hereby incorporated by reference in its entirety.

INCORPORATION BY REFERENCE OF MATERIAL SUBMITTED ELECTRONICALLY

[0002] Incorporated by reference in its entirety is a computer-readable sequence listing submitted concurrently herewith and identified as follows: one 28,672 byte ASCII (text) file named "225450_SEQ_LISTING_ST25.txt", created on May 28, 2013.

FIELD OF THE DISCLOSURE

[0003] The disclosure relates generally to plant breeding. Methods are provided for determining the presence of the *fl2* mutation and the zygosity of plants for this mutation. Methods of the disclosure are useful for selecting for plants bearing the floury2 (*fl2*) trait and for *fl2* trait introgression into plants, such as maize.

BACKGROUND

[0004] Corn silage is a popular forage for ruminant animals because of its high energy and digestibility. Corn grain is often added to ration formulations to improve nutritional balance. Kernel hardness and texture play an important role in starch digestibility. A softer kernel or a hybrid that is harvested when it is less mature is easier to digest in the rumen. Naturally-occurring maize mutations, such as brown midrib (*BMR*), floury2 (*fl2*), and opaque2 (*O2*) have become a focus of silage product development because they are associated with a softer kernel. *BMR* germplasms, for example, have reduced cell wall lignin content. Cherney *et al.* (1991) Adv. Agron. 46:157-98. Plants with the *fl2* or *O2* mutations produce soft, starchy endosperm with irregularly shaped protein bodies and higher lysine content than wild type. Coleman *et al.* (1997) PNAS 94:7094-97. Adding grain with the *fl2* or *O2* mutations to rations may increase digestibility in ruminants, reducing the amount of grain needed for nutritional requirements and reducing the need for kernel processing at harvest. *See* Ladely *et al.* (1995) J. Anim. Sci. 73:228-235.

[0005] The floury2 trait is reportedly associated with a mutation in one of the members of the zein gene family, the major prolamin storage proteins in maize seed. Song *et al.* (2001) Gen. Res. 11:1817-25. Introgression of the *fl2* mutation into corn lines is a time-consuming process. Since the *fl2* mutant allele is semidominant, phenotyping based on kernel vitreousness is difficult and often ambiguous. Coleman *et al.* (1997). A rapid, gene-specific molecular assay is needed to detect the *fl2* mutant allele and determine zygosity in candidate plants. This assay will greatly facilitate breeding efforts by reducing the time needed to select plants with desirable features.

SUMMARY OF THE DISCLOSURE

[0006] Described herein are methods for high-throughput PCR-based molecular characterization of floury2 corn varieties (*e.g., fl2* mutants) that may greatly enhance the breeding process for introgression of corn lines containing *fl2*. 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 an *fl2* mutant and the wild type alpha-zein alleles. Thus, a fluorescent probe PCR-based zygosity assay (referenced herein as the KASPar® assay) is provided that specifically detects and tests the zygosity status at the *fl2* locus and is capable of distinguishing a unique SNP variant in the gene between segregating populations. Also disclosed is the nucleotide sequence comprising this unique SNP, and plants and germplasm selected using these methods.

[0007] In a particular embodiment, a method for determining the zygosity and/or presence /absence of an allele using corn plant tissue includes: obtaining a sample of isolated genomic DNA from the corn plant tissue; contacting the isolated genomic DNA with at least one nucleic acid molecule comprising a nucleotide sequence capable of hybridizing to SEQ ID NO:9 under high stringency conditions and at least one nucleic acid molecule capable of hybridizing to SEQ ID NO:10 under high stringency conditions; and determining zygosity of a *fl2* mutation in the isolated genomic DNA.

[0008] In another embodiment, a method for reliably and predictably introgressing a trait for high lysine content into plant germplasm includes: crossing a plant having a mutation in the *fl2* gene with another plant; obtaining a sample of isolated genomic DNA from a progeny plant produced by the cross; contacting the isolated nucleic acid with at least one nucleic acid molecule having a nucleotide sequence capable of hybridizing to SEQ ID NO:10 under high stringency conditions; and selecting progeny from the cross that includes a mutation in the *fl2*

gene by reproducing a plant from which a sample was obtained that binds at least one nucleic acid molecules with high stringency, thereby producing a genetically engineered plant wherein a trait for high lysine content that has been introgressed into the germplasm of the genetically engineered plant.

BRIEF DESCRIPTION OF THE FIGURES

[0009] **FIG. 1** shows the alignment of the 22 kDa α-zein genomic sequences from the wild-type corn line B73 (B73), wild-type corn line 1 (NF7_3), L3430 is a publicly available floury2 corn line 1 (L3430), floury2 corn line 2 (FF1_1) and floury2 corn line 3 (FF2_1). 72 SNPs and 7 InDels are identified.

[0010] **FIG. 2** shows the alignment of the predicted 22-kDa α-zein protein sequences from B73, wild-type corn line 1 (NF7_3), floury2 corn line 1 (L3430), floury2 corn line 2 (FF1_1) and floury2 corn line 3 (FF2_1). The lighter color font indicates the A to V substitution at the -1 signal peptide.

[0011] **FIG. 3** shows the KLIMS Data analysis of 23 maize inbred lines genotyped with KASPar® assay (FAM signal for mutant allele as x-axis and CAL signal for wild type allele as y-axis) based on the alanine to valine substitution on -1 signal peptide.

[0012] **FIG. 4** shows the KLIMS Data analysis of 23 maize inbred lines genotyped with KASPar® assay based on the alanine to valine substitution at the 39^{th} amino acid (FAM signal for mutant allele as x-axis and CAL signal for wild type allele as y-axis).

[0013] **FIG. 5** shows a confirmation test with maize inbred lines genotyped with KASPar® assay based on the alanine to valine substitution at the 39^{th} amino acid (FAM signal for mutant allele as x-axis and CAL signal for wild type allele as y-axis).

[0014] **FIG. 6** shows a validation test with segregating populations genotyped with KASPar® assay (*fl2*_zygo) based on the alanine to valine substitution at the 39^{th} amino acid (FAM signal for mutant allele as x-axis and CALs signal for wild type allele as y-axis).

SEQUENCE LISTING

[0015] 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 being included by any reference to the displayed strand. In the accompanying sequence listing:

[0016] SEQ ID NO:1 shows a forward primer sequence (Zein_68F) used to amplify the 22 kDa B73 *α-zein* gene: GAGATCATGCATGTCATTCCA.

[0017] SEQ ID NO:2 shows a reverse primer sequence (Zein_68R) used to amplify the 22 kDa B73 *α-zein* gene: TTGGTGTTGTTAAGTTCACATGC.

[0018] SEQ ID NO:3 shows an oligonucleotide sequence (Zein_513F) from the *α-zein* gene used to sequence a B73 *α-zein* partial gene: AATCCTTGGCACATCTAA.

[0019] SEQ ID NO:4 shows an oligonucleotide sequence (Zein-23R) from the *α-zein* gene used to sequence a B73 *α-zein* partial gene: TAGGTGGCTCAGTGATGGCAGAA.

[0020] SEQ ID NO:5 shows shows an oligonucleotide sequence (385_R) from the *α-zein* gene used to sequence a B73 *α-zein* partial gene: CTAAAAGATGGCACCTCCAA.

[0021] SEQ ID NO:6 shows an allele-specific primer sequence (A1).

[0022] SEQ ID NO:7 shows an allele specific primer sequence (A2).

[0023] SEQ ID NO:8 shows a common (reverse) primer sequence (C1).

[0024] SEQ ID NO:9 shows the nucleotide sequence of the 22 kDa *α-zein* gene from plant line B73.

[0025] SEQ ID NO:10 shows the nucleotide sequence of the 22 kDa *α-zein* gene from plant line L34340.

[0026] SEQ ID NO:11 shows the nucleotide sequence of the 22 kDa *α-zein* gene from plant line FF2_1.

[0027] SEQ ID NO:12 shows the nucleotide sequence of the 22 kDa *α-zein* gene from plant line FF1_1.

[0028] SEQ ID NO:13 shows the nucleotide sequence of the 22 kDa *α-zein* gene from plant line NF7_3.

[0029] SEQ ID NO:14 shows the predicted amino acid sequence of the *α-zein* gene from plant line B73.

[0030] SEQ ID NO:15 shows the predicted amino acid sequence of the *α-zein* gene from plant line L34340.

[0031] SEQ ID NO:16 shows the predicted amino acid sequence of the *α-zein* gene from plant line FF2_1.

[0032] SEQ ID NO:17 shows the predicted amino acid sequence of the *α-zein* gene from plant line FF1_1.

[0033] SEQ ID NO:18 shows the predicted amino acid sequence of the *α-zein* gene from plant line NF7_3.

[0034] SEQ ID NO:19 shows the primer sequence A1-1.

[0035] SEQ ID NO:20 shows the primer sequence A2-1.

[0036] SEQ ID NO:21 shows the primer sequence C1-1.

[0037] SEQ ID NO:22 shows the primer sequence A1-2.

[0038] SEQ ID NO:23 shows the primer sequence A2-2.

[0039] SEQ ID NO:24 shows the primer sequence C1-2.

[0040] SEQ ID NO:25 shows the primer sequence A1-3.

[0041] SEQ ID NO:26 shows the primer sequence A2-3.

[0042] SEQ ID NO:27 shows the primer sequence C1-3.

[0043] SEQ ID NO:28 shows the primer sequence A1-4.

[0044] SEQ ID NO:29 shows the primer sequence A2-4.

[0045] SEQ ID NO:30 shows the primer sequence C1-4.

[0046] SEQ ID NO:31 shows the primer sequence A1-5.

[0047] SEQ ID NO:32 shows the primer sequence A2-5.

[0048] SEQ ID NO:33 shows the primer sequence C1-5.

[0049] SEQ ID NO:34 shows the primer sequence A1-6.

[0050] SEQ ID NO:35 shows the primer sequence A2-6.

[0051] SEQ ID NO:36 shows the primer sequence C1-6.

DETAILED DESCRIPTION

I. Overview of several embodiments

[0052] Disclosed herein are methods for PCR-based gene-specific molecular assays to detect the presence of the floury2 (*fl2*) trait in maize.

[0053] In embodiments herein, the method may disclose a method to determine the zygosity of the plant for *fl2* mutant alleles.

[0001] Embodiments herein describe the cloning of the α-zein gene from multiple maize lines. In some embodiments, several mutations associated with the floury phenotype are identified.

[0002] In a preferred embodiment, a gene-specific assay for the *fl2* trait is described. In particular embodiments, a method for using the *fl2* gene specific assay to select plant germplasm is described.

[0003] In some embodiments, a method for using a *fl2* gene specific assay to facilitate introgression of the *fl2* mutant gene is described. The introgression of *fl2* is not as complicated as *O2*.

