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REPEATS OF AN OLEATE DESATURASE REGION CAUSE SILENCING OF THE NORMAL GENE EXPLAINING THE HIGH OLEIC PERVENETS SUNFLOWER MUTANT

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SUMMARY

This work aims to clone the genomic region of the sunflower genome that may carry the Pervenets mutation causing high oleic content of seed oil. We previously reported that the oleHOS allele at the oleate-desaturase locus (oleHL) carries or is genetically closely linked to Pervenets mutation. We pointed out that the fragments that border the 5.8 kb *Eco*RI fragments carry the Pervenets mutation. The oleHOS reference physical map was constructed. It displays a common part made of the 5.8 kb *Eco*RI fragment, also present in oleLOR allele, and a specific region with the 7.9 kb *Eco*RI fragment, also carrying oleate-desaturase sequences.

A genomic library was constructed in λfixII with an average insert size of 15 kb. Two millions clones were screened enabling to isolate clones from Group I, which were entirely sequenced and revealed carrying a gene for an oleate probably located in the RE. This corresponds to the invariant part of the oleHL locus. Two clones of Group II (11.1, 27.1) are overlapping but 11.1 is characterized by new specific restriction fragments and instability leading to a smear when probed with the oleate cDNA. Consequently, 11.1 sequence probably carries oleate repeated sequences that cause instability of the clone. The clone 11.1 is a good candidate to carry a part of the specific oleHOS allele, but due to its organization in repeats it is not yet sequenced. This organization leads to speculate on the mechanisms that could disturb oleate function in Pervenets [HOAC] genotypes.

Key words: **cloning, high oleic mutant, oil composition, oleate desaturase, sunflower**

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INTRODUCTION

Soldatov (1976) has performed a chemical mutagenesis on seeds of the Russian population VNIIMK 8931. He screened one mutant enabling him to construct the Pervenets population that displays a high oleic acid content [HOAC] in achene oil. Several pollen bulks from 6,000 M_3 mutagenized progenies were used to pollinate a linoleic or normal line ([LO]). Only one progeny, out of 200 issued from pollen bulks, contained plants which display an oleic acid content (OAC) higher than 40%. The sister [HOAC] plants were gathered constituting the Pervenets population (Soldatov, pers. comm). In three cycles Soldatov enhanced the OAC to about 65% with a variation range between 50 to 80% and this population was the source of the [HOAC] trait for breeding worldwide.

The [LO] sunflower usually ranges between 20 to 30% OAC whereas [HOAC] cultivars are above 83%. Several agronomic, biochemical, genetic and molecular information have been cumulated on the [HOAC] trait derived from the Pervenets population by different groups.

1. Agronomic information

The [LO] and [HOAC] commercial hybrids are equivalent for yield and oil content (Lagravčre *et al.*, 1997). The conversion process of an [LO] line into an [HOAC] line is not as regular as it is thought, and, broadly, more [LO] plants than expected appear in the generation. Moreover, some [LO] progenies may lead to unexpected [HOAC] plants (Demurin & Škorić, 1996). It appeared that through conversion process some [LO] parent might appear more or less recalcitrant for conversion into a [HOAC] line. Commercial [HOAC] hybrids from different origins but from the same source, Pervenets, display a variation range for OAC (Lagravčre *et al.*, 1997). The NewSun is obtained by crossing [LO] (for linoleic content) by [HOAC] lines.

2. Biochemical informations

The [HOAC] trait is embryo-specific and is associated with a lack of oleate-desaturase activity (Garcés & Mancha, 1991). The OAC has been reported to be influenced by environmental factors such as temperature, light, and drought stress (Lacombe and Bervillé, 2000 for review).

3. Genetic information

Genetic analyses performed on ([LO] x [HOAC]) or ([HOAC] x [LO]) progenies using backcross (BC_1) or F_2 segregating populations have led to heterogeneous conclusions dealing with the number of factors controlling OAC (1 to 5), with the dominance of Pervenets [HOAC] trait versus [LO] and with maternal effects. Several reviews have already reported the details of the results and this is not developed longer here (see Lacombe and Bervillé, 2000). In conclusions of these studies, it

appeared that the dissociation between the Pervenets effect from the other factors acting on OAC is required to perform any genetic approach of the [HOAC] trait.

