Variability and genetic analysis of sterols content in sunflower seeds

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ABSTRACT

Phytosterols are triterpenoid molecules naturally present in plants that are involved in the functioning of cell membranes and embryogenesis. These bioactive molecules are of interest due principally to their natural properties decreasing the Low Density Lipoprotein cholesterol, but other properties have also been highlighted. These findings have greatly increased industrial demands for sterols but present extraction methods imply toxic chemical products. Therefore, increased plant sterol contents could not only help to meet industrial demands but also to develop environmentally friendly extraction methods. The aims of this study were to evaluate genotypic variability and to analyze genetic determination of sterol content in sunflower seed. Seventeen genotypes and a population of 200 recombinant inbred lines (RIL) derived from a cross XRQ x PSC8 were grown at INRA Clermont Ferrand (France) in 2005. The seed harvested was used for sterol analyses in the Lipochemistry platform, INRA Toulouse (France). Results showed a large variability among the genotypes studied, which was confirmed within the RIL population. Values of total sterols varied twofold between extreme genotypes. QTL analyses showed several zones detected for all traits. One or two QTL were detected for the most abundant sterols. For example, one QTL was identified in LG 1 explaining 10% of the total variability. Possible further investigations and use of the results to start breeding programs aiming at improvement in sterol content are discussed.

Key words: bioaccumulation – genotypic variability – phytosterols – QTL - sunflower.

INTRODUCTION

Sterols, minor compounds, are naturally present in plants. They are involved in membrane fluidity and permeability (Schaller, 2003). The role of sterols in embryogenesis has also been demonstrated (Clouse, 2000). During the last decade, there has been interest in sterols due to their potential benefits for human health. They may reduce Low Density Lipoprotein cholesterol (LDL) (Miettinen et al., 1990; Bosner et al., 1999; Ostlund, 2007) and several studies have highlighted other interesting properties such as anti-cancer (Awad et al., 2003), anti-inflammatory (Bouic, 2001) and anti-oxidation activities (Van Rensburget et al., 2000). Consequently, these bioactive molecules are now used for various industrial applications. They are used in nutrition as functional food, in particular in enriched margarines (Moreau et al., 2002). By chemical modification, phytosterols could also be used as raw materials in the production of pharmaceuticals as a source of steroids (Van Dansik, 2000); or in cosmetics (Folmer, 2003). Sterols have more recently been used in liquid crystals in the optics industry (Zhang et al., 2005).

The wide uses of these molecules require a specific composition of sterols in oil depending on the applications which are used in native statement or chemically modified. Phytosterols are commercialized mostly as by-products from seed oil processing industries and deodoriser distillates from industrial seed oil refiningl (Daguet and Coïc, 1999). This source of production suffers from two major problems. The first concerns the traceability of seeds samples whose origin is not well known. The second, linked to the methods of extraction, results from the use of chemical substances damaging to human health and to the environment.

Phytosterols are present in different plant parts and mostly in seeds. Their level depends on species and sunflower seeds contain quite a high concentration (Mouloungui et al., 2006). Nevertheless, these minor compounds are present in low thresholds which considerably limit their extraction. The improvement in phytosterol concentration could improve accessibility of molecules and, therefore, could help the development of extraction methods. Moreover, the diversification of sterol uses has led to an increase in industrial demands. Levels of seed sterol contents can be maximized by crop management (Roche et al., 2006). Whereas the effects of the genotype on seed oil and protein contents are widely reported, studies of genotypic effects on sterol content in sunflower seeds are lacking. Similarly, genetic

determination of oil content has been investigated but, information on genomic regions governing sterols in sunflower seeds is not available.

The aim of this study was focused on the determination of genotype effect on sterol accumulation and also on the genetic analysis of this trait in sunflower seeds.

MATERIALS AND METHODS

Sunflower genotypes: Seventeen diverse cultivated genotypes were used in this study. These genotypes (from INRA Clermont-Ferrand, France) are contrasted for their seed yield and oil content (Table 1). Two hundred recombinant inbred lines (RIL), from a population obtained by single seed descent from a cross of INRA lines XRQ (bred from a cross of USDA line HA89 and the Russian open pollinated variety Progress) and PSC8 (bred from a populations under recurrent selection for *Sclerotinia* resistance) were used for the genetic study. These RIL were genotyped with SSR and RFLP for construction of a genetic linkage map (Vear et al., 2008).