II. Abbreviations

[0004]	*BMR*	brown midrib mutation
[0005]	*fl2*	floury2 mutation
[0006]	*O2*	opaque2 mutation
[0007]	PCR	polymerase chain reaction

III. Terms

[0008] Base position: A "base position," as used herein, refers to the location of a given base or nucleotide residue within a designated nucleic acid. The designated nucleic acid may be defined by alignment (see below) with a reference nucleic acid.

[0009] Elite line: As used herein, the term "elite line" means any line that has resulted from breeding and selection for superior agronomic performance. An elite plant is any plant from an elite line.

[0010] Introgression: As used herein refers to a genomic segment that has moved from one individual, species, variety or cultivar into the genome of another individual, species, variety or cultivar, by crossing those individuals, species, varieties or cultivars. As used herein, the terms "introgressing", "introgress" and "introgressed" refer to both a natural and artificial process whereby individual genes or entire traits are moved from one individual, species, variety or cultivar into the genome of another species, variety or cultivar, by crossing those species, varieties or cultivars. In plant breeding, the process usually involves selfing or backcrossing to the recurrent parent to provide for an increasingly homozygous plant having essentially the characteristics of the recurrent parent in addition to the introgressed gene or trait.

[0011] Line: As used herein, a “line” or a "plant line" refers to a group of plants that display little genetic variation (*e.g.*, no genetic variation) between individuals for at least one trait. Inbred lines may be created by several generations of self-pollination and selection or, alternatively, by vegetative propagation from a single parent using tissue or cell culture techniques. As used herein, the terms “cultivar,” “variety,” and “type” are synonymous, and these terms refer to a line that is used for commercial production.

[0012] Nucleic acid molecule: As used herein, the term “nucleic acid molecule” (or “nucleic acid” or “polynucleotide”) may refer to a polymeric form of nucleotides, which may include both sense and anti-sense strands of RNA, cDNA, genomic DNA, and synthetic forms and mixed polymers of the above. A nucleotide may refer to a ribonucleotide, deoxyribonucleotide, or a modified form of either type of nucleotide. A “nucleic acid molecule” as used herein is synonymous with “nucleic acid” and “polynucleotide.” A nucleic acid molecule is usually at least 10 bases in length, unless otherwise specified. The term may refer to a molecule of RNA or DNA of indeterminate length. The term includes single- and double-stranded forms of DNA. A nucleic acid molecule may include either or both naturally-occurring and modified nucleotides linked together by naturally occurring and/or non-naturally occurring nucleotide linkages.

[0013] 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.

[0014] Sequence identity: The term “sequence identity” or “identity,” as used herein in the context of two nucleic acid or polypeptide sequences, may refer to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window.

[0015] As used herein, the term “percentage of sequence identity” may refer to the value determined by comparing two optimally aligned sequences (*e.g.*, nucleic acid sequences, amino acid sequences) over a comparison window, wherein the portion of the sequence in the comparison window may comprise additions or deletions (*i.e.*, gaps) as compared to the reference sequence

(which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleotide or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the comparison window, and multiplying the result by 100 to yield the percentage of sequence identity.

[0016] Methods for aligning sequences for comparison are well-known in the art. Various programs and alignment algorithms are described in, for example: Smith and Waterman (1981) Adv. Appl. Math. 2:482; Needleman and Wunsch (1970) J. Mol. Biol. 48:443; Pearson and Lipman (1988) Proc. Natl. Acad. Sci. U.S.A. 85:2444; Higgins and Sharp (1988) Gene 73:237-44; Higgins and Sharp (1989) CABIOS 5:151-3; Corpet *et al.* (1988) Nucleic Acids Res. 16:10881-90; Huang *et al.* (1992) Comp. Appl. Biosci. 8:155-65; Pearson *et al.* (1994) Methods Mol. Biol. 24:307-31; Tatiana *et al.* (1999) FEMS Microbiol. Lett. 174:247-50. A detailed consideration of sequence alignment methods and homology calculations can be found in, *e.g.*, Altschul *et al.* (1990) J. Mol. Biol. 215:403-10.

[0017] The National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST™; Altschul *et al.* (1990)) is available from several sources, including the National Center for Biotechnology Information (Bethesda, MD), and on the internet, for use in connection with several sequence analysis programs. A description of how to determine sequence identity using this program is available on the internet under the "help" section for BLAST™. For comparisons of nucleic acid sequences, the "Blast 2 sequences" function of the BLAST™ (Blastn) program may be employed using the default parameters. Nucleic acid sequences with even greater similarity to the reference sequences will show increasing percentage identity when assessed by this method.

[0018] Single-nucleotide polymorphism (SNP): As used herein, the term "single-nucleotide polymorphism" may refer to a DNA sequence variation occurring when a single nucleotide in the genome (or other shared sequence) differs between members of a species or paired chromosomes in an individual.

[0019] Trait or phenotype: The terms "trait" and "phenotype" are used interchangeably herein. For the purposes of the present disclosure, a trait of particular interest is seed coat color. Some canola varieties exhibit a yellow seed coat, while further varieties exhibit a dark (*e.g.*, black, dark, and mottled) seed coat.

[0020] Zein: As used herein, the term "zein" shall refer to any member of a class of genes, and their gene products, gene fragments, partial genes, protein products, and RNA products,

in or derived from the zein superfamily in maize and other plants. Zeins may include the following known classes or family members: α-zeins, β-zeins, γ-zeins and δ-zein. In embodiments, the α-zein gene, gene segment, partial gene, or its protein or RNA products is referenced.

[0021] 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."

[0022] 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).

[0023] Unless indicated otherwise, the terms "a" and "an" as used herein refer to at least one.

[0024] All references, including publications, patents, and patent applications, cited herein are hereby incorporated by reference to the extent they are not inconsistent with the explicit details of this disclosure, and are so incorporated to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein. The references discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention.

IV. Gene-specific molecular assay for genotype and zygosity determination of fl2

A. Overview

[0025] Described herein is a gene-specific molecular assay for characterizing the floury2 (*fl2*) trait in plants. Particular examples utilize the KASPar® PCR-based assay system to detect specific *fl2* mutations, and compare to the corresponding wild-type sequences in the same assay. In some examples, the zygosity of the plant may be determined by the presence or absence of an *fl2* mutant allele and the wild-type α-zein allele. Particular examples describe a

unique alanine to valine substitution at the 39 amino acid position, which may be used for molecular characterization. This assay can differentiate the *fl2* mutant allele from the wild type allele and has been confirmed with segregating populations. In examples, the assay may be used for introgression of a desired trait, such as the floury2 trait, into plant lines bearing other desirable features. This gene-based marker/assay will be a powerful tool for accelerating molecular breeding processes with *fl2*.

B. Floury2 (fl2) corn

[0026] Zeins are the major prolamin storage proteins in maize seeds synthesized starting between 10 and 45 days after pollination, and represent 60-70% of total seed protein. Coleman *et al.* (1997); Kodrzycki *et al.* (1989) Plant Cell 1:105-114. Wild type zein proteins are glutamine and proline rich, but deficient in lysine and tryptophan, making the grain nutritionally inferior, especially for monogastric animals. Coleman (1995) PNAS 92:6828-31.

[0027] Four distinct classes of zein proteins have been named, classified as α-, β-, γ-, and δ-zein. Coleman *et al.* (1997). The alpha zeins, the most abundant class, are 19 and 22 kDa. Hagen and Rubenstein (1981) Gene 13(3):239-49. Song *et al.* isolated and sequenced all 23 members of the maize 22 kDa alpha zein gene family. (2001) Gen. Res. 11:1817-25. Twenty-two of the α-zein genes are located in a tandem array on the short arm of chromosome 4S, with the twenty-third gene located at a more proximal location on the same chromosome. Song (2001). Only seven of these genes are reportedly expressed, based on cDNA profiles, with the expressed genes interspersed among the nonexpressed genes. Song (2001).

[0028] The floury2 (*fl2*) and opaque2 (*O2*) traits in maize are associated with a softer kernel, almost twice the lysine content of normal maize, and increased digestibility in ruminants and chickens. Nelson *et al.* (1965) Science 150:1469-70. Chickens fed with *fl2* corn grow faster. Cromwell *et al.* (1968) Poul. Sci. 57:840-47. Calves fed a high lysine corn diet are 10% more efficient and degrade crude protein as much as 27% faster than calves fed normal corn. Ladely *et al.* (1995) J. Anim. Sci. 73:228-35. Because of its soft kernel, floury2 seeds are subject to mechanical damage, making them susceptible to insect and fungal stresses, but introgression of the *fl2* allele into plant lines bearing other desirable features may alleviate this problem.

[0029] The floury2 phenotype is caused by mutations in the zein gene family. Coleman *et al.* (1997) PNAS 94:7094-97. The wild type allele associated with the *fl2* mutation is one of the expressed alpha zein genes. Coleman *et al.* identified an alanine to valine substitution at the -1

position of the signal peptide (a [T/C] SNP) in the maize 22-kDa alpha zein gene thought to be responsible for the *fl2* mutation. (1995 and 1997). The signal peptide normally targets the alpha zein protein to the lumen of the rough endoplasmic reticulum (RER). The mutation prevents cleavage of the signal peptide and results in a mature protein that is 2 kDa larger than expected. The defective 24 kDa protein triggers improper assembly and packaging of zein family proteins during endosperm development, leading to a reduction in prolamin storage protein synthesis and an accumulation of lysine. Coleman *et al.* (1997).

C. Phenotypic selection for floury2 trait

[0030] Breeders have attempted to introduce the *fl2* and *O2* mutations into high performance *BMR* lines, but selecting for these mutants is complicated. Vitreousness can be used to phenotypically select for mutations that influence the opacity of the maize kernel, including the *fl2* and *O2* mutations. Unfortunately, vitreousness is also affected by the morphology and natural discoloration of the kernel, making phenotypic characterization using a light box time-consuming and often misleading. Phenotypic selection for *O2* is also complicated by the presence of multiple modifier genes. Holding *et al.* (2010) Theor. App. Gen. 122:783-94. The *fl2* mutant allele is semi-dominant, with the severity of the phenotype determined by the number of copies of the mutant allele. Maize endosperm contains two copies of genetic material from the female parent and one copy from the male parent. The phenotype of progeny seeds thus varies significantly, depending on which parent the *fl2* mutant allele comes from.

D. Molecular detection assay

[0031] Gene-specific molecular assays for the *BMR* and *fl2* traits would greatly facilitate programs to produce low lignin and soft kernel silage by improving selection accuracy and decreasing the breeding cycle. A gene-specific assay has previously been developed for brown midrib 1 (*bm1*) and brown midrib 3 (*bm3*), but no gene-specific assay was previously available for the *fl2* mutation.

