Theoretical and Applied Genetics
https://doi.org/10.1007/s00122-018-3061-2

ORIGINAL ARTICLE

Characterization of a new *GmFAD3A* allele in Brazilian CS303TNKCA soybean cultivar

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Received: 24 October 2017 / Accepted: 19 January 2018

Abstract

Key Message We molecularly characterized a new mutation in the *GmFAD3A* gene associated with low linolenic content in the Brazilian soybean cultivar CS303TNKCA and developed a molecular marker to select this mutation.

Abstract Soybean is one of the most important crops cultivated worldwide. Soybean oil has 13% palmitic acid, 4% stearic acid, 20% oleic acid, 55% linoleic acid and 8% linolenic acid. Breeding programs are developing varieties with high oleic and low polyunsaturated fatty acids (linoleic and linolenic) to improve the oil oxidative stability and make the varieties more attractive for the soy industry. The main goal of this study was to characterize the low linoleic acid trait in CS303TNKCA cultivar. We sequenced CS303TNKCA *GmFAD3A*, *GmFAD3B* and *GmFAD3C* genes and identified an adenine point deletion in the *GmFAD3A* exon 5 (delA). This alteration creates a premature stop codon, leading to a truncated protein with just 207 residues that result in a non-functional enzyme. Analysis of enzymatic activity by heterologous expression in yeast support delA as the cause of low linolenic acid content in CS303TNKCA. Thus, we developed a TaqMan genotyping assay to associate delA with low linolenic acid content in segregating populations. Lines homozygous for delA had a linolenic acid content of 3.3 to 4.4%, and the variation at this locus accounted for 50.83 to 73.70% of the phenotypic variation. This molecular marker is a new tool to introgress the low linolenic acid trait into elite soybean cultivars and can be used to combine with high oleic trait markers to produce soybean with enhanced economic value. The advantage of using CS303TNKCA compared to other lines available in the literature is that this cultivar has good agronomic characteristics and is adapted to Brazilian conditions.

Introduction

Soybean (*Glycine max* (L.) Merrill) is the most important legume cultivated in the world due to its high productivity, low production cost and its high protein and oil content. The 2016/2017 harvest had 120.48 million hectares

Luiz Claudio Costa Silva, Rafael Delmond Bueno, and Loreta Buuda da Matta have contributed equally to this work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00122-018-3061-2) contains supplementary material, which is available to authorized users.

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Published online: 03 February 2018

planted worldwide with a productivity of 351.74 million tons (USDA 2017b). Brazil is the second largest producer with 114.04 million tons of grain, nearly a third of the global production (CONAB 2017).

Soybean oil represents 29% of all vegetable oil consumed in the world and is the second in the world ranking in terms of production and consumption among vegetable oils (USDA 2017a). Almost eight million tons of soybean oil was produced in Brazil during 2016, and over 80% was intended for domestic consumption (ABIOVE 2017). The significant increase in consumption over the past few years was mainly due to the amount of biodiesel blended in diesel oil, which rose from 5 to 8% and growing to 10% by 2019, according to the Brazilian government (Brazil 2017).

The soybean seed accumulates about 20% of oil and contains on average 13% of palmitic acid (16:0), 4% stearic acid (18:0), 20% oleic acid (18:1 Δ^9), 55% linoleic acid (18:2 $\Delta^{9,12}$) and 8% linolenic acid (18:3 $\Delta^{9,12,15}$) (Goettel et al. 2014; Pham et al. 2010). The fatty acid composition determines the oil quality and influences its physical and chemical properties, such as melting point and oxidative stability (Fehr 2007; Yadav 1996). The high concentration of polyunsaturated fatty acids in soybean oil is the main cause of low oxidative stability, resulting in rancidity, a rapid decline of optimal flavor and shortened storage time of manufactured food products (Warner and Fehr 2008).

In order to increase the oxidative stability of soybean oil, the food industry has used chemical hydrogenation to reduce linoleic and linolenic acid and increase oleic acid, but this process generates trans isomers of fatty acids. The consumption of partially hydrogenated compounds is directly related to the incidence of certain heart disease, high cholesterol levels and development of type 2 diabetes (Hu et al. 1997; Mozaffarian et al. 2006; Pham et al. 2014; Yadav 1996). This fact is so significant that the FDA (US Food & Drug Administration) made a final ruling that partially hydrogenated oils (PHOs) are no longer considered as safe (FDA 2015), and established a compliance period of 3 years for food manufacturers to comply or make a request to use PHOs (FDA 2016).

Conventional breeding approaches and genetic engineering have been used to obtain soybean varieties with low polyunsaturated fatty acid content, improving the oxidative stability without the need for chemical hydrogenation (Fehr 2007; Pham et al. 2014; Warner and Fehr 2008). The first soybean breeding program aimed at changing the fatty acid composition to improve quality gains started in the 1950s (Dunton et al. 1951). Over the years, it was determined that the biosynthesis of polyunsaturated fatty acids in soybeans depends mainly on the endoplasmic reticulum desaturase enzymes (microsome) (Baud and Lepiniec 2010). The primary substrates for these enzymes are the fatty acid residues linked to phosphatidyl choline (PC) or coenzyme A (CoA). The ω-6 desaturase converts oleyl (18:1) into linoleyl (18:2), while the ω-3 desaturase is responsible for the conversion of linoleyl (18:2) into linolenyl (18:3) (Baud and Lepiniec 2010).

The gene encoding ω-3 desaturase enzyme in soybean is called *GmFAD3*. The reduction of linolenic acid content in soybean genotypes is controlled by mutations in some alleles at loci *fan1*, *fan2*, *fan3* and *fanx* related to ω-3 desaturase microsomal enzyme (Anai et al. 2005; Bilyeu et al. 2005, 2006; Chapell 2006). Initially, three independent loci controlling linolenic acid production in soybean, designated *GmFAD3-A*, *GmFAD3-B* and *GmFAD3-C*, were identified (Bilyeu et al. 2003). Currently, *GmFAD3* makes up a small family of four genes, designated *GmFAD3-1a* (equivalent to *GmFAD3B*), *GmFAD3-1b* (equivalent to *GmFAD3A*), *GmFAD3-2a* (equivalent to *GmFAD3C*) and *GmFAD3-2b* (Anai et al. 2005). *Glyma.14g194300* is the gene encoding by *GmFAD3A/fan1* locus in linkage group (LG) B2/chromosome 14; *Glyma.02g227200* encodes *GmFAD3B/fan3* in LG-D1b/chromosome 2; and *Glyma.18g062000* encodes *GmFAD3C/fan2* in GL-G/chromosome 18 (Bilyeu et al. 2011; Cardinal et al. 2011; Goodstein et al. 2012).

