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ORIGINAL PAPER

An insertion of oleate desaturase homologous sequence silences via siRNA the functional gene leading to high oleic acid content in sunflower seed oil

Séverine Lacombe · Irénée Souyris · André J. Bervillé

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Abstract Classical sunflower varieties display a high linoleic acid content in their seeds [low oleic (LO) varieties] whereas genotypes carrying the Pervenets mutation display an increased oleic acid content of above 83% [high oleic (HO) varieties]. Despite the advantage in health terms of oleic acid, the nature of the mutation was still unknown. Previous work reported that HO genotypes carried a specific oleate desaturase (OD) allele. This enzyme catalyses the desaturation of oleic acid into linoleic acid. The present work demonstrates that this allele is organised in two parts: the first section present in both HO and LO genotypes carries a normal OD gene, the second section is specific to HO genotypes and carries OD duplications. The study of mRNA accumulation in LO and HO seeds revealed that the mutation is dominant and induces an OD mRNA down-regulation. Furthermore, OD small interfering RNA, characteristic of gene silencing, accumulated specifically in HO seeds. Considered together, these observations show that the mutation is associated with OD duplications leading to gene silencing of the OD gene and consequently, to oleic acid accumulation. This finding allowed the development of molecular markers characterising the mutation that can be used in breeding programmes to facilitate the selection of HO genotypes.

Keywords High oleic acid oil · Pervenets mutation · Sunflower · PTGS

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S. Lacombe
Monsanto SAS, Croix de Pardies, BP 21,
40305 Peyrehorade Cedex, France

I. Souyris · A. J. Bervillé (✉)
Ressources Génétiques du tournesol, UMR-DIAPC,
INRA, 2 Place P Viala, Bât 33, 34060 Montpellier Cedex 1, France
e-mail: Andre.Berville@supagro.inra.fr

Present Address:
S. Lacombe
UMR UP-IRD-CNRS 5096, Centre IRD, 911 Av Agropolis,
BP 94501, 34394 Montpellier Cedex 5, France

Introduction

Vegetable oils are composed of triacylglycerol carrying saturated, mono-unsaturated and poly-unsaturated fatty acids. The first desaturation of stearic acid (18:0) into oleic acid (18:1) is catalysed by the stearoyl-ACP desaturase (stearate desaturase). The oleoyl-PC desaturase (oleate desaturase, OD) catalyses the desaturation of oleic acid (18:1) into linoleic acid (18:2). The linoleoyl-PC desaturase catalyses the third desaturation of linoleic acid (18:2) into linolenic acid (18:3) (Ohlrogge and Browse 1995; Somerville and Browse 1996; Schwartzbeck et al. 2001). Complementary DNAs corresponding to genes encoding for these enzymes were first isolated in *Arabidopsis thaliana* (for review see Somerville and Browse 1996). These cDNAs have also been isolated for most of the oil seed crops including sunflower (*Helianthus annuus* L.) (Hongtrakul et al. 1998a,b; Martinez-Rivas et al. 2001).

The nutritional qualities of vegetable oils depend mainly on their fatty acid composition. Monounsaturated oleic acid (18:1) only reduces cholesterol's atherogenic fraction level and consequently, preserves the cholesterol anti-atherogenic fraction, which is beneficial for cardiovascular disease prevention. Thus, diets containing vegetable oil with

high oleic acid content (OAC) have been reported to be the most effective to prevent cardiovascular diseases (Delplanque et al. 1997, 2000; Broun et al. 1999). Consequently, the increase of OAC has become one of the major goals to improve vegetable oil quality. Each different species displays in nature a specific spectrum in fatty acid composition. Breeders exploit the natural genetic diversity to modify the fatty acids ratio in order to fulfil objectives defined by the need to have a healthy diet and by industrial application requirements. In addition, mutagenesis has also been used to select new varieties with increased OAC for the major oil seed crops such as rapeseed (*Brassica napus* L.) and peanut (*Arachis hypogaea* L.) (Schierholt et al. 2001; Jung et al. 2000a,b; Patel et al. 2004). Transgenic strategies based on posttranscriptional gene silencing have also been used to increase OAC in soybean [*Glycine max* (L.) Merr.], rapeseed and cotton (*Gossypium hirsutum* L.) (Kinney 1996; Stoutjesdijk et al. 2000; Liu et al. 2002).

Sunflower oil is naturally rich in linoleic acid (55–70%) and consequently poor in oleic acid (20–25%). Classic varieties are qualified as low oleic (LO). Up to the 1970s, mutagenesis programmes were conducted to produce varieties with an increased OAC compared to the traditional LO varieties. The Pervenets sunflower population was obtained by chemical mutagenesis using dimethyl sulphate (DMS) on the population Vniimk 8931 and it displayed OAC in seed oil higher than 65% (Soldatov 1976). This Pervenets population was then used in breeding programmes to obtain varieties with OAC higher than 80% [high oleic (HO)]. This fatty acid composition modification of oil is specifically located in embryo tissues (Garcés et al. 1989). Due to the increased interest in health terms of oleic acid and the similar agronomic performance of the HO compared to the traditional LO varieties, HO varieties are now widely used around the world covering about 1.2 million ha (CETIOM 2003). In these varieties, there is a reduction of the OD-mRNA accumulation compared to the LO genotypes leading to a decrease of OD activity in the seeds during the lipid accumulation (Garcés and Mancha 1989, 1991; Kabbaj et al. 1996; Hongtrakul et al. 1998b). Using a candidate gene approach in a diversity analysis study, we found linkage disequilibrium between the Pervenets mutation and a HO specific OD allele. This allele was not found in any of the LO genotypes tested (Lacombe and Bervillé 2001). Genetic studies performed on F2 and recombinant inbred lines populations revealed that this linkage disequilibrium was due to a close genetic linkage between the Pervenets mutation and the HO specific OD allele (Hongtrakul et al. 1998b; Lacombe et al. 2001; Lacombe and Bervillé 2001). Recently, Schuppert et al. (2006) studied the HO specific OD allele using PCR based approaches and identified part of this allele. They showed that it carries a full and an incomplete duplication of the OD gene. This result allowed them to develop dominant markers linked to the mutation but due to the lack of DNA polymorphism in the region tested, no codominant markers were found between wild type and mutant oilseed inbred lines. However, all these approaches were not able to determine whether the HO specific OD allele carries or is genetically linked to the Pervenets mutation. Consequently, the nature of the mutation is still unknown.

