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ORIGINAL PAPER

Nonsense-mediated mRNA degradation of *CtFAD2-1* and development of a perfect molecular marker for *olol* mutation in high oleic safflower (*Carthamus tinctorius* L.)

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Abstract There are two types of safflower oil, high oleic (HO) with 70–75 % oleic acid and high linoleic (HL) with about 70 % linoleic acid. The original HO trait in safflower, found in an introduction from India, is controlled by a partially recessive allele *ol* at a single locus (Knowles and Bill 1964). In the lipid biosynthesis pathway of developing safflower seeds, microsomal oleoyl phosphatidylcholine desaturase (FAD2) is largely responsible for the conversion of oleic acid to linoleic acid. In vitro microsomal assays indicated drastically reduced FAD2 enzyme activity in the HO genotype compared to conventional HL safflower. A previous study indicated that a single-nucleotide deletion was found in the coding region of *CtFAD2-1* that causes premature termination of translation in the HO genotypes, and the expression of the mutant *CtFAD2-1Δ* was attenuated in the HO genotypes compared to conventional HL safflower (Guan et al. 2012). In this study, we hypothesise that down-regulation of *CtFAD2-1* expression in the HO genotype may be explained by nonsense-mediated RNA decay (NMD). NMD phenomenon, indicated by gene-specific RNA degradation of defective *CtFAD2-1Δ*, was subsequently confirmed in *Arabidopsis thaliana* seed as well as in the transient expression system in *Nicotiana benthamiana* leaves. We have developed a perfect molecular marker corresponding to the *olol* mutation that can facilitate a rapid screening and early detection of genotypes carrying the *olol* mutation for use in marker-assisted selection for the management of the HO trait in safflower breeding programmes.

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Introduction

Traditional safflower oil contains about 6–8 % palmitic acid, 2–3 % stearic acid, 16–20 % oleic acid and 71–75 % linoleic acid; hence, it is regarded as one of most highly polyunsaturated vegetable oils (Velasco and Fernandez-Martinez 2001). However, there are natural mutants and breeding lines with various levels of elevated oleic acid mainly at the expense of linoleic acid (Fernández-Martinez et al. 1993). Consumption of oils with high oleic acid content (HO) is regarded as desirable because of its cholesterol-lowering effect and enhanced oxidative stability that reduces the need for hydrogenation, a process known to generate nutritionally undesirable trans fatty acids. Further, the combination of high oxidative stability and low melting point characteristics of HO safflower oil also renders it more suitable for biodiesel applications and biodegradable replacements for mineral oils, such as hydraulic oils and lubricants (Kinney and Clemente 2005). Furthermore, purified oleic acid could be used to manufacture a range of oleochemicals such as diacids that are found in the formulations of hundreds of different oleochemical products, offering an interesting suite of properties including elasticity, flexibility, impact strength and hydrolytic stability (Crandall 2002; Hill 2000).

The original HO trait in safflower, found in an introduction from India, is controlled by a partially recessive

allele *ol* at a single locus OL (Knowles and Bill 1964), with oleic acid content of *olol* homozygous genotypes reaching 70–75 % (Knowles 1989). The *ol* allele was incorporated into safflower breeding programmes and the first HO safflower variety "UC-1" was released in 1966 in the USA, which was followed by the releases of "Oleic Leed" and the Saffola series including Saffola 317 (S-317), S-517 and S-518. The *ol* allele has also been used as the background genetic material for further enhancement of oleic acid content in safflower breeding programmes worldwide (Mündel and Bergman 2009; Weiske 1999).

Fatty acid biosynthesis is a highly regulated and compartmentalised process in higher plants (Ohlrogge and Jaworski 1997). Saturated fatty acids and monounsaturated fatty acids, mostly oleic acid, are synthesised in plastids. Following its exit from plastids, oleic acid can be further modified on phosphatidylcholine (PC) by the microsomal oleate Δ12 desaturase (FAD2; EC1.3.1.35), using NADH, NADH-cytochrome b_5 reductase and cytochrome b_5 as electron donors (Shanklin and Cahoon 1998). Fatty acids formed on PC are exchanged with the acyl-CoA pool prior to incorporation into storage lipids, mainly triacylglycerol (TAG) via the Kennedy pathway (Ohlrogge and Browse 1995). TAG can also be formed via an acyl-CoA independent pathway catalysed by phospholipid:diacylglycerol acyltransferase (PDAT) (Dahlqvist et al. 2000). Numerous biochemical, genetic and transgenic studies have clearly indicated that FAD2 is the key enzyme controlling the relative accumulation of oleic acid versus linoleic acid in safflower seed oils (Stymne and Appelqvist 1978).

To explore the biochemical nature of the *olol* mutation, we carried out in vitro microsomal FAD2 enzyme assay comparing HO and HL genotypes. This revealed a defective *FAD2* as the *ol* allele. We have isolated an exceptionally large *FAD2* gene family from safflower consisting of at least 11 members, including the seed-specific *CtFAD2-1* (Cao et al. 2013). We have confirmed a previous finding that a single-nucleotide deletion in the coding region of *CtFAD2-1* would cause a frame shift and lead to attenuated transcription (Guan et al. 2012). In this study, we carried out in vitro biochemical assays of FAD2 enzyme activity, directly associating the high oleic *ol* allele with reduced FAD2 activity. We also propose that the molecular basis of the *olol* genotype is mediated by nonsense-mediated mRNA decay (NMD) of *CtFAD2-1*, a process that typically degrades transcripts containing a premature termination codon (PTC). Further, we have designed a set of PCR-based perfect molecular markers for the identification of the *ol* allele, which may allow for rapid screening and early detection of genotypes carrying the *ol* allele for the management of the HO trait in safflower breeding programmes.

Materials and methods

Plant materials

A wild-type or HL safflower 'SU' and five HO safflower varieties, including 'S-317', 'S-517', 'LeSaf486', 'CW99-OL' and 'Ciano-OL', were used in this study. SU is commonly grown as a bird seed crop in Australia, and it was obtained from Heffernan seeds in NSW. A few HO varieties including LeSaf486 (PI603208, ATC 120562), CW99-OL (ATC 120561) and Ciano-OL were obtained from Australian Temperate Field Crops Collections in Horsham, Victoria, Australia. Other HO varieties, including S-317 and S-517, were supplied by Devexco International. The plants were grown from seeds and maintained under glasshouse conditions with day/night cycle of 25/22 °C and 16 h/8 h photoperiod.

Microsomal assay of the FAD2 enzyme activity

Safflower microsomes from S-317 and SU developing seeds 15 days after anthesis (DAA) were freshly prepared as described previously (Stymne and Appelqvist 1978). The assay mixture (100 μL) contained 40 μg microsomal protein, 2 nmol [1-^{14}C]oleoyl-CoA (10 K dpm/nmole), 10 nmole CoA and 1 % BSA in 0.1 mM potassium phosphate buffer pH 7.2 with or without 5 mM NADH. The incubations were carried out in a water bath at 30 °C with constant shaking for 10 min, followed by another 5, 10 and 20 min, respectively, after adding NADH. The reactions were stopped by adding 225 μL of methanol:$CHCl_3$:HAc = 50:50:1 (V:V:V). The lower $CHCl_3$ phases were recovered, dried and loaded onto a silica gel 60 thin layer chromatography (TLC) plate (MERCK, Dermstadt, Germany) and developed with a solvent mixture containing $CHCl_3$:Methanol:HAc:H_2O in the ratio of 90:15:10:3. The PC fraction was isolated and methylated with methanolic-HCl at 80 °C for 2 h as previously described (Zhou et al. 2006). The resultant fatty acid methyl esters (FAMEs) were separated on $AgNO_3$-treated argentation TLC plate with hexane:diethyl ether:HAc (85:15:1). The plates were exposed and analysed by a Fujifilm FLA-5000 phosphorimager (Fujifilm, Fuji, Tokyo, Japan). The radioactivity of each sample was quantified with Fujifilm Multi Gauge software.