4. Molecular approaches

A reduction of the oleate-desaturase transcript accumulation in [HOAC] compared with [LO] genotypes has been reported (Kabbaj *et al.*, 1996). Temperature effects where detected for stearate-desaturase and oleate-desaturase transcript accumulation: an enhancement of temperature causes an increase of both transcript accumulation, higher for stearate-desaturase (Δ9-desaturase) than for oleate-desaturase (Δ12-desaturase). Hongtrakul *et al.* (1998), Lacombe *et al.* (2000b) have shown that the lack of the oleate transcript accumulation is strictly associated with the [HOAC] trait. Moreover, Lacombe *et al.* (2000) showed that the Pervenets allele prevents the [LO] allele to function in ([HOAC] x [LO]) or (LO x [HOAC]) F_1 embryos. Hongtrakul *et al.* (1998) and Lacombe and Bervillé (2001a) revealed a [HOAC] specific RFLP with an oleate cDNA as a probe. It corresponds to a duplication of an oleate gene and it was named oleHOS (Lacombe *et al.*, 2001b).

5. Molecular genetics

Lacombe *et al.* (2000) and Perez-Vick *et al.* (2000) showed that the Pervenets mutation is closely linked to the oleHOS allele at the oleate desaturase locus (oleHL). Moreover, Lacombe *et al.* (2001b) have recently shown that the [HOAC] trait due to the Pervenets mutation could be suppressed in the presence of the sup [HOAC] allele at a supole locus genetically independent from the oleate locus. The conditional expression of the Pervenets mutation depends on the sup0 and supole alleles which cause no effect on [HOAC] and [LO] genotypes and restore the [LO] level of [HOAC] genotypes and had no effect on [LO] genotypes, respectively. The suppression mechanism explains most of the troubles met by breeders along the conversion process of [LO] into [HOAC] lines.

All these data were verified in different studies performed in Montpellier and we decided to undertake the cloning of oleate regions in sunflower genomes in order to understand what happened in the Pervenets mutant. We exposed the strategy and the organization of the Pervenets mutation deduced from several clones. We discussed the possible effects of the mutation on the oleate function.

This work aims to clone the genomic region of the sunflower genome that may carry the Pervenets mutation. We previously reported that the oleHOS allele at the oleate desaturase locus (oleHL) carries or is genetically closely linked to Pervenets mutation. Moreover, the physical organization of the oleHL locus is for oleLOR a 5.8 *Eco*RI and a 8 *Hind*III fragments whereas oleHOS shows an extra 7.9 kb fragment with *Eco*RI, but with *Hind*III the 8 kb fragment lengthens to 15 kb. This led us to consider that the fragments that border the 5.8 *Eco*RI fragments carry the Pervenets mutation. Hybridization signal intensity of the 7.9 kb *Eco*RI extra fragment and PCR analyses suggest that the fragment carry at least one copy of the oleate-desatu-

rase sequence. The reference for oleHOS physical map was constructed. It displays a common part made of the 5.8 kb *Eco*RI fragment also present in oleLOR allele and a specific region with the 7.9 kb EcoRI fragment, also carrying oleate-desaturase sequences.

A genomic library was constructed in λfixII with the DNA from the RHA345 [HOAC] line with an average insert size of 15 kb. Two millions clones were screened enabling to isolate clones ranging in four classes. Genomic clones from two of the classes display only faint signal with the oleate cDNA as a probe and were therefore not further studied. Eight clones from the other two classes displaed RFLP fragments with intense signals. Two clones (15.4, 44.1) from Group I were entirely sequenced and revealed carrying a gene for an oleate probably located in the RE. This corresponds to the invariant part of the oleHL locus. Further sequence studies showed that the Group I clone carries apparently a functional oleate gene (probably seed-specific) with a 1678 bp intron between nt 87 and 88 of the transcript sequence, 29 nt upstream of the initiation codon ATG (5' UTR). Two clones of Group II (11.1, 27.1) are overlapping but 11.1 is characterized by an instability leading to a smear when probed with the oleate cDNA. Consequently, the 11.1 sequence probably carries oleate repeated sequences that cause the instability of the clone. We showed that the 11.1 clone carries the whole cDNA sequence.

In conclusion, the clone 11.1 is a good candidate to carry partly of the specific oleHOS part, but due to its organization it is not yet sequenced. This organization leads to speculate on the mechanisms that could disturb oleate function in Pervenets [HOAC] genotypes.