The work reported here was carried out to identify the nature of the Pervenets mutation. Based on a detailed study of the HO specific OD allele, we proposed and experimentally validated that the Pervenets mutation corresponds to the OD duplication and induces gene silencing on the normal OD gene. Furthermore, this work allowed the identification of molecular markers corresponding to the Pervenets mutation. This type of molecular markers can be useful tools in breeding programmes, as they will allow for a rapid screening and early detection of genotypes carrying the Pervenets mutation and for the use of marker-assisted selection to introduce the HO trait into breeding programmes.

Materials and methods

Plant material

Several lines and varieties were used to supply either genomic DNA for genotyping or RNA for northern studies (Table 1). This set of genotypes includes Pervenets and Vniimk 8931 corresponding to the population used for the Pervenets mutagenic treatment. The F2 segregating population used in this study is the same as the one reported by Lacombe et al. (2001). Briefly, the LO line *BD40713* (Monsanto) was used as a female in a cross with the HO line *BE78079* (Monsanto). One F1 plant was self-fertilised to produce the F2 progenies composed of 107 plants. To determine oil composition half a cotyledon from each seed was analysed before germination. Each plant was also genotyped with the OD cDNA probe and with PCR markers. For mRNA and small RNA accumulation studies in immature seeds, self-progenies and controlled crosses were obtained using a protected paper bag set up a few days before flowering. Crosses were then performed by transferring pollen from the male to the female under the paper bag to prevent illicit fertilisation.

Probes and hybridisation

The stearate desaturase probe corresponds to a 5′ fragment of 780 bp cloned and sequenced by Kabbaj et al. (1996). This nt sequence corresponded to the GenBank accession number U91340 (Hongtrakul et al. 1998a). The OD-cDNA

Table 1 List of plant material used with their high oleic (HO)/low oleic (LO) phenotype and their country origin or company provider

Genotype	Phenotype	Origin	Genotyping[a]	Northern[a]
BE78079	HO	Monsanto	+	+
BD40713	LO	Monsanto	+	+
HOC 97	HO	Monsanto	+	+
HOC	HO	Monsanto	+	+
HOC 98	HO	Monsanto	+	+
BD 70080	LO	Monsanto	+	+
BE 78078	HO	Monsanto	+	+
BD 70032	LO	Monsanto	+	+
BE 73201	HO	Monsanto	+	+
RHA345	HO	USDA	+	+
83 HR 4	LO	INRA	+	+
90 R 19	LO	INRA	+	+
63	LO	INRA	+	+
Ha Ol 9	HO	CSIC	+	+
Santiago	LO	Novartis	+	+
Trisun 870	HO	Mycogen	+	+
Olbaril	HO	Pioneer	+	+
LO1	LO	Monsanto	+	–
LO2	LO	Monsanto	+	–
LO3	LO	Monsanto	+	–
LO4	LO	Monsanto	+	–
LO5	LO	Monsanto	+	–
LO11	LO	Monsanto	+	–
VNIIMK 8931	LO	Russia	+	–
HO1	HO	Monsanto	+	–
HO2	HO	Monsanto	+	–
HO5	HO	Monsanto	+	–
HO9	HO	Monsanto	+	–
HO19	HO	Monsanto	+	–
HO22	HO	Monsanto	+	–
HO24	HO	Monsanto	+	–
HO26	HO	Monsanto	+	–
HO37	HO	Monsanto	+	–
HO39	HO	Monsanto	+	–
OPA1	HO	INRA	+	–
OPA2	HO	INRA	+	–
R-OL1	HO	CSIC	+	–
RHA346	HO	USDA	+	–
RHA347	HO	USDA	+	–
LG26	HO	Russia	+	–
Pervenets	HO	Russia	+	–

[a] Genotypes used for both genotyping and northern studies (+/+) or only for genotyping studies (+/−)

used as a probe corresponds to the full length cDNA referenced in GenBank as accession number U91341. These fragments were labelled with [^{32}P] dCTP by random primer extension (Sambrook et al. 1989). Hybridisation and washes were performed under high stringency (i.e., hybridisation in 0.25 M sodium phosphate, 7% SDS at 65°C for 16 h, three washes in 2× SSC and one with 0.3% SSC, each at 65°C for 20 min).

Southern analysis

DNA preparation, restriction analyses, Southern blotting transfers, and hybridisation, were done according to Gentzbittel et al. (1995). DNAs from HO and LO lines, hybrids and population genotypes were restricted either by *Eco*RI and/or *Hin*dIII and the Southern transfers of the gels were probed with the OD-cDNA as described above.

Genomic library

A lambda FIXII (Stratagene) genomic library was constructed from 2-week-old green seedlings DNA from the cultivar *RHA345* (USDA-Fargo) restricted with *Sau*3A following the provider recommendations. Two million clones were screened with the OD cDNA probe under high stringency as described above to select six genomic clones with strong hybridisation signals.

PCR amplifications

The sequence of primers used for PCR amplifications are listed in Table 2. F1 to F8 and R1 to R8 primers were designed on OD cDNA (U91341). F1_R1 to F8_R8 primer pairs were chosen to amplify overlapping fragments of about 240 bp covering all OD cDNA sequence. SSR-F and SSR-R primers were designed to amplify the SSR motif located in the intron of the OD gene. $F_{sp\text{–}a}$ and $F_{sp\text{-}b}$ were designed on the spacer fragment between the OD gene and the HO specific part of the OD-HO allele.