Ectopic expression of *CtFAD2-1* and *CtFAD2-1Δ* in *Saccharomyces cerevisiae*

The entire coding regions of *CtFAD2-1* and *CtFAD2-1Δ* were first PCR amplified and ligated behind the GAL1 promoter in a pYES2 vector in sense orientation for inducible gene expression. The oligo primers used to amplify the entire coding region of *CtFAD2-1* and

CtFAD2-1Δ were: s1: 5′-TGAAAGCAAGATGGGAG GAGG-3′ and a1: 5′-TCACAACTTTACTTATTCTTGT-3′. The resulting plasmids and the empty pYES2 vector (negative control) were introduced into bakers' yeast (*Saccharomyces cerevisiae*) YPH499 cells by lithium acetate-mediated transformation. The transformed cells derived from a single colony were grown for 2 days at 28 °C in synthetic dropout medium lacking uracil, but supplemented with glucose (SD-glucose) liquid medium. The expression of *CtFAD2-1* and *CtFAD2-1Δ* was induced by transferring the cells to a fresh SD liquid medium supplemented with galactose instead of glucose and grown with shaking for an additional 2 days. The cells were harvested by centrifugation. FAMEs were prepared by transesterification of the total fatty acids in yeast cell pellets by adding 750 µL of 1 N MeOH–HCl at 80 °C for a minimum of 2 h prior to adding 500 µL of 0.9 % NaCl. FAMEs were extracted with 300 µL of hexane and analysed by Agilent 7890A gas chromatography (GC) with a 30-m BPX70 column essentially as described (Zhou et al. 2011). Each experiment was carried out in triplicate. Total RNAs of the yeast cells expressing either *CtFAD2-1* or *CtFAD2-1Δ* were isolated using Trizol reagent (Invitrogen, Carlsbad, USA) and real-time quantitative RT-PCR (RT-qPCR) was carried out for gene expression studies.

Transient expression of *CtFAD2-1* and *CtFAD2-1Δ* in *Nicotiana benthamiana* leaves

The entire coding regions of *CtFAD2-1* derived from SU and *CtFAD2-1Δ* derived from S-317 were each cloned in sense orientation into a modified pORE04 binary vector between the double CaMV-35S promoter and an *Agrobacterium tumefaciens* NOS terminator containing the polyadenylation signal sequence (Coutu et al. 2007), forming the vectors *35S:CtFAD2-1* and *35S:CtFAD2-1Δ*, respectively. A vector constitutively expressing the viral suppressor protein, P19, was obtained from Dr Peter Waterhouse's laboratory (University of Sydney). *A. tumefaciens* strain AGL1 harbouring the *35S:CtFAD2*-1 or *35S:CtFAD2-1Δ* was co-infiltrated with the *35S:P19* culture into the underside of the fully expanded *N. benthamiana* leaves as previously described (Voinnet et al. 2003; Wood et al. 2009). Following a period of 5 days of further growth at 24 °C, the infiltrated leaf regions were excised and immediately subjected to RNA isolation using an RNeasy Mini Kit (Qiagen, Hilden, Germany).

Ectopic expression of *CtFAD2-1* and *CtFAD2-1Δ* in Arabidopsis

Constructs expressing either *CtFAD2-1* derived from SU or *CtFAD2-1Δ* derived from S-317, each driven by the seed-specific promoter *Fp1* derived from a truncated *Brassica napus* napin gene (Stalberg et al. 1993), were transformed to *Arabidopsis thaliana* ecotype Col-0 via the *A. tumefaciens* dipping method. Inoculations were performed by dipping the aerial parts of plants at flowering stage for a few seconds in 300 mL of a solution containing 5 % (w/v) sucrose, 10 mm $MgCl_2$, resuspended *A. tumefaciens* cells from a 150 mL overnight culture and Silwet L-77 (Lehle Seeds, TX, USA) following Bent and Clough (1998). The *A. thaliana* plants were maintained in a glasshouse with constant 22 °C and 16 h photoperiod until seed maturity had been reached. Twelve lines of *A. thaliana* independently transformed with each of the aforementioned constructs were established following selection on 50 mg/L kanamycin on Murashige and Skoog medium. The siliques containing middle maturity developing embryos from these transgenic plants were harvested and total RNAs were isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany).

Real-Time quantitative PCR analysis of *CtFAD2* expression

Total RNAs from yeast, developing *A. thaliana* siliques and *N. benthamiana* leaves were isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany). Contaminating DNA was removed by digestion with TURBO RNA-free DNaseI (Ambion, TX, USA) according to the manufacturer's protocol. RNA concentrations were determined using a Nanodrop® spectrophotometer ND1000 (Thermo Fisher Scientific, Victoria, Australia), and concentrations were equalised before analysis. To verify RNA integrity, 1 µg of total RNAs from each sample was visualised on an ethidium bromide-stained 1.5 % agarose gel following electrophoresis.

The gene expression patterns were studied with RT-qPCR carried out in triplicate using Platinum SYBR Green qPCR SuperMix (BioRad, CA, USA) and run on ABI 7900HT Sequence Detection System. Each PCR reaction contained 20 ng of total RNA template, 800 mM each of the forward and reverse primers, 0.25 µL of reverse transcriptase and 5 µL One-Step RT-PCR Master Mix reagents, increased to 10 µL total volume with nuclease-free water. PCR was carried out with an initial cycle at 48 °C for 30 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The primers for *CtFAD2-1* are sense: 5′-GTGTATGTCTGCCTCC GAGA-3′; antisense: 5′-GCAAGGTAGTAGAGGACGA AG-3′. A constitutively expressed reference gene from safflower, *CtKASII,* was used to normalise the relative quantities. KASII is responsible for the elongation of C16:0-ACP to C18:0-ACP in de novo fatty acid biosynthesis in plants. Safflower *KASII* gene (*CtKASII*) has been

previously used as an internal reference gene because of its high expression stability in various tissues and developmental stages (Cao et al. 2013). The primers for *CtKASII* are sense: 5′-CTGAACTGCAATTATCTAGG-3′; and antisense: 5′-GGTATTGGTATTGGATGGGCG-3′. The calculations were made using the comparative CT method as reported (User Bulletin #2, Applied Biosystems). The data are presented as mean ± SD of three reactions performed on independent 96-well plates.

Small RNA Northern blot analysis

Approximately, 10 µg of total RNAs from 15 DAA developing embryos of SU and S-317 were separated using a 17 % denaturing polyacrylamide gel and blotted onto Hybond-N+ membranes (GE Healthcare, NJ, USA). The membranes were UV cross-linked and pre-hybridised at 42 °C for 3 h in hybridisation buffer containing 50 % formamide, 5 × SSPE (3 M NaCl, 0.2 M NaH_2PO_4, and 0.02 M EDTA, pH7.4), 5× Denhardt's solution, 1 mM EDTA, 1 % BSA and 1 % SDS. DNA oligos antisense to *CtFAD2-1* and *CtFAD2-1Δ* were end labelled by the forward reaction using 10 units of T4 polynucleotide kinase (Roche Molecular Biochemicals, Indianapolis, IN, USA) with the supplied buffer, to which 300 nM [γ-32P] ATP (3,000 Ci/mmol) was added. The reaction was incubated for 1 h at 37 °C. Unincorporated ^{32}P-label was removed using a G-25 microcolumn (GE Healthcare, NJ, USA). Probes were added to the hybridisation buffer and hybridisation was allowed to proceed at 42 °C overnight. The membranes were then washed twice, 30 min each in 2× SSC and 0.2 % SDS at 40 °C. Hybridisation signal was detected and analysed by a Fujifilm FLA-5000 phosphorimager (Fujifilm, Tokyo, Japan).

