Theor Appl Genet (2012) 125:1589–1601
DOI 10.1007/s00122-012-1937-0

ORIGINAL PAPER

Genetic analysis of phytosterol content in sunflower seeds

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Received: 11 January 2012 / Accepted: 28 June 2012 / Published online: 24 July 2012

Abstract Interest in phytosterol contents due to their potential benefits for human health has been largely documented in several crop species. Studies were focused mainly on total sterol content and their concentration or distribution in seed. This study aimed at providing new insight into the genetic control of total and individual sterol contents in sunflower seed through QTL analyses in a RIL population characterized over 2 years showing contrasted rainfall during seed filling. Results indicated that 13 regions on 9 linkage groups were involved in different phytosterol traits. Most of the QTL mapped were stable across years in spite of contrasted growing conditions. Some of them explained up to 30 % of phenotypic variation. Two QTL, located on LG10, near *b1*, and on LG14, were found to co-localize with QTL for oil content, indicating that likely, a part of the genetic variation for sterol content is only the result of genetic variation for oil content. However, three other QTL, stable over the 2 years, were found on LG1, LG4 and LG7 each associated with a particular class of sterols, suggesting that some enzymes known to be involved in the sterol metabolic pathway may determine the specificity of sterol profiles in sunflower seeds. These results suggest that it may be possible to introduce these traits as criteria in breeding programmes for quality in sunflower. The molecular markers linked to genetic factors controlling phytosterol contents could help selection during breeding programs.

Communicated by A. Bervillé.

In memory of Andrée Bouniols who died in 2008.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-012-1937-0) contains supplementary material, which is available to authorized users.

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Introduction

Sunflower (*Helianthus annuus* L.) is among the most important oilseed crops around the world. This crop can be of special interest for its adaptation to high temperatures and to limited water availability (Rondanini et al. 2003; Roche et al. 2004; Anastasi et al. 2010). Breeding programs have focused particularly on yield, resistance to diseases and oil quantity and quality (Vear 2010). So far, few studies have been devoted to minor lipids including phytosterols, which are present at interesting levels in sunflower (Mouloungui et al. 2006).

Plants contain a complex mixture of sterols, the most abundant being β-sitosterol, campesterol and stigmasterol while cholesterol occurs only in trace amounts (Benveniste

2002). These compounds are involved in membrane fluidity and permeability (Hartmann 1998; Schaller 2003) and in embryogenesis (Clouse 1996; Schrick et al. 2011). As plant hormone-precursors, they play a crucial role in plant growth and developmental processes such as cell division, polarity and morphogenesis (Bajguz and Tretyn, 2003; Lindsey et al. 2003). Phytosterols are products of the isoprenoid biosynthetic pathway (Fig. 1), which occurs only in the cytoplasm and consists of more than 25 enzyme-catalyzed reactions (Benveniste 2002).

In recent years, there has been an increasing interest in sterols due to their potential benefits for human health. They have been shown to reduce total plasma cholesterol and low-density lipoprotein cholesterol (LDL) levels in human subjects (Ostlund 2007; Brufau et al. 2008). Like cholesterol, to which they are related both structurally and biosynthetically, phytosterols present a tetracyclic ring and side chains linked to C-17 that differ according to sterol compound. Sitosterol and campesterol have an ethyl and a methyl group at C-24, respectively (Fig. 1). The absence of the double bond at the D5 position represents the saturated form of sterols, called stanols, which occur in small amounts, mainly in cereals.

Several studies have highlighted other interesting properties such as anti-cancer, anti-inflammatory, anti-oxidation activities and prevention of cardiovascular diseases (Hansel et al. 2011; Valerio and Awad 2011). Consequently, these bioactive molecules are now used for various industrial applications and they are also used in nutrition as functional foods (Chen et al. 2008). 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). Overall, the diversification of sterol uses has led to an increase of industrial demands.

Phytosterols are present in several plant parts and their levels depend on species (Mouloungui et al. 2006). Often, these minor compounds are present in low concentrations, which limit considerably their extraction. Improvement of phytosterol concentration could improve accessibility of molecules and therefore could help the development of extraction methods. Levels of seed sterol contents can be maximized by crop management (Roche et al. 2006, 2010a), or by harvesting before maturity (Roche et al. 2010b) but knowledge of the available genetic variability is essential to make possible studies and mapping of genes controlling sterol content as well as for breeding.

There have so far been few studies on genotypic effects and Quantitative Trait Loci (QTL) mapping of phytosterols in sunflowers or other oil crops. In winter rapeseed, Amar et al. (2008a) found three QTL explaining 60 % of the observed variance of phytosterol content. In sunflower, Roche et al. (2006) reported significant differences of total sterol contents between three genotypes but sterol compounds were not measured individually. More recently, among a collection of sunflower inbred lines and hybrids, Roche et al. (2010a) observed strong genotypic effects both on individual and total sterol levels. Among the genotypes studied by these authors, two inbred lines (XRQ and PSC8) with highly contrasting sterol contents—304 and 229 mg/100 g SDM (seed dry matter), respectively—are parents of a set of recombinant inbred lines (RIL), suggesting that it would be possible to map QTL for sterol contents. A preliminary study based on single RIL samples and a 200 RFLP-SSR marker map indicated QTL concerning five sterol compounds, each explaining 10–14 % of variance (Alignan et al. 2008). Also in sunflower, Haddadi et al. (2012) reported four QTL for total phytosterol content in a different set of RIL (RHA266 × PAC2) grown in France and in Iran, but they explained only 4–13 % of observed variation, and did not provide information on individual sterol compounds.

Although this study was focused on mapping QTL of individual sterols in a RIL population, it appeared of interest to also take into account the mapping of QTL involved in oil content in the same background, as sterols are lipids and therefore are likely affected by the whole lipid metabolism. Seed oil content, a major character for the sunflower crop, is highly heritable (Fick 1975). Since direct measurements of whole seed are possible by Nuclear Magnetic Resonance (NMR), breeding for high oil content is quite easy. Studies of QTL controlling oil content have been made since 1995 and the linkage groups (LG) involved appear quite stable. Mestries et al. (1998) detected three QTL for oil content in an F3/F4 population, on LG6, 10 and 17. Bert et al. (2002), using the F2/F3 population from which the "INEDI" Recombinant Inbred Lines (RIL) used in the present study were developed, showed QTL on the same LG (6, 10 and 17) and also on LG14. More recently, on unbranched hybrids, Bachlava et al. (2010) studied the QTL on LG10 in the region of the recessive branching gene *b1*, which has a strong pleiotropic effect on seed size, yield and oil content. They showed that there was probably a QTL for oil content close to *b1* but still active in unbranched hybrid plants.

This paper presents genetic and QTL analyses of total and individual phytosterol contents in the INEDI RIL population, and of their per se oil contents and those of hybrids between two tester lines and these RIL, using a map with 482 markers.

