



Development and in-house validation of two real-time PCR methods for the detection of genome-editing events in soybean *FAD2* gene variants

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Abstract

Since the commercialization of the first genetically modified (GM) crops, their relevance in agricultural applications has increased. Soybean (*Glycine max*) ranks as the most widely cultivated GM crop globally. With the advancements in site-specific genome-editing tools, the development of GM organisms (GMO) using new genomic techniques has accelerated, particularly in agriculture. In the US, a genome-edited soybean variety, Calyno, was developed with altered fatty acid composition by inactivating two members of the *fatty acid desaturase 2 (FAD2)* gene family, *FAD2-1A* and *FAD2-1B*, using TALEN technology. Although deregulated in the US, Calyno soybeans are not authorized for cultivation or import in the European Union and therefore cannot be placed on the European market. To address the need for reliable detection methods in routine analysis for GMO by public authorities, two event-specific qPCR methods were developed to detect the *FAD2-1A-Δ63bp* and *FAD2-1B-Δ23bp* gene variants in the Calyno soybeans. In the absence of reference material, methods were validated in-house using synthetic plasmids carrying the target regions' sequences, following European Network of GMO Laboratories (ENGL) guidelines. Both assays meet the minimum performance requirements for GMO testing, demonstrating high sensitivity, qPCR efficiency, specificity and robustness. An interlaboratory comparison study further confirmed the reliability of these methods. These qPCR assays provide an effective tool for detecting the *FAD2-1A-Δ63bp* and *FAD2-1B-Δ23bp* gene variants in Calyno soybeans, supporting GMO testing requirements in the European Union and ensuring accurate monitoring of GM crops.

Keywords Genome editing · Real-time PCR · Soybean · GMO analysis · GMO detection · *FAD2* gene variants

1 Introduction

Since the commercialization of the first genetically modified (GM) crop in 1996, the global cultivation of GM crops has expanded to cover 190.4 Mha by 2019 (ISAAA 2019). Among these, soybean (*Glycine max*) is the most widely cultivated GM crop, followed by maize, cotton and canola

(ISAAA 2019). The development of site-specific genome-editing tools, particularly zinc finger nucleases, transcription activator-like effector nucleases (TALEN), and the clustered regularly interspaced short palindromic repeats (CRISPR/Cas) system, has significantly accelerated the development of GM organisms (GMOs), using new genomic techniques (NGTs). Genome editing, particularly in agriculture, has gained prominence due to its ability to create new crop varieties more quickly, affordably, and precisely compared to traditional genetic engineering methods (Yang et al. 2022).

The US biotech company Calyxt has developed and commercialized a genome-edited soybean variety (Calyno) with altered fatty acid composition by targeting as a result of knockouts of two members of the *fatty acid desaturase 2 (FAD2)* gene family: *FAD2-1A* and *FAD2-1B* (Haun et al. 2014). Using TALEN technology, Calyno soybeans were engineered to carry short deletions of 63 bp in *FAD2-1A*

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(*FAD2-1A-Δ63bp*) and 23 bp in *FAD2-1B* (*FAD2-1B-Δ23bp*; Fig. 1; Haun et al. 2014). The FAD2 enzyme is responsible for converting monounsaturated oleic acid into the polyunsaturated linoleic acid. *FAD2-1A* and *FAD2-1B* are highly expressed in soybean seeds (Schlueter et al. 2007), their knockout significantly alters the fatty acid profile of soybean oil, containing 80% oleic acid compared to 20% in conventional soybean oil, with a corresponding reduction in linoleic acid from 50% to 4% (Haun et al. 2014). Consequently, the resulted oil, marketed as Calyno High Oleic Soybean Oil. Similar effects have been observed in *FAD2-1A* / *FAD2-1B*

mutant soybean lines generated through conventional mutagenesis and/or breeding techniques (Pham et al. 2011).

In 2018, the European Court of Justice ruled that all organisms developed using NGTs are classified as GMOs under Directive 2001/18/EC in the European Union (EU; Court of Justice of the European Union 2018). Consequently, they are subject to the EU's GMO authorization process. In contrast, several non-EU countries, including the US, do not classify NGT-generated organisms as GMOs if no transgenic DNA is introduced during the genome-editing process. As a result, the Calyno soybean variety is deregulated in the US, and its