[0032] In experiments, the 22-kDa α-zein gene was cloned and sequenced from two floury2 donors originated from the J15 and Oh-43 lines, as well as a wild type maize inbred line. The SNP mutation at the -1 position was confirmed and zygosity assays were designed. However, this assay failed to differentiate mutants from the wild type due to the high homology of the zein family genes. A unique alanine to valine substitution at the 39 amino acid position was found. A molecular assay was designed targeting the associated SNP and was found to be useful for differentiating the *fl2* mutant allele from the wild type allele. The validity of the assay was confirmed with segregating populations.

[0033] Described herein is a gene specific KASPar® PCR assay, generally useful for zygosity analysis of *fl2* corn or putative *fl2* corn. In particular embodiments, a gene (allele) specific KASPar® PCR assay may be used to analyze the zygosity of corn for as *fl2* mutation. In embodiments, the gene specific assay may provide a high throughput system and method for rapidly screening plants for a selected genotype including, for example, the floury2 (fl2) trait. In

some of these embodiments, for example, a microarray system or well plate containing 96, 384 or 1536 well samples may be assayed simultaneously.

[0034] Primers and probes for use in a gene specific KASPar® PCR assay may be designed based on a known mutation in the gene of interest. The mutation may be, for example, a single nucleotide polymorphism (SNP) or an insertion or deletion mutation. In certain embodiments, primers and probes for an *fl2*-specific assay may be designed based on a SNP (C/T) at nucleotide 495 that is associated with a substitution from alanine to valine at the -1 signal peptide of the *fl2* gene. In a preferred embodiment, primers and probes may be designed based on a SNP associated with an alanine to valine substitution at the 39 amino acid position of the gene. In some embodiments, the gene specific KASPar® PCR assay may target other mutations that may be associated with a trait of interest, such as the floury2 trait.

[0035] In embodiments, two allele-specific primers may be used with a single common (reverse) primer to selectively amplify an allele-specific fragment of the *fl2* gene. In some embodiments, an *fl2*-specific assay amplifies a fragment that is unique to one allelic form of *fl2*, such as the allele bearing the C/T SNP at position 495. In certain embodiments, a target-specific oligonucleotide probe hybridizes under high stringency conditions to a target sequence in a genomic DNA sample between two PCR primers.

[0036] 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 an *fl2* mutation. Positive control samples may comprise genomic DNA from a corn variety with a known *fl2* mutation. Control hemizygous samples may comprise either genomic DNA from a corn variety predetermined to be hemizygous for an *fl2* mutation; or a hemizygous sample may comprise equal proportions of negative control DNA to DNA from a corn variety predetermined to be homozygous for *fl2*.

[0037] 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.

[0038] Once primers, probes, and genomic DNA sample(s) have been prepared or otherwise made available, a competitive allele specific PCR (KASPar®) assay may be designed using commercial software, such as Dow AgroSciences Kraken workflow manager, available through KBiosciences (KBiosciences, Hoddesdon, Hertfordshire, UK) to identify nucleic acid sequences of interest (*e.g.*, sequences particular to a *fl2* 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 KASPar® reaction comprising primers and gene-specific probes for *fl2* mutant and wild-type corn, the reaction mixture may comprise an assay mix containing two allele-specific (forward) primers, a common (reverse) primer, buffer, reaction mix, 50mM $MgCl_2$, and water. To this mix may be added genomic DNA, and PCR cycles may be initiated according to standard protocols to amplify fragments of interest.

[0039] In some embodiments, a PCR (e.g. KASPar®) 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 *fl2* 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 94° C for 15 minutes, then 20 cycles of denaturation (94° C for 10 seconds) and annealing (57° C for 5 seconds) and extension (72° C for 40 seconds), followed by 22 additional cycles with longer annealing (denaturation at 94 ºC for 10 seconds; annealing at 57ºC for 20 seconds, extension at 72 ºC for 40 seconds). Those of skill in the art understand that PCR cycle conditions may be varied according to the practitioner's discretion or the specific primer/oligonucleotide sequences involved, and comparable results obtained.

E. Determination of genotype and/or zygosity for the floury 2 (fl2) trait

[0040] A gene-based (allele-specific) molecular assay may be used for genotype and/or zygosity analysis of plants. In some embodiments, a unique SNP may be targeted using a

KASPar® PCR-based assay system. In a preferred embodiment, the SNP that is targeted may be associated with an alanine to valine substitution at the 39 amino acid position of the 22 kDa α-zein gene. For assays using the KASPar® method, PCR products may be analyzed using fluorometric detection. For example, a fluorescent dye may be measured with an excitation wavelength at 485 nm and emission wavelength at 520 nm for fluorescent signal of FAM and 520 nm and 560 nm for CAL.

[0041] 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 and/or known genotypic backgrounds may serve as positive or negative 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.

[0042] 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.

[0043] 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.

[0044] 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).

VI. Introgression of a trait for high lysine content into plant germplasm

[0045] Introgression of a desirable trait in plants may be facilitated by repeated backcrossing.

[0046] Described herein are methods for producing a corn plant with high lysine content (*e.g.*, *fl2* 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 fl2 mutation to a second parent corn plant, so as to produce F_1 progeny. The first plant can be any *fl2* corn plant or varieties including, for example, *Oh-43* or *J15*. 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.

[0047] In some embodiments, progeny plants are subjected to a genotype and/or zygosity determination, as outlined in the disclosure. Once progeny plants have been genotyped, and/or their zygosity 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 *fl2* 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).

[0048] The following Examples are provided to illustrate certain particular features and/or aspects. These Examples should not be construed to limit the disclosure to the particular features or aspects described.

EXAMPLES

EXAMPLE 1: Cloning of the 22 kDa α-zein gene from DAS germplasms

[0049] Select maize plant lines *J15 and Oh-43* were requested from the maize genetic stock center and propagated by DAS plant breeders. Corn plants were derived from the *J15* line and are homozygous for the *floury2* trait. Likewise, additional corn plants were derived from the *Oh-43* line and contain the *floury2* trait. In addition, another group of corn plants lacking the floury phenotype (wild type) were used to develop the assay.

[0050] DNA Isolation

[0051] For DNA extraction, embryos dissected from seeds or fresh leaf punches were ground to a fine powder using a Genogrinder 2000™ and extracted with a standard MagAttract 96 DNA Plant Core Kit™ (Qiagen, Valencia, CA) using the customized BioCel robot system™ from Agilent Technologies (Santa Clara, CA). Prior to PCR, DNA samples were quantified with the Quant-iT™ PicoGreen® Quantification Kit (Invitrogen, Carlsbad, CA) using the manufacturer's instructions.

[0052] PCR amplification and sequencing of 22-kDa *α-zein*

[0053] The maize B73 inbred genomic sequence of 22-kDa α-zein, containing approximately 1.7 kb with one single exon, was retrieved from NCBI (AC229981.2). Primers were designed based on the B73 sequence Zein_68F (SEQ ID NO:1 5'-GAGATCATGCATGTCATTCCA-3') Zein_68R (SEQ ID NO:2 5'-TTGGTGTTGTTAAGTTCACATGC-3') and PCR was performed in 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), 400nM of dNTP, 200nM of forward and reverse primer and 30 ng of genomic DNA. The following PCR program was used: PCR started with 2 minutes of denaturing at 94°C as recommended, followed by 30 cycles of 94 °C for 45 seconds, 55 °C for 45 seconds and 72 °C for 2 minutes. PCR products were visualized on a 2% E-Gel™ and then extracted using the Purelink™ Quick Gel Extraction kit (Invitrogen, Carlsbad, CA). Purified PCR products were then cloned into the pCR4-TOPO vector™ (Invitrogen, Carlsbad, CA) per 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 $MgSO_4 \cdot 7H_2O$ and 12.5 μg/ml chloramphenicol (added immediately before use) with 50 μg/ml Kanamycin and outsourced to an external sequencing provider (Houston, TX) for sequencing with primers designed to bind to the T7 and T3 promoters (which flank the pCR4-TOPO™ vector insertion site) as well as oligos Zein513F (SEQ ID NO:3 5' AATCCTTGGCACATCTAA 3'), Zein-23R (SEQ ID NO:4 5' TAGGTGGCTCAGTGATGGCAGAA 3') and Zein-385_R (SEQ ID NO:5 5' CTAAAAGATGGCACCTCCAA 3') within the 22-kDa α-zein gene. Sequences were analyzed using the Sequencher 4.8™ sequence alignment computer program.

[0054] KASPar® assay design and cycling conditions

[0055] The KBiosciences Competitive Allele Specific PCR® (KASPar®) assays based on SNPs or insertion and deletions (In/Dels) were designed (KBiosciences, Hoddesdon, Hertfordshire, UK). KASPar® reactions were set up according to **Table 1 and Table 2**. PCR cycles started at 94°C for 15 minutes; then for 20 cycles with 10 seconds of denaturing at 94 °C and 5 seconds of annealing at 57°C; then 10 seconds of extension at 72 °C; followed by 22 cycles with 10 seconds of denature at 94 °C and 20 seconds of annealing at 57°C; then 40 seconds of extension at 72 °C. ABI GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA) was used for amplification. PCR products were measured by PheraStar

spectrofluorometer™ (BMG LABTECH Inc., Cary, NC) with excitation wavelength at 485 nm and emission wavelength at 520 nm for fluorescent signal of FAM and 520 nm and 560 nm for CAL.

[0056] **Table 1**: Recipe for assay mix set up.

	Concentration in Assay Mix (μM)	Volume in Assay Mix (μl)
Allele Specific Primer 1 (A1, 100μM)	12	12
Allele Specific Primer 2 (A2, 100μM)	12	12
Common (reverse) Primer (C1, 100μM)	30	30
H_20 / TrisHCl (10mM, pH8.3)	-	**46**
TOTAL		**100**

[0057] **Table 2**: Recipe for KASPar® reaction in a 5 μl final volume.

Component	volume (μl)
DNA (5ng/μl):	1
2X Reaction Mix:	2.5
Assay Mix:	0.07
*$MgCl_2$ (50mM):	0.04
H_2O:	1.39
TOTAL:	**5**

[0058] The 1.7 kb 22-kDa α-zein genomic fragments were successfully PCR-cloned from two floury lines and a wild type corn line. Sequences were aligned with B73 (NCBI accession AC229981.2) and a public *fl2* allele (L34340, *floury 2* mutant from *W64fl2*). A total of 72 SNPs and 7 insertion/deletion (InDels) were identified (**Fig. 1**) with 24 variations within the exon region. The substitution fro**m alani**ne to valine at the -1 signal peptide, the cause of the floury appearance, is present in both *fl2* corn line 2 and *fl2* corn line 3 (**FIG. 2**), which originated from J15 and Oh-43, respectively.

[0059] Neither corn line contained the histidine insertion between the 164th and 165th amino acid or the alanine to threonine substitution at the 173rd amino acid, the other two mutations reported to also be responsible for the *floury2* phenotype. Efforts were focused on gene-specific assays to detect mutations from J15 and Oh43 that correlated with the *floury2* phenotype. Comparison of *fl2* corn line 2 with *fl2* corn line 3 at the protein level revealed one change from histidine (H) to glutamine (Q) at amino acid position 159 (**FIG. 2**). Since there was no phenotypic variation reported between these two sources of *floury2* mutations, no further characterization was conducted on this mutation.