Materials and methods

Sunflower genotypes

The INEDI RIL population was obtained by single seed descent (self-pollination to at least F8) from a cross of

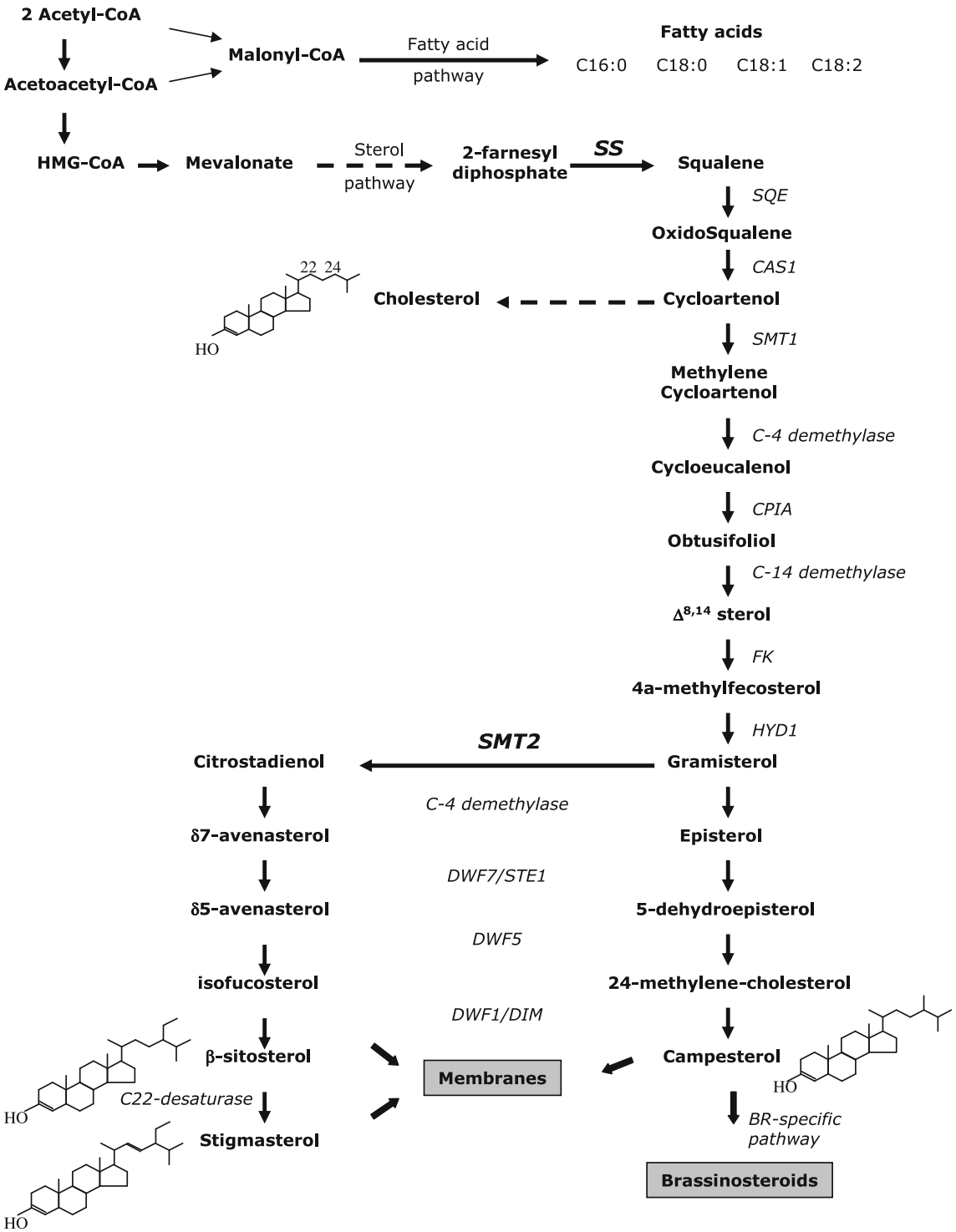


Fig. 1 Hypothetical simplified representation of sterol and fatty acid biosynthesis pathways in sunflower. *SMT1* sterol-methyl transferase 1, *SMT2* sterol-methyl transferase 2, *C16:0* palmitic acid, *C18:0* stearic acid, *C18:1* oleic acid, *C18:2* linoleic acid, *SS* squalene synthetase, *SQE* squalene epoxidase, *CAS1* cycloartenol synthase, *CPI1* cyclopropyl isomerase, *FK* C14 reductase, *HYD1* C8,7 isomerase, *DWF7/STE1* Δ^7 sterol C5 reductase, *DWF5* Δ^7 sterol C-7 reductase, *DWF1/DIM* C24 reductase

INRA lines XRQ (bred from a cross of USDA line HA89 and the Russian open pollinated variety Progress) and PSC8 (bred from a population under recurrent selection for *Sclerotinia* resistance). Both these parents have relatively high seed oil contents, very similar to the USDA line HA89, over 10 years at Clermont-Ferrand, after open pollination HA89: 50.5 % (of dry matter), XRQ: 51.0 %; PSC8: 49.5 %. They both produce conventional high linoleic acid oil. The RIL population is made up of 270 lines, which are maintained and multiplied by self-pollination.

Per se value for oil content and phytosterol profiles

The RIL were grown in a breeding nursery at INRA Clermont-Ferrand in 2001 to determine oil content in open pollination, and in 2004 and 2005 to characterize sterol profiles. For each genotype, there was 1 row of 13 plants, of which 5–10 were bagged before flowering to obtain seed by self-pollination. In 2001, four plants were left under open pollination to ensure good seed set and then bagged after flowering to protect from bird damage. Seed was dried at 30 °C, to about 6 % moisture content.

Values in hybrid combination for oil content

Hybrids between the RIL and two tester lines, firstly a cytoplasmic male sterile, unbranched line (Cms PGF650) and secondly a restorer line with apical branching and recessive genic male sterility (83HR4gms, INRA), were produced by hand cross pollination in 2000. The objective was to obtain male fertile unbranched hybrids, resembling modern sunflower varieties; so crosses were made according to the branching and restoration genotypes of the RIL. 181 hybrids were obtained with PGF650 and 131 with 83HR4gms. These hybrids were grown in four locations chosen in the sunflower cultivation area of France (Charentes/SC01, Eure et Loir/RN01, Gers/SL01 and Puy de Dôme/CF01) in 2001 and the last three locations in 2002 (RN02, SL02, CF02). The whole set of hybrids was divided into trials each of 28 hybrids and two check varieties (PRODISOL and MELODY) with two blocks. INEDI, the F1 hybrid from which the RIL were developed, was present in all the trial locations. 50 g samples were taken from each plot during harvesting and dried at 100 °C for 48 h.

Oil content was measured by NMR (Bruker Minispec 10, Wissembourg, France) on 3–5 g samples of five plants under open pollination for each of 270 RIL grown at Clermont-Ferrand in 2001 or for all the hybrid plots from the samples taken at harvest in 2001 and 2002. For the RIl, statistical analyses were made on oil contents of the four plants measured. For hybrids, after ANOVA to check validity of trials, results were expressed as percentages of the two checks, in order to group results from different trials.