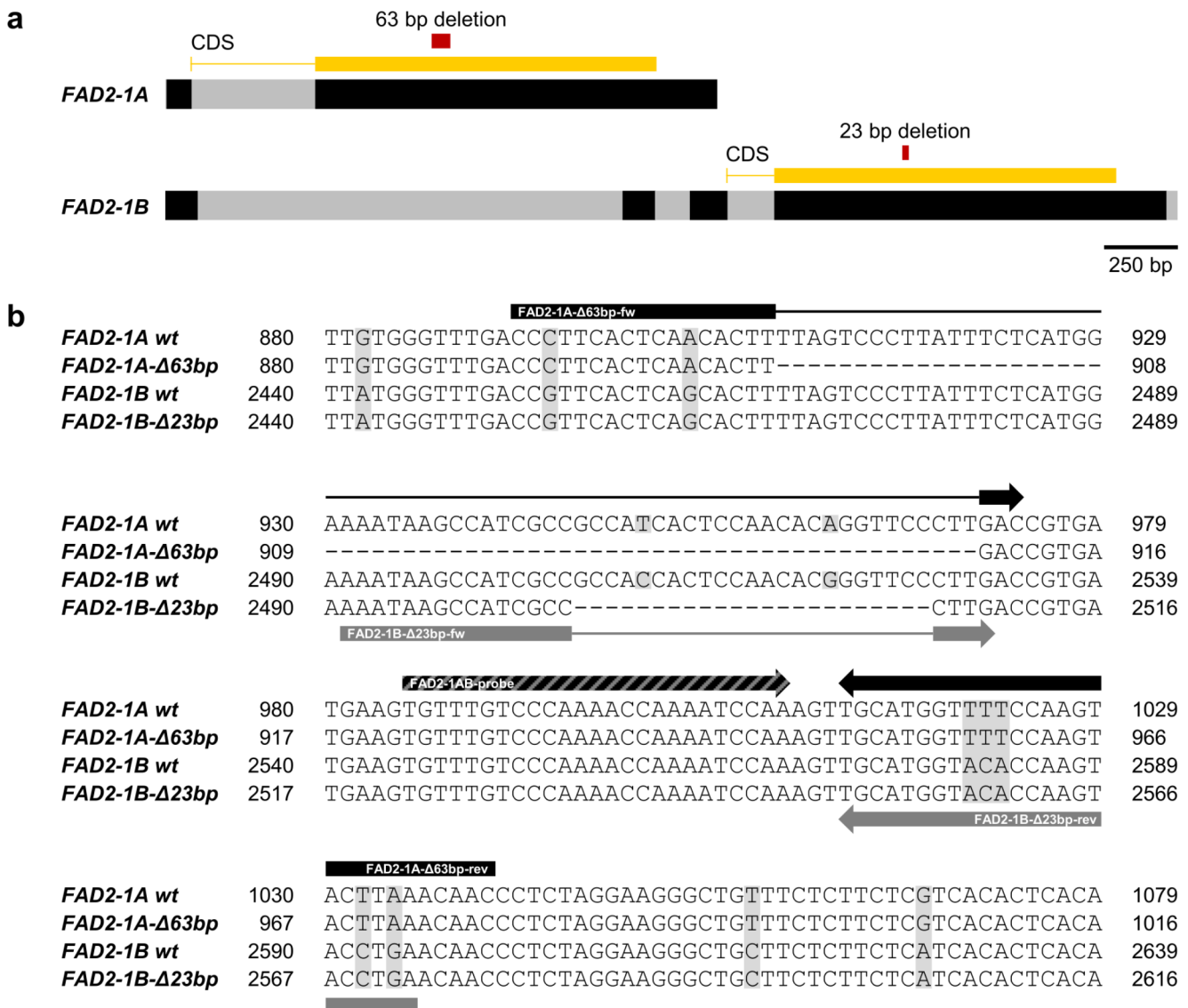


Fig. 1 Genetic structure of *FAD2-1A* and *FAD2-1B* variants. **a** Schematic illustration of the genetic organization of *FAD2-1A* and *FAD2-1B* genes. Exons are displayed in black, whereas introns are depicted in grey. The coding sequences (CDS) and deletion sites in Calyno soybean variety of both genes are shown above in yellow and red, respectively. **b** sequence alignment of wild-type (wt) and genome-edited *FAD2-1A* and *FAD2-1B* gene variants. Primer and probe placement for

FAD2-1A-Δ63bp and *FAD2-1B-Δ23bp* detection assays are indicated above and below the aligned sequences, respectively. Illustrations and alignment are based on sequence data obtained from the NCBI gene database (<https://www.ncbi.nlm.nih.gov/gene/>; Sayers et al. 2022) and can be found under the following accession numbers: *FAD2-1A*: GeneID 547814, NM_001251413.1; *FAD2-1B*: GeneID 100805777, XM_006605820.4

oil entered the market in 2019 (Calyxt 2019). This divergence in regulatory frameworks highlights the critical role of public authorities in the EU in monitoring crops grown domestically or imported into the region. This underlines the need to develop sensitive and specific detection methods for GM crops. To ensure consistency and reliability across surveillance laboratories, the European Network of GMO Laboratories (ENGL) has established minimum performance requirements for GMO detection methods during the validation process (ENGL 2015).

Since Calyxt has not sought EU approval for the Calyno soybean or its oil, it is the responsibility of local authorities to develop suitable detection methods to fulfill their legal obligations. This study reports the development and in-house validation of 2 independent, event-specific real-time PCR (qPCR) methods for detecting the *FAD2-1A-Δ63bp* and *FAD2-1B-Δ23bp* gene variants in the Calyno soybean variety, in accordance with ENGL guidelines. Additionally, an interlaboratory comparison study conducted among member laboratories of the § 28b GenTG working group, coordinated by the German Federal Office of Consumer

Protection and Food Safety (BVL), demonstrates the applicability of these methods.

2 Materials and methods

2.1 Sample material and DNA extraction

Synthetic plasmids pFAD2-1A-Δ63bp and pFAD2-1B-Δ23bp carrying genome-edited sequences (in pUCIDT; Table S1, Supplementary Material) were obtained from Integrated DNA Technologies (IDT, Coralville, IA, USA) and provided by the BVL. Sequences were derived from Haun et al. (2014) and NCBI gene database entries (Sayers et al. 2022; *FAD2-1A*: NM_001251413.1; *FAD2-1B*: XM_006605820.4). Certified GM reference materials (Table S2, Supplementary Material) were purchased from IRMM (Geel, Belgium) and AOCS (Urbana, IL, USA). DNA extracted from non-GM soybean varieties (Table S7, Supplementary Material), routinely screened at the Bavarian Health and Food Safety Authority (LGL) in 2022–2023, was included in specificity testing. DNA extraction was performed using a Maxwell 16 system with the Maxwell 16 FFS Nucleic Acid Extraction System Custom Kit (both Promega, Fitchburg, WI, USA), following Guertler et al. (2013). RNase A (25 μL, 4 mg/mL) and thioglycerol (20 μL for rapeseed) were added to the CTAB lysis buffer. DNA extracts were quantified using the Quantus Fluorometer and QuantiFluor ONE dsDNA System (both Promega). Samples were stored at 4 °C (short-term) or -20 °C (long-term).