Deep sequencing of safflower small RNAs

Small RNAs were extracted from the mid-maturity developing seeds at 15 DAA of SU and S-317 plants using the mirVana™ miRNA Isolation Kit (Ambion, CA, USA) following the manufacturer's instructions. Subsequently, small RNAs were subjected to 15 % (w/v) denaturing polyacrylamide gel electrophoresis (PAGE), and 18- to 25-bp portions were excised from the gel and purified. The purified small RNA molecules were then ligated to the Solexa 5′ and 3′ adaptor sequentially and converted to cDNA following the Illumina protocol. Deep sequencing was performed on the Illumina HiSeq2000 and the samples were run side by side at the Beijing Genome Institute (BGI, Shenzhen, China). After removing the low-quality reads and those that were less than 18 nt in length, the small RNA data derived from both SU and S-317 were "Blast" searched for sequences corresponding to *CtFAD2-1* and *CtFAD2-1Δ*, respectively.

Developing a perfect PCR marker and PCR for *ol* allele

A large intron located in the 5′UTR of *CtFAD2-1* in SU or *CtFAD2-1Δ* in S-317 was amplified by PCR using forward oligo primer intron-s1: 5′-GAGATTTTCAGAGAGCAAG CGCTT-3′ and reverse oligo primer: intron-a1: 5′-CTTTG GTCTCGGAGGCAGACATA-3′. Based on the unique DNA sequences of *CtFAD2-1Δ*, a pair of oligo primers was designed to amplify a specific band of 300-bp long from the genomic DNA of the HO genotypes, including S-317, S-517, CW99-OL, LeSaf496 and Ciano-OL. The sequences of these primers are: HO-S1, 5′-ATAAGGCTGTGTTCAC GGGTTT-3′; and HO-A1, 5′-GCTCAGTTGGGGATACA AGGAT-3′. A pair of oligo primers specific for the conventional HL safflower SU is designated as follows: HL-S1, 5′-AGTTATGGTTCGATGATCGACG -3′; and HL-A1, 5′-TTGCTATACATATTGAAGGCACT-3′. The primers derived from the safflower *CtKASII* gene, which were also used in RT-qPCR, were used as the positive control and to ensure equal loading in this experiment. PCR was conducted using HotStar mix following the manufacturer's instructions (Qiagen, Hilden, Germany). The PCR cycle was 94 °C for 15 min, followed by 40 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min. The reaction products were separated by electrophoresis on a 1 % agarose gel and visualised under UV light following EtBr staining. The amplicon's identity was confirmed by DNA sequencing.

Results

In vitro microsomal analysis of oleate desaturase activity

Microsomal oleate desaturation assays were performed on developing embryos of 15 DAA. After argentation TLC and autoradiography, examination of the autoradiographs indicated that the microsomal extracts derived from developing safflower embryos of HL safflower SU were able to rapidly desaturate [^{14}C]oleoyl-CoA in the presence of NADH. As shown in Fig. 1, upon the addition of NADH, rapid appearance of [^{14}C] linoleate in the PC fraction was observed following 5 min reaction, indicating efficient biosynthesis of [^{14}C] linoleate from oleate in the HL safflower microsomes in vitro. In contrast, in microsomes prepared from developing embryos of S-317, the biosynthesis of linoleic acid via desaturation of oleate was significantly less. The ratio of [^{14}C] oleate versus [^{14}C]

linoleate in the microsomal in vitro reaction throughout the time course was also compared and is shown in Table 1.

CtFAD2-1Δ codes for a non-functional microsomal Δ12 oleate desaturase

To verify the functionality loss of the CtFAD2-1Δ from HO genotypes, its corresponding ORF was cloned into the expression vector pYES2 behind the inducible GAL1 promoter and transformed into *S. cerevisiae*. *CtFAD2-1* derived from the conventional HL safflower SU was used as a positive control. FAMEs were prepared from transgenic yeast cells that were induced with galactose and grown to stationary phase. Fatty acid composition of the harvested yeast cells was analysed by GC (Fig. 2). As expected, the yeast cells transformed with *CtFAD2-1* showed the presence of two dienoic acids, palmitolinoleic acid (C16:2) and linoleic acid (C18:2), which were not present in the untransformed yeast or in the control cells transformed with the empty vector. In contrast, the expression of the *CtFAD2-1Δ* did not result in accumulation of any dienoic acid, indicating that *CtFAD2-1Δ* had lost its functionality as a microsomal Δ12 oleate desaturase. We also measured the transcript levels of both *CtFAD2-1* and *CtFAD2-1Δ* in yeast cells, which were at comparable levels as shown in Fig. 3. The transcript reduction of *CtFAD2-1Δ* as demonstrated in HO safflower developing embryos by Guan et al. (2012) and our own experiment (data not shown) was not observed in transgenic yeast cells.

Northern blot and deep sequencing analysis of small RNAs

Northern blot analysis of small RNAs isolated from mid-maturity developing embryos of both HL safflower (SU) and HO genotypes S-317 and LeSaf486 revealed no discernible level of small RNA corresponding to *CtFAD2-1* or *CtFAD2-1Δ* (Fig. 4).

Table 1 Percentage of linoleate derived from oleate in safflower microsomes

Time (min)	SU		S-317	
	Oleate	Linoleate	Oleate	Linoleate
0	99.2	0.8	100.0	0.0
5	79.6	20.4	99.4	0.6
10	69.6	30.4	95.4	4.6
20	60.6	39.4	95.1	4.9

The lack of significant levels of small RNAs corresponding to *CtFAD2-1* or *CtFAD2-1Δ* was confirmed by small RNA deep sequencing. Solexa sequencing of small RNAs derived from SU and S-317 generated 3,741,194 and 3,585,498 sequences ranging from 18 to 30 nucleotides (nt), respectively. These two small RNA databases were BLAST searched with *CtFAD2-1* and *CtFAD2-1Δ,* respectively. A comparable low number of small RNAs corresponding to the *CtFAD2-1* in conventional HL safflower SU and *CtFAD2-1Δ* in HO genotype S-317 were detected. The small RNAs corresponding to the *CtFAD2-1Δ* sequence in S-317 were not significantly higher than those in SU (Fig. 5). The small RNAs corresponding to *CtFAD2-1Δ* were not expected to be sufficient to generate any substantial effect of posttranscriptional gene silencing in S-317.

Transient expression of *CtFAD2-1Δ* in *N. benthamiana* leaves

To test whether the ectopic expression of *CtFAD2-1Δ* harbouring a PTC would result in NMD-mediated mRNA degradation in a plant system, we first investigated its expression level measured by RT-qPCR in a heterologous

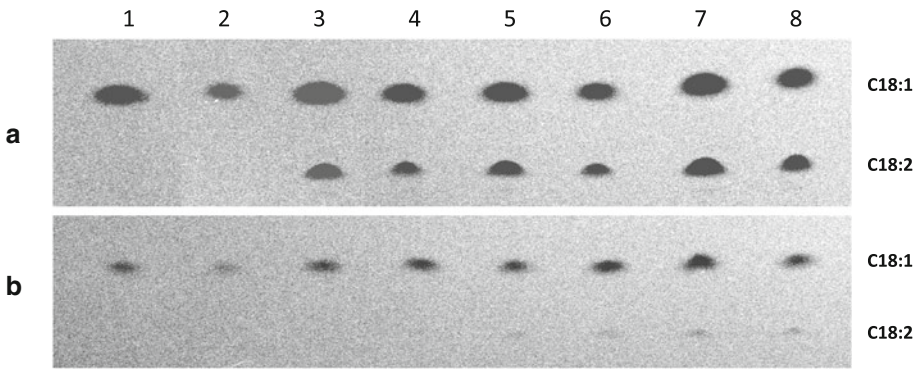


Fig. 1 Argentation TLC analysis of FAMEs derived from PC isolated from in vitro microsomal reactions comparing SU and S-317. *Lanes 1 and* 2 were duplicated reactions without NADH; *lanes 3 and 4* were duplicated reactions with NADH for 5 min; *lanes 5 and 6* were duplicated reactions with NADH for 10 min; *lanes 7* and *8* were duplicated reactions with NADH for 20 min. **a** SU; **b** S-317