Phytosterol contents were measured on 202 RIL grown in 2004 and 201 RIL grown in 2005 (per se value), using 10 g seed samples obtained by pooling 3–5 g of seed from 2–4 self-pollinated plants according to quantities available. Time, labour and seed requirements limited the number of replications which could be made.

Determination of sterol contents and composition using a small-scale sample extraction method

Sterol extraction and determination

The method of phytosterol extraction used in this study was based on the current literature (Toivo et al. 2000; Alignan et al. 2009; Roche et al. 2010b). One hundred microgram of cholestanol (Dihydrocholesterol, Aldrich Chem. Co.) from a stock solution of 5 mg diluted in 2.5 ml of chloroform was accurately weighed into a 10 ml Pyrex glass tube with a Teflon screw cap. Each sample of 10 g of sunflower seeds was ground to a powder. After chloroform evaporation, 250 mg of sunflower seed powder were saponified with 3 ml of KOH (1 M) (Titrinorm™, Prolabo) for 60 min at 75 °C and mixed every 30 min. The tube was cooled at room temperature for 20 min and 1 ml of distilled water was added. The non-saponifiable fraction was extracted from saponified lipids with 6 ml of iso-hexane (Merk). Tubes were shaken for 1 min using a vortex-mixer. Forty microlitre of silylation reagent (a mix of 1 ml of *N*-methyl-*N*-trimethylsilyl-heptafluorobutyramide (MSHFBA, Macherey-Nagel) and 50 µl of 1-methyl imidazole (Sigma) was added to 160 µl of sterol extracted phase and heated 3 min at 103 °C.

One microlitre of sterol trimethylsilyl ether derivatives was injected into Perkin-Elmer GC equipped with a CPSIL 8CB 30 m column (D: 0.25 mm, film thickness: 0.25 µm) and FID detector. The thermal regime was the following: 160 °C (0.5 min), 10 °C/min until 260 °C, 2.5 °C/min until 300 °C, 25 °C/min until 350 °C, and 350 °C (1.5 min) for the oven temperatures, 55 °C (0.5 min), 200 °C/min until 320 °C, 30 °C/min until 350 °C, and 350 °C (2.5 min) for the injector temperatures and 365 °C for the detector temperature. Total phytosterols detected included desmethylsterols (*β*-sitosterol, campesterol, stigmasterol, Δ7-stigmastenol, Δ5-avenasterol, Δ7-avenasterol), methylsterols (24-ethylidene lophenol also called citrostadienol, 24-methylen lophenol also called gramisterol) and dimethylsterols (cycloartenol and methylencycloartanol).

The determination of sterols was calibrated against a GC/MS method previously released. Individual sterols and squalene were identified by comparison of their retention time and by comparison of their mass spectra with those recorded in GC/MS.

Statistical data analyses

Phytosterol data are expressed as a weight percentage of seed dry matter (mg of sterol per 100 g of seed dry matter). There were three technical replications. Analyses of variance (General Linear Models Procedure, SAS Institute 1988) were performed in order to assess effects of years and RIL. Components of variance were computed using the mean squares expectation obtained from the SAS procedure. Estimates of the variance components σ^2RIL (genotypic variance) and σ^2 RIL × year (error variance) allowed the calculation of the broad-sense heritabilities of the mean of genotypes (h^2) for all the traits as:

$$h^2 = \sigma^2\text{RIL}/[\sigma^2\text{RIL} + (\sigma^2\text{RIL} \times \text{year}/y)],$$

where y is the number of years

The genetic map was built with genotyping data obtained for 482 markers exhibiting polymorphism between the two parental lines, including 214 SSR, 235 SNP detected on sunflower genes, 9 Resistance Gene candidates (RGC), 13 BAC End Sequence derived markers and 4 Mendelian phenotypic traits (*P12, P15, Rf1,* and *b1*). Genotyping methods and information on markers were those described by Vincourt et al. (2012). The map was built using the CARTHAGENE software (de Givry et al. 2005; http://www.inra.fr/internet/Departements/MIA/T//CartaGene/cartagene.html) with the commands "group 0.3 7", then "buildfw 3 3 {} 0" to built a framework for each group and finally "buildfw 0 0 {…} 0" to add the remnant markers. The map spanned over 1,762 cM. Detailed information on this map is provided in Supplementary File 1 and is also available at http://www.heliagene.org/Web/INRA/INEDI_mapping/mapping_public_2011-09.html. QTL detection was performed with the software MCQTL (Jourjon et al. 2005) under the "forward" algorithm and with the "iQTLm" option (Charcosset et al. 2001), with a threshold corresponding to a Type I error rate of 1 % at the genome wide level, as determined after 3,000 replications of the resampling process for each trait. As several phytosterol fractions were analysed, we used the software BIOMERCATOR® (Arcade et al. 2004) to map the different QTL and to check the hypothesis of a unique QTL associated with different related traits. Moreover, in a similar approach aiming to map the genetic factors having a pleiotropic effect on the individual phytosterol contents, a Principal Component Analysis was performed on the phytosterol traits, using R (version 2.9.2) with the function PCA from the FactoMineR package and initial data scaled to unit variance.

Results

Oil contents

Genetic variation between RIL in per se and hybrid combination

The oil contents of RIL (per se) showed highly significant differences (data not shown) with many lines much lower than either of the two parents and a fewer lines exceeding them. There were significant or highly significant differences between hybrids in each trial. Their mean values over the five locations did not exceed the level of the hybrid between the two parental lines and many had lower mean oil contents.

QTL detection

QTL for seed oil content were found highly consistent across years and locations for the evaluation in hybrid combinations for a given tester, but highly different between testers: while QTL explaining between 15 and 32 % of the phenotypic variability were located on LG15 and LG17 with the tester 83HR4gms, QTL explaining from 11 to 21 % of the phenotypic variability were detected on LG13 and LG14 with the tester CmsPGF650 (Table 1). Other QTL were occasionally detected on LG6 and LG7. In per se evaluation, in addition to a QTL explaining 51 % of the phenotypic variability and co-localizing with the *b1* locus, the QTL previously detected in hybrid combination on LG14 and on LG6 were also detected (Table 1).

Sterol content

Effect of climatic conditions and of genetic variation between RIL on phytosterol contents (per se evaluation)

In an ANOVA taking into account a year effect (two different climatic conditions) and a RIL effect (genetic variation), there were significant differences between seed harvested in 2004 and 2005, with higher values observed in 2005 for total sterols, dimethyl and methylsterols, when rainfall was much lower during the seed filling period (2004: 124.8 mm; 2005: 17.0 mm). In contrast, desmethylsterols, which represent the most important part of total sterols, were higher in 2004 than in 2005. The greatest variation was observed for cycloartenol and for squalene, which is the sterol precursor, their 2004 contents being only half those in 2005 (Table 2).