EXAMPLE 2: Gene-specific molecular assay for *fl2*

[0060] The alanine to valine substitution at the -1 position of the signal peptide is sufficient in creating the 24 kDa unprocessed *α-zein* and the *floury2* phenotype. To develop gene-specific molecular assays for *fl2*, a KASPar® assay was designed based on this single nucleotide polymorphism from C to T. It was tested with a diverse panel of 23 maize inbred lines, including six lines of *floury2* samples. The assay was effective at distinguishing the *floury2* lines from the rest of the inbreds based on copy number variations of the mutant allele, which contains the SNP basepair C. However, wild type alleles with basepair T amplified from all samples with equal intensity, indicating the assay cannot be used for gene-specific detection (**FIG. 3**). This appeared to be mainly due to the high homology of 22-kDa α-zein with other zein family members. The *α-zein* family contains 75 members with more than 75% amino acid identity.

[0061] Four additional substitutions and two Ins/Dels with amino acid changes in the single exon of the 22-kDa α-zein gene (**Table 3**) were tested as candidates using the KASPar® molecular assay described. Most of the trials demonstrated the same cluster pattern as shown in **FIG. 3** tested with 23 inbred maize lines. Although these assays could detect copy number variations, they were not sufficiently robust for large-scale zygosity analysis, where a clear separation between *fl2* allele and the wild type allele is crucial.

[0062] One KASPar® assay, however, based on the second alanine to valine substitution at the 39th amino acid, produced distinct clusters between *floury2* and wild type samples, with the FAM signal indicating the *fl2* gene (**FIG. 4**). This assay is described in **Table 3** as Assay Number 3. To confirm the specificity of the assay, an additional 82 non-floury maize inbred lines were then tested. All 82 wild type lines tested negative for the targeted *fl2* allele, strongly indicating that the assay is highly selective for the *floury2* gene. The assay was then named ***fl2*_zygo**.

[0063] Table 3: KASPar® assays designed based on the variations within the exon of 22 kDa α-zein gene.

Based on 1687 bp Nucleotide Sequence Alignment			Based on 243 Amino Acid Sequence Alignment		KASPar® assay		
Assay number	Position	mutation	Position	mutation	Oligos	SEQ ID NO:	Oligo sequences
1	378	C to T	-1	Alanine to Valine	A1-1	19	GAAGGTGACCAAGTTCATGCTCCCTTTTAGTGAGCGCAACAAATGT
					A2-1	20	GAAGGTCGGAGTCAACGGATTCCTTTTAGTGAGCGCAACAAATGC
					C1-1	21	GGAGCAAGTGAGCACTGTGGAATAA
2	417	G to T	13	Serine to Isoleucine	A1-2	22	GAAGGTGACCAAGTTCATGCTCACTTGCTCCTAGTGCCAT
					A2-2	23	GAAGGTCGGAGTCAACGGATTCTCACTTGCTCCTAGTGCCAG
					C1-2	24	AACTGGTGGGAGGAACTGTGGAATA
3	495	C to T	39	Alanine to Valine	A1	6	GAAGGTGACCAAGTTCATGCTGCAAGCCTATAGGCTACAACTAGT
					A2	7	GAAGGTCGGAGTCAACGGATTCAAGCCTATAGGCTACAACTAGC
					C1	8	TGTTGCAATTGGGCAATTGGTTGTTGTAA
4	638	A to G	88	Threonine to Alanine	A1-3	25	GAAGGTGACCAAGTTCATGCTCAGCTGCTGTTGCAAGTAGGC
					A2-3	26	GAAGGTCGGAGTCAACGGATTGCAGCTGCTGTTGCAAGTAGGT
					C1-3	27	TGAGCCACCTAGCCGTGGTGAA
5	870	TCA insertion	164	Histidine insertion	A1-4	28	GAAGGTGACCAAGTTCATGCTATTGTACCAGCTCTGACTCAT
					A2-4	29	GAAGGTCGGAGTCAACGGATTATTGTACCAGCTCTGACTCAG
					C1-4	30	CAATTGGTTGAATGGAAGCA
6	899	G to A	173	Alanine to Threonine	A1-5	31	GAAGGTGACCAAGTTCATGCTAATGGAAGCAACTGTTGTAAGTAGGT
					A2-5	32	GAAGGTCGGAGTCAACGGATTATGGAAGCAACTGTTGTAAGTAGGC
					C1-5	33	CGTACCTACAACAACAGTTGCTGCAA
7	1036	CAA deletion	219	Glutamine deletion	A1-6	34	GAAGGTGACCAAGTTCATGCTGGTTGTATGGCAGCAATTGTTGC
					A2-6	35	GAAGGTCGGAGTCAACGGATTCTGGTTGTATGGCAGCAATTGTTGT
					C1-6	36	CAGTGGCTAACCCATTGGTCGCT

EXAMPLE 3: Assay validation with segregating populations

[0064] Validation with segregating populations

[0065] The *fl2*_zygo gene-specific assay was validated using segregating samples from two genetic backgrounds. Each pool contained 25 samples. Ten seeds from each sample were dissected and DNA was extracted from embryos, with a total of 500 seeds (50 ears with 10 seeds per ear), and tested with *fl2*_zygo (FIG. 6). Samples with discrepancies were further evaluated with field experiments: ten seeds from each sample were planted in Arlington, IL. Fresh leaf punches were harvested for DNA extraction and genotype testing. Plants were then self pollinated and the ears harvested. Phenotype data was collected by placing the kernels on a light box and scored based on a vitreouseness scale: translucent -wildtype; partial opaque-heterozygotes and complete opaque-homozygotes.

[0066] The assay clearly distinguished among the homozygous, heterozygous and wild type genotypes. For homozygous *floury2* as well as wild type ears, 97% had matching genotype and phenotype data, with more than half of the heterozygous ears (15 out of 26) showing discrepancies between genotype and phenotype results.

[0067] Validating the zygosity assay

[0068] With the ambiguity of the light box method, one would expect low accuracy when the phenotype relies on a small number of seeds. An ideal zygosity assay would, however, require a perfect match.

[0069] To further validate the specificity of the *fl2*_zygo assay, ten seeds from each ear with discrepancies were planted in Arlington, IL. DNA was extracted from leaf punches collected from 2 month old plants and genotyped with *fl2*_zygo. The plants were then self pollinated and seeds were harvested. The seeds were subjected to light box phenotyping after drying.

[0070] Comparison of the visual scoring with the genotype data from the *fl2*_zygo assay revealed a 95% match (145 out of 152), with the remaining seven ears all segregating with questionable phenotypes. These were either originated from Iodent lines with bronze discoloration, or had round, bulky shapes, and thus extremely difficult to score with a light box. If the phenotype was correct, there could be gene(s) in addition to the 22-kDa α-zein contributing to the *floury2* trait, since all seven ears tested as wild type genetically. Using marker assisted selection, null plants were excluded from advancement. The alanine to valine substitution in the 22-kDa α-zein is sufficient to cause the *floury2* mutation. Extended use of the gene-specific assay *fl2*_zygo should select against other mutations associated with the *floury2*

phenotype, given the high specificity of the assay for the 39 amino acid associated mutation. All *fl2* donor lines currently in conversion contained the mutated 22-kDa α-zein and tested positive for the *fl2* allele using *fl2*_zygo (data not shown).

[0071] Genotype and zygosity determinations from this *fl2* gene-specific molecular matched very closely with phenotypic data collected from the field. This assay provides an easy, rapid, and accurate way to characterize the *fl2* zygosity status using a high-throughput, molecular-based assay system.

EXAMPLE 4: Introgression of the floury2 (fl2) trait into plant lines

[0072] Introgression of fl2

[0073] Mutation fl2_zygo can be used as a robust assay for *fl2* introgression. The zygosity of a corn plant with respect to the *fl2* mutation may be determined as described in Examples 1-2. A selected corn plant determined homozygous for the *fl2* mutation may be crossed through conventional plant breeding with a corn plant known to be homozygous for a desired trait, such as *BMR* or *COMT*. F_1 progeny may then be selfed to produce F_2 progeny. Samples of genomic DNA of the F_2 progeny may be prepared and the zygosity of the F_2 progeny plants determined as described, supra. F_2 progeny plants that are homozygous for the *fl2* mutation may then be selected. The selected progeny are then assayed for high lysine content, reduced cell wall lignin content and/or increased digestibility, and those progeny that exhibit desirable traits may be further reproduced by crossing and selfing, with the resulting progeny are cultivated.

[0074] 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.

WHAT IS CLAIMED IS:

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

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

contacting the isolated genomic DNA with at least one nucleic acid molecule comprising a nucleotide sequence capable of hybridizing to SEQ ID NO:9 under high stringency conditions and at least one nucleic acid molecule capable of hybridizing to SEQ ID NO:10 under high stringency conditions; and

determining zygosity of a *fl2* mutation in the isolated genomic DNA.

2. The method of claim 1, further comprising:

contacting the isolated genomic DNA with two nucleic acid molecules each comprising a nucleotide sequence capable of hybridizing to SEQ ID NO:9 under high stringency conditions, and two nucleic acid molecules capable of hybridizing to SEQ ID NO:10 under high stringency conditions;

amplifying the nucleotide sequence of the isolated genomic DNA between nucleotide sequences of the isolated genomic DNA that hybridize to the two nucleic acid molecules each comprising a nucleotide sequence capable of hybridizing to SEQ ID NO:9 under high stringency conditions;

amplifying the nucleotide sequence of the isolated genomic DNA between nucleotide sequences of the isolated genomic DNA that hybridize to the two nucleic acid molecules each comprising a nucleotide sequence capable of hybridizing to SEQ ID NO:10 under high stringency conditions;

including in the amplifying reaction at least one nucleic acid molecule comprising a nucleotide sequence capable of hybridizing to SEQ ID NO:9 under high stringency conditions that is labeled with a first reporter, and at least one nucleic acid molecule capable of hybridizing to SEQ ID NO:10 under high stringency conditions that is labeled with a second reporter; and

detecting the levels of the first and second reporter.

3. The method of claim 2, wherein the first reporter and the second reporter are fluorescent dyes with distinguishable excitation/emission spectra.

4. The method of claim 3, wherein the first reporter is FAM, and the second reporter is VIC.

5. The method of claim 1, wherein the at least one nucleic acid molecule comprising a nucleotide sequence capable of hybridizing to SEQ ID NO:9 is between 10 and 35 nucleotides in length.

6. The method of claim 5, wherein the at least one nucleic acid molecule comprising a nucleotide sequence capable of hybridizing to SEQ ID NO:9 is between 15 and 30 nucleotides in length.