Recurrent selection using genetic materials with low levels of linolenic acid (White et al. 1961) as a gene donor has made it possible to reduce linolenic acid content to 4.2% (Wilson et al. 1981). More recently, the use of genetic changes induced by physical irradiation and chemical mutagenesis produced mutants with low linolenic acid content: A5, C1640, RG10, M5, IL8, A23 and A26 (Bubeck et al. 1989; Fehr and Hammond 2000; Hammond and Fehr 1983; Rahman et al. 1996; Stojsin et al. 1998; Wilcox et al. 1984). The sequencing of *GmFAD3* genes in soybean lines A5, A26 and A23, together with the marker–trait association analysis identified mutations in three *GmFAD3* genes, which directly contributes to low linolenic acid content in these soybean lines (Bilyeu et al. 2003, 2006; Pham et al. 2014; Thompson et al. 2002). The combination of alleles *fan1* (A5), *fan2* (A23) and *fan3* (A26) produced the line A29 with only 1% linolenic acid (Fehr and Hammond 2000; Pham et al. 2014; Ross et al. 2000).

Through a breeding program, we developed a variety line CS303TNKCA containing low linolenic acid content (3.5–4%), the absence of lipoxygenase (*lox1*, *lox2* and *lox3*) and the Kunitz protease inhibitor. In the present study, we evaluated the genetic structure of *GmFAD3A*, *GmFAD3B* and *GmFAD3C* in CS303TNKCA soybean line to identify mutations. The data were used to develop a TaqMan genotyping assay, and the observed causative mutation was associated with a decreased content of linolenic acid in a segregating population evaluated in field and greenhouse trials. The methodology developed in this study will assist in the selection of individuals obtained from CS303TNKCA with low linolenic acid content. This variety has good agronomic characteristics and is adapted to Brazilian conditions, which

is a great advantage in comparison with other lines reported in the literature because this will accelerate gene introgression in other cultivars.

Materials and methods

Plant material

The present study used two contrasting soybean lines for linolenic acid content. The CS303TNKCA variety is a soybean cultivar developed by Programa de Melhoramento da Qualidade da Soja (PMQS/BIOAGRO/UFV), with middle oil content in the seed (20–23%), low linolenic acid content (about 3.5–4.0%), absence of lipoxygenase (*lox1*, *lox2* and *lox3*) and Kunitz protease inhibitor. This variety is an isoline obtained by backcrossing BARC-12 (Leffel 1994a) into the recurrent parent variety, Monarca (COOPADAP, Brazil). The FA22 line was developed by chemical mutagenesis in Iowa State University, with average oleic acid (50%) and normal linolenic acid content (about 5.0–6.0%) (Alt et al. 2005).

Population development

The cultivar CS303TNKCA and FA22 line were used to develop the study population. The crossing between the two lines was carried out in greenhouses (GH) at the Universidade Federal de Viçosa (Viçosa, Minas Gerais (MG), Brazil; 20°45′14″S, 42 52′55″W) to obtain the F2 population. F2:3 families were grown in field trials at Universidade Federal de Viçosa (Visconde do Rio Branco (VRB), MG, Brazil; 21 00′37″S, 42 50′26″W). The experiment was conducted in a randomized block design with two replications. Each plot was planted with 25 seeds per 1.5-meter row and a row spacing of 0.5 meters. Families F2:4 were planted in Viçosa (VIC) and twice in São Gotardo (SG) (São Gotardo, MG, Brazil; 19 18′39″S, 46 02′ 56″W) under similar conditions as the previous experiment.

DNA extraction, sequencing and mutation identification

Leaf samples from CS303TNKCA, FA22, F1 and F2 plants were collected at V2 stage (Flores et al. 2008), frozen in liquid nitrogen and then lyophilized. Genomic DNA was extracted using the methodology proposed by Doyle and Doyle (1990). The DNA concentration was determined using NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the quality checked by 0.8% agarose gel electrophoresis.

The complete gene sequences of *GmFAD3A* (*Glyma.14G194300*), *GmFAD3B* (*Glyma.02G227200*) and *GmFAD3C* (*Glyma.18G062000*) were obtained from the PHYTOZOME database (Goodstein et al. 2012; Schmutz et al. 2010). Using Primer3 Input Program (Rozen and Skaletsky 2000), we designed sets of primers for each selected gene in order to amplify the entire gene region (Supplementary Table S1). Each set of primers were tested for producing a single amplification product, which was purified using ExoSAP IT kit (USB Corporation, Cleveland, Ohio, USA) and then sequenced by Macrogen company (Gasan-dong, Geumchun-gu, Seoul, Korea). The resulting sequences were edited using the Sequencer 4.1.4 program (Gene Codes Corporation), aligned using the ClustalW program (Thompson et al. 2002) and the polymorphisms were analyzed in ExPASy platforms (Artimo et al. 2012) and NetPlantGene Server (Goodstein et al. 2012). We used the reference genes from Williams 82 to identify and compare mutations.