Table 1. Characteristics of seventeen genotypes studied (inbred lines and hybrids)

Genotype	Characteristics	Origin	Genotype	Characteristics	Origin
OF	High C18:1	France	OEG	High C18:1	Spain
83HR4	Standard	France	HA382.LS2	Low C18:0[1]	USA
VHQ	Standard	France	OPA3	High C18:1	France
OSQ	High C18:1	France	59259	Dwarf	France
XRQ	Standard	France	Trisun (hybrid)	High C18:1	USA
PSC8	Standard	France	HA300	High sterol content	USA
83HR4OL	High C18:1	France	R105	High sterolcontent	France
RHA345	High C18:1	USA	Olbaril (hybrid)	High oleic	France
HA821.LP1	Low C16:0[1]	USA			

[1]C18:1 standard

Field crop conditions: The inbred lines and RIL were grown in a breeding nursery at INRA Clermont-Ferrand (45°46'59'' N, 3°4'56'' E latitude) 2005. For each genotype, there was 1 row of 13 plants, 5 to 10 of which were bagged to obtain seed by self-pollination. Local climate data (mean temperatures, rainfall and evapotranspiration) were checked at the weather station at INRA Clermont-Ferrand. The weather data are summarized in Table 2.

Table 2. Weather conditions during plant cycle of 17 contrasted genotypes and 200 RIL of sunflower cultivated in INRA station of Clermont-Ferrand in 2005.

Months	June	July	August	September (1-10)	Mean or sum
Mean temperatures (°C)	19.8	20.9	18.3	20.5	19.9
Rainfall (mm)	56.6	29.6	17.0	15.8	62.4
Evapotranspiration (TP) (mm)	140.5	159.0	125.7	33.1	317.8
Rainfall / TP	0.40	0.19	0.14	0.48	0.20

Climate conditions prevailing during the cropping season were very stressing for plant development. The evaporative demand during the plant cycle greatly exceeded the rainfall. In contrast, the grain filling stage (August) coincided with a drought period (Table 2) as shown by the low rainfall /ETP ratio noticed in *August 2005.*

Determination of sterol contents and composition using a small-scale sample extraction method: Biochemical analyses were performed at the Lipid platform of the Agro-Industrial Chemistry laboratory. A small-scale sample extraction method was developed for reliable and economic analysis of sterols in sunflower seeds. Cholestanol (Dihydrocholesterol, Aldrich Chem. CO.) was used as an internal standard. Sunflower seed samples were saponified with ethanolic KOH (1M) (Titrinorm™, Prolabo). The non-saponifiable fraction was extracted with iso-hexane (Merck). Sterols were silylated by 1ml of N-methyl-N-trimethylsilyl-heptafluorobutyramide (MSHFBA, Macherey-Nagel) mixed with 50µl of 1-methyl imidazole (Sigma) called silylation reagent. 1 µl of sterol trimethylsilyl ether derivatives were injected in a Perkin-Elmer GC equipped with a CPSIL 5CB 30m (D: 0.25mm), FID detector. The thermal regime

was the following: 160°C (0.5min), 10°C/min until 260°C, 2.5°C/min until 300°C, 25°C/min until 350°C, and 350°C (1.5min) for the oven temperatures, 55°C (0.5min), 200°C/min until 320°C, 30°C/min until 350°C, and 350°C (2.5min) for the injector temperatures and 365°C for the detector temperature. Total phytosterols detected included desmethylsterols (β-sitosterol, stigmasterol, campesterol, Δ7-stigmastenol, Δ5-anenasterol, Δ7-avenasterol), methylsterols (24-ethyldiene lophenol, 24-methylen lophenol) and dimethylsterol (cycloartenol and methylencycloartanol).