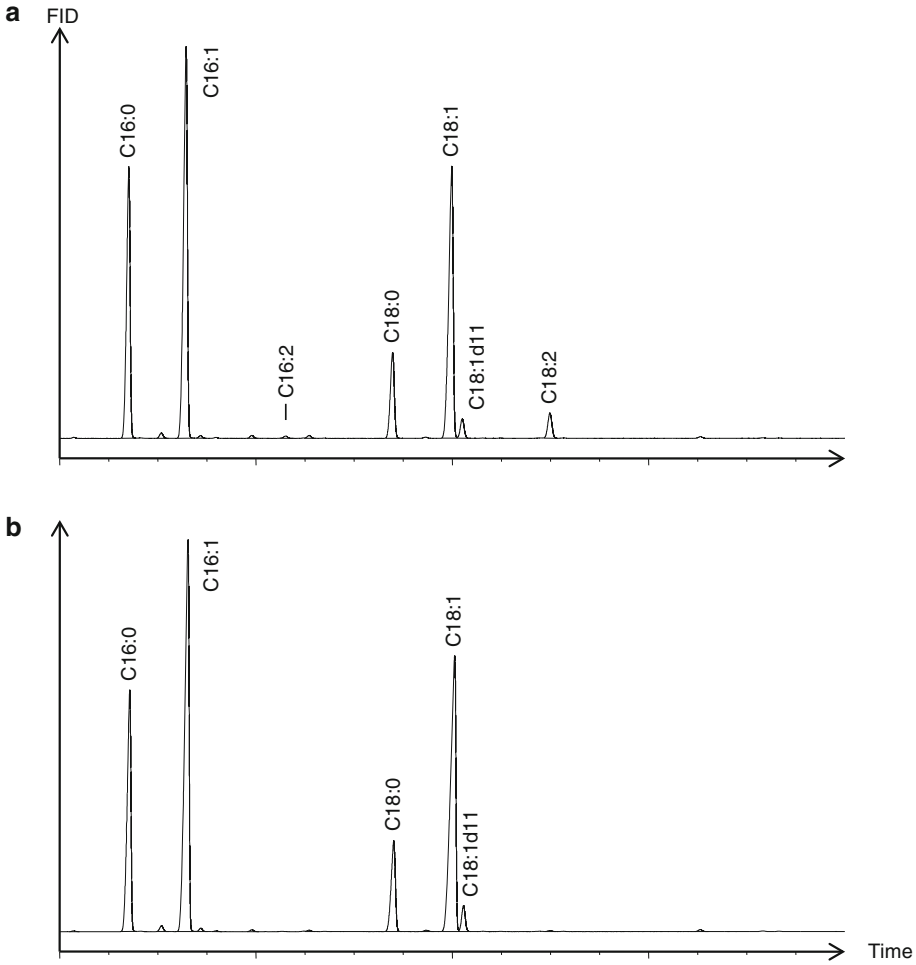


Fig. 2 Functional identification of *CtFAD2-1* and *CtFAD2-1Δ* by ectopic expression in yeast. **a**. *CtFAD2-1;* **b**. *CtFAD2-1Δ*

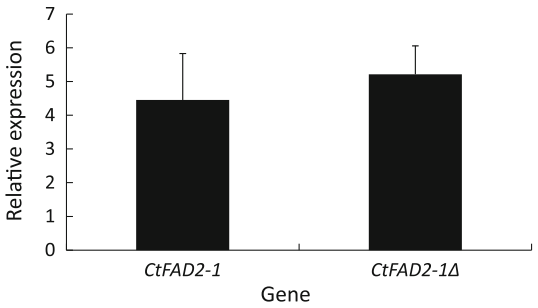


Fig. 3 RT-qPCR analysis of gene expression in yeast transformed with *CtFAD2-1* and *CtFAD2-1Δ*

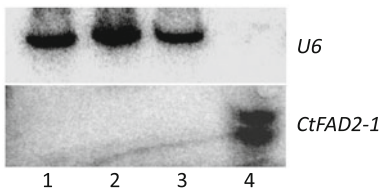


Fig. 4 Northern blot analysis of small RNAs corresponding to *CtFAD2-1* and *CtFAD2-1Δ*. The *top panel* was hybridised with *U6* RNA as a positive control. The *lower panel* was hybridised with *CtFAD2-1*. *Lane* 1 SU, *lane 2* S-317, *lane 3* Lesaff496, *lane 4*, RNA ladder

plant host *N. benthamiana* transient expression system. *N. benthamiana* leaves infiltrated with *A. tumefaciens* culture containing either the *35S:CtFAD2-1* or the *35S:CtFAD2-1Δ* expression vectors were harvested 5 days after infiltration and total RNAs were isolated for RT-qPCR analysis. As shown in Fig. 6, the expression level of *CtFAD2-1Δ* derived from S-317 was significantly lower than that of *CtFAD2-1*, with the former expressed at levels that were observed to be at least twofold lower than the latter.

Seed-specific expression of *CtFAD2-1Δ* in Arabidopsis

A. thaliana transgenic lines were produced to investigate whether the NMD phenomenon observed in the expression of *CtFAD2-1Δ* could also be observed in the developing seeds of a heterologous host *A. thaliana*. A truncated *B. napus* napin promoter *Fp1* was used to drive the seed-specific expression of the transgenes. The *Fp1:CtFAD2-1* and *Fp1:CtFAD2-1Δ* plasmids were introduced into *A. tumefaciens* that were used to transform wild-type

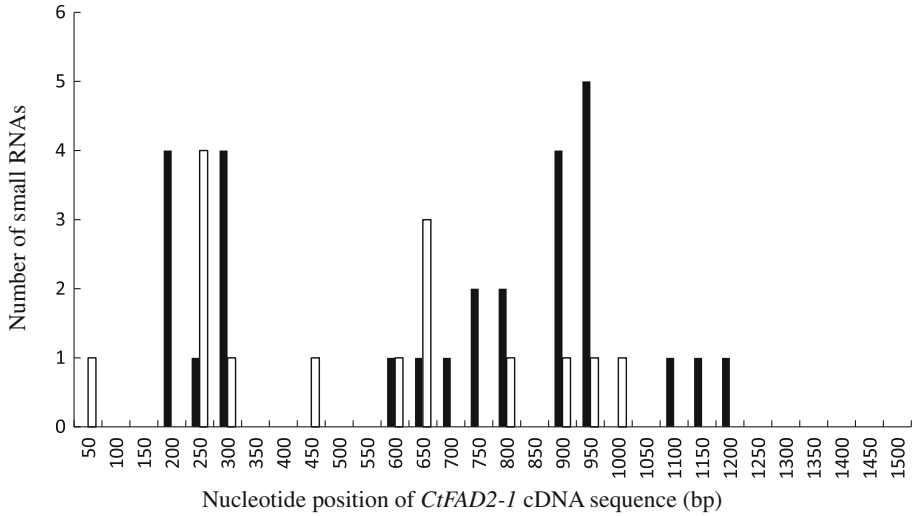


Fig. 5 The number of small RNAs corresponding to the cDNA sequence in conventional HL safflower SU *CtFAD2-1* (*filled circle*) and HO genotype S-317 *CtFAD2-1Δ* (*open circle*)

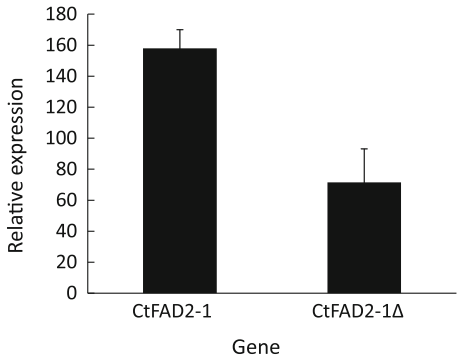


Fig. 6 Real-time RT-qPCR analysis of *CtFAD2-1* and its mutant *CtFAD2-1Δ* that were transiently expressed in *N. benthamiana* leaves, controlled by a 35S promoter. *CtFAD2-1* (*filled circle*) and *CtFAD2-1Δ* (*open circle*)