The RIL showed significant differences for individual and total phytosterol contents (Table 2). The largest

Table 1 QTL of oil content detected in hybrid combinations of "INEDI" RIL crossed with the two testers 83HR4gms (in bold) and with Cms PGF650 (in italic), and in "INEDI" RIL per se (open pollination, in bolditalic)

Trait	LG[a]	QTL position[b]	Support interval of the QTL		R^2 (%)[e]	LOD	XRQ-PSC8[f]
			Mini[c]	Maxi[d]			
oil_01CF_83HR4	LG15	19.8	14.4	22.4	26	8.1	2.2
oil_01SL_83HR4	LG17	97.5	88.3	100.6	24	7.6	−2.0
oil_01RN_83HR4	LG15	19.8	13.3	32.4	18	5.6	1.5
oil_01SC_83HR4	LG15	19.8	7.2	28.9	16	5.1	1.3
oil_01aver_83HR4	LG14	31.1	30.2	47.7	17	5.1	1.0
	LG15	19.2	12.8	22.2	27	8.2	1.4
	LG17	95.9	88.6	101.9	20	5.9	−1.2
oil_02CF_83HR4	LG15	17.2	3.4	42.8	15	4.6	1.3
	LG17	95.5	89.8	101.4	22	6.9	−1.8
oil_02SC_83HR4	LG15	31.7	7.2	39.7	22	6.7	1.5
	LG17	107.8	91.9	115.9	20	6.1	−1.5
oil_02RN_83HR4	LG15	15.2	5.6	30.9	21	6.5	1.8
oil_02aver_83HR4	LG15	17.2	9.3	36.0	27	8.3	1.5
	LG17	95.5	90.1	110.1	21	6.6	−1.3
oil_aver_83HR4	LG15	19.2	14.2	22.4	32	10.2	1.5
	LG17	95.5	88.8	102.4	22	6.7	−1.2
oil_01CF_PGF650	LG14	30.2	6.3	50.5	14	5.9	1.2
oil_01SC_PGF650	LG06	30.7	21.1	41.1	11	4.6	1.0
oil_01SL_PGF650	LG13	108.3	105.6	113.3	14	6.0	2.5
oil_01RN_PGF650	LG13	105.8	95.6	111.0	11	4.9	1.8
oil_01aver_PGF650	LG07	49.2	41.4	55.8	13	5.5	−0.7
	LG13	108.3	105.2	111.0	19	7.7	1.8
	LG14	30.2	6.3	50.5	12	5.1	0.7
oil_02CF_PGF650	LG13	110.3	95.2	114.7	14	5.6	1.8
	LG14	30.2	12.5	33.5	19	7.4	1.2
oil_02SC_PGF650	LG14	31.1	7.3	51.3	13	7.3	0.9
oil_02RN_PGF650	None						
oil_02_aver_PGF650	LG13	110.3	96.1	113.6	13	5.2	1.5
	LG14	31.1	7.1	51.1	14	5.6	0.9
oil_aver_PGF650	LG07	49.2	38.4	59.1	11	4.5	−0.6
	LG13	108.3	105.8	111.4	21	8.3	1.8
	LG14	31.1	11.7	35.8	16	6.3	0.8
oil_01_FL_RIL	LG06	34.7	26.0	38.5	9	5.8	1.5
	LG10	108.0	108.0	109.4	51	37.2	−5.5
	LG14	33.1	25.8	57.0	11	6.7	1.6

The trait "oil_yyLL_…" is designating the oil content for year "yy" in location "LL"

[a] Linkage group

[b] Position of the QTL expressed as the distance from the first marker in cM

[c,d] Minimum and maximum of the support interval of the QTL

[e] Percentage of variance explained by the QTL

[f] Represents the difference between XRQ and PSC8 alleles for the given QTL

variations within the RIL population were found for dimethyl and methylsterol contents, which varied ten times between extreme RILs. Individual, total desmethylsterol and total sterol contents varied more than twofold. Extreme RIL values showed both positive and negative transgressions compared with previous values of the two parents

Table 2 Variation in phytosterol contents (in mg/100 g SDM, Seed Dry Matter) for the INEDI RIL population grown in 2004 and 2005 at INRA Clermont-Ferrand (France)

Trait	Source of variation[a]		h^2	Year average		RIL average		XRQ	PSC8
	Years	RIL		2004	2005	Mean	Range		
Squalene	89.32***	1.54**	0.35	3.4	6.9	5.1	1.0–20.2	4.6	2.1
Cycloartenol	245.67***	1.76***	0.43	10.1	21.0	15.6	6.2–38.5	32.8	5.0
Methylen-cycloartenol	75.22***	2.48***	0.59	8.4	10.9	9.7	4.6–20.3	8.2	2.5
Dimethylsterols	220.34***	1.88***	0.46	18.6	31.9	25.3	12.0–55.1	41.0	7.5
Gramisterol	168.58***	2.48***	0.59	2.2	3.7	2.9	0.7–6.6	2.6	1.1
Citrostadienol	105.86***	4.32***	0.76	13.0	16.8	14.9	6.5–35.6	18.5	7.6
Methylsterols	134.24***	4.03***	0.75	15.2	20.5	17.9	7.3–41.4	21.1	8.7
δ7-Avenasterol	7.50**	4.03***	0.75	10.6	11.2	10.9	5.3–24.7	12.0	8.5
δ7-stigmastenol	28.83***	5.60***	0.82	29.5	32.5	31.0	14.5–62.9	59.1	25.6
δ5-Avenasterol	2.39	5.42***	0.81	8.3	8.0	8.1	3.4–22.5	5.2	4.7
Campesterol	69.42***	11.03***	0.90	17.4	15.7	16.6	7.8–28.5	20.6	16.5
β-Sitosterol	34.56***	3.23***	0.69	156.7	146.0	151.4	101.3–218.8	149.3	120.0
Stigmasterol	9.44**	2.89***	0.65	16.8	16.1	16.4	9.8–25.3	20.1	17.0
Desmethylsterols	13.34***	3.18***	0.68	239.3	229.5	234.4	152.3–249.1	266.3	192.3
Total sterols	8.54**	3.61***	0.72	273.1	282.0	277.5	179.7–414.0	328.4	208.5

, * Significance at 0.01 and 0.001 probability levels, respectively

[a] For each trait F value for RIL and year effects are displayed

(Table 2) High broad sense heritability values were found for most of the sterol traits (Table 2).

QTL detection

Table 3 presents the QTLs detected, the additive effect of alleles of each parent, the LOD score and confidence interval for all sterols traits. Before meta-analysis, 45 QTLs were detected on 9 linkage groups, using a type I genome wide error rate of 1 % for each trait. This conservative approach led to LOD scores generally >4, depending on trait and according to MCQTL procedure detection (see "Materials and methods"). For each sterol trait, at least one QTL was detected in each year, except for methylene-cycloartenol for which no QTL was mapped (Table 3). A meta-analysis was performed in order to check the hypothesis of a unique QTL associated with different related traits when QTLs concerning different characters co-localized.