7. The method of claim 5, wherein one of the at least one nucleic acid molecules comprising a nucleotide sequence capable of hybridizing to SEQ ID NO:9 is at least 95% identical to between 10 and 35 contiguous nucleotides of SEQ ID NO:9.

8. The method of claim 7, wherein the at least one nucleic acid molecule comprising a nucleotide sequence capable of hybridizing to SEQ ID NO:9 is selected from the group consisting of SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:8.

9. The method of claim 1, wherein at least one nucleic acid molecule comprising a nucleotide sequence capable of hybridizing to SEQ ID NO:9 under high stringency conditions is labeled with a fluorescent dye, and at least one nucleic acid molecule capable of hybridizing to SEQ ID NO:10 under high stringency conditions is labeled with a second fluorescent dye with a distinguishable excitation/emission spectra.

10. The method of claim 9, wherein at least one nucleic acid molecule comprising a nucleotide sequence capable of hybridizing to SEQ ID NO:9 under high stringency conditions is labeled with FAM, and at least one nucleic acid molecule capable of hybridizing to SEQ ID NO:10 under high stringency conditions is labeled with VIC.

11. The method of claim 1, wherein amplifying the nucleotide sequence comprises amplifying in a PCR reaction.

12. The method of claim 1, wherein amplifying the nucleotide sequence comprises amplifying in a non-PCR based reaction.

13. The method of claim 1, wherein the *fl2* mutation comprises a SNP (C/T) at nucleotide position 495.

14. The corn plant comprising the *fl2* mutation of claim 13.

15. Germplasm comprising the *fl2* mutation of claim 13.

16. A method for reliably and predictably introgressing a trait for high lysine content into plant germplasm, said method comprising:

crossing a plant having a mutation in the *fl2* gene with another plant;

obtaining a sample of isolated genomic DNA from a progeny plant produced by the cross;

contacting the isolated nucleic acid with at least one nucleic acid molecule having a nucleotide sequence capable of hybridizing to SEQ ID NO:10 under high stringency conditions; and

selecting progeny from the cross that includes a mutation in the *fl2* gene by reproducing a plant from which a sample was obtained that binds the at least one nucleic acid molecules with high stringency, thereby producing a genetically engineered plant wherein a trait for high lysine content that has been introgressed into the germplasm of the genetically engineered plant.

17. A corn plant with a zygosity determined by the method of claim 16.

18. A genetically engineered plant exhibiting high lysine content produced by the method of claim 16.

19. A corn plant with a specific allele detection test determined by the method of claim 16.

20. A corn plant with a specific allele detection test determined by the method of claim 16.

21. A genetically modified corn plant, wherein the modification is a (C/T) SNP at nucleotide position 495.

22. The nucleotide sequence of SEQ ID NO:10.

23. A corn plant comprising germplasm, wherein said germplasm comprises the nucleotide sequence of SEQ ID NO:10.

FIG. 1

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B73(SEQ ID NO:9)        GAGATCATGCATGTCATTCCACATAAATGAAAAGAATTCCTATATAAAAATGACATGTTT 60
NF7_3(SEQ ID NO:13)     GAGATCATGCATGTCATTCCACATAAATGAAAAGAATTCCTATATAAAAATGACATGTTT 60
L34340(SEQ ID NO:10)    GAGATCATGCATGTCATTCCACATAAATGAAAAGAATTCCTATATAAAAACGACATGTTT 60
FF2_1(SEQ ID NO:11)     GAGATCATGCATGTCATTCCACATAAATGAAAAGAATTCCTATATAAAAACGACATGTTT 60
FF1_1(SEQ ID NO:12)     GAGATCATGCATGTCATTCCACATAAATGAAAAGAATTCCTATATAAAAACGACATGTTT 60
                        ********************************************** *********

B73             TGTTGTAGGTAGTGGAAATTATCTTTCCAGCAAAGACCATATAATCCGATAAAGCTGATA 120
NF7_3           TGTTGTAGGTAGTGGAAATTATCTTTCCAGCAAAGACCATATAATCCGATAAAGCTGATA 120
L34340          TGTTGTAGGTAGTGGAAACTATCTTTCCAGCAAAGACCATATAATCCGATAAAGCTGATA 120
FF2_1           TGTTGTAGGTAGTGGAAACTATCTTTCCAGCAAAGACCATATAATCCGATAAAGCTGATA 120
FF1_1           TGTTGTAGGTAGTGGAAACTATCTTTCCAGCAAAGACCATATAATCCGATAAAGCTGATA 120
                ****************** ****************************************

B73             ACTAAATGTCAAAATCGAGTAAGTGCCATATCATCTATATCTTATCTGTTGTTTGGAAAA 180
NF7_3           ACTAAATGTCAAAATCGAGTAAGTGCCATATCATCTATATCTTATCTGTTGTTTGGAAAA 180
L34340          ACTAAATGTCGAAATCGAGTAGGTGCCATATCATCTATATCTTATCTGTTGTTTGGAAAA 180
```

FIG. 1 (continued)

```
FF2_1           ACTAAATGTCGAAATCGAGTAGGTGCCATATCATCTATATCTTATCTGTTGTTTGGAAAA 180
FF1_1           ACTAAATGTCGAAATCGAGTAGGTGCCATATCATCTATATCTTATCTGTTGTTTGGAAAA 180
                ********** ********** ****************************************

B73             AGACAAAATCCAAAAAAAATATATGAGATCTCACATGTATAAATAGCTCCCAAATCAGTA 240
NF7_3           AGACAAAATCCAAAAAAAATATATGAGATCTCACATGTATAAATAGCTCCCAAATCAGTA 240
L34340          AGACAAAATCCAAAAAAAATATATGAGATCTCACCTGTATAAATAGCTCCCAAATCAGTA 240
FF2_1           AGACAAAATCCAAAAAAAATATATGAGATCTCACTTGTATAAATAGCTCCCAAATCAGTA 240
FF1_1           AGACAAAATCCAAAAAAAATATATGAGATCTCACTTGTATAAATAGCTCCCAAATCAGTA 240
                ********************************** ************************

B73             GTTAATACATCTCCCATAATATTTTCAGCATTCAAAAACACACCAAGCGAAGCGCACTAG 300
NF7_3           GTTAATACATCTCCCATAATATTTTCAGCATTCAAAAACACACCAAGCGAAGCGCACTAG 300
L34340          GTTAATACATCTCCCATAATATTTTCAGCATTCAGAAACACACCAAGCGAA-CG-ACTAG 298
FF2_1           GTTAATACATCTCCCATAATATTTTCAGCATTCAGAAACACACCAAGCGAAGCGCACTAG 300
FF1_1           GTTAATACATCTCCCATAATATTTTCAGCATTCAGAAACACACCAAGCGAAGTGCACTAG 300
                ********************************** ***************  * *****
```

FIG. 1 (continued)

```
B73             CAACGACCTAACACCAATGGCTACCAAGATATTAGCCCTCCTTGCGCTTCTTGCCCTTTT 360
NF7_3           CAACGACCTAACACCAATGGCTACCAAGATATTAGCCCTCCTTGCGCTTCTTGCCCTTTT 360
L34340          CAACGACCTAACAACAATGGCTACCAAGATATTAGCCCTCCTTGCGCTTCTTGCCCTTTT 358
FF2_1           CAACGACCTAACAACAATGGCTACCAAGATATTAGCCCTCCTTGCGCTTCTTGCCCTTTT 360
FF1_1           CAACGACCTAACAACAATGGCTACCAAGATATTAGCCCTCCTTGCGCTTCTTGCCCTTTT 360
                ************ ********************************************

B73             AGTGAGCGCAACAAATGCGTTCATTATTCCACAGTGCTCACTTGCTCCTAGTGCCAGTAT 420
NF7_3           AGTGAGCGCAACAAATGCGTTCATTATTCCACAGTGCTCACTTGCTCCTAGTGCCAGTAT 420
L34340          AGTGAGCGCAACAAATGTGTTCATTATTCCACAGTGCTCACTTGCTCCTAGTGCCATTAT 418
FF2_1           AGTGAGCGCAACAAATGTGTTCATTATTCCACAGTGCTCACTTGCTCCTAGTGCCATTAT 420
FF1_1           AGTGAGCGCAACAAATGTGTTCATTATTCCACAGTGCTCACTTGCTCCTAGTGCCATTAT 420
                ***************** ************************************ ***

B73             TCCACAGTTCCTCCCACCAGTTACTTCAATGGGCTTCGAACATCCAGCCGTGCAAGCCTA 480
NF7_3           TCCACAGTTCCTCCCACCAGTTACTTCAATGGGCTTCGAACATCCAGCCGTGCAAGCCTA 480
L34340          TCCACAGTTCCTCCCACCAGTTACTTCAATGGGCTTCGAACATCCAGCCGTGCAAGCCTA 478
```

FIG. 1 (continued)

```
FF2_1           TCCACAGTTCCTCCCACCAGTTACTTCAATGGGCTTCGAACATCCAGCCGTGCAAGCCTA 480
FF1_1           TCCACAGTTCCTCCCACCAGTTACTTCAATGGGCTTCGAACATCCAGCCGTGCAAGCCTA 480
                ************************************************************

B73             CAGGCTACAACTAGCGCTTGCGGCGAGCGCCTTACAACAACCAATTGCCCAATTGCAACA 540
NF7_3           CAGGCTACAACTAGCGCTTGCGGCGAGCGCCTTACAACAACCAATTGCCCAATTGCAACA 540
L34340          TAGGCTACAACTAGTGCTTGCGGCGAGCGCCTTACAACAACCAATTGCCCAATTGCAACA 538
FF2_1           TAGGCTACAACTAGTGCTTGCGGCGAGCGCCTTACAACAACCAATTGCCCAATTGCAACA 540
FF1_1           TAGGCTACAACTAGTGCTTGCGGCGAGCGCCTTACAACAACCAATTGCCCAATTGCAACA 540
                ************* ********************************************

B73             ACAATCCTTGGCACATCTAACCCTACAAACCATTGCAACGCAACAACAACAACAACAGTT 600
NF7_3           ACAATCCTTGGCACATCTAACCCTACAAACCATTGCAACGCAACAACAACAACAACAGTT 600
L34340          ACAATCCTTGGCACATCTAACCCTACAAACCATCGCAACGCAACAACAACAACAT---TT 595
FF2_1           ACAATCCTTGGCACATCTAACCCTACAAACCATCGCAACGCAACAGCAACAACAG---TT 597
FF1_1           ACAATCCTTGGCACATCTAACCCTACAAACCATCGCAACGCAACAACAACAACAG---TT 597
                ********************************** *********** ********    **
```

FIG. 1 (continued)

