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GENETIC AND MOLECULAR CHARACTERIZATION OF THE HIGH OLEIC CONTENT OF SUNFLOWER OIL IN PERVENETS

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RÉSUMÉ :

Le facteur génétique majeur de la teneur en TG-oléique de l'huile de tournesol a pour origine la population *Pervenets*. Le caractère à haute teneur oléique (HO) a été transféré de *Pervenets* à des variétés, mais de nombreuses difficultés persistent dans la compréhension et donc dans l'utilisation en sélection, pour produire des variétés à haute teneur stables et performantes. Pour identifier des marqueurs moléculaires spécifiques de la mutation HO *Pervenets*, l'approche gène candidat a été réalisée, en considérant les 2 enzymes intervenant dans la synthèse de l'acide oléique : les Δ9- et Δ12-désaturases. Une étude de diversité portant sur 180 génotypes (populations, lignées et hybrides) HO et LO a révélé un profil de restriction spécifique des génotypes HO, avec l'ADNc de la Δ12-désaturase utilisé comme sonde. Il est strictement associé au caractère HO. Dans une population F2 de 107 individus obtenue d'une F1 HO X LO, nous avons observé une distribution bi modale en accord avec la ségrégation 3:1 du facteur dominant HO bien que quelques individus aient une teneur intermédiaire. Les individus homozygotes pour le Δ12-RFLP LO ont une teneur oléique moyenne de 50,7 % (15,5 – 61,3 %) ; les individus homozygotes pour le Δ12-RFLP HO ont une teneur oléique moyenne de 90,6% (85,2 – 91,9 %) ; les individus hétérozygotes pour le Δ12-RFLP HO/LO ont une teneur oléique moyenne de 83,7 % (67,5 – 91,3 %). Les proportions sont en accord avec une ségrégation 1:2:1 qui est donc due à l'allèle dominant du Δ12-RFLP spécifique du caractère HO. Ce Δ12-RFLP est associé à une forte réduction de l'accumulation du transcrit de la Δ12-désaturase. Pour comprendre l'organisation moléculaire de cette région nous avons entrepris de la cloner.

SUMMARY:

The main genetic factor modifying oil composition in sunflower originated from the *Pervenets* population that displays a TG-oleic content over 70 %. This HO trait has been transfered from Pervenets to all present HO varieties, but several troubles were met to use the trait in breeding programs to obtain efficient and stable varieties. A candidate gene approach was used to identify the mutated gene, considering the two enzymes involved in stearic and oleic desaturation. In diversity analysis performed on 180 HO or LO populations, lines and hybrids, a specific Δ12-RFLP for the HO trait was recognised. It was strictly associated with to HO trait. In 107 F2 plants derived from a HO x LO F1 we observed a bimodal distribution that agreed with a 3:1 segregation ratio for a dominant HO factor at one locus, although few individuals with an intermediate oleic level were found. The individuals homozygous for the LO Δ12-RFLP contained in average 50.7 % of oleic acid (15.5 – 61.3 %); the individuals homozygous for the HO Δ12-RFLP contained in average 90.6 % of oleic acid (85.2 – 91.9 %) ; the individuals heterozygous for the HO/LO Δ12-RFLP contained in average 83.7 % of oleic acid (67.5 – 91.3 %). The ratio agreed with a 1 :2 :1 proportion that is therefore due to a dominant allele associated with the Δ12-RFLP specific of the HO trait. This Δ12-RFLP was associated to a strong reduction in the level of the Δ12-desaturase transcript accumulation. To understand the molecular organisation of this region we undertook to clone it.