2.2 qPCR

Standard qPCR was conducted using the Magnetic Induction Cycler (MIC, Bio Molecular Systems, Upper Coomera, Australia). Optimization involved testing annealing/extension temperature, oligo type (with/without locked nucleic acid [LNA] bases), and oligo concentrations. Optimal conditions were chosen based on the amplification curve, amplification efficiency and C_q value. A double-quenched probe was initially used for optimization but later replaced with a single-quenched variant for better availability. Reaction mixtures included 12.5 μL GoTaq Probe qPCR Master Mix (Promega), primers, probe (Table 1), 5 μL DNA or control, and water to 25 μL. The thermal profile was: 95 °C for 3 min; 45 cycles of 95 °C for 15 s and 62 °C for 60 s. Herring sperm DNA (20 ng/μL; Promega) in 1× TE buffer (pH 8.0, low EDTA; AppliChem, Darmstadt, Germany) was used for template dilutions and negative controls. Negative controls were measured in duplicates or triplicates and consistently yielded negative results. Primers and probes were designed using CLC Genomics Workbench 11 (Qiagen, Venlo, Netherlands) and

Table 1 Oligos used in event-specific detection assays for deletions in the *FAD2-1A-Δ63bp* and *FAD2-1B-Δ23bp* gene variants of the genome-edited calyno soybean variety

Assay	Oligo	Sequence [5'-3']	PCR conc.	Amplification size
<i>FAD2-1A-Δ63bp</i>	FAD2-1A-Δ63bp-fw	CCCTTC ACTCAA C+ACCTT+GAC	300 nM	86 bp
	FAD2-1A-Δ63bp-rev	GTTGTTTAAG TACTTGGA ACCATGCA	500 nM	
<i>FAD2-1B-Δ23bp</i>	FAD2-1B-Δ23bp-fw	AAATAAGC CATC+GCCC TTG	300 nM	82 bp
	FAD2-1B-Δ23bp-rev	TCAGGTACTT GGTGTACCA TGCA	500 nM	
<i>FAD2-1A-Δ63bp</i> <i>FAD2-1B-Δ23bp</i>	FAD2-1A/B-probe (single-quenched) ^{a, b}	FAM-TGTTT GTC+CCAA+ AACC AAA A+TCCAA- IBFQ	150 nM	
	FAD2-1A/B-probe (double-quenched) ^b	FAM-TG TTTGTC/ ZEN/+CCAA+ AACC AAA A+TCCAA- IBFQ	150 nM	

The same probes were used in both real-time PCR detection methods. FAM: 6-carboxyfluorescein; ZEN: internal quencher; IBFQ: iowa black fluorescent quencher; +N: LNA nucleotide

^a Used for optimization, in-house validation and the interlaboratory comparison study involving 2 laboratories

^b Used for optimization and both interlaboratory comparison studies

NCBI Primer-BLAST (Ye et al. 2012), followed by in silico analysis in NCBI BLASTN (Altschul et al. 1997). qPCR analyses adhered to MIQE guidelines (Bustin et al. 2009).

2.3 Droplet digital PCR (ddPCR)

Plasmids and genomic DNA were quantified using the QX200 Droplet Digital PCR System (Biorad, Hercules, CA, USA) to determine their copy number. Reactions were prepared with 11 μ L ddPCR Supermix for Probes (No dUTP; Biorad), 700 nM forward (fw) and reverse (rev) primers (Table 1), 200 nM probe (Table 1), sample DNA and PCR-grade water to 22 μ L. PCR cycling on a Mastercycler X50 instrument (Eppendorf, Hamburg, Germany) followed these conditions: 95 °C for 10 min; 45 cycles of 94 °C for 30 s and 62 °C for 60 s; 98 °C for 10 min. DNA quantification methods for DNA extracts from seed samples are listed in Table S2 (Supplementary Material), the GMOMETHODS database (Bonfini et al. 2012), and in the collection of method validations of the European Union Reference Laboratory for Genetically Modified Food and Feed (<https://gmo-crl.jrc.ec.europa.eu/>).

2.4 In-house validation

In-house validation was performed in accordance to published guidelines (BVL 2016; ENGL 2015).

2.4.1 LOD and qPCR efficiency

LOD_{95%} was assessed on QuantStudio 7 FLEX (Thermo Fisher Scientific) and MIC using serial dilutions (1,000 cp/ μ L to 0.02 cp/ μ L) of pFAD2-1A- Δ 63bp and pFAD2-1B- Δ 23bp plasmids. 5 μ L of diluted plasmids were used in qPCR in 4, 6 or 12 replicates (Tables S3–S6, Supplementary Material). Theoretical LOD_{95%} was calculated using the QuoData web tool (Uhlig et al. 2015). qPCR efficiency and R² were determined from dilutions containing 5,000 to 10 cp/qPCR.

2.4.2 Specificity

DNA samples from various GM crops were tested for amplification using *FAD2-1A- Δ 63bp* and *FAD2-1B- Δ 23bp* detection methods to verify specificity. Genomic DNA extracts (5,000 cp/qPCR) from wild-type soybean and GM events in soybean, maize, and rapeseed were analyzed (Table S2, Supplementary Material). Additionally, pFAD2-1A- Δ 63bp and pFAD2-1B- Δ 23bp (40 cp/qPCR) plasmids were tested in wild-type soybean DNA background (200 ng/qPCR, equivalent to 0.02% cp/cp GMO content). All samples were run in triplicates. Furthermore, 20 routine non-GM soybean samples tested at LGL in 2022 and 2023 (200 ng/qPCR)

were included in the specificity analysis, with qPCR runs performed in duplicates. For 3 varieties (Abaca, Aurelina, ES Compositor), solutions containing 0.9%, 0.5%, and 0.1% pFAD2-1A- Δ 63bp or pFAD2-1B- Δ 23bp plasmid DNA (% cp/cp) were processed alongside identical plasmid DNA samples without non-GM background DNA.

2.4.3 Robustness

Robustness was assessed using a multi-factorial design, testing various combinations of assay parameters: annealing/elongation temperature (± 1 °C), primer concentrations (-30%), probe concentration (-30%), instruments (QuantStudio 7, MIC, Mx3005P [Stratagene, La Jolla, CA, USA]), master mixes (GoTaq Probe qPCR Master Mix; Luna Universal Probe qPCR Master Mix [New England Biolabs]), and reaction mix volumes ($\pm 5\%$) as per BVL (2016). Each combination of parameters was tested with plasmid DNA (20 cp/qPCR) in triplicates.