Enzymatic activity

The enzymatic activity of the *GmFAD3A* was investigated through its heterologous expression in *Saccharomyces cerevisiae*. RNA samples of CS303TNKCA and the accession PL04 (wild type for *GmFAD3A*) were extracted from seeds in R5 stage and quantified using Picogreen (Thermo Fisher Scientific) in SpectraMax M5 instrument (Molecular Devices). The RNA quality was checked on a 1.5% agarose gel. Subsequently, 1 µg of RNA sample was treated with DNAse I Amplification Grade (Thermo Scientific) and then subjected to reverse transcription using Superscript III (Invitrogen). *GmFAD3A* was cloned using specific sets of primers that include sites for BamHI (*GmFAD3A*/Fw: CCC GGATCCATGGTTAAAGACACAAAGCCTT) or XhoI (*GmFAD3A*/Rv: GGGGCTCGAGTCAGTCTCGGTG CGAGTGA and *GmFAD3A*-CS303/Rv: GGGGCTCGAG CTATCCCTTTCTCTCACTGG) restriction enzymes. The CDS region was amplified in reactions containing 2.0 µL of cDNA, 3.0 µL of 10× PCR Rxn Buffer (Invitrogen), 1.5 mM of $MgSO_4$, 200 µM of each deoxyribonucleotide, 0.33 µM of each primer, and 0.5 U of Platinum Taq DNA Polymerase High Fidelity (Invitrogen), with a total volume of 30 µL. PCR was performed under the following conditions: 94 °C for 2 min; 40 cycles initiated at 94 °C for 15 s, 55 °C for 30 s, and 68 °C for 2 min; and a final step at 68 °C for 10 min. Amplification products were visualized by 1.2% agarose gel electrophoresis and purified using PureLink Quick Gel Extraction Kit (Thermo Fisher Scientific). These products were digested with BamHI and XhoI restriction enzymes (New England Biolabs), as suggested by the manufacturer, and cloned into a pYES2 Yeast Expression Vector (Thermo Fisher Scientific). The cloning reaction was performed using 90 fmol of fragment, 30 fmol of pYES2, 1.0 µL of T4 DNA

Ligase (New England Biolabs), and 2.0 µL of T4 DNA Ligase Reaction Buffer, in a total volume of 20 µL.

The constructed vectors were transformed into *Escherichia coli* strain DH5α for validation and multiplication, and then transformed into *S. cerevisiae* strain W303, as described by Gietz and Schiestl (2007). Recombinant yeasts were grown in 7 mL of SD mid containing galactose at 28 °C for 12–16 h followed by 26 °C for around 48 h in three to five replicates. Fatty acids from recombinant yeasts were extracted and analyzed by gas chromatography. ANOVA was performed to analyze the variance in each fatty acid content, and the Tukey test was used to compare means (5% of significance level).

Expression analysis

Real-time PCR was performed on a 7500 Real-Time PCR Systems (Applied Biosystems). The sequences of *GmFAD3A* (*Glyma.14g194300*), *GmFAD3B* (*Glyma.02g227200*) and *GmFAD3C* (*Glyma.18g062000*) transcripts were obtained from the PHYTOZOME database (Goodstein et al. 2012). The primers were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA), and the specificity confirmed by BLAST in the PHYTOZOME database (Supplementary Table S2). The real-time PCR reactions were performed using 1 µL of 1:10 diluted cDNA, 5 µL of primer Forward and Reverse mixed at 1.5 µM (each primer) and 6 µL of SYBR Green PCR Master Mix. The experiment was conducted using three biological replicates for each genotype and two technical replicates using the following conditions: 95 °C for 10 min; 40 cycles at 95 °C for 15 s and 60 °C for 1 min. We analyzed *GmFAD3* gene expression in the CS303TNKCA variety, and the soybean lines FA22, SUPREMA, BARC8 and BR8014887. We used lines with differences in genetic background and in fatty acid content to make a better evaluation of *GmFAD3* expression levels. SUPREMA have high oil content and normal levels of oleic and linolenic acids (about 20% oleic, 58% linoleic and 8% linolenic). BARC8 (about 19% oleic, 59% linoleic and 8% linolenic) and BR8014887 (about 15% oleic, 61% linoleic and 11% linolenic) are both high protein content lines with normal levels of oleic and linolenic acids. All lines are commonly used in our breeding program as allele donors.

A literature review was conducted to find endogenous controls already used and validated in soybean (Hu et al. 2009; Le et al. 2012; Li et al. 2012; Libault et al. 2008; Miranda Vde et al. 2013; van de Mortel et al. 2007). We tested the stability in our samples and by statistical testing using the GeNorm (Vandesompele et al. 2002), BestKeeper (Pfaffl et al. 2004) and NormFinder software (Andersen et al. 2004) we selected CONS7 and UKN2 (GenBank ID AW310136 and BE330043, respectively; data not shown). To evaluate desaturase expression, we used the analysis described in Hellemans et al. (2007). The results and the statistical analysis were plotted using GraphPad Prism.

RT-PCR

The RT-PCR was performed in 15 µL, containing 2 µL of reverse transcription reaction product, 0.33 µM of each primer, 0.5 U of Platinum® *Taq* DNA Polymerase (Invitrogen), 200 µM of each deoxyribonucleotide, 1.5 µL PCR Buffer (Invitrogen), 1.5 mM of $MgCl_2$, under the following conditions: 94 °C for 2 min; 38 cycles initiated at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min; and a final step at 72 °C for 7 min. The PCR products were visualized in 1.2% agarose gel electrophoresis. The primers used to amplify *GmFAD3* genes were: *GmFAD3A*/B-Fw: ATGGTTAAAGACACAAAGCCTT (*GmFAD3A* and *GmFAD3B*), *GmFAD3C*-Fw: AAGGCACAATGTTTTGGGCAC (*GmFAD3C*), Rv-*GmFAD3A*: TCACTCGCACCGAGACTGA (*GmFAD3A*), Rv-*GmFAD3B*: CCACTCGCAACGAGACTGA (*GmFAD3B*), Rv-*GmFAD3C*: CTTGGACCCAGTCCAACTAA (*GmFAD3C*).

Genotyping

The genotyping of an F2 segregating population for low linolenic acid content was performed using the TaqMan genotyping system using the following primers and allele-specific probes: forward TCTCAAGAAGCCCCGGAAAG; reverse CCATAGAGCTTGAGCAATAGAACTG; probe1/FAM CAGTGAGAGAAAGGGAATA; probe2/VIC CCAGTGAGAGAAAGGGATAG). The experiment was conducted on 7500 Real Time PCR Systems (Applied Biosystems) using 96-well plates at 10 µL per well containing 50 ng of DNA, 5 µL of TaqMan Genotyping MasterMix (Thermo Fisher Scientific), 250 nM of each probe, and 300 nM of each primer. PCR was started at 95 °C for 10 min, and 60 cycles at 95 °C for 20 s, and 63 °C for 1 min. The genotypes were identified using 7500 Software version 2.3 with the Quality Value of 90%.