MATERIAL AND METHODS

Several [HOAC] and [LO] lines were used to construct the physical map of the oleate region. They are listed in Table 1. We used the line RHA345 from USDA-NDSU (USA) as source of the genomic DNA. Genomic DNA was partially restricted with Sau3A and sized for 15 kb fragments on average. The 15 kb fraction was ligated to the arms of Lambda fixII phage (Stratagene) restricted with *Xho*I. The final phage library was 2 x 10^6 phages per ml.

Molecular techniques

Two million clones were spread on recipient bacteria and plates were Southern-transferred to Nylon membranes for screening with an oleate cDNA. Finally, 10 clones hybridizing with the oleate cDNA were retained and further studied.

Plant DNA preparation, restriction, Southern transfer and labeling of probes were done according to Lacombe and Bervillé (2001 MB). Phage DNA preparation, and subsequent treatments were done according to the protocol given by the provider.

RESULTS

Construction of the oleate region physical map

We first constructed the physical map of the oleate region in both [LO] and [HOAC] genotypes knowing that at the oleHL locus in the [LO] there is the oleLOR allele corresponding to 5.8 *Eco*RI and 8 *Hind*III fragments (Figure 1).

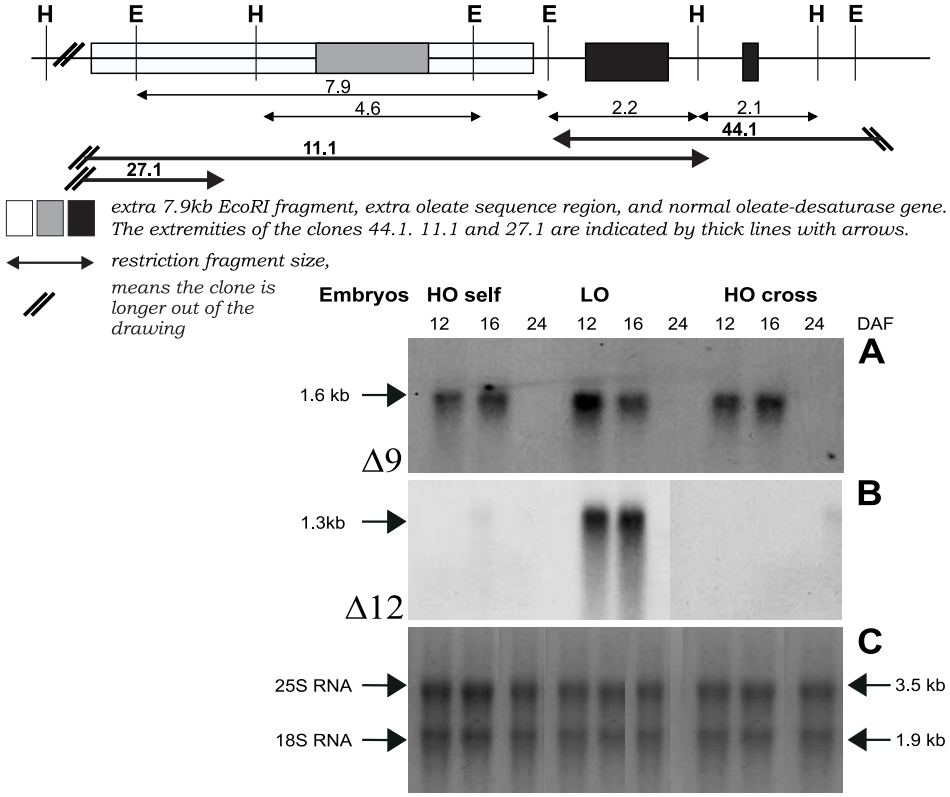

Figure 1: Physical maps of the oleHOS region carrying an oleate-desaturase gene and an extra fragment of 7.9 kb carrying oleate-desaturase similar sequences

The double restriction with *Eco*RI and *Hind*III led to a 2.2 kb fragment carrying apparently an oleate-similar sequence strongly hybridized by the probe. In the map for the [HOAC] mutant, the oleHOS profile with *Eco*RI the 5.8 kb *Eco*RI fragment is still present but another extra fragment of 7.9 kb fragment is also present (Figure 1). With *Hind*III, the 8 kb fragment in the [LO] lengthens to 15 kb in the [HOAC]. The double restriction led also in the [HOAC] to the 2.2 kb fragment. Moreover, the 7.9 *Eco*RI fragment is still present. This led us to consider that one of the fragments that border the 5.8 *Eco*RI fragments, carries or was due to the Pervenets mutation. Hybridization signal intensity of the 7.9 kb *Eco*RI extra fragment and PCR analyses

suggest that the fragment carry at least one copy of the Δ12 oleate sequence. These data enabled us to construct the ole HL region reference map with *Eco*RI and *Hind*III sites and to indicate oleate sequence positions.