INTRODUCTION:

Sunflower oil is of economic importance because fatty acids that compose this oil are used in food, feed, chemical and food industries. In average, this oil, composed by triacylglycerol contains 10 to 14 % of saturated fatty acids (palmitic and stearic acids), 20 % of monounsaturated fatty acid (oleic acid) and 70 % of polyunsaturated fatty acids (mainly linoleic acid). To increase the number of sunflower oil applications, breeding effort has focused on the modification of the proportion of the different fatty acids in seed oil. Chemical mutagenesis has been used on *VNIIMK 8931* population to produce a new population, *Pervenets* (Soldatov, 1976). The seeds in this mutant population present an increase of oleic acid content compared to the normal or linoleic sunflower (LO). Breeding programs have been developed from *Pervenets* to produce « High Oleic » genotypes (HO) all around the world. These HO genotypes present an oleic acid content in seeds up to 80 % without modification of membrane fatty acid composition (Garcès et al., 1989). Hypotheses explaining the oleic acid accumulation focus on the 2 enzymes leading to the oleic acid accumulation (Somerville and Browse, 1991):

- the stearoyl-ACP-desaturase (Δ9-desaturase) which catalyses the first desaturation of the stearoyl-ACP in oleoyl-ACP (Mc Keon et Stumpf, 1982),
- the oleoyl-phosphatidyl-choline-desaturase (Δ12-desaturase) which catalyses the second desaturation of the oleoyl-PC in linoleoyl-PC (Slack et al., 1979; Stymme and Appelqvist, 1980).

Kabbaj et al. (1996a; 1996b), Hongstrakul et al. (1998), and Lacombe and Bervillé (2000) have revealed a correlation between the HO phenotype and a strong reduction of Δ12-desaturase transcript accumulation in HO seeds during critical stages of lipid reserve synthesis. However, mechanisms that might lead to this strong reduction of the Δ12-desaturase transcript accumulation are numerous and therefore still unknown in this mutant sunflower origin. On the other hand, a lot of genetic studies on the inheritance of the HO phenotype have been carried out, but contradictory results have been obtained. Urie (1985) revealed a single partially dominant gene controlling the HO phenotype. Miller et al. (1987) showed a major dominant gene plus a minor recessive gene controlling the HO phenotype. Fernàndez-Martinez et al. (1989) revealed that the HO character was controlled by three dominant complementary genes. According to Fernandez et al. (1999), a two-gene interaction model could explain the HO phenotype. Thus, the mode of inheritance for the trait leading to the oleic acid accumulation in *Pervenets* germplasm, is still not well understood.

Our aims were firstly, to find molecular markers specific of the HO mutation derived from *Pervenets* germplasm. For this purpose, the RFLP profiles obtained with candidate genes coding either for the Δ9-desaturase or the Δ12-desaturase, were studied among a set of HO and LO genotypes. This study revealed that all the HO genotypes were characterized by a specific RFLP carrying a Δ12-desaturase homologous sequence. Furthermore, we confirmed the co-segregation between this specific RFLP and the HO trait in a F2 population segregating for the HO / LO phenotype.

The results of our studies allowed us to reveal a strict linkage between the HO-specific RFLP in a region of a Δ12-desaturase gene and the HO trait. To unravel the mechanism leading to the HO phenotype, the molecular study of this region was undertaken.

MATERIALS AND METHODS

Materials

Diversity study

In order to represent the main diversity available in cultivated HO and LO sunflower, genotypes were chosen to represent diverse germplasm pools by their pedigree and their country origin. Populations, inbred lines and commercial hybrids were studied. Eighty-four LO genotypes (3 populations including *VNIIMK 8931*, 56 inbred lines and 25 commercial hybrids) and ninety-six HO genotypes (2 population including *Pervenets*, 61 inbred lines and 33 commercial hybrids) were grown in the glasshouse or the field.

F2 segregating population

The HO female inbred line BE 78079 was crossed with the LO restorer inbred line BD 40713 (Monsanto). One F1 plant was selfed to produce F2 seeds. This F2 segregating population, represented by 107 individuals, was grown in the glasshouse.