Phenotyping analysis

Lipids of CS303TNKCA, FA22 and all F2 individuals were extracted using the following method: 1 mL of hexane was added to 15 mg of powdered soybean seed, mixed with N_2 and stored at 4 °C for 16 h. The hexane solution was collected into another tube and evaporated. Then, 0.4 mL of 1 M sodium methoxide was added to the tube, shaken and incubated at 30 °C for 1 h. Then, 1 mL of milliQ water and 1 mL of hexane was added, shaken and incubated at room temperature for 1 h. Finally, 0.75 mL of the organic phase was collected, added to anhydrous sodium sulfate to remove the moisture and transferred to a vial tube. The fatty acid

composition was performed by gas chromatography using the Model GC-2010 Plus (Shimadzu), as described by Burkey et al. (2007). The fatty acid content was determined as a proportion of total fatty acids represented in g/kg of oil. The seed oil content was determined by nuclear magnetic resonance spectrometry (NM—Resonance Instruments, Witney, Oxfordshire, UK).

Statistical analysis

To assess the mutation segregation on *GmFAD3A* gene in the F2 population, the Chi square test was performed. We calculated averages, standard deviation, and maximum and minimum content of palmitic, stearic, oleic, linoleic and linolenic acids for each experiment. We performed ANOVA to analyze the variance between the genotypes in each fatty acid, and the Tukey test was performed to compare means (5% significance level). Linear regression was performed to verify the association between the mutation and the linolenic acid content, as well to check the additive and dominant effects. All analyses were performed using GENES software (Cruz 2013). Graphs of "linolenic acid content x frequency of occurrence" were constructed using Microsoft Office Excel and Microsoft Office Power Point, separating individuals by genotype, and grouping the values in intervals of standard deviation.

Results

Identifying mutations in *GmFAD3* genes

The sequencing of *GmFAD3A* gene (*Glyma.14G194300*) covered a total of 3721 bp in CS303TNKCA, including approximately 56 bp upstream and 408 bp downstream of the ORF. The sequence comparison between CS303TNKCA-amplified *GmFAD3A* and from the reference variety Wm82 revealed the presence of eight mutations in *GmFAD3A* gene: two mutations in exon region, five mutations in introns and a single mutation in 3′UTR (Table 1). We observed an exchange (A>G) at position 34 in exon 2. The intron 3 harbored a T insertion at position 94, while exon 5 contained an A deletion at position 79. Analysis of the intron 6 sequences revealed a C insertion at position 66 and an exchange (G>T) at position 383. Furthermore, in intron 7, an exchange (T>C) at position 184 and an insertion of an A in position 252 were detected. In the 3′UTR region, an A>G change was discovered at position 140. None of the mutations caused alterations in splicing sites according to NetPlantGene Server platform (Goodstein et al. 2012). The exchange (A>G) at position 34 of exon 2 does not result in amino acid change. However, the deletion of an A at position 79 of exon 5 (delA), position 622 counting from the first ATG, resulted in a premature stop codon, leading to a truncated protein with just 207 residues compared to the 376 residues of the standard genotype protein in Williams 82 (Fig. 1). The missing fragment of 169 amino acid residues in CS303TNKCA harbors a region rich in histidine, and it is considered essential for the catalytic function of the desaturase enzyme (Shanklin et al. 1994).

The sequencing of the *GmFAD3B* gene (*Glyma.02G227200*) covered a total of 2670 bp in CS303TNKCA, in which 62 bp were upstream and 203 bp downstream from the ORF. The sequencing of *GmFAD3C* genes (*Glyma.18G062000*) covered a total of 2803 bp, which includes 44 bp upstream and 225 bp downstream of the ORF. The sequence comparisons between the corresponding loci of CS303TNKCA and Wm82 revealed no polymorphism in the sequenced regions of the *GmFAD3B* and *GmFAD3C* genes. The exons were completely sequenced for all three genes, while intronic regions were partially sequenced in *GmFAD3A* and *GmFAD3B* (Supplementary Fig. S1). As *GmFAD3* is highly expressed in seed (Bilyeu et al. 2005), we performed an RT-PCR to verify if there were any non-identified intron mutations that could result in different transcripts due to splice variants (Supplementary Fig. S2). The results indicate that CS303TNKCA seeds do not hold different splice forms of *GmFAD3A*, *GmFAD3B* or *GmFAD3C*.

Enzymatic activity

The complete *GmFAD3A* CDS sequences of CS303TNKCA and PL04 individuals (*GmFAD3A* wild-type allele) were cloned, transformed and expressed in *S. cerevisiae* strain W303. No significant variance was found in palmitic, palmitoleic, stearic and oleic acids. Yeasts transformed with delA *GmFAD3A* allele produced 2.42% of linoleic acid and no detectable levels of linolenic acid, values similar to yeasts transformed by empty vector (2.30% of linoleic acid and no detectable linolenic acid), while yeasts transformed with

Table 1 Variants in DNA sequences between CS303TNKCA and Williams 82 in *GmFAD3A* gene

Soybean line	Exon 2	Intron 3	Exon 5	Intron 6		Intron 7		3′ UTR
Position	34	94	**79**	66	383	184	252	140
Williams 82	A	–	**A**	–	G	T	–	A
CS303 TNKCA	G	T	**–**	C	T	C	A	G