2.5 Interlaboratory comparison study

Oligos, plasmids, genomic wild-type soybean DNA, and optimized qPCR conditions were supplied by the LGL to participating laboratories. A double-quenched probe was used for the interlaboratory comparison study (Table 1). Each laboratory prepared dilution series for pFAD2-1A- Δ 63bp and pFAD2-1B- Δ 23bp with their standard laboratory diluent and conducted qPCR analyses using the provided oligos, their respective in-house master mix, and qPCR instrument (Table 4). Serial dilutions were measured in at least 3 replicates, and results were returned to the LGL for analysis. Theoretical LOD_{95%} values, qPCR efficiencies, and R² were evaluated following in-house validation protocols. Similarly, the performance of single- and double-quenched probes was compared by 2 laboratories using fewer number of dilutions measured in triplicates for both *FAD2-1A- Δ 63bp* and *FAD2-1B- Δ 23bp* methods. qPCR efficiency and R² calculations were based on dilutions ranging from 5,000 cp/qPCR to 5 cp/qPCR.

3 Results

3.1 Method development

The *FAD2-1A* and *FAD2-1B* gene variants in the Calyno soybean variety exhibit deletions of several base pairs (Haun et al. 2014). To design qPCR methods specific for these deletions, forward (fw) primers were selected to span the deletion sites, ensuring specific primer binding to the genome-edited gene variants and preventing alignment with the wild-type genes

(Fig. 1). Locked nucleic acid (LNA) bases were incorporated into the *FAD2-1A-Δ63bp-fw* and *FAD2-1B-Δ23bp-fw* primers to enhance specificity (Table 1; You et al. 2006). Reverse (rev) primers were placed in regions with multiple sequence variations between *FAD2-1A* and *FAD2-1B* for further discrimination (Fig. 1). A single hydrolysis probe complementary to an identical region in both *FAD2-1* gene variants downstream of the deletions was used for both assays, reducing cost and potential errors. This has the advantage that the same hydrolysis probe can be used for both methods. In silico analysis using NCBI Primer-BLAST or BLASTN confirmed no other significant matches for the primers or probe sequences in soybean or other plants (Altschul et al. 1997; Ye et al. 2012). Optimal conditions were determined through gradient-based testing, resulting in primer concentrations of 300 nM (fw) and 500 nM (rev), a probe concentration of 150 nM, and an annealing/elongation temperature of 62 °C.

As no plant reference material for Calyno soybeans was available despite repeated requests to Calyxt by the BVL, synthetic plasmids carrying parts of the genome-edited *FAD2-1A-Δ63bp* and *FAD2-1B-Δ23bp* variants were designed for the development and validation of the detection methods according to published information (Table S1, Supplementary Material; Haun et al. 2014). Due to the lack of genomic DNA from actual reference material, the procedure was referred to

as “cold validation”. Plasmids were quantified by ddPCR to determine their exact copy numbers and subsequently were subjected to in-house validation.

3.2 In-house validation

3.2.1 Sensitivity

The sensitivity of *FAD2-1A-Δ63bp* and *FAD2-1B-Δ23bp* assays was determined using dilution series ranging from 5,000 to 0.1 cp/qPCR on both QuantStudio 7 and MIC qPCR cyclers (Fig. 2; Tables S3–6, Supplementary Material). Analysis with the QuoData web tool yielded LOD_{95%} values of 3.7 cp/qPCR and 4.2 cp/qPCR for the *FAD2-1A-Δ63bp* method using QuantStudio 7 and MIC, respectively (Fig. 3a + c; Table 2). For *FAD2-1B-Δ23bp*, LOD_{95%} values were 3.2 cp/qPCR for QuantStudio 7 and 3.6 cp/qPCR for MIC (Fig. 3b + d; Table 2).

To ease laboratory handling, the LOD for both assays was set to 5 cp/qPCR, the lowest dilution where all 12 replicates returned positive results. qPCR efficiencies were 96.6% and 96.4% for *FAD2-1A-Δ63bp* and 96.6% and 95.5% for *FAD2-1B-Δ23bp* on QuantStudio 7 and MIC cyclers, respectively (Table 2). R² values were 0.999 for *FAD2-1A-Δ63bp* and