```
B73             TCTGCCATCACTGAGCCACCTAGCCGTGGTGAACCCTGTCACCTACTTGCAACAGCAGCT 660
NF7_3           TCTGCCATCACTGAGCCACCTAGCCGTGGTGAACCCTGTCACCTACTTGCAACAGCAGCT 660
L34340          TCTGCCATCACTGAGCCACCTAGCAGTGGTGAACCCTGTCGCCTACTTGCAACAGCAGCT 655
FF2_1           TCTGCCATCACTGAGCCACCTAGCCGTGGTGAACCCTGTCGCCTACTTGCAACAGCAGCT 657
FF1_1           TCTGCCATCACTGAGCCACCTAGCCGTGGTGAACCCTGTCGCCTACTTGCAACAGCAGCT 657
                ************************ *************** ******************

B73             GCTTGCATCCAACCCACTTGCTCTGGCGAACGTAGCTGCATACCAGCAACAACAACAGCT 720
NF7_3           GCTTGCATCCAACCCACTTGCTCTGGCGAACGTAGCTGCATACCAGCAACAACAACAGCT 720
L34340          GCTTGCATCCAACCCACTTGCTCTGGCGAACGTAGCTACATACCAGCAACAACAACAGCT 715
FF2_1           GCTTGCATCCAACCCACTTGCTCTGGCAAACGTAGTTGCAAACCAGCAACAACAACAGCT 717
FF1_1           GCTTGCATCCAACCCACTTGCTCTGGCAAACGTAGTTGCAAACCAGCAACAACAACAGCT 717
                *************************** ******* * ** ******************

B73             GCAACAGTTTATGCCAGTGCTCAGTCAACTAGCCATGGTGAACCCTGCCGTCTACCTACA 780
NF7_3           GCAACAGTTTATGCCAGTGCTCAGTCAACTAGCCATGGTGAACCCTGCCGTCTACCTACA 780
L34340          GCAACAGTTTATGCCAGCGCTCAGTCAACTAGCCATGGTGAACCCTGCCGTCTACCTACA 775
```

FIG. 1 (continued)

```
FF2_1           GCAACAGTTTCTGCCAGCGCTCAGTCAACTAGCCATGGTGAACCCTGCCGCCTACCTACA 777
FF1_1           GCAACAGTTTCTGCCAGCGCTCAGTCAACTAGCCATGGTGAACCCTACCGCCTACCTACA 777
                ********** ****** **************************** *** *********

B73             AC---------TACTTTCATCTAGCCCGCTCGCGGTGGGCAATGCACCTACGTACCTACA 831
NF7_3           AC---------TACTTTCATCTAGCCCGCTCGCGGTGGGCAATGCACCTACGTACCTACA 831
L34340          AC---------TGCTTTCATCTAGCCCGCTCGCTGTGGGCAATGCACCTACGTACCTACA 826
FF2_1           ACAGCAACAACTGCTTTCATCTAGCCCGCTCGCTGTGGCCAATGCACCTACATACCTGCA 837
FF1_1           ACAGCAACAACTGCTTTCATCTAGCCCGCTCGCTGTGGCCAATGCACCTACATACCTGCA 837
                **          * ******************** **** ************ ***** **

B73             ACAACAGTTGCTGCAACAAATTGTACCAGCTCTGACTCA---GCTAGCTGTGGCAAACCC 888
NF7_3           ACAACAGTTGCTGCAACAAATTGTACCAGCTCTGACTCA---GCTAGCTGTGGCAAACCC 888
L34340          ACAACAGTTGCTGCAACAGATTGTACCAGCTCTAACTCATCAGCTAGCTATGGCAAACCC 886
FF2_1           ACAACAATTGTTGCAACAGATTGTACCAGCTCTAACTCA---GCTAGCTGTGGCAAACCC 894
FF1_1           ACAACAATTGTTGCAACATATTGTACCAGCTCTGACTCA---GCTAGCTGTGGCAAACCC 894
                ****** *** ******* ************** *****   ******* **********
```

FIG. 1 (continued)

```
B73             TGCTGCCTACTTACAACAGTTGCTTCCATTCAACCAACTGGCTGTGTCAAACTCTGCTGC 948
NF7_3           TGCTGCCTACTTACAACAGTTGCTTCCATTCAACCAACTGGCTGTGTCAAACTCTGCTGC 948
L34340          TGCTACCTACTTACAACAGTTGCTTCCATTCAACCAATTGGCTGTGTCGAACTCTGCTGC 946
FF2_1           TGCTGCCTACTTGCAACAGCTGCTTCCATTCAACCAACTGACTGTGTCGAACTCTGCTGC 954
FF1_1           TGCTGCCTACTTGCAACAGCTGCTTCCATTCAACCAACTGACTGTGTCGAACTCTGCTGC 954
                **** ******* ****** ****************** ** ******* ***********

B73             GTACCTACAACAGCGACAACAGTTACTTAATCCATTGGCAGTGGCTAACCCATTGGTCGC 1008
NF7_3           GTACCTACAACAGCGACAACAGTTACTTAATCCATTGGCAGTGGCTAACCCATTGGTCGC 1008
L34340          GTACCTACAACAGCGACAACAATTACTTAATCCATTGGCAGTGGCTAACCCATTGGTCGC 1006
FF2_1           GTACCTACAACAGCGACAACAGTTACTTAATCCATTGGCAGTGGCTAACCCATTGGTCGC 1014
FF1_1           GTACCTACAACAGCGACAACAGTTACTTAATCCACTGGCAGTGGCTAACCCATTGGTCGC 1014
                ********************* ************ *************************

B73             TACCTTCCTGCAGCAGCAACAACAATTGCTGCCATACAACCAGTTCTCTTTGATGAACCC 1068
NF7_3           TACCTTCCTGCAGCAGCAACAACAATTGCTGCCATACAACCAGTTCTCTTTGATGAACCC 1068
L34340          TACCTTCCTGCAGCAGCAACAA---TTGCTGCCATACAACCAGTTCTCTTTGATGAACCC 1063
```

FIG. 1 (continued)

```
FF2_1           TGCCTTCCTACAGCAGCAACAA---TTGCTGCCATACAACCAGTTCTCTTTGATGAACCC 1071
FF1_1           TGCCTTCCTACAGCAGCAACAA---TTGCTGCCATACAACCAGTTCTCTTTGATGAACCC 1071
                * ******* ************   ************************************

B73             TGCCTTG------CAGCAACCCATCGTTGGAGGTGCCATCTTTTAGATTACATATGAGAT 1122
NF7_3           TGCCTTG------CAGCAACCCATCGTTGGAGGTGCCATCTTTTAGATTACATATGAGAT 1122
L34340          TGCCTTG------CAGCAACCCATCGTTGGAGGTGCCATCTTTTAGATTACATATGAGAT 1117
FF2_1           TGTCTTGTCGAGGCAGCAACCCATCGTTGGAGGTGCCATCTTTTAGATTACATATGAGAT 1131
FF1_1           TGTCTTGTCGAGGCAGCAACCCATCGTTGGAGGTGCCATCTTTTAGATTACATATGAGAT 1131
                ** ****      ********************************************

B73             GTACTCGACAATGGTGCCCTCATACCGACATGTGTTTCCTAGAAATAATCAATATATTGA 1182
NF7_3           GTACTCGACAATGGTGCCCTCATACCGACATGTGTTTCCTAGAAATAATCAATATATTGA 1182
L34340          GTACTCGACAATGGTGCCCTCATACCGGCATGTGTTTCCTAGAAATAATCAATATATTGA 1177
FF2_1           GTACTCGATAATGGTGCCCTCATACCGGCATGTGTTTCCTAGAAATAATCAATATATTGA 1191
FF1_1           GTACTCGATAATGGCGCCCTCATACCGGCATGTGTTTTCTAGAAATAATCAATATATTGA 1191
                ******** ***** ************ ********* ***********************
```

FIG. 1 (continued)

```
B73             TTGAGATTTATCTCGATATATTTCTGAACTATGTTCATCATATAAATAATTGAAAACATC 1242
NF7_3           TTGAGATTTATCTCGATATATTTCTGAACTATGTTCATCATATAAATAATTGAAAACATC 1242
L34340          TTGAGATTTATCTCGATATATTTCTGAACTATGTTCATCATATAAATAACTGAAAACATC 1237
FF2_1           TTGAGATTTATCTCGATATATTTCTGAACTATGTTCATCATATAAATAATTGAAAACATC 1251
FF1_1           TTGAGATTTATCTCGATATATTTCTGAACTATGTTCATCATATAAATAATTGAAAACATC 1251
                ************************************************** **********

B73             AAATCATAATTTTAAA-CTCATGCTTGGTCAATACATAGATAATACAATATTACTTCATC 1301
NF7_3           AAATCATAATTTTAAA-CTCATGCTTGGTCAATACATAGATAATACAATATTACTTCATC 1301
L34340          AAATCGTAATTTTAAAGCTCATGCTTGGTCAATACATAGATAATACAATATTACTTCATC 1297
FF2_1           AAATCGTAATTATAAA-CTCATGCTTGGTCAATACATAGATAATACAATATTACTTCATC 1310
FF1_1           AAATCGTAATTATAAA-CTCATCCTTGGTCAATACATAGATAATACAATATTACTTCATC 1310
                ***** ***** **** ***** ************************************

B73             ATCCCAATGATGTCCTAGCCCAACCTATTGAATGTTAATGTTTGGTTGTGTGAGGGTGTG 1361
NF7_3           ATCCCAATGATGTCCTAGCCCAACCTATTGAATGTTAATGTTTGGTTGTGTGAGGGTGTG 1361
L34340          ATCCCAATGATGTCCTAGCACAACCTATTGAATGTTAATGTTTGGTTGTGTGGGGGTGTG 1357
```

FIG. 1 (continued)

```
FF2_1           ATCCCAATGATGTCCTAGCACAACCTATTGAATGTTAATGTTTGGTTGTGTGAGGGTGTG 1370
FF1_1           ATCCCAATGATGTCCTAGCCCAACCTATTGAATGTTAATGTTTGGTTGTGTGAGGGTGTG 1370
                ********************* ********************************** *******

B73             TTTATAACATAGATGTGATTATTTGCGCTTTTTGTTGAGTATATACATATATGGTATGTT 1421
NF7_3           TTTATAACATAGATGTGATTATTTGCGCTTTTTGTTGAGTACATACATATATGGTATGTT 1421
L34340          TTTATAACATAGATGTGATTATTTGTGCTTTTTGTTGAGTATATACATATATGGTATGTT 1417
FF2_1           TTTATAACATAGATGTGATTATTTGCGCTTTTTGTTGAGTACATACATATATGGTATGTT 1430
FF1_1           TTTATAACATAGATGTGATTATTTGCGCTTTTTGTTGAGTACATACATATATGGTATGTT 1430
                ************************** *************** ******************