Bold characters in Exon 5 indicate the 1-bp deletion that leads to a frameshift

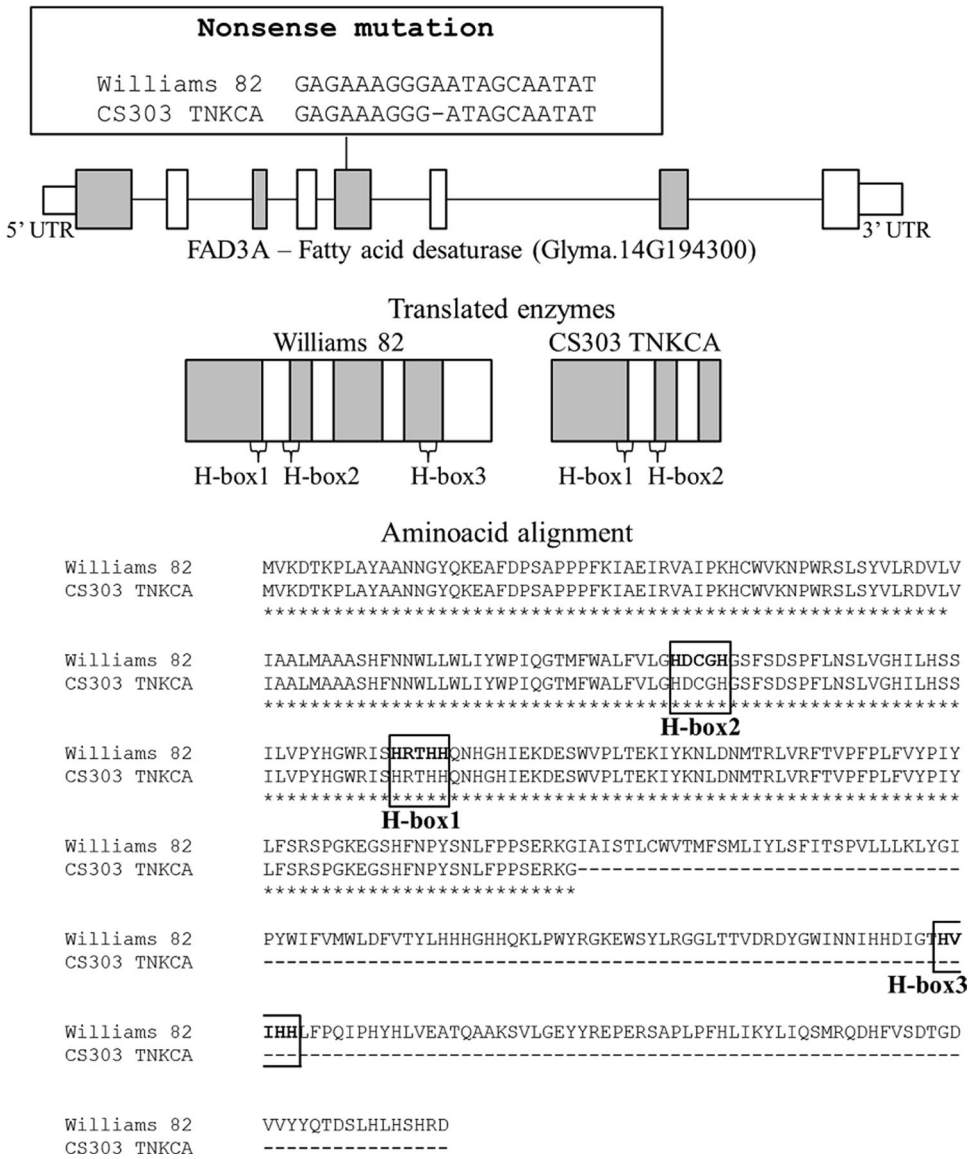

Fig. 1 A 1-bp deletion in the 5th exon of *GmFAD3A* on CS303TNKCA soybean cultivar leads to a stop codon and the loss of a histidine box3

Table 2 Average fatty acid content of empty vector and *GmFAD3A*—transformed yeasts

Colonies	Allele donor	Fatty acid content (%)					
		Palmitic	Palmitoleic	Stearic	Oleic	Linoleic	Linolenic
Yeast transformed by empty vector	–	14.66 ± 3.55	36.27 ± 8.86	7.73 ± 2.62	39.04 ± 7.37	2.3 ± 2.3[b]	ND[b]
GmFAD3A wild type	PL04	16.92 ± 4.45	22.30 ± 6.45	13.71 ± 2.72	34.04 ± 5.2	8.48 ± 1.28[a]	4.55 ± .49[a]
GmFAD3A delA	CS303TNKCA	15.53 ± 1.91	36.35 ± 8.09	10.57 ± 4.41	35.14 ± 4.07	2.42 ± 2.84[b]	ND[b]

Tukey test was performed for linoleic and linolenic acid contents for traits with significant variance ($P < 0.05$). For the test, ND was considered as "zero"

Values obtained from three to five quantifications, each one from three colonies

The means are followed by standard deviation

ND non-detectable

wild-type *GmFAD3A* allele produced 8.48% of linoleic acid and 4.55% linolenic acid, on average (Table 2).

Expression analysis

To evaluate the expression of *GmFAD3A*, *GmFAD3B* and *GmFAD3C* desaturases genes, we first selected endogenous controls validated in soybean, tested the stability in our samples and by statistical test selected CONS7 and UKN2 (data not shown) to normalize the desaturase expression. We found no variation in gene expression between the five lines evaluated (Fig. 2). This result indicates that delA mutation has no interference on transcription level of *GmFAD3A*, *GmFAD3B* and *GmFAD3C*.

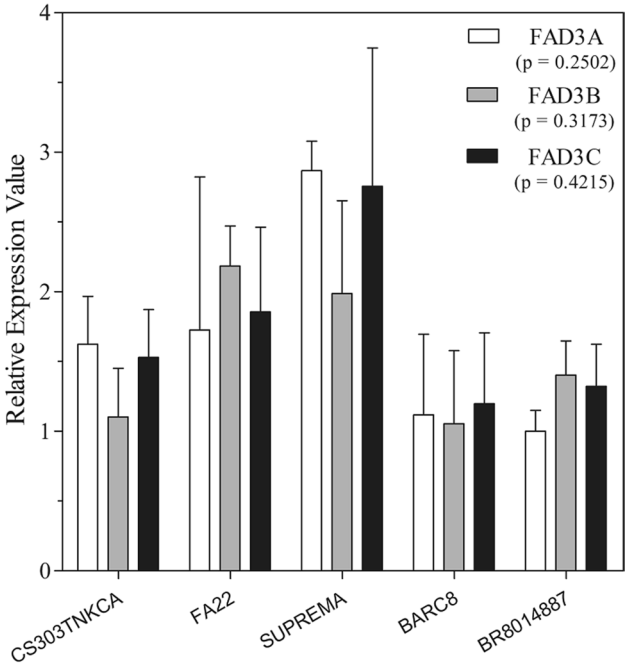

Fig. 2 *GmFAD3A*, *GmFAD3B* and *GmFAD3C* gene expression patterns from CS303TNKCA, FA22, SUPREMA, BARC8 and BR8014887 soybean lines using CONS7 and UKN2 genes as endogenous controls. A one-way ANOVA with Tukey's test was performed to compare expression between lines in the same gene. *P*-values < 0.05 were considered significant

Genotyping analysis

We designed a diagnostic marker flanking the delA mutation in order to differentiate contrasting individuals for linolenic acid content. A total of 187 F2 plants were genotyped using the TaqMan marker (Fig. 3). We observed 44 homozygous mutants, 38 wild-type homozygous and 105 heterozygous plants, obtaining a Chi square equal to 3.21 (P equal 0.2005), indicating that the marker behaves as expected for Mendelian segregation.