Long PCR amplifications were performed with the Expend Long Template PCR System (Roche Applied Science) according to the provider instructions. Amplification products were ran on a 1% agarose gel as described previously to detect polymorphism between *83HR4* (LO) and *RHA345* (HO) genotypes. The $F_{sp\text{-}a}$_R7 PCR fragment obtained using *RHA345* DNA was cloned into pGEM-T Easy Vector (Promega) following the manufacturer's instructions and sequenced.

Amplifications of the SSR motives were performed with SSR-F_SSR-R primer pair in a 30 µl reaction mixture containing 40 ng template DNA, 1 U of *Taq* DNA polymerase, 1× reaction buffer (both from Sigma), 200 µM of dNTPs; 1 µM of each primer. SSR amplifications were carried out with an initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 50°C for 1 min and 72°C for 1 min and a final 5 min extension at 72°C. Amplification products were loaded onto 6% denaturing polyacrylamide

Table 2 List of primers used with the name and sequence of each primer

Primers	Sequence (5′–3′)
F1	ACCCTAAGCCTCTGTCGCTC
R1	AGCGGTTATGGTGAGGTCAG
F2	TCGCTAACCCGTTCGTTCTC
R2	ACAAAGCCCACAGTGTCGTC
F3	CCATGCGTTTAGTGATTATC
R3	GAGTGAGAGTGACGAACATAC
F4	TCCCGTGGTACTCGAAATA
R4	GCCATAGCAACACGATAAAG
F5	AGCCCTATGTACAATGAACGT
R5	AATGCTCCCTTTAACCATTC
F6	TCTTCAACATACTCACCCTG
R6	TGTCAAACCGATAATACTCC
F7	ATGCGATGGAAGCACAGAAG
R7	AACCAGACCCAGACCCAAAC
F8	AGATGATGAAGGGAAAGGAG
R8	TCTAAAACACACCCAACACG
$F_{sp\text{-}a}$	CAAACCACCACCCACTAAC
$F_{sp\text{-}b}$	AGAAGAGGGAGGTGTGAAG
SSR-F	TTGGAGTTCGGTTTATTTAT
SRR-R	TTAGTAAACGAGCCTGAAC

gels containing 7.5 M urea, 6% acrylamide and 1× TBE buffer (Tris–HCl 90 mM pH 8, boric acid 90 mM, EDTA pH 8 2 mM). Gels were run in a 1× TBE buffer for 90 min at 60 V. SSR amplifications were visualised by silver staining with a commercial kit from Promega.

The PCR reactions performed to amplify the HO specific part of the OD-HO allele were carried out using the same reaction mixture described above. PCR amplifications were performed with an initial denaturation at 94°C for 4 min followed by 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min and a final 5 min extension at 72°C. PCR products were separated by electrophoresis on 1% agarose gel and visualised under UV light after EtBr staining of the gel.

RNA gel blot analyses

Total RNA was extracted from immature seeds, 10 to 24 days after pollination (DAP) using TriReagent (Sigma) according to the manufacturer's instructions. Northern blots for mRNA studies were performed according to Sambrook et al. (1989). Northern blots were hybridised sequentially with the stearate desaturase and with the OD-cDNA probes as described above. Northern blots for the small RNA study were performed as described previously (Herr et al. 2005). Briefly, between 50 and 80 µg of total RNA from *RHA345* and *83HR4* immature seeds was separated in a 15% polyacrylamide gel and transferred to a Zeta-Probe GT nylon membrane (Bio-Rad). Membranes were cross-linked under UV illumination and hybridised overnight at 40°C with a ^{32}P labelled T7-OD RNA probe in PerfectHyb buffer (Sigma). The T7-OD RNA probe was generated by T7 transcription (Promega) of a 1,176-bp fragment that was amplified with a forward OD primer carrying a T7 extension (5′-ATAATACGACTCACTATAGGGTCGCTAACCCGTTCGTTCTC-3′) and a reverse OD primer (5′-TCTAAAACACACCCAACACG-3′). As a loading control, each blot was hybridised with labelled U6 oligonucleotide (5′-GCAGGGGCCATG-CTAATCTTCTCTGTATCGT-3’) designed on the *Arabidopsis thaliana* U6-1 snRNA gene (accession number X52527). Hybridisations were washed once at 50°C with 2× SSC, 0.1% SDS for 10 min and exposed to a phosphor screen (Amersham Bioscience).

Oleic acid content measurement

For each plant head used for northern studies, oil composition measurements were performed on mature seeds using the gas chromatography method (Conte et al. 1989). Routine OAC measurements were also performed using a refractometer (OPL Sopelem, Paris) and a range of OAC for oil samples. The concentration in oleic acid was estimated by comparison of a sample with reference OAC concentrations.

Accession numbers

Sequences reported are related to the patent WO2005 1060022 and were deposited in the GenBank data library under accession numbers: CS201179 to CS201197 (Bervillé et al. 2005).

Results

Physical organisation of the OD genomic region in HO and LO genotypes

Physical maps of the HO specific and the LO-OD alleles (OD-HO and OD-LO alleles, respectively) were established according to the *Eco*RI, *Hin*dIII, *Eco*RI/*Hin*dIII RFLP profiles revealed with the OD probe in a set of 27 HO and 14 LO genotypes (Table 1, Fig. 1a). As previously reported (Lacombe and Bervillé 2001), all the LO genotypes including Vniimk 8931 corresponding to the population used for mutagenic treatment, displayed the same profiles characteristic of the OD-LO allele whereas the HO genotypes carried the same OD-HO allele. Using *Hin*dIII, the OD-LO allele displayed an 8-kb fragment whereas, for the OD-HO allele, the hybridised fragment size was over 16 kb. This suggests