Methods

Restriction analysis and Southern hybridizations

DNA from plants of both diversity study and F2 offspring was prepared from leaf tissue frozen at - 80°C and ground to a fine powder in liquid nitrogen as described by Gentzbittel et al. (1992). DNA, restricted by either *Eco*RI or *Hind*III was subjected to electrophoresis in TBE on a 0.8% TBE-agarose horizontal slab gel at 1V/cm for 16 h. The DNA was Southern blotted onto Hybond-N+ membrane (Amersham) under alkaline condition. The Δ9-desaturase cDNA (Kabbaj et al., 1996a, 1996b) and the Δ12-desaturase cDNA (Abbott et al. unpublished) were used to produce labelled probes with [^{32}P] α-dCTP by random priming reaction, as described by Feinberg and Vogelstein (1982). Different length of exposure for membranes onto films was performed depending on the level of radioactive spots on the membranes.

Measure of oleic acid content

After selfing, each F2 seed from the F1 plant head was analysed for fatty acid composition. Oil was extracted from F2 half seed. The methylated fatty acids were quantified using gas chromatography. The oleic acid content of each F2 seed was deduced.

RESULTS AND DISCUSSIONS

HO and LO genotype RFLP profiles using a Δ9-desaturase cDNA as a probe

The *Eco*RI and *Hind*III RFLP profiles using a Δ9-desaturase cDNA as a probe, were revealed in eighty-four LO genotypes (3 populations, 56 inbred lines and 25 commercial hybrids) and in ninety-six HO genotypes (2 populations, 61 inbred lines and 33 commercial hybrids), (Fig. 1).

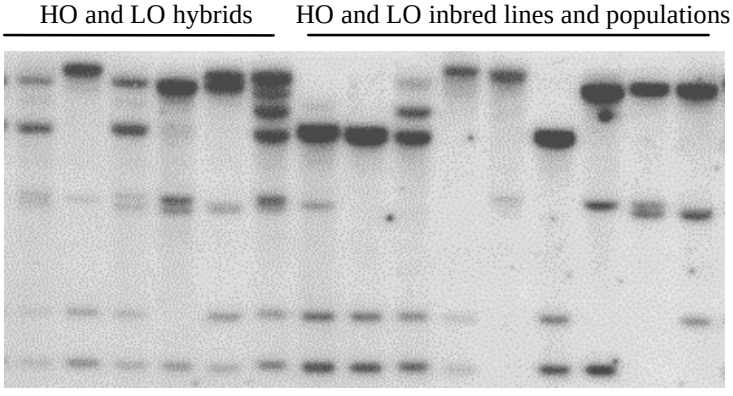


Fig. 1: Autoradiography film of *Eco*RI restricted DNA of hybrids, inbred lines and populations transfered onto a Southern blot and hybridized with the Δ9-desaturase cDNA used as a probe.

The restriction profiles of lines and populations displayed in average 3 and 2 *Eco*RI and *Hind*III fragments, respectively. Moreover, the restriction profiles of hybrids displayed in average 5 and 4 *Eco*RI and *Hind*III fragments, respectively. These profiles were polymorphic but none of the polymorphism was specific of all the HO genotypes.

HO and LO genotype RFLP profiles using a Δ12-desaturase cDNA as a probe

The *Eco*RI and *Hind*III RFLP profiles using a Δ12-desaturase cDNA as a probe, were revealed in eighty-four LO genotypes (3 populations, 56 inbred lines and 25 commercial hybrids) and in ninety-six HO genotypes (2 populations, 61 inbred lines and 33 commercial hybrids), (Fig. 2).