B73             GATTTGATATAGTGATGGACACATGCTTTGGCCTTGGATATTCAAATCACTTGTACTTGC 1481
NF7_3           GATTTGATATAGTGATGGACACATGCTTTGGCCTTGGATATTCAAATCACTTGTACTTGC 1481
L34340          GATTTGATATAGTGATGGACACATGCTTTGGCCTTGGATATTCAAATCACTTGTACTTGC 1477
FF2_1           GATTTGATATAGTGATGGACACATGCTTTGACCATGAATATTCAAATCAGTTGTATTTGC 1490
FF1_1           GATTTGATATAGTGATGGACACATGCTTTGACCATGAATATTCAAATCAGTTGTACTTGC 1490
                ****************************** ** ** ************ ***** ****
```

FIG. 1 (continued)

```
B73             ACGAAGCAAAACATAATATAAGTTTAGAAGTAAACTTGTAACTATGTCCAAACATGCTCA 1541
NF7_3           ACGAAGCAAAACATAATATAAGTTTAGAAGTAAACTTGTAACTATGTCCAAACATGCTCA 1541
L34340          ACGAAGCAAAACATAATATAAGTTTAGAAGTAAACTTGTAACTGTGTCCAAACATGCTCA 1537
FF2_1           ACGAAGCAAAACATAATATAAGTTTAGGAGTACACTTATAACTGTGTCCAAACATGCTCA 1550
FF1_1           ACGAAGCAAAGCATAACATAAGTTTAGGAGTAGACTTATAACTATGTCCAAACATGCTCA 1550
                ********** ***** ********** **** **** ***** ****************

B73             CACAAAGTCATACCGCATTATAATTTTTTGGTAAATATTCAACACATGTATTTTTTACAA 1601
NF7_3           CACAAAGTCATACCGCATTATAATTTTTTGGTAAATATTCAACACATGTATTTTTTACAA 1601
L34340          CACAAAGTCATATCGCATTATATTTTTTTGGTAAATATTCAACACATGTATTTTTTACAA 1597
FF2_1           CACAAATTCATACCACATTATAATTTTTTGGTAAATATTCCACACATGTATTTTTTACAA 1610
FF1_1           CACAAATTCATACCACATTATAATTTTTTGGTAAATATTCCACACATGTATTTTTTACAA 1610
                ****** ***** * ******* ****************** ********************

B73             GAACCCAAATTTTACAGACAAATGCAGCATTGTAGACATGTAGAATTCTTTGAAGCATGT 1661
NF7_3           GAACCCAAATTTTACAGACAAATGCAGCATTGTAGACATGTAGAATTCTTTGAAGCATGT 1661
L34340          GAACCCAAATTTTACAGACAAATGCAGCATTGTAGACATGTAGAATTCTTTGAAGCATGT 1657
```

FIG. 1 (continued)

```
FF2_1           GAACCCAAATTTTACAGACAAATGCAGCATTGTAGACATGTAGAATTCTTTGAAGCATGT 1670
FF1_1           GAACCCAAATTTTACAGACAAATGCAGCATTGTAGACATGTAGAATTCTTTGAAGCATGT 1670
                ************************************************************


B73             GAACTTAACAACACCAA 1678
NF7_3           GAACTTAACAACACCAA 1678
L34340          GAACTTAACAACACCAA 1674
FF2_1           GAACTTAACAACACCAA 1687
FF1_1           GAACTTAACAACACCAA 1687
                *****************
```

FIG. 2

```
B73(SEQ ID NO:9)      -21 MATKILALLALLALLVSATNAFIIPQCSLAPSASIPQFLPPVTSMGFEHPAVQAYRLQLA 39
NF7_3(SEQ ID NO:13)       MATKILALLALLALLVSATNAFIIPQCSLAPSASIPQFLPPVTSMGFEHPAVQAYRLQLA
L34340(SEQ ID NO:10)      MATKILALLALLALLVSATNVFIIPQCSLAPSAIIPQFLPPVTSMGFEHPAVQAYRLQLV
FF2_1(SEQ ID NO:11)       MATKILALLALLALLVSATNVFIIPQCSLAPSAIIPQFLPPVTSMGFEHPAVQAYRLQLV
FF1_1(SEQ ID NO:12)       MATKILALLALLALLVSATNVFIIPQCSLAPSAIIPQFLPPVTSMGFEHPAVQAYRLQLV
                          ********************1***********2**************************3

B73              LAASALQQPIAQLQQQSLAHLTLQTIATQQQQQQFLPSLSHLAVVNPVTYLQQQLLASNP 99
NF7_3            LAASALQQPIAQLQQQSLAHLTLQTIATQQQQQQFLPSLSHLAVVNPVTYLQQQLLASNP
L34340           LAASALQQPIAQLQQQSLAHLTLQTIATQQQQ-HFLPSLSHLAVVNPVAYLQQQLLASNP
FF2_1            LAASALQQPIAQLQQQSLAHLTLQTIATQQQQ-QFLPSLSHLAVVNPVAYLQQQLLASNP
FF1_1            LAASALQQPIAQLQQQSLAHLTLQTIATQQQQ-QFLPSLSHLAVVNPVAYLQQQLLASNP
                 ******************************** :**************4***********

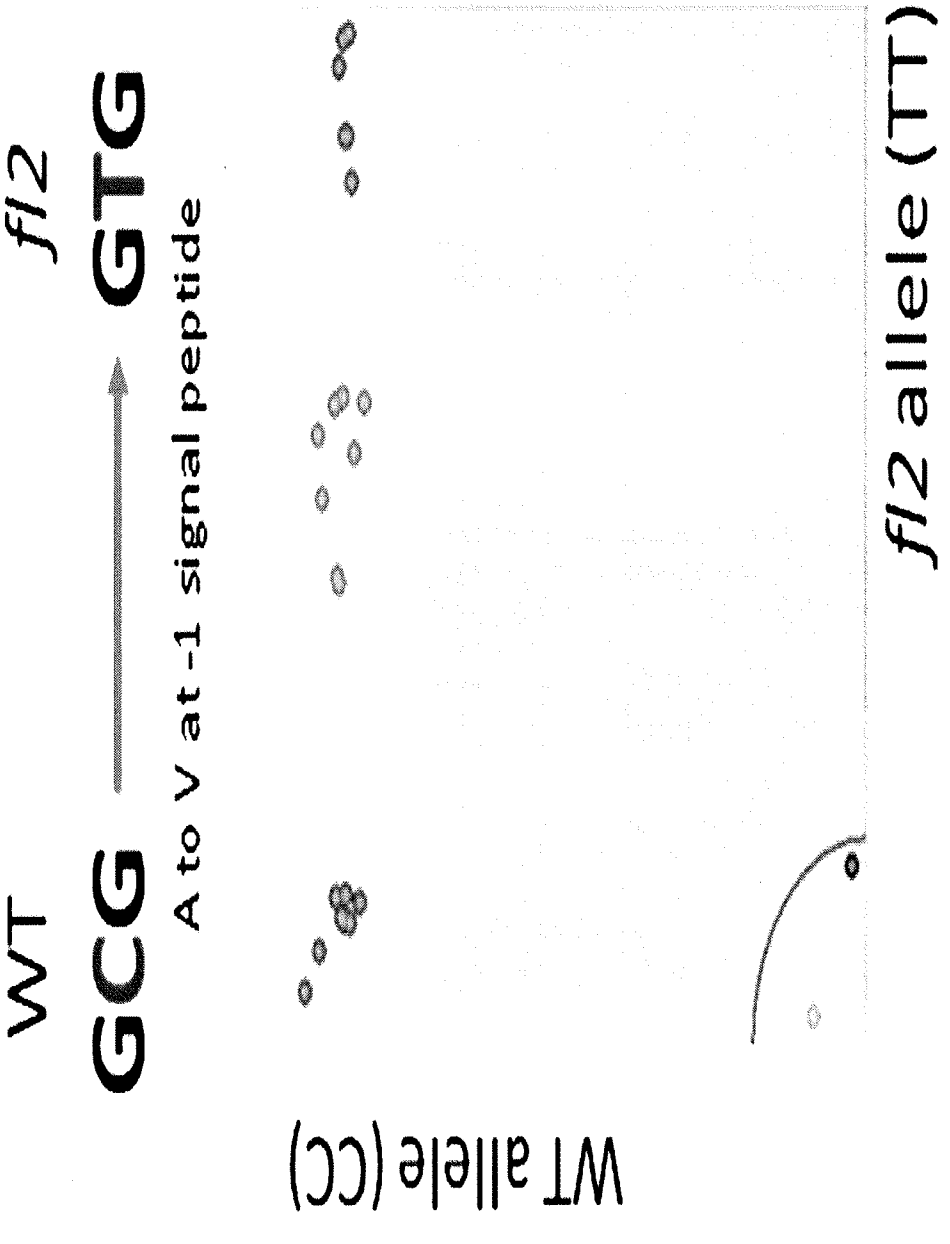
B73              LALANVAAYQQQQQLQQFMPVLSQLAMVNPAVYLQ---LLSSSPLAVGNAPTYLQQQLLQ 157
NF7_3            LALANVAAYQQQQQLQQFMPVLSQLAMVNPAVYLQ---LLSSSPLAVGNAPTYLQQQLLQ
L34340           LALANVATYQQQQQLQQFMPALSQLAMVNPAVYLQ---LLSSSPLAVGNAPTYLQQQLLQ
```

FIG. 2 (continued)

```
FF2_1           LALANVVANQQQQQLQQFLPALSQLAMVNPAAYLQQQQLLSSSPLAVANAPTYLQQQLLQ
FF1_1           LALANVVANQQQQQLQQFLPALSQLAMVNPTAYLQQQQLLSSSPLAVANAPTYLQQQLLQ
                ******.  *********:*.*********:.***   *********.************

B73             QIVPALT-QLAVANPAAYLQQLLPFNQLAVSNSAAYLQQRQQLLNPLAVANPLVATFLQQ 216
NF7_3           QIVPALT-QLAVANPAAYLQQLLPFNQLAVSNSAAYLQQRQQLLNPLAVANPLVATFLQQ
L34340          QIVPALTHQLAMANPATYLQQLLPFNQLAVSNSAAYLQQRQQLLNPLAVANPLVATFLQQ
FF2_1           QIVPALT-QLAVANPAAYLQQLLPFNQLTVSNSAAYLQQRQQLLNPLAVANPLVAAFLQQ
FF1_1           HIVPALT-QLAVANPAAYLQQLLPFNQLTVSNSAAYLQQRQQLLNPLAVANPLVAAFLQQ
                :******5***:****6**********:**************************:****