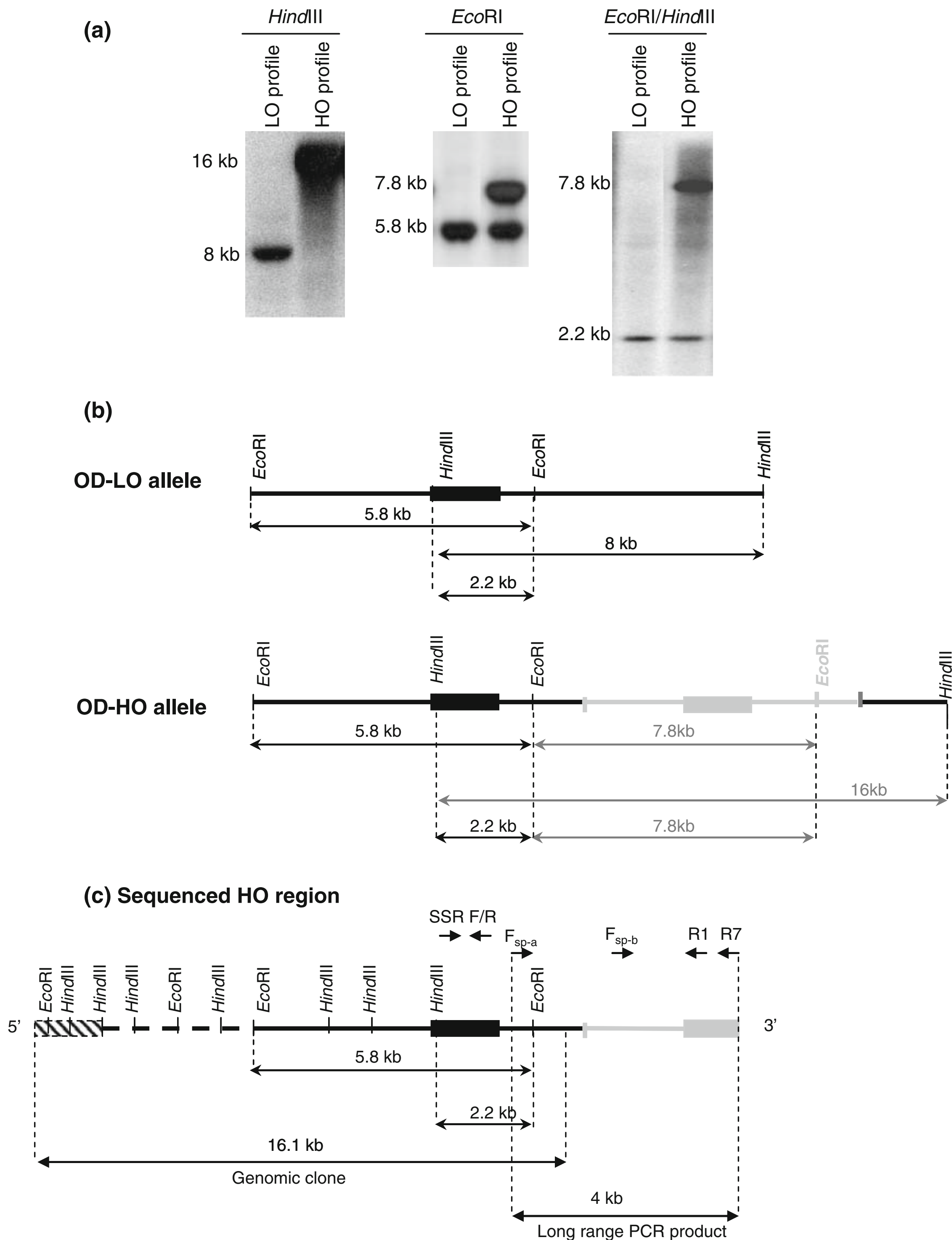

Fig. 1 High oleic (HO) and low oleic (LO) oleate desaturase (OD) alleles. **a** HO and LO RFLP profiles revealed with the OD probe. RFLP profiles were obtained after *Hin*dIII and/or *Eco*RI restriction. All the LO and HO genotypes tested displayed the LO and HO profiles, respectively. Sizes of the hybridised fragments are noted. **b** OD-LO and OD-HO alleles physical maps established according to *Eco*RI and/or *Hin*dIII RFLP profiles revealed with the OD cDNA. *Boxes* indicate OD like sequences. The *black parts* correspond to OD-LO/OD-HO common elements whereas the *grey part* corresponds to the HO specific element. **c** Representation of the sequenced HO genomic region. Parts corresponding to the 16.1-kb genomic clone and to the 4-kb long range PCR fragment are indicated. The *striped box* corresponds to the retrotransposon putative sequences and as for **b**, the *other boxes* indicated OD like sequences. Positions of the primers used are indicated by *arrows* in the upper part of the figures

that the OD-HO allele has either an insertion of about 8 kb in the OD genomic region or a mutation on at least one of the *Hin*dIII sites in this region. Using *Eco*RI, the OD-HO and OD-LO alleles carried a common 5.8-kb fragment and in addition, the OD-HO allele carried an extra 7.8-kb fragment. This supports the hypothesis that there is an 8-kb insertion in the OD-HO allele compared to the OD-LO allele and suggests that this inserted fragment carries one *Eco*RI restriction site and OD-like sequences. The double *Eco*RI–*Hin*dIII restriction LO revealed a common 2.2-kb fragment in both HO and LO genotypes and a 7.8-kb fragment unique to HO genotypes. This data was used to construct physical maps of the OD-LO and OD-HO alleles (Fig. 1b). The OD-LO allele displayed only one region with the OD sequence carrying the 5.8-kb *Eco*RI fragment. The OD-HO allele displayed two adjacent regions with OD sequences, a common part with the OD-LO allele carrying the 5.8-kb *Eco*RI fragment and an HO specific part carrying the 7.8-kb *Eco*RI fragment. The 16-kb *Hin*dIII fragment carried these two adjacent regions (Fig. 1b). Because the Vniimk 8931 population does not carry this HO specific part, this fragment might be a consequence of the mutagenic treatment. This physical map agrees in part with results reported previously (Schuppert et al. 2006). However, the HO specific part of the allele described by Schuppert et al. (2006) carries two *Eco*RI sites leading to a 4.2-kb fragment (accession number DQ075691). This result does not agree with our RFLP observations suggesting that the allele described here might not correspond to the one described by Schuppert et al. (2006).