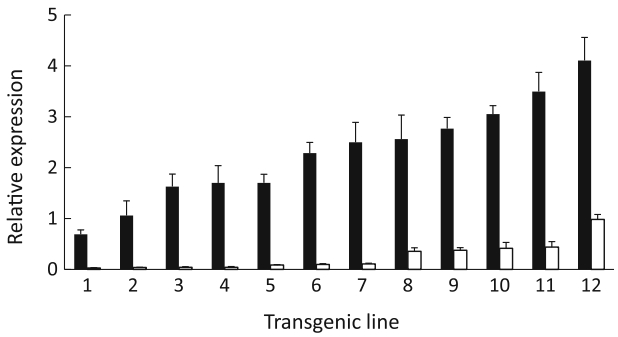
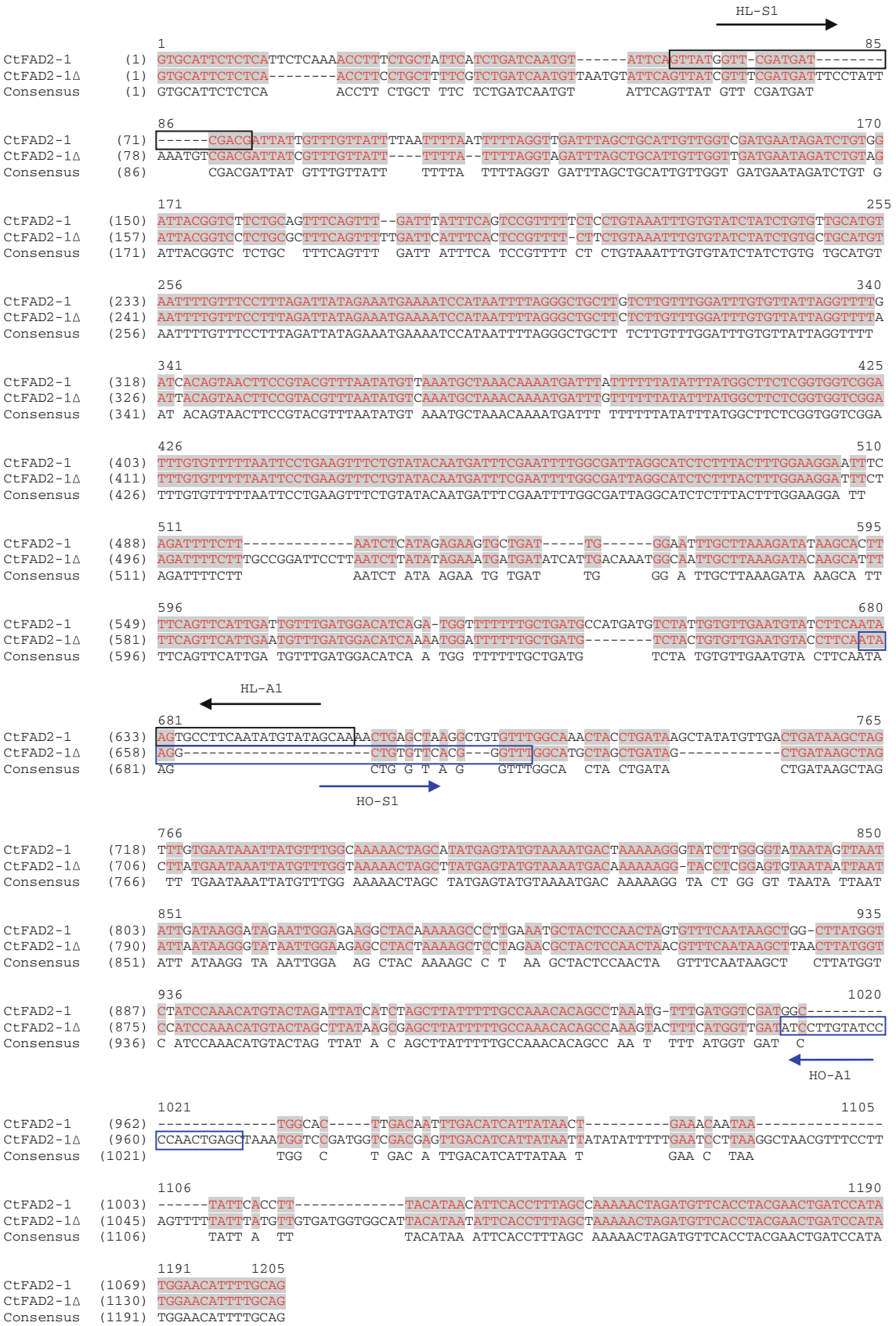


Fig. 7 Real-time RT-qPCR analysis of *CtFAD2-1* and its mutant form *CtFAD2-1Δ* expressed in *A. thaliana* embryos, controlled by a seed-specific Fp1 promoter. Twelve transgenic lines were renumbered according to their expression levels of *CtFAD2-1*. *CtFAD2-1* (*filled circle*) and *CtFAD2-1Δ* (*open circle*)

A. thaliana. Based on kanamycin selection, 12 primary transgenic lines were generated from the transformations involving each plasmid. Kanamycin-resistant T_1 plants were grown to flowering and developing siliques containing T_2 immature embryos were harvested for further analysis. The gene expression patterns of *CtFAD2-1* and *CtFAD2-1Δ* were comparatively analysed by RT-qPCR. As shown in Fig. 7, in the majority of the 12 independently transformed *A. thaliana* lines, the average expression level of *CtFAD2-1* was significantly higher than *CtFAD2-1Δ* in developing *A. thaliana* siliques.

Perfect PCR markers for *ol* allele controlling the HO trait

The sequence polymorphism between *CtFAD2-1* and *CtFAD2-1Δ* was exploited to develop a highly efficient molecular marker for tracking the HO mutant *ol* allele. The coding regions of *CtFAD2-1* and *CtFAD2-1Δ* share 98 % nucleotide sequence identity. For *CtFAD2-1Δ*, in agreement with previous finding by Guan et al. (2012), the deletion of a single base pair "C" was identified at the position 606 bp downstream of the ATG initiation codon. This deletion causes a shift in the open reading frame that creates a stop codon and generates a putatively truncated protein of 245 amino acids in length. In addition, there are 22 nucleotide substitutions in S-317 *CtFAD2-1Δ* compared to SU *CtFAD2-1*. This is different from the earlier observation by Guan et al. (2012) that the single base pair "C" deletion was the sole sequence variation between *CtFAD2-1* and *CtFAD2-1Δ*. Interestingly, the nucleotide substitutions seem not to be randomly distributed within the 1,142-bp long putative coding region of *CtFAD2-1Δ* in that 13 nt of the 22 (63.6 %) substitutions occur within 123 bp downstream of the deletion site. Nevertheless, it is apparent that the single-nucleotide polymorphisms (SNPs) revealed in the coding region of *CtFAD2-1* and *CtFAD2-1Δ* may not be sufficient to design PCR-based molecular markers. However, the DNA sequence of a large intron situated in


HL-S1
HL-A1
HO-S1
HO-A1

◀ **Fig. 8** Alignment of DNA sequences of the 5′ UTR intron of *CtFAD2-1* derived from HL safflower SU (GenBank accession number KC886425) and *CtFAD2-1Δ* derived from HO genotype (GenBank accession number KC886424). Oligo primers designated for the amplification of genotype-specific fragment is *boxed* and orientation of the primer is indicated by *arrow*

the 5′ UTR of *CtFAD2-1* and *CtFAD2-1Δ,* which is 1,083-bp long in *CtFAD2-1*, and 1,144-bp long in *CtFAD2-1Δ*, was found to be highly divergent (Fig. 8). The two intron sequences share about 76.8 % sequence identity. Several short stretches of highly variable sequences between HL and HO genotypes enabled the designing of PCR primers specific for each of the two genes (Fig. 8). The gene-specific PCR products are shown in Fig. 9. The amplicon of the *CtFAD2-1Δ* intron was a 315-bp fragment that was present in all the five HO varieties including S-317, S-517, CW99-OL, LeSaf496 and Ciano-OL, while it was absent in the conventional HL safflower SU. The amplicon of the *CtFAD2-1* intron, 603 bp in length, was present in the conventional HL safflower SU, while absent in all five HO varieties tested. A 198-bp band derived from the *CtKASII* gene was present in all the safflower varieties tested, acting as a positive control to distinguish between PCR failures and absence of a target gene-specific band.