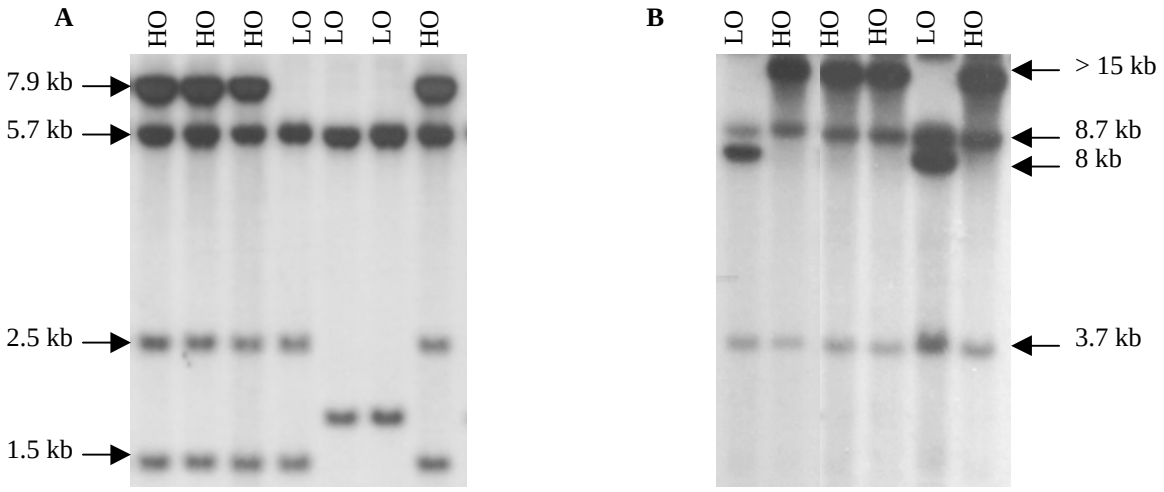


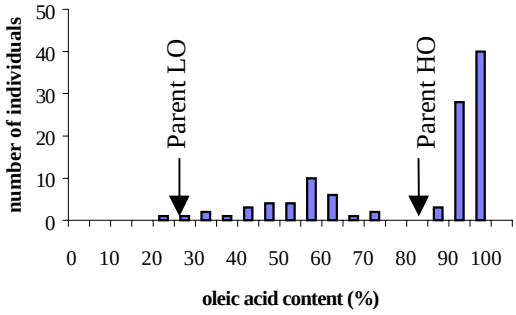
Fig. 2: Autoradiography film of *Eco*RI (**A**) and *Hind*III retricted DNA (**B**) of hybrids, inbred lines and populations transferred onto a Southern blot and hybridized with the Δ12-desaturase cDNA used as a probe.

The restriction profiles for hybrids, populations and inbred lines displayed 3 or 4 *Eco*RI fragments and 3 *Hind*III fragments. They were less polymorphic than profiles revealed by the Δ9-desaturase cDNA used as a probe. However, one *Eco*RI profile and one *Hind*III profile, were present in all the HO genotypes. The *Eco*RI HO profile was characterized by an extra fragment of about 7.9 kb in comparison of all *Eco*RI LO profiles (Fig. 2A). The *Hind*III HO profile was characterized by a fragment of more than 15 kb which replaced the 8 kb fragment present in all the *Hind*III LO profiles (Fig. 2B). The two *Eco*RI and *Hind*III HO-specific profiles, hybridizing the Δ12-desaturase cDNA as a probe, were always revealed in the HO hybrids, inbred lines and population whereas, they were never revealed in any of the LO genotypes.

Segregation of the oleic acid content in the F2 segregating population

Oleic acid content was quantified in each F2 half seed. The oleic acid content histogram of the F2 offspring was shown in Fig. 3.

Fig. 3 : Distribution of F2 individuals for the oleic acid content in % off the total seed oil



The threshold for HO and LO classes could be placed either between 60 and 70 % or between 70 and 85 %. We would underline that there are only three individuals which ranked between 65 to 85 %. We have no means to determine which is the actual threshold for HO and LO classes.