B73             QQQLLPYNQFSLMNPAL--QQPIVGGAIF 243
NF7_3           QQQLLPYNQFSLMNPAL--QQPIVGGAIF
L34340          QQ-LLPYNQFSLMNPAL--QQPIVGGAIF
FF2_1           QQ-LLPYNQFSLMNPVLSRQQPIVGGAIF
FF1_1           QQ-LLPYNQFSLMNPVLSRQQPIVGGAIF
                **7************.*  **********
```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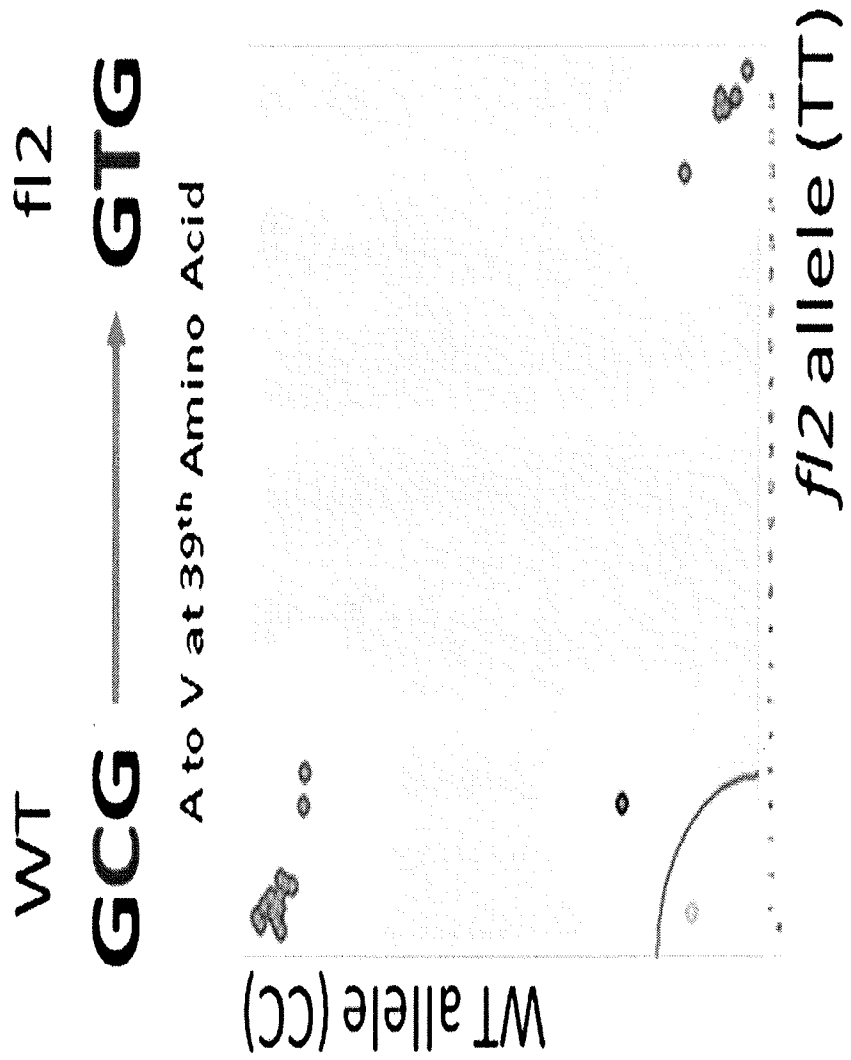
GCG
fl2
GTG
A to V at -1 signal peptide
WT allele (CC)
fl2 allele (TT)

FIG. 3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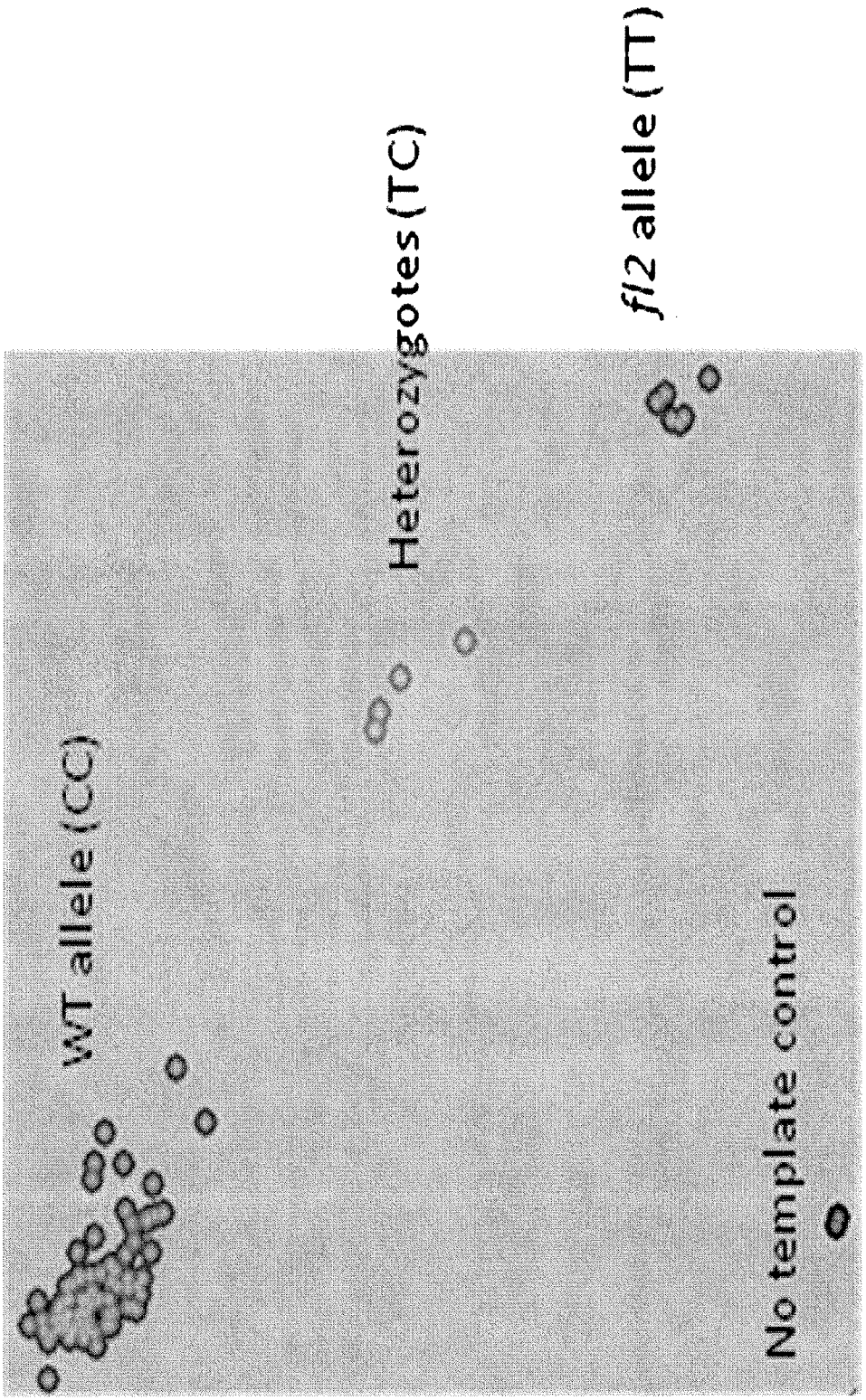
GCG
fl2
GTG
A to V at 39th Amino Acid
WT allele (CC)
fl2 allele (TT)

FIG. 4

FIG. 5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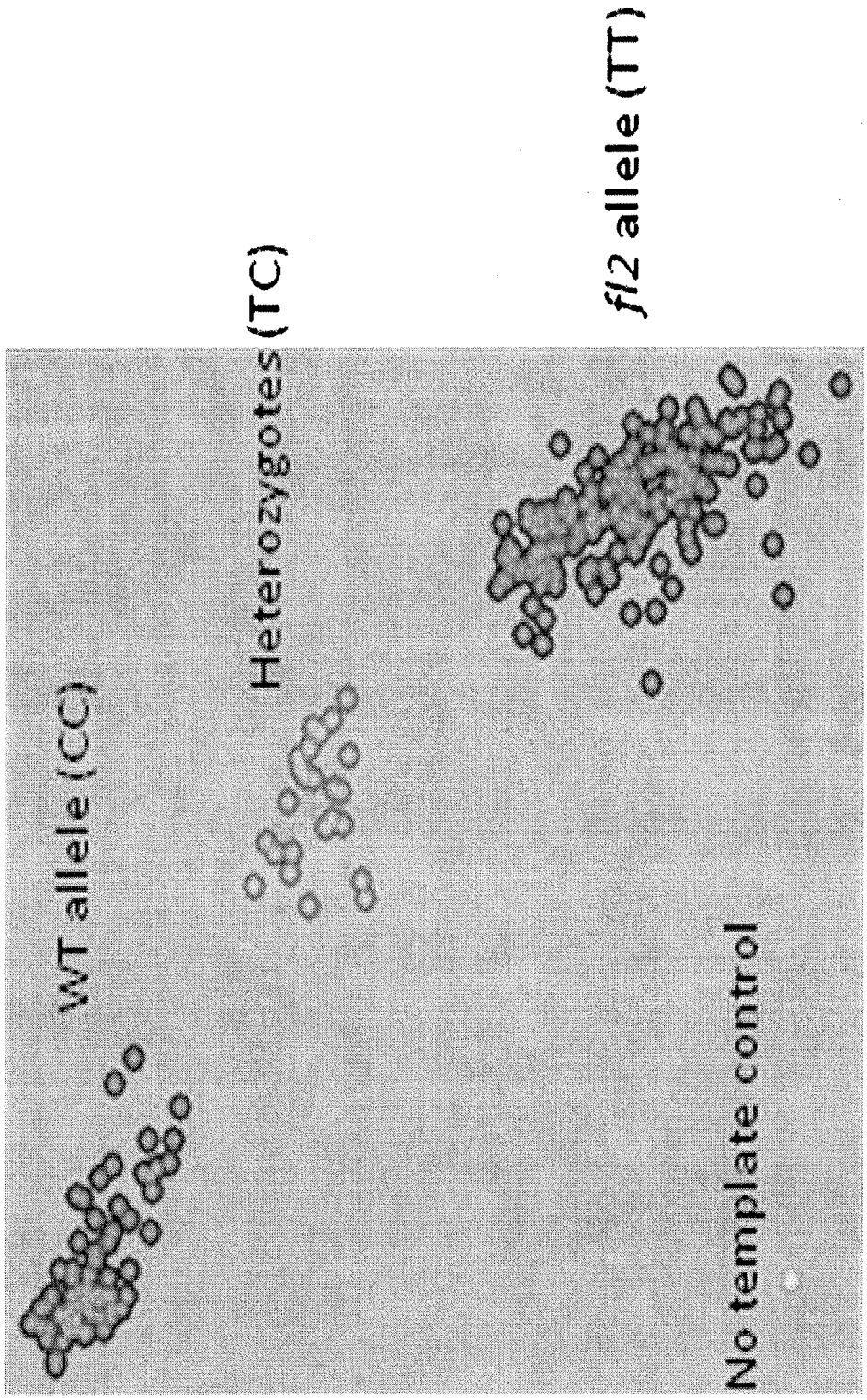
Heterozygotes (TC)
f/2 allele (TT)
No template control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Heterozygotes (TC)
fl2 allele (TT)
No template control

FIG. 6