Statistical data analysis: Phytosterol data are expressed as a weight percentage of seed dry matter (mg of sterol per 100g of seed dry matter). Analysis of variance and Student-Newman & Keuls test were applied to the experiment results to determine the significance between (General Linear Models Procedure, SAS Institute, 1988); genotypes and for the different measured traits. The map built for QTL detection included 39 RFLP, 162 SSR, and 4 mendelien traits (P12, P15, Rf1, and b1). It was developed with CARTHAGENE software (de Givry et al., 2005) with the commands [group 0.4 4], then [builtdfw 3 3 {} 0] to build a framework for each of the groups identified, and, finally, [build] to add the remnant markers. It spans over 1666 cM, with an average of 12.2 markers per linkage group. QTL detection was performed with the software MCQTL (Jourjon et al., 2005) under the "forward" algorithm and with the iQTL" option (Charcosset et al., 2001). The level of significance was determined through 3000 permutations for each trait. As several phytosterols were recorded, we used the software BIOMERCATOR ® (Arcade et al., 2004) to map the different QTLs and to check the hypothesis of a single QTL associated with different related traits.

RESULTS

Genotypic variability for sterol content in sunflower seeds: A large genotypic variability was shown for the traits measured (Fig 1). The mean value of total seed sterols within the collection studied was more than 280 μmol.g^{-1}DM. The difference between the extreme genotypes for this trait was nearly two-fold (Fig. 1). The most abundant sterols (70% of total) were desmethysterols which were mostly constituted by β-sitosterol (Fig. 1a and 1d). Methyl and dimethylsterols represented only 19% (Fig. 1b and 1c). The highest values of the total and desmethylsterols were obtained for XRQ which is one of the parents of the recombinant inbred lines used for the QTL study. The lowest values were noted for Trisun, a high oleic hybrid variety and, as expected for 59259, a dwarf genotype. PSC8 the second parent of RIL population showed an intermediate values for sterol content. Although, they present the same sterol profile, the two parents were contrasted for the seed sterol content.

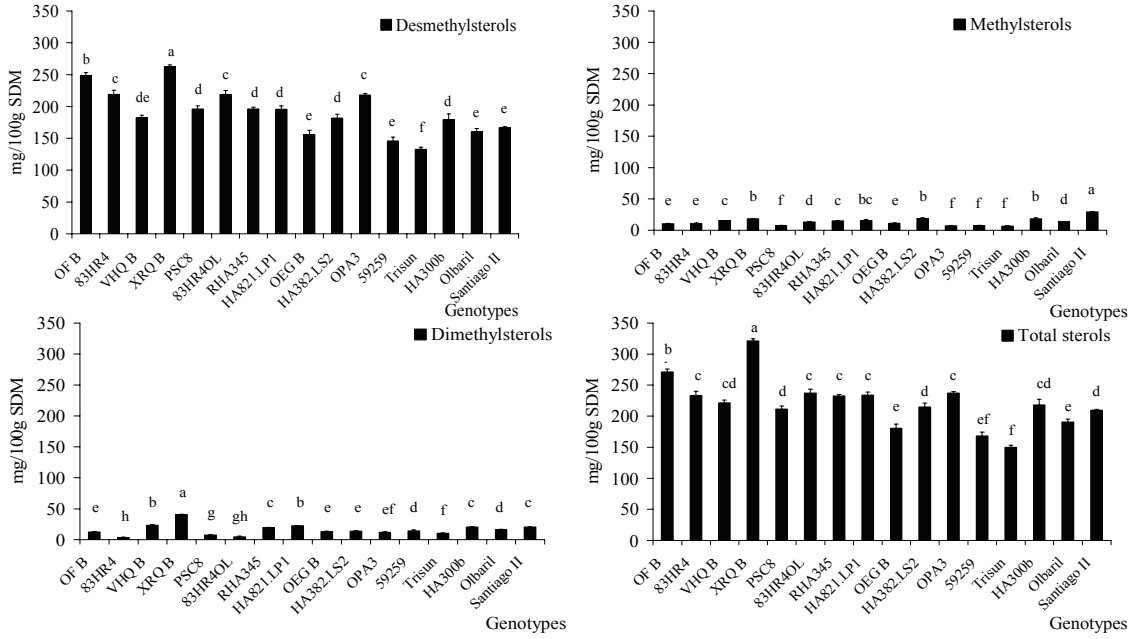

Fig. 1. Variability for total sterol (a), desmethylsterol (b), methylsterol (c) and dimethylsterol contents (d) among seventeen genotypes cultivated under rainfed conditions in 2005. Within each figure, means followed by a different letter are significantly different at P = 0.05.