Discussion

The original HO trait with oleic acid content of up to 75 % of total fatty acids in safflower seed oil is controlled by a partially recessive allele *ol* at a single locus OL (Knowles and Bill 1964). The *ol* allele has been commercially exploited and new safflower oil with elevated oleic acid content is widely available for food and industrial uses. In the current study, we have explored the biochemical and molecular features of the *ol* allele and established perfect molecular markers for tracking the *ol* allele in both homozygous and heterozygous states.

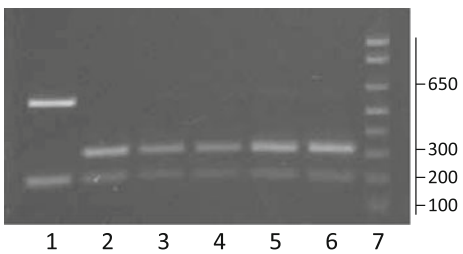


Fig. 9 Development of a perfect DNA marker for *ol* allele safflower varieties. A ~300-bp band is distinctive in all five high oleic genotypes including S-317, S-517, CW99-OL, Ciano-OL and LeSaf496, while it is absent in the wild-type SU. A 600-bp fragment is specific for wild-type SU. A 198-bp band derived from *CtKASII* gene is present in all the lines tested and is used as a positive control

The biochemical analysis of oleate desaturase activity as determined by TLC and autoradiography analyses clearly indicated that the microsomal oleate desaturase enzyme CtFAD2 was defective in S-317. Among the numerous FAD2 enzymes, *CtFAD2-1* was expressed strongly and exclusively in the developing embryos of the conventional HL safflower and, therefore, it likely plays an essential role in the biosynthesis of linoleic acid in developing seeds (Guan et al. 2012; Cao et al. 2013). This is direct evidence that *CtFAD2-1* is the site of the *ol* allele as proposed by Hamdan et al. (2012). When mutated, the *CtFAD2-1Δ* disrupts the biosynthesis of linoleic acid, leading to a buildup of oleic acid in the seeds, as has been shown in various other oilseed crops such as sunflower (*Helianthus annuus* L.), peanut (*Arachis hypogaea* L.) and soybean (*Glycine max* L.) (Falentin et al. 2007; Lacombe et al. 2009; Perez-Vich et al. 2002; Pham et al. 2010; Schuppert et al. 2006).

It is noticeable that some residual linoleic acid still exists in the seed oil of the *olol* genotype, despite *CtFAD2-1,* which makes a major contribution to the production of linoleic acid, being defective. It is likely that genetic redundancy amongst the *CtFAD2* genes, such as *CtFAD2-2* and/or some other *CtFAD2* genes, contributes to the accumulation of the residual linoleic acid in S-317. The chloroplast-localised oleate desaturase FAD6 could also be a contributor to the residual linoleic acid. However, its role is likely minor, if any, as safflower embryo is white and the number of mature and fully functional chloroplasts in developing embryos are relatively low (Martínez-Rivas et al. 2001).

Additional germplasm with even higher level of oleic acid than the *olol* genotype has also been reported (Fernández-Martinez et al. 1993; Mündel and Bergman 2009). Oleic acid content up to 89 % in safflower seed oil was reported by Fernandez-Martinez et al. (1993) in the germplasm accession PI401472 originally sourced from Bangladesh. The Montola series developed by safflower breeders in the USA (Mündel and Bergman 2009) contains more than 80 % oleic acid, clearly beyond the upper level of oleic acid of the *olol* genotype. An EMS mutant S901 containing up to 90 % oleic acid in its seed oil has also been obtained (Weiske 1999). Crossing and segregation analysis of the HO and very high oleic lines suggested that the very high oleic acid content is generated by the combination of *ol* allele and modifying gene(s) with a small positive effect on oleic acid (Hamdan et al. 2009, 2012).

The coding regions of *CtFAD2-1Δ* differed from that of the conventional HL safflower *CtFAD2-1* in one nucleotide deletion and 22 nucleotide substitutions in S-317 *CtFAD2-1Δ*. The single-nucleotide deletion in the middle of the coding region in *CtFAD2-1Δ* caused a shift in the ORF that created a PTC and a non-functional microsomal oleate

desaturase. A single base pair insertion or deletion (Indel) in the coding region of *FAD2* leads to the formation of PTC in several oilseed crops. For example, in *G. max*, a single-nucleotide deletion 232 bases downstream of the initiation codon ATG of *GmFAD2-1a* was found in an HO mutant resulting from X-irradiation (Anai et al. 2008). This deletion resulted in a frame-shift mutation and led to increased oleic acid content from 27 % in wild type to 47 % in the HO mutant. A PTC was created by EMS mutagenesis in the seed-expressed *FAD2* gene in *Camelina sativa*, which caused a moderate increase of oleic acid from 17 % in the wild type to 27 % in the mutant (Kang et al. 2011). In allotetraploid (AABB) *A. hypogaea*, the homoeologous *FAD2-A* and *FAD2-B* sequences share a high degree (99 %) of identity with a few polymorphic SNPs among normal and HO lines. In the HO variety with 80 % oleic acid, a missense mutation (G448A) was identified in *FAD2-A* and a single base pair insertion (442insA) in *FAD2-B* producing a frame shift and downstream PTC (Lopez et al. 2000). Both of these mutations (G448A and 442insA) are necessary to produce an HO peanut phenotype (Jung et al. 2000).

Interestingly, the expression of *CtFAD2-1Δ* in HO genotypes was drastically reduced in developing seeds, compared to the conventional HL safflower indicating specific down-regulation of *CtFAD2-1Δ* operating in the HO genotypes (Guan et al. 2012). In eukaryotes, gene silencing involves 21 and 24-nt siRNA produced from double-strand RNA resulting from transcription of antisense or hairpin RNA (Brodersen and Voinnet 2006). We carried out Northern blot analysis of small RNAs that indicated the absence of *CtFAD2-1Δ* siRNA in HO developing embryos. This was also verified by deep sequencing of small RNAs isolated from developing embryos. Only a negligible amount of small RNA corresponding to *CtFAD2-1Δ* could be detected. This was also the case for *CtFAD2-1* in the conventional HL safflower. Further, previous research indicated that the *ol* allele is semi-recessive, which is distinct from the small interference RNA (siRNA)-mediated gene silencing system in which the HO trait would act as the dominant phenotype as is the case in the HO *H. annuus* genotype Pervenets (Lacombe et al. 2009).