Characterisation of the OD-HO allele

To characterise the OD-HO allele, a genomic library of the *RHA345* HO line was constructed. Six genomic clones with strong hybridisation signal were isolated. According to their restriction and hybridisation profiles, they carried the same genomic region corresponding to the common part of the OD-HO allele (data not shown). One of them was sequenced and covered 16.1 kb, carrying the expected common 5.8-kb *Eco*RI fragment (Fig. 1c, Supplementary Figure 1). This fragment carries the whole OD cDNA sequence without any mutation compared to the reference cDNA sequence (U91341). The consensus promoter elements 5′-TATAAA-3′ and 5′-CAAT-3′ were revealed at position −42 bp and −92 bp upstream of the putative transcription start point, respectively. A 5′-AATGAA-3′ sequence was found 16 nt before the end of the putative transcript sequence. This motif is identical to a 5′-AA TAAA-3′ poly-A signal except for one nucleotide. One single intron of 1,684 nt was detected between 92 and 93 nt in the reference cDNA sequence (U91341) in the putative 5′ untranslated region, 29 nt before the ATG start codon. The consensus splicing sequences 5′-GT-3′ and 5′-AG-3′ were present at the extremities of the intron. A 16-nt repeat of a 5′-ATT-3′ SSR motif was revealed in the intron between 784 and 832 nt from the transcription start (Supplementary Figure 1). All these observations confirm results previously reported by Schuppert et al. (2006) and, in addition, suggest that the common part of the OD-HO allele may carry a functional OD gene.

On the 5′ border, 7.4 kb far from the putative OD gene, three adjacent 1,600, 1,000 and 800 nt regions encoded polypeptides displaying between 45 and 61% similarity with retrotransposons-like proteins from *Arabidopsis thaliana* (Q9FXB7), *Oryza longistaminata* (O24438) and *Nicotiana tabacum* (Q9ZRJ0; Fig. 1c, Supplementary Figure 1).

Six *Hin*dIII sites were found on the 5′ border of the putative gene. The closest to the OD putative gene is located 722 nt upstream of the transcription start. Another *Hin*dIII site is present in the intron. These two sites defined a 2.1-kb *Hin*dIII fragment carrying a 83-nt OD sequence. This fragment was never revealed in RFLP profiles of HO or LO genotypes probably due to the small size of the OD sequence on this fragment which was not long enough to allow its detection using a classical RFLP technique. No other *Hin*dIII site was detected in the rest of the putative gene or in the 1.3 kb sequence on its 3′ border (Fig. 1c, Supplementary Figure 1). According to the RFLP profiles, the OD-HO allele is characterised by an 8-kb insertion in the OD genomic region carried by a 16-kb *Hin*dIII fragment. The presence of *Hin*dIII sites in the 5′ border of the common fragment and their absence in the 3′ border suggest that the HO specific insertion is beyond this sequenced region on the 3′ side of the common fragment.

None of the six clones selected in the genomic library carried the HO specific part of the OD-HO allele suggesting that this genomic region was not represented in our library. To isolate the HO specific fragment, a different strategy was followed based on the data reported above showing that this insertion carried OD sequences and was adjacent to the common part of the OD-HO allele. A primer was designed on the 3′ extremity of the genomic region already sequenced corresponding to the spacer fragment between the OD gene and the HO specific part ($F_{sp\text{-}a}$), 1,218 nt downstream of the end of the sequence (Fig. 1c). Long range PCRs were performed on DNA from the *RHA345* HO line and the *83HR4* LO line using the $F_{sp\text{-}a}$ primer in combination with primers designed along the entire OD cDNA sequence in both orientations (F1 to F8 and R1 to R8, Table 2). PCR products were separated on an agarose gel. For the LO line, no PCR product was detected with any of the primer sets (data not shown). For the HO line, products were detected with the $F_{sp\text{-}a}$ primer in combination with R1, R2, R3, R4, R5, R6 and R7 (Fig. 2). For each primer set, the PCR yielded one major amplification

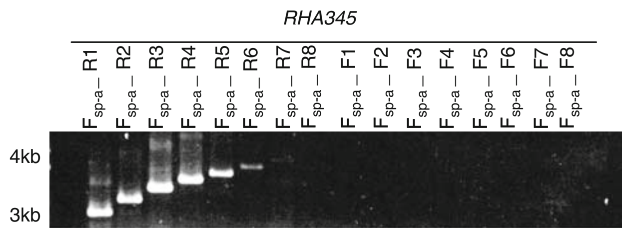

Fig. 2 RHA345 long PCR amplifications using F_{sp-a} primer in combination with OD primers. The name of the OD primer used is indicated as R1 to R8 and F1 to F8

fragment of 3, 3.2, 3.4, 3.6, 3.7, 3.9 and 4 kb, respectively. The primer sets F_{sp-a}_R8 and F_{sp-a}_F1 to F8 did not generate any visible amplification product for the HO line (Fig. 2). The 4-kb F_{sp-a}_R7 PCR fragment from the *RHA345* HO line was cloned and sequenced (Fig. 1c, Supplementary Figure 2). The organisation of this fragment from 5′ to 3′ is as follows: (1) 1,218 nt overlapping with the previously sequenced OD region as expected according to the F_{sp-a} primer position; (2) 1,357 nt without any similarity with database sequences nor with previously sequenced regions; (3) 1,507 nt identical to the putative OD gene corresponding to 244 nt of intron and the following 1,263 nt of the exon 2. The 5′ extremity of the putative gene was not detected. The absence of PCR amplification with F_{sp-a}_R8 primers suggest that the 3′extremity of the OD gene was not present in the duplicated fragment. No *Eco*RI or *Hin*dIII sites were detected in the F_{sp-a}_R7 fragment. These results are consistent with the physical map of the OD-HO allele established from the RFLP profiles (Fig. 1b, c) and show that the HO specific part of the OD-HO allele carries an incomplete duplication of the OD gene. This duplication was not identical to the one proposed by Schuppert et al. (2006) (accession number DQ075691). In fact, the insertion described by these authors comprised the 3′ extremity and the adjacent part of the OD gene carrying one *Eco*RI site. This insertion is surrounded by two *Eco*RI restriction sites leading to a 4.2-kb fragment carrying the OD duplication (Schuppert et al. 2006) that does not agree with the physical map established within this paper.