Among the thirteen QTLs detected on LG1, four were related to total and individual methylsterols (citrostadienol); and nine were involved in specific desmethylsterols (δ7-avenasterol, δ7-stigmastenol, β-sitosterol), total desmethylsterols and total sterols contents (Table 3; Fig. 2). The meta-analysis showed them all to be at a single position except for δ7-avenasterol. QTL for campesterol were found on LG4 and 7, on LG4 close to a QTL one for squalene content (Table 3) and on LG 7, co-localizing with one for δ7-avenasterol content. QTL involved in total and individual methylsterols, individual desmethylsterol and squalene contents were identified on LG10 (Fig. 2), grouped in two metaQTL with slightly different positions according to year. Additional QTL for methylsterols were found on LG16 (Table 3). For total dimethylsterols and cycloartenol, QTL were identified on LG14, apparently co-localizing with control of δ5-avenasterol content Alleles of XRQ provided the favourable alleles for all QTL except those on LG10 (Table 3).

Discussion

Overall genetic variation on sterol content in the INEDI RIL population

The values of sterol content observed in sunflower seeds are quite similar to those reported by Anastasi et al. (2010), Roche et al. (2010a) but lower than the results obtained by Roche et al. (2006), Haddadi et al. (2012). This difference may partly result from the different genotypes used, but may also be due to climatic conditions during the cropping seasons. Variations of temperatures during grain filling are known to induce differences in phytosterols content (Alignan et al. 2009; Roche et al. 2010b). Mean temperatures that prevailed during 2004 and 2005 in Clermont-Ferrand were 2–4 °C lower than those reported by Roche et al. (2006) in Toulouse in 2002 and 2003. Unfortunately, climatic conditions were not detailed in Haddadi et al. (2012) which limits comparison.

Table 3 QTL detected for seed phytosterol contents in the sunflower RIL population INEDI, grown at INRA Clermont-Ferrand (France) in 2004 and 2005

Trait	LG[a]	QTL position[b]	Support interval of the QTL		R^2 (%)[e]	LOD	XRQ-PSC8[f]	MetaQTL[g]
			Mini[c]	Maxi[d]				
Citrostadienol_04	LG01	25.5	20.3	33.4	14	6.5	0.8	Meta_LG1
Citrostadienol_05	LG01	24.5	21.1	28.1	18	8.9	1.3	
δ7_Stigmastenol_04	LG01	25.5	19.9	41.8	12	5.2	1.5	
δ7_Stigmastenol_05	LG01	25.5	21.9	35.3	17	8.8	2.2	
Desmethylsterols_tot_04	LG01	28.4	18.4	44.5	12	5.3	6.7	
Desmethylsterols_tot_05	LG01	24.5	21.4	40.9	12	5.6	6.5	
Methylsterol_04	LG01	25.5	20.3	34.0	15	6.5	0.9	
Methylsterol_05	LG01	24.5	21.8	28.0	16	7.7	1.4	
β_Sitosterol_04	LG01	28.4	18.5	43.0	13	5.7	4.8	
β_Sitosterol_05	LG01	24.5	20.4	41.9	9	4.1	3.8	
Total sterol_04	LG01	28.4	19.9	42.7	14.2	6.4	7.9	
Total sterol_05	LG01	24.5	21.6	36.2	13.6	6.6	8.8	
δ7_Avenasterol_04	LG01	79.1	72.2	95.5	10	4.3	0.5	
Campesterol_04	LG04	104.3	99.2	108.4	9	3.8	0.7	
Campesterol_05	LG04	104.3	101.0	108.5	12	5.5	0.8	
Squalene_04	LG04	105.4	92.1	110.2	13	5.5	0.3	
δ5_Avenasterol_05	LG06	38	31.5	51.6	17	8.4	0.5	
Gramisterol_05	LG06	12.3	2.1	19.5	9	4.1	−0.3	
Campesterol_04	LG07	50.3	43.9	66.1	13	5.7	0.9	Meta_LG7
Campesterol_05	LG07	50.3	44.3	60.8	12	5.7	0.8	
δ5_Avenasterol_04	LG07	57.7	47.3	77.4	13	5.6	0.8	
Methylsterol_05	LG09	62.6	54.7	106.4	9	4.1	−1.1	
Citrostadienol_04	LG10	109.1	107.7	117.3	26	13.7	−1.1	Meta_LG10_1
δ5_Avenasterol_04	LG10	109.1	104.7	118.3	11	4.9	0.5	
Gramisterol_04	LG10	109.1	106.5	126.0	11	4.8	−0.1	
Methylsterol_04	LG10	109.1	107.8	117.3	25	13.1	−1.2	
Squalene_04	LG10	102.7	99.9	105.7	31	17.1	−0.5	
Citrostadienol_05	LG10	116.5	113.6	122.2	20	10.6	−1.4	Meta_LG10_2
δ7_Stigmastenol_04	LG10	115.5	105.5	118.2	18	8.7	−1.9	
δ7_Stigmastenol_05	LG10	116.5	112.2	119.3	14	6.8	−1.9	
Gramisterol_05	LG10	116.5	107.5	123.5	9	4.2	−0.3	
Methylsterol_05	LG10	116.5	113.5	118.2	20	10.0	−1.7	
Stigmasterol_05	LG10	115.5	107.5	128.2	12	5.6	−0.6	
Squalene_05	LG10	94.6	85.7	103.3	20	10.9	−1.2	
Dimethylsterol_04	LG13	14.8	0	47.9	4	4.5	−0.8	
Dimethylsterol_04	LG14	74.3	55.6	89.1	10	4.2	1.3	Meta_LG14_1
Total sterol_04	LG14	74.3	63.1	89.1	9	3.7	6.4	
Cycloartenol_04	LG14	33.1	27.7	37.2	25	13.2	1.0	Meta_LG14_2
Cycloartenol_05	LG14	32.1	24.7	37.0	18	9.0	2.3	
δ5_Avenasterol_04	LG14	34.1	29.2	48.5	17	8.0	0.8	
Dimethylsterol_05	LG14	31.1	24.8	75.5	14	6.6	2.5	
δ5_Avenasterol_05	LG14	8.0	0.0	58.3	13	5.9	0.6	
Citrostadienol_04	LG16	119.8	108.9	119.8	9	3.6	0.6	
Gramisterol_04	LG16	116.3	107.9	119.8	13	5.9	0.2	

Table 3 continued

Trait	LG[a]	QTL position[b]	Support interval of the QTL		R^2 (%)[e]	LOD	XRQ-PSC8[f]	MetaQTL[g]
			Mini[c]	Maxi[d]				
Methylsterol_04	LG16	119.3	109.7	119.8	11	4.6	0.8	

[a] Linkage group

[b] Position of the QTL expressed as the distance from the first marker in cM

[c,d] Minimum and maximum of the support interval of the QTL

[e] Percentage of variance explained by the QTL

[f] Represents the difference between XRQ and PSC8 alleles for the given QTL

[g] MetaQTL obtained from Biomercator (see "Materials and methods")

A significant difference was found in seed sterol contents between the 2 years of the present study (Table 2) although these were more similar than their means compared with the results of Anastasi et al. (2010); Roche et al. (2010a). In 2005, the drier year, dimethylsterols, methylsterols, δ7-avenasterol and δ7-stigmastenol contents were higher than in 2004; whereas, the other demesthylsterols were lower (Table 2). Thus, the contents of sterols produced at the beginning of the enzyme pathway were higher in the dry year whereas the final compounds, mostly desmethylsterols, were greater in 2004, the rainy year. Limited water availability could have reduced desmethylsterols in 2005 or increased their synthesis in 2004 as reported by Anastasi et al. (2010).