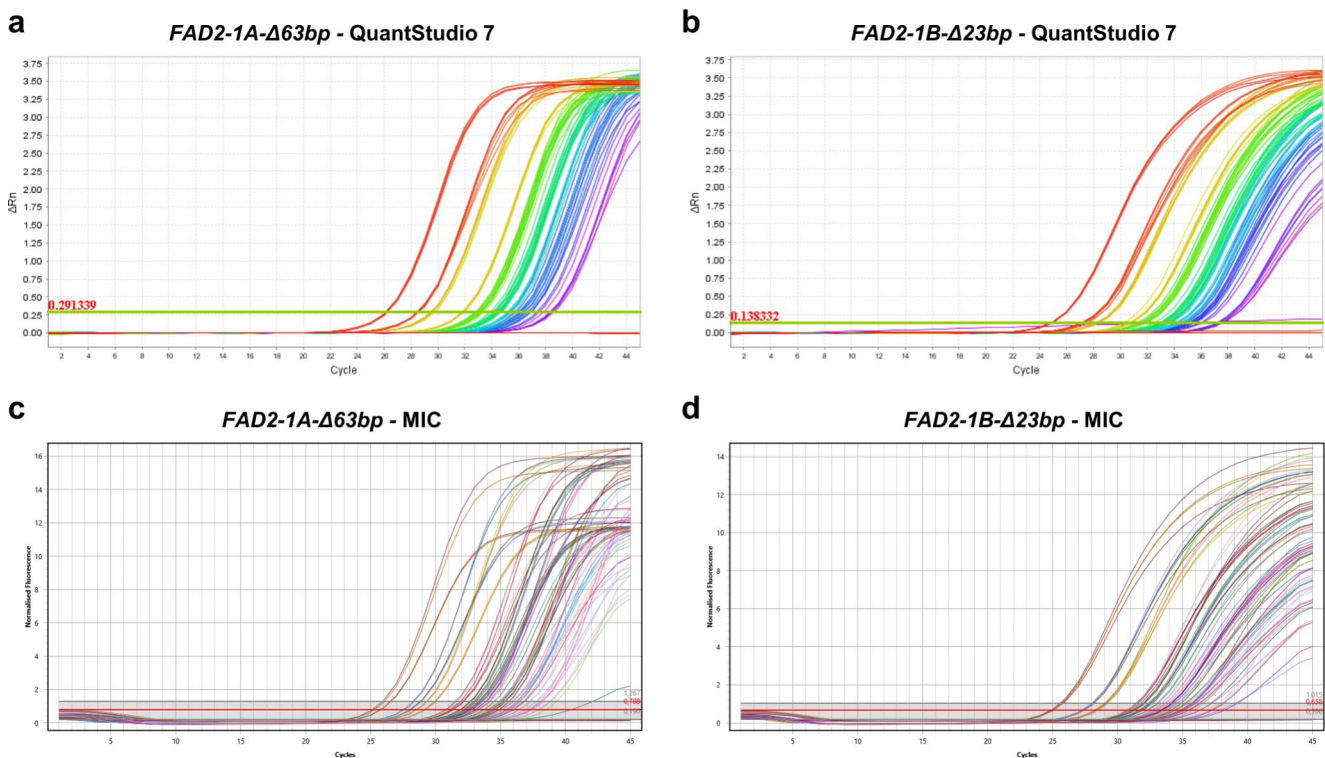


Fig. 2 Determination of sensitivity for *FAD2-1A-Δ63bp* and *FAD2-1B-Δ23bp* assays. Amplification curve data from qPCR runs for sensitivity determination of *FAD2-1A-Δ63bp* (a + c) and *FAD2-1B-Δ23bp* (b + d) detection assays using QuantStudio 7 (a + b) and MIC (c + d) cyclers.

Thresholds were automatically set by the software of each respective instrument. Detailed information for individual samples is provided in Tables S2–S5 (Supplementary Material)

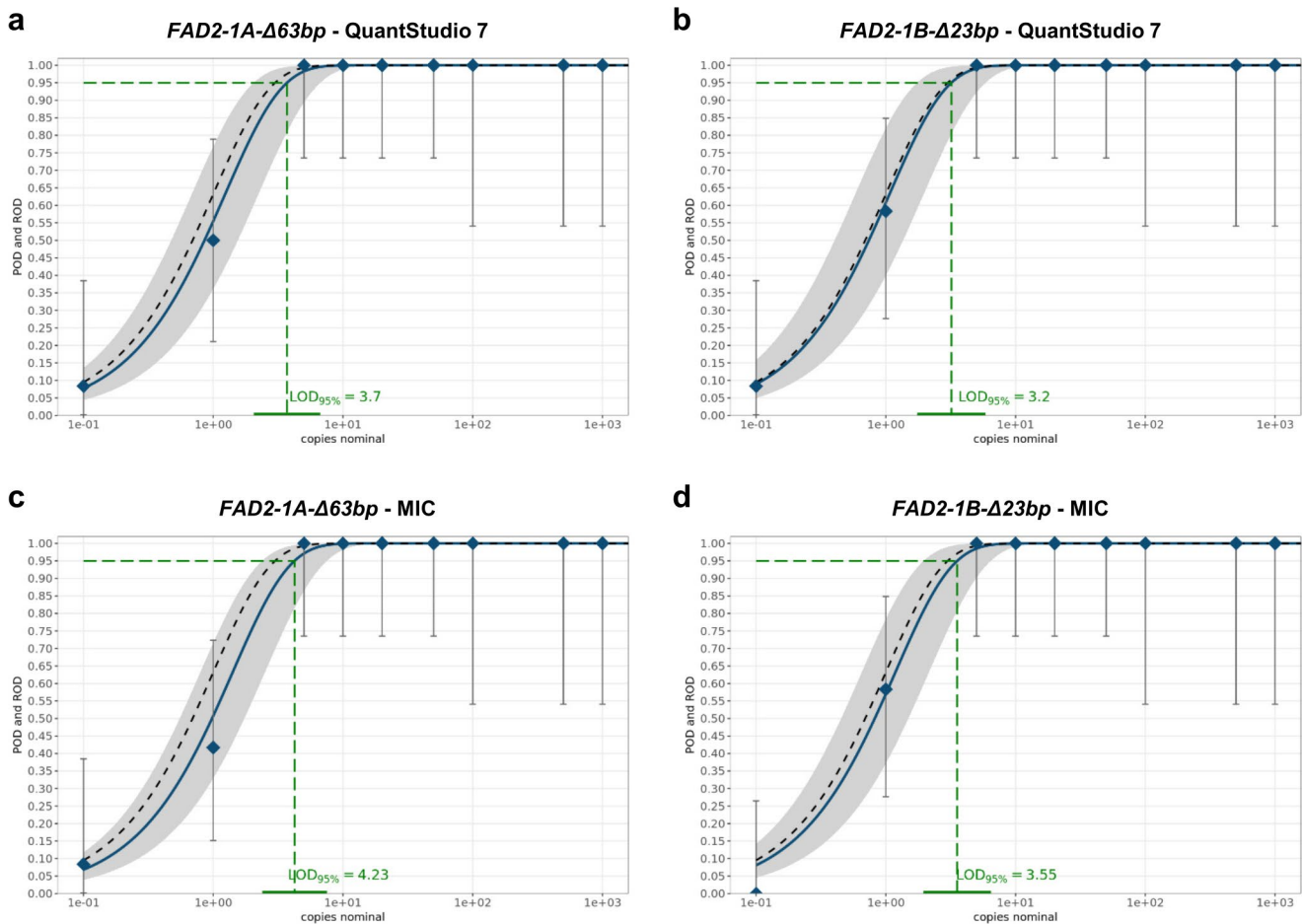


Fig. 3 $LOD_{95\%}$ of *FAD2-1A-Δ63bp* and *FAD2-1B-Δ23bp* assays. LODs were determined using the QuoData web tool according to Uhlig et al. (2015). Corresponding qPCR runs for *FAD2-1A-Δ63bp* (a + c) and *FAD2-1B-Δ23bp* (b + d) detection assays using QuantStudio 7 (a + b)

Table 2 Sensitivity of qPCR assays for detecting genome-edited *FAD2-1* genes

Assay	qPCR cyler	$LOD_{95\%}$	Efficiency	R^2
<i>FAD2-1A-Δ63bp</i>	QuantStudio 7	3.7 cp/qPCR	96.6%	0.999
	MIC	4.2 cp/qPCR	96.4%	0.999
<i>FAD2-1B-Δ23bp</i>	QuantStudio 7	3.2 cp/qPCR	96.6%	1.000
	MIC	3.6 cp/qPCR	95.5%	1.000

Parameters were determined using dilution series ranging from 5,000–0.1 cp/qPCR on 2 different instruments for both assays. $LOD_{95\%}$ values were calculated using the QuoData web tool (Uhlig et al. 2015). qPCR efficiencies and R^2 values were calculated based on dilutions containing at least 10 cp/qPCR

1.000 for *FAD2-1B-Δ23bp* on both instruments, meeting ENGL's minimum performance requirements (ENGL 2015).