Dominant and co-dominant markers for the OD-HO allele

PCR tests were set up to detect the HO specific insertion. Polymorphism of the SSR locus located in the intron of the putative OD gene was evaluated in a set of 27 HO and 14 LO genotypes (Table 1). A primer pair, SSR-F_SSR-R was selected to amplify a 240-bp PCR fragment carrying the SSR motives (Fig. 1c). Three different alleles were found according to the size of the PCR amplification product (Fig. 3a). These three alleles were revealed in both the HO and LO genotypes showing that no linkage disequilibrium

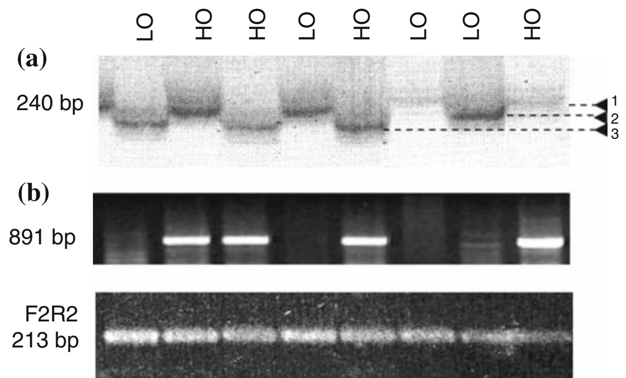

Fig. 3 High oleic (HO) specific PCR amplifications in a set of four HO and four low oleic (LO) genotypes. **a** Amplification of the SSR motives using primer pair combination SSR-F_SSR-R. **b** Amplification of a part of the OD HO specific OD duplication using primer pair combination F_{sp-b}_R1. Profiles displayed by these LO and HO genotypes represent polymorphisms found in the set of 27 HO and 14 LO genotypes. PCR amplification of the OD gene using F2_R2 primers is used as a positive PCR control

exists between the Pervenets mutation and a specific SSR allele despite the genetic linkage between the two loci. Because the fidelity of replication is much lower in the case of SSR structure compared to nonrepetitive DNA (Sia et al. 1997), this observation suggests that natural variation at the SSR locus has occurred since the Pervenets mutation event. The use of these primers revealed a 3-nt polymorphism between the LO line *BD40713* (16 TTA repeats) and the HO line *BE78079* (17 TTA repeats). An F2 population of 107 progenies has been created from a cross between these two lines (Lacombe et al. 2001). The HO:LO segregation of 72:35 was consistent with a 3:1 ratio (χ^2 test, $P > 0.1$), which provides evidence for one dominant allele controlling the HO trait in this population. This allele corresponds to the Pervenets mutation characterised by the OD-HO allele (HO specific 7.8 kb *Eco*RI RFLP) (Lacombe et al. 2001). In the 107 F2 population, the 17-TTA repeat allele strictly co-segregated with the HO specific 7.8 kb *Eco*RI RFLP and consequently with the HO phenotype. These observations agree with a tight genetic linkage between the Pervenets mutation and the SSR locus.

A new primer, F_{sp-b}, was designed on the F_{sp-a}_R7 fragment to amplify a 891-bp region of the HO specific fragment in combination with R1 (Fig. 1c). This primer combination was tested in PCR experiments involving the 27 HO and 14 LO genotypes. They led to PCR amplification at the expected size for all the HO genotypes tested (Fig. 3b). No amplification was detected for the LO genotypes. Therefore, linkage disequilibrium exists between the Pervenets mutation and these HO specific PCR fragments generated by the F_{sp-b}_R1 primer set. Using these primers, amplification was detected at the expected size for the HO line *BE78079* whereas no amplification was detected for

Table 3 Oleic acid content (OAC), stearate and oleate desaturase (OD) mRNA accumulation in seeds obtained from genotypes selfing or crosses

Genotype	OAC (%)	Parents phenotype	Embryos production	Stearate desaturase[a]	OD[a]
BE78079	75	HO	Lines selfing	+	−
BD40713	25	LO	Lines selfing	+	+
HOC97	84	HO	Lines selfing	+	−
HOC	86	HO	Lines selfing	+	−
HOC98	85	HO	Lines selfing	+	−
BD70080	36	LO	Lines selfing	+	+
BE78078	85	HO	Lines selfing	+	−
BD70032	35	LO	Lines selfing	+	+
BE73201	84	HO	Lines selfing	+	−
RHA345	75	HO	Lines selfing	+	+
83HR4	25	LO	Lines selfing	+	+
90R19	23	LO	Lines selfing	+	+
2603	29	LO	Lines selfing	+	+
HaOl9	86	HO	Lines selfing	+	−
Santiago	38	LO	Hybrid selfing	+	+
Trisun 870	91	HO	Hybrid selfing	+	−
Olbaril	84	HO	Hybrid selfing	+	−
83HR4 × RHA345	61	LO × HO	Lines crosses	+	−
2603 × HOC	78	LO × HO	Lines crosses	+	−
HOC × 2603	88	HO × LO	Lines crosses	+	−

[a] + and − indicate the detection and the absence of detection of a desaturase transcript in northern blot experiments, respectively

the LO line *BD40713*. In the 107 F2 population, a strict co-segregation was revealed between this PCR amplification and the HO specific 7.8 kb *Eco*RI RFLP that confirmed that the PCR fragment is carried by the OD-HO allele. Furthermore, as previously described for the SSR marker, all the 72 HO F2 lines displayed the PCR amplification in agreement with the strict genetic linkage between the Pervenets mutation and OD-HO allele (data not shown).