Genotypic variation of sterol contents varies according to crop species. Yamaya et al. (2007) showed weak genetic differences in sterol composition of soya-bean between Japanese and non-Japanese genotypes, whereas Alignan et al. (2009) and Amar et al. (2008b) reported strong genotypic effects in wheat and rapeseed, respectively. Broad variation was found within our RIL population, greater than the differences observed among the genotype collection reported by Roche et al. (2010b). The transgressive segregation shown by some RIL for total sterols (Table 2) is in agreement with Haddadi et al. (2012).

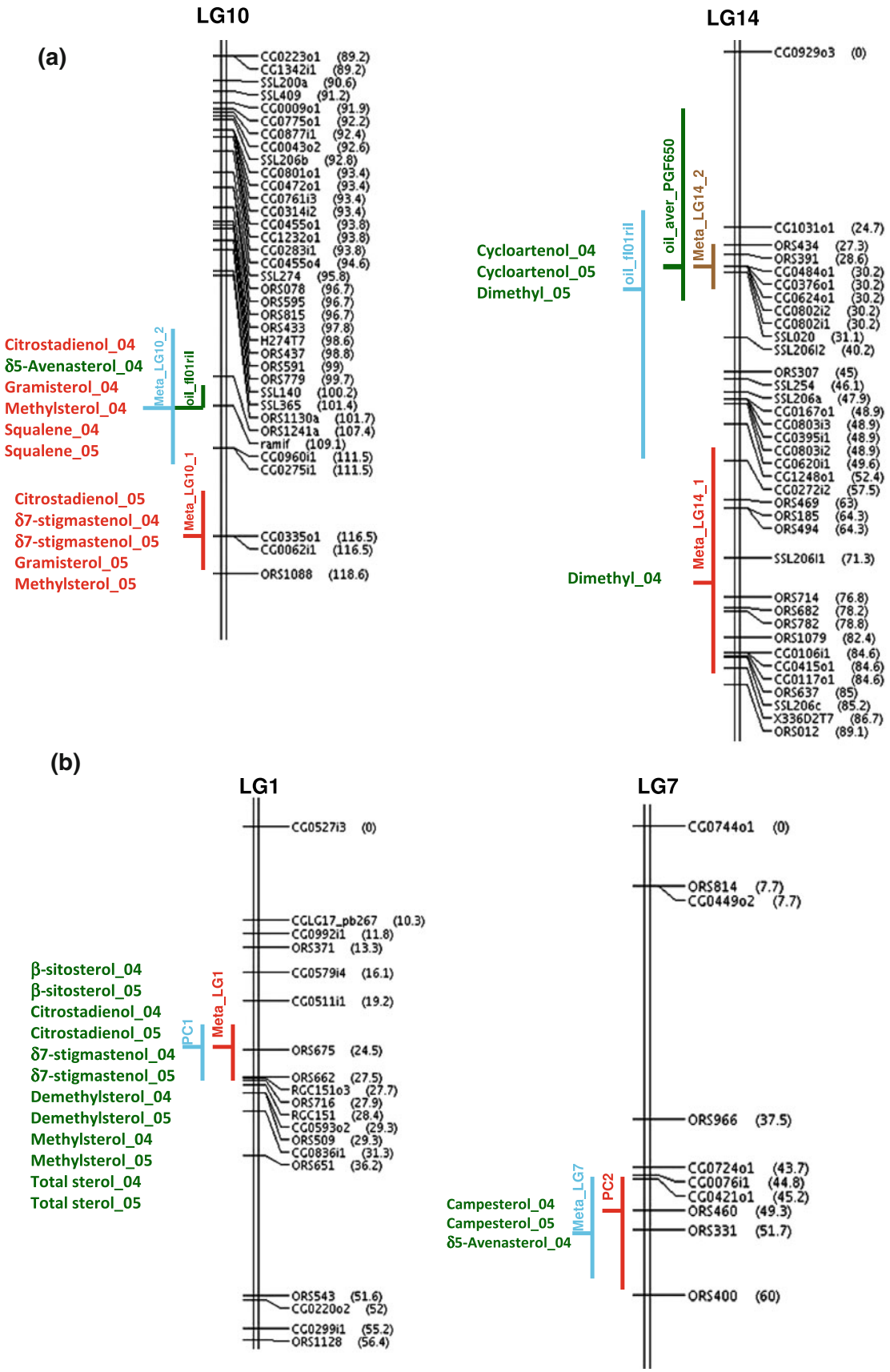
Co-localization for oil and individual sterol contents

Shared QTL positions were found on LG10 (meta_LG10_2, near of the *b1* locus, Fig. 2a) and on LG14 (meta_LG14_2, Fig. 2a), for seed oil content both in per se value and in hybrid combination and individual sterol contents. These QTL positions accounted for the largest part of phenotypic variability for sterol content (Table 3). The implication of the *b1* region on LG10 has been already discussed by Bachlava et al. (2010), and the QTL position on LG14 for oil content confirmed the previous results of Bert et al. (2002).

Two QTL were actually mapped on LG10, each appearing to be specific to a particular year, suggesting that, according to climatic conditions, different genes reflected the limiting factors for each year in the phytosterol biosynthetic pathway. As the QTL meta_LG10_2 was also found to co-localize with oil content, it could be moreover suggested that in 2004, the limiting factor for phytosterol content was associated with oil synthesis, while in 2005, a factor more specifically associated with phytosterol content was involved.

The QTL for squalene content was found at the same position (meta_QTL_2) in 2004 and 2005, suggesting that genes encoding enzymes involved in squalene accumulation, mainly squalene synthase, may be localized at this position. Over expression of squalene synthase increases squalene or phytosterol contents in *Eleutherococcus senticosus* and *Withania coagulans*, (Seo et al. 2005; Mirjalili et al. 2009).