We therefore propose that the specific attenuation of *CtFAD2-1* transcripts in the *olol* genotypes is governed by nonsense-mediated mRNA degradation (NMD), an mRNA surveillance pathway involved in the degradation of aberrant mRNAs that contain a PTC resulting from mutation, transcriptional errors or alternative splicing. NMD protects eukaryotic cells from potentially harmful effects of truncated proteins that might have dominant negative effects on functional homologues. While only sporadically reported in plants, NMD is universally present in eukaryotes and has been extensively studied in yeast and mammals (Culbertson 1999; Conti and Izaurralde 2005; Trcek et al. 2013). One of the most defining features of NMD substrates is a PTC that gives rise to a long 3′ UTR that is sensed in a translation-dependent manner by the evolutionarily conserved RNA helicase UPF1 (Hogg and Goff 2010). In yeast, the presence of a downstream instability element relative to the PTC was found to be required for NMD (Culbertson 1999). In mammals, splicing of at least one intron is required for NMD and occurs only if the PTC is more than 50 nucleotides upstream of the last intron (Nagy and Maquat 1998). In plants, several studies have shown that PTC-harbouring genes both with and without an intron can trigger NMD. The NMD phenomenon was initially reported in the *G. max* Kunitz trypsin inhibitor gene (Kti3), phytohemagglutinin gene (PHA) from common bean (*Phaseolus vulgaris* L.) (Jofuku et al. 1989; Voelker et al. 1990) and pea (*Pisum sativa* L.) ferredoxin gene (FED1) (Dickey et al. 1994), none of which contain an intron. In contrast, the splicing of an intron upstream of the PTC in the rice (*Oryza sativa* L.) *waxy* gene affected the efficiency of NMD (Isshiki et al. 2001). *CtFAD2-1Δ* contains a reasonably large intron of approximately 1.1 kb in length in the 5′ UTR that is more than 600 bp upstream of the PTC. We were able to reproduce the NMD phenomenon by expressing the coding region of the *CtFAD2-1Δ* without its 5′ UTR intron in both *N. benthamiana* leaves and transgenic *A. thaliana* seeds. This suggests that an intron located at 5′ UTR is not required in the activation and maintenance of NMD in HO safflower. However, the expression of the intron-less *CtFAD2-1Δ* in yeast failed to generate NMD, indicating that a mere PTC present in the *CtFAD2-1Δ* coding region was not sufficient to induce NMD in transgenic yeast. The fact that the *CtFAD2-1Δ* transcript containing a PTC was able to be detected in the HO varieties of safflower indicates that not all these defective transcripts have been subjected to NMD degradation. This is conceivable since NMD is not 100 % efficient, but generally reduces the abundance of nonsense-containing mRNAs to ~5–25 % of the normal level (Isken and Maquat 2007). Whether an RNA molecule will be degraded or not is determined by competition between cytoplasmic poly(A)-binding protein 1 and UPF1 for binding to translation release factors at the terminating ribosome (Silva et al. 2008). This interaction may be modulated by numerous structural features within the mRNA. In mammals, the presence of splicing boundaries downstream of a stop codon acts as a strong enhancer of NMD (Nicholson et al. 2010). In plants, NMD is activated by either a long 3′ UTR or by a premature termination codon in close proximity to an exon junction (Kertesz et al. 2006). By introducing PTCs at various positions in the trypsin proteinase inhibitor (TPI) gene of *Nicotiana attenuate*, it was observed that

NMD efficiency was correlated with PTC location (Wu et al. 2007).

We have proposed that NMD is the molecular basis of the high oleic genotype of *olol* mutant based on the following observations. Firstly, as the result of single-nucleotide deletion in the middle of its coding region, *CtFAD2-1Δ* contains an extra long 3′ UTR that is the prerequisite for the NMD phenomenon. Secondly, we have ruled out the possibility of siRNA or microRNA-mediated RNA degradation because of the lack of high-level presence of siRNA corresponding to *CtFAD2-1Δ*. Thirdly, we have successfully re-produced the NMD phenomenon in *N. benthamiana* leaves using an *A. tumefaciens* infiltration transient assay of *CtFAD2-1Δ*. Transgenic *A. thaliana* plants expressing the PTC-containing *CtFAD2-1Δ* without the 5′ UTR intron in a seed-specific manner also showed more highly attenuated mRNA levels than did the plants expressing the wild-type *CtFAD2-1* gene.

Conventional breeding selects HO genotypes based on the analysis of fatty acid composition on safflower half seeds. This phenotype-based method is time consuming and environmentally sensitive as temperature influences the FAD2 oleate desaturase activity. Further, since the *ol* allele controlling the HO trait is partially recessive, the heterozygote is not clearly distinguishable from the wild-type homozygote. This fact makes it difficult to select plants carrying the *ol* allele in backcrossing programmes focusing on the HO trait. Therefore, the use of molecular marker-assisted selection can overcome such a limitation and greatly support the introgression of the *ol* allele into elite safflower lines. Recently, an SSR-based molecular marker for the OL locus has been described (Hamdan et al. 2012). In the current study, we have developed a dominant Indel marker that is not only diagnostic for the OL locus, but also able to resolve the *Ol* and *ol* alleles.

Using a pair of oligo primers that are based on the specific regions of the *CtFAD2-1Δ* intron sequence that lacks homology with the conventional HL safflower *CtFAD2-1* intron, the PCR amplification products are produced only for genotypes carrying the *ol* allele, in either homozygous or heterozygous states. Such a perfect molecular marker is completely linked to the HO mutation and is able to detect the *ol* allele regardless of genetic background. Because such a molecular marker is dominant, it does not allow the distinction between homozygous and heterozygous genotypes at the OL locus. We have subsequently used the HL-specific PCR marker in both wild-type and heterozygous HO lines. It is anticipated that both HO-specific and HL-specific markers would be present in the heterozygous (*Olol*) plants simultaneously. This combination of both *Ol* and *ol* molecular markers can be useful in breeding programmes, as they will allow for rapid screening and early detection of not only the genotypes carrying the *ol* allele, but also the progenies with *olol* homozygotes.

In conclusion, this research reveals the molecular mechanism of the HO trait generated by the *olol* mutation originally discovered by Knowles and Bill (1964). The combination of in vitro microsomal FAD2 enzyme assays and gene expression studies indicated the seed-specific *CtFAD2-1* as the OL locus. A single-nucleotide deletion in the coding region of *CtFAD2-1Δ* as previously described (Guan et al. 2012) possibly triggers an NMD phenomenon leading to highly attenuated transcript levels of *CtFAD2-1Δ* in the HO varieties carrying the *olol* mutation. We have explored the NMD mechanism by ectopic expression in yeast, *N. benthamiana* leaves and *A. thaliana* seeds. We have developed molecular marker assays that allow for the selection of the desired *ol* allele, differentiating its presence in either homozygous or heterozygous states. It is anticipated that the development of such a perfect molecular marker will make it possible for safflower breeders to quickly incorporate the HO trait in their breeding programme.

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References

Anai T, Yamada T, Hideshima R, Kinoshita T, Rahman S, Takagi Y (2008) Two high-oleic-acid soybean mutants, M23 and KK21, have disrupted microsomal omega-6 fatty acid desaturase, encoded by *GmFAD2-1a*. Breeding Sci 58:447–452

Bent A, Clough S (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735–743

Brodersen P, Voinnet O (2006) The diversity of RNA silencing pathways in plants. Trends Genet 22:268–280

Cao S, Zhou X-R, Wood C, Green A, Singh S, Liu L, Liu Q (2013) A large and functionally diverse family of Fad2 genes in safflower (*Carthamus tinctorius* L.). BMC Plant Biol 13:5

Conti E, Izaurralde E (2005) Nonsense-mediated mRNA decay: molecular insights and mechanistic variations across species. Curr Opin Cell Biol 17:316–325

Coutu C, Brandle J, Brown D, Brown K, Miki B, Simmonds J, Hegedus D (2007) pORE: a modular binary vector series suited for both monocot and dicot plant transformation. Transgenic Res 16:771–781

Crandall L (2002) Bioplastics: a burgeoning industry. INFORM 13: 626–628

Culbertson M (1999) RNA surveillance. Unforeseen consequences for gene expression, inherited genetic disorders and cancer. Trends Genet 15:74–80

Dahlqvist A, Stahl U, Lenman M, Banas A, Lee M, Sandager L, Ronne H, Stymne S (2000) Phospholipid:diacylglycerol acyltransferase: an enzyme that catalyzes the acyl-CoA-independent formation of triacylglycerol in yeast and plants. Proc Natl Acad Sci USA 97:6487–6492

Dickey LF, Nguyen TT, Allen GC, Thompson WF (1994) Light modulation of ferredoxin mRNA abundance requires an open reading frame. Plant Cell 6:1171–1176

Falentin C, Bregeon M-O, Lucas M, Deschamps F, Leprince M-T, Fournier R-D, Renard M (2007) Identification of *fad2* mutants and development of allele-specific markers for high oleic acid content in rapeseed (*Brassica napus* L.).In: Proceedings of the 12th International Rapeseed Congress 2:117–119

Fernández-Martinez J, Rio M, Haro A (1993) Survey of safflower (*Carthamus tinctorius* L.) germplasm for variants in fatty acid composition and other seed characters. Euphytica 69:115–122