Genetic analysis of sunflower seed sterol content

- Variability within the RIL population: The two parental lines were different for most traits (Fig. 1). Wide phenotypic variability was observed within the RIL population. Extreme RIL values showed both positive and negative transgression compared with parents (Fig. 2).

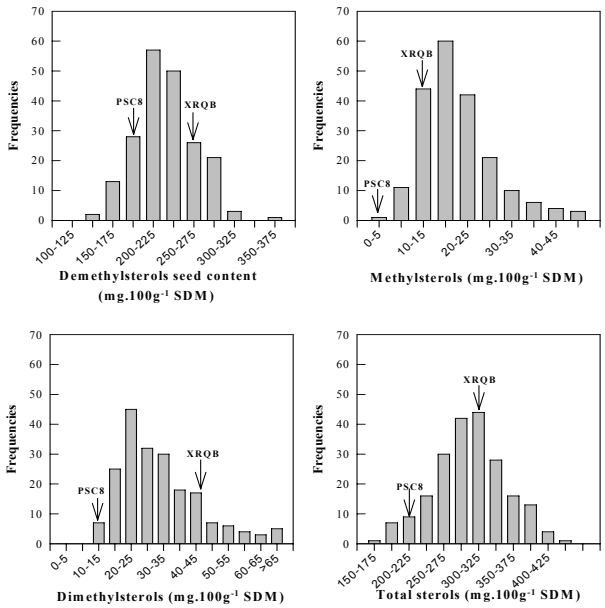

Fig. 2. Distribution of total sterol (a), desmethylsterol (b), methylsterol (c) and dimethylsterol contents (d) within a population of 200 RIL grown under rainfed conditions in 2005. Arrows indicate the parental values.

--QTL detection

Table 3 presents QTLs detected, their additive effect of alleles of each parent and the LOD likelihood confidence interval for the most important sterols traits. 24 QTLs were detected for all measured traits, at the level 1% for each trait (LOD>2.88). For some ones two regions have been identified while for others only one has been noted. One QTL was detected for sitosterol on LG1 explaining more than 10% of the variability of this trait. In the same LG QTLs were detected for desmethylsterols and total sterols content. Three QTLs were found for campesterol on LG7 and LG4 (nearly 20%) and stigmasterol on LG10 (12%).

Table 3. QTLs detected for the most important sterol seed content in sunflower.

Trait	Linkage group	R^2 (%)	LOD
β-Sitosterol	LG1	10.4	4.99
Campesterol	LG7	10.1	4.83
	LG4	9.6	4.57
Stigmasterol	LG10	12.3	7.08
Demethylsterols	LG1	13.9	6.98
Total sterols	LG1	14.3	7.21

DISCUSSSION

Genotypes were chosen in order to give a large range of sterol variability. Variance analyses show a very highly significant effect of the factor genotype for all traits measured (Fig. 1). Generally, high oleic genotypes produce lower content of phytosterols than conventional lines. Highest values were obtained for the genotype XRQ and the lowest ones for Trisun and for the dwarf genotype 59259. The values

observed of sterol content in sunflower seed are similar to those reported by Anastasi et al. (2000) but lower than the results obtained by Roche et al. (2006) in our laboratory. This difference is partly due to the germplasm used in the two studies but mostly due to the climate conditions during the cropping season. Mean temperatures which have prevailed during 2005 in Clermont-Ferrand were 2 to 4 °C lower than those reported by Roche et al. (2006) in their study in Toulouse in 2002 and 2003, respectively. Delayed sowing in the latter region may have increased sterols content.