3.2.2 Specificity

Specificity of the *FAD2-1A-Δ63bp* and *FAD2-1B-Δ23bp* detection methods was assessed using DNA extracts from different GM and non-GM crop species and varieties. Ten

or MIC (c + d) cyclers are shown in Fig. 2. Template copy numbers are plotted against probability of detection (POD) and rate of detection (ROD) to calculate the theoretical $LOD_{95\%}$, which is the quantity of target DNA copies needed to achieve a 95% likelihood of detection

soybean, three maize, and two rapeseed GM lines were analyzed, alongside the target plasmid DNA (Table 3; Fig. S1a–d, Supplementary Material). No unspecific or false-positive signals were observed, and amplification occurred exclusively for the respective plasmid targets. No cross-reactivity was detected between the *FAD2-1A-Δ63bp* and *FAD2-1B-Δ23bp* assays (Table 3). Hence, both assays were considered specific for their respective targets.

Routine non-GM soybean seed samples tested at LGL in 2022–2023 were also analyzed for both event-specific methods. Furthermore, three soybean varieties were mixed with 0.9%, 0.5% or 0.1% pFAD2-1A-Δ63bp or pFAD2-1B-Δ23bp plasmid DNA (% cp/cp). Again, specific signals were observed only in samples containing the target plasmid DNA, confirming assay specificity in routine GMO testing (Fig. S1e + f; Table S7, Supplementary Material).

Table 3 Specificity of event-specific *FAD2-1* deletion assays

Template DNA	Species	<i>FAD2-1A-Δ63bp</i>	<i>FAD2-1B-Δ23bp</i>
pFAD2-1A-Δ63bp	Plasmid	+	-
pFAD2-1B-Δ23bp	Plasmid	-	+
routine seed sample (non-GM)	Soybean	-	-
DP-305423	Soybean	-	-
MON87701	Soybean	-	-
MON87705	Soybean	-	-
MON87708	Soybean	-	-
MON87769	Soybean	-	-
MON89788	Soybean	-	-
DAS-44406	Soybean	-	-
DAS-81419	Soybean	-	-
GTS40-3-2	Soybean	-	-
MON810	Maize	-	-
MON88017	Maize	-	-
MON87427	Maize	-	-
MON88302	Rapeseed	-	-
DP-73496	Rapeseed	-	-

Genomic DNA extracts from listed crop varieties, as well as pFAD2-1A-Δ63bp and pFAD2-1B-Δ23bp plasmids, were tested for amplification using *FAD2-1A-Δ63bp* or *FAD2-1B-Δ23bp* assays to assess specificity. Samples were measured in triplicates. Amplification of template DNA for the *FAD2-1A-Δ63bp* or *FAD2-1B-Δ23bp* assays is denoted by a “+” symbol, while a “-” symbol indicates the absence of amplification

3.2.3 Robustness

Robustness of both assays was tested according to the ENGL guidelines by introducing variations in the qPCR master mix, its volume, the annealing temperature, the primer and probe concentrations, and the qPCR cyclers. Expected minor differences were observed across conditions, e.g. for qPCR instruments, master mixes or annealing temperatures (Fig. 4). Nonetheless, amplification signals were consistently obtained for all samples containing pFAD2-1A-Δ63bp or pFAD2-1B-Δ23bp, whereas negative controls showed no amplification. Therefore, the performance of the methods is considered reliable and robust enough to withstand minor variations in experimental setup and/or conditions. In conclusion, in-house validation confirmed that *FAD2-1A-Δ63bp* and *FAD2-1B-Δ23bp* assays meet ENGL (2015) minimum performance requirements for sensitivity, specificity, and robustness.

3.3 Interlaboratory comparison study

The applicability of the detection methods was further evaluated in an interlaboratory comparison study involving 6 laboratories coordinated by the LGL as part of the § 28b GenTG working group. Participants were provided with

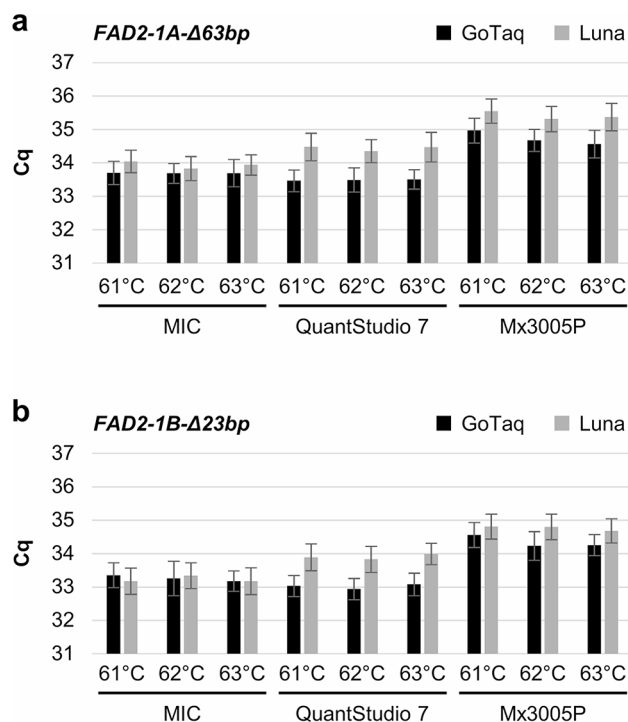


Fig. 4 Robustness of *FAD2-1A-Δ63bp* and *FAD2-1B-Δ23bp* assays. Detection method parameters (master mix volume and supplier, annealing temperature, primer and probe concentration, qPCR instrument) were varied and examined in regard to the robustness of *FAD2-1A-Δ63bp* (a) and *FAD2-1B-Δ23bp* (b) assays. The Cq values categorized according to master mix (GoTaq Probe qPCR Master Mix, Luna Universal Probe qPCR Master Mix), annealing temperature and qPCR cycler are presented as the mean $\pm$ SD, encompassing all other variations

reagents and performed qPCR experiments on pFAD2-1A-Δ63bp or pFAD2-1B-Δ23bp in order to verify both assays using their respective standard laboratory setup, i.e. qPCR instrument and master mixes. Unlike the single-quenched probe used in in-house validation, a double-quenched probe with the identical sequence was used for the interlaboratory comparison study (Table 1).