Segregation of the Δ12-desaturase polymorphism in the F2 segregating population

The Δ12-desaturase cDNA used as a probe, revealed two *Hind*III profiles for the two parents of the F2 population. The HO inbred line BE 78079 was characterized by the HO-specific *Hind*III profile that displayed the fragment of more than 15 kb and the absence of a 8 kb fragment. The *Hind*III profile of the LO inbred line BD 40713 was characterized by the absence of the fragment of more than 15 kb and the presence of the 8 kb fragment. The other fragments hybridizing with the Δ12-desaturase cDNA were not polymorphic between the two parents (Fig. 4).

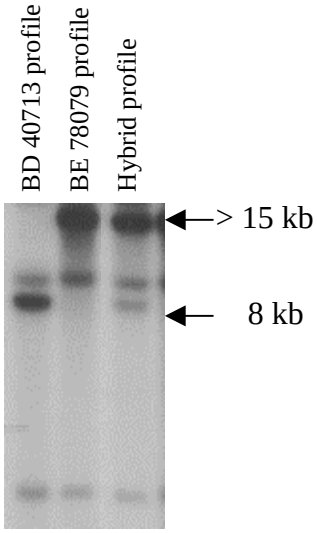


Fig. 4: Autoradiography film showing the three different profiles found in the F2 population. DNA, restricted by *Hind*III, were transferred onto a of the Southern blot and hybridized with the Δ12-desaturase cDNA used as a probe.

The segregation of the HO-specific Δ12-desaturase RFLP was studied in 107 individuals of the F2 population: 24 individuals displayed the HO parent BE 78079 profile, 33 individuals displayed the LO parent BD 40713 profile and 50 individuals displayed the sum of these two profiles. This agrees to a 1:2:1 segregation (χ^2=1.97; for df=2, $\chi^2_{(\alpha=0,95)}$ =5.99) and suggests that the HO-specific RFLP be at one locus.

Co-segregation in the F2 of the oleic acid content and the Δ12-desaturase RFLP

The F2 individuals, characterized by the homozygous HO parent BE 78079 profile, displayed an oleic acid content between 85.2 % and 91.9 % with an average of 90.6%. The F2 individuals, characterized by the homozygous LO parent BD 40713 profile, displayed an oleic acid content between 15.5 % and 61.3 % with an average of 50.7 %. The individuals, characterized by the hybrid profile, displayed an oleic acid content between 67.5 % and 91.3 % with an average of 83.7 %. Moreover, the Δ12-desaturase RFLP heterozygous individuals ranked between 85 to 91.3 % except the three individuals of the classes 65 and 70 %. Thus, we suspected that these three individuals could be due to illicit fertilisation. So, in this case, individuals characterized by the HO specific *Hind*III profile with the fragment of more than 15 kb at the homozygous or heterozygous state, displayed a oleic acid content of more than 85 %. This is in agreement with a strict 3:1 segregation for a dominant allele leading to a High Oleic content.

CONCLUSION

The result of the diversity study on a high number of different HO and LO genotypes permitted to us to revealed *Hind*III and *Eco*RI RFLP specific to HO genotypes derived from *Pervenets*. Moreover, the *Hind*III HO-RFLP co-segregated with the HO trait in our F2 segregating offspring. These results point out the strict linkage between the HO-specific RFLP in a Δ12-desaturase region and the HO trait. A strong reduction of Δ12-desaturase transcript accumulation have been already reported for HO in comparison with LO seeds (Kabbaj et al., 1996a; 1996b; Hongstrakul et al., 1998; Lacombe and Bervillé, 2000). We suggest that the mutation that has occurred in *Pervenets* affected the structure of the HO specific Δ12-desaturase region, and also affected the Δ12-desaturase transcript accumulation. This may be due to either a lack of transcription or to early transcript decay. Moreover, in our F2 segregating population, this character appears strictly dominant.

Thus, the model to explain the lack of transcript accumulation has to take into account the trans-effect of the HO mutation. However, several mechanisms of gene regulation are still possible to explain these facts. The simple way to understand the failure of this seed specific Δ 12-desaturase gene function should be to look at the structure and organization of this specific region by cloning approach. This was performed and the results will be presented.

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