The QTL meta_LG10_2 was also found to account for variation in methylsterols, and particularly citrostadienol and its precursor gramisterol, which are the most abundant methylsterols. Enzymatic modifications resulting in transformation of gramisterol in β-sitosterol are described in Fig. 1 and imply several enzymes. This may explain QTL co-localization of total and individual desmethylsterols and methylsterols on LG1 and LG10 (Fig. 3). However, QTL for δ7-avenasterol and δ5-avenasterol did not map at the same position. In addition, three QTL were identified for δ5-avenasterol in 2004 and two in 2005 (Table 3). δ5-avenasterol is accumulated mainly after two enzymatic modifications involving Δ7-sterol C5 desaturase and Δ7-sterol C7 reductase. Our results indicate a year effect on sterol contents, probably due to different rainfall levels during grain filling, which affected seed development. In 2005, dimethylsterols, methylsterols, δ7-avenasterol and δ7-stigmastenol contents were higher than in 2004, whereas, the other desmethylsterol and total sterol contents were lower in 2005 (Table 2). This result supports that a probable regulation of Δ7-sterol-C7-desaturase (DWF7/STE1) and Δ7 sterol C7 reductase (DWF5), which are involved in the transition between δ7-avenasterol and δ5-avenasterol (Fig. 1). These multiple steps involved in δ5-avenasterol synthesis may explain the numerous QTL


(a)
LG10
CG0223o1 (89.2)
CG1342i1 (89.2)
SSL200a (90.6)
SSL409 (91.2)
CG0009o1 (91.9)
CG0775o1 (92.2)
CG0877i1 (92.4)
CG0043o2 (92.6)
SSL206b (92.8)
CG0801o1 (93.4)
CG0472o1 (93.4)
CG0761i3 (93.4)
CG0314i2 (93.4)
CG0455o1 (93.8)
CG1232o1 (93.8)
CG0283i1 (93.8)
CG0455o4 (94.6)
SSL274 (95.8)
ORS078 (96.7)
ORS595 (96.7)
ORS815 (96.7)
ORS433 (97.8)
H274T7 (98.6)
ORS437 (98.8)
ORS591 (99)
ORS779 (99.7)
SSL140 (100.2)
SSL365 (101.4)
ORS1130a (101.7)
ORS1241a (107.4)
ramif (109.1)
CG0960i1 (111.5)
CG0275i1 (111.5)
CG0335o1 (116.5)
CG0062i1 (116.5)
ORS1088 (118.6)
Citrostadienol_04
δ5-Avenasterol_04
Gramisterol_04
Methylsterol_04
Squalene_04
Squalene_05
Meta_LG10_2
oil_fl01ril
Citrostadienol_05
δ7-stigmastenol_04
δ7-stigmastenol_05
Gramisterol_05
Methylsterol_05
Meta_LG10_1
LG14
CG0929o3 (0)
CG1031o1 (24.7)
ORS434 (27.3)
ORS391 (28.6)
CG0484o1 (30.2)
CG0376o1 (30.2)
CG0624o1 (30.2)
CG0802i2 (30.2)
CG0802i1 (30.2)
SSL020 (31.1)
SSL206l2 (40.2)
ORS307 (45)
SSL254 (46.1)
SSL206a (47.9)
CG0167o1 (48.9)
CG0803i3 (48.9)
CG0395i1 (48.9)
CG0803i2 (48.9)
CG0620i1 (49.6)
CG1248o1 (52.4)
CG0272i2 (57.5)
ORS469 (63)
ORS185 (64.3)
ORS494 (64.3)
SSL206l1 (71.3)
ORS714 (76.8)
ORS682 (78.2)
ORS782 (78.8)
ORS1079 (82.4)
CG0106i1 (84.6)
CG0415o1 (84.6)
CG0117o1 (84.6)
ORS637 (85)
SSL206c (85.2)
X336D2T7 (86.7)
ORS012 (89.1)
Cycloartenol_04
Cycloartenol_05
Dimethyl_05
oil_aver_PGF650
Meta_LG14_2
oil_fl01ril
Dimethyl_04
Meta_LG14_1
(b)
LG1
CG0527i3 (0)
CGLG17_pb267 (10.3)
CG0992i1 (11.8)
ORS371 (13.3)
CG0579i4 (16.1)
CG0511i1 (19.2)
ORS675 (24.5)
ORS662 (27.5)
RGC151o3 (27.7)
ORS716 (27.9)
RGC151 (28.4)
CG0593o2 (29.3)
ORS509 (29.3)
CG0836i1 (31.3)
ORS651 (36.2)
ORS543 (51.6)
CG0220o2 (52)
CG0299i1 (55.2)
ORS1128 (56.4)
β-sitosterol_04
β-sitosterol_05
Citrostadienol_04
Citrostadienol_05
δ7-stigmastenol_04
δ7-stigmastenol_05
Demethylsterol_04
Demethylsterol_05
Methylsterol_04
Methylsterol_05
Total sterol_04
Total sterol_05
PC1
Meta_LG1
LG7
CG0744o1 (0)
ORS814 (7.7)
CG0449o2 (7.7)
ORS966 (37.5)
CG0724o1 (43.7)
CG0076i1 (44.8)
CG0421o1 (45.2)
ORS460 (49.3)
ORS331 (51.7)
ORS400 (60)
Campesterol_04
Campesterol_05
δ5-Avenasterol_04
Meta_LG7
PC2

◀**Fig. 2** Main QTL for phytosterols traits in the XRQ × PSC8 recombinant inbred lines population cultivated in field conditions in 2004 and 2005. Individual phytosterol traits are listed in front of the major MetaQTL detected in using Biomercator (see "Materials and methods") and of QTL associated with the principal components. **a** QTL detected both for sterol content and oil content; **b** QTL specifically detected for sterol content

(Table 3; Fig. 2) observed for this trait and its regulation which was probably affected by weather conditions.

As both individual sterol and oil content are measured as a fraction of seed dry matter, these two common QTL could be considered as not particularly relevant to identify particular genetic profiles giving increased production of sterols for a given seed oil content. However, this co-localization confirms the overall interest for sunflower breeding to identify the genes behind those two QTL.

QTLs for cycloartenol were identified in 2004 and 2005 and were co-localized on LG14 (meta_LG14_2), while QTL for dimethylsterols were mapped at different positions on LG14 (meta_LG14_1 and meta_LG14_2). In the sterol biosynthesis pathway (Fig. 1), cycloartenol synthase (CAS) transforms oxidosqualene to cycloartenol, which is the substrate for production of methylene-cycloartenol catalysed by sterol methyltransferase 1 (SMT1). In the present study, only QTLs for cycloartenol and total dimethylsterols were identified. It may be suggested that the QTL meta_LG14_2 cover CAS genes, while its regulation may be affected by a regulator underlying meta_LG14_1 under the influence of the environment. Holmberg et al. (2002) over expressed the SMT1 gene in seed tissues of tobacco resulting in a large increase in phytosterols and modulation of their composition. This increase was most important for isofucosterol (δ5-avenasterol), campesterol and sitosterol.

QTL specifically involved in sterol profiles in the INEDI RIL population

Assuming that QTL involved in both sterol content and oil content (meta_LG10_2, meta_LG14_2) are not the most informative to understand the genetics of sterol profiles, we will focus here on QTL detected for sterol content across the 2 years, but not for oil content, i.e. those detected on LG1, LG4, and LG7.