Phenotyping analysis

The levels of palmitic, stearic, oleic, linoleic and linolenic acids were determined for 187 F2 seeds derived from CS303TNKCA × FA22 planted in the greenhouse (GH), and for 187 F2 derived families grown in Visconde do Rio Branco (VRB) (F2:3), Viçosa (VIC) (F2:4) and São Gotardo (SG1 and SG2) (F2:4) (Table 3). In all evaluated experiments, the linolenic acid content ranged between 2.67 and 9.45%. No significant variance was found in palmitic and oleic acid content. For linoleic acid, significant variance was found only in GH. For stearic acid, we found significant variance in VRB, VIC, SG1 and SG2. For linolenic acid content, variance was significant in all evaluations.

Means and frequency of linolenic acid content for each genotype among the 187 lines are shown in Fig. 4. The

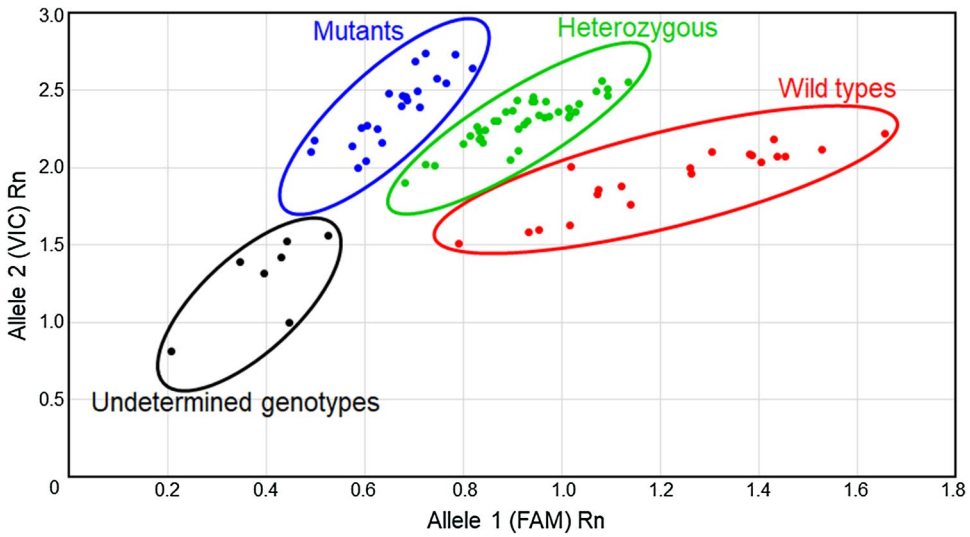

Fig. 3 Taqman marker genotyping graph of 187 individuals from a CS303TNKCA × FA22 F2 population developed based on a 1-bp deletion associated with low linolenic content. The *x*-axis is the fluorescence data from the FAM-allele (Wild-type), whereas the *y*-axis is the fluorescence data from the VIC-allele (delA)

Table 3 Average fatty acid profile in g kg^{-1} of a segregating population derived from crossing between CS303TNKCA and FA22 evaluated in different locations: GH (F2 in the greenhouse, Vicosa, MG, Brazil); VRB (F2:3 on Visconde do Rio Branco, Vicosa, MG, Brazil); VIC (F2:4 on Vicosa, MG, Brazil); SG1 (F2:4 on Vicosa, MG, Brazil); SG2 (F2:4 on Vicosa, MG, Brazil)

	Palmitic	Stearic	Oleic	Linoleic	Linolenic
F test	ns	ns	ns	**	**
GH					
Mutant	104.62	35.47	214.57	602.66[a]	42.68[a]
Heterozygous	102.92	34.53	214.58	587.37[ab]	60.59[b]
Wild type	101.41	33.12	213.70	573.31[b]	78.45[c]
Mean	103.02 ± 10.05	34.47 ± 4.54	214.4 ± 45.47	588.11 ± 42.69	60.01 ± 14.42
Minimum	85.29	25.30	157.17	390.39	26.73
Maximum	190.63	63.62	426.14	651.79	94.52
VRB					
F test	ns	**	ns	ns	**
Mutant	102.88	42.64[a]	284.61	525.87	44.00[a]
Heterozygous	102.78	41.18[b]	281.63	516.93	57.48[b]
Wild type	101.98	38.83[c]	274.80	515.38	69.01[c]
Mean	102.64 ± 4.52	41.05 ± 3.14	280.94 ± 35.26	518.72 ± 31.71	56.65 ± 9.68
Minimum	90.59	33.10	214.71	405.19	38.60
Maximum	115.92	51.39	411.56	590.74	80.22
VIC					
F test	ns	**	ns	ns	**
Mutant	100.76	32.43[a]	419.89	413.44	33.47[a]
Heterozygous	100.33	31.76[a]	424.12	403.07	40.72[b]
Wild type	100.20	30.41[b]	404.62	416.03	48.74[c]
Mean	100.41 ± 5.42	31.64 ± 3	419.16 ± 62.17	408.14 ± 53.66	40.65 ± 6.79
Minimum	87.90	24.32	256.86	272.46	27.06
Maximum	116.19	40.10	577.42	540.32	63.46
SG1					
F test	ns	**	ns	ns	**
Mutant	104.07	35.40[a]	318.49	498.47	43.57[a]
Heterozygous	103.24	34.12[b]	316.77	491.85	54.02[b]
Wild type	102.28	32.46[c]	312.61	488.53	64.12[c]
Mean	103.24 ± 5.37	34.09 ± 2.75	316.33 ± 61.43	492.73 ± 53.29	53.62 ± 9.26
Minimum	89.52	26.21	188.86	352.90	37.19
Maximum	118.23	42.77	475.07	609.85	82.30
SG2					
F test	ns	**	ns	ns	**
Mutant	104.43	35.18[a]	316.39	500.70	43.31[a]
Heterozygous	103.20	34.09[a]	318.85	489.92	53.95[b]
Wild-type	102.39	32.40[b]	309.72	490.59	64.90[c]
Mean	103.33 ± 5.91	34 ± 2.97	316.41 ± 66.81	492.59 ± 58.36	53.67 ± 10.01
Minimum	86.71	26.75	192.50	308.31	36.58
Maximum	121.12	43.81	523.70	609.73	84.05