Similarly, the variability observed within the RIL population was greater than the difference observed within the genotype collection. Moreover, the extreme values of some RIL were higher than the highest parent value or were lower than the lowest parent value, indicating transgressive segregation.

Studies on genetic determination of seed sterol content in sunflower have not so far been published. Several QTLs were revealed for the traits measured. Our results showed that for most abundant sterols, only one or two QTLs were detected. For β-sitosterol, which constitutes more than 75% of total sterol content, one QTL, explaining 10% of the observed variability, was detected on LG1 (Fig. 3). As checked with BIOMERCATOR, a unique metaQTL was found in this linkage group for β-sitosterol, demethylsterols and total sterols. In the same linkage group 1, another QTL position was detected for citrostadeniol and Δ7-stigmasterol. In linkage group 10, a unique metaQTL was also detected for citrostadenol, Δ7-stigmastenol and gramisterol.

The results show the potentialities existing within cultivated sunflower for sterol accumulation in seeds and this could help breeders for parental choice in order to initiate breeding programmes for sterol content improvement. These data need other investigations in multi-year experiments to assess the stability of QTLs identified across environments and genetic backgrounds in sunflower. Moreover, the genetic map used in this study should be improved by mapping more molecular markers and gene candidates as enzymes involved in sterol biosynthesis in sunflower. For example, starting from Arabidopsis amino acid sequence of cycloartenol-C-24-methyltransferase (EC 2.1.1.41), several potential SNP positions are found across the *Helianthus* EST sequences, and some of them can be used to check candidate co-localizes with some of the QTLs.

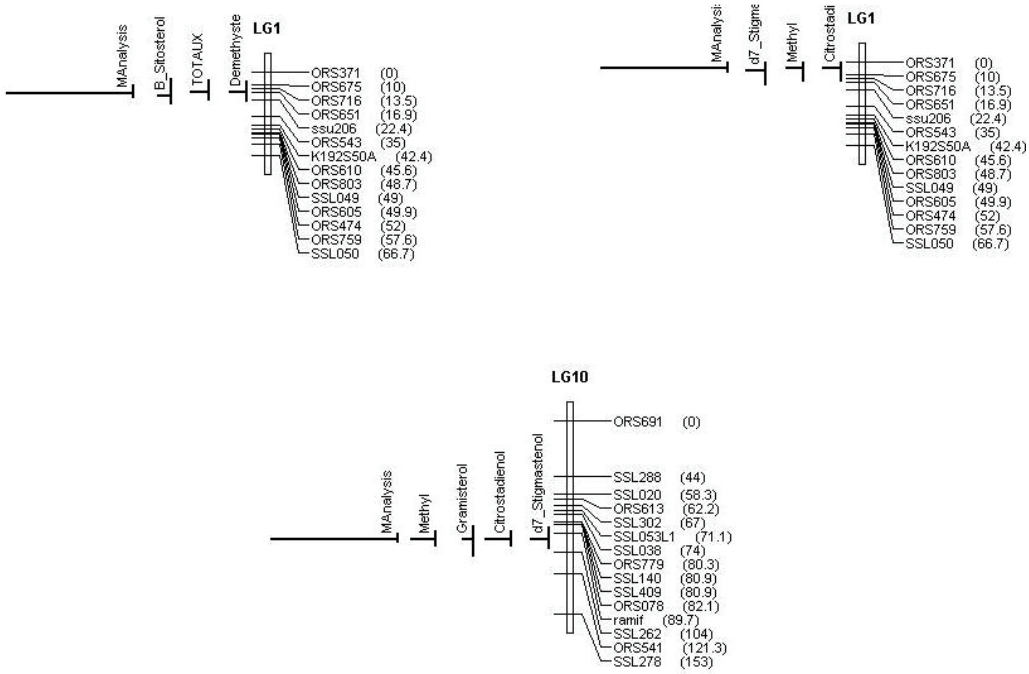

Fig. 3. MetaQTL mapping for some of the phytosterols data recorded on the XRQ x PSC8 RIL population (detailed results on http://lipm-helianthus.toulouse.inra.fr/Web/QTL/synthese-phytosterols.xml).

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