For *FAD2-1A-Δ63bp*, LOD_{95%} values ranged from 3.35 cp/qPCR to 7.83 cp/qPCR, with efficiencies between 90.7% and 100.1% (Table 4; Table S8, Supplementary Material). Thus, both parameters were in compliance with the minimum performance requirements (ENGL 2015). LOD_{95%} values of the *FAD2-1B-Δ23bp* assay were determined between 2.72 cp/qPCR and 11.2 cp/qPCR (Table 4; Table S9, Supplementary Material). However, values for qPCR efficiency of labs 1, 5 and 6 were found to be slightly outside of the 90–110% range set by ENGL. All participants determined the R² value of both assays at 0.990 or higher, which meets the minimum performance requirements.

Additionally, specificity was tested by including non-GM soybean DNA. All laboratories, except for one *FAD2-1A-Δ63bp* replicate in laboratory 1, returned negative results

Table 4 Interlaboratory comparison study for *FAD2-1A-Δ63bp* and *FAD2-1B-Δ23bp* assays

Lab no.	Master mix	qPCR instrument	<i>FAD2-1A-Δ63bp</i>			<i>FAD2-1B-Δ23bp</i>		
			LOD _{95%} [cp/qPCR]	Efficiency [%]	R ²	LOD _{95%} [cp/qPCR]	Efficiency [%]	R ²
1	GoTaq Probe qPCR Master Mix	QuantStudio 7	4.79	90.7	0.998	6.58	89.6	0.999
2	QuantiTect Multiplex PCR NoROX	QuantStudio 5	3.35	98.5	0.998	4.65	96.5	0.999
3	TaqMan Universal PCR Master Mix	ABI 7500	4.94	90.7	0.999	11.2	91.2	0.997
4	PerfeCTa MultiPlex qPCR Tough Mix Low ROX	QuantStudio 5	7.83	91.1	0.999	4.09	95.5	0.999
5	TaqMan Universal PCR Master Mix	QuantStudio 5	7.09	100.1	0.997	2.72	113.3	0.997
6	TaqMan Universal PCR Master Mix	ABI 7500	7.83	100.0	0.992	5.23	87.5	0.990

Results from 6 independent laboratories are presented. LOD_{95%} values were calculated using the QuoData web tool (Uhlig et al. 2015). The qPCR instruments used included QuantStudio 7, QuantStudio 5, and the Applied Biosystems (ABI) 7500 PCR system (Thermo Fisher Scientific). Master mixes utilized were GoTaq Probe qPCR Master Mix (Promega), QuantiTect Multiplex PCR NoROX (Qiagen), PerfeCTa MultiPlex qPCR ToughMix Low ROX (Quantabio, Beverly, MA, USA), and TaqMan Universal PCR Master Mix (Thermo Fisher Scientific). Detailed results are available in Tables S7 and S8 (Supplementary Material)

(Table S8, Supplementary Material). The C_q value (43.74) for this replicate was found to be higher than for samples containing low amounts of target DNA. Therefore, it was deemed a non-specific amplification signal, unrelated to the target DNA.

Additionally, a second interlaboratory comparison tested single- and double-quenched probes to evaluate their performance. Since few oligonucleotide manufacturers produce probes combining two quenchers and LNA modifications, the use of a single quencher probe was further investigated, particularly with regard to the transferability of the methods to other laboratories. Two laboratories performed qPCR experiments, and results for LOD_{95%}, qPCR efficiency, and R² were similar for both probe types (Table S10, Supplementary Material). A single R² value for *FAD2-1A-Δ63bp* using the single-quenched probe was slightly below ENGL requirements. As this did not occur during the in-house validation, it was likely due to limited number of dilutions and replicates.

4 Discussion and conclusion

The event-specific *FAD2-1A-Δ63bp* and *FAD2-1B-Δ23bp* detection methods targeting the alterations in the genome-edited Calyno soybean variety meet the ENGL minimum performance requirements for assays in GMO testing (ENGL 2015). In-house validation confirmed the assays' sensitivity, qPCR efficiency, specificity, and robustness, which are necessary for reliable GMO detection.

The use of a universal probe for both assays simplifies detection of the Calyno soybean variety, reducing costs and minimizing errors. The primers ensure the specificity of the *FAD2-1A-Δ63bp* and *FAD2-1B-Δ23bp* assays, as they target homologous gene variants with high sequence similarity. This allows for the use of a universal probe, as no cross-talk between the methods has been observed. However, attempts

to combine the assays into a duplex qPCR method were unsuccessful, likely due to primer dimer formation.

For initial assay development, optimization approaches and the interlaboratory comparison study, a ZEN-IBFQ double-quenched version of the probe was used. However, a single-quenched probe (IBFQ) was applied for in-house validation. The second interlaboratory study confirmed that either probe yields comparable results regarding qPCR performance. Consequently, a single-quenched probe is recommended due to the wider range of suppliers, as options for double-quenched probes with LNA bases are limited.

With regard to the interlaboratory comparison study, minor deviations in qPCR efficiency for the *FAD2-1B-Δ23bp* method were slightly outside of ENGL requirements in a few laboratories, highlighting challenges in plasmid dilution handling, which proved to be more error-prone than the handling of genomic DNA. Differences also occurred in the number of replicates, use of laboratory-specific master mixes and devices, including the respective software for automatic vs. manual threshold setting. Despite these differences, overall results demonstrated the robustness of the assays. The use of genomic DNA might further enhance the reliability and accuracy of the validation process.