Nature of the Pervenets mutation

Given that the HO specific fragment carries the OD sequence duplication, the hypothesis that this duplication could induce a gene silencing mechanism on the OD gene was tested. This could mean that this duplication would correspond to the Pervenets mutation itself. To test this hypothesis, the OD mRNA accumulation was analysed in HO compared to LO genotypes. Selfing of seven LO and ten HO genotypes was performed and three crosses between HO and LO genotypes were developed, two of which were reciprocal (Table 3). Seeds from LO and HO selfing genotypes displayed an OAC of 23–38% and 75–91%, respectively. Hybrids displayed an OAC between 61 and 88%. The OAC in reciprocal crosses was around the same range between 78 and 88% (Table 3). Stearate desaturase- and OD-mRNA accumulations were investigated by Northern blot. RNA was isolated from developing seeds at 10 and 15 DAP, during lipid reserve elaboration. A 1.6-kb mRNA was revealed with the stearate desaturase probe at 10 and 15 DAP. However, no difference in signal intensity was detected between LO or HO seeds resulting from selfing or crosses involving *RHA345* and *83HR4* genotypes (Fig. 4a). The OD probe hybridised a 1.4-kb mRNA for LO seeds at 10 and 15 DAP. No hybridisation signal was detected in HO seeds resulting from selfing or crosses (Fig. 4b). Similar results were obtained with the other genotypes tested (not shown). These results show that the Pervenets mutation leading to the HO trait is correlated to an absence or a weak OD mRNA accumulation in seeds and acts as dominant which is consistent with a gene silencing mechanism. To confirm that the Pervenets mutation leads to gene silencing on the OD mRNA, the presence of OD small interfering RNA (siRNA), characteristic of gene silencing, were investigated in *RHA345* and *83HR4* immature seeds. Twenty-one nt and 24 nt OD siRNA were specifically detected in HO seeds at 10 and 15 DAP. No OD siRNA were detected in LO embryos (Fig. 4c).

Discussion

A gene silencing mechanism associated with the Pervenets mutation

We present evidence that supports the Pervenets mutation leading to the HO phenotype does not directly modify the

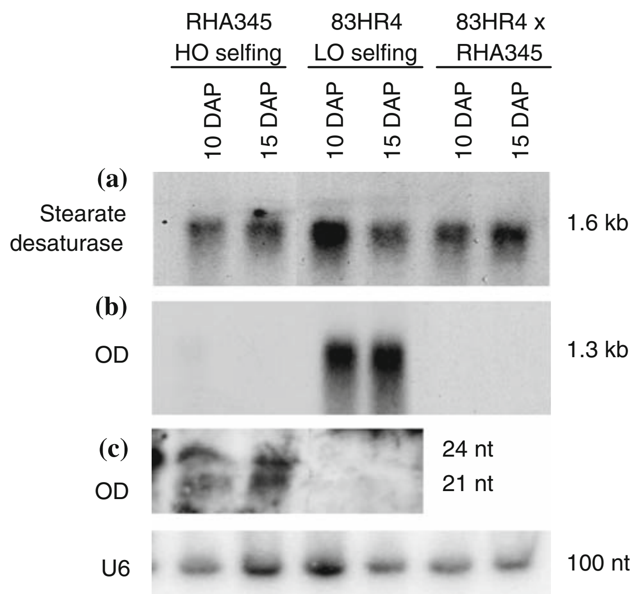

Fig. 4 mRNA (**a** and **b**) and siRNA (**c**) accumulations in seeds obtained from *RHA345* and *83HR4* selfing or cross between *83HR4* and *RHA345*. Northern blots were sequentially hybridised with (**a**) stearate desaturase and (**b**) oleate desaturase (OD) probes. Similar results were obtained for all the seven LO, the ten HO and the three hybrid samples (not shown). (**c**) OD siRNA accumulations were studied in *RHA345* HO and *83HR4* LO seeds. Sizes of RNAs and probes used to hybridise northern blots are indicated. U6 hybridisation intensity was used as a loading control

OD gene sequence but corresponds to OD duplications. In other plant species that have been characterised, the mutations leading to an increase of OAC directly affect OD genes (Jung et al. 2000a,b; Patel et al. 2004; Okuley et al. 1994). Sunflower HO genotypes carrying the Pervenets mutation display two adjacent genomic regions with OD sequences (Fig. 1b). One of them is also present in LO genotypes and may contain a functional gene. However, we showed that OD-mRNA did not accumulate in HO seeds whereas it accumulated in LO seeds confirming results reported previously (Fig. 4b; Kabbaj et al. 1996; Hongtrakul et al. 1998b). Furthermore, hybrid seeds obtained by crossing LO and HO genotypes presented OAC higher than 61% and the same reduced accumulation of OD mRNA. These show that the Pervenets mutation acts *in trans* to prevent OD mRNA accumulation. A mutation in the OD gene could not explain this dominance behaviour whereas a gene silencing mechanism is supported by all the observations. The detection of OD siRNA specifically in HO seeds confirmed the gene silencing of the OD gene (Fig. 4c). Considered together, all these observations show that the Pervenets mutation is associated with OD duplications leading to gene silencing of the OD gene.

In eukaryotes, gene silencing is a process that affects gene expression through sequence specific interactions. It involves 21 and 24 nt siRNA produced from double strand RNA resulting from transcription of antisense or hairpin RNA and can act as dominant or semi dominant (Baulcombe 2004; Brodersen and Voinnet 2006). Antisense and invert repeat RNA can directly induce double strand RNA formation, whereas direct repeat RNA can also be an efficient inducer of double strand RNA through the action of an RNA dependant RNA polymerase (Dalmay et al. 2000; Ma and Mitra 2002; Wesley et al. 2001).

Strategies based on invert or direct repeat induced gene silencing against OD genes have been previously reported in crops to obtain transgenic plants with increased OAC. High OAC soybean and rapeseed were obtained through antisense and co-suppression mediated OD gene silencing (Kinney 1996; Stoutjesdijk et al. 2000). Cotton transformed with an OD inverted repeat construct also showed high OAC (Liu et al. 2002). For the Pervenets mutant, the duplicated fragment has only been partially sequenced, thus we cannot predict whatever antisense or hairpin RNAs are involved in the process. Sequencing of the entire OD duplication could tell us about the nature of the gene silencing inducer as a direct or invert OD repeat. Classical cloning strategies failed to isolate this region. In fact, clones carrying such fragments with repeats tend to recombine in the bacteria during clone multiplication suggesting that conventional cloning strategies are not adapted to clone this type of region (Boë and Masson 1996). Using PCR based strategies we still only managed to isolate part of this duplication, suggesting that the presence of the duplication might have affected the PCR amplification. PCR derived methods have to be improved in order to isolate the full duplication and to further characterise the OD gene silencing induced by the Pervenets mutation.