Lambda clones carrying oleate-like sequences

A genomic library was constructed in λfixII with the DNA from the RHA345 [HOAC] line with an average insert size of 15 kb. Two millions clones were screened enabling to isolate ten clones ranging in four classes.

Class I genomic clones from two of the four classes displayed only faint signal with the oleate cDNA as a probe and were therefore not further studied. The eight clones from the two other classes displayed RFLP fragments hybridizing the oleate cDNA as a probe with intense signals. Two clones (15.4 & 44.1) from Group I, were entirely sequenced and thus revealed that they carry a functional oleate gene probably located in the endoplasmic reticulum (ER). It is carried by the 5.8 kb *Eco*RI fragment and therefore it corresponds to the invariant part of the oleateHL locus. Further sequence studies show that the clones of Group I carry apparently a functional oleate gene (probably seed-specific) interrupted by a 1678 bp intron between nt 87 and 88 of the transcript sequence, 29nt upstream of the initiation codon ATG (5' UTR). The intron is located at 4nt in the 5'UTR in *Arabidopsis* but it is 1,300 bp (Okuley *et al*., 1994).

Class II clones also hybridized strongly with the oleate-desaturase cDNA as a probe, but they differ from Class I by carrying another oleate-similar sequence on another *Eco*RI-*Hind*III fragment differing from the previous 2.2 kb fragment. The clones 27.1 and 11.1 overlapped, but the clone 11.1 was characterized by an instability leading to a smear when probed with the oleate cDNA. Consequently, the 11.1 sequence probably carries oleate repeated sequences that cause instability of the clone.

PCR amplification products obtained with the DNA from the clone 11.1 with 8 primer pairs covering the oleate cDNA hybridized with the oleate cDNA as a probe showing that the clone 11.1 carries approximately a whole copy of an oleate cDNA corresponding sequence. However, the instability of this clone does not allow its sequencing.

Alignment of classes I and II clones on the physical map

The clones were tentatively aligned on the genomic physical map and thus we obtained a refined map for this region, locating the *Hind*III site in the intron. Furthermore, the clones 11.1 and 15.4 appeared adjacent in the map and although we did not obtain another clone overlapping the two preceding ones, we cannot eliminate that is true (Figure 1).

Consequently, in the [HOAC] Pervenets mutant, the organization of this region appears with a tandem repeat of the oleate gene whereas there is a single copy in the [LO] genotypes. We have already reported that the main effect of the Pervenets muta-

tion is to decrease the oleate transcript accumulation leading to prevent enzyme activity during the key stage for lipid deposit causing oleic acid accumulation. Such an organization at the DNA level has already been found to cause disturbances in gene expression in different plant species (Morel *et al*., 2000; Matzke *et al*., 2001). The extra oleate-desaturase copy should cause silencing of the normal oleate-desaturase gene, although both could be structurally functional. At present, it remains to determine whether the mechanism of silencing runs at the transcriptional or post-transcriptional level.

In *Arabidopsis*, several genes may affect the silencing mechanisms and even suppressor alleles have been identified. Those genes are looked for in sunflower to check whether one may correspond to the suppressor locus.

In conclusion, a duplication of an oleate region appeared in the genomic organization of the [HOAC] Pervenets mutant. This oleate-desaturase gene has the characteristic for being targeted in the RE and thus to have seed specific expression. Two clones, 15.4 and 11.1, that fit adjacent locations in the genomic map revealed this organization. Moreover, the corresponding RFLP is strictly required for the expression of the [HOAC] phenotype. However, another locus can suppress the expression of the mutation. Consequently, the Pervenets mutation is probably the duplicated region. However, another oleate gene in this region causes silencing of the normal oleate gene. Moreover, the suppressor locus could correspond to a gene that could prevent silencing, thus leaving the normal oleate-desaturase allele to function. Further studies are undertaken to verify whether gene silencing acts at the transcriptional or posttranscriptional level. Nevertheless, the suppressor may also correspond to an alternative pathway with an oleate-desaturase compensating for the normal oleate-desaturase function.