QTLs detected for β-sitosterol, desmethylsterols and total sterols content co-localized on LG1. This result was expected since β-sitosterol represents more than 67 % of desmethylsterols and 55 % of total sterols in the present study and is widely distributed throughout the plant kingdom. Moreover, QTL for δ7-stigmastenol and its precursor δ7-avenasterol, two other desmethylsterols (Fig. 1), were also at the same position on LG1 (Fig. 2). Meta-analyses revealed that this chromosomal region probably covers a

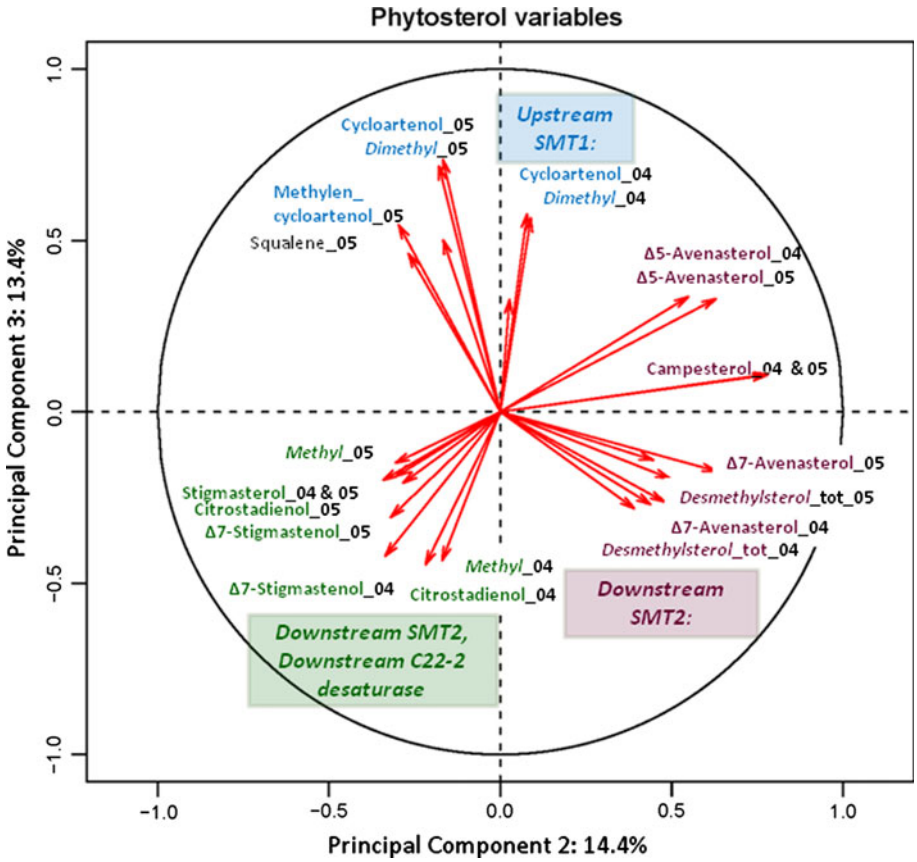


Fig. 3 Second and third component of the principal component analysis are grouping phytosterol families according to their places in the hypothetical simplified representation of sterol and fatty acids biosynthesis pathways

single QTL controlling the content of several different compounds. On LG1, Haddadi et al. (2012) found a QTL for total phytosterol content and also one for total tocopherol content co-localizing with a homologue of GST (glutathione *S*-transferase, AT1G02930, HuCL00790C003 at http://www.heliagene.org). This QTL was mapped between the SSR markers 0RS803 and ORS509, quite far from the location we found in this study. However, considering that the map of LG1 in Haddadi et al. (2012) was less accurate (6 markers on LG1, 123 RIL for mapping and QTL detection), the two results are probably not contradictory. Amar et al. (2008a) identified two regions involved in most abundant desmethylsterols and total phytosterols in winter rapeseed. The allopolyploid nature of *B. napus* might explain why two QTL were found in this species.

Stable QTL across years were found on LG4 and LG7 for campesterol content (Table 3). Campesterol represents a tiny amount of the desmethylsterol content (7 %) compared to β-sitosterol. However, campesterol is downstream in the sterol pathway and the precursor of the BR-specific specific pathway leading to brassinolide, which is now recognized as a major plant hormone. Our results suggest that, in this genetic background, there is an independent regulation of campesterol content.

Pleiotropic effects of QTL involved in sterol content

In order to test a possible pleiotropic effect of the QTL identified, a principal component analysis was performed. The first four principal components (PC) representing 69 % of the total variability were used as entries, and QTL were found only for the first three PC representing 60 % of the total variability. PC1 mainly reflected the total phytosterols, but was not correlated with campesterol and δ5-avenasterol contents, while QTL on LG7 and LG10 were associated with PC2 and those on LG14 with PC3. Results allowed the identification of three groups of QTL with two major effects on enzymes regulation (Fig. 3). The first group was composed by QTL identified for squalene, individual and total dimethylsterols which probably results from enzymes between squalene synthetase and SMT1 activity (see Fig. 1), accounting for the regulation upstream of SMT1. In contrast, the two other groups of QTL appear to be involved in accumulation of several methyl and desmethylsterols and downstream regulation of enzymes involved in their synthesis (SMT2, C4 methylase to C22 desaturase, see Fig. 1). These results highlight the interest of this RIL population in a gene network approach.

The genes controlling the sterol pathway have not yet been mapped in the INEDI RIL population. However, consistent genome sequencing information has been recently obtained on the XRQ genotype thanks to the new sequencing technologies (unpublished results). The sequences of some of these genes (SQE1/SQE2, CAS, SMT2, DWF1, STE1/DWF7,GST) for which an annotation based on EuGene (Schiex et al. 2001) has been at least partially obtained are provided (Supplementary File 2).

Conclusion

This study has shown that wide genetic variation exists for seed phytosterol contents, associated with, or independent from, variation in oil content. Weather conditions during grain filling influenced individual sterol contents but had little effect on QTL detection and heritability was high. The preliminary results of Alignan et al. (2008) for 2004 were confirmed for the five sterol compounds they analysed, with increased precision in QTL positioning and percentage explanation of variance. The stability across years of QTL mapped for each sterol trait indicates that molecular markers of QTL should be valid in most conditions. This study on the INEDI RIL population suggests that there is sufficient diversity in cultivated sunflower to obtain genotypes quantitatively and qualitatively different for seed phytosterol content and that markers associated with QTL specific to sterol content such as those mapped on LG1 and LG7 could be useful in breeding programs to modify the sterol profiles. In addition, the co-localization of QTL for several individual sterol contents suggests that the regions concerned may contain putative genes encoding enzymes involved in the sterol biosynthesis pathway. Fine mapping studies would help to determine the genes involved.

Acknowledgments We would like to thank the European Union for financially supporting this study (EFDR 7440 and 12688). Thanks are due to Elodie Gestas for her help in phytosterol analyses. The participation of Dr. O. Merah was supported by IUT A, Paul Sabatier University of Toulouse, France. This work also benefited from the support of the Genoplante 1999-2004 and OLEOSOL projects, funded respectively by the French Agence Nationale pour la Recherche (ANR) and the Midi-Pyrénées Region, French governmental support to competitive clusters, and the EDFR, for the establishment of the genetic map.

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