The standard deviation is shown after the mean value for each trait

The letter following values are based on Tukey test (only for traits with significant variance for $P < 0.01$)

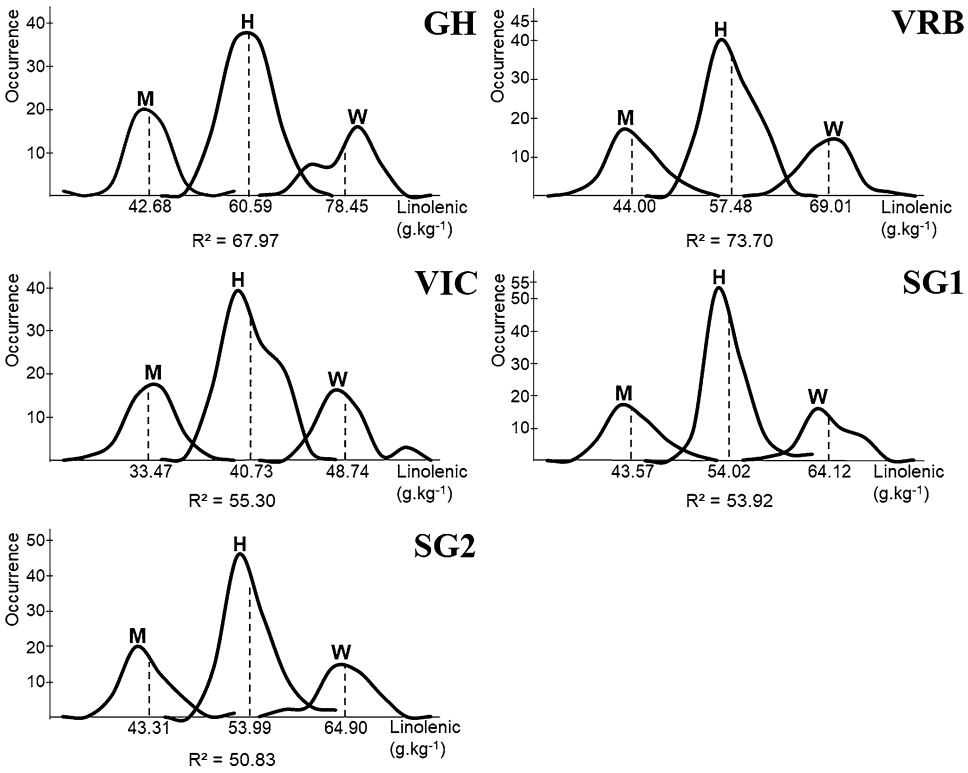

Fig. 4 Distribution graphs of linolenic acid content for 187 soybean lines in a segregating population separated by *GmFAD3A* genotypes in different locations. The determination coefficient of "linolenic acid content × *GmFAD3A* genotype" on each local and the average of linolenic acid content of each genotype are shown. The genotype letters mean: mutant (M), heterozygous (H), wild type (W). The locations indicate: GH (F2 on greenhouse, Vicosa, MG, Brazil); VRB (F2:3 on Visconde do Rio Branco, MG, Brazil); VIC (F2:4 on Vicosa, MG, Brazil); SG1 (F2:4 on São Gotardo, MG, Brazil) and SG2 (F2:4 on São Gotardo, MG, Brazil)

linolenic acid content values for each genotype presented normal distribution (data not shown). Linear regression analysis showed that delA effect on linolenic acid content is additive, and no significant dominance effect was detected (Supplementary Table S3). The delA mutation explained 50.83–73.70% of the linolenic acid variation (Fig. 4).

Discussion

Soybean varieties with desirable characteristics have a higher value because they bring greater benefit for consumers and the soybean industry. Reducing levels of polyunsaturated fatty acid in soybean oil is one of the main goals in breeding programs to increase the oil oxidative stability, preventing changes in the odor and taste, and reducing problems created by trans fatty acids in hydrogenation process (Bilyeu et al. 2006; Li et al. 2007; Pham et al. 2012; Shanklin et al. 1994). In the soy industry, this market was discovered by an initiative of American producers who recognized the importance of investing in soybean varieties that have reduced polyunsaturated fatty acid content. The goal to use these varieties in 25–30% of the American territory by 2023 has been established (http://qualisoy.com/); however, to achieve this goal, studies need to be conducted to obtain new varieties that meet this criterion. The multinational Monsanto recently developed Vistive Gold (MON 87705), a genetically modified variety with high oleic and low linolenic content by silencing *FatB* and *FAD2* genes using RNAi (http://www.monsanto.com/products/pages/vistive-gold-soybeans.aspx). DuPont Pioneer has Plenish, another variety developed from genetic modification that has high oleic and low linolenic acid content (below 3%) (Waltz 2010). On the other hand, Pham et al. (2012) produced soybean with 85% oleic acid and 2% linolenic acid content using mutations in genes *GmFAD2-1A*, *GmFAD2-1B*, *GmFAD3A* and *GmFAD3C*.

Our research group has a breeding program that aims to develop soybean varieties with reduced linolenic and high oleic acid content, which are desirable factors for the food and biodiesel industry in Brazil. We developed CS303TNKCA, a cultivar with low linolenic acid content (about 3.5–4%) derived from Monarca (COOPADAP, Brazil) and BARC-12 (Leffel 1994b). CS303TNKCA also shows the absence of three lipoxygenases genes (*lox1*, *lox2* and *lox3*) and the Kunitz protease inhibitor, a middle oil content (about 20–23%) and yields around 3200 kg per hectare.