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EXPLICACION DE LA MUTACION CON ALTA OLEINA EN PERVENEC A BASE DEL EFECTO DE AREAS REPETIDAS DE OLEATO-DESATURASE SOBRE LA INHIBICION DEL GEN NORMAL

RESUMEN

El objetivo de este trabajo es de clonar la area de genoma en el genoma del girasol para cual se supone que contiene la mutacion de Pervenec que causa el alto contenido de acido oleico en el oleo. Hemos previamente informado que el alelo oleHOS al locus de oleato-desaturase contiene o es estrechamente ligado con la mutacion de Pervenec. Hemos acentuado que los fragmentos que se limitan con los fragmentos 5.8 kb *Eco*RI contienen la mutacion de Pervenec. Para oleHOS fue construida la mapa fisica referente. La mapa mostra la parte comun que hacen el fragmento 5.8 kb *Eco*RI, tambien presente al alelo oleLOR, asi como la area especifica con el fragmento 7.9 kb *Eco*RI que contiene tambien la secuencia de oleato-desaturase.

Dentro de λfixII fue construida la biblioteca de genoma con la cantidad media del insert de 15 kb. El repaso de dos miliones de clones facilito de aislar los clones del grupo I, que fueron totalmente secuenciados y para que fue constatado que contienen el gen para oleato-desaturase que es situado por lo mas probable en RE. Eso corresponde a la parte invariable del locus oleHL. Los clones del grupo II (11.1, 27.1) se traslapan, pero el clon 11.1 posee nuevos fragmentos restrictivos especificos y la inestabilidad que llevan a la aparicion de cinta derramada en la pasta con oleato-desaturase cDNK. Parece que el clon

11.1 contiene por lo mas probable la parte del alelo especifico oleHOS, pero a causa de la organizacion de segmentos que se repiten este clon no es aun secuenciado. Este modo de organizacion provoca la reflexion sobre los mecanismos por los cuales seria posible influir sobre la funcion de oleato-desaturase en los genotipos que contienen la mutacion de Pervenec [HOAC].

EXPLICATION DE LA FORTE MUTATION OLÉIQUE DANS LE PERVENETS PAR L'EFFET D'INHIBITION DES RÉPÉTITIONS DE LA RÉGION OLÉATE SATURASE SUR LE GÈNE NORMAL

RÉSUMÉ

Le but de ce travail était le clonage de la région génomique du génome du tournesol qui peut être porteur de la mutation Pervenets causant un contenu oléique élevé dans l'huile. Nous avions fait part précédemment du fait que l'allèle oleHOS au locus oléate-désaturase (oleHL) portait ou était génétiquement étroitement lié à la mutation Pervenets. Nous soulignions que les fragments qui limitent les fragments 5.8 kb *Eco*RI portaient la mutation Pervenets. La carte de référence physique oleHOS a été établie. Elle montre une partie commune faite de fragment 5.8 kb *Eco*RI aussi présent dans l'allèle oleLOR et une région spécifique avec le fragment 7.9 kb *Eco*RI portant lui aussi une séquence oléate désaturase.

Une bibliothèque génomique a été construite dans le cadre λfixII d'une dimension moyenne d'insert de 15 kb. L'examen de deux millions de clones a permis l'isolement de clones du groupe I qui etaient devenus entièrement séquentiels et pour lesquels il a été établi qu'ils étaient porteurs du gène d'un oléate situé probablement dans RE. Ceci correspond à la partie invariable du locus oleHL. Deux clones du groupe II (11.1, 27.1) sont correspondants mais le clone 11.1 possède de nouveaux fragments de restriction spécifiques et une instabilité qui conduit à l'apparition d'une traînée dans le test au cADN oléate-désaturase. Il semble que le clone 11.1 porte des séquences répétées d'oléate qui causent l'instabilité du clone. Le clone 11.1 est un bon candidat porteur pour une partie de l'allèle spécifique oleHOS mais à cause de l'organisation des segments qui se répètent il n'a pas encore été rangé dans une séquence. Cet aspect de l'organisation porte à la réflexion sur les mécanismes qui pourraient avoir une influence sur la fonction d'oléate-désaturase dans les génotypes qui contiennent la mutation Pervenets [HOAC].