The lack of plant reference material for Calyno soybean remains a limitation, preventing the detection methods from achieving official validation under ENGL and BVL guidelines (BVL 2016; ENGL 2015). Nevertheless, besides the use of plasmid DNA, the assays and their validations were treated as official methods. The pFAD2-1A-Δ63bp and pFAD2-1B-Δ23bp insert sequences resemble the genetic situation of the *FAD2* gene variants in the genome-edited Calyno soybean variety, which have been described in detail in Haun et al. (2014). Moreover, the assays' performance was not affected by the presence of excess amounts of genomic non-GM soybean background DNA as demonstrated by specificity tests. Therefore, data from this study did not indicate any experimental limitations for the assays that might occur when encountering

genomic Calyno soybean DNA in a routine GMO testing environment carrying *FAD2-1A-A63bp* and *FAD2-1B-A23bp*. Additionally, as the number of NGT or classical GM crops continues to grow, the reluctance of companies to provide reference materials may become a more prevalent issue. Nevertheless, public authorities remain obligated to formulate and implement event-specific detection methods in GMO testing. Therefore, our “cold validation” approach utilizing plasmid DNA provides a straightforward and sophisticated solution to address this challenge.

Additional genome editing events of *FAD3A* in Calyno background (Demorest et al. 2016) lack sequence details on the mutations. As both varieties, described in Haun et al. (2014) and Demorest et al. (2016), lack approval in the EU, positive results would indicate the presence of a genome-edited soybean, even if a definitive differentiation of the lines is not possible.

Since Calyxt’s merger with Cibus in 2023, the BVL has attempted to obtain reference material under new management but was again denied. Yet, the § 28b GenTG working group plans a collaborative ring-trial to validate the detection methods for both *FAD2-1A-A63bp* and *FAD2-1B-A23bp*. As long as we do not obtain plant material or genomic DNA, this ring-trial will involve a “cold validation” using plasmid DNA. Should plant material or genomic DNA become available, further validation of the *FAD2-1A-A63bp* and *FAD2-1B-A23bp* detection methods using Calyno soybean DNA will be undertaken.

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Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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References

- Altschul SF, Madden TL, Schäffer AA et al (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25:3389–3402. <https://doi.org/10.1093/nar/25.17.3389>
- Bonfini L, van den Bulcke MH, Mazzara M et al (2012) GMOMETH-ODS: the European Union database of reference methods for GMO analysis. *J AOAC Int* 95:1713–1719. <https://doi.org/10.5740/jaoacint.12-050>
- Bustin SA, Benes V, Garson JA et al (2009) The MIQE guidelines: minimum Information for publication of quantitative real-time PCR experiments. *Clin Chem* 55:611–622. <https://doi.org/10.1373/clinchem.2008.112797>
- BVL (2016) Guidelines for the single-laboratory validation of qualitative real-time PCR methods. BVL, Braunschweig
- Calyxt (2019) First Commercial sale of calyxt high oleic soybean oil on the U.S. market. <https://www.businesswire.com/news/home/20190226006176/en>. Accessed 04 June 2024
- Court of Justice of the European Union (2018) Press Release 111/18-Judgment in case C-528/16. https://curia.europa.eu/jcms/jcms/p1_1217550/en/. Accessed 04 June 2024
- Demorest ZL, Coffman A, Baltes NJ et al (2016) Direct stacking of sequence-specific nuclease-induced mutations to produce high oleic and low linolenic soybean oil. *BMC Plant Biol* 16:225. <https://doi.org/10.1186/s12870-016-0906-1>
- ENGL (2015) Definition of minimum performance requirements for analytical methods for GMO Testing. http://gmo-crl.jrc.ec.europa.eu/doc/MPR%20Report%20Application%2020_10_2015.pdf. Accessed 04 June 2024
- Guertler P, Harwardt A, Eichelinger A et al (2013) Development of a CTAB buffer-based automated gDNA extraction method for the surveillance of GMO in seed. *Eur Food Res Technol* 236:599–606. <https://doi.org/10.1007/s00217-013-1916-y>
- Haun W, Coffman A, Clasen BM et al (2014) Improved soybean oil quality by targeted mutagenesis of the fatty acid desaturase 2 gene family. *Plant Biotechnol J* 12:934–940. <https://doi.org/10.1111/pbi.12201>
- ISAAA (2019) Global status of commercialized biotech/GM crops in 2019: biotech crops drive socio-economic development and sustainable environment in the new frontier. ISAAA Brief 55, ISAAA, Ithaca, NY
- Pham A-T, Lee J-D, Shannon JG et al (2011) A novel *FAD2-1 A* allele in a soybean plant introduction offers an alternate means to produce

- soybean seed oil with 85% oleic acid content. *Theor Appl Genet* 123:793–802. <https://doi.org/10.1007/s00122-011-1627-3>
- Sayers EW, Bolton EE, Brister JR et al (2022) Database resources of the national center for biotechnology information. *Nucleic Acids Res* 50:D20–D26. <https://doi.org/10.1093/nar/gkab1112>
- Schlueter JA, Vasylenko-Sanders IF, Deshpande S et al (2007) The *FAD2* gene family of soybean: insights into the structural and functional divergence of a paleopolyploid genome. *Crop Sci* 47:S–14. <https://doi.org/10.2135/cropsci2006.06.0382tpg>
- Uhlig S, Frost K, Colson B et al (2015) Validation of qualitative PCR methods on the basis of mathematical–statistical modelling of the probability of detection. *Accredit Qual Assur* 20:75–83. <https://doi.org/10.1007/s00769-015-1112-9>
- Yang Y, Xu C, Shen Z et al (2022) Crop quality improvement through genome editing strategy. *Front Genome Ed*. <https://doi.org/10.3389/fgeed.2021.819687>
- Ye J, Coulouris G, Zaretskaya I et al (2012) Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinform* 13:134. <https://doi.org/10.1186/1471-2105-13-134>
- You Y, Moreira BG, Behlke MA et al (2006) Design of LNA probes that improve mismatch discrimination. *Nucleic Acids Res* 34:e60. <https://doi.org/10.1093/nar/gkl175>
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