Origin of OD duplications

Chemical mutagenesis with DMS instead of transgenic techniques was used to obtain the Pervenets mutations leading to the high OAC character (Soldatov 1976). Several gene-silencing mechanisms not associated with transgenic methods have already been reported in plants. In soybean, spontaneous dominant mutants displaying duplication of a chalcone synthase (CHS) gene, express gene silencing of the CHS gene leading to a reduced mRNA accumulation. Nearly all commercial varieties carry one of these mutations and display a yellow seed coat colour whereas the seeds of wild *Glycine* accessions are black or brown (Wang et al. 1994; Todd and Vodkin 1996; Senda et al. 2004; Tuteja et al. 2004). The flower pigment biosynthesis in *Antirrhinum majus* is controlled by the *Nivea* locus encoding for the CHS enzyme. Mutants that have arisen following the movement of a transposable element in this locus displayed inverted duplications of the CHS sequence leading to a gene silencing against the CHS genes. Flowers of

these mutants present a reduced pigmentation intensity compared to the wild type (Coen and Carpenter 1988; Bollmann et al. 1991). The *low glutelin content 1* mutation obtained by ethyleneimine mutagen treatment reduces the glutelin content in rice grains. The mutation is associated with a deletion between two highly similar glutelin genes. It induces gene silencing of glutelin genes through the synthesis of a double strand RNA molecule (Iida et al. 1993; Kusaba et al. 2003). In sunflower, the Pervenets mutation associated with gene silencing of the OD gene may represent a new example of non-transgenic induced gene silencing in plants.

Chemical treatment generates mainly point mutations but can also induce the movement of transposable elements. In peanut, a diethyl sulphate mutagenesis treatment induced the insertion of a miniature inverted repeat transposable element in an OD gene resulting in a putatively truncated protein sequence (Patel et al. 2004). The *frizzi panicle* (*fzp*) mutations associated with abnormal inflorescence development in rice affects an ethylene-responsive-element binding factor (ERF). One of the *ftz* mutants obtained after an EMS mutagenesis treatment presents an insertion of a *Houba Copia*-type retro-element in the ERF gene resulting in the formation of a putative premature stop codon (Komatsu et al. 2003). Moreover, it has been reported that the movement of transposable elements can be associated with sequence duplications. In *Antirrhinum majus*, the inverted duplication found in the CHS gene of mutants affected in flower pigmentation has arisen following the excision of a transposable element (Coen and Carpenter 1988). In *Arabidopsis* and rice, some mutator-like transposable elements (MULES) have been reported as harbouring fragments of host genes. They are referred as Pack-MULE (Yu et al. 2000; Turcotte et al. 2001). In the rice genome, the average copy number of the 3000 Pack-MULE studied is three, suggesting transposition activities of these elements and consequently duplications of the associated fragments (Jiang et al. 2004). The DMS treatment performed to obtain the Pervenets mutation may have induced the movement of transposable elements causing duplications of an OD sequence. Although an exact mechanism cannot be proposed, such a hypothesis can be supported by the fact that retrotransposon-like sequences have been found adjacent to the OD putative gene.

PCR based molecular markers linked to the Pervenets mutation

The conventional methods used to select HO genotypes in breeding programmes consist mainly of OAC quantification on sunflower half seeds (Conte et al. 1989). This phenotypic based method is time consuming and environmentally affected as temperature influences the OD activity and consequently the OAC (Garcés et al. 1992). Molecular markers linked to the Pervenets mutation would represent a valuable and useful tool in breeding programmes, as they will allow for a rapid screening and early detection of genotypes carrying the mutation. The detailed study of the HO specific OD allele presented here allowed the identification of two different types of PCR based molecular markers linked to the Pervenets mutation. First, co-dominant molecular markers have been developed based on the polymorphism of the SSR locus located in the intron of the OD gene. These markers do not mark the mutation itself but are genetically tightly linked to the mutation. Due to the close genetic linkage between the two loci, such markers can be used to identify genotypes carrying the Pervenets mutation in breeding programmes. Furthermore, because of their co-dominance, they can indicate the homozygous or heterozygous status of the Pervenets mutation locus. However, because of the absence of linkage disequilibrium between the Pervenets mutation and the SSR allele in the diversity analysis performed, these markers can only be used in segregating populations coming from HO and LO parents carrying two polymorphic SSR alleles. The second type of PCR based molecular markers has been developed on the mutation itself as the PCR amplification fragment carries parts of the OD duplication. Consequently, amplification products are produced only for genotypes carrying the Pervenets mutation. Unlike the markers described above, these markers are completely linked to the Pervenets mutation and can be used to select genotypes carrying the mutation whatever their genetic background. However, such molecular markers are dominant and consequently, cannot allow the distinction between homozygous and heterozygous genotypes at the Pervenets mutation locus. Due to the size of the OD insertion, a pair of PCR primers surrounding it are expected to lead to the amplification of only the wild type locus. Consequently, the use of such LO specific primers in combination with Pervenets specific PCR markers should allow to distinguish between homozygous and heterozygous genotypes at the Pervenets mutation locus. Sequencing of the corresponding region in a LO genotype is in progress and should allow to select for LO specific PCR markers (C. Granier, personal communication).

Until now, the nature of the Pervenets mutation was still unknown. Our work demonstrated that the mutation displays an original behaviour as it corresponds to oleate desaturase duplications inducing gene silencing of the corresponding gene. In addition to this fundamental finding, this work allowed the development of PCR markers of the Pervenets mutation that can be used in breeding programmes for rapid screening and early detection of genotypes with high OAC.

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