The present study successfully identified a 1-bp (delA) deletion in omega-3 fatty acid desaturase gene (*GmFAD3A*) in the CS303TNKCA genotype, which causes a premature stop codon (Fig. 1). The delA mutation results in a truncated protein missing 169 amino acid residues, a fragment that comprises the third histidine box present in the enzyme. A similar result was detected by Reinprecht et al. (2009) when characterizing a G>A substitution at position 798 in the coding region on RG10 line. This mutation leads to a stop codon and a truncated protein missing 111 amino acid residues, including the third histidine box, which is responsible for

reducing linolenic acid content to about 3.3%. The omega-3 fatty acid desaturase is responsible for the synthesis of linolenic acid from linoleic acid and belongs to a family of enzymes characterized by the presence of a di-iron cofactor that interacts with three regions of conserved histidine motifs (H-Box) in the protein (Shanklin et al. 1994). These membrane-bound desaturase histidine boxes are essential for coordinating the di-iron cofactor required for catalysis (Byrum et al. 1997; Shanklin et al. 1994), and the change of even a single residue within these histidine boxes can reduce enzymatic activity (Bilyeu et al. 2006; Byrum et al. 1997; Gietz and Schiestl 2007; Reinprecht and Pauls 2016).

Heterologous expression is usually performed to characterize new functional isoforms in many species, such as soybean (Li et al. 2007), *Physaria fendleri* (Lozinsky et al. 2014) and *Elaeis guineenses* (Sun et al. 2016). This method was used to evaluate the effect of an aspartate for asparagine exchange at position 150 of one *FAD2* desaturase in *Arachis hypogaea* (Bruner et al. 2001). Using a similar approach, we evaluated the enzymatic activity of mutant and wild-type *GmFAD3A* alleles. The results showed that delA allele codes for a nonfunctional enzyme expressing polyunsaturated fatty acid levels similar to yeasts transformed by empty vector (Table 2). Additionally, Real-Time PCR analysis showed practically no variation at gene expression levels at *GmFAD3A, GmFAD3B* and *GmFAD3C* (Fig. 2). These results support the hypothesis that the CS303TNKCA *GmFAD3A* allele is not functional and no compensatory mechanism at the transcription level occurs to overcome the inactive enzyme.

We noted a decrease in palmitoleic acid content in yeasts transformed with wild-type *GmFAD3A* despite no significant variance being found using F test (Table 2), which was probably due to replicate variation. In the fatty acid pathways in yeasts, palmitic acid (16:0) can be dehydrogenated to palmitoleic acid (16:1), or converted into stearic acid (18:0), which is dehydrogenated into oleic acid (18:1). Inserting a new enzyme at the end of the 18-carbon branch, can activate this pathway, shifting the balance for the production of 18-carbon fatty acids. As the content of palmitic acid seems to remain equal, the activation of 18-carbon branch will reduce the activity in the 16-carbon branch, decreasing the palmitoleic acid content (Table 2). A similar consideration can explain the fatty acid content in evaluated populations (Table 3). As expected, we observed a variance in linolenic acid content between genotypes in all trials. However, in the mutant genotype, we observed an increase in stearic acid in all field trials due the inactivation of *GmFAD3A*, while in the greenhouse trial, we observed the increase of linoleic acid, instead of stearic acid. The mutation effect in linolenic acid content was the same for all evaluations, but the environment showed differences in the accumulated compounds in the pathway when we decreased linolenic acid production.

We developed a diagnostic marker that detects the delA mutation observed in the *GmFAD3A* gene in CS303TNKCA using TaqMan (Fig. 3), a technique that has been used in other studies to associate SNPs with fatty acid levels (Bachleda et al. 2016; Pham et al. 2014; Shi et al. 2015). The genotyping of F2 plants derived from the cross between CS303TNKCA and FA22 was used to associate delA with linolenic acid content across three generations. The mutation reduced the linolenic acid content by 3.3–4.4% in segregating populations evaluated at different locations (Fig. 4). Precedents in the literature have reported similar results. A G>A substitution in the first nucleotide of intron 6 in CX1512-44 line leads to an alteration in a splice form (Bilyeu et al. 2005); the G>A substitution at position 798 in the coding sequence of RG10 line leads to a premature stop codon (Reinprecht et al. 2009); a 6.4-kbp deletion of the *GmFAD3A* gene was observed in the A5 line (Pham et al. 2014); a G>A substitution in the second splice site of PE1690 line leads to an early stop codon (Li et al. 2007). In the present investigation, the delA mutation was responsible for reducing 31.33–45.60% of the linolenic acid content in the soybean seed, explaining 50.83–73.70% the phenotypic variation.

The delA mutation marker for low linolenic acid proved efficient for marker-assisted selection in breeding programs. Currently, we are developing a backcrossing program to develop improved soybean varieties with the combination of delA mutation from the variety CS303TNKCA and mutations identified in *GmFAD2-1A* and *GmFAD2-1B* genes of a plant with 80% oleic acid developed by crossing PI283327 and PI603452 (Pham et al. 2011). Mutant lines containing non-functional *GmFAD2-1A*, *GmFAD2-1B* and *GmFAD3A* were sufficient to produce plants with 80% oleic acid, 1.5–4.0% linoleic acid and 1.8–2.6% linolenic acid (Pham et al. 2012). In this particular case, the use of only the *GmFAD3A* gene was enough because this gene has a greater effect on the characteristic (Pinto et al. 2013), and we have to consider that decreasing metabolic flux in linoleic acid production by *GmFAD2-1A* and *GmFAD2-1B* mutation will affect the amount of available substrate for omega-3 fatty acid desaturase (*GmFAD3* genes). We expect in the future to produce soybean cultivars with high oleic acid and low levels of linoleic and linolenic acids, generating oil with good oxidative stability and greater benefits for human health and the Brazilian Biodiesel Industry.

Author contribution statement LBM developed populations and performed statistical and phenotype analysis. RDB and PHSP designed primers, sequenced *GmFAD3* genes, identified mutations and performed bioinformatics analysis. LCCS and DBM designed primers, and conducted enzymatic and expression analysis. LCCS screened genotypes with TaqMan method. NDP, EPBF, CSS, AJC and MD provided

experiment oversight, interpreted the results and reviewed the manuscript. LCCS, RDB and MD generated tables and figures and wrote the manuscript.

Acknowledgements This work was funded by the CNPq (Grant 455812/2014-4), graduate fellowships (L.C.C.S. and L.B.M), post-doctoral fellowships (R.D.B) and a science initiation scholarship (D.B.M. and P.H.S.P.); FAPEMIG (Grant APQ-00077-13 and in part by APQ-01416-16). *In memoriam* professor Maurilio Alves Moreira.

Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author declares that there is no conflict of interest.

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