Guan LL, Xu YW, Wang YB, Chen L, Shao JF, Wu W (2012) Isolation and characterization of a temperature-regulated microsomal oleate desaturase gene (*ctFAD2-1*) from safflower (*Carthamus tinctorius* L.). Plant Mol Biol Rep 30:391–402

Hamdan Y, Pérez-Vich B, Velasco L, Fernández-Martínez J (2009) Inheritance of high oleic acid content in safflower. Euphytica 168:61–69

Hamdan YAS, Garcia-Moreno MJ, Fernandez-Martinez JM, Velasco L, Perez-Vich BP (2012) Mapping of major and modifying genes for high oleic acid content in safflower. Mol Breed 30: 1279–1293

Hill K (2000) Fats and oils as oleochemical raw materials. Pure and Appl Chem 72:1255–1264

Hogg J, Goff S (2010) Upf1 senses 3′UTR length to potentiate mRNA decay. Cell 143:379–389

Isken O, Maquat L (2007) Quality control of eukaryotic mRNA: safeguarding cells from abnormal mRNA function. Gene Dev 21:1833–1856

Isshiki M, Yamamoto Y, Satoh H, Shimamoto K (2001) Nonsense-mediated decay of mutant waxy mRNA in rice. Plant Physiol 125:1388–1395

Jofuku K, Schipper R, Goldberg R (1989) A frameshift mutation prevents Kunitz trypsin inhibitor mRNA accumulation in soybean embryos. Plant Cell 1:427–435

Jung S, Swift D, Sengoku E, Patel M, Teulé F, Powell G, Moore K, Abbott A (2000) The high oleate trait in the cultivated peanut [*Arachis hypogaea* L.]. I. Isolation and characterization of two genes encoding microsomal oleol-PC desaturases. Mol Gen Genet 263:796–805

Kang JL, Snapp AR, Lu CF (2011) Identification of three genes encoding microsomal oleate desaturases (FAD2) from the oilseed crop *Camelina sativa*. Plant Physiol Biochem 49: 223–229

Kertesz S, Kerenyi Z, Merai Z, Bartos I, Palfy T, Barta E, Silhavy D (2006) Both introns and long 3 '-UTRs operate as cis-acting elements to trigger nonsense-mediated decay in plants. Nucleic Acids Res 34:6147–6157

Kinney AJ, Clemente TE (2005) Modifying soybean oil for enhanced performance in biodiesel blends. Fuel Process Technol 86:1137–1147

Knowles PF (1989) Safflower. In: Robbelen G, Downey RK, Ashri A (eds) Oil crops of the world: their breeding and utilization. McGraw-Hill, New York, pp 363–374

Knowles PF, Bill AB (1964) Inheritance of fatty acid content in the seed oil of a safflower introduction from Iran. Crop Sci 4: 406–409

Lacombe S, Souyris I, Berville AJ (2009) An insertion of oleate desaturase homologous sequence silences via siRNA the functional gene leading to high oleic acid content in sunflower seed oil. Mol Genet Genomics 281:43–54

Lopez Y, Nadaf HL, Smith OD, Connell JP, Reddy AS, Fritzf AK (2000) Isolation and characterization of the Δ12-fatty acid desaturase in peanut (*Arachis hypogaea* L.) and search for polymorphisms for the high oleate trait in Spanish market-type lines. Theor Appl Genet 101:1131–1138

Martínez-Rivas JM, Sperling P, Lühs W, Heinz E (2001) Spatial and temporal regulation of three different microsomal oleate desaturase genes (FAD2) from normal-type and high-oleic varieties of sunflower (*Helianthus annuus* L.). Mol Breeding 8:159–168

Mündel HH, Bergman JW (2009) Safflower. In: Vollmann J, Rajcan I (eds) Oil Crops, Handbook of Plant Breeding 4. Springer-Verlag, Berlin, pp 423–447

Nagy E, Maquat LE (1998) A rule for termination-codon position within intron-containing genes: when nonsense affects RNA abundance. Trends Biochem Sci 23:198–199

Nicholson P, Yepiskoposyan H, Metze S, Orozco RZ, Kleinschmidt N, Muhlemann O (2010) Nonsense-mediated mRNA decay in human cells: mechanistic insights, functions beyond quality control and the double-life of NMD factors. Cell Mol Life Sci 67:677–700

Ohlrogge J, Browse J (1995) Lipid biosynthesis. Plant Cell 7:957–970

Ohlrogge JB, Jaworski JG (1997) Regulation of fatty acid synthesis. Annu Rev Plant Physiol Plant Mol Biol 48:109–136

Perez-Vich B, Fernandez-Martinez JM, Grondona M, Knapp SJ, Berry S (2002) Stearoyl-ACP and oleoyl-PC desaturase genes cosegregate with quantitative trait loci underlying high stearic and high oleic acid mutant phenotypes in sunflower. Theor Appl Genet 104:338–349

Pham AT, Lee JD, Shannon JG, Bilyeu KD (2010) Mutant alleles of FAD2-1A and FAD2-1B combine to produce soybeans with the high oleic acid seed oil trait. BMC Plant Biol 10:195

Schuppert GF, Tang S, Slabaugh MB, Knapp SJ (2006) The sunflower high-oleic mutant Ol carries variable tandem repeats of FAD2-1, a seed-specific oleoyl-phosphatidyl choline desaturase. Mol Breed 17:241–256

Shanklin J, Cahoon EB (1998) Desaturation and related modifications of fatty acids. Annu Rev Plant Physiol Plant Mol Biol 49: 611–641

Silva AL, Ribeiro P, Inacio A, Liebhaber SA, Romao L (2008) Proximity of the poly(A)-binding protein to a premature termination codon inhibits mammalian nonsense-mediated mRNA decay. RNA 14:563–576

Stalberg K, Ellerstrom M, Josefsson LG, Rask L (1993) Deletion analysis of a 2S seed storage protein promoter of *Brassica napus* in transgenic tobacco. Plant Mol Biol 23:671–683

Stymne S, Appelqvist L-A (1978) The biosynthesis of linoleate from oleoyl-coA via oleoyl-phosphatidylcholine in microsomes of developing safflower seeds. Eur J Biochem 90:223–229

Trcek T, Sato H, Singer RH, Maquat LE (2013) Temporal and spatial characterization of nonsense-mediated mRNA decay. Genes Dev 27:541–551

Velasco L, Fernandez-Martinez J (2001) Breeding for oil quality in safflower. In: Proceedings of the 5th Int safflower conference Bergman JW and HH Mundel (eds). Sidney, MO, USA. pp. 133–137

Voelker TA, Moreno J, Chrispeels MJ (1990) Expression analysis of a pseudogene in transgenic tobacco: a frameshift mutation prevents mRNA accumulation. Plant Cell 2:255–261

Voinnet O, Rivas S, Mestre P, Baulcombe D (2003) An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. Plant J 33:949–956

Weiske AC (1999) Safflower products with very high levels of unsaturated fatty acids. US patent US5912416

Wood CC, Petrie JR, Shrestha P, Mansour MP, Nichols PD, Green AG, Singh SP (2009) A leaf-based assay using interchangeable

design principles to rapidly assemble multistep recombinant pathways. Plant Biotechnol J 7:914–924

Wu JQ, Kang JH, Hettenhausen C, Baldwin IT (2007) Nonsense-mediated mRNA decay (NMD) silences the accumulation of aberrant trypsin proteinase inhibitor mRNA in *Nicotiana attenuata*. Plant J 51:693–706

Zhou X-R, Singh SP, Liu Q, Green AG (2006) Combined transgenic expression of Δ12-desaturase and Δ12-epoxygenase in high linoleic acid seeds leads to increased accumulation of vernolic acid. Funct Plant Biol 33:585–592

Zhou X-R, Green AG, Singh SP (2011) *Caenorhabditis elegans* Δ12-desaturase FAT-2 is a bifunctional desaturase able to desaturate a diverse range of fatty acid substrates at the Δ12 and Δ15 positions. J Biol Chem